Steady-state kinetics and chemical mechanism of octopus hepatopancreatic glutathione transferase

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The kinetic mechanism of glutathione *S*-transferase (GST) from *Octopus vulgaris* hepatopancreas was investigated by steady-state analysis. Initial-velocity studies showed an intersecting pattern, which suggests a sequential kinetic mechanism for the enzyme. Product-inhibition patterns by chloride and the conjugate product were all non-competitive with respect to glutathione or 1-chloro-2,4-dinitrobenzene (CDNB), which indicates that the octopus digestive gland GST conforms to a steady-state sequential random Bi Bi kinetic mechanism. Dead-end inhibition patterns indicate that ethacrynic acid {[2,3-dichloro-4-(2-methylenebutyl) phenoxy]acetic acid} binds at the hydrophobic H-site, norophthalmic acid (γ -glutamylalanylglycine) binds at the glutathione G-site, and glutathione-ethacrylate conjugate occupied both H- and G-sites of the enzyme. The chemical mechanism of the enzyme was examined by pH and kinetic

solvent-isotope effects. At pH (and p^2H) = 8.011, in which k_{cat} was independent of pH or p^2H , the solvent isotope effects on V and V/K_{mGSH} were near unity, in the range 1.069–1.175. An inverse isotope effect was observed for V/K_{mCDNB} (0.597), presumably resulting from the hydrogen-bonding of enzyme-bound glutathione, which has pK_a of 6.83 ± 0.04 , a value lower by 2.34 pH units than the pK_a of glutathione in aqueous solution. This lowering of the pK_a value for the sulphhydryl group of the bound glutathione was presumably due to interaction with the active site Tyr⁷, which had a pK_a value of 8.46 ± 0.09 that was raised to 9.63 ± 0.08 in the presence of glutathione thiolate. Subsequent chemical reaction involves attacking of thiolate anion at the electrophilic substrate with the formation of a negatively charged Meisenheimer complex, which is the rate-limiting step of the reaction.

INTRODUCTION

Glutathione *S*-transferase (GST; EC 2.5.1.18) constitutes one of the major detoxification systems in cells [1–4]. A representative GST-catalysed nucleophilic aromatic substitution between GSH and 1-chloro-2,4-dinitrobenzene (CDNB) is shown in Scheme 1. On the basis of amino acid sequence similarities, substrate specificities and immuno-cross-reactivity with human GST, the cytosolic GST isoenzyme supergene family is grouped into four gene classes: GST-Alpha, GST-Mu, GST-Pi and GST-Theta. The kinetic mechanism of the GST-catalysed conjugation reaction is quite complex and isoenzyme-dependent [5–9]. While Pi-type GST conformed to a rapid equilibrium random Bi Bi kinetic mechanism [8–10], a steady-state random Bi Bi mechanism was proposed for Mu-type GST to account for the non-Michaelian substrate rate curves, which produced a biphasic double reciprocal plot [6,9]. The biphasic nature of the double reciprocal plot of initial-velocity data observed for Alpha-type GST was less pronounced [7,9]. Detailed studies on kinetic and chemical mechanisms have been focused mostly on Mu-type GST. However, since the kinetic mechanism of GSTs may provide a further criterion to distinguish various GST isoenzyme classes, it is of paramount importance to characterize the detailed kinetic mechanism of a newly isolated GST.

We have purified the octopus digestive gland GST to apparent homogeneity and have performed preliminary characterization of the enzyme [11]. In the present paper, we describe the kinetic mechanism of the octopus GST studied by steady-state techniques. From the initial-velocity and inhibition studies we deduced a steady-state random Bi Bi kinetic mechanism for the enzyme. The less pronounced biphasic nature of the double

reciprocal plots suggests that the octopus GST is kinetically similar to an Alpha-type isoenzyme. Kinetic solvent isotope and pH effects were also studied to investigate the possible chemical mechanism of the enzyme.

