The initial-rate kinetics of mouse glutathione *S*-transferase YfYf

Evidence for an allosteric site for ethacrynic acid

Michael F. PHILLIPS and Timothy J. MANTLE

Department of Biochemistry, Trinity College, Dublin 2, Ireland

Mouse glutathione *S*-transferase GST YfYf (an orthologue of GST P or 7–7 in the rat and of GST π in the human) was found to have a subunit M_r of 24 500 and cross-reacted with anti-(rat GST YfYf). *N*-Terminal analysis showed a close similarity to the rat, human and bovine orthologues. On isoelectric focusing the native enzyme had a pI of 8.3 and a pI of 7.3 in the presence of urea. Initial-rate studies with 1-chloro-2,4-dinitrobenzene (CDNB) and GSH as substrates and inhibition studies with the product of the enzyme-catalysed conjugation of CDNB and GSH, *S*-(2,4-dinitrophenyl)glutathione, indicated a rapid-equilibrium random mechanism for the enzyme. The diuretic drug ethacrynic acid was found to be simultaneously a competitive inhibitor and an uncompetitive activator of the enzyme (with CDNB as the substrate whose concentration was varied). By using a computer simulation program (EKPLLOT) a model was developed that would explain the experimental data. It is proposed that ethacrynic acid can compete with CDNB at the active site but simultaneously bind to an allosteric site on the enzyme, causing an elevation in the $V_{\max}$ for the conjugation of CDNB and GSH. The implications of such an activation mechanism for an enzyme potentially conjugating a range of xenobiotic compounds are discussed.

INTRODUCTION

The cytosolic glutathione *S*-transferases (GSTs) exist in multiple forms that can be grouped into three species-independent families that have been termed Alpha, Mu and Pi (Mannervik *et al.*, 1985; Ålin *et al.*, 1986). Although structural and immunological relationships between the major forms have been well delineated (Hayes & Mantle, 1986a; Rothkopf *et al.*, 1986; Okuda *et al.*, 1987; Lai *et al.*, 1988) and substrate and inhibitor profiles have been described (Mannervik *et al.*, 1985; Hayes & Mantle, 1986b), detailed kinetic studies have until recently been restricted to members of the Mu family. The initial-rate pattern for the rat form GST A [now known to be a homodimer of Yb₁ subunits (subunit 3 in the alternative nomenclature of Jakoby *et al.*, 1984)] was recognized as complex by Pabst *et al.* (1974), who proposed a model that changed from a sequential mechanism at high concentrations of GSH to a ping-pong mechanism at low concentrations of GSH. However, a detailed analysis of all the available data (including the original data) showed that the complex initial-rate pattern was better explained as being a steady-state random mechanism (Jakobson *et al.*, 1977). More recently Danielson & Mannervik (1988) have proposed a similar mechanism for the other major rat Mu homodimer, GST Yb₂Yb₂ (4–4 in the alternative nomenclature). Insect forms of GST have also been shown to conform to a steady-state random mechanism (Clark *et al.*, 1984, 1986). Schramm *et al.* (1984) suggested that rat GST YaYa (1–1 in the alternative nomenclature), a member of the Alpha family, obeyed a rapid-equilibrium random mechanism, although they did not rule out a steady-state random mechanism.

There has been little kinetic work reported for the Pi family, although Ivanetich & Goold (1989) have proposed a rapid-equilibrium random mechanism for human placental GST π . In some respects this is surprising, as this form [described variously as the YfYf, YpYp or 7–7 homodimer in the rat (Kitahara *et al.*, 1984; Jensson *et al.*, 1985; Hayes & Mantle, 1986a)] has received considerable interest as a possible early marker for cancer, as it

is markedly increased in preneoplastic nodules in rat liver (for a review see Sato, 1989).

The regulation of expression of the mouse Yf orthologue in liver is known to be up-regulated by testosterone (Hatayama *et al.*, 1986; McLellan & Hayes, 1987), so that high concentrations are expressed in the male. Since this is a rich source of the enzyme, we have examined the reaction mechanism with the model substrate 1-chloro-2,4-dinitrobenzene (CDNB). During this study we obtained evidence consistent with the existence of an allosteric site on the enzyme for the diuretic drug ethacrynic acid (which is also a substrate), and details of these experiments are reported in the present paper.

MATERIALS AND METHODS

Materials

S-(2,4-Dinitrophenyl)glutathione (DNPG) was synthesized by the method of Vince *et al.* (1971). *S*-Hexylglutathione–Sepharose was prepared by the method of Mannervik & Guthenberg (1981). Glutathione, *S*-methylglutathione and ethacrynic acid were purchased from Sigma Chemical Co. Hydroxyapatite was purchased from Bio-Rad Laboratories. All other reagents were of analytical standard.

