ARTICLE

OBC
www.rsc.org/obc

Structure–function relationships in glutathione and its analogues

Artur Krężel[a] **and Wojciech Bal***[b]

[a] *Faculty of Chemistry, University of Wrocław, F. Joliot-Curie 14, 50-383 Wrocław, Poland. E-mail: arti@wcheto.chem.uni.wroc.pl; Fax: +48 71 3282348; Tel: +48 71 3757264*

[b] *Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawińskiego 5a, 02-106 Warsaw, Poland. E-mail: wbal@ibb.waw.pl; Fax: +48 22 6584636; Tel: +48 22 6597072 ext. 2353*

Received 8th August 2003, Accepted 17th September 2003
First published as an Advance Article on the web 6th October 2003

The results are presented of measurements of protonation constants (potentiometry and NMR), UV spectroscopic properties and redox potentials of GSH and its five analogues, which are modified at the C-terminal glycine residue (γGlu–Cys-X, X = Gly, Gly–NH_2, Gly–OEt, Ala, Glu, Ser). Strong linear correlations were found between various properties of the thiol and other functions of these peptides. These results allow discussion of the relationships between the structures and properties in glutathione and its analogues, and provide a novel chemical background for the issue of control of GSH reactivity.

Introduction

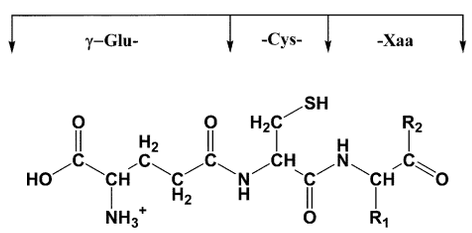
Glutathione (GSH, γGlu–Cys–Gly) is one of the most ubiquitous and important small biomolecules, present in cells of all organisms at millimolar concentrations, and possessing a multitude of physiological functions.[1] It is also one of the most widely studied molecules, with more than 120 000 references in the literature.[2] The major, well established functions of glutathione include redox-buffering of the cell environment, detoxication of xenobiotics (including drug resistance in cancer cells) and antioxidant activity, including the maintenance of biological membranes. Novel roles of glutathione are being recognized continuously. The recent additions include the release of zinc from metallothionein (a specific function for glutathione disulfide, GSSG),[3] protein glutathionylation as an element of intracellular signal transduction,[4] and intracellular nitric oxide storage and transport.[5] Glutathione is also a versatile chelator of many different metal ions, with consequences in toxicology and homeostasis.[6,7] Virtually all of these functions depend on the reactivity of the thiol group of glutathione.

The presence of a γ-peptidic bond between Glu and Cys residues is the most distinct structural feature of glutathione. It is thought to protect GSH from intracellular aminopeptidases.[1] It has two structural consequences: it separates the thiol from the functional groups of the Glu residue and it yields an α-amino acid-like domain at the Glu residue, which is absent from α-peptides. One well established consequence of these features is the above-mentioned versatility of metal ion binding.[6] Another may be the high conformational flexibility of GSH, important for its interactions with enzymes.[8]

Many studies were devoted in the past to the acid–base properties of GSH, mainly as a prerequisite for metal binding experiments. The IUPAC Stability Constants Database lists 19 references, published between 1953 and 1994,[9] with newer ones coming up at a pace of *ca*. one a year. Those relevant for our results are referred to in the Discussion section.

The redox potential of the 2GSH–GSSG pair was established accurately, with various methods[10,11] and was discussed recently in depth in the context of cellular physiology.[12]

We have re-established acid–base and redox properties of GSH and compared them with those of its several analogues, which were modified at the C-terminus by substituting the Gly carboxyl function, or replacing Gly with another amino acid residue. These analogues are presented in Scheme 1. Such comparative studies were not performed previously and several of these derivatives are novel. Our approach provided a new look at the properties of GSH, in particular by delivering evidence for the conformational preferences present in the molecule, which control its acid–base and redox properties.



γECG (GSH) R_1 = H, R_2 = OH
γECG-OEt R_1 = H, R_2 = OC_2H_5
γECG-am R_1 = H, R_2 = NH_2
γECA R_1 = CH_3, R_2 = OH
γECS R_1 = CH_2OH, R_2 = OH
γECE R_1 = $(CH_2)_2$-COOH, R_2 = OH

Scheme 1 Structures of γGlu–Cys–Xaa peptides studied in this work, presented in their fully protonated forms.

Materials and methods

Materials

Glutathione (GSH), glutathione glycine ethyl ester (γGlu–Cys–Gly–OEt, γECGOEt), glutamic acid (Glu), sodium (3-trimethylsilyl)-2,2,3,3-tetradeuteriopropionate (TSP), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), sodium phosphates and NaOD (40% w/v in D_2O) were obtained from Sigma. KNO_3, HNO_3, $NaClO_4$, and NaOH volumetric solution (0.1 M) were purchased from Merck. D_2O (99.9%) and DCl (35% solution in D_2O) were from Cambridge Isotope Laboratories.

Peptide synthesis

The peptides γGlu–Cys–Ala, γGlu–Cys–Ser, and γGlu–Cys–Glu were synthesized in the solid state on a 2-chlorotrityl chloride resin, while γGlu–Cys–Gly-am was synthesized using the H-linker-2-chlorotrityl resin. Fmoc strategy was used.[13,14] The *N*-Fmoc-protected amino acids *N*–Fmoc–Gly–OH, *N*–Fmoc–Ala–OH, *N*–Fmoc–Ser(*t*Bu)–OH, *N*–Fmoc–Glu-(γ-*t*Bu)–OH and *N*–Fmoc–Cys–(Mtt)–OH were obtained from

DOI: 10.1039/b309306a

Nova Biochem, while the N-terminal *N*-*t*Boc–Glu-(α-*t*Bu) was obtained from Sigma. The coupling agents 1-hydroxybenzotriazole (HOBt) and dicyclohexylcarbodiimide (DCC) were purchased from Merck, solvents trifluoroacetic acid, 2,2,2-trifluoroethanol, piperidine, acetonitrile, *N,N*-dimethylformide (DMF) and dichloromethane (DCM) were obtained from Riedel-de Haën GmbH. Fmoc protection groups were removed by 25% piperidine in DMF. The coupling was monitored by the Keiser (ninhidrin) test and TLC. The scale of the synthesis was *ca*. 1.3 mmol in terms of resin substitution for γECA, γECS, γECE and 0.6 for γECGam, 2.5-fold excess of amino acids were used for additions. The cleavage was effected using a mixture of trifluoroacetic acid, trifluoroethanol, DCM, and dimercaptoglycol (v/v/v/v = 3 : 1 : 5 : 1) over a period of 24 h, followed by precipitation with diethyl ether, peptide cleavage from the resin, removal of the protecting groups Mtt, Boc, and *t*Bu and washing with diethyl ether. The yields of crude unprotected peptides, complexed with TFