molecule amphiphilic. Our results presented here indicate that whereas the topographical proximity of the Tyr[1] and Phe[4] aromatic rings in DPDPE and the overall amphiphilic properties of the peptide may be critical for interaction with the δ receptor, the μ opioid receptors seem to bind ligands with significant separation of the aromatic (e.g., [D-Tic[1]]CTP (**2**)).

Further efforts in our laboratory are concentrated on design and synthesis of amino acids with constrained conformational properties that are complimentary to these already exhibited by 1,2,3,4-tetrahydroisoquinolinecarboxylate derivatives.

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Registry No. 1, 103335-28-0; **2**, 115981-69-6; **3**, 115962-17-9; **4**, 131904-39-7; Boc-Thr(*O*-Bzl), 15260-10-3; Boc-Pen(*S*-4-MeBzl), 104323-41-3; Boc-Orn(*N*-Cbz), 2480-93-5; Boc-D-Trp, 5241-64-5; Boc-Tic, 78879-20-6; Boc-Cys(*S*-4-MeBzl), 61925-77-7; Boc-D-Phe, 18942-49-9; Boc-D-Tic, 115962-35-1.

Supplementary Material Available: Full sets of data on the transverse and longitudinal cross-relaxation rates ($\sigma_\perp$, $\sigma_\parallel$), internuclear distances (r^{ij}), and correlation times (τ_c) for peptides **1-4**, stereoviews of the conformations derived from the methods discussed for 1, *3*, and *4*, and plots of the normalized (using eqs 4 and 5—see text) cross-peak intensities vs mixing times or spin-lock times in longitudinal and cross-relaxation experiments (11 pages). Ordering information is given on any current masthead page.

Contributions of Thiolate "Desolvation" to Catalysis by Glutathione *S*-Transferase Isozymes 1-1 and 2-2: Evidence from Kinetic Solvent Isotope Effects

Su-Er W. Huskey,*,† W. Phillip Huskey,*,‡ and Anthony Y. H. Lu†

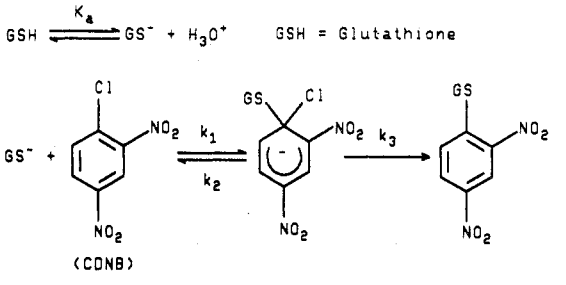
Contribution from the Department of Animal and Exploratory Drug Metabolism, Merck Sharp and Dohme Research Labs, Rahway, New Jersey 07065, and Department of Chemistry, Rutgers, The State University, Newark, New Jersey 07102. *Received June 19, 1990*

Abstract: Kinetic solvent isotope effects on the reaction of glutathione with 1-chloro-2,4-dinitrobenzene catalyzed by rat liver glutathione *S*-transferase isozymes 1-1 and 2-2 (as expressed in Escherichia *coli*) have been measured. At pH (and pD) = 8.0, the isotope effects (H_2O/D_2O) ranged from 0.79 to 1.05 under various conditions of substrate concentrations. Solvent isotope effects were also measured for the nonenzymic reactions of glutathione or dithiothreitol with 1-chloro-2,4-dinitrobenzene. For reactions with the respective thiolate anions, solvent isotope effects of 0.84 and 0.87 were observed. For both the enzymic and nonenzymic reactions, the solvent isotope effects were interpreted in terms of hydrogen bond changes about the thiolate anion.

As catalysts for the nucleophilic addition of glutathione to a variety of electrophilic aromatic compounds, the glutathione *S*-transferases (EC 2.5.1.18) play an important role in the metabolism of endogenous and xenobiotic compounds.[1] The purification of individual isozymes has been complicated by the complexities of multiple forms (the enzymes exist as homo- or heterodimers[2]) and their microheterogeneities.[3] However, recent developments in molecular biology have made it possible to express cDNA of several glutathione transferase isozymes in *Escherichia coli* and make available large amounts of the enzyme for detailed mechanistic studies.[4]

Armstrong et al.[5] have studied the mechanisms of isozymes 3-3 and 4-4 in detail. Substantial evidence has been accumulated suggesting that these enzymes bind the protonated form of glutathione (referring to the thiol function), and that the anionic form is generated on the enzyme in the catalytic mechanism. Direct evidence has also been found for the formation of a Meisenheimer complex in the reaction of enzyme-bound glutathione anion with 1,3,5-trinitrobenzene.[5c] Substituent effects on rates measured with substituted chlorobenzenes and leaving-group effects are consistent with rate-limiting C–S bond formation for k_{cat}. An important feature[5a] of the mechanism is the apparent increase in the acidity of glutathione bound to the enzyme by roughly 2 pK_a units.

Scheme I



We suspected that the factors associated with the pK_a shift might involve stabilization of the anionic form of enzyme-bound

† Merck Sharp & Dohme Research Labs.
‡ Rutgers, The State University.

(1) (a) Jakoby, W. B. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1978**, *46*, 383-414. (b) Mannervik, B. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1985**, *57*, 357-417. (c) Armstrong, R. N. *CRC Crit. Rev. Biochem.* **1987**, *22*, 39-88. (d) Pickett, C. B.; Lu, A. Y. H. *Annu. Rev. Biochem.* **1989**, *58*, 743-764.

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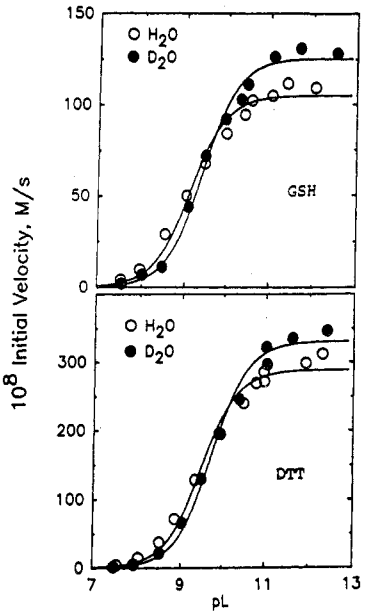

Figure 1. pH rate profiles for reactions of glutathione (GSH) and dithiothreitol (DTT) with 1-chloro-2,4-dinitrobenzene in H_2O and D_2O. pL is either pH or pD. Reactions were conducted at 25 °C in 0.25 M bis-tris propane buffer (4% ethanol) using 0.3 mM GSH or DTT and 1.5 mM 1-chloro-2,4-dinitrobenzene. Parameters for each of the curves plotted are given in Table I.

