Spectroscopic and Kinetic Evidence for the Thiolate Anion of Glutathione at the Active Site of Glutathione S-Transferase†

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ABSTRACT: Ultraviolet difference spectroscopy of the binary complex of isozyme 4-4 of rat liver glutathione S-transferase with glutathione (GSH) and the enzyme alone or as the binary complex with the oxygen analogue, γ-L-glutamyl-L-serylglycine (GOH), at neutral pH reveals an absorption band at 239 nm (ϵ = 5200 M^{-1} cm^{-1}) that is assigned to the thiolate anion (GS^-) of the bound tripeptide. Titration of this difference absorption band over the pH range 5–8 indicates that the thiol of enzyme-bound GSH has a pK_a = 6.6, which is about 2.4 pK units less than that in aqueous solution and consistent with the kinetically determined pK_a previously reported [Chen et al. (1988) *Biochemistry 27*, 647]. The observed shift in the pK_a between enzyme-bound and free GSH suggests that about 3.3 kcal/mol of the intrinsic binding energy of the peptide is utilized to lower the pK_a into the physiological pH range. Apparent dissociation constants for both GSH and GOH are comparable and vary by a factor of less than 2 over the same pH range. Site occupancy data and spectral band intensity reveal large extinction coefficients at 239 nm (ϵ = 5200 M^{-1} cm^{-1}) and 250 nm (ϵ = 1100 M^{-1} cm^{-1}) that are consistent with the existence of either a glutathione thiolate ($E{\cdot}GS^-$) or ion-paired thiolate ($EH^+{\cdot}GS^-$) in the active site. The observation that GS^- is likely the predominant tripeptide species bound at the active site suggested that the carboxylate analogue of GSH, γ-L-glutamyl-(D,L-2-aminomalonyl)glycine, should bind more tightly than GSH. Replacement of the CH_2SH group of GSH with CO_2^- results in a mixture of diastereomeric peptides, comprising an effective competitive inhibitor ($K_{i(obs)}$ = 0.74 μM) of the enzyme and binding 30–90-fold more tightly at pH 6.5 than GSH (K_D = 22 μM). Inasmuch as the γ-glutamyl-(D,L-2-aminomalonyl)glycine could only be synthesized as a mixture of the L,D and L,L diastereomers, the stereochemical preference for binding was examined with the configurationally stable aspartate analogues, γ-L-glutamyl-L-aspartylglycine and γ-L-glutamyl-D-aspartylglycine. The L,L diastereomer (K_i = 0.93 μM) binds about 25 times more tightly than GSH, whereas the incorrectly configured L,D diastereomer (K_i = 47 μM) binds 2-fold less tightly than GSH. The carboxylate anion is, therefore, a sufficiently good mimic of the methylene thiolate group, such that roughly 2.0–2.6 kcal/mol of the 3.3 kcal/mol intrinsic binding energy of GSH that is utilized to destabilize the sulfhydryl group can be realized as enhanced binding of the "preionized" carboxylate peptide. The results suggest the existence of a positively charged electrostatic field in the active site of the enzyme that functions to destabilize the thiol of enzyme-bound GSH.

The glutathione S-transferases are a family of prototypic detoxication enzymes involved in the metabolism of electrophilic compounds. Recent reviews of their properties and participation in the metabolism of endogenous and xenobiotic compounds are available (Jakoby & Habig, 1980; Mannervik, 1985; Armstrong, 1987). Although the enzymes exhibit broad, overlapping structural specificity for the electrophilic substrate, they are, not surprisingly, quite specific for the physiologic cosubstrate glutathione (GSH).[1] Reasonable chemical intuition suggests that the active nucleophilic species in the enzyme-catalyzed reaction is the thiolate anion, GS^-. However, no direct and very little indirect evidence concerning the existence of GS^- in the active site has been forthcoming (Jakoby & Habig, 1980; Armstrong, 1987). Probably the strongest indirect evidence for GS^- as the active nucleophilic species in the enzyme-catalyzed reaction is the dependence of k_c/K_m^s (for 1-chloro-2,4-dinitrobenzene at saturating GSH concentration) on a single ionization with an apparent pK_a = 6.6, which has been assigned to the thiol in the binary enzyme–GSH complex (Chen et al., 1988 This apparent shift in the pK_a of the thiol from about 9.0 in aqueous solution (Reuben & Bruice, 1976; Jung et al., 1972) to 6.6 on the enzyme surface requires that some 3.3 kcal/mol of the intrinsic binding energy of GSH be utilized for that purpose.

In this paper we report the direct observation, by UV absorption spectroscopy, of GS^- in binary complexes with isozyme 4-4 of GSH transferase from rat liver. The pK_a of the bound thiol can be determined by direct titration of the ultraviolet absorption band at 239 nm assigned to GS^-. Furthermore, synthesis of the carboxylate analogues of GSH, γ-L-glutamyl-(D,L-2-aminomalonyl)glycine, γ-L-glutamyl-L-as-

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[1] Abbreviations: GSH, glutathione; GOH, γ-L-glutamyl-L-serylglycine; $GSCH_3$, S-methylglutathione; GH, γ-L-glutamyl-L-alanylglycine; t-BOC, *tert*-butyloxycarbonyl; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Tris, tris(hydroxymethyl)aminomethane; TMS, tetramethylsilane; TAPS, 3-[[tris(hydroxymethyl)methyl]-amino]propanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid.

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partylglycine, and γ-L-glutamyl-D-aspartylglycine, and their use as competitive inhibitors of the enzyme permit the observation of a portion of the intrinsic binding energy (Jencks, 1975), normally utilized to lower the pK_a of GSH, as enhanced binding of inhibitors that have the correct stereochemistry.

Experimental Procedures

Materials

Reagents and Buffers. Reduced glutathione, *S*-methylglutathione, and buffer materials were obtained from Sigma Chemical Co. Chemical reagents were purchased from either Aldrich Chemical Co. or Sigma. Tritiated glutathione [*glycine*-2-^{3}H], 1000 Ci/mol was from New England Nuclear. The oxygen, γ-L-glutamyl-L-serylglycine, and desthio, γ-L-glutamyl-L-alanylglycine, analogues of GSH were synthesized as described by Chen et al. (1985).