MATERIALS AND METHODS

Materials

CDNB, ethacrynic acid {EA; [2,3-dichloro-4-(2-methylenebutyl) phenoxy]acetic acid}, GSH and 2H_2O (99.9 atom % 2H) were purchased from Sigma (St. Louis, MO, U.S.A.). Norophthalmic acid (GH; γ -glutamylalanylglycine) was a gift from Senju Pharmaceutical Co. (Itami, Japan). *S*-(2,4-dinitrophenyl)-glutathione [S-(DNP)GS] was synthesized non-enzymically from GSH and CDNB [12]. Other chemicals were as described [11].

Conjugation of GSH and EA was performed according to Awasthi et al. [13] with modifications. GSH (50 mg in 0.1 M sodium phosphate buffer, pH 7.15) and EA (50 mg in ethanol) were mixed in a 1:1 ratio (v/v) at room temperature (27 °C) for 2 h, which is enough to complete the reaction. Purification of *S*-(ethacrylate)glutathione [S-(EA)GS] was performed by passing the reaction mixture through a HPLC column (Waters μ Bondapak C₁₈) and isocratically eluting with 40 % methanol at a flow rate of 1 ml/min. The peak that was positive with both ninhydrin (for GSH) and A_{270} (for EA) was collected. Re-injecting the purified S-(EA)GS into the same column produced a single symmetric sharp peak eluted at 3.758 min with purity > 99 % as judged from the integration of peak areas. A molar absorption coefficient of $5.0 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 270 nm [14] for S-(EA)GS was used in the calculation.

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; EA, ethacrynic acid {[2,3-dichloro-4-(2-methylenebutyl) phenoxy]acetic acid}; GH, norophthalmic acid (γ -glutamylalanylglycine); GST, glutathione *S*-transferase; S-(DNP)GS, *S*-(2,4-dinitrophenyl)glutathione; S-(EA)GS, *S*-(ethacrylate)glutathione; 2H V, kinetic solvent isotope effect on V , i.e. $V_{HOH}/V_{H_2O^2H}$; 2H V/K, kinetic solvent isotope effect on V/K , i.e. $(V/K)_{HOH}/(V/K)_{H_2O^2H}$.
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Scheme 1 GST-catalysed nucleophilic aromatic substitution of GSH and CDNB

Purification and assay of octopus GST

Octopus digestive gland GST was purified by a single GSH-Sepharose 4B affinity chromatography as described [11]. The dimeric enzyme was judged to be apparently homogeneous by SDS/PAGE with a subunit M_r of 24000. Protein concentration was determined by the protein-dye binding method [15].

The GST activity was assayed at 25 °C with a Perkin-Elmer Lambda 3B spectrophotometer with appropriate correction for the minor non-enzymic conjugation as described by Chang et al. [12]. In a standard assay, the reaction mixture (1 ml) contained $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer (100 mM, pH 6.5), GSH (1 mM), CDNB (1 mM) (added as dimethyl sulphoxide solution) and an appropriate amount of the enzyme (23 ng/assay). The formation of S-(DNP)GS conjugate was continuously monitored at 340 nm. A molar absorption coefficient of $9.6 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [14] for the conjugate was used in calculations. Under the standard assay conditions, the relationship between the enzymic reaction rate and the amount of GST used in the assay was linear up to 3 μg of GST.

Initial-velocity studies

Initial-velocity studies were performed by varying the concentration of GSH from 0.032 to 1.0 mM and of CDNB from 0.1 to 1.0 mM. Concentrations of other components were held constant.

