Dissection of the Catalytic Mechanism of Isozyme 4–4 of Glutathione *S*-Transferase with Alternative Substrates†

Wen-Jian Chen, Gerard F. Graminski, and Richard N. Armstrong*
Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742
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ABSTRACT: The kinetic and chemical mechanism of isozyme 4–4 of rat liver glutathione (GSH) *S*-transferase was investigated by using several alternative peptide substrates including *N*-acetyl-GSH, γ-L-glutamyl-L-cysteine (γ-GluCys), N^4-(malonyl-D-cysteinyl)-L-2,4-diaminobutyrate (*retro*-GSH), and N^4-(*N*-acetyl-D-cysteinyl)-L-2,4-diaminobutyrate (decarboxylated *retro*-GSH). The enzyme, which is normally stereospecific in the addition of GSH to the oxirane carbon of *R* absolute configuration in arene oxide substrates, loses its stereospecificity toward phenanthrene 9,10-oxide with the retro peptide analogues, giving a 2:1 mixture of the *S,S* and *R,R* stereoisomeric 9,10-dihydro-9-(*S*-peptidyl)-10-hydroxyphenanthrenes. The analogues with normal peptide bonds (*N*-acetyl-GSH and γ-GluCys) show normal stereospecific addition. The kinetic mechanism of the enzyme was investigated by using the alternative substrate diagnostic with several 4-substituted 1-chloro-2-nitrobenzenes and GSH, *N*-acetyl-GSH, and γ-GluCys. Varying the concentration of electrophile vs the identity of the GSH analogue and the concentration of GSH vs the identity of the electrophile gave two sets of intersecting reciprocal plots, a result consistent with a random sequential kinetic mechanism. The pH profiles of k_c and k_c/K_m^s [saturating GSH, variable 1-chloro-2,4-dinitrobenzene (**1**)] exhibit a dependence on a deprotonation in the enzyme–GSH–**1** and enzyme–GSH complexes with molecular pK_a's of 6.1 and 6.6, respectively. This result is consistent with spectroscopic studies that suggest the pK_a of the thiol of bound GSH is ≤6.8. In similar experiments with fixed, saturating **1** and variable peptide, kinetic pK_a's and limiting turnover numbers at high pH (k_c^{lim}) of 6.1 (2.5 s^{-1}), 7.5 (0.33 s^{-1}), and 7.7 (0.10 s^{-1}) were obtained from plots of log k_c vs pH for GSH, *N*-acetyl-GSH, and γ-GluCys, respectively. A plot of log k_c^{lim} vs the apparent pK_a of the nucleophile gave an anomalous Brønsted β_{nuc}(obsd) = −0.79 ± 0.15. In contrast, the specific-base-catalyzed reaction exhibited a β_{nuc} = 0.16 ± 0.02 for thiolate anions with conjugate acid pK_a's in the range of 7.3–9.5. The anomalous β_{nuc}(obsd) for the enzyme-catalyzed reaction is interpreted to suggest that the pK's observed in pH–rate profiles of k_c for *N*-acetyl-GSH and γ-GluCys do not reflect the ionization of the peptide thiol in the ternary complexes of the alternative substrates. Hammett plots of log k_c vs σ^- for a series of 4-substituted 1-chloro-2-nitrobenzenes give ρ values of 1.2, 1.9, and 2.5 for GSH, *N*-acetyl-GSH, and γ-GluCys, respectively, as compared to 3.4 for the specific-base-catalyzed reaction with GSH. The results indicate the importance of the peptide structure for the correct orientation of the thiol in the active site and suggest that one major contribution of the enzyme to catalysis is to lower the pK_a of the bound nucleophile. Finally, 4-fluoro-3-nitro-1-(trifluoromethyl)benzene was found to be a much better substrate than the 4-chloro substrate in both the specific-base- and enzyme-catalyzed reactions. This observation suggests that both reactions have similar rate-determining steps, the formation of a σ-complex intermediate.

The glutathione *S*-transferases (EC 2.5.1.18) are a group of enzymes that function primarily as catalysts in the nucleophilic addition of the thiol of glutathione (GSH)[1] to molecules containing electrophilic functional groups. In doing so they act as catalytic scavengers of potentially toxic substances (Jakoby & Habig, 1980). The importance of this family of proteins is most clearly evinced by their high concentrations in liver tissue. Recent reviews are available (Jakoby & Habig, 1980; Mannervik, 1985; Armstrong, 1987).

The GSH transferases most thoroughly studied with respect to structure and mechanism are those isolated from rat liver cytosol. The enzymes are homo- or heterodimeric proteins with subunit types designated 1, 2, 3, 4, 5, 6, etc. (Jakoby et al., 1984). Although there is considerable evidence for microheterogeneity in subunit types (Tu & Reddy, 1985; Satoh et al., 1985; Rothkoph et al., 1986), the six major isozymes in rat liver appear to occur in two families with binary subunit combinations of 1–1, 1–2, 2–2, 3–3, 3–4, and 4–4 (Mannervik & Jensson, 1982; Jakoby et al., 1984).

Even though the enzyme has received considerable attention in the last several years, relatively little is known concerning the details of its catalytic mechanism. Isozymes 1–1 and 3–3 have been characterized with respect to their kinetic mechanisms. Initial velocity and product inhibition studies of iso-

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* Address correspondence to this author. Recipient of a National Institutes of Health Research Career Development Award (ES 00133).

[1] Abbreviations: GSH, glutathione; rGSH, *retro*-glutathione [N^4-(malonyl-D-cysteinyl)-L-2,4-diaminobutyrate]; dcrGSH, N^4-(*N*-acetyl-D-cysteinyl)-L-2,4-diaminobutyrate; NAcGSH, *N*-acetylglutathione; γ-GluCys, γ-L-glutamyl-L-cysteine; CysOEt, L-cysteine ethyl ester; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); MES, 2-(*N*-morpholino)ethanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); MOPS, 3-(*N*-morpholino)propanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; TAPS, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; $K_m^{peptide}$, Michaelis constant for GSH or analogues; K_m^s, Michaelis constant for electrophilic substrate; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography; CD, circular dichroism.