Enzyme. Isoenzyme 4-4 of rat liver GSH transferase was purified and characterized as previously described (Chen et al., 1988). Concentration of active sites was determined spectrophotometrically from the molar extinction coefficient [ϵ_{270}(native) = 36 700 M^{-1} cm^{-1}] calculated for the polypeptide (M_r = 26 000). This value was obtained by calculating ϵ_{270} for the denatured protein (Demchenko, 1986b) from the known tyrosine and tryptophan content (Ding et al., 1986) and relating this value to the optical densities of native and denatured (6 M guanidine hydrochloride) stock protein solutions by ϵ_{270}(native) = ϵ_{270}(denatured) [A_{270}(native)/A_{270} (denatured)].

Synthesis of N-t-BOC-2-aminomalonic Acid Dibenzyl Ester (**4**). The protected amino acid **4** was prepared by *tert*-butyloxycarbonylation of 2-aminomalonic acid (Kinugasa et al., 1969) according to the general method of Nagasawa et al. (1973) followed by benzyl esterification by the method of Wang et al. (1977). To a solution of 1.2 g (10 mmol) of 2-aminomalonic acid and 4.2 mL (30 mmol) of triethylamine in 5.5 mL of water was added a solution of 2.6 g (11 mmol) of *tert*-butyl *S*-(4,6-dimethylpyrimidin-2-yl)thiocarbonate (Aldrich) in 5.5 mL of dimethylformamide. After the mixture was stirred 5 h at room temperature, 15 mL of water was added and the unreacted carbonate was extracted with two 20-mL portions of ethyl acetate. The aqueous layer was cooled to 0 °C, adjusted to pH 2 with 5 N HCl, and extracted with one 20-mL and two 10-mL portions of ethyl acetate. The extracts were combined and washed twice with 10 mL of 5% HCl and twice with 10 mL of saturated NaCl and dried over anhydrous Na_2SO_4. The solvent was evaporated in vacuo to give 1.3 g (59%) of *N*-t-BOC-2-aminomalonic acid as an oil. The crude material, which was unstable to heating, was used without further purification.

One gram (4.6 mmol) of *N*-t-BOC-2-aminomalonic acid was dissolved in 20 mL of methanol and 2 mL of water. A solution of 1.49 g (4.6 mmol) of Cs_2CO_3 in 10 mL of water was added. The mixture was evaporated to dryness and the residue reevaporated twice from 12 mL of DMF at room temperature. The cesium salt was then stirred with 1.56 g (9.2 mmol) of benzyl bromide in 12 mL of DMF for 6 h. After evaporation to dryness, the product was dissolved in 20 mL of ethyl acetate, washed twice with 10 mL of H_2O, and dried over anhydrous Na_2SO_4. After evaporation of the solvent and chromatography on silica gel (Merck, grade 60) with hexane–benzene, 1.4 g (77%) of **4** was obtained: mp 86–87 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.49 (s, 9 H, t-BOC), 5.08 (d, 1 H, *J* = 7.7 Hz, CH), 5.13 and 5.22 (ABq, 4 H, *J* = 12.2 Hz, benzylic CH_2), 5.60 (br d, 1 H, *J* = 7.7 Hz, NH), 7.2–7.4 (m, 10 H). Anal. Calcd for $C_{22}H_{25}NO_6$: C, 66.15; H, 6.31; N, 3.51. Found: C, 66.40; H, 6.47; N, 3.44.

Synthesis of (N-t-BOC-O-benzyl-2-aminomalonyl)glycine Benzyl Ester (**5**). The protected dipeptide **5** was prepared by partial hydrolysis of **4** followed by coupling to glycine benzyl ester. A solution of 0.14 g (2.5 mmol) of KOH in 4 mL of benzyl alcohol was gradually added to a solution of 1.0 g (2.5 mmol) of **4** in 4 mL of benzyl alcohol. After stirring 16 h at room temperature, 30 mL of H_2O and 50 mL of ethyl acetate were added to the solution. The aqueous layer was washed twice with 20 mL of ethyl acetate, cooled to 0 °C, adjusted to pH 2 with 5 M HCl, and extracted with one 20-mL and two 10-mL portions of ethyl acetate. The combined ethyl acetate extract was washed with two 10-mL portions of 5% HCl and twice with 10 mL of aqueous NaCl and dried over anhydrous Na_2SO_4. The solvent was evaporated to give 0.34 g (44%) of crude racemic *N*-t-BOC-2-aminomalonic acid monobenzyl ester, which was used without further purification.

To a solution of 0.34 g (1.1 mmol) of the monobenzyl ester, 0.37 g (1.1 mmol) of glycine benzyl ester *p*-tosylate (Sigma) and 0.11 g (1.1 mmol) of triethylamine in 10 mL of CH_2Cl_2 was added to 0.25 g (1.2 mmol) of dicyclohexylcarbodiimide. The mixture was stirred 16 h at room temperature. After addition of 0.1 mL of glacial acetic acid, the dicyclohexylurea was removed by filtration and washed with 5 mL of methylene chloride. The combined filtrates were washed with two 10-mL portions of 5% HCl, 5% $NaHCO_3$, and saturated NaCl and dried over anhydrous Na_2SO_4. Evaporation of solvent and chromatography on silica gel eluted with benzene–ethyl acetate gave 0.41 g (82%) of **5**: mp 73–75 °C; ^{1}H NMR (200 MHZ, $CDCl_3$) δ 1.42 (s, 9 H, t-BOC), 4.04 (d, 2 H, *J* = 5.3 Hz, Gly-CH_2), 4.96 (d, 1 H, *J* = 7.0 Hz, CH), 5.17 (s, 2 H, benzylic CH_2), 5.17 and 5.25 (AB q, 2 H, *J* = 12.2 Hz, benzylic CH_2), 5.79 (br d, 1 H, *J* = 7.0 Hz, N*H*CH), 6.94 (br t, 1 H, N*H*CH_2), 7.3–7.5 (m, 10 H). High-resolution FAB mass spectrum for $C_{24}H_{28}N_2O_7 + H_1$, calcd 457.1975. Found *m/z* (rel intensity) 457.1959 (6%).

