Purification and kinetic mechanism of the major glutathione *S*-transferase from bovine brain

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The major glutathione *S*-transferase isoenzyme from bovine brain was isolated and purified approx. 500-fold. The enzyme has a pI of 7.39 ± 0.02 and consists of two non-identical subunits having apparent M_r values of 22000 and 24000. The enzyme is uniformly distributed in brain, and kinetic data at pH 6.5 with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate suggest a random rapid-equilibrium mechanism. The kinetics of inhibition by product, by GSH analogues and by NADH are consistent with the suggested mechanism and require inhibitor binding to several different enzyme forms. Long-chain fatty acids are excellent inhibitors of the enzyme, and values of $\ln K_i$ for hexanoic acid, octanoic acid, decanoic acid and lauric acid form a linear series when plotted as a function of alkyl chain length. A free-energy change of -1900 J/mol (-455 cal/mol) per CH_2 unit is calculated for the contribution of hydrophobic binding energy to the inhibition constants. The turnover number of the purified enzyme dimer is approx. 3400/min. When compared with the second-order rate constant for the reaction between CDNB and GSH, the enzyme is providing a rate acceleration of about 1000-fold. The role of entropic contributions to this small rate acceleration is discussed.

INTRODUCTION

The glutathione *S*-transferases (GSTs; EC 2.5.1.18) represent a ubiquitous and diverse family of isoenzymes that have been suggested to participate in a wide variety of biotransformations, including xenobiotic detoxification, ligand binding and transport, and the synthesis and modification of prostaglandins, leukotrienes and steroids (Jakoby, 1978; Mannervik, 1985). Initially, the enzymes from rat liver received the most attention and these were originally described as combinations of four different subunits into six dimeric proteins (Bass *et al.*, 1977; Mannervik & Jensson, 1982). More recently, however, 11 major forms have been identified in rat liver (Sato *et al.*, 1985; Tu & Reddy, 1985), and evidence for extensive microheterogeneity within a single subunit has been presented (Wang *et al.*, 1986). The multigene family encoding for the GSTs has been widely studied (Pemble *et al.*, 1986), and the isoenzyme heterogeneity can be accounted for by multiple copies of the gene having diversity in regions associated with the 'second-substrate'-binding site (Rothkopf *et al.*, 1986; Telakowski-Hopkins *et al.*, 1986). Despite attempts to clarify nomenclature, the heterogeneity of GST isoenzymes among various tissues and the potential for electrophoretic anomalies displayed by some of the subunits (Hayes & Mantle, 1986) has left the nomenclature issue somewhat confused. Mannervik *et al.* (1985) have suggested a simplification of the nomenclature by establishing the categories 'acidic', 'near-neutral' and 'basic' (Alpha, Mu and Pi). Each of these groups shares common specificities, in addition to their pI, and it has been suggested that the three groups represent evolutionary ancestors. There are, however, newly reported isoenzymes that possibly do not adhere to the

three groupings (Awasthi & Singh, 1984; Singh & Awasthi, 1986).

In spite of the abundance of the proteins in virtually all cells, there is still no clear role for the enzymes in cellular metabolism. Xenobiotic protection is the most popular concept for a role *in vivo*, and it has been reported that overexpression of the Ya GST subunit in *Cos* cells leads to increased resistance to benzo[*a*]pyrene-*anti*-diol epoxide (Manoharan *et al.*, 1987). In other cases, however, increases in GST concentrations in tumour-cell lines do not correlate well with the resistance of the cells to particular alkylating agents (Evans *et al.*, 1987). The GST isoenzymes have been reported to catalyse the conjugation of GSH to epoxyeicosatrienoic acids (Spearman *et al.*, 1985), the transformation of prostaglandin H_2 into prostaglandin F_2 (Burgess *et al.*, 1987) and the conversion of leukotriene A_4 into leukotriene C_4 (Söderström *et al.*, 1985; Wu, 1986). This last-mentioned reaction, however, has been found to be catalysed more efficiently by a distinct leukotriene C_4 synthetase (Abe *et al.*, 1985; Yoshimoto *et al.*, 1985).