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Chart I

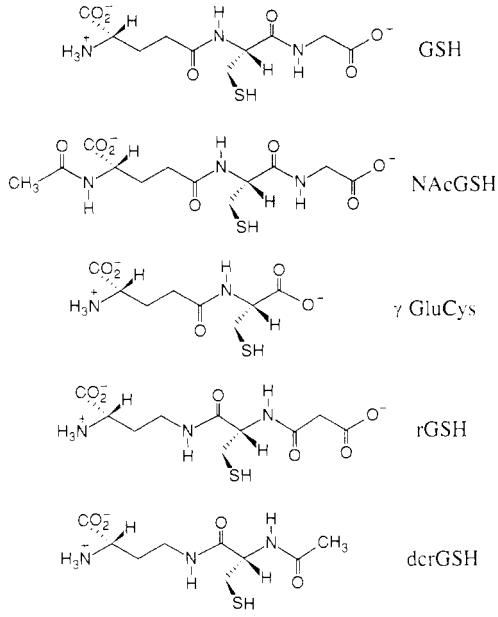
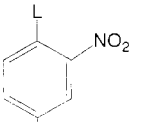


Chart II



	L	X
1	Cl	NO_2
2	Cl	SO_2CH_3
3	Cl	$COCH_3$
4	Cl	CHO
5	Cl	CF_3
6	Cl	SO_3^-
7	F	CF_3

zymes 1–1 (Schramm et al., 1984) and 3–3 (Jakobson et al., 1977, 1979a,b) clearly suggest random sequential mechanisms for both isozymes. Dead-end inhibition patterns of isozyme 3–3 by the oxygen and dethio analogues of GSH are also consistent with a random sequential mechanism (Chen et al., 1985). Less is understood about the chemical mechanism of catalysis. It is certainly reasonable to assume and some experimental evidence, with different electrophilic substrates, suggests that the protein enhances the nucleophilic reactivity of the thiol of bound GSH relative to that free in solution (Keen et al., 1976; Jakoby & Habig, 1980). In principle, the enzyme could accomplish this by increasing either the fraction of nucleophile (thiolate anion) or its nucleophilicity or both when GSH is bound to the protein surface.

Of the rat liver enzymes, isozyme 4–4 has been shown to be unique in its catalytic efficiency and stereoselectivity toward arene oxide substrates (Cobb et al., 1983; Boehlert & Armstrong, 1984; deSmidt et al., 1987). However, essentially nothing is known concerning its kinetic or chemical mechanism of catalysis. Dead-end inhibition studies are consistent with either a random sequential mechanism or an ordered mechanism with GSH adding first (Chen et al., 1985). Preliminary spectroscopic evidence indicates that the pK_a of the thiol of GSH bound to isozyme 4–4 is ≤ 6.8 or at least 2.0 pK_a units below that in aqueous solution (Graminski et al., 1987). The effect of this apparent shift in pK_a of the bound thiol on the nucleophilicity of the thiolate is not clear.

The use of alternative substrates is an attractive approach for the investigation of both the kinetic and chemical mechanisms of catalysis of isozyme 4–4. In the first instance, it has been demonstrated by Northrop (Radika & Northrop, 1984) that alternative substrates provide a simple diagnostic tool for distinguishing ping-pong, Theorell–Chance, ordered, and ranodm mechanisms for two-substrate enzymic reactions. Second, manipulation of the electronic character of the electrophilic substrate can yield, in principle, from linear free energy relationships, information on the rate-limiting step(s) and transition state of the reaction. In addition, it is reasonable to expect that the enhancement of the nucleophilic reactivity of glutathione by the enzyme must involve specific recognition of the tripeptide by the protein surface and the utilization of intrinsic binding energy. Therefore, synthetic alterations of the tripeptide structure should provide an indirect method for changing the nucleophilicity (or pK_a) of the putative enzyme-bound thiolate.

In this paper we report the use of four peptide analogues of GSH (see Chart I) and several 4-substituted 1-halo-2-nitrobenzenes (see Chart II) in an investigation of the catalytic mechanism of isozyme 4–4 of rat liver GSH transferase in nucleophilic aromatic substitution reactions. The results indicate that the enzyme follows a random sequential kinetic mechanism. Structural alterations of the peptide substrate appear to affect both the effective pK_a of the bound thiol and the relative orientation of the thiol with respect to the electrophilic substrate. Substituent and leaving group effects on k_c and k_c/K_m^s for substituted halonitrobenzenes are consistent with a mechanism involving rates limiting or partially rate limiting formation of an enzyme-bound σ- or Meisenheimer-complex intermediate between glutathione and the haloarene.

Experimental Procedures

Materials

Enzyme. Isozyme 4–4 of rat liver GSH transferase was purified by the following modification of previously described procedures. Approximately 160-g male Wistar rat livers (Pel-Freez) were homogenized, centrifuged, and chromatographed on a 5 × 55 cm DEAE-cellulose column (Whatman DE-52) as described by Habig et al. (1974). Most GSH transferases, except isozyme 4–4, were eluted from the column with 10 mM Tris-HCl (pH 8.0). Isozyme 4–4 was eluted from the column with the same buffer containing 0.1 M KCl. This fraction was further purified by precipitation with 96% saturated ammonium sulfate, dialysis, and chromatography on an *S*-hexylglutathione affinity matrix (Guthenberg & Mannervik, 1979). Finally, the protein was chromatographed on a 2 × 50 cm bed of hdyroxylapatite equilibrated with 10 mM KH_2PO_4 (pH 6.7) and eluted with a 2-L linear gradient of 10–300 mM KH_2PO_4 (pH 6.7). Isozyme 4–4 was concentrated to about 2 mg/mL, dialyzed against 10 mM KH_2PO_4 (pH 6.7), and flash frozen for storage at −80 °C.

The purified protein was judged to be ≥95% homogeneous by the criteria of SDS gel electrophoresis and high-performance anion-exchange chromatography on a 0.5 × 5 cm Pharmacia Mono-Q column eluted at 1 mL/min with 10 mM Tris-HCl, pH 8.0, for 2 min followed by a linear gradient of 0–0.4 M NaCl in 30 min. Retention time for isozyme 4–4 was 5.6 min. Identity of the enzyme was confirmed by amino-terminal sequence analysis, which gave PMTLGYWDIRGLAHAIRLFLEYTDTSYEDKKYSMGDAPDY?R, a sequence consistent with that known for isozyme 4–4 (Mannervik, 1985; Ålin et al., 1986).