The data were fitted to eqns. (1) and (2) respectively for a rapid-equilibrium random Bi Bi or a steady-state random Bi Bi kinetic mechanism, using the non-linear least square program DNRP-53 of Duggleby [16].

$$v = \frac{V_m [\text{GSH}][\text{CDNB}]}{K_{s\text{GSH}}K_{m\text{CDNB}} + K_{m\text{GSH}}[\text{CDNB}] + K_{m\text{CDNB}}[\text{GSH}] + [\text{GSH}][\text{CDNB}]} \quad (1)$$

$$v = \frac{V_1[\text{GSH}][\text{CDNB}] + V_2[\text{GSH}]^2[\text{CDNB}] + V_3[\text{GSH}][\text{CDNB}]^2}{K_1 + K_2[\text{GSH}] + K_3[\text{CDNB}] + [\text{GSH}][\text{CDNB}] + K_4[\text{GSH}]^2 + K_5[\text{CDNB}]^2 + K_6[\text{GSH}]^2[\text{CDNB}] + K_7[\text{GSH}][\text{CDNB}]^2} \quad (2)$$

in which v is the observed initial reaction rate; V_m is the maximum reaction rate; $K_{s\text{GSH}}$, $K_{m\text{GSH}}$ and $K_{m\text{CDNB}}$ are defined in Table 1. V_i ($i = 1-3$) and K_j ($j = 1-7$) are the constants involving the various rate constants shown in Scheme 2.

Inhibition studies

Inhibition studies were performed in a similar manner as described for initial-velocity studies with S-(DNP)GS or NaCl as product inhibitor, and EA, S-(EA)GS or GH as dead-end inhibitor. The inhibitor was held at several fixed concentrations close to its inhibition constant (K_i).

The data were fitted to eqn. (3) and (4) respectively for competitive or non-competitive inhibition.

$$v = \frac{V_m}{1 + (K_m/[S])(1 + [I]/K_i)} \quad (3)$$

$$v = \frac{V_m}{(1 + K_m/[S])(1 + [I]/K_i)} \quad (4)$$

Fitting of experimental data to the respective equation was carried out by EZ-FIT [17], a curve-fitting microcomputer program using the Melder-Mead Simplex and Marquardt non-linear regression algorithms sequentially.

The Gibbs free energy (ΔG°) of binding for the inhibitor was estimated from the inhibition constant according to eqn. (5).

$$\Delta G^\circ = -RT \ln K_i \quad (5)$$

in which R is the gas constant and T is the absolute temperature in Kelvin.

pH studies

Assuming that the K_m values do not vary substantially with pH, we assayed the enzyme activity at various pH values with high and low substrate concentrations. At high substrate concentration, the GSH and CDNB concentrations were fixed at 1.2 and 1.5 mM respectively, which was approx. 10 times the $K_{m\text{GSH}}$ or $K_{m\text{CDNB}}$. When the enzyme was assayed at low substrate concentration, the GSH and CDNB concentrations used were

both 40 μM . The buffer used was Bistris propane {1,3-bis[tris(hydroxymethyl)-methylamino] propane} throughout the whole pH range. The actual pH values were measured before initiation of each assay.

The data were analysed by a generally applicable non-linear regression program, SigmaPlot (Jandel Scientific, San Rafael, CA, U.S.A.). pH profiles were fitted to eqn. (6) to estimate K_1 and K_2 values.

$$\text{Log } Y = \log \{C/(1 + [\text{H}^+]/K_1 + K_2/[\text{H}^+])\} \quad (6)$$

in which Y is the value of the parameter of interest measured at

Values shown are averages $\pm$ S.E.M. ($n = 66$). The enzyme amount used was 23 ng/assay.

Reaction in the grey area is the conjugation of GSH and CDNB catalysed by GST, while outside this area shows the reactions of GSH with alternative substrate EA or binding of inhibitors GH, S-(EA)GS or S-(DNP)GS with the enzyme. Non-covalent binding is represented by a dot and covalent bond is represented by a vertical line. Since no mixed-type non-competitive inhibition pattern was observed in this study, $\beta = 1$ in this particular case.

Figure 1 Initial-velocity pattern for octopus hepatopancreatic GST

The varied-concentration substrate was GSH and the fixed-concentration substrate was CDNB. The concentration of CDNB was (○) 0.1, (●) 0.15, (□) 0.2, (■) 0.3, (△) 0.5, and (▲) 1.0 mM respectively. The amount of GST used was 23 ng/assay. Lines are computer fitted to eqn. (1). Computer fitting to eqn. (2) results in large standard errors for this parameter (not shown).