Table I. Kinetic Parameters[a] for the Nonenzyme Addition of Two Thiols to 1-Chloro-2,4-dinitrobenzene at 25 °C

thiol	k_s, M^{-1} s^{-1}	$10^{10}K_a$, M	pK_a
glutathione, H_2O	2.333 ± 0.056	7.47 ± 1.2	9.13 ± 0.07
glutathione, D_2O	2.787 ± 0.057	3.73 ± 0.45	9.43 ± 0.05
ratio	0.837 ± 0.026	2.00 ± 0.39	
dithiothreitol, H_2O	6.41 ± 0.15	1.63 ± 0.26[b]	9.79 ± 0.07
dithiothreitol, D_2O	7.36 ± 0.16	0.89 ± 0.11[b]	10.05 ± 0.05
ratio	0.871 ± 0.029	1.83 ± 0.46	

[a] Parameters were obtained from least-squares fits of the data shown in Figures 1 and 2 to eq 1. Error limits are least-squares estimates of standard deviations. See Figure 1 for more details. [b] K_a shown for dithiothreitol is half the value obtained from a least-squares fit of the data to eq 1. k_s is exactly as obtained from the fit.

glutathione through hydrogen bond formation sufficiently strong as to be detected by using the effects of isotopic solvents. We report here solvent isotope effects (H_2O/D_2O) measured on the rates of the reaction of glutathione with 1-chloro-2,4-dinitrobenzene catalyzed by glutathione transferase isozymes 1-1 and 2-2. Isotope effects on nonenzymic reactions were also measured to aid in the interpretation of the enzymic results. Our observations on isozymes 1-1 and 2-2 support the general mechanistic ideas and results of Armstrong et al.[5] obtained with isozymes 3-3 and 4-4.

Results

pH/pD Rate Profiles for the Chemical Reactions. Initial velocities for the nonenzymic reactions of glutathione and dithiothreitol with 1-chloro-2,4-dinitrobenzene were determined over a range of pH or pD values (Figure 1). The pL ($L^+ = H^+$ or D^+) dependence of the velocities is well described by eq 1, obtained

$$v_0 = [RSH]_0[CDNB]_0k_sK_a/(K_a + [L^+]) \quad (1)$$

$$k_s = k_1k_3/(k_2 + k_3) \quad (2)$$

(4) (a) Board, P. G.; Pierce, K. *Biochem. J.* **1987**, *248*, 937–941. (b) Lai, H. C. J.; Qian, B.; Grove, G.; Tu, C. P. D. *J. Biol. Chem.* **1988**, *263*, 11389–11395. (c) Abramovitz, M.; Ishigaki, S.; Felix, A. M.; Listowsky, I. *J. Biol. Chem.* **1988**, *263*, 17627–17631. (d) Wang, R. W.; Pickett, C. B.; Lu, A. Y. H. *Arch. Biochem. Biophys.* **1989**, *269*, 536–543.

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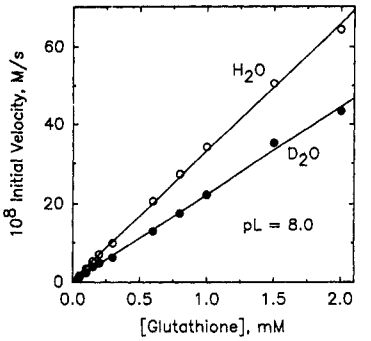


Figure 2. Initial velocity (25 °C) for the reaction of glutathione with 1-chloro-2,4-dinitrobenzene (1.5 mM) at pH (or pD) = 8.0 as a function of glutathione concentration in H_2O or D_2O buffers of 0.25 M bis-tris propane (4% ethanol). The least-squares lines through the points have slopes of (32.70 ± 0.42) × 10^{-5} (H_2O) and 21.18 ± 0.40) × 10^{-5} s^{-1} (D_2O) and intercepts of (0.50 ± 0.38) × 10^{-8} M/s (H_2O) and (0.01 ± 0.36) × 10^{-8} M/s (D_2O).

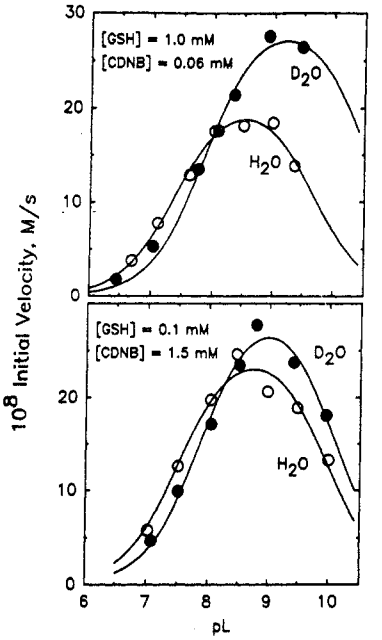


Figure 3. pH and pD rate profiles for the reaction of glutathione (GSH) with 1-chloro-2,4-dinitrobenzene (CDNB) catalyzed by isozyme 1-1 of rat liver glutathione transferase (expressed by *E. coli*). Each 1-mL run contained 1.625 μg of the enzyme. Reactions were conducted at 25 °C in 0.25 M bis-tris propane (4% ethanol) using the substrate concentrations shown on the figure. Background nonenzymic reaction rates have been subtracted from observed rates to obtain the enzymic rates. Parameters for the curves on the figure are shown in Table II.

from the mechanism of Scheme I by assuming the acid–base equilibrium is rapid, and by treating the intermediate Meisenheimer complex as a steady-state intermediate. $[RSH]_0$ and $[CDNB]_0$ are the respective initial concentrations of the thiol and the aromatic reactant. Least-squares fits of $v_0/([RSH]_0[CDNB]_0)$ vs pL were used to obtain estimates of k_s and K_a, shown in Table I. Solvent isotope effects on these parameters are also shown in the table.

Solvent Isotope Effects on the Chemical Reaction at pH = 8.0. Measurements of initial velocities as a function of glutathione concentration were made at pH = 8.0, where the dominant form of the thiol group is protonated. The measurements shown in Figure 2 confirm that the reaction is first order in glutathione concentration. A kinetic solvent isotope effect determined from the ratio of slopes in the figure is 1.474 ± 0.032.

pH/pD Rate Profiles for Glutathione Transferase Catalyzed Reactions. Figure 3 shows the pH and pD rate profiles for reactions catalyzed by glutathione transferase isozyme 1-1. Reaction conditions were such that the enzymes were always saturated with one substrate while the concentration of the remaining substrate

Table II. Kinetic Parameters[a] and Solvent Isotope Effects[b] for the Reaction of Glutathione and 1-Chloro-2,4-dinitrobenzene Catalyzed by Glutathione Transferase Isozymes 1-1 and 2-2