Intracellular binding of hydrophobic substrates by GST has also been widely studied, and the enzymes have been noted to bind haem derivatives (Senjo *et al.*, 1985), steroids and bile acids, leukotrienes (Sun *et al.*, 1986*a,b*) etc. As above with the leukotriene C_4 synthetase, some of these ligands have since been found to bind more tightly to specific carrier proteins (Takikawa *et al.*, 1986*b*), and in several cases ligands have been found to be displaced from the enzyme by physiological concentrations of GSH (Clark & Carrol, 1986; Takikawa *et al.*, 1986*a*).

In the course of other work we noted that the supernatant fraction from bovine brain contained a high GST activity. A cursory examination of the activity indicated that at pH 6.5 the enzyme obeyed simple

Abbreviations used: GST, glutathione *S*-transferase; CDNB, 1-chloro-2,4-dinitrobenzene.

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Michaelis–Menten kinetics at GSH concentrations up to $17 \times K_m$. This is an important point, since previous kinetic studies on the rat liver isoenzyme A (3–3; Yb₁Yb₂) had shown significant deviations from linearity (Pabst *et al.*, 1974; Askelöf *et al.*, 1975), limiting the utility of kinetic studies that might help elucidate the physiological role of the enzyme. In order to assess the utility of the bovine brain enzyme for kinetic and binding studies, we have isolated the major isoenzyme and here report the purification and kinetic characterization.

MATERIALS AND METHODS

Materials

All chemicals used were readily available commercial products and were used without further purification unless otherwise noted. GSH was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and was used as supplied. Stock solutions were prepared in glass-distilled water containing 2% (w/v) dithiothreitol; all solutions were stored at 4 °C under an atmosphere of N₂. Stock solutions of 1-chloro-2,4-dinitrobenzene (CDNB) were prepared in ethanol at 0.25 M; the ethanol concentration in enzyme assays was generally less than 1% and never exceeded 4%.

The product of the conjugation reaction, S-(2,4-dinitrophenyl)glutathione, was prepared by incubating CDNB with a slight excess of GSH in aq. 60% (v/v) methanol containing 0.01 M-potassium phosphate buffer, pH 7.2. The reaction was followed to completion by monitoring the absorbance change at 340 nm (about 6 h). The final concentration of product was determined from the absorption coefficient (Habig *et al.*, 1974) of $9.6 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ at 340 nm. A small amount of GSH remaining in the solution was quantified by reaction with DTNB and included when calculating GSH concentrations in inhibition experiments. The tripeptide γ -glutamylserylglycine (Chen *et al.*, 1985) was prepared by using the enzyme γ -glutamyltransferase (EC 2.3.2.2) from bovine kidney (Sigma Chemical Co.) and L- γ -glutamic acid *p*-nitroanilide. A mixture containing 500 μmol of Tris/HCl buffer, pH 9.0, 100 μmol of MgCl₂, 200 μmol of L- γ -glutamic acid *p*-nitroanilide, 400 μmol of serylglycine and 10 units of enzyme was incubated at 37 °C for 45 min with shaking. The reaction was terminated by the addition of 20 ml of 1.5 M-acetic acid and the mixture was freeze-dried. The solid was extracted with small volumes of water, and the product was separated by gel filtration on a Sephadex G-10 column (1.5 cm $\times$ 125 cm) with 0.05 M-Tris/HCl buffer, pH 8.4, as eluent and monitoring at 220 nm.

Determination of enzyme activity

Enzyme activity was determined by monitoring the change in absorbance at 340 nm with a Hitachi 100-60 spectrophotometer equipped with a thermostatically controlled cell compartment. The background reaction between GSH and CDNB was monitored for approx. 1 min before the addition of enzyme, and all the rates are corrected for the background reaction. Assays were carried out in 1 ml cuvettes with 0.1 M-potassium phosphate buffer, pH 6.5 at 25 °C. Initial rates were linear functions of enzyme concentration and of time for at least 2 min at a rate of 0.05 A_{340} unit/min or less. One unit of activity is defined as the formation of 1 μmol

of product/minute, corresponding to an absorbance change of 9.6 A_{340} units/min at 340 nm in 1 ml total volume in a 1 cm cell. Specific activity is defined as μmol of product formed/min per mg of protein, as measured by absorbance at 280 nm (by using 1.0 A_{280} unit/mg, in good agreement with protein concentration as measured by the biuret method). Average values for kinetic constants (common intercepts etc.) were obtained by standard non-linear least-squares analysis of the appropriate data by using the computer program EK-Analyst (Pewter Scientific, P.O. Box 388-838, Chicago, IL, U.S.A.); representative plots of these data (Fig. 1) are constructed by using these average values.