Synthesis of [(N-t-BOC-O-benzyl-γ-L-glutamyl)-O-benzyl-2-aminomalonyl]glycine Benzyl Ester (**6**). To a suspension of 2.38 g (5.22 mmol) of **5** in 20 mL of glacial acetic acid cooled to 0 °C was added 40 mL of anhydrous CF_3CO_2H. The mixture was stirred 30 min at room temperature. The solvents were removed under vacuum. The residue was lyophilized three times with H_2O. The crude hygroscopic product was dissolved in 20 mL of CH_2Cl_2 to which was added 1.76 g (5.22 mmol) of *N*-t-BOC-α-L-glutamic acid benzyl ester (Sigma), 0.63 g (6.23 mmol) of triethylamine, and 1.40 g (6.8 mmol) of dicyclohexylcarbodiimide. The mixture was stirred 10 h at room temperature. Acetic acid (0.5 mL) was added, and the dicyclohexylurea was removed by filtration and washed with 5 mL of CH_2Cl_2. The combined filtrates were washed with two 15-mL portions of 5% HCl, 5% $NaHCO_3$, and saturated NaCl and dried over Na_2SO_4. Evaporation of solvent and chromatography on silica gel with benzene–ethyl acetate gave 2.11 g (60%) of **6** as a 2:1 mixture of the two diastereomers: mp 118–122 °C; $[\alpha]^{27}_D$ −6.1° ($CH_3CO_2CH_2CH_3$, *c* 1); ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.40 and 1.42 (2 s, 9 H, t-BOC), 1.7–2.5 (m, 4 H, $CHCH_2CH_2$), 3.9–4.2 (m, 2 H, $NHCH_2CO_2Bz$), 4.25–4.45 (br t, 1 H, $CHCH_2CH_2$), 5.1–5.4 (m, 8 H, 3 × benzylic CH_2 + t-BOC-N*H* + NHC*H*CONH), 6.81 and 6.92 (2 br d, 1 H, N*H*CHCONH), 6.99 and 7.22 (2 br t, 1 H, N*H*CH_2), 7.3–7.5 (m, 15 H). Anal. Calcd for $C_{36}H_{41}N_3O_{10}$: C, 63.99; H, 6.12; N, 6.22. Found: C, 63.71; H, 6.30; N, 6.25.

Synthesis of γ-L-Glutamyl-(D,L-2-aminomalonyl)glycine (**1**). To a suspension of 0.5 g (0.74 mmol) of **6** in 5 mL of glacial acetic acid at 0 °C was added 10 mL of anhydrous

CF_3CO_2H. The mixture was stirred 30 min at room temperature and the solvent removed. The residue was dissolved in a mixed solvent of 10 mL of ethanol and 10 mL of glacial acetic acid. The solution was hydrogenated at room temperature (70 psi of H_2) over 200 mg of 5% palladium on charcoal for 12 h. The catalyst was removed by filtration. After evaporation of the solvent in vacuo, the oily residue was washed three times with ether and dried in vacuo. The residue was purified by chromatography on a 2.5 × 35 cm bed of Bio-Rad AG1-X2 acetate eluted with 200 mL of 50 mM acetic acid followed by a 2-L linear gradient of 50 mM acetic acid to 2 M formic acid. The peptide, which eluted between 1.2 and 1.3 L, was lyophilized three times from H_2O to give 120 mg (53%) of **1**: $[\alpha]^{27}_D$ −0.2° (H_2O, pH 7, *c* 1); ^1H NMR (200 MHz, D_2O, pD ≈ 2.3) δ 1.9–2.1 (m, 2 H, $CHCH_2CH_2$), 2.40 (t, 2 H, *J* = 7.3 Hz, $CHCH_2CH_2$), 3.76 (t, 1 H, *J* = 6.0 Hz, $CHCH_2CH_2$), 3.83 (s, 2 H, $NHCH_2$), 4.85 (s, 1 H, NHCHCONH) (after 2 h in D_2O, resonance at δ 4.85 is absent); ^{13}C NMR (100 MHz, 75% H_2O, 25% D_2O, pH ≈ 2.3) δ 28.4 ($CHCH_2CH_2$), 33.7 ($CHCH_2CH_2$), 44.3 ($NHCH_2$, minor isomer), 44.4 ($NHCH_2$, major isomer), 55.9 ($CHCH_2CH_2$), 60.76 (NHCHCONH, minor isomer), 60.84 (NHCHCONH, major isomer), 171.4 (minor isomer), 171.5 (major isomer), 172.8, 175.3, 175.8, 176.9 (minor isomer) 177.0 (major isomer); ^{13}C NMR (100 MHz, D_2O pD ≈ 7.0) δ 29.4, 34.6, 46.9, 57.5, 62.7, 172.4, 174.6, 177.2, 177.3, 179.1. Resonance at 62.7 ppm collapses to at 1:1:1 triplet at 62.4 ppm, $^1J_{^2H,^{13}C}$ = 24 Hz upon deuterium exchange. High-resolution FAB mass spectrum for $C_{10}H_{15}O_8N_3 + H_1$, calcd 306.0933. Found *m/z* (rel intensity) 306.0937 (4%).

*Synthesis of (N-t-BOC-β-benzyl-*L*-aspartyl)glycine Benzyl Ester* (**7**). *N*-t-BOC-L-aspartic acid β-benzyl ester (Sigma) was coupled to glycine benzyl ester *p*-tosylate by the procedure described above for **5**. The dipeptide **7** was obtained in 77% yield: mp 68–70 °C; $[\alpha]^{27}_D$ −12.6° ($CH_3CO_2C_2H_5$, *c* 1); ^1H NMR (200 MHz, $CDCl_3$) δ 1.45 (s, 9 H, t-BOC), 2.73 (dd, 1 H, *J* = 6.2 and 17.2 Hz, CHCHH), 3.08 (dd, 1 H, *J* = 4.4 and 17.2 Hz, CHCHH), 4.05 (d, 2 H, *J* = 5.4 Hz, $NHCH_2CO_2Bz$), 4.58 (m, 1 H, $CHCH_2$), 5.10 and 5.17 (AB q, 2 H, *J* = 12.3 Hz, benzylic CH_2), 5.18 (s, 2 H, benzylic CH_2), 5.68 (br d, 1 H, *J* = 8.2 Hz, t-BOC–NH), 7.01 (br t, 1 H, $NHCH_2$), 7.3–7.4 (m, 10 H). Anal. Calcd for $C_{25}H_{30}N_2O_7$: C, 63.82; H, 6.43; N, 5.95. Found: C, 63.79; H, 6.38; N, 5.95.