Purification of GST YfYf from mouse liver

GST YfYf was purified from the livers of adult male mice (Laca strain) by the method of Hayes *et al.* (1987), by using *S*-hexylglutathione–Sepharose chromatography followed by hydroxyapatite chromatography.

Analysis of purified protein

SDS/PAGE was carried out by using the method of Laemmli (1970). Isoelectric focusing was performed in flat-bed polyacrylamide gels in an LKB Multiphor apparatus under native and denaturing (8 M-urea) conditions. Immunoblotting was carried out as described previously (Hayes & Mantle, 1986a).

Protein concentration was determined by measuring the absorbance at 280 nm and by the method of Markwell *et al.* (1978). The *N*-terminal amino acid sequence for mouse GST YfYf was determined by using an Applied Biosystems 477A sequencer.

Assay conditions for kinetic studies

Initial rates with CDNB as the electrophilic substrate were measured with a Gilford 240 spectrophotometer by following the appearance of the product, DNPG, at 400 nm ($\epsilon = 2.15 \text{ mM}^{-1} \cdot \text{cm}^{-1}$). Although the standard assay for CDNB activity is carried out at 340 nm (Habig *et al.*, 1974), it was necessary to change the assay wavelength to 400 nm for two reasons: (i) to allow the measurement of CDNB activity in the presence of ethacrynic acid without measuring the formation of the GSH-ethacrynic acid adduct ($\lambda_{\text{max.}} = 270 \text{ nm}$) and (ii) to allow the use of higher concentrations of DNPG in the product-inhibition studies. The reaction was carried out in 0.1 M-sodium phosphate buffer, pH 6.5, at 30 °C. The concentration of ethanol, the solvent for the stock solution of CDNB, was kept constant at 3.5% (v/v). The reaction was initiated by the addition of enzyme. A linear relationship was found for the initial velocity as a function of the concentration of enzyme in the range 0.5–15 $\mu\text{g/ml}$ and the concentration of enzyme used for initial-rate studies was 2 $\mu\text{g/ml}$. The concentration of GSH was varied in the range 0.05–2 mM and the concentration of CDNB was varied in the range 0.1–2 mM (the concentration of CDNB cannot be increased above 2 mM for reasons of solubility). For product-inhibition studies, DNPG was added in the concentration range 0.02–0.8 mM with the concentration of one substrate varied as above and the other substrate kept at a fixed concentration. For inhibition studies with ethacrynic acid, the concentration of ethacrynic acid was varied in the range 5–40 μM . The formation of the GSH-ethacrynic acid adduct is not detected at 400 nm, so that the changes in absorption monitor only the formation of DNPG.

The binding of GSH to free enzyme was determined by monitoring the quenching of protein fluorescence upon addition of GSH with the use of Perkin-Elmer MPP-44B fluorimeter. The excitation and emission wavelengths were 280 nm and 340 nm respectively.

Statistical analysis of data

Initial-rate data were fitted to the equation for a rectangular hyperbola. The inhibition constants were obtained from linear fits of appropriate replots. Where appropriate, the data for some plots of apparent inhibition constant (Y) against the fixed substrate were fitted to the hyperbola:

$$Y = \frac{qK_iS + K_iK_s}{S + qK_s} \quad (1)$$

where S is substrate concentration, K_i and K_s are dissociation constants for inhibitor and substrate respectively and q is an

arbitrary constant. All fitting was carried out by using the computer program ENZFITTER (Leatherbarrow, 1987), which uses an iterative Gauss-Newton procedure.

RESULTS AND DISCUSSION

Analysis of purified protein

The purified protein appeared as a single band on SDS/PAGE with an apparent M_r of 24500. On isoelectric focusing the enzyme had a pI of 8.3 under native conditions and 7.3 under urea denaturing conditions. Immunoblotting analysis with antisera to rat liver GSTs revealed cross-reactivity with anti-(rat GST YfYf) serum but not with anti-(Ya subunit), anti-(Yc subunit), anti-(Yk subunit), anti-(Yb₁ subunit) or anti-(Yb₂ subunit) serum.

Fig. 1 shows the *N*-terminal sequence for the first 25 residues. The analysis revealed differences at positions 11, 12 and 13 when compared with the mouse liver GST YfYf (M-II) *N*-terminal sequence reported by Mannervik *et al.* (1985). Although this may be evidence of variants of the mouse Yf subunit, it is noteworthy that our *N*-terminal sequence shows identity with the rat, human and bovine sequences at positions 12 and 13 and with the rat and human sequences at position 11. We have also purified a form of GST of mobility identical with that of GST YfYf on SDS/PAGE, which cross-reacts with antiserum to mouse GST YfYf but which appears later in the elution gradient on hydroxyapatite chromatography. The relationship between this fraction and the major form of GST YfYf is unclear, and all kinetic work reported in the present study was conducted with the major form.