[GSH], mM	[CDNB], mM		$10^8 v$, M/s	pK_1	pK_2
			Isozyme 1-1[c]		
1.00	0.06	H_2O	21.5 ± 1.1	9.18 ± 0.11	6.89 ± 0.07
		D_2O	29.5 ± 2.0	10.08 ± 0.49	7.36 ± 0.09
0.10	1.50	H_2O	25.8 ± 1.6	9.48 ± 0.12	7.01 ± 0.11
		D_2O	30.6 ± 1.7	9.61 ± 0.10	7.37 ± 0.08
1.00	varied		$^{DOD}(V/K_C)$ = 0.793 ± 0.013	^{DOD}V = 0.971 ± 0.014	
varied	1.50		$^{DOD}(V/K_G)$ = 1.054 ± 0.014	^{DOD}V = 1.032 ± 0.014	
			Isozyme 2-2[d]		
0.06	1.00	H_2O	16.7 ± 0.4		6.89 ± 0.05
		D_2O	25.1 ± 0.4		7.18 ± 0.03
varied	1.5[e]		$^{DOD}(V/K_C)$ = 0.813 ± 0.024	$^{DOD}(V/K_0)$ = 1.048 ± 0.020	

[a] Parameters v, K_1, and K_2 were obtained from least-squares fits of the data in Figure 3 to eq 3 of the text for isoenzyme 1-1, and a two-parameter equation for isoenzyme 2-2. Error limits are least-squares estimates of standard deviations. [b] Isotope effects, shown as ratios of parameters in light/heavy solvents, were obtained from the intercepts of the least-squares lines shown in Figure 4. Error limits are estimates of standard deviations based on least-squares error estimates and uncertainties in the apparent K_m values used to compute the fractional saturation of the enzyme. Isotope effects were measured at 25 °C in 0.25 M bis-tris propane buffer (4% ethanol) at pH (or pD) = 8.0. [c] Each 1-mL run contained 1.625 μg of isozyme 1-1. [d] Each 1-mL run contained 6.0 μg of isozyme 2-2. [e] The apparent K_m for 1-chloro-2,4-dinitrobenzene with isozyme 2-2 at saturating concentrations of glutathione is much higher (ca. 7 mM) than the same value for isozyme 1-1 (0.3 mM). Thus a concentration of 1.5 mM for the aromatic compound is "high" for isozyme 1-1 but "low" for isozyme 2-2.

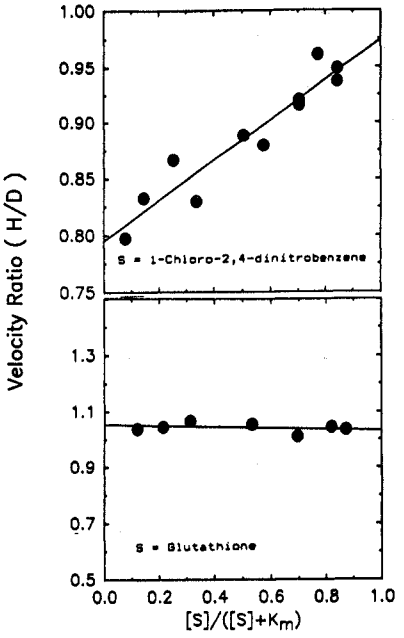


Figure 4. Solvent isotope effects at pH (or pD) = 8.0 on initial velocities of the enzymic reaction (isozyme 1-1) as functions of the fractional saturation of the enzyme by the substrate shown on the plot. The substrate not varied was maintained constant at saturating concentrations (1.5 mM for 1-chloro-2,4-dinitrobenzene and 1 mM for glutathione). The apparent K_m values used to compute fractional levels of saturation were 0.293 ± 0.049 mM for the aromatic compound and 0.219 ± 0.032 mM for glutathione. Consult Table II for further details.

was maintained at less than a saturating level. When both substrates are at saturating concentrations, the nonenzymic reaction overwhelms the enzymic reaction making measurements under such conditions very difficult. The velocities shown in Figure 3 have been corrected for the nonenzymic reaction. pL rate data (L^+ is either H^+ or D^+) for the reaction catalyzed by isozyme 1-1 can be expressed in terms of the two-pK_a model of eq 3.

$$v_0 = v[L^+]K_2/(K_1K_2 + (K_1 + K_2)[L^+] + [L^+]^2) \quad (3)$$

Least-squares estimates of the parameters in eq 3 are shown in Table II. pL rate profiles for the same reaction catalyzed by isozyme 2-2 are shown in Figure 5. Initial velocities were fit to a simple one-pK_a model. Parameters from the fits are shown in Table II.

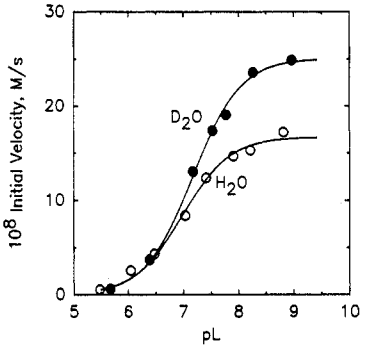


Figure 5. pL (L^+ = H^+ or D^+) rate profiles for the reaction of glutathione (0.06 mM) and 1-chloro-2,4-dinitrobenzene (1 mM) catalyzed by glutathione transferase isozyme 2-2. Each 1-mL run contained 6 μg of the enzyme in 0.25 M bis-tris propane buffer (4% ethanol) at 25 °C. Under these conditions, the apparent K_m for glutathione is near 0.1 mM and for 1-chloro-2,4-dinitrobenzene, the apparent K_m is at least 4 mM. The curves are least-squares fits to $v_0 = vK_a/([H^+] + K_a)$. The parameters for the curves are given in Table II.

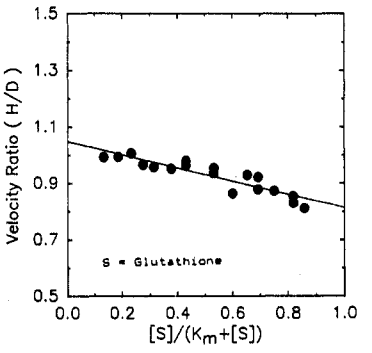
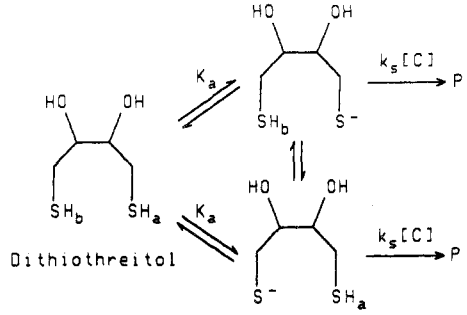


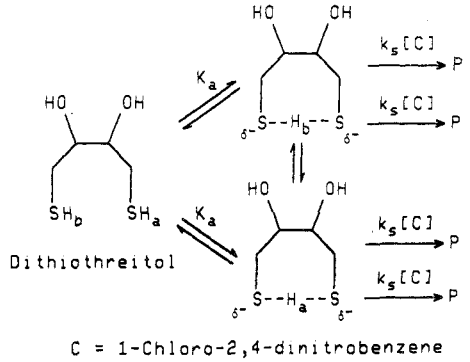
Figure 6. Solvent isotope effects on initial velocities for the reaction catalyzed by glutathione transferase isozyme 2-2. Each 1-mL run contained 6 μg of enzyme in 0.25 M bis-tris propane buffer (4% ethanol) (pL = 8.0) and 1.5 mM 1-chloro-2,4-dinitrobenzene (apparent K_m = 6.6 ± 3.5 mM in the presence of 1 mM glutathione) at 25 °C. The fractional satuation of the enzyme with glutathione was calculated by using K_m = 132 ± 59 μM (measured with 1.5 mM 1-chloro-2,4-dinitrobenzene). The least-squares line shown was used to estimate the isotope effects listed for isozyme 2-2 in Table II.