*Synthesis of (N-t-BOC-β-benzyl-*D*-aspartyl)glycine Benzyl Ester* (**8**). The D enantiomer was prepared as described above in 75% yield from *N*-t-BOC-D-aspartic acid β-benzyl ester: $[\alpha]^{27}_D$ +12.8° ($CH_3CO_2C_2H_5$, *c* 1). Spectra properties and melting point were identical with those of **7**. Anal. Calcd for $C_{25}H_3N_2O_7$: C, 63.82; H, 6.43; N, 5.95. Found: C, 64.00; H, 6.37; N, 5.90.

*Synthesis of [(N-t-BOC-O-benzyl-γ-*L*-glutamyl)-β-benzyl-*L*-aspartyl]glycine Benzyl Ester* (**9**). Deprotection of **7** followed by coupling to *N*-t-BOC-α-L-glutamic acid benzyl ester by the procedure described for **6** gave the fully protected L,L tripeptide **9** in 56% yield: mp 110–113 °C; $[\alpha]^{27}_D$ −26.4° ($CH_3CO_2C_2H_5$, *c* 1); ^1H NMR (200 MHz, $CDCl_3$) δ 1.40 (s, 9 H, t-BOC), 1.8–2.5 (m, 4 H, $CHCH_2CH_2$), 2.76 (dd, 1 H, *J* = 6.3 and 17.0 Hz, CHCHH), 2.98 (dd, 1 H, *J* = 5.1 and 17.0 Hz, CHCHH), 3.97 (dd, 1 H, *J* = 5.6 and 18.1 Hz, NHCHH), 4.07 (dd, 1 H, *J* = 5.6 and 18.1 Hz, NHCHH), 4.34 (br t, 1 H, $CHCH_2CH_2$), 4.89 (m, 1 H, $NHCHCH_2CO_2Bz$), 5.0–5.2 (m, 6 H, three benzylic CH_2), 5.40 (br d, 1 H, *J* = 8.0 Hz, t-BOC–NH), 7.03 (br d, 1 H, *J* = 8.0 Hz, $NHCHCH_2CO_2Bz$), 7.2–7.5 (m, 16 H, aromatic 15 H + $NHCH_2$). Anal. Calcd for $C_{37}H_{43}N_3O_{10}$: C, 64.43; H, 6.28; N, 6.09. Found: C, 64.55; H, 6.41; N, 6.17.

*Synthesis of [(N-t-BOC-O-benzyl-γ-*L*-glutamyl)-β-benzyl-*D*-aspartyl]glycine Benzyl Ester* (**10**). The fully protected L,D tripeptide **10** was prepared from **8** in 62% yield as described above for **9**: mp 104–106 °C; $[\alpha]^{27}_D$ +12.1° ($CH_3CO_2C_2H_5$, *c* 1); ^1H NMR (200 MHz, $CDCl_3$) δ 1.38 (s, 9 H, t-BOC), 1.6–2.5 (m, 4 H, $CHCH_2CH_2$), 2.70 (dd, 1 H, *J* = 5.9 and 17.1 Hz, CHCHH), 3.11 (dd, 1 H, *J* = 4.6 and 17.1 Hz, CHCHH), 3.96 (dd, 1 H, *J* = 5.7 and 17.9 Hz, NHCHH), 4.08 (dd, 1 H, *J* = 5.7 and 17.9 Hz, NHCHH), 4.35 (m, 1 H, $CHCH_2CH_2$), 4.95 (m, 1 H, $NHCHCH_2CO_2Bz$), 5.0–5.3 (m, 6 H, three benzylic CH_2), 5.33 (br d, 1 H, *J* = 8.3 Hz, t-BOC–NH), 6.87 (br d, 1 H, *J* = 8.6 Hz, $NHCHCH_2CO_2Bz$), 7.2–7.5 (m, 15 H), 7.61 (br t, 1 H, $NHCH_2$). Anal. Calcd for $C_{37}H_{43}N_3O_{10}$: C, 64.43; H, 6.28; N, 6.09. Found: C, 64.35; H, 6.44; N, 5.95.

*Synthesis of γ-*L*-Glutamyl-*L*-aspartylglycine* (**2**). The protected tripeptide **9** (1.0 g) was dissolved in 50 mL of anhydrous CF_3CO_2H at 0 °C under N_2. Anhydrous HBr was bubbled through the solution for 30 min at 0 °C and then at room temperature for 2 h. The solution was then purged with N_2 for 30 min. CF_3CO_2H was removed in vacuo to give a tan oil that solidified upon addition of diethyl ether. The solid was washed three times with ether and dried in a vacuum desiccator. The product was purified by chromatography on a 2.5 × 35 cm bed of Bio-Rad AG1-X2 acetate eluted with 200 mL of 50 mM CH_3CO_2H followed by a 2-L linear gradient of 50 mM CH_3CO_2H to 2 M formic acid. The product, which eluted between 600 and 700 mL, was lyophilized three times with H_2O to give 350 mg (76%) of **2**: $[\alpha]^{27}_D$ −4.3° (H_2O, pH 7, *c* 1), ^1H NMR (200 MHz, D_2O, pD ~ 3.5) δ 1.9–2.1 (m, 2 H, $CHCH_2CH_2$), 2.35 (t, 2 H, *J* = 7.3 Hz, $CHCH_2CH_2$), 2.65 (dd, 1 H, *J* = 8.0 and 16.9 Hz, CHCHH), 2.81 (dd, 1 H, *J* = 5.5 and 16.9 Hz, CHCHH), 3.68 (t, 1 H, *J* = 6.1 Hz, $CHCH_2CH_2$), 3.81 (s, 2 H, $NHCH_2$), 4.65 (dd, 1 H, *J* = 5.5 and 8.0 Hz, $CHCH_2$); ^{13}C NMR (100 MHz, D_2O, pD ~ 7.0) δ 29.1, 34.4, 41.9, 46.4, 54.7, 57.2, 176.1, 176.6, 177.7, 179.2, 180.7.

*Synthesis of γ-*L*-Glutamyl-*D*-aspartylglycine* (**3**). The protected L,D tripeptide **10** was deprotected and the product purified as described above for **2** to give **3** in 82% yield: $[\alpha]^{27}_D$ +6.7° (H_2O, pH 7, *c* 1); ^1H NMR (200 MHz, D_2O, pD ~ 3.0) δ 1.9–2.1 (m, 2 H, $CHCH_2CH_2$), 2.36 (t, 2 H, *J* = 7.1 Hz, $CHCH_2CH_2$), 2.65 (dd, 1 H, *J* = 8.0 and 16.9 Hz, CHCHH), 2.80 (dd, 1 H, *J* = 5.6 and 16.9 Hz, CHCHH), 3.67 (t, 1 H, *J* = 6.1 Hz, $CHCH_2CH_2$), 3.81 (s, 2 H, $NHCH_2$), 4.65 (dd, 1 H, *J* = 5.6 and 8.0 Hz, $CHCH_2$); ^{13}C NMR (100 MHz, D_2O, pD ~ 7.0) δ 29.2, 34.5, 42.0, 46.5, 54.8, 57.3, 176.1, 177.0, 177.8, 179.3, 180.8. High-resolution FAB mass spectrum for $C_{11}H_{17}N_3O_8 + H_1$, calcd 320.1094. Found *m/z* (rel intensity) 320.1107 (1%).