Initial-rate studies with GST YfYf

Double-reciprocal plots of the initial-rate pattern for murine GST-YfYf with GSH and CDNB as substrates were linear and converging, indicating a sequential mechanism. Whether CDNB or GSH was the substrate whose concentration was varied, the same intersection point was observed in the lower left quadrant of a double-reciprocal plot. In order to discriminate between an ordered mechanism and a rapid-equilibrium random mechanism, product-inhibition studies were carried out with DNPG. All product-inhibition patterns were linear. Fig. 2 shows the relationship between inhibition constants (the subscripts *i* and *ii* refer to slope and intercept constants respectively) and the fixed substrate concentration.

The product (DNPG) was found to be a competitive-type inhibitor with respect to GSH and the K_{is} varied hyperbolically with the concentration of CDNB. DNPG was found to be a mixed-type inhibitor with respect to CDNB and both the K_{is} and the K_{ii} increased linearly with the concentration of GSH.

These data are consistent with the rapid-equilibrium random mechanism shown in Scheme 1, where there is formation of an enzyme-GSH-DNPG complex but not of an enzyme-CDNB-DNPG complex. The discriminatory points between this

	1	10	20
Mouse	:	P P Y T I V Y F P V	R G R C E A M R M L L A D Q G
Mouse*	:	P P Y T I V Y F P V	V D G C E A M
Rat †	:	P P Y T I V Y F P V	R G R C E A T R M L L A D Q G
Human ‡	:	P P Y T V V Y F P V	R G R C A A L R M L L A D Q G
Bovine §	:	P P Y T I V Y F P V	Q G R C E

Fig. 1. *N*-Terminal amino acid sequences for mouse, rat, human and bovine Pi GSTs

The *N*-terminal amino acid sequences of mouse, rat, human and bovine Pi GSTs are aligned for comparison. Any differences that occur are outlined by boxes. Key to references: *Mannervik *et al.* (1985); †Sugioka *et al.* (1985); ‡Kano *et al.* (1987), Ålin *et al.* (1985) and Dao *et al.* (1984); §Schaffer *et al.* (1988).

Fig. 2. Product (DNPG) inhibition of mouse GST YfYf

(a) DNPG was found to be a competitive-type inhibitor with respect to GSH. The relationship between the K_{is} , with respect to GSH, and the concentration of CDNB is shown. The curve is a fit to eqn. (1). The asymptote is equivalent to βK_p and the intercept equivalent to K_p in eqn. (2). (b) DNPG was found to be a mixed-type inhibitor with respect to CDNB. The relationship between the K_{is} (●) and the K_{ii} (■), with respect to CDNB, and the concentration of GSH is shown. The intercepts for the K_{is} and K_{ii} plots are equivalent to βK_p and K_p respectively in eqn. (3). All points plotted are inhibition constants $\pm$ S.E.M. ($n = 5$) obtained from linear regression as described in the text.

Scheme 1. Proposed rapid-equilibrium random mechanism for mouse GST YfYf with CDNB and GSH as substrates

A refers to GSH, B to CDNB and P to DNPG. The steady-state rate equation (eqn. 4) is given in the text. The release of products is indicated as a single step since the order of release of DNPG and Cl^- cannot be determined from the available data.

mechanism and an ordered mechanism are: (i) there is a hyperbolic relationship between the K_{is} [with respect to GSH (A)] and the concentration of CDNB (B), whereas for an ordered mechanism the K_{is} would be insensitive to the concentration of B; (ii) there is a difference in the intercept values for the plots of the K_{is} and K_{ii} [with respect to CDNB (B)] against the concentration of GSH (A), whereas for an ordered mechanism these would be identical. The initial-rate equation for Scheme 1 is given below, in terms of A:

$$v_0 = \frac{V_{\max} A}{\alpha K_A \left(1 + \frac{K_B}{B} + \frac{K_B P}{K_p B} + \frac{P}{\beta K_p} \right) + A \left(1 + \frac{\alpha K_B}{B} \right)} \quad (2)$$

and in terms of B:

$$v_0 = \frac{V_{\max} B}{\alpha K_B \left(1 + \frac{K_A}{A} + \frac{K_A P}{K_p A} \right) + B \left(1 + \frac{\alpha K_A}{A} + \frac{\alpha K_A P}{\beta K_p A} \right)} \quad (3)$$

where A, B and P represent concentrations of GSH, CDNB and DNPG respectively. This shows that the difference found in the

value for K_{is} and K_{ii} , when extrapolated to zero GSH concentration, is explained by the factor β in the equation. The intercepts from the plots of K_{is} and K_{ii} (with respect to CDNB) against the concentration of GSH (Fig. 2b) are equal to K_p and βK_p respectively (see Scheme 1). This same factor operates in defining the hyperbola observed in the plot of K_{is} (with respect to GSH) against the concentration of CDNB (Fig. 2a). The hyperbola has an intercept equal to K_p and approaches the asymptote βK_p . Table 1 summarizes the values obtained from computer fits. It is significant that Ivanetich & Goold (1989) have described an identical mechanism for the human form of GST YfYf (GST π).