Isolation of the enzyme

Bovine brains were obtained at a local slaughterhouse approx. 30 min after death. Whole brain was dissected to be essentially free of grey matter and circulatory debris. The enriched white matter was cut into small pieces and either used fresh or rapidly frozen in liquid N₂ and stored at –25 °C. Frozen brains retained 80–90% of the activity as compared with fresh brain.

In a typical preparation, brain tissue was homogenized in a Waring blender for about 1 min in 0.05 M-Tris/HCl buffer, pH 7.5, containing 10 μM -dithiothreitol, 1 μM -EDTA and 2.5% (w/v) mannitol (buffer A); 3 ml of buffer was used per g of tissue. The crude homogenate was further treated with a Virtis homogenizer for about 3 min and then centrifuged at 13000 *g* for 15 min. The supernatant was clarified by centrifugation at 100000 *g* for 60 min. A calcium phosphate gel suspension of 25 mg/ml was added to the 100000 *g* supernatant at a ratio of 3 mg of gel/mg of protein. Concentrated potassium phosphate buffer (1.0 M) was added to give a final concentration of 0.01 M-potassium phosphate buffer, pH 6.5. The suspension was stirred for 5 min in an ice bath and then centrifuged at 13000 *g* for 15 min; the pellet was discarded. Finely divided (NH₄)₂SO₄ was slowly added (20 g/100 ml of enzyme solution) with stirring in an ice bath. After all the salt had dissolved, the suspension was centrifuged at 30000 *g* for 20 min; the pellet was discarded. Additional (NH₄)₂SO₄ (28 g/100 ml of enzyme solution) was added and the suspension was centrifuged again at 30000 *g* for 20 min. The pellet was dissolved in a minimum amount of 0.05 M-Tris/HCl buffer, pH 8.0, containing 10 μM -dithiothreitol, 1 μM -EDTA and 5% (w/v) mannitol (buffer B).

The (NH₄)₂SO₄-treated enzyme was applied to a Sephadex G-100 column (2.5 cm $\times$ 45 cm) and eluted with buffer B; fractions were assayed for protein concentration and activity. The fractions containing the highest specific activity were applied to a DEAE-cellulose column (6 cm $\times$ 12 cm) equilibrated in buffer B. The column was eluted with 250 ml of this buffer followed by 150 ml each of 0.05 M- and 0.1 M-potassium phosphate buffers containing dithiothreitol, EDTA and mannitol as described for buffer B. Two activity peaks were observed. The first, containing most of the activity, was adjusted to pH 7.2 with 1 M-potassium phosphate buffer, pH 6.5, and applied to the affinity column described below.

Affinity chromatography

With the use of the general procedure of Simons & Vander Jagt (1977), 2 g of epoxy-activated Sepharose 6B (Sigma Chemical Co.) was washed with glass-distilled water and 0.01 M-potassium phosphate buffer, pH 7.0,

and then degassed briefly with N_2 . A 0.1% solution of GSH (2 ml) was added and coupling was allowed to proceed for 20 h at 37 °C in a shaking incubator. The coupled gel was washed and remaining groups were blocked by the addition of 1.0 M-ethanolamine. After 4 h the gel was washed with (1 litre each) 0.5 M-KCl in 0.10 M-sodium acetate buffer, pH 4.0, 0.10 M-sodium borate buffer, pH 8.0, and finally with 0.10 M-potassium phosphate buffer, pH 8.0. The gel was poured in a glass column (0.5 cm $\times$ 10 cm). In general, up to 30 mg of DEAE-cellulose-purified protein could be applied to the column with little loss in separating efficiency. Protein was applied in 0.02 M-potassium phosphate buffer, pH 7.0, containing 2.5% (w/v) mannitol and washed with this buffer until no more protein was eluted. The enzyme was eluted with 0.05 M-Tris/HCl buffer, pH 8.0, containing 5 mM-GSH, 1 μ M-EDTA and 2.5% (w/v) mannitol. Collection tubes contained 0.05 ml of 1.0 M-potassium phosphate buffer, pH 6.5, containing 10% (w/v) mannitol; 1 ml fractions were collected. Active fractions were combined and dialysed against 0.01 M-potassium phosphate buffer, pH 7.0, containing 10 μ M-dithiothreitol, 1 μ M-EDTA and 5% (w/v) mannitol. All kinetic determinations were performed on this enzyme fraction.