Methods

Equilibrium Dialysis. The rapid equilibrium dialysis technique of Cheng and Carlson (1983) using conical cells (Type D cell with 76-μL capacity) was used. All buffers contained 1 mM EDTA and 1 mM DTT and were degassed before use. Experiments were performed at three pH values: 0.1 M MES (pH 5.5), 0.1 M KH_2PO_4 (pH 6.5), and 0.1 M Tris (pH 8.0). Dialysis chambers were separated with 76 mm diameter disks of Spectra Por 2 membranes (Spectrum Medical Industries) pretreated as described by McPhie (1971). Experiments were initiated by placing 50 μL of enzyme solution (~25 μM active sites) in one compartment and 50 μL of [^{3}H]GSH (ca 10000 cpm) in the second compartment with

initial concentrations ranging from 2 to 70 μM. Dialysis chambers were rotated at 30–50 rpm for 2 h at 23 °C after which time 20-μL aliquots from each compartment were analyzed by liquid scintillation counting. Scatchard plots were constructed to obtain the observed K_D values and stoichiometries of binding.

UV Difference Spectroscopy. UV difference spectra were recorded on a Perkin-Elmer Lambda 4B UV–visible double-beam spectrometer equipped with a double-monochromator with stray light of <0.0005%. Spectra were recorded with a bandwidth of 1 nm in 1-cm cells under conditions (40 μM active sites and 120 μM GSH) where 80–90% of the active sites were occupied. Spectra of the binary enzyme–GSH complexes were recorded between 320 and 235 nm and stored. Difference spectra were recorded through subtraction of either the spectrum of enzyme alone or the binary enzyme–GOH complex. Difference spectra were obtained at 0.5 pH unit intervals between pH 5 and 8 by using the following buffers (0.1 M) containing 1 mM EDTA: pH 5.0, 5.5, and 6.0, MES; pH 6.5 and 7.0, KH_2PO_4; pH 7.5, MOPS; pH 8.0, Tris. Difference spectra at each pH were recorded at least twice. The observed ϵ_{239} at each pH was calculated for the binary complex from the intensity of the difference absorption band combined with the site occupancy calculated from actual and interpolated values of K_D^{GSH} obtained from equilibrium dialysis. Absorption intensities at pH 7.5 and 8 were corrected for contributions from unbound thiolate by using a pK_a for GSH(aq) of 9.0 and a ϵ_{239} = 4400 M^{-1} cm^{-1} for GS^-(aq). The pH dependence of log $\epsilon_{239(obs)}$ for the E·GSH complex was analyzed with the program HABELL (Cleland, 1979) to obtain both the pK_a of E·GSH and ϵ_{239} for E·GS^-. Difference spectrophotometric titration of GSH (260 μM) in aqueous solution was performed between pH 8 and 11 by using $GSCH_3$ as the reference with the following buffers (0.04 M): pH 8.0, Tris; pH 8.5, TAPS; pH 9.0 and 9.5, CHES; pH 10.0, 10.5, and 11.0, CAPS.

Kinetic Analysis. All kinetic data were collected at 25 °C as previously described (Chen et al., 1985) with 1-chloro-2,4-dinitrobenzene as the electrophilic substrate. Unless otherwise stated, reactions were run in 0.1 M KH_2PO_4 (pH 6.5) with 1-chloro-2,4-dinitrobenzene fixed at 200 μM. Inhibition experiments were conducted with at least four different, fixed levels of inhibitor with variable concentrations of GSH (20–500 μM). Initial velocities were determined at least in triplicate, corrected for any spontaneous reaction, and analyzed by using the programs HYPER, LINE, and COMP (Cleland, 1979). The pH dependence of K_i^{GOH} was determined between pH 5 and 8 by using the following buffers at 0.1 M concentrations: pH 5.0, 5.5, and 6.0, MES; pH 6.5, PIPES or KH_2PO_4; pH 7.0, KH_2PO_4; pH 7.5 HEPES; and pH 8.0, Tris.

Instrumental Methods. ^1H and ^{13}C NMR spectra were recorded on either an IBM WP-200 or a Bruker AM 400 spectrometer. Chemical shifts are reported relative to external TMS. High-resolution FAB mass spectra were obtained on a VG 7070E instrument in the EI mode. Optical rotations were obtained on a Perkin-Elmer Model 241 polarimeter.

Results and Discussion

UV Difference Spectroscopy. Ionization of the sulfhydryl group of cysteine results in the appearance of an intense absorption band (ϵ = 4500 M^{-1} cm^{-1}) in the ultraviolet between 235 and 240 nm associated with the solvated thiolate anion (Demchenko, 1986a). It was reasoned, then, that it should be possible to observe any significant fraction of GSH bound as GS^- to the enzyme by UV difference spectroscopy between the binary enzyme–GSH complex and the uncomplexed enzyme. At concentrations of enzyme and GSH that thermodynamically favor >80% binding-site occupancy it is possible to observe a distinct absorption band at 239 nm in the UV difference spectrum (E·GSH – E) at neutral pH as illustrated in Figure 1. The wavelength and apparent intensity (ϵ_{239} = 5200 M^{-1} cm^{-1}, vide infra) are consistent with that expected for a thiolate anion. A weak difference absorption band is also observed at 294 nm ($\epsilon \approx$ 600 M^{-1} cm^{-1}), suggesting that there is some conformational difference between the complexed and uncomplexed enzyme.