Solvent Isotope Effects on the Enzymic Reactions. Kinetic solvent isotope effects are shown as a function of fractional substrate saturation of isozyme 1-1 and isozyme 2-2 in Figures

Scheme II



Scheme III



4 and 6, respectively. Each set of data was fit by the method of least squares to the line of eq 4, where f is the fractional saturation

$$^{DOD}v_0 = f^{DOD}(V) + (1 - f)^{DOD}(V/K) \quad (4)$$

$$f = [S]/(K_{m(HOH)} + [S])$$

of the isozyme for a particular substrate, and kinetic parameters with leading superscripts of DOD are various H_2O/D_2O solvent isotope effects. For convenience, the isotope effects observed have been defined in terms of eq 5, a rate law known to be at least

$$v_0^{-1} = V^{-1} + (V/K_G)^{-1}/[GSH] + (V/K_C)^{-1}/[CDNB] + (V/K_0)^{-1}/[GSH][CDNB] \quad (5)$$

approximately correct for many two-substrate enzymic reactions. Isotope effects obtained in this way are shown in Table II.

Discussion

Kinetics of the Nonenzymic Reactions. The rate constant k_s for the reaction of glutathione with 1-chloro-2,4-dinitrobenzene and the estimated pK_a for glutathione are in good agreement with previous observations of Gan[6] and Chen, Graminski, and Armstrong.[5a] k_s can be estimated to be 2.1 M^{-1} s^{-1} for the reaction at 25 °C from a plot in Gan's work, and Chen et al. reported a similar value, 2.02 ± 0.02 M^{-1} s^{-1}. Both values compare favorably with our value of 2.33 ± 0.06 M^{-1} s^{-1}. From their kinetics, Chen at al. estimated the glutathione thiol pK_a to be 8.86 ± 0.04 in fair agreement with 9.13 ± 0.07 reported in Table I.

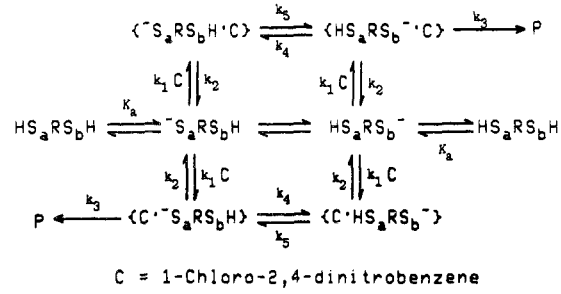
The apparent value of k_s (6.41 M^{-1} s^{-1}) for the reaction with dithiothreitol is about twice as large as the value estimated from a published[5a] Brønsted plot for the reaction. The mechanism shown in Scheme II predicts a pH dependence on the rate according to eq 6. The apparent K_a determined from a fit of the

$$v_0 = [RSH]_0[CDNB]_0k_s(2K_a)/(2K_a + [L^+]) \quad (6)$$

data to eq 1 should be twice the value of the K_a for a single thiol group. By use of the pK_a for a single thiol group of 9.79, k_s is predicted from the Brønsted plot to be 2.7 M^{-1} s^{-1}, roughly half the value observed. The rate constant for the dithiothreitol reaction

(6) Gan, L. H. *Aust. J. Chem.* **1977**, *30*, 1475–1479.

Scheme IV



can be reconciled with the previous observations if the monoanion has the symmetrical structure shown in Scheme III. An identical anion would be generated in this scheme from deprotonation of either thiol group. The scheme shows two routes to the anion, and from each route, the anion generated has two identical reacting sulfur centers. The appropriate rate expression for the mechanism of Scheme III (eq 7) shows that the apparent value of k_s obtained

$$v_0 = [RSH]_0[CDNB]_0(2k_s)(2K_a)/(2K_a + [L^+]) \quad (7)$$

by fitting data to eq 1 should be twice the value of k_s for reaction at a single sulfur center of the anion. If half of the apparent k_s (6.41 M^{-1} $s^{-1}/2$) is used, the dithiothreitol reaction falls on the Brønsted line reported by Chen et al.[5a] With the new point, the slope is not significantly different (0.18 ± 0.02) from the previously reported value (0.16 ± 0.02). The small Brønsted slopes are consistent with rate-limiting addition of the thiol to the aromatic ring. All models involving changes in the rate-limiting step as the origin of the anomalous apparent k_s for dithiothreitol can be eliminated, as these models predict that the apparent rate constant should fall below the Brønsted line described by the reactions with the remaining thiols.

An alternative explanation for the apparent doubling of k_s for the reaction with dithiothreitol anion is depicted by the mechanism shown in Scheme IV. On this scheme, HS_aRS_bH represents dithiothreitol. The species shown in brackets are diffusional encounter complexes consisting of the anion of the thiol compound and 1-chloro-2,4-dinitrobenzene. Complexes are shown with the aromatic compound adjacent to either the protonated or unprotonated thiol groups of a molecule of the dithiothreitol anion. Assuming that the encounter complexes are steady-state intermediates, the rate equation is given by eq 8 (DDT, dithiothreitol).

$$v_0 = [CDNB]_0[DTT]_0\frac{k_1k_3}{k_2[1+k_4/(k_2+k_5)] + k_3} \times \left(1 + \frac{k_5}{k_2 + k_5}\right)\frac{2K_a}{2K_a + [L^+]} \quad (8)$$

Assuming further that $k_5 \gg k_2$ and $k_2 \gg k_3$ (proton transfer within the complex is faster than diffusion out of the complex, but diffusion out is faster than C–S bond formation), and with the condition that $k_4/k_5 < 1$, the rate equation for the mechanism of Scheme IV is given by eq 9. Although the mechanisms of

$$v_0 = [CDNB]_0[DTT]_0(2k_3k_1/k_2)(2K_a)/(2K_a + [L^+]) \quad (9)$$

Schemes III and IV have the same form for their rate expressions, a distinction between the mechanisms can be made by use of solvent isotope effects.

Solvent Isotope Effects on the Nonenzymic Reactions. The inverse kinetic solvent isotope effects on k_s for the reactions with glutathione (0.837 ± 0.026) and dithiothreitol (0.872 ± 0.029) can be explained by using a model with an inverse reactant-state isotopic fractionation factor. Kinetic solvent isotope effects can be expressed (eq 10) in terms of running products of isotopic

$$^{DOD}k = k_{HOH}/k_{DOD} = \prod_i^{RS}\phi_i/\prod_j^{TS}\phi_j \quad (10)$$

fractionation factors (ϕ) for reactant-state (RS) and transition-state (TS) sites rapidly exchanging protons with the solvent.[7-10]

All fractionation factors discussed here are relative to isotopic exchange with water. The reactant state for the rate constant k_s is the thiolate form of the reactant, and it is the thiolate site that undergoes a major change during the reaction. The inverse isotope effects are consistent with a mechanism involving solvation changes at the thiolate sulfur as the sulfur–carbon bond is formed.