Electrophilic Substrates. Phenanthrene 9,10-oxide was prepared by the method of Krishnan et al. (1977). 1-

Chloro-2,4-dinitrobenzene (**1**) and 4-chloro-3-nitrophenyl methyl sulfone (**2**) were from Eastman and ICN Biochemicals, respectively. 4-Chloro-3-nitroacetophenone (**3**), 4-chloro-3-nitrobenzaldehyde (**4**), 4-chloro-3-nitro-1-(trifluoromethyl)-benzene (**5**), 4-chloro-3-nitrobenzenesulfonic acid (**6**), and 4-fluoro-3-nitro-1-(trifluoromethyl)benzene (**7**) were obtained from Aldrich.

Glutathione Analogues. retro-Glutathione (rGSH) and decarboxylated *retro*-glutathione (dcrGSH) were synthesized as described earlier (Chen et al., 1986).

N-Acetylglutathione (NAcGSH) was prepared as follows. A solution of 0.3 g (0.49 mmol) of glutathione disulfide in 5 mL of H_2O was adjusted to pH 9.5 with NH_4OH. After addition of 1.5 equiv of acetic anhydride the solution was strirred at room temperature for 4 h with addition 1.5-equiv aliquots of anhydride added at 0.5-h intervals. The pH was maintained at 9.5 by periodic addition of NH_4OH. The reaction was stopped by adjusting the pH to 7.0 with acetic acid and lyophilizing. The residue, which was dissolved in 4 mL of H_2O, was stirred with 1 equiv of dithiothreitol under N_2 for 1.0 h. The reaction mixture was immediately applied to a 2.4 × 70 cm bed of DEAE-cellulose equilibrated with 0.1 M ammonium acetate (pH 7.0). The column was eluted with a 1-L linear gradient of 0.1–0.5 M ammonium acetate (pH 7.0). Chromatography and fraction collection were performed under N_2. Fractions containing NAcGSH were lyophilized repeatedly from water to give 74 mg (0.27 mmol) of NAcGSH as a white hygroscopic powder. The product was judged ≥98% pure by ^1H NMR and contained no detectable (<0.2%) GSH. ^1H NMR (400 MHz, D_2O, ref DSS) δ 1.98 (s, 3 H, acetyl-CH_3), 1.91–2.10 (m, 2 H, Glu-β-CH_2), 2.35 (m, 2 H, glu-γ-CH_2), 2.84 (dd, 1 H, ^2J = 14.5 Hz, ^3J = 6.9 Hz, H_A, Cys-β-CH_2), 2.88 (dd, 1 H, ^2J = 14.5 Hz, ^3J = 5.4 Hz, H_B, Cys-β-CH_2), 3.67 (d, 1 H, ^2J = 17.2 Hz, H_A, Gly-CH_2), 3.72 (d, 1 H, ^2J = 17.2 Hz, H_B, Gly-CH_2), 4.19 (dd, 1 H, ^3J = 4.6 Hz, ^3J = 9.1 Hz, Glu-α-CH), 4.51 (dd, 1 H, ^3J = 6.8 Hz, ^3J = 5.4 Hz, Cys-α-CH).

The dipeptide γ-L-glutamyl-L-cysteine (γ-GluCys) was prepared by the following modification of an earlier procedure. The disulfide of γ-GluCys was prepared from GSSG by digestion with carboxypeptidase and purified by chromatography on Dowex 1 as described by Strumeyer and Block (1962). A solution of the dipeptide disulfide (0.7 g, 1.4 mmol) in 4 mL of H_2O was adjusted to pH 7.0 with NH_4OH, and 30.9 mg (1 equiv) of dithiothreitol was added. The solution was stirred 1 h under N_2 and immediately applied to a 2.5 × 105 cm bed of DEAE-cellulose equilibrated with 0.1 M ammonium acetate (pH 7.0). The column was eluted with a 4-L linear gradient of 0.1–0.5 M ammonium acetate (vide supra). Fractions containing the reduced dipeptide were combined and lyophilized 5 times from H_2O to give 0.609 g (2.43 mmol) of γ-GluCys. ^1H NMR (400 MHz, D_2O, ref DSS) δ 2.11 (m, 2 H, Glu-β-CH_2), 2.48 (m, 2 H, Glu-γ-CH_2), 2.85 (dd, 1 H, ^2J = 14.4 Hz, ^3J = 6.1 Hz, H_A, Cys-β-CH_2), 2.89 (dd, 1 H, ^2J = 14.4 Hz, ^3J = 4.7 Hz, H_B, Cys-β-CH_2), 3.72 (dd, 1 H, ^3J = 5.7 Hz, ^3J = 6.7 Hz, Glu-α-CH), 4.32 (dd, 1 H, ^3J = 4.9 Hz, ^3J = 6.1 Hz, Cys-α-CH).

Other Materials. Carboxypeptidase, glutathione, glutathione disulfide, dithiothreitol, 5,5′-dithiobis(2-nitrobenzoic acid), L-cysteine ethyl ester, and buffer salts were obtained from Sigma.

Methods

Enzyme Kinetics. Enzyme-catalyzed reactions were run at 25 °C and the appropriate pH by use of 0.1 M buffer solutions. Solutions of glutathione or analogues were prepared fresh each day and stored on ice under a blanket of N_2 or Ar. Thiol concentrations were determined by titration with DTNB (Ellman, 1959). Reactions were followed on a Perkin-Elmer 552 or Lambda 4B UV–visible spectrometer at the following wavelengths for the various substrates: **1**, 340 nm, $\Delta\epsilon$ 9600 M^{-1} cm^{-1}; **2**, 281 nm, $\Delta\epsilon$ 7850 M^{-1} cm^{-1}; **3**, 297 nm, $\Delta\epsilon$ 11 900 M^{-1} cm^{-1}; **4**, 304 nm, $\Delta\epsilon$ 10 000 M^{-1} cm^{-1}; **5**, 365 nm, $\Delta\epsilon$ 2200 M^{-1} cm^{-1}; **6**, 370 nm, $\Delta\epsilon$ 2300 M^{-1} cm^{-1}; **7**, 365 nm, $\Delta\epsilon$ 2700 M^{-1} cm^{-1}. In general, a solution (975 μL) of the appropriate concentrations of enzyme and glutathione (or analogue) in buffer was equilibrated to 25 °C and the reaction initiated by addition of the electrophile in 25 μL of CH_3CN. Initial velocity data were analyzed by the program HYPER (Cleland, 1979). All initial velocities were determined in triplicate. Turnover numbers were calculated on the basis of one active site per subunit of M_r 26 000. Enzyme concentrations were determined spectrophotometrically, with $E_{280}^{0.1\%}$ = 1.11.