Table 2 Inhibition patterns for various inhibitors of the octopus hepatopancreas GST

Values shown are averages $\pm$ S.E.M. ($n = 15$). The enzyme amount used was 23 ng/assay. Abbreviations: NC, non-competitive; C, competitive.

Varied-concentration substrate	Fixed-concentration substrate	Inhibitor	Inhibition pattern	Inhibition constant
Product inhibition				
GSH	CDNB	S-(DNP)GS	NC	$39.6 \pm 2.42 \mu\text{M}$
CDNB	GSH	S-(DNP)GS	NC	$53.7 \pm 3.93 \mu\text{M}$
GSH	CDNB	Cl^-	NC	$357 \pm 3.5 \text{ mM}$
CDNB	GSH	Cl^-	NC	$348 \pm 2.8 \text{ mM}$
Alternative-substrate inhibition				
GSH	CDNB	EA	NC	$2.59 \pm 0.26 \mu\text{M}$
CDNB	GSH	EA	C	$0.94 \pm 0.14 \mu\text{M}$
Alternative-product inhibition				
GSH	CDNB	S-(EA)GS	C	$9.70 \pm 0.62 \text{ nM}$
CDNB	GSH	S-(EA)GS	C	$12.99 \pm 0.47 \text{ nM}$
Dead-end inhibition				
GSH	CDNB	GH	C	$2.08 \pm 0.26 \text{ mM}$
CDNB	GSH	GH	NC	$2.96 \pm 0.15 \text{ mM}$

concentration substrate, non-competitive inhibition patterns were obtained. With NaCl as the inhibitor and GSH or CDNB as the varied-concentration substrate, non-competitive inhibition patterns were obtained again. The inhibition constants obtained by fitting the data to eqn. (4) are listed in Table 2.

Dead-end inhibition studies

Further kinetic data for the kinetic mechanism of octopus GST came from dead-end inhibition studies. EA, an alternative substrate for octopus GST, gave a non-competitive inhibition pattern versus GSH but a competitive inhibition pattern versus CDNB. The conjugate of EA and GSH, S-(EA)GS, an alternative

Figure 2 pH- and $p^2\text{H}$ -effects of octopus hepatopancreatic GST catalysed reaction

Log (V/S) versus pH (○) or $p^2\text{H}$ (●) plots for CDNB or GSH are shown in (a) and (b) respectively. Curves are computer fitted to eqn. (6). The substrates concentrations used were $[\text{GSH}] = 2 \text{ mM}$, $[\text{CDNB}] = 10 \mu\text{M}$ in (a); and $[\text{GSH}] = 10 \mu\text{M}$, $[\text{CDNB}] = 2 \text{ mM}$ in (b).

product, on the other hand, gave competitive inhibition patterns versus both GSH and CDNB with very small K_i values ($\sim 10 \text{ nM}$). GH, a desthio analogue of GSH, gave a competitive inhibition pattern versus GSH, but non-competitive inhibition versus CDNB. The inhibition constants for the above dead-end inhibition studies are summarized in Table 2.

The sulphhydryl group of GSH is the group to be conjugated with electrophilic substrates. The binding energies calculated from K_{sGSH} and K_{iGH} were 36.9 and 32.4 kJ/mol respectively. The contribution of the SH-group in the GSH binding is approx. 4.5 kJ/mol (1.08 kcal/mol), which is close to the value determined for GST 3-3 (7.11 kJ/mol) or GST 4-4 (6.28 kJ/mol) [21].

pH studies

Purified octopus digestive gland GST is stable between pH 5.0 and 10.0 for at least 20 min under the assay conditions used. Detailed pH effects on the enzyme activity were studied in this pH range. Since the non-enzymic conjugation between GSH and CDNB was extremely rapid at $\text{pH} > 8$ when both substrates were saturated, only one substrate was kept at saturation in the pH studies.