Distribution of enzyme activity in brain

One whole fresh bovine brain was dissected into eight anatomical regions (Table 2). Each region was weighed and homogenized as described above. Enzyme activities were determined on the 100000 g supernatants; protein determinations were by the biuret method, with bovine serum albumin as standard. Pellets from 100000 g centrifugation were washed with buffer A and with buffer A containing 0.1 M-KCl. Enzyme activities were measured for these washings and for a suspension of the microsomal pellet, also in buffer A.

Table 1. Purification of GST from bovine brain

Data are for fresh brain; the purification from frozen brain is larger since the initial specific activity is less and the final specific activity is approximately the same. Values for total protein are based on absorbance at 280 nm, after dialysis. Specific-activity units are μ mol/min per mg of protein, determined in 1.0 ml volume with [GSH] = 0.20 mM and [CDNB] = 0.625 mM.

	Total protein (mg)	Specific activity (μ mol/min per mg)
100000 g supernatant	1755	0.060
Calcium phosphate gel	1209	0.085
(NH_4) ₂ SO ₄ (35–75% satn.) followed by Sephadex G-100	93	0.58
DEAE-cellulose		
Peak I	15	1.98
Peak II	30	0.20
GSH-Sepharose	2	14.60
6B affinity column		
Repeat GSH-Sepharose 6B affinity column	0.86	32.50

Physical characterization

Apparent M_r values were determined by gel filtration and by SDS/polyacrylamide-gel electrophoresis. A calibrated Sephadex G-100 column was prepared by the use of bovine serum albumin (M_r 66000), egg albumin (M_r 45000), trypsinogen (M_r 24000) and lysozyme (M_r 14300) as standards. Purified protein was applied to the column and eluted with 0.05 M-potassium phosphate buffer, pH 7.2, containing 5% (w/v) mannitol. The relative elution volume of the standard proteins was calculated and plotted against the logarithm of the M_r . Calibration proteins for SDS/polyacrylamide-gel electrophoresis included bovine serum albumin, egg albumin, pepsin (M_r 34700), trypsinogen, β -lactoglobulin (subunit M_r 18400) and lysozyme.

Preparative isoelectric focusing was done in an LKB-110 column with Ampholine carriers, pH 7–9. Dialysed enzyme was introduced into the column with the sucrose gradient by standard methods (Stenersen *et al.*, 1979). A 2% Ampholine concentration was used; the temperature was maintained at 4 °C. The column was run at an initial voltage of 580 V for 48 h. Fractions (1 ml) were eluted and the pH and activity were determined.

RESULTS AND DISCUSSION

Isolation of the enzyme

As shown in Table 1, GST was purified about 540-fold from calf brain supernatant, with an overall yield of 27%. Isoelectric focusing after the first GSH-Sepharose 6B affinity column resulted in the same purification; however, the yield was only 3%. The purification scheme is unremarkable and involves calcium phosphate-gel and (NH_4)₂SO₄ precipitations, chromatography over Sephadex G-100 and DEAE-cellulose and finally affinity chromatography on GSH immobilized through the sulphur atom to epoxy-activated Sepharose 6B, as described by Simons & Vander Jagt (1977). Elution from DEAE-cellulose gave two active fractions; the major peak, comprising 83% of the total activity, represents the major isoenzyme from the soluble fraction and was carried through the remaining purification steps. Affinity chromatography after partial purification, as described above, proceeded with higher recoveries than if the affinity steps were utilized early in the purification. The distribution of the enzyme in bovine brain appears to be quite uniform (Table 2), with a slightly higher activity in the corpus collasum and the thalamus and the lowest activity in the spinal cord. The fraction of the total GST activity that is microsomal or particulate-bound seems to be quite small in bovine brain (less than 0.5%), and the specific activities of the crude extracts of bovine brain appear to be about 10-fold higher than those previously reported for monkey brain (Asaoka *et al.*, 1977).