The pH dependence of k_c and k_c/K_m was determined by using the following buffers at the indicated pH: citrate, 5.0; MES, 5.5 and 6.0; PIPES, 6.5; MOPS, 7.0; KH_2PO_4, pH 7.3; HEPES, 7.5; Tris, 8.0; TAPS, 8.5; CHES, 9.0 and 9.5; CAPS, 10.0, 10.5, and 11.0. Reactions were carried out with saturating GSH and variable **1** or, in the case of NAcGSH and γ-GluCys as well as GSH, with saturating **1** and variable peptide. Observed reaction velocities were corrected for spontaneous reaction rates when necessary. The pH dependence of kinetic parameters was analyzed by the program HABELL (Cleland, 1979).

Substitutent effects on the kinetic parameters k_c and k_c/K_m^s were determined at either pH 6.5 or pH 7.5 under conditions of saturating GSH or analogue and variable electrophile.

Kinetics of Uncatalyzed Reactions. The pH dependence of the uncatalyzed reaction of GSH or analogues was obtained from observed first-order rate constants at 0.5 pH unit intervals with 100 μM **1** and GSH concentrations of 5, 10, 15, and 20 mM. Second-order rate constants were derived from the slopes of plots of the observed pseudo-first-order rate constants vs the concentration of GSH or analogue. Substituent effects on the specific-base-catalyzed reaction of GSH with substituted halonitrobenzenes were determined in a similar manner at pH 11.0. All reactions were performed at 25 °C in degassed buffers under an atmosphere of Ar.

Stereoselectivity toward Phenanthrene 9,10-Oxide. The stereoselective formation of GSH analogue conjugates by isozyme 4–4 with phenanthrene 9,10-oxide was determined essentially as described by Cobb et al. (1983). Reactions containing 0.1 mM phenanthrene 9,10-oxide, 0.5–5 mM GSH or analogue, and approximately 5 μM enzyme were run for 3 h at room temperature. The diastereomeric conjugates were isolated by reversed-phase HPLC on a 4.6 mm × 25 cm Rainin Microsorb ODS column eluted at 0.5 mL/min with the following gradients. For conjugates of GSH, NAcGSH, and γ-GluCys the column was eluted with 0.1 M ammonium acetate and a linear gradient of 0–30% CH_3OH in 40 min. For the conjugates of rGSH, isocratic conditions, 0.2 M ammonium acetate, pH 3.6, containing 35% CH_3OH, were used. Stereoisomeric conjugates of dcrGSH were separated on a 1.0 × 25 cm Altex Ultrasphere ODS column eluted at 2.0 mL/min with 0.1 M ammonium acetate (pH 3.6) containing 30% CH_3OH. Absolute configurations of the diastereomers were determined by the sign of the CD transition of the biphenyl chromophore at 230 nm (Cobb et al., 1983; Armstrong & Lewis, 1985). Retention times for the 9*S*,10*S* and 9*R*,10*R* stereoisomers of the various conjugates under the above conditions are respectively GSH, 59.9 and 63.9 min; NAcGSH,

Table I: Kinetic Constants for Enzyme-Catalyzed Reactions of GSH and Analogues with 1-Chloro-2,4-dinitrobenzene (**1**)[a] and Stereoselectivity toward Phenanthrene 9,10-Oxide

peptide	k_c (s^{-1})	$k_c/K_m^{peptide}$ (M^{-1} s^{-1})	mol % *S,S* stereoisomeric product[b]
GSH	0.63 ± 0.02	(1.1 ± 0.1) × 10^4	≥99
NAcGSH	0.030 ± 0.002	(2.7 ± 0.2) × 10^1	≥99
γ-GluCys	0.028 ± 0.002	(1.0 ± 0.2) × 10^1	≥99
rGSH	0.027 ± 0.007	3.5 ± 0.4	68 ± 2
dcrGSH	0.019 ± 0.002	9.0 ± 0.7	67 ± 2

[a] Kinetic constants were obtained at pH 6.5 with subsaturating (200 μM) **1** and variable peptide. [b] From enzyme-catalyzed reaction with phenanthrene 9,10-oxide as described under Experimental Procedures. Values are corrected for any contribution of spontaneous reaction.

66.4 and 68.8 min; γ-GluCys, 55.3 and 57.9 min; rGSH, 68.1 and 74.3 min; and dcrGSH, 35.8 and 40.7 min. Control reactions without enzyme indicated <2% of products were generated by nonenzymic reaction.

Instrumental Methods. ^{1}H NMR spectra were obtained on a Bruker AM 400 spectrometer at 400 MHz. Amino acid sequence analysis was performed on an Applied BioSystems 477A sequencer. Circular dichroism spectra were obtained on a Jasco J-500C spectropolarimeter at room temperature.

Results

Glutathione Analogues as Alternative Substrates. Structural modifications to GSH are found to have rather pronounced effects on the kinetic constants for the enzyme-catalyzed reaction with 1-chloro-2,4-dinitrobenzene. Although all four GSH analogues were found to be alternative substrates, turnover numbers under the conditions employed were 20–30-fold smaller than that for GSH (Table I). Michaelis constants for the analogues are between 1 and 8 mM or about 10^2–10^3 larger than for GSH. A striking contrast is observed in the stereoselectivity of the enzyme-catalyzed reaction toward phenanthrene 9,10-oxide with GSH, γ-GluCys, and NAcGSH and the two retro-peptide substrates (Table I). Clearly, reversal of the direction of the peptide bonds has the net effect of lowering the difference in the free energies of activation of the two possible diastereomeric transition states. Due to this clear distinction in substrate properties, the somewhat higher K_m values, and limited availability of the retro peptides, only GSH, NAcGSH, and γ-GluCys were used in subsequent investigations.