From the plot of $\log (V/K_{\text{mCDNB}})$ versus pH, two molecular pK_a values of 6.83 ± 0.04 and 9.63 ± 0.08 were suggested in the E·GSH binary complex (Figure 2a). The former has to be deprotonated and the latter protonated to give the optimum reaction rate. Similarly, molecular pK_a values of 7.48 ± 0.09 and 8.46 ± 0.09 respectively were detected in the E·CDNB binary complex (Figure 2b).

Table 3 Kinetic parameters and solvent isotope effects for the octopus hepatopancreatic GST catalysed conjugationValues shown are averages $\pm$ S.E.M. ($n = 16$). The enzyme amount used was 115 ng/assay.

Molecular forms	Solvent	$k_{\text{cat}}/K_{\text{m}}$ ($\text{mM}^{-1} \cdot \text{s}^{-1}$)	$\text{p}K_1$	$\text{p}K_2$
E · GSH	H ₂ O	24.88 ± 1.20	6.83 ± 0.04	9.63 ± 0.08
	² H ₂ O	27.33 ± 1.52	6.90 ± 0.05	10.65 ± 0.02
	² H/ $V/K_{\text{mCDNB}} = 0.597 \pm 0.030$		$^{2}\text{H}V = 1.069 \pm 0.034$	
E · CDNB	H ₂ O	95.63 ± 13.52	7.48 ± 0.09	8.46 ± 0.09
	² H ₂ O	61.75 ± 7.46	7.33 ± 0.09	9.42 ± 0.13
	² H/ $V/K_{\text{mGSH}} = 1.175 \pm 0.057$		$^{2}\text{H}V = 1.140 \pm 0.068$

Figure 3 Kinetic solvent isotope effects on the octopus hepatopancreatic GST catalysed reaction

Conjugation of GSH and CDNB was assayed in H₂O or ²H₂O media at pH (or p²H) 8.011 at various substrate concentrations, GSH in (a) and CDNB in (b). Solvent kinetic isotope effects were expressed as function of fractional substrate saturation. Lines are computer fitted to eqn. (8).

Solvent kinetic isotope effects

In ²H₂O medium, $\text{p}K_1$ for both E · GSH and E · CDNB complexes were, within experimental errors, similar to those in H₂O. Both $\text{p}K_2$ values however, increased by about one pH unit (Table 3).

Kinetic solvent isotope effects are shown as a function of fractional substrate saturation of the enzyme in Figure 3. The solvent isotope effects on various kinetic parameters are summarized in Table 3. Inverse isotope effect (0.597 ± 0.03) was found for $^{2}\text{H}(V/K_{\text{mCDNB}})$. Isotope effects for other kinetic parameters were small and near unity (1.069 – 1.175).

DISCUSSION

Kinetic mechanism of octopus GST

We have fitted our initial-velocity data to two kinetic models. Although it is not difficult to fit data to the eight parameter, steady-state random Bi Bi model, our data did not seem to require this complexity. The data can be equally well explained by a much simpler four parameter rapid-equilibrium random Bi Bi model. Fitting the initial-velocity data to eqn. (1) assumes that $K_s = K_m$, thus, K_{mGSH} , K_{mCDNB} and K_{sGSH} in Table 1 are all simply dissociation constants and K_{sCDNB} was obtained from the relationship of $K_{\text{mGSH}} \cdot K_{\text{sCDNB}} = K_{\text{sGSH}} \cdot K_{\text{mCDNB}}$. However, the fact that the data fit to a simplified equation (eqn. 1) does not prove that the mechanism is a rapid-equilibrium one. Product inhibition patterns conclusively indicate that the octopus GST conforms to a steady-state random Bi Bi kinetic mechanism instead of a rapid equilibrium one [20]. Furthermore, solvent isotope effects on V/K also indicate that the binding of GSH cannot be completely random. In our case, the non-competitive inhibition nature of S-(DNP)GS is probably because the conjugate contains the majority of both substrates and therefore this product very likely binds to both the GSH G-site and CDNB H-site. The inhibition of octopus GST by Cl^- also suggests the formation of an E · GSH · Cl^- dead-end complex, although the possibility that the high concentration of NaCl produced an ionic-strength effect on the kinetics was not completely ruled out. The possible reason that our initial-velocity data do not fit a steady-state random Bi Bi kinetic model might be due to an artifact of the fitting routine; presumably the key point is that the data for initial velocities are not good enough to distinguish the two models. Since we do not have enough data to sufficiently constrain all of these constants, fitting ratios of constants might be a better way. However, it should be pointed out that a steady-state random Bi Bi mechanism leads to non-linearity in the reciprocal plots. Since the curvature of our reciprocal plots is small, we always had difficulty in fitting a steady-state mechanism.