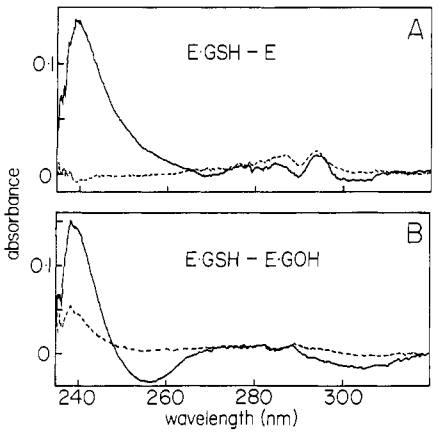


FIGURE 1: UV difference spectra of E·GSH complexes at pH 5.0 (---) and pH 7.0 (—). (A) Difference spectra between E·GSH and E, where [E] = 41.2 μM active sites and $[GSH]_{total}$ = 120 μM. (B) Difference spectra between E·GSH and E·GOH under the same conditions. The residual absorbance at 239 nm in the pH 5 spectrum persists to at least pH 4.

That the absorption band at 239 nm was not due simply to a conformational difference between the binary complex and the uncomplexed enzyme was examined by using the spectroscopically transparent oxygen analogue of GSH, GOH. UV difference spectra between the two binary complexes (E·GSH – E·GOH) also gave a clear indication of an absorption band at 239 nm (Figure 1). It is also apparent, however, that there are conformational differences between these two binary complexes as well. This is evinced by the negative difference band at 255 nm ($\epsilon \approx -850$ M^{-1} cm^{-1}) in the spectrum recorded at pH 7.0 (Figure 1B). Although neither the enzyme alone nor the E·GOH complex is a perfect conformational mimic of the E·GSH complex, the intense difference band at 239 nm seen in both cases is most reasonably assigned to the existence of a substantial amount of GS^- in the binary complex. The absorption band in both instances disappears upon protonation of the complex at pH 5 (Figure 1).

Integrity of Binary Complexes as a Function of pH. That the disappearance of the chromophore at 239 nm was not due to desorption of the peptide from the enzyme surface at low pH was confirmed by determination of the dissociation constants for GSH and GOH as a function of pH. The K_D^{GSH} was determined at three pH values by rapid equilibrium dialysis. The K_D^{GOH} was measured as the K_i for the competitive inhibition of the enzyme with GSH as the variable substrate and a fixed, subsaturating concentration of 1-chloro-2,4-dinitrobenzene. As illustrated in Figure 2, the affinity (pK_D or pK_i) of the enzyme for both peptides was comparable and varied by a factor of about 2 over the entire pH range. The affinity was minimum at pH 6.5 where K_i^{GOH} = 48 ± 4 μM and K_D^{GSH} = 22 ± 3 μM. The stoichiometry of binding of GSH was 1.1 ± 0.2 per subunit over the pH range 5.5–8. The K_D^{GSH} for isoenzyme 4-4 is comparable to that previously determined for isoenzyme 3-3 (Jakobson et al.,

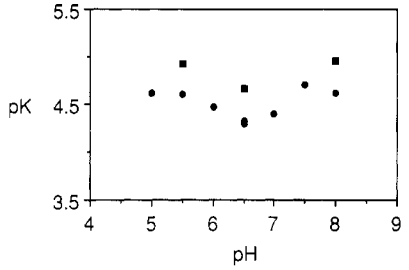


FIGURE 2: pH dependence of pK_i^{GOH} (●) and pK_D^{GSH} (■).

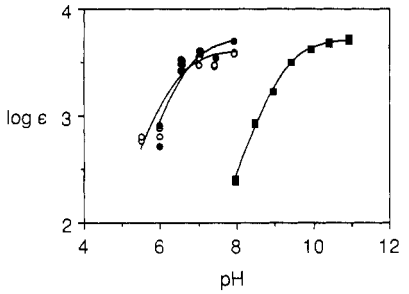


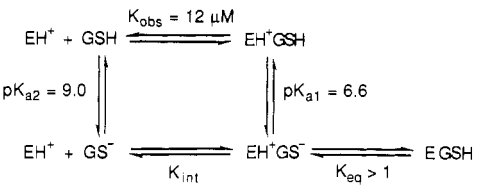
FIGURE 3: pH dependence of the difference absorption band expressed as $\epsilon_{233(obs)}$ for the difference spectra between GSH(aq) and $GSCH_3$(aq) (■) and as $\epsilon_{239(obs)}$ for the difference spectra between E·GSH and E (●) and E·GSH and E·GOH (○). The $\epsilon_{239(obs)}$ in the latter case was corrected for the residual absorbance at 239 nm (see Figure 1B). Solid lines are computer fits using the program HABELL with pK_a = 6.69 ± 0.04 and ϵ_{239} (E·GS⁻) = 5230 ± 280 M^{-1} cm^{-1} (●), pK_a = 6.39 ± 0.05 and ϵ_{239} (E·GS⁻) = 4010 ± 190 M^{-1} cm^{-1} (○), and pK_a = 9.22 ± 0.01 and ϵ_{233} [GS^- (aq)] = 5070 ± 40 M^{-1} cm^{-1} (■).

1979). The somewhat higher affinity (larger pK_D) of the enzyme for the peptides at the pH extremes, particularly at lower pH (pH 5.5) where K_D^{GSH} = 12 ± 1 μM and K_i^{GOH} = 25 ± 3 μM, eliminates the possibility that the loss of the chromophore at lower pH is due to loss of GSH from the enzyme surface.

Titration of the Binary Complex. Titrations of the chromophore in the binary complex at 239 nm between pH 5 and 8 are shown in Figure 3. Observed values of ϵ_{239} were obtained from the quotient of the optical density at 239 nm, and the concentration of occupied sites was calculated by using the K_D^{GSH}. The titration curves reveal that the disappearance of the chromophore is associated with a single ionization with an apparent pK_a of 6.4–6.7. The close agreement between this spectroscopically determined value and that previously derived from the pH dependence of k_c/K_m^s for 1-chloro-2,4-dinitrobenzene at saturating GSH (Chen et al., 1988), which, under ideal conditions, is a direct reflection of kinetically significant ionizations occurring in the binary E·GSH complex, strongly suggests that the sulfhydryl group of GSH has a pK_a of about 6.6 in the binary complex with isoenzyme 4-4 and that the thiolate is the nucleophilic species. It is, in principle, not possible to rule out entirely that the loss of chromophore at 239 nm is not due to protonation of the thiolate but rather due to protonation of some other group in the binary complex that profoundly decreases the ϵ_{239} of bound GS^-.[2] Strictly speaking, it can only be concluded that the pK_a of the bound thiol is ≤6.6.