Kinetic Mechanism of the Enzyme by Use of the Alternative Substrate Diagnostic. Given at least two glutathione analogues and a large selection of electrophilic substrates, the kinetic diagnostic of Northrop (Radika & Northrop, 1984) using alternative substrates provides an efficient and sensitive approach to determining the kinetic mechanism of the enzyme. The substrate combinations GSH, NAcGSH, and γ-GluCys and haloarenes **1**, **2**, and **3** were chosen both for ease of saturation of the enzyme with the fixed substrate and to give

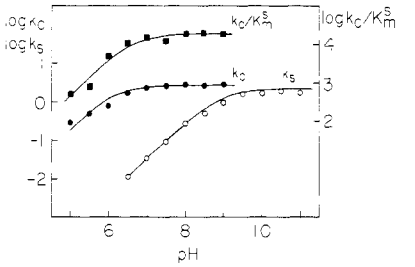


FIGURE 1: Dependence of kinetic constants k_c (●) and k_c/K_m^s (■) on pH for the enzyme-catalyzed reaction of GSH with **1** under conditions of saturating GSH and variable **1** and k_s (○) for the spontaneous (specific-base-catalyzed) reaction. Solid lines are computer fits using the program HABELL with pK_a's of 6.10 ± 0.05, 6.57 ± 0.05, and 8.86 ± 0.04 for k_c, k_c/K_m^s, and k_s, respectively.

different maximal velocities. When GSH is varied against fixed, saturating concentrations of **1**, **2**, and **3**, a pattern of intersecting or, more precisely, nonparallel lines is observed (plots not shown). An intersecting pattern is also apparent when **1** is varied against the identity of the three peptide substrates. This unique pair of kinetic patterns requires a random sequential kinetic mechanism for isozyme 4–4, at least in nucleophilic aromatic substitution reactions.

Dependence of Reactions on pH. The influence of pH on the reaction of enzyme-bound GSH (saturating GSH) with **1** indicates a dependence of k_c on an apparent molecular pK_a of 6.1 and of k_c/K_m^s on a pK_a of 6.6 (Figure 1). This result, together with the recent spectroscopic observation of the thiolate anion of GSH with a pK_a ≤6.8 when bound to isozyme 4–4 (Graminski et al., 1987), suggests that the pK_a observed in the pH–rate profiles may be good estimates for that of the thiol group of enzyme-bound GSH. In contrast, the kinetically determined pK_a for the sulfhydryl group of GSH in aqueous solution of 8.9 (Figure 1, Table II), which is within experimental error of that determined by potentiometry or NMR spectroscopy (Reuben & Bruice, 1976; Jung et al., 1972), is approximately 2.5 units higher.

The pH–rate behavior of the spontaneous and enzyme-catalyzed reactions of NAcGSH and γ-GluCys is analogous to, but quantitatively different from, that for GSH as summarized in Table II. It should be noted that, because of very high spontaneous reaction velocities, it was technically impossible to obtain pH–rate data under conditions of saturating peptide and variable **1**. This is primarily due to the much higher K_m values for the two alternative substrates as well as the need to collect data at higher pH. Thus the data in Table II were, of necessity, obtained under conditions of saturating **1** and variable peptide. Three observations are worth noting. First, the kinetic pK_a's obtained from log k_c vs pH for the enzyme-catalyzed reactions with NAcGSH and γ-GluCys are 1.4–1.6 pK units higher than that observed for GSH. Second, as the apparent pK_a *increases*, the limiting turnover number of high pH, k_c^{lim}, *decreases*. In fact, the data, though some-

Table II: pH Dependence of Specific-Base- and Enzyme-Catalyzed Reactions of Peptides with 1-Chloro-2,4-dinitrobenzene

	specific base		enzyme			
			pK_a(app)			
peptide	k_s (M^{-1} s^{-1})	pK_a (–SH)	log k_c vs pH	log ($k_c/K_m^{peptide}$) vs pH	k_c^{lim} (s^{-1})	effective molarity[a]
GSH	2.02 ± 0.02	8.86 ± 0.04	6.11 ± 0.05	6.42 ± 0.06	2.49 ± 0.10	1.2
			(6.10 ± 0.05)[b]	(6.57 ± 0.05)[b]	(2.59 ± 0.13)[b]	(1.3)[b]
NAcGSH	2.47 ± 0.04	9.41 ± 0.01	7.49 ± 0.01	7.61 ± 0.02	0.332 ± 0.005	0.14
γ-GluCys	2.20 ± 0.05	9.47 ± 0.05	7.67 ± 0.05	8.11 ± 0.02	0.100 ± 0.004	0.045
CysOEt	1.05 ± 0.05	7.30[c]				

[a] Ratio of k_c^{lim}/k_s. [b] Values in parentheses are from Figure 2 and were obtained with saturating GSH and variable **1**. Thus the apparent pK_a is derived from log k_c/K_m^s vs pH. [c] Value from Reuben and Bruice (1976).

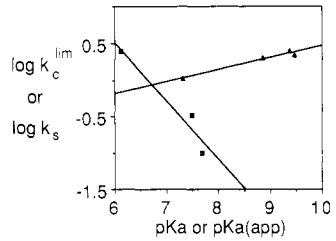


FIGURE 2: Brønsted plots of log k_c^{lim} vs pK_a(app) (■) of the nucleophile derived from log k_c vs pH for the enzyme-catalyzed reaction of log k_s vs pK_a (▲) for the base-catalyzed reaction of thiolate anions with **1**. Lines are least-square fits of the data points to the equations log k_c^{lim} = 5.23 (±1.07) − [0.79 (±0.15)] pK_a for (■) and log k_s = −1.16 (±0.15) + [0.16 (±0.02)] pK_a for (▲).

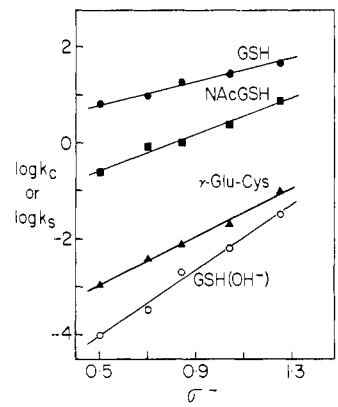


FIGURE 3: Hammett plots of log k_c vs σ^- for enzyme-catalyzed reactions of GSH (●), NAcGSH (■), and γ-GluCys (▲) and the specific-base-catalyzed reaction of GSH (○). Electrophilic substrates used and the Hammett (σ^-) substituent constants are **1** (1.25), **3** (0.84), **4** (1.04), **5** (0.70), and **6** (0.50). Lines are least-square fits to the data points with the slopes (ρ values) indicated in Table III.