Dominey et al. [22] have isolated a Pi-class GST from salmonid livers that appears to be similar to those from *O. vulgaris*. Among five compounds (with GST-type specificity in parenthesis), i.e. CDNB (Alpha/Mu/Pi), EA (Pi), bromosulphophthalein (Mu), *trans*-4-phenyl-3-buten-2-one (Mu) and cumene hydroperoxide (Alpha), only CDNB and EA showed activity with octopus GST [11]. This seems to suggest that the octopus GST is a Pi-type isoenzyme. However, the initial-velocity pattern reveals that the enzyme is kinetically similar to an Alpha-type GST, which also produces small curvature of the $1/[S]$ versus $1/v$ double reciprocal plots. Inhibition patterns of EA (non-competitive with respect to GSH and competitive with respect to CDNB) also suggest octopus GST to be an Alpha-type isoenzyme [23]; Pi-type GST was reported to give a non-competitive inhibition pattern for both substrates [13]. Furthermore, amino acid sequences deduced from cDNA of octopus S-crystallin, a lens structural protein with an amino acid sequence very similar to the octopus digestive gland GST, corroborate that the octopus S-crystallin (and maybe hepatopancreatic GST as well) is an Alpha-type GST [23a].

Inhibition studies can be very informative. As a rule, when an inhibitor occupies the same portion of an active site as the variable substrate, the inhibitor is competitive. When an inhibitor combines with a different portion of the same active site as the variable substrate, the inhibition is non-competitive [24]. EA, a diuretic drug, reacts with the sulphhydryl group of GSH through a Michael addition reaction with the β -carbon of the α,β -unsaturated ketone moiety of EA [13]. The competitive-inhibition patterns observed for S-(EA)GS with respect to both substrates are in agreement with the results of Awasthi et al. [13] who

suggested a separate active site for GST-catalysed conjugation of GSH to the α,β -unsaturated ketone of EA than that required for CDNB. An allosteric site for EA has also been proposed by Phillips and Mantle [10]. However, we prefer to think of the H-site of GST as a rather flexible domain. This binding domain can accommodate various hydrophobic substances. An aromatic ring occupies only part of it. EA, with its side chain providing an extra binding ligand to Tyr¹¹⁵ [25], has much larger binding affinity than CDNB, as reflected in the approx. 400-fold reduction of K_{EA} as compared with K_{CDNB} . The proposed kinetic mechanism of octopus GST is summarized in Scheme 2.

Ligand binding to the glutathione G-site and hydrophobic H-site show a synergistic effect with an interaction factor of 2.31 (estimated from K_{SGSH} and K_{mGSH} values), which is greatly enforced when two substrates are covalently linked. S-(DNP)GS and S-(EA)GS have much smaller K_i values. S-(EA)GS, with a K_i value of only ~ 10 nM, is one of the most potent inhibitors for GST and may have potential pharmacological applications. The binding energy of S-(EA)GS, estimated from its K_i value, was 62.8 kJ/mol. Such a high affinity between the conjugate and the enzyme suggests that the conjugate binds to the enzyme at an extra binding motif.