The enzyme is stable at 4 °C for up to 2 months in 0.1 M-potassium phosphate buffer, pH 7.0, containing 10 μ M-dithiothreitol, 1 μ M-EDTA and 5% (w/v) mannitol. The enzyme rapidly loses activity above pH 7.5, as it does if the dithiothreitol, EDTA and mannitol are omitted. High concentrations of sucrose and glycerol (25–30%, w/v) may be substituted for the 5% (w/v) mannitol. The sensitivity of the enzyme to high pH presents a problem in the affinity-chromatography step. According to the procedure of Simons & Vander Jagt (1977), elution of the enzyme from the

Table 2. Distribution of GST in bovine brain

Anatomical regions are as defined by Leach (1961). Specific-activity units are $\mu\text{mol}/\text{min}$ per mg, determined in 1 ml volume with $[\text{GSH}] = 0.20 \text{ mM}$ and $[\text{CDNB}] = 0.625 \text{ mM}$.

	Wet wt. (g)	Specific activity ($\mu\text{mol}/\text{min}$ per mg)
Cortex	114	0.061
Corpus callosum	62	0.072
Thalamus	4.7	0.070
Midbrain (including hypothalamic region)	19	0.065
Pons	0.8	0.062
Cerebellum	26	0.060
Medulla oblongata	8	0.060
Spinal cord	2.3	0.044
Activity in fractions of whole-brain homogenate ($\mu\text{mol}/\text{min}$)		
100 000 g supernatant	22.2	
Mitochondrial suspension	0.9	
Microsomal suspension	2.0	
Microsomal wash I	1.5	
Microsomal wash II	0.4	

column is best accomplished with 5 mM-GSH at pH 9.6. Unfortunately, the bovine brain enzyme rapidly loses activity under these conditions. This can be largely overcome by eluting at pH 8.0 and by layering a small amount of concentrated phosphate buffer, pH 6.5, containing mannitol, in each of the collection tubes to lower the pH of each fraction rapidly as it is collected. The large activity loss that occurs on isoelectric focusing is no doubt a result of the long incubation under conditions of non-ideal pH.

Physical properties

The M_r of GST from bovine brain was determined by gel filtration over a calibrated Sephadex G-100 column. On gel filtration the protein migrates as a single band very close to the position of egg albumin (M_r 45 000). On the basis of interpolation on linear plots of $\log M_r$ versus R_f , the apparent M_r for the enzyme is 48 000. When subjected to SDS/polyacrylamide-gel electrophoresis after incubation of the enzyme in 6 M-urea in the presence of 2% 2-mercaptoethanol for more than 12 h, the protein migrated as two sharp bands very close to one another. Interpolation on linear plots of $\log M_r$ versus R_f gives apparent M_r values of 22 000 and 24 000 for the two subunits. The protein is relatively resistant to incubation in SDS, and three bands appear on the gels (apparent M_r values 40 000, 24 000 and 22 000) if the incubation is carried out for 5 h or less. This tight association of the subunits is unusual for a GST heterodimer and has, thus far, made it impossible to separate the subunits for kinetic or physical characterization. The pI of the enzyme from bovine brain is 7.39 ± 0.22 , as determined by isoelectric focusing. Human

brain has been found to contain one cationic (pI 8.3) and two anionic (pI 5.5 and 4.6) forms of GST (Theodore *et al.*, 1985). The acidic isoenzyme has a subunit composition that is very similar to the bovine enzyme (M_r 22 500 and 24 500), but obviously differs markedly in pI. The higher-pI isoenzymes in human brain are homodimers of 26 500- M_r subunits, which are immunologically and catalytically distinct. Abramovitz & Listowsky (1987) have described the selective expression of a unique GST gene (termed Yb₃) that comprises the major GST subunit in rat brain. This gene is also observed as a minor component in liver and heart, and as a very minor component in spleen, lung and kidney, again suggesting that the brain GST enzymes may be slightly different from those observed in other tissues.