The term $\prod_i\phi_i$ (hereafter called ϕ_S-) for the reactant state can be estimated by using known fractionation factors for glutathione[11] ($\phi_{SH} = 0.456$) and a single hydronium ion site[12] ($\phi_l = 0.69$) and the kinetic solvent isotope effect observed on the reaction with glutathione and 1-chloro-2,4-dinitrobenzene at pH = 8 (1.474 ± 0.032). At pH = 8.0, the reactant state is 93% protonated and 7% thiolate. With reference to the mechanism of Scheme I, the observed isotope effect at a given pH is a weighted average of the isotope effects for reactions with protonated and unprotonated thiol reactant states. Accordingly, the observed isotope effect of 1.474 gives $^{DOD}(k_sK_a) = 1.52$. In terms of isotopic fractionation factors, this isotope effect is expressed in eq 11. With the known

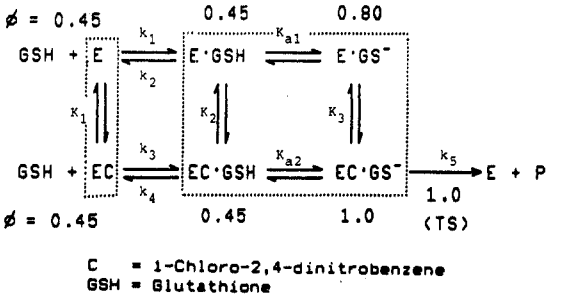
$$^{DOD}(k_sK_a) = (k_sK_a)_{HOH}/(k_sK_a)_{DOD} = \phi_{SH}/\phi_{TS}(\phi_l)^3 \quad (11)$$

fractionation factors described above, ϕ_{TS} is estimated to be 0.91. From $^{DOD}k_s = 0.837 = \phi_{S^-}/\phi_{TS}$, the fractionation factor ϕ_{S^-} is estimated to be 0.76 for the thiolate form of glutathione. The extent of desolvation of the thiolate at the transition state can be estimated[13,14] to be 34% by assuming that complete desolvation would give a solvent isotope effect of 0.76. The modest extent of desolvation is readily compatible with the small Brønsted slope[5a] for the reaction with a series of thiolate ions. By use of the same extent of desolvation and $^{DOD}k_s = 0.871$, ϕ_{S^-} is estimated to be 0.81 for dithiothreitol.

The similarity between ϕ_{S^-} estimated for glutathione and dithiothreitol argues against the symmetrical anion structure shown in Scheme III as the deprotonated form of dithiothreitol. The fractionation factor for the symmetrical hydrogen bond is expected to be substantially lower than 0.8, perhaps in the range of 0.2–0.5.[15] Since the reactant-state dithiothreitol thiolate structure in Scheme IV is not distinguished in any way from the thiolate structure of glutathione, ϕ_{S^-} is predicted to be similar for the two thiolate anions. For this reason, the apparent doubling of k_s for the dithiothreitol reaction is best accounted for by the symmetry of the mechanism in Scheme IV, when the proton switch (k_5) is faster than separation of the encounter complex (k_2). Because the proton transfer is likely to be an intermolecular reaction involving normal acid and base centers, it is reasonable[16] to suggest that $k_5 \gg k_2$. In addition, the apparent doubling of the rate constant is reproduced in the mechanism of Scheme IV only when $k_4/k_5 < 1$. This condition can be justified by invoking a stabilizing influence on the thiolate anion by an adjacent aromatic compound in the encounter complexes.

A thiolate fractionation factor of 0.76–0.81 agrees well with previous measurements of solvent isotope effects on the ionization of various thiols. Jencks and Salvesen[17] reported solvent isotope effects over a range of 2.0–2.5 for the ionization of six thiols, and they noted that these effects were larger than expected if the thiolate fractionation factor is unity. With the Jencks and Salvesen isotope effect of 2.2 for the ionization of mercaptoethanol, the hydronium ion fractionation factor, and the fractionation factor for mercaptoethanol[18] (0.55 ± 0.02), ϕ_{S^-} for the thiolate anion of mercaptoethanol is estimated to be 0.76. Similarly, the solvent isotope effect on an ionization constant in Table I yields $\phi_{S^-} = 0.69 \pm 0.14$ for the glutathione anion, using 0.456 for the fractionation factor of the thiol.[7] By analogy with fractionation factors for hydroxide ion and methoxide ion, the origin of the inverse thiolate fractionation is likely to arise from stronger hydrogen bonding between thiolate ions and water than the hydrogen bonding of the reference bulk solvent. However, the reactant-state contribution to solvent isotope effects on reactions involving methoxide ion[9] is considerably larger [$\prod_i\phi_i = (0.74)^3 = 0.41$] than the same contribution from thiolate ion (ca. 0.8). Thiolate fractionation factors similar to those seen in the nonenzymic reactions are invoked below to explain the kinetic solvent isotope effects on the enzymic reaction.

Scheme V



Solvent Isotope Effects on the Glutathione Transferase Reactions. At pL values corresponding to maximal velocities in the pL–rate profiles, kinetic solvent isotope effects (see Table II) on the reaction catalyzed by isozyme 1-1 are small but in the same range as values observed for the nonenzymic reaction. Isotope effects on V and V/K_G are close to unity, suggesting that reactant-state and transition-state structures in the vicinity of the thiol group are very similar for each of these parameters. In contrast, the isotope effect on V/K_c (0.79) is very similar to the isotope effect observed on the chemical reaction (0.84), suggesting a common origin for the solvent isotope effect among the enzymic and chemical reactions. The isotope effects on each kinetic parameter correspond to effective reactant-state and transition-state structures that must be part of a consistent mechanism for the reaction.

The mechanism of Scheme V represents a mechanism we believe offers the simplest explanation for our observations consistent with the observations of others. The scheme shows random addition of the two substrates in agreement with the conclusions reached by Schramn at al.[19] in their studies of isozyme 1-1. Chen, Graminski, and Armstrong have also interpreted their observations with isozyme 4-4 in terms of a random addition of substrates.[5a] Isotopic fractionation factors appropriate for the thiol function and consistent with our observations are shown above various points in the mechanism. For reasons of parsimony we have presumed all species within a dotted-line box to be rapidly equilibrating. The initial velocity for the mechanism of Scheme V is given by eq 12. As will be discussed below, our observations

$$v_0 = [(k_1K_1 + k_3[CDNB])k_5K_{a2}[CDNB] \times [GSH]]/[D_2(k_1K_1 + k_3[CDNB])[GSH] + D_1(k_2K_2[H^+] + k_4[CDNB][H^+] + k_5K_{a2}[CDNB])] \quad (12)$$