Proposed chemical mechanism of octopus GST

The three-dimensional structures of mammalian GST-Pi, GST-Mu and GST-Alpha have been elucidated recently [26–28]. Although the amino acid sequence identity between different gene-type subunits is only $\sim 30\%$, the structural topology is surprisingly similar and these proteins utilize the same catalytic mechanism as implicated by active-site geometry as well as kinetic and spectral analyses [26,27,29–31]. The N-terminal amino acid sequence of octopus GST shows high homology with those of Alpha/Pi-GST. Our experimental data on pH and isotope effect studies suggest that octopus GST conforms to a similar mechanism as Alpha-type GST. In general, $\text{p}K_{\text{a}}$ values deduced from the pH-log (V/K) plot reveal the dissociating groups of the enzyme or substrate. Since the co-substrate is saturated in our studies, the enzyme forms being titrated are the E·GSH and E·CDNB complexes respectively (Figures 2a and 2b). Some caution is required in interpreting the results of the pH studies. The pH-rate profile cannot be used directly to determine $\text{p}K_{\text{a}}$ values of the enzyme when different steps in the overall process depend on different levels of protonation. The assignment of $\text{p}K_{\text{a}}$ values thus must remain tentative. With this caveat in mind, we try to interpret our results.

For GST-Pi, the substrate is in hydrophobic contact within 4 Å (1 Å = 0.1 nm) of Tyr⁷, Phe⁸, Val¹⁰, Val³⁵ and Tyr¹⁰⁶ [26]. Recently, Tyr¹¹⁵, located in the H-site of GST-Mu, was demonstrated to participate in both the chemical and physical steps of the catalytic mechanism [25,32–34]. However, none of these groups seem to be the candidate with a $\text{p}K_{\text{a}}$ of 6.83. On the other hand, spectroscopic and kinetic evidence suggested that thiolate anion (GS^-) is the predominant enzyme-bound GSH [30, 31]. Thus the molecular $\text{p}K_{\text{a}}$ of 6.83 in Figure 2(a) more likely represents that of the thiol group of bound GSH in the E·GSH binary complex. The small shifts (0.07 pH unit) of this $\text{p}K_{\text{a}}$ value in $^2\text{H}_2\text{O}$ corroborate this conclusion [35]. A similar conclusion was drawn by Chen et al. for GST-Mu [30]. In the absence of GST, we found that the $\text{p}K_{\text{a}}$ value of the SH group of GSH was 9.169 ± 0.115 , which is in good agreement with the value (9.13 ± 0.07) reported by Huskey et al. [19]. When measurements were performed under V/K_{mGSH} conditions (GSH is not bound) the $\text{p}K_{\text{a}}$ of 7.48 determined in Figure 2(b) should be mostly attributed to free GSH whose $\text{p}K_{\text{a}}$ is kinetically perturbed in the

presence of GST; or else this ionization might reflect another unidentified group.

Glutathione thiolate anion was demonstrated to be the active GSH species during catalysis and Tyr⁷ was found to be involved in ionization of the enzyme-bound GSH. Arg¹³, Gln⁶² and Asp⁹⁶ were involved in polar interactions with GSH [26]. N-terminal amino acid sequence analysis indicates that Tyr⁷ and Arg¹³ are conserved in octopus GST [11]. On the basis of chemical modification and site-directed mutagenesis results [29,36–38], the most attractive candidate for the group with $\text{p}K_{\text{a}}$ shown in Figure 2 will be Tyr⁷ (Tyr⁸ in Alpha-type GST and Tyr⁶ in Mu-type GST), which when hydrogen-bonded with the enzyme-bound GSH may help in lowering the $\text{p}K_{\text{a}}$ of -SH in the enzyme-substrate complex. The $\text{p}K_{\text{a}}$ value (8.46) determined for E·CDNB is identical with the $\text{p}K_{\text{a}}$ value (8.4) of Tyr⁸ determined by Atkins et al. [29] in an Alpha-type GST. In the E·GSH form with the presence of GS^- , the $\text{p}K_{\text{a}}$ value of this tyrosine is raised to 9.63, which provides further evidence to support the involvement of Tyr⁷ in GSH binding. Ionization of this tyrosine residue gives lower activity, which is in agreement with the proposed role for the hydroxyl group of Tyr⁷ in stabilizing the thiolate anion of GSH.