The apparent extinction coefficient for enzyme-bound GS^- obtained from the plateau of the titration curves, ϵ_{239} = 4000–5200 M^{-1} cm^{-1}, is quite close to that found for the aquated thiolate of GSH (ϵ_{233} = 5100 M^{-1} cm^{-1}, ϵ_{240} = 4400 M^{-1} cm^{-1}) determined by UV difference spectroscopy (GSH − $GSCH_3$) between pH 8 and 11 in this work (Figure 3) as well as in previous work (Benesch & Benesch, 1955). It has been pointed out by Polgar (1974, 1975) that the extinction coefficients (ϵ_{250}) for the intramolecular ion-paired thiolates in the active sites of papain, glyceraldehyde-3-phosphate dehydrogenase, and thiosubtilisin are found to be between 1000 and 1200 M^{-1} cm^{-1}, about half that of the corresponding unpaired thiolate anions. The ϵ_{250} for E·GS^- determined from difference spectra with the uncomplexed enzyme is 1100 M^{-1} cm^{-1}, close to that expected for an ion-paired thiolate. However, it should be noted that ϵ_{250} for E·GS^- could not be measured from difference spectra with E·GOH because of interference from the negative difference band at 255 nm (Figure 1). Given the uncertainties of this and other environmental influences on the extinction coefficients at 239 and 250 nm and on absorption band shape, it is not possible to conclude from the UV spectra if E·GS^- exists as an ion-paired species at neutral pH. However, the rather large values for both ϵ_{239} and ϵ_{250} clearly suggest that if an ion-paired species does exist, then the equilibrium constant (K_{eq}) between it and the corresponding neutral species (Scheme I) largely favors the former ($K_{eq} > 1$).

Scheme I



From the evidence discussed above, it appears that the pK_a of the sulfhydryl group of GSH is shifted from 9.0 in aqueous solution to about 6.6 in the active site of GSH transferase. It is most probable that the enzyme utilizes part of the intrinsic binding energy of GSH to destabilize the thiol by placing it in a positively charged electrostatic field, represented in Scheme I by EH^+. This field may be supplied by a positively charged conjugate acid of a basic residue(s) in the active site or perhaps wholly or in part by dipoles from the secondary structure of the protein (Chen et al., 1988). In either case the shift in pK_a of GSH requires that, as illustrated in Scheme I, the intrinsic dissociation constant for GS^-(K_{int}) be considerably smaller than the observed dissociation constant, K_{obs}, for GSH. If the major uncomplexed species in solution at pH 6.5 are the protonated enzyme EH^+ and GSH, then the intrinsic dissociation constant for GS^- and EH^+ is given by $K_{int} = K_{obs}(K_{a2}/K_{a1})$ = 0.05 μM, where K_{obs} is the dissociation constant of EH^+·GSH measured at pH < pK_{a1} and pK_{a2}. The fraction of the intrinsic binding energy of GSH utilized to destabilize the bound thiol is given by $\Delta G_D = RT \ln (K_{a1}/K_{a2})$ = 3.3 kcal/mol. It can be concluded, then, that a good structural mimic of GSH which has a stabilized anionic group replacing the side chain of cysteine should bind to the protein surface considerably more tightly than GSH, since some portion of ΔG_D normally used to destabilize the bound thiol should be translated into observed binding energy, e.g., enhanced binding of the analogue.

Anionic Inhibitors of GSH Transferase. To test the above hypothesis, three carboxylate analogues of GSH were synthesized. In the first, replacement of the entire cysteine side chain with a carboxylate group was accomplished by substitution of a 2-aminomalonyl residue as in γ-L-glutamyl-(D,L-2-aminomalonyl)glycine (**1**). This tripeptide is easily prepared

[2] For example, protonation of an adjacent basic residue in the active site to form an ion-paired species (E·GS^- + H^+ ⇌ EH^+·GS^-) might be expected to lower the extinction coefficient of the thiolate by about 50% (Polgar, 1974).

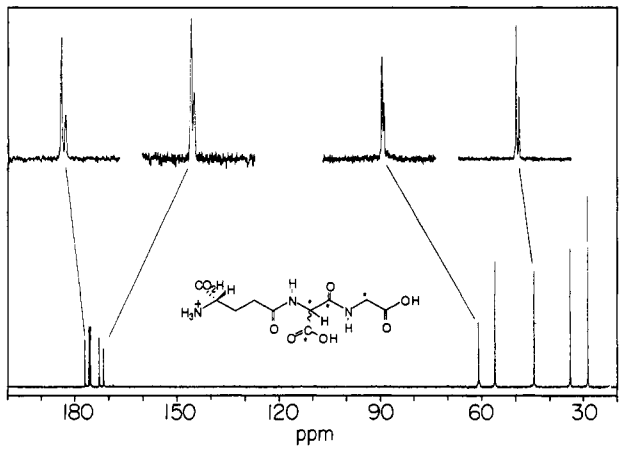


FIGURE 4: ^{13}C NMR spectrum of **1** at 100 MHz in 75% H_2O, 25% D_2O (pH ≈ 2.3). Expanded regions show two resonances for the four carbons indicated with an asterisk as evidence for a 2:1 mixture of the two diastereomers.

Table I: Inhibition Constants and Differential Binding Energies for Analogues of Glutathione with Isoenzyme 4-4 of GSH Transferase

peptide (γ-L-Glu-X-Gly)	X	K_i or K_D (μM)	$\delta\Delta G_{obs}$[a] (kcal/mol)
GSH	L-Cys	22 ± 1.4	0
1[b]	2-aminomalonyl	0.74 ± 0.06	−2.0
		(0.25, 0.50)[c]	(−2.6, −2.2)[c]
2	L-Asp	0.93 ± 0.04	−1.9
3	D-Asp	47 ■ 2	0.4
GOH	L-Ser	48 ± 4	0.4
GH[d]	L-Ala	160 ± 10	1.2
$GSCH_3$	*S*-methyl-L-Cys	200 ± 20	1.3

[a] $\delta\Delta G_{obs} = -RT \ln (K_D^{GSH}/K_i^{peptide})$. [b] Observed value for the mixture of the L,L and L,D diastereomers. [c] Values calculated by assuming a 2:1 mixture of diastereomers where one is inactive as an inhibitor. [d] Value from Chen et al. (1985).