what limited, would appear to obey the Brønsted equation such that

$$\log k_c^{lim} = \log G + (\beta)(pK_a) \quad (1)$$

as shown in Figure 2. If it is assumed that the kinetic pK_a's obtained from the pH–rate profiles of k_c are an approximate measure of the basicities of the nucleophiles (thiolate anion) in the active site of the ternary complex, then an apparent β_{nuc}(obsd) = −0.79 ± 0.15 is obtained for the thee peptides in the enzyme-catalyzed reaction (Figure 2). The fact that β_{nuc}(obsd) for the enzyme-catalyzed reaction is negative suggests that the kinetic pK_a may not directly reflect the thermodynamic pK_a's of the thiolate anions in the three ternary complexes (vide infra). Quite in contrast, the second-order rate constants for reaction of the thiolate anion of the three peptides and L-CysOEt with **1** show a small positive dependence on the basicity of the nucleophile (β_{nuc} = 0.16 ± 0.02) in aqueous solution (Table II) over a pK_a range of 7–9.5 (Figure 2). Finally, the pH dependence of $k_c/K_m^{peptide}$, which should reflect the effect of ionizations on the free enzyme or free peptide substrate, is different for the three peptides and parallel to that observed for k_c.

Substituent Effects. It is well established that the sensitivity of the second-order rate constants for the reaction of anions, including GS^-, with 4-substituted 1-halo-2-nitrobenzenes is described by the Hammett relationship (eq 2) with the σ^- substituent constant. The reaction constant ρ = 3.4 (Figure

$$\log k_s = \log k_0 + \rho\sigma^- \quad (2)$$

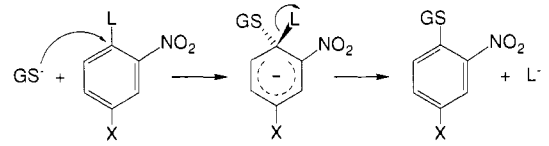
3) for reaction of GS^- with the series of substrates used here is the same as that previously described by Keen et al. (1976). In contrast, ρ values for the turnover numbers of the enzyme-catalyzed reactions with the three peptide substrates are significantly lower than that of the specific-base-catalyzed reaction (Figure 3, Table III). It is notable that, as the peptide substrate becomes poorer, the ρ value for the enzyme-catalyzed reaction approaches that of the specific-base-catalyzed reaction for GSH (Table III).

Table III: Reaction Constants (ρ Values) for Specific-Base- and Enzyme-Catalyzed Additions to 4-Substituted 1-Chloro-2-nitrobenzenes

		ρ values from		
peptide	catalysts	k_s	k_c	k_c/K_m^s
GSH	OH^-	3.4 ± 0.2 (3.1)[a]		
GSH	isozyme 4–4		1.2 ± 0.1	2.4 ± 0.6
NAcGSH	isozyme 4–4		1.9 ± 0.1	2.5 ± 1.2
γ-GluCys	isozyme 4–4		2.5 ± 0.1	2.7 ± 0.5

[a] Value from Keen et al. (1976).

Scheme I



Somewhat surprisingly, the kinetic constant k_c/K_m^s also appears to follow the Hammett relationship, though the correlation with σ^- is not nearly as good as it is with k_c (Table III).[2] It is clear, however, that the ρ values for k_c/K_m^s are generally greater than those for k_c. This is particularly true for GSH. Furthermore, unlike the reaction constants for k_c, the ρ values for k_c/K_m^s with the three peptide substrates are indistinguishable from one another and approach that for the uncatalyzed reaction.

Effect of Leaving Group. Variation of the leaving group is a well-established approach in defining the rate-limiting step in bimolecular nucleophilic aromatic substitution reactions that proceed through a σ-complex intermediate as illustrated in Scheme I. Thus, rate-limiting formation of a σ-complex intermediate should be accelerated by substitution of F for Cl due to the creation of a more electropositive aryl substrate carbon. If decomposition of the σ-complex to product is rate-limiting, then the opposite effect is expected because of the stronger C–F bond. The ratio of the second-order rate constants for reaction of GS^- with **5** and **7** in the specific-base reaction is much greater than 1: k_s^F/k_s^{Cl} = 47. This behavior is mirrored in both kinetic constants for the enzyme-catalyzed reactions of GSH with **5** and **7** where k_c^F/k_c^{Cl} = 40 and $(k_c/K_m^s)^F/(k_c/K_m^s)^{Cl}$ = 14. By this criterion the overall rate-limiting step appears to be σ-complex formation (bond making) in both the specific-base and enzyme-catalyzed reactions. Interestingly, when NAcGSH is used as the peptide substrate, the sensitivity of k_c to the leaving group is considerably smaller, with k_c^F/k_c^{Cl} = 1.4 and $(k_c/K_m^s)^F/(k_c/K_m^s)^{Cl}$ = 6.9, compared to k_s^F/k_s^{Cl} = 62.

DISCUSSION

Utility of Alternative Peptide Substrates. It is clear from this and previous work (Habig et al., 1974; Sugimoto et al.,

[2] That the correlations with k_c are considerably better than those for k_c/K_m^s is not unusual since the two steady-state kinetic constants are composed of different rate constants and reflect free energy differences between different ground states and perhaps different transition states. The problems of superposition of geometrical and solvation factors on the electronic rate effects in enzyme-catalyzed reactions are well documented (Kirsch, 1972).

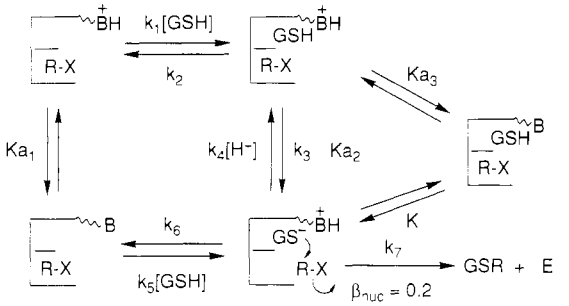
1985; Chen et al., 1986) that GSH transferases will accept as substrates a number of modified, cysteine-containing peptides. The pronounced effects of structural alterations on the stereoselectivity and kinetic constants of catalysis are potentially quite useful in investigations of the kinetic and chemical mechanisms of the enzyme. For instance, it is known that isozyme 4–4 of GSH transferase is stereospecific in the addition of GSH to arene oxide substrates, catalyzing attack at the oxirane carbon of *R* absolute configuration, and that this stereospecificity is sensitive to the nature of the hydrophobic surface of the electrophile (Cobb et al., 1983; Boehlert & Armstrong, 1984; deSmidt et al., 1987). The distinct difference noted (Table I) in the stereospecificity of the enzyme-catalyzed reactions with "normal" and retro-peptide substrates demonstrates that enzyme–peptide interactions can also influence the geometrical preference and relative stabilities of the two diastereomeric transition states in the enzyme-catalyzed reactions. Although this phenomenon appears to depend on the direction of the peptide bonds in the substrate, the exact structural cause in the enzyme–peptide complex remains obscure.