Kinetic characterization

Initial-velocity studies with either GSH or CDNB as the varied-concentration substrate at fixed concentrations of the other gave linear double-reciprocal plots with a common intersection point on the abscissa in both cases (for example, Fig. 1); intercept replots of these data were linear in both cases. Fig. 2 is a double-logarithmic plot of $v_0/V_{\max}$ versus $[\text{GSH}]$ and clearly demonstrates that this enzyme is not showing the deviations from Michaelis-Menten kinetics that are observed with the rat liver transferase A (3-3; Yb₁ Yb₁) when $[\text{GSH}]$ is varied (Pabst *et al.*, 1974; Askelöf *et al.*, 1975; Jakobson *et al.*,

Fig. 1. Double-reciprocal plots of $1/v_0$ versus $1/[\text{GSH}]$ for the reaction between CDNB and GSH catalysed by bovine brain GST at various fixed concentrations of CDNB.

The fixed concentrations of CDNB were: $\blacksquare$, 0.0625 mM; $\triangle$, 0.125 mM; $\circ$, 0.25 mM; $\bullet$, 0.5 mM; $\square$, 0.75 mM. Assay mixtures contained 0.25 μg of enzyme in 1.0 ml of 0.10 M-potassium phosphate buffer, pH 6.5, at 25 °C; rates have been corrected for background reaction. Inset: replot of ordinate intercept versus $1/[\text{CDNB}]$; $K_m = 0.41 \text{ mM}$.

Fig. 2. Effect of GSH concentration on the rate of the reaction between CDNB and GSH catalysed by bovine brain GST

The continuous line is drawn for the theoretical approach to $V_{\max}$ with a K_m of 0.06 mM. Assay mixtures contained 0.25 mM-CDNB and 0.25 μ g of enzyme in 1.0 ml of 0.10 M-potassium phosphate buffer, pH 6.5 at 25 °C; rates have been corrected for background reaction.

1977). In Fig. 2 a linear approach to $V_{\max}$ is observed for GSH concentrations 17-fold the apparent K_m at this CDNB concentration. Non-linear behaviour is observed, however, if this same experiment is performed at pH 7, suggesting that the non-linearity at higher pH is arising from some step in the reaction sequence that is pH-sensitive. It should also be noted that, at pH 6.5, there is no 'time-dependent' effect on enzyme activity such as that reported by Vander Jagt and co-workers for several GST enzymes (Vander Jagt *et al.*, 1982). The fact that this GST enzyme is kinetically simple at pH 6.5 means that the binding and inhibition properties can be investigated by using standard kinetic methods.

There are several possible kinetic models for an enzyme reaction that is bimolecular in both directions. The observation of a mixed-type slope-intercept pattern in double-reciprocal plots of each variable substrate at fixed concentrations of the other (Fig. 1) is inconsistent with the mechanism requiring ordered addition of substrates in which the binding is rapid equilibrium, and inconsistent with a double-displacement ('ping-pong') mechanism. The observation is consistent, however, with the ordered mechanism if the binding of the second substrate is a steady-state process, or if the entire binding scheme is random and rapid equilibrium. By both models, the fact that the common intersection point of the double-reciprocal plots in Fig. 1 (and the comparable plot, not shown, for varied CDNB concentrations) lies on the abscissa means that the subunits are catalytically equivalent and that the binding of the second substrate does not affect the binding of the first. The apparent Michaelis constant for the first substrate is equal to the

true dissociation constant only if the kinetic mechanism is random rapid equilibrium.

Assuming that Cl^- ion is not specifically bound by the enzyme and that it is therefore released first, the order of substrate binding in an ordered mechanism can be distinguished by product-inhibition studies (Segal, 1975). The product *S*-(2,4-dinitrophenyl)glutathione was prepared and found to be a competitive inhibitor with respect to CDNB and non-competitive with respect to GSH. For the ordered mechanism this requires that CDNB binds first, GSH second. For the random rapid-equilibrium mechanism the product would be expected to have the greatest affinity for the free enzyme and thus be competitive with respect to both substrates. In order to accommodate the observed non-competitive inhibition with respect to GSH in this mechanism, the product must also be able to bind to the E·GSH form of the enzyme. This is not entirely unlikely, as the dinitrophenyl sulphide would be quite hydrophobic and could compete with CDNB for a non-specific hydrophobic binding site. Consistent with this notion, slope replots of double-reciprocal plots for product inhibition show upward curvature at high concentrations of product, requiring a parallel mechanism in which product can bind twice to, presumably, the free enzyme.