$$D_1 = K_1 + [CDNB]$$

$$D_2 = [H^+][CDNB] + K_2[H^+] + K_{a2}K_3 + K_{a2}[CDNB]$$

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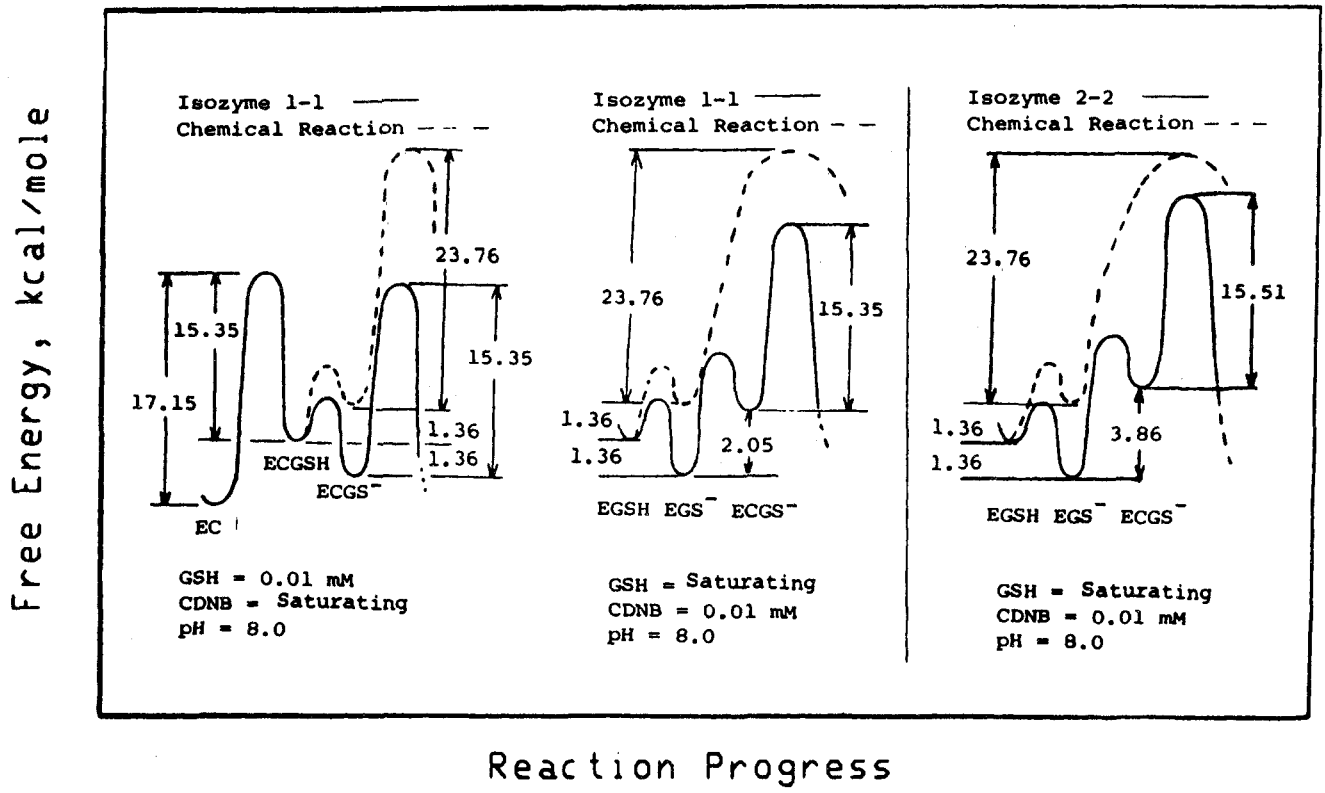

Figure 7. Free energy profiles for uncatalyzed and glutathione transferase catalyzed reactions of glutathione addition to 1-chloro-2,4-dinitrobenzene. The choice of standard state (at 25 °C) is shown below each profile with the abbreviations GSH for glutathione and CDNB for 1-chloro-2,4-dinitrobenzene. The diagram is not drawn to scale. The enzymic and nonenzymic profiles have been drawn with a common point at the stage of glutathione bound to the enzyme to emphasize the effect of the pK_a shift on the enzyme. $pK_a = 9.0$ was used for the uncatalyzed reaction, and $pK_a = 7.0$ was used for glutathione ionization on either isozyme. $k_s = 2.33\ M^{-1}\ s^{-1}$ (Table I) was used for the uncatalyzed reaction. Rate constants for the isozyme 1-1 reactions used to construct the diagram are $k_{cat} = 34\ s^{-1}$ (measured 32.0 ± 1.7 and $35.9 \pm 1.6\ s^{-1}$ in separate experiments), $k_{cat}/K_G = (1.64 \pm 0.18) \times 10^5\ M^{-1}\ s^{-1}$, and $k_{cat}/K_c = (1.09 \pm 0.13) \times 10^5\ M^{-1}\ s^{-1}$. In the first profile, the barrier for release of glutathione from the enzyme is shown equal to the barrier for k_{cat} because the mechanism of Scheme V requires that $k_5 = k_4$ in order for the kinetically observed K_a to correspond exactly to the ionization of glutathione on the enzyme. For the isozyme 2-2 profile, rate constants used were $k_{cat} = 26 \pm 11\ s^{-1}$ and $k_{cat}/K_c = 3880 \pm 390\ M^{-1}\ s^{-1}$. To obtain these rate constants, an enzyme molecular mass (for the dimer) of 50 000 g/mol was used for both isozymes.

suggest that the rapid equilibrium assumption is not valid for binding of glutathione in this kinetic mechanism. Under the conditions of the isotope effect measurements (pL = 8.0), free glutathione should be substantially protonated, and in agreement with the conclusions reached[5b,c] concerning isozymes 3-3 and 4-4, the enzyme is shown binding the protonated form of the substrate.

Beginning with the isotope effect on V/K_c (0.793 ± 0.013), the mechanism proposed in Scheme V can be justified in terms of our observations. V/K_c corresponds to conditions of saturating concentrations of glutathione and infinitely dilute 1-chloro-2,4-dinitrobenzene. Under these conditions with the rapid equilibrium assumption applied to the boxed species, the initial velocity expression reduces to the hyperbolic form of the Michaelis–Menten equation, and V/K_c is determined by the reactant state E·GS⁻ and one or more transition states appearing after EC·GS⁻. Because the isotope effect is inverse, the all-protonic sites undergoing changes must give rise to a product of fractionation factors that is closer to unity than the reactant state. It is convenient to decompose the observed isotope effect of 0.79 on V/K_c into a fractionation factor of 1 for the effective transition state and a value of 0.80 for the reactant state because it resembles the thiolate ion of the chemical reactions described above.

The near-unit solvent isotope effect on V can be explained by assigning a reactant-state fractionation factor that is identical with the appropriate transition-state value. At pH = 8.0 with saturating levels of both substrates, the reactant state according to Scheme V is EC·GS⁻ and the effective transition state is identical with the transition state corresponding to V/K_c. Having previously assigned a value of 1 for the transition-state fractionation factor, we must assign the same value for EC·GS⁻. In principle, if we removed the rapid equilibrium assumption for binding of the aromatic compound, the initial state for V could be an enzyme–product state and the final state could be the transition state for product release. We have chosen a reactant state that precedes the chemical reaction on the enzyme to maintain consistency with the good Hammett correlation ($\rho^- = 1.2$) seen[5a] with V, albeit using a different isozyme.