The observed quenching of the protein fluorescence upon addition of GSH to the enzyme indicated that GSH forms a complex with free enzyme. A plot of the change in fluorescence against the concentration of GSH was hyperbolic and gave $K_d = 0.19 \pm 0.02$ mM. Further evidence in support of a random mechanism could in theory come from binding experiments with CDNB and free enzyme. Binding experiments with CDNB are not straightforward, as this substrate is a fairly reactive electrophile, so that reversible binding has to be distinguished from covalent incorporation. Some evidence that CDNB exhibits saturable binding has been presented by Corrigan *et al.* (1989). On the assumption that the mechanism is rapid-equilibrium random, the initial-rate data were interpreted according to the equation:

$$v_0 = \frac{V_{\max} AB}{\alpha K_A K_B + \alpha K_B A + \alpha K_A B + AB} \quad (4)$$

where A and B represent concentrations of GSH and CDNB respectively. Table 1 summarizes the kinetic constants. These constants compare very closely with those reported by Ivanetich & Goold (1989) for human GST π . Although a full-length mouse sequence has not yet been reported, there is 85.6% identity between the rat and human sequences (Kano *et al.*, 1987), and it would seem therefore that all the major Yf forms of GST will probably exhibit a highly conserved kinetic mechanism.

An important point to notice is the value for $\alpha = 2.1$ (Table 1). The fact that the initial-rate patterns for GSH and CDNB intersect below the [substrate] axis also implies that $\alpha > 1$ and therefore that the binding of either substrate to free enzyme

Table 1. Summary of the initial-rate kinetic constants for mouse GST YfYf with GSH and CDNB as substrates

The parameters are evaluated from eqn. (4) where *A* and *B* refer to concentrations of GSH and CDNB respectively. Values given are means $\pm$ S.E.M. (*n* = 5).

K_A	0.086 ± 0.006 mM
K_B	0.68 ± 0.27 mM
α	2.1 ± 0.18
$V_{\max}$	0.13 ± 0.01 μ mol/min per μ g

hinders binding of the other substrate. Similarly the fact that $\beta > 1$ (Table 2) indicates that it is more difficult for DNPG to bind to the enzyme-CDNB complex than to the free enzyme. The data suggest some conformational change or steric hindrance. Principato *et al.* (1988) have shown that binding of *S*-methylglutathione to rat GST Yb₂Yb₂ (4-4) enables the enzyme to utilize 2-mercaptoethanol as the thiol substrate, indicating conformational changes at the active site. We have observed the same phenomenon for mouse GST YfYf (M. F. Phillips & T. J. Mantle, unpublished work), indicating that *S*-alkylated glutathione binding may induce conformational changes.

The initial-rate pattern for mouse GST YfYf with GSH and ethacrynic acid as substrates gives a value for $\alpha = 0.83 \pm 0.09$, indicating a marginal enhancement of binding of the second substrate (McCusker *et al.*, 1990). We have also carried out studies with ethacrynic acid as an inhibitor of the GST-YfYf-catalysed conjugation of GSH and CDNB. Ethacrynic acid was found to be a mixed-type inhibitor with respect to GSH. The inhibition with respect to CDNB was unusual in that it gave rise to an inhibition pattern that intersected to the right of the $1/v_0$ axis, in the upper quadrant, on a double-reciprocal plot. Fig. 3 shows, for example, the double-reciprocal pattern for CDNB in the presence of ethacrynic acid at 4 mM-GSH.

As the concentration of ethacrynic acid was increased, the computer fits to a rectangular hyperbola yielded negative values for both the K_m and the $V_{\max}$. If the data were plotted as simply v_0 versus the concentration of CDNB it was clear that at higher concentrations of ethacrynic acid and initial velocity-substrate concentration curve appeared to be linear. Where the fit to a rectangular hyperbola yielded positive values for the K_m and $V_{\max}$, the $K_m/V_{\max}$ ratio was taken as the reciprocal of the initial slope. Where a negative value was obtained for the K_m , the data (v_0 versus [CDNB]) were instead fitted to a straight line and the reciprocal of the slope was taken. Replots of these reciprocal slopes against the concentration of ethacrynic acid were found to be linear and a K_{is} value was taken from the intercept on the [ethacrynic acid] axis. A plot of K_{is} against the concentration of GSH was found to be hyperbolic (see Fig. 4).