Since the sulphhydryl group has an unusual affinity with protium over deuterium, with a fractionation factor of 0.456 [39], the above hypothesis may be tested by examining the solvent isotope effects on various kinetic parameters [18,40–42]. The kinetic isotope effect represents the ratio of a ground state and transition state fractionation factor $v_{\text{H}}/v_{\text{D}} = \phi/\phi^\ddagger$. When examining the kinetic solvent isotope effect on V/K , the effective reactant state is free substrate and enzyme, and the effective transition state is that for the Meisenheimer complex, in which GS^- and chloride are attached to the same carbon atom in the CDNB molecule that break down the aromaticity, and thus is unstable (Scheme 3). An isotope effect on V represents decomposition of the complex. Direct evidence for such a σ -complex has been provided by Graminski et al. [43].

The absence of isotope effects on V suggests that reactant-state and transition-state structures in the vicinity of the glutathione sulphur atom are very similar. Decomposition of Meisenheimer complex (step 'b' in Scheme 3) or desorption of S-(DNP)GS as the rate-determining step can be ruled out. Isotope effect on V/K_{mGSH} is also close to unity. The reactants in this case are E·CDNB and free GSH, and transition state is realized during GSH binding. The transition states beyond the Meisenheimer complex will not contribute to V/K_{mGSH} provided that the proton removed from the -SH is present in the solvent and not in the enzyme molecule [19].

Scheme 3 Proposed chemical mechanism of octopus hepatopancreatic GST

Step 'a' shows attacking of glutathione thiolate anion (GS^-) at the carbon bearing the chlorine atom of CDNB with formation of a negatively charged Meisenheimer transition state. Step 'b' shows the chloride ion leaving the transition state to give the conjugate product.

On the other hand, an inverse isotope effect was observed for V/K_{mCDNB} , which monitors the formation of the Meisenheimer complex transition state, and is determined by reactant state $\text{E} \cdot \text{GS}^-$ and one or more transition states appearing after the $\text{CDNB} \cdot \text{E} \cdot \text{GS}^-$ ternary complex [44]. An inverse isotope effect means the net binding at the contributing hydrogenic site is tighter in the effective transition state than in the effective reactant state [18]. The inverse isotope effect on V/K_{mCDNB} is consistent with a mechanism involving solvation changes at the thiolate sulphur as the σ -bond is formed, and were interpreted to arise from stronger hydrogen-bonding between thiolate anion and Tyr^7 than the hydrogen-bonding of bulk water [19]. Under the experimental conditions at pH 8.011, a pH (and $p^2\text{H}$)-independent region of k_{cat} , the rate-limiting step should be the formation of a σ -bond in the Meisenheimer complex transition state (step 'a' in Scheme 3). The non-enzymic conjugation between GSH and CDNB at high pH contributes to ionization of GSH and the electron-withdrawing nitro groups at *o*- and *p*-positions, which strongly stabilize the negatively charged transition state. Arg^{11} , Arg^{13} or Arg^{18} , all invariant amino acid residues for GST, and other essential groups involved may have contributed in stabilizing the transition state for the enzyme-catalysed reaction.

In conclusion, our experimental results suggest that the octopus GST follows the following catalytic mechanism: GSH binds to the enzyme in the unionized form. Tyr^7 , by promoting the ionization of the SH group of enzyme-bound GSH, lowering its pK_a by 2.34 pH units, resulting in a more reactive thiolate anion toward the electrophilic substrate. The other critical amino acid residues may be involved in the stabilization of the Meisenheimer complex transition state in the nucleophilic aromatic substitution reaction.

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