A near-unit isotope effect was also observed for V/K_G. According to the mechanism of Scheme V, in the limit of saturating 1-chloro-2,4-dinitrobenzene and infinitely dilute glutathione, the initial state must be EC and free glutathione, and the transition state must be realized during glutathione binding. The relevant isotopic fractionation factors for these states should both be near the fractionation factor for glutathione,[7] 0.456. The transition states beyond EC·GS⁻ do not contribute to V/K_G at limiting values of pH provided that the proton removed from the thiol is present in the solvent (as opposed to being bound to the enzyme) at the states E·GS⁻, EC·GS⁻, and beyond in the Scheme V mechanism. Consistent with this provision are the studies of Chen et al.[5a] using glutathione analogues and isozyme 4-4, which show that the pK_a obtained from fits of V/K_G vs pH varies with the identity of the thiol, and each apparent pK_a is significantly lower than the pK_a of a given glutathione analogue. If the apparent pK_a's derived in a simple way from the acid–base properties of the enzyme, there would be no dependence on the identity of the glutathione analogue. The pH dependence of V/K_G (the ascending arm) was explained by Chen et al.[5a] in terms of ionization of glutathione on the enzyme, and according to the mechanism of Scheme V, the proton must be located in the solvent at E·GS⁻ and EC·GS⁻. Contributions to kinetic solvent isotope effects from nonunit fractionation factors associated with the proton will cancel between reactant-state and transition-state terms for V/K_c and V.

Solvent isotope effects on the reaction catalyzed by isozyme 2-2 are similar in magnitude to those of isozyme 1-1 and are

readily explained in terms of the Scheme V mechanism. Because the K_m for 1-chloro-2,4-dinitrobenzene is large for this isozyme (ca. 7 mM), and at concentrations above ~2 mM the background chemical reaction overwhelms the enzymic reaction, isotope effects could not be measured at saturating concentrations of this substrate. Instead, isotope effects on initial velocity were measured as a function of glutathione concentration at a constant, but sub-K_m concentration of 1-chloro-2,4-dinitrobenzene (Figure 6). In the limit of very low levels of saturation of the enzyme by glutathione, the kinetic isotope effect corresponds closely to the isotope effect on V/K_0 (refer to eq 5). According to the mechanism of Scheme V, V/K_0 is determined by k_1 for binding glutathione to the free enzyme. The isotope effect measured (1.048 ± 0.020) is similar to $^{DOD}V/K_G$ measured for isozyme 1-1, which was interpreted above in terms of glutathione binding to enzyme saturated with 1-chloro-2,4-dinitrobenzene. At the other extreme of glutathione concentration, again with relatively low concentrations of the aromatic compound, the isotope effect of 0.813 ± 0.024 on the reaction catalyzed by isozyme 2-2 corresponds to $^{DOD}V/K_c$. This value is nearly identical with the value seen for the isozyme 1-1 reaction (0.793 ± 0.013). It appears that the two isozymes are characterized by the same rate-controlling features for the reaction between glutathione and 1-chloro-2,4-dinitrobenzene.

Catalytic Efficiency of Glutathione Transferase. Comparisons between the reactions catalyzed by the two isozymes and the nonenzymic reaction can be made by using the free-energy profiles shown in Figure 7. Kinetic constants used to generate the diagrams are listed in the figure legend. For isozyme 1-1, free energy profiles are shown for two standard states, one corresponding to conditions of a saturating concentration of 1-chloro-2,4-dinitrobenzene and a relatively low concentration of glutathione, and another standard state reversing the relative concentration levels. With the aromatic compound saturating, the first profile shows glutathione binding, followed by ionization of the thiol and, finally, C–S bond formation. The dotted line shows a two-step nonenzymic reaction involving ionization of the thiol and C–S bond formation. When glutathione is at a saturating concentration (the second profile in the figure), the curve for the enzymic reaction traces thiol ionization, 1-chloro-2,4-dinitrobenzene binding, and C–S bond formation.

By use of the profiles, the catalytic efficiency of the enzyme can be artificially separated into two components. The first component includes all the of the features of the protein associated with reducing the pK_a of the thiol. If all of these features are carried into the transition state for C–S bond formation, the maximum rate acceleration for this factor (seen at pH values less than ~6) is given by the ratio of the enzymic to nonenzymic K_a's (ca. 100). At pH = 8.0, this limiting factor is diminished to 10, which corresponds to 1.36 kcal/mol. The second component of the catalytic efficiency can be assigned to all remaining features of the transition state for C–S bond formation. The effective molarity (k_{cat}/k_s) is a convenient way to express the effect of these factors. For isozyme 1-1, the effective molarity is modest at 14 M and may reflect in large part the energy required to assemble the thiolate ion and the aromatic compound in an optimal arrangement for reaction.

Additional transition-state stabilization is also revealed in the solvent isotope effects as described by Scheme V. The change in the thiol-related fractionation factor from 0.80 for E·GS$^-$ to 1.0 for EC·GS$^-$ and the subsequent transition state suggests a change in the hydrogen bonding to the thiolate ion, similar to the desolvation of the ion seen in the chemical reaction. However, the extent of desolvation appears to be nearly complete in the enzymic transition state,[20] as opposed to 34% complete in the nonenzymic case. Because K_{a1} and K_{a2} (see Scheme V) appear to be similar (the apparent K_a's measured extracted from the kinetic data under the diverse conditions of Figure 3 are similar), the stability of the thiolate in E·GS$^-$ and EC·GS$^-$ must be comparable. The change in fractionation factor without an associated change in thiolate stability may reflect a change in the anion-stabilizing factors from hydrogen bonding (to solvent or protein residues) to electrostatic interactions, which might offer more favorable stabilization of the transition state for C–S bond formation. The negative Brønsted coefficient measured by Chen et al.[5a] for reactions with isozyme 4-4 using a series of glutathione analogues is in agreement with this mechanism. Their measurements were made under conditions of saturating concentrations of glutathione analogue and subsaturating concentrations of 1-chloro-2,4-dinitrobenzene, conditions that closely correspond to our V/K_c (reactant state is E·GS$^-$ in Scheme V). Chen, et al.[5a] suggested that a desolvation phenomenon might explain their observations, and through our analysis of solvent isotope effects, we find additional support for this idea.