The observed increase in the $K_m/V_{\max}$ ratio can be attributed

Table 2. Summary of the inhibition constants for product inhibition of mouse GST YfYf with DNPG

Values given are means $\pm$ S.E.M. (*n* = 5).

	From inhibition with respect to GSH	From inhibition with respect to CDNB
K_P	3.2 ± 0.4 μ M	3.2 ± 2.7 μ M
β	7.2 ± 4.2	3.4 ± 2
K_B	0.58 ± 0.23 mM	—
βK_P	—	11.0 ± 3.6 μ M

Fig. 3. Anomalous inhibition of mouse GST YfYf by ethacrynic acid

A double-reciprocal plot for inhibition by ethacrynic acid with respect to CDNB shows a linear pattern intersecting to the right of the $1/v_0$ axis. Each line represents a fixed concentration of ethacrynic acid indicated as follows: 0 μ M ($\bullet$), 6.25 μ M ($\circ$), 12.5 μ M ($\blacksquare$), 20 μ M ($\square$) and 35 μ M ($\blacktriangledown$). The concentration of GSH was fixed at 4 mM throughout. All points plotted represent initial velocities in duplicate.

to an increase in the K_m for CDNB in the presence of ethacrynic acid. However, the double-reciprocal plots also show that $1/V_{\max}$ decreases as the concentration of ethacrynic acid increases. It would seem therefore that ethacrynic acid is acting simultaneously as a competitive inhibitor and as an uncompetitive activator, giving the observed intersection of lines seen in Fig. 3.

In order to find a possible mechanism that would provide a reasonable model for the observed behaviour, we utilized the computer program EKPLLOT (M. F. Phillips, unpublished work), which allows a simulation of the behaviour of any equation with more than one independent variable and presents the user with *Y*-versus- x_1 or $1/Y$ -versus- $1/x_1$ plots for one variable as the second variable (x_2) is altered. Equations were constructed, first

Fig. 4. Relationship between the K_{is} for inhibition by ethacrynic acid and the concentration of GSH

The K_{is} was determined for inhibition by ethacrynic acid with respect to CDNB as described in the text. A hyperbolic relationship was observed between the K_{is} and the concentration of GSH. The curve drawn is a fit to eqn. (1). All points plotted are inhibition constants $\pm$ S.E.M. (*n* = 5) obtained from linear regression as described in the text.

Scheme 2. Proposed mechanism for the interaction of ethacrynic acid with mouse GST YfYf

A refers to GSH, B to CDNB and I to ethacrynic acid. The rate equation (eqn. 6) is given in the text. In this scheme ethacrynic acid can act as an inhibitor of the enzyme-catalysed conjugation of CDNB and GSH by competing with CDNB at the active site. However, ethacrynic acid can also bind to an allosteric site on the enzyme, thereby altering the $V_{\max}$ and possibly the affinity for CDNB and GSH. The altered enzyme (EI) is capable of binding both substrates and catalysing product formation.

in terms of a single substrate and inhibitor and then in terms of two substrates and inhibitor, with terms in the form of [ligand(s)]/dissociation constant(s). The equations were also constructed along the same general form as 'standard' initial-rate equations, i.e. a numerator containing one or more $V_{\max}$ terms and a denominator containing a series of equilibrium terms. The behaviour of these equations was simulated and the resulting initial-rate patterns were compared with the experimental data. The basic equation:

$$v_0 = \frac{N_1 \frac{AB}{c_1} + N_2 \frac{ABI}{c_2}}{1 + \frac{A}{c_3} + \frac{B}{c_4} + \frac{AB}{c_1} + \frac{I}{c_5} + \frac{I^2}{c_6} + \frac{AI}{c_7} + \frac{BI}{c_8} + \frac{ABI}{c_2} + \frac{AI^2}{c_9}} \quad (5)$$

where A , B and I are ligand concentrations, N_1 and N_2 are arbitrary rate constants and c_1 to c_9 are arbitrary dissociation constants, was the simplest equation constructed that produced a similar pattern to that observed experimentally. This equation was then examined in terms of a possible enzyme mechanism and the model shown in Scheme 2 was devised. The rapid-equilibrium equation was then derived for the model shown in Scheme 2 and is given below:

$$v_0 = \frac{V_{\max,1} \frac{AB}{\alpha K_A K_B} + V_{\max,2} \frac{IAB}{\beta K_1 K'_1 K'_2}}{1 + \frac{A}{K_A} + \frac{B}{K_B} + \frac{AB}{\alpha K_A K_B} + \frac{I}{K_1} + \frac{I^2}{K_1^2} + \frac{AI}{\gamma K_A K'_1} + \frac{IA}{K_1 K'_1} + \frac{IB}{K_1 K'_2} + \frac{IAB}{\beta K_1 K'_1 K'_2} + \frac{I^2}{K_1 K'_1} + \frac{AI^2}{\delta K_A K'_1 K'_2}} \quad (6)$$

where A , B and I represent concentrations of GSH, CDNB and ethacrynic acid respectively. This equation was then examined by using the computer program EKPLLOT and produced an initial-