The best distinction between the two possible pathways is provided by GSH analogues that would be expected to bind to both the free enzyme and to the E·CDNB complex. We have examined inhibition by γ -glutamylserine, γ -glutamylglutamate and γ -glutamylserylglycine (Chen *et al.*, 1985). For all of these the observed inhibition is competitive with respect to GSH and non-competitive with respect to CDNB. This is the dependence predicted by the random rapid-equilibrium mechanism; the ordered mechanism requires un-competitive inhibition with respect to CDNB.

The data are consistent with an enzyme having two non-identical but catalytically equivalent and non-co-operative subunits functioning by a random rapid-equilibrium mechanism in which the binding of one substrate does not affect the binding constant of the second (Scheme 1). It is unusual to find non-identical subunits that are catalytically equivalent, and the possibility must be considered that only one of the subunits in the present enzyme might be catalytically active. Non-co-operativity between GST subunits has been previously demonstrated with heterodimer combinations of rat liver isoenzymes (Danielson & Mannervik, 1985), in which each subunit has been found to function independently of the second, the observed rate and catalytic properties being simply additive.

The common abscissa intercept observed in Fig. 1 and in the comparable plot for varied CDNB concentrations give the dissociation constants for the binding of the substrates to the free enzyme. Mannervik and co-workers have suggested that the rat liver 3-3 isoenzyme exhibits non-ideal Michaelis-Menten kinetic behaviour resulting from a random steady-state mechanism (Askelöf *et al.*, 1975; Jakobson *et al.*, 1977). If this is correct, the non-linearity observed with the bovine enzyme at higher pH could simply reflect a change in the rate-limiting step from arylation at low pH to substrate binding or product release at higher pH values.

It is widely believed that GST enzymes function as detoxifiers (Jakoby, 1978; Mannervik, 1985), alkylating noxious compounds as a step in their excretion. Although

Scheme 1.

such a role may seem appropriate for liver enzymes, the central nervous system, in general, has very little reputation as a detoxifying organ. In order to gain a clearer understanding of the nature of the second-substrate-binding site we have examined inhibition by a wide variety of compounds (Table 3). The most powerful inhibition by a carboxylic acid in Table 3 is the C_{12} lauric acid. The inhibition occurs well below the critical micelle concentration, suggesting the inhibition is from acid monomer. The steady decrease in inhibition constant for other carboxylates as the chain length of the acid is increased (Table 3) suggests that an orderly binding in an appropriate site may be occurring. The incremental binding energy of each methylene unit at this site can be easily quantified by measuring the fraction of the initial rate that is inhibited at increasing concentrations of the fatty acid inhibitors. The slopes of these plots, $[(v_0 - v_i)/v_i]/[\text{fatty acid}]$, are directly proportional to the K_i for each fatty acid. The slope of the secondary plot of $\ln(\text{slope})$ versus chain length (Fig. 3) is equal to the quantity $\Delta G/RT$ (Young & Hou, 1979; Gitler & Ochoa-Solano, 1968) for the binding equilibrium. At 25 °C the free-energy change, ΔG° , is equal to 1900 J/mol (455 cal/mol) per CH_2 unit. This corresponds to the utilization of approx. 80% of the available hydrophobic binding energy (Gitler & Ochoa-Solano, 1968) and suggests an 'orderly' binding of methylene units is occurring, consistent with the existence of a fatty acyl-binding site.

The observation of a fatty acid-type-binding site is consistent with the numerous reports of GST enzymes binding leukotrienes (Sun *et al.*, 1986a), prostaglandins (Burgess *et al.*, 1987) and their precursors (Spearman *et al.*, 1985), although there are specific transport and

Table 3. Inhibition of GST from bovine brain

	K_i (mM)
Competitive with CDNB ($K_m^{\text{CDNB}} = 0.41 \text{ mM}$)*	
Lauric acid	0.2
Decanoic acid	0.9
Octanoic acid	4.4
Hexanoic acid	20
Benzoic acid	1.4
Styrene oxide†	4.2
Nitrobenzene	5.0
Competitive with GSH ($K_m^{\text{GSH}} = 0.06 \text{ mM}$)*	
γ -Glu-Ser-Gly	0.01
γ -Glu-Glu	0.8
Ser-Gly	8.0
NADH	0.05
NAD ⁺	2.8
NMN	8.1
UDP-glucose	0.75

* Turnover number per dimer was 3430/min.