A comparison of the middle and right panels of Figure 7 demonstrates that isozyme 2-2 is a poorer catalyst than isozyme 1-1 for the reaction with 1-chloro-2,4-dinitrobenzene when the aromatic compound is in low concentration, but at saturating concentrations, the catalytic efficiencies appear to be similar. k_{cat} is difficult to measure for isozyme 2-2 because the K_m for the aromatic compound is large (ca. 7 mM), and at higher concentrations, the rate of the background reaction overwhelms the enzymic reaction. Using data collected up to 2.0 mM of the substrate, we estimate k_{cat} for isozyme 2-2 (26 ± 11 s^{-1}) to be similar to the better determined value for isozyme 1-1 (32.0 ± 1.7 and 35.9 ± 1.6 s^{-1} in different experiments). The essential difference between the isozymes appears to reside in the binding site for the aromatic compound. The similar k_{cat} values and the similar solvent isotope effects suggest that the reactivity of the thiolate ion on the proteins are nearly identical on the two isozymes. The various glutathione transferase isozymes may show common strategies for stabilizing the thiolate region of transition states and achieve their specificity merely through differences in the binding sites for the second substrate.

Experimental Section

Materials. Reduced glutatione (γ-Glu-Cys-Gly) and DL-dithiothreitol (*threo*-1,4-dimercapto-2,3-butanediol) were purchased from Calbiochem; 1-chloro-2,4-dinitrobenzene and bis-tris propane buffer [1,3-bis[tris(hydroxymethyl)methylamino]propane] were obtained from Sigma Chemical Co.; ethyl-d_5 alcohol-*d* and deuterium chloride were from Aldrich; deuterium oxide was obtained from Merck Sharp and Dohme Isotopes. Glutathione transferase isozymes 1-1 and 2-2 were expressed in *Escherichia coli* from plasmide pKK-GTB34[4d] and pKK-GTB2[21] encoding cDNA of the respective glutathione transferase isoenzymes. The purified isoenzymes were identified by SDS gel electrophoresis and HPLC elution profile.[22] Protein concentrations were determined by modified Lowry assay.[23]

Initial Velocity Measurements. Initial velocities were measured by monitoring the absorbance of the product (ϵ = 9600 cm^{-1} M^{-1})[24] at 340 nm as a function of time with a UV–visible spectrophotometer (Varian Model 2290). The extinction coefficient showed no isotope effect under the conditions of our experiments ($\epsilon_{HOH}/\epsilon_{DOD}$ = 1.009 ± 0.014). The analog chart-recorder signal was digitized and stored on a microcomputer, where least-squares estimates of the slopes of lines were used to obtain intitial velocities. For experiments over the course of a day, fresh solutions of the thiol were prepared and separated into several aliquots, each stored under nitrogen. A new aliquot was opened for use every hour over the course of the experiments. pD was determined for D_2O solutions by adding 0.4 to the reading measured on a pH meter with a combination glass electrode.[7] 1-Chloro-2,4-dinitrobenzene at an appropriate concentration was always prepared in ethanol (or ethanol-d_6), and for each run

(20) The solvent isotope effects are consistent with many choices for the rate-limiting transition state among all the processes we have lumped into k_5, including the choice of rate-limiting Meisenheimer complex formation discussed by Chen et al.[5a] The essential feature of our interpretation is that the thiolate anion appears to resemble a desolvated thiolate anion at the complex EC·GS$^-$ (see Scheme V), and no changes in protonic sites sufficient to alter the solvent isotope effects appear in the rate-limiting step of k_5.

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40 μL of this solution was used for each 1-mL kinetic run. The solvent for all experiments is thus 4% ethanol/96% water, by volume. Exact conditions for experiments are listed in the tables and figure legends.

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Registry No. GSH, 70-18-8; DL-DTT, 27565-41-9; CDNB, 97-00-7; GSH transferase, 50812-37-8.

Bleomycin-Mediated Degradation of Aristeromycin-Containing DNA. Novel Dehydrogenation Activity of Iron^{II}–Bleomycin

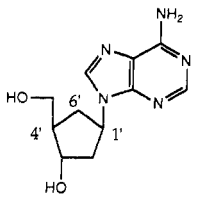
Hiroshi Sugiyama,† Takashi Sera,† Yukihiro Dannoue,† Ryuji Marumoto,‡ and Isao Saito*,†

Contribution from the Department of Synthetic Chemistry, Faculty of Engineering, Kyoto University, Kyoto 606, Japan, and the Central Research Division, Takeda Chemical Industries, Ltd., Osaka 532, Japan. *Received July 13, 1990*

Abstract: The antitumor antibiotic bleomycin (BLM) induces C–H bond scission at the C4′ of deoxyribose moiety of DNA in the presence of Fe(II) and O_2. To investigate the primary oxidation step, 2′-deoxyaristeromycin (Ar) possessing a cyclopentane ring instead of a ribofuranose ring was incorporated into the BLM-cleaving site of synthetic oligonucleotide d(GGArAGG). It was found that an unprecedented dehydrogenation occurs effectively at the C4′ and C6′ positions of the Ar moiety to give 2′-deoxyneplanocin A containing oligonucleotide together with a minor but stereospecific C4′ hydroxylation in the Fe^{II}–BLM-mediated degradation of duplex d(GGArAGG)–d(CCTTCC). An intermediate C4′ carbocation derived from one-electron oxidation of initially formed C4′ radical has been proposed for the dehydrogenation reaction.

While various types of site specifically modified oligonucleotides have been used as a probe for DNA–ligand interactions, e.g., DNA–protein and DNA–drug interactions,[1] the use of such modified oligonucleotides as a probe for the mechanism of DNA degradation by antitumor antibiotics has attracted only little attention. Antitumor antibiotic bleomycins (BLMs) are known to induce C–H bond scission at the C4′ of the deoxyribose moiety of DNA in the presence of Fe(II) and O_2.[2] Under aerobic conditions, O_2-dependent products such as base propenals and 5′-phosphate and 3′-phosphoglycolate termini are produced as the major products,[3] whereas at limiting O_2 concentrations C4′ hydroxylation occurs preponderantly to give alkali-labile products with concomitant release of free bases.[3,4] While the structures of these ultimate degradation products have been well established,[3b,5] the exact nature of the initial step of the C4′ oxidation is still a matter of debate.[2,6] One major difficulty in getting closer insight into the mechanism of the primary oxidation step is due to the inherent instability of the initial product of hemiketal structure derived from deoxyribose oxidation (Scheme I).

We reasoned that if 2′-deoxyaristeromycin (Ar, **1**), a carbocyclic analogue of 2′-deoxyadenosine possessing a cyclopentane ring instead of a ribofuranose ring, is incorporated into the BLM-cleaving site of a synthetic oligonucleotide, such modified oligonucleotide would then serve as a useful probe for investigating the primary oxidation step, e.g., the stereochemical outcome of C4′ hydroxylation of the deoxyribose moiety. Such an Ar-containing oligonucleotide would also serve as a useful probe for an O_2-labeling experiment due to the lack of the exchange of functional groups of sugar oxidation products with solvent water, which is inevitable in the oxidation of normal DNA.[3c,7]



2′-deoxyaristeromycin (**1**)

We describe here our results on the degradation of an Ar-containing deoxyhexanucleotide induced by Fe^{II}–peplomycin

† Kyoto University.
‡ Takeda Chemical Industries, Ltd.

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