Table 3. Values given to parameters in simulation of eqn. (6) with the computer program EKPLLOT

Parameter	Value used	Comments
A	0.05–4.0 mM	Variables
B	0.05–2.0 mM	
I	0.007–0.032 mM	
K_A	0.2 mM	Can be larger but not smaller than K_A , K_B
K_B	0.68 mM	
K'_A	0.04–1.0 mM	
K'_B	0.14–3.4 mM	Better at upper end of range (finally set at 0.06)
K_1	0.01–2.0 mM	
K'_1	0.001–0.005 mM	Better at lower end of range (finally set at 0.001)
K'_2	0.001–0.005 mM	Set equal to K'_1
α	2.1	Not varied
β	2.1	
γ	10	
δ	10	
$V_{\max,1}$	0.2 $\mu\text{mol/min per } \mu\text{g}$	$V_{\max,1}$ must be less than $V_{\max,2}$
$V_{\max,2}$	0.02–2.0 $\mu\text{mol/min per } \mu\text{g}$	Finally set at 0.5

rate pattern identical with that found experimentally. However, it was found that $V_{\max,2}$ must be greater than $V_{\max,1}$ and both the K'_1 and K'_2 must be less than the K_A .

There are two important terms in the equation. The first is the second numerator term, containing $V_{\max,2}$ and I , which is responsible for the observed increase in the apparent $V_{\max}$ in the presence of ethacrynic acid, provided that $V_{\max,2} > V_{\max,1}$. In Scheme 2 this is seen as an alternative (faster) flux for DNPG formation in the presence of ethacrynic acid. The second crucial feature is the I^2 terms in the denominator, which are responsible for the observed decrease in the apparent $V_{\max}/K_m$ ratio in the presence of ethacrynic acid, provided that the enzyme has a greater affinity for I than that for A . In Scheme 2 this is seen as the formation of enzyme-(ethacrynic acid)₂ complexes. It is also evident that the binding of ethacrynic acid to free enzyme governed by the equilibrium [ethacrynic acid]/ K_1 does not preclude the further binding of CDNB and GSH. It is therefore assumed that ethacrynic acid is binding at a site on the enzyme that is distinct from the active site. Since the binding of ethacrynic acid to all other enzyme forms excludes the binding of CDNB but not of GSH, it is assumed here that ethacrynic acid is competing with CDNB at the active site. This is expected, as ethacrynic acid is an alternative substrate to CDNB for YfYf.

The computer program EKPLLOT proved to be very useful for analysing a complex equation such as eqn. (6). It was possible to alter the values for parameters and see the effect graphically and in a form directly comparable with the observed experimental

results. Once the mechanism shown in Scheme 2 had been identified and eqn. (6) had been derived, values for parameters were constrained within realistic bounds. Table 3 gives the range

Fig. 5. Comparison between a plot of K_m/V_{max} ratio against the concentration of I (ethacrynic acid) from a simulation of eqn. (6) and from experimental observation

The output from EKPLLOT for K_m/V_{max} ratio against [I] for eqn. (6) is shown in (a) where parameters were given the following values: $A = 4$, $B = \text{variable } 0.05\text{--}2$, $I = \text{variable } 0\text{--}0.032$, $V_{max,1} = 0.2$, $V_{max,2} = 0.5$, $K_A = K'_A = 0.2$, $K_B = K'_B = 0.68$, $K_1 = 0.06$, $K'_1 = K''_1 = 0.001$, $\alpha = \beta = 2.1$ and $\gamma = \delta = 10$. The broken line is a linear regression. A plot of K_m/V_{max} ratio against the concentration of ethacrynic acid (inhibition with respect to CDNB) at 4 mM-GSH, as determined experimentally, is shown in (b) for comparison. The line drawn is a linear regression.

of values used and the restrictions on parameter values necessary to produce the experimentally observed pattern.