The availability of alternative substrates that mimic the physiologic cofactors of xenobiotic metabolizing enzymes should greatly facilitate the efficient elucidation of the kinetic mechanisms of enzyme-catalyzed reactions as illustrated here with GSH transferase. That isozyme 4–4 is shown to have a random sequential kinetic mechanism by the alternative substrate diagnostic is not surprising and is consistent with results from dead-end inhibition studies (Chen et al., 1985). Thus, the three most extensively studied isozymes from rat liver (1–1, 3–3, and 4–4) all appear to follow similar kinetic mechanisms. As discussed in more detail below, the utility of alternative substrates can be extended to investigations of the chemical mechanism of catalysis.

Activation of the Thiol of GSH. A central question in the catalytic chemistry of GSH transferase is exactly how the protein enhances the nucleophilic reactivity of thiol of GSH. It is tempting to conclude from the pH dependence of k_c and k_c/K_m^s that the protein serves to lower the pK_a of the thiol of GSH by 2–2.5 pK units relative to that in aqueous solution, when bound at the active site. Although the pitfalls of identifying kinetically determined pK_a values with thermodynamic acid dissociation constants are numerous, this is nonetheless an attractive proposal. Further support for this suggestion has been recently obtained from UV difference spectroscopic studies of the enzyme–GSH complex and complexes containing *S*-methyl-GSH or the oxygen analogue γ-L-glutamyl-L-serylglycine (Graminski et al., 1987). The UV difference spectrum shows an absorption band for bound GS^- at 239 nm ($\epsilon \approx 2200$) that disappears on protonation with a pK_a ≤6.8. Both the kinetic and preliminary spectroscopic results are consistent with the hypothesis that either GS^- or a (GS^-, BH^+) ion pair is the nucleophilic species in the active site of the enzyme at neutral pH.

It is instructive to compare the reactivity of the thiolate anion in the active site of the enzyme to that in aqueous solution. One way to do this is to calculate the effective molarity of the enzyme-catalyzed reaction at high pH from the ratio of k_c^{lim}/k_s. The effective molarity (Table II) at high pH for the reaction of GS^- with **1** is very low by most standards (Jencks, 1969) and in the range of that expected for a simple encounter complex. This suggests two things. First, the nucleophilic reactivity of the thiolate bound to the enzyme is not significantly greater than that in aqueous solution. Second, a good deal of the catalytic potential of the enzyme at neutral

Scheme II



pH is derived from its apparent ability to lower, by about 2.5 pK units, the acid dissociation constant of the sulfhydryl group.

One distinct mechanistic possibility for lowering the pK_a of the thiol of the bound peptide is by positioning the group in the appropriate electrostatic field such as that of a positively charged basic residue or the amino-terminal end of one or more α-helix dipoles of the protein.[3] This proposal would be an intermolecular example of features thought important in the activation of the active site sulfhydryl groups of a number of enzymes including glyceraldehyde phosphate dehydrogenase, papain, and rhodanese (Polgar, 1974; Schlesinger & Westley, 1974; Hol et al., 1978). Moreover, it has recently been suggested that charge–charge interactions acting through space may lower the pK_a of the conjugate acid of an anionic nucleophile such as RS^- without lowering the nucleophilic reactivity (Roberts et al., 1986). Inasmuch as it is reasonable to expect that GSH transferase would utilize some of the intrinsic binding energy of GSH to promote the proper electrostatic interaction, a corollary to this proposition is that any critical alteration in the ability of the enzyme to recognize the peptide substrate should alter the ionization and behavior of the bound nucleophile. This does seem to be the situation with NAcGSH and γ-GluCys. In particular, the molecular pK_a's observed in the pH vs rate profiles increase significantly.

The most curious and perhaps telling observation is the anomolous β_{nuc}(obsd) value of −0.8 relating the kinetic pK_a of the bound nucleophile and log k_c^{lim}. In the absence of any preequilibrium effects, a $\beta_{nuc} \geq 0$ would be expected. Several possible scenarios are consistent with this behavior. One trivial possibility is that the decreased reactivity of the analogues is due to geometric or orientational effects and that any relationship to the apparent pK_a is fortuitous. On the other hand, it may be that the reaction of the thiolate in the active site with **1** has a small intrinsic $\beta_{nuc} \approx 0.2$ (as is observed in aqueous solution) and that the negative β_{nuc}(obsd) is due to a preequilibrium with a large dependence on basicity ($\beta_{pe} \approx 1.0$) such that

$$\beta_{nuc}(\text{obsd}) = \beta_{nuc} - \beta_{pe} \quad (3)$$

Such a preequilibrium might involve solvation (Jencks et al., 1986), hydrogen bonding, or coordination of the thiolate to a functional group on the protein. This explanation is not entirely satisfactory since β_{pe} would probably be too small to account for the large negative value observed.

Alternatively, it must be considered that the kinetic pK_a's for k_c may not, in all cases, approximate the thermodynamic value of the peptide sulfhydryl group in the ternary complex. For example, if the major and reactive species in the ternary

[3] In the absence of any structural information these propositions are, of course, speculative and are drawn only by analogy with other proteins. The structure of isozyme 3–3 of the rat liver enzyme is under investigation by X-ray crystallography (Sesay et al., 1987).

complex with GSH is a (GS^-, BH^+) ion pair (Scheme II), then it is conceivably that with the alternative peptides the neutral, unreactive (or less reactive) species (GSH, B) predominates. In this situation, where $\log K = pK_{a_3} - pK_{a_2} < 0$ (Scheme II), the observed kinetic pK_a will be that of BH^+, not GSH, in the ternary complex. The spectroscopic observation of GS^- in the binary enzyme–GSH complex is a good indication that, at least for GSH, the predominate species contains GS^-. That is, the equilibrium constant K between the neutral and zwitterionic species is large ($pK_{a_3} > pK_{a_2}$, $\log K \gg 0$). The anomalous β_{nuc}, therefore, leads to the conclusion that the kinetic pK_a for k_c with NAcGSH and γ-GluCys is not that of the sulfhydryl group but that of another group in the ternary complex, perhaps a protonated base (BH^+) on the enzyme with a $pK_a \approx 7.5$–7.7.