by condensation of racemic *N*-t-BOC-2-aminomalonic acid monobenzyl ester with glycine benzyl ester, deprotection of the amino group, and coupling of the dipeptide to *N*-t-BOC-L-glutamic acid α-benzyl ester. Deprotection gives **1** as a mixture of the two diastereomers epimeric at the α-carbon of the 2-aminomalonyl residue. The lability of this residue to epimerization under acidic conditions is most clearly evinced by the exchange behavior of the α-proton that occurs in D_2O with a $t_{1/2}$ = 6.2 min at 25 °C and pD ≈ 2.3 as judged by proton NMR. The equilibrium ratio of the two diastereomers at pH 2.3 is about 2:1 as can be seen in the ^{13}C NMR spectrum of the peptide (Figure 4) in which four resonances including the α-carbon, peptide carbonyl, and carboxylate carbons of the 2-aminomalonyl residue and the α-carbon of glycine appear as two resonances. Although it is not known which isomer predominates, the observed ratio allows limits to be set on the concentration of each isomer. Unfortunately, the two diastereomers are not evident in either the ^1H or ^{13}C NMR spectra at neutral pH, indicating that, under these conditions, the two molecules either have very similar spectral properties or that one isomer predominates to the extent of ≥95%.

The mixture of diastereomers act as a potent competitive inhibitor of GSH transferase vs GSH (Table I). If it is assumed that the equilibrium ratio of the two diastereomers observed at pH 2.5 is relevant at pH 6.5 and that only the L,L-configured isomer is responsible for the inhibition in the concentration range used, then a K_i of between 0.25 μM (if the L,D isomer predominates) and 0.5 μM (if the L,L isomer predominates) can be calculated. Regardless of the stereochemical question, it is clear that the carboxylate analogue, **1**, binds at least 30-fold and perhaps as much as 90-fold more tightly to the enzyme than does GSH. Given a peptide analogue that very nearly approximates the structural and electrostatic characteristics of GS^-, it could be expected that almost all of ΔG_D would appear as observed binding energy (e.g., $\delta\Delta G_{obs} \approx -\Delta G_D = -3.3$ kcal/mol). The peptide is a sufficiently good structural and electrostatic mimic of GS^- such that 60–80% of ΔG_D normally utilized to ionize GSH can be observed in enhanced binding of **1** relative to GSH (Table I). Structural differences between **1** and GS^- due to the shorter C–O bond and smaller ionic radius of oxygen make **1** a somewhat less than perfect analogue.

The diastereomeric aspartate-containing peptides provide further insight into the structural requirements for tight-binding anionic inhibitors of GSH transferase. The two diastereomers γ-L-glutamyl-L-aspartylglycine (**2**) and γ-L-glutamyl-D-aspartylglycine (**3**) are easily assembled from the appropriately protected amino acids. Both peptides are competitive inhibitors of GSH transferase vs GSH (data not shown). Several observations are worth noting from the binding data summarized in Table I. First, the L,L isomer (**2**) of the configurationally stable analogues binds 50-fold more tightly than does **3**, suggesting that the L,L-configured diastereomer of **1** is the effective isomer of the 2-aminomalonyl-containing peptides. Second, **2** is almost as good as an inhibitor as **1** in spite of the added methylene group that probably places the carboxylate a bit too far from the peptide backbone. Finally, the incorrect configuration of isomer **3** does not prevent the peptide from binding. In fact, **3** binds considerably better than correctly configured peptides having small hydrophobic groups such as the desthio analogue, GH, or *S*-methylglutathione. These results are fully consistent with the postulate that the active-site surface has a positively charged electrostatic field in the vicinity of the cysteine side chain of bound GSH that is utilized to destabilize and hence lower the pK_a of the thiol.

Thiolate Anion and Catalysis. There are essentially two positions that have been taken with respect to exactly how GSH transferases activate the thiol of the bound peptide for nucleophilic attack. They, in fact, correspond to two extreme positions of the thiol proton in the transition state, that is, whether the proton remains on the sulfur or whether it is elsewhere. In the first instance, that of general base catalysis discussed by Douglas (1987), the sulfur remains essentially protonated but hydrogen bonds to a general base in the active site (e.g., EB···H–SG). The nucleophilicity of the sulfur is increased in the binary complex by a combination of the general base tugging on the thiol proton and desolvation of the thiol in the putative hydrophobic environment of the active site. The evidence obtained in the present work suggests the other alternative, that the predominant species in the active site is the thiolate anion (e.g., $EBH^+ \cdot {}^-SG$ or $E \cdot GS^-$). It has been correctly pointed out (Douglas, 1987) that lowering the pK_a of the thiol of GSH in the binary complex will, depending on the sensitivity of the transition state, decrease to a greater or lesser extent the effective nucleophilicity of the thiolate anion. It should be noted that the high polarizability and hence nucleophilicity of sulfur generally results in rather early transition states that are relatively insensitive to the basicity of the nucleophile; i.e., they have small Bronsted coefficients. For example, thiolate anion additions to arene oxides (Bruice et al., 1976) and aryl halides (Chen et al., 1988), both good substrates for isoenzyme 4-4, occur in aqueous solution with $\beta_{nuc} \leq 0.2$. In instances where β_{nuc} is small, considerable catalytic advantage can be realized by lowering the pK_a of the

conjugate acid of the nucleophile and increasing the fraction of thiolate in the active site. Finally, it is possible that through-space charge–charge interactions as in the ion-paired species $EB^+H\cdot^-SG$ may alter the pK_a of a thiol with minimal effect on the nucleophilicity of the thiolate anion. This has been suggested to occur in the intramolecular example of Cys-25, in the active site of papain (Roberts et al., 1986).

Conclusions. Both spectroscopic evidence and the binding of anionic analogues of GSH strongly suggest that GS^- is the predominant species of glutathione bound at the active site of GSH transferase at neutral pH. It is indeed reasonable to expect that the GSH transferases have evolved such that the reactive nucleophilic species (GS^-) is also the major species in the active site of the enzyme under physiological conditions. The results are also consistent with the notion that the enzyme uses some of the intrinsic binding energy of GSH to position the thiol in the appropriate electrostatic field, perhaps a positively charged conjugate acid of a basic residue, to lower the effective pK_a of the nucleophile. Investigations to identify the structural features that contribute to this electrostatic field are under way (Sesay et al., 1987).

ACKNOWLEDGMENTS

This paper is dedicated to the memory of Professor Emil Thomas Kaiser, whose untimely death cut short a brilliant career dedicated to the investigation of the chemistry of life. We are grateful to John N. Darnow for his help in obtaining NMR spectra.

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