After simulation of the inhibition by ethacrynic acid with respect to CDNB, the slope replot was found to be hyperbolic. However, the curvature was not pronounced and, depending on the choice of parameter, values could easily be interpreted experimentally as a linear function (see Fig. 5). If a linear approximation was taken, it was found that the intercept on the [ethacrynic acid] axis (K_{is}) showed a hyperbolic relationship with GSH concentration, as was found experimentally (see Fig. 4). This hyperbola had an intercept equal to K'_1 and approached the asymptote $\delta K''_1$, i.e. as the concentration of GSH is increased the inhibition shifts from the enzyme-(ethacrynic acid)₂ complex to the enzyme-GSH-(ethacrynic acid)₂ complex, as one might expect. The simulation also indicated that the intercept replot was markedly hyperbolic. This was also seen experimentally. For some of the data, however, $1/V_{max} = 0$. This was due to the occurrence of apparently linear v_0 -versus-[CDNB] plots, presumably due to a shift in the K_m for CDNB well beyond the maximum concentration of CDNB used in the assay.

The simulation also predicted mixed-type inhibition by ethacrynic acid with respect to GSH, under the conditions presented in Table 3. Furthermore, this inhibition was abolished by increasing the concentration of CDNB. This was also observed experimentally. If the concentration of CDNB was increased further in the simulation it was found that there was a shift from inhibition to activation by ethacrynic acid with respect to GSH.

The fact that the enzyme can catalyse the conjugation of ethacrynic acid and GSH to form an alternative product need not be considered. The formation of this product is not being measured with the assay system used and therefore, in a rapid-equilibrium system, ethacrynic acid will behave as though it were a dead-end inhibitor (Segel, 1975). Interestingly, ethacrynic acid was found to be a potent inhibitor of the enzymic conjugation of CDNB and GSH by mouse GST YbYb but not an activator (M. F. Phillips & T. J. Mantle, unpublished work).

There is a similarity between the effects of ethacrynic acid on the enzymic conjugation of CDNB and GSH by GST YfYf and the effects of ADP on the oxidative deamination of glutamate by glutamate dehydrogenase with NAD⁺ as cofactor (Frieden, 1959). ADP acts as a competitive inhibitor at the nucleotide-binding site on the enzyme and as an activator, increasing V_{max} ,

by destabilizing an enzyme-NAD(P)H-glutamate abortive complex (Iwatsubo & Pantaloni, 1967). Furthermore, this effect is pH-dependent (Markau *et al.*, 1972). This results in a similar intersection of lines in the right upper quadrant of double-reciprocal plots to that observed for mouse GST YfYf.

There is good evidence for the presence of a binding site on GST YaYc for bilirubin (Vander Jagt *et al.*, 1982) and lithocholate (Strange *et al.*, 1977) at a site distinct from the catalytic centre. The presence of such a binding site supports the view that some GSTs have an important role in the intracellular transport of hydrophobic ligands. Hayes & Mantle (1986b) observed activation/inhibition effects for both chenodeoxycholate (on GST YcYc, Yb₁Yb₁ and Yb₂Yb₂) and lithocholate (on GST Yb₂Yb₂) that were pH-dependent. Caccuri *et al.* (1990) have identified two distinct binding sites for haemin on placental GST π , and Xia & Chen (1989) have reported the allosteric inhibition of GST π by bilirubin. Clearly there is at least one binding site on GST π , distinct from the catalytic centre, that may play a role in the regulation of the enzyme. It would seem therefore that allosteric regulation of GSTs might be a more common occurrence than has been thought up to now. Further work is necessary to determine the significance of the observed allosteric activation, but perhaps this activation allows the enzyme to act more efficiently in the presence of more than one electrophilic substrate, as must surely be the case *in vivo*. Mutual activation by alternative electrophilic substrates would help to alleviate or cancel out to some extent the inhibition resulting from competition at a single site.

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REFERENCES

- Ålin, P., Mannervik, B. & Jörnvall, H. (1985) FEBS Lett. **182**, 319–322
- Ålin, P., Mannervik, B. & Jörnvall, H. (1986) Eur. J. Biochem. **156**, 343–350
- Caccuri, A. M., Aceto, A., Piemonte, F., Di Ilio, C., Rosato, N. & Federici, G. (1990) Eur. J. Biochem. **189**, 493–497
- Clark, A. G., Dick, G. L. & Smith, J. N. (1984) Biochem. J. **217**, 51–58
- Clark, A. G., Dick, G. L. & Carrol, N. (1986) Biochem. J. **233**, 325–331
- Corrigall, A. V., Bhargava, M. M., Ivanetich, K. M., Ehlers, M. R. W. & Kirsch, R. E. (1989) Biochim. Biophys. Acta **991**, 399–404