Inasmuch as the pK_a's obtained from $\log k_c/K_m^{peptide}$ must reflect ionizations on the free enzyme (actually, in this case, the enzyme–electrophile complex) or the free peptide substrate, the fact that different pK_a's are seen with the three different peptides suggests that the observed pK_a's in the $k_c/K_m^{peptide}$ profiles are due to kinetic perturbation of the molecular acid dissociation constant(s). Whether the ionizable group is on the substrate or enzyme cannot, of course, be ascertained from the profiles. One possibility is that the observed pK_a's reflect the kinetically perturbed ionization of a protonated group on the enzyme as illustrated in Scheme II. For example, in the situation where GS^- is a sticky substrate ($k_7 > k_6$) and $k_1 \approx k_5$ and proton release is more rapid than release of peptide from the protonated complex ($k_3 > k_2$), it can be shown that the apparent pK_a and $k_c/K_m^{peptide}$ profiles is given by eq 4 (Cleland, 1977), where K_{a_1} is the true acid dissociation constant of the group on the enzyme.

$$pK_a(app) = pK_{a_1} - \log (1 + k_7/k_6) \quad (4)$$

It is reasonable to assume that the substrate analogues NAcGSH and γ-GluCys are less sticky than GS^- such that as k_6 becomes larger relative to k_7 the observed pK_a(app) approaches pK_{a_1}. If this proposition is correct, then it can be estimated that $pK_{a_1} > 7.6$. A ratio $k_7/k_6 \geq 15$ is therefore required to account for the kinetic perturbation. Similar arguments can be made for the case that the observed pK_a's are due to kinetic perturbation of the ionization of, for instance, the thiol group on the free peptide substrate. In any case, it is premature to suggest the identity of a protonated base in the active site of the enzyme.

GSH Transferase Catalyzed Nucleophilic Aromatic Substitutions. Most bimolecular aromatic nucleophilic substitution reactions on activated substrates are thought to proceed through a σ- or Meisenheimer-complex intermediate as illustrated in Scheme I (Miller, 1968). In the majority of instances these reactions proceed in solution with rate-limiting formation of the σ-complex. Exceptions have been documented, particularly with thiolate anion nucleophiles and substrates with fluoride as the leaving group (Ho et al., 1966). It is relevant, then, to ascertain if reactions involving the enzyme-bound thiolate occur with rate-limiting addition of the nucleophile to form a σ-complex intermediate or if some other step, such as decomposition of the σ-complex or product release, is rate-determining. That the fluoro substrate **7** is more than an order of magnitude more reactive than the corresponding chloro compound, **5**, in both the specific-base- and enzyme-catalyzed reactions strongly suggests that both occur with rate-limiting formation of a σ-complex intermediate. The situation is less clear with NAcGSH. The much lower sensitivity of k_c for NAcGSH to the identity of the leaving group suggests that the rate-determining steps in turnover of the species that accumulates at saturation in the enzyme-catalyzed reactions of GSH and NAcGSH are different.

The rather marked differences in the sensitivity of the rate constants for the specific-base- (k_s) and enzyme-catalyzed reactions (k_c) to electron-withdrawing substituents on the aromatic substrate tend to suggest differences in the transition states for the two reactions. The catalytic rate constant k_c, which, in a simple kinetic situation, is related to the free energy difference between the state in which the enzyme accumulates at saturation and the highest free energy barrier following that state, can be influenced by a number of steps in the reaction sequence. It is certainly possible that a step other than formation of a σ-complex is partially rate determining. A couple of possibilities can be considered. It is unlikely that desorption of product, 1-(*S*-glutathionyl)-2-nitro-4-X-benzene, is rate-limiting. Were this the case, **5** and **7** would be expected to have similar turnover numbers and linear Hammett plots would not be expected (Keen et al., 1976). Alternatively, partial, rate-limiting desorption of a σ-complex intermediate as the initial product off the enzyme surface might be expected to be related to the Hammett σ^- substituent constant since the stability of the enzyme–intermediate complex and the transition state for desorption could be influenced by the electron density and distribution in the σ-complex. It is interesting to note that enzyme-bound σ-complexes have apparently not been considered as possible kinetically significant species in previous kinetic studies of GSH transferases.

Perhaps the smaller ρ value of the enzymatic reaction simply reflects a more reactant-like, earlier transition state for the nucleophilic attack of the thiolate on the enzyme surface. This view is certainly consistent with the observation that the less reactive GSH analogues have ρ values in the enzymatic reaction which begin to approach that for the reaction of thiolates in aqueous solution. In this regard, it is interesting to consider the apparent correlation of $\log (k_c/K_m^s)$ with σ^-. Since k_c/K_m^s is related to the free energy difference between the enzyme–GSH complex and free electrophile and the transition state for the first irreversible step (perhaps formation of the σ-complex), the fact that ρ values for all three peptide substrates are close to that for the spontaneous reaction suggests that the transition states for the first irreversible step in bimolecular reactions of electrophile with enzyme-bound or free GSH (or analogue) are similar. Thus differences in ρ values for the kinetic constant k_c must reflect either differences in the reactivity of the thiolates in the ternary enzyme–peptide–electrophile complex or the contribution of other steps on the reaction coordinate, perhaps desorption of a σ-complex intermediate.

Conclusions. Alternative substrates provide an interesting approach to the study of the kinetic and chemical mechanisms of the GSH transferases. The results of this study suggest that a great deal of the catalytic potential of isozyme 4–4 of GSH transferase is due to is ability to lower the effective pK_a of the sulfhydryl group of GSH. The rate-limiting step for both bimolecular specific-base- and enzyme-catalyzed reactions of GSH with haloarenes appears to be formation of a σ-complex intermediate although the transition state for turnover of the enzyme is influenced by other steps on the reaction coordinate. Several questions such as the identity of catalytic residues and the kinetic significance of σ-complex intermediates in the enzyme-catalyzed reactions are under active investigation.

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