† No activity towards styrene oxide as a substrate was detected (< 2% of CDNB rate).

receptor proteins for most of these compounds with higher affinities than are typically displayed by the GST enzymes (Clark & Carrol, 1986; Takikawa *et al.*, 1986a,b). Nonetheless, binding of leukotriene C_4 by cytoplasmic GST enzymes *in vivo* is sufficiently strong that it represents a substantial portion of the total leukotriene C_4 -binding sites in the cell (Sun *et al.*, 1986b).

Fig. 3. Plot depicting the dependence on number of methylene units of the inhibition constant for carboxylate inhibition of the reaction between CDNB and GSH catalysed by bovine brain GST

Kinetic terms: v_0 and v_1 , initial velocity in the absence and in the presence of carboxylates respectively. Assay mixtures contained 0.25 mM-CDNB and 0.25 μ g of enzyme in 1.0 ml of 0.10 M-potassium phosphate buffer, pH 6.5, at 25 °C; rates have been corrected for background reaction. The slope gives a value of $\Delta G^0 = -1900$ J/mol per CH₂ unit.

Danielson *et al.* (1987) have described the kinetics of the detoxification of a series of 4-hydroxyalkenals, ranging from C₅ to C₁₅ in chain length, by several GST isoenzymes. The presence of fatty acid-type-binding sites such as these that accept methylene units in an orderly and systematic fashion could lend further credence to the suggestion that the GST enzymes may function to protect against lipid-peroxidation products (Ålin *et al.*, 1985; Scott & Kirsch, 1987).

The reaction of GSH with CDNB is a bimolecular reaction that can be monitored in solution under exactly the same conditions as for the enzyme-catalysed reaction. The second-order rate constant for this reaction is 3.7 M⁻¹·min⁻¹ at pH 6.5 and 25 °C. The turnover number per dimer molecule for GST from bovine brain is about 3400/min under the same conditions, giving a rate acceleration for the enzyme-catalysed reaction of approx. 1000-fold; this is only slightly larger than values reported for other GST enzymes (Douglas, 1987). Page & Jencks (1971) and Jencks (1975) have estimated that, simply from entropic considerations, an enzyme can accelerate a bimolecular reaction by up to a factor of 10⁸-fold; the 1000-fold acceleration in the present case pales by comparison. In order to achieve this large acceleration of about 10⁸-fold, it is necessary to lose completely translational, rotational and internal entropies for both reactants. In brain GST there is little doubt that GSH is one of the natural substrates, and hence a 'perfect' enzyme (Knowles & Alberly, 1977) would be expected to bind GSH so that all translational, rotational and internal entropies were minimized. There is also little doubt that CDNB is bound by the enzyme and that it reacts; hence the translational and internal entropies must also be lost

for this substrate. However, since CDNB is probably unrelated to the 'natural' substrate for the brain enzyme, it is unlikely that the binding site is complementary enough to the structure to allow a complete loss of rotational entropy. If the substrate is completely rotationally disordered, then none of the 84 kJ·mol⁻¹·K⁻¹ entropy units available from rotational-entropy loss is available for rate acceleration. Since the factor of 10⁸-fold represents about -145 kJ·mol⁻¹·K⁻¹, a change of +84 kJ·mol⁻¹·K⁻¹ will leave only about -60 kJ·mol⁻¹·K⁻¹ available for rate acceleration. At 25 °C this amounts to a factor of just under 2000-fold. The agreement between the predicted value based on rotational entropy loss by only one reactant and the observed value of 1000-fold suggests that the enzyme may be doing little chemically to accelerate the reaction and that the GST-catalysed arylation of GSH by CDNB may be an example of an enzyme-catalysed reaction that is totally entropy-driven.

This work was supported by Grant NS-17094 from the National Institutes of Health and by Research Career Development Award NS-00775 (to P. R. Y.).

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Received 18 January 1988/29 July 1988; accepted 8 August 1988