- Danielson, U. H. & Mannervik, B. (1988) *Biochem. J.* **250**, 705–711
- Dao, D. D., Partridge, C. A., Kurosky, A. & Awasthi, Y. C. (1984) *Biochem. J.* **221**, 33–41
- Frieden, C. (1959) *J. Biol. Chem.* **234**, 815–820
- Habig, W. H., Pabst, M. J. & Jakoby, W. B. (1974) *J. Biol. Chem.* **249**, 7130–7139
- Hatayama, I., Satoh, K. & Sato, K. (1986) *Biophys. Biochem. Res. Commun.* **140**, 581–588
- Hayes, J. D. & Mantle, T. J. (1986a) *Biochem. J.* **233**, 779–788
- Hayes, J. D. & Mantle, T. J. (1986b) *Biochem. J.* **233**, 407–415
- Hayes, J. D., Coulthwaite, R. E., Stockman, P. K., Hussey, A. J., Mantle, T. J. & Wolf, C. R. (1987) *Arch. Toxicol. (suppl.)* **10**, 136–146
- Ivanetich, K. M. & Goold, R. D. (1989) *Biochim Biophys. Acta* **998**, 7–13
- Iwatsubo, M. & Pantaloni, D. (1967) *Bull. Soc. Chim. Biol.* **49**, 1563–1571
- Jakobson, I., Askelöf, P., Warholm, M. & Mannervik, B. (1977) *Eur. J. Biochem.* **77**, 253–262
- Jakoby, W. B., Ketterer, B. & Mannervik, B. (1984) *Biochem. Pharmacol.* **33**, 2539–2540
- Jensson, H., Eriksson, L. C. & Mannervik, B. (1985) *FEBS Lett.* **187**, 115–120
- Kano, T., Sakai, M. & Muramatsu, M. (1987) *Cancer Res.* **47**, 5626–5630
- Kitahara, A., Satoh, K., Nishimura, K., Ishikawa, T., Ruike, K., Sato, K., Tsuda, H. & Ito, N. (1984) *Cancer Res.* **44**, 2698–2703
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lai H.-C. J., Qian, B., Grove, G. & Tu, C.-P. D. (1988) *J. Biol. Chem.* **263**, 11389–11395
- Leatherbarrow, R. J. (1987) *ENZFITTER*, Elsevier Biosoft, Cambridge
- Mannervik, B. & Guthenberg, C. (1981) *Methods Enzymol.* **77**, 231–235
- Mannervik, B., Ålin, P., Guthenberg, C., Jensson, H., Tahir, M. K., Warholm, M. & Jörnvall, H. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 7202–7206
- Markau, K., Schneider, J. & Sund, H. (1972) *FEBS Lett.* **24**, 32–26
- Markwell, M., Hass, S. M., Burke, L. & Tolber, N. (1978) *Anal. Biochem.* **87**, 206–210
- McCusker, F. M., Phillips, M. F., Boyce, S. J. & Mantle, T. J. (1990) in *Glutathione S-Transferases and Drug Resistance* (Hayes, J. D., Pickett, C. B. & Mantle, T. J., eds.), pp. 419–429, Taylor and Francis, London, New York, and Philadelphia
- McLellan, L. I. & Hayes, J. D. (1987) *Biochem. J.* **245**, 399–406
- Okuda, A., Sakai, M. & Muramatsu, M. (1987) *J. Biol. Chem.* **262**, 7140–7150
- Pabst, M. J., Habig, W. H. & Jakoby, W. B. (1974) *J. Biol. Chem.* **249**, 7140–7150
- Principatio, G. B., Danielson, U. H. & Mannervik, B. (1988) *FEBS Lett.* **231**, 155–158
- Rothkopf, G. S., Telakowski-Hopkins, C. A., Stotish, R. L. & Pickett, C. B. (1986) *Biochemistry* **25**, 993–1002
- Sato, K. (1989) *Adv. Cancer Res.* **52**, 105–155
- Schaffer, J., Gallay, O. & Ladenstein, R. (1988) *J. Biol. Chem.* **263**, 17405–17411
- Schramm, V. L., McCluskey, R., Emig, F. A. & Litwack, G. (1984) *J. Biol. Chem.* **259**, 714–722
- Segel, I. H. (1975) *Enzyme Kinetics*, pp. 283–293, John Wiley and Sons, New York, London, Sydney and Toronto
- Strange, R. C., Cramb, R., Hayes, J. D. & Percy-Robb, I. W. (1977) *Biochem. J.* **165**, 425–429
- Sugioka, Y., Kano, T., Okuda, A., Sakai, M., Kitagawa, T. & Muramatsu, M. (1985) *Nucleic Acids Res.* **13**, 6049–6057
- Vander Jagt, D. L., Wilson, S. P., Dean, V. L. & Simons, P. C. (1982) *J. Biol. Chem.* **257**, 1997–2001
- Vince, R., Daluge, S. & Wadd, W. B. (1971) *J. Med. Chem.* **14**, 402–404
- Xia, C. L. & Chen, H. L. (1989) *Shengwu Huaxue Zazhi* **5**, 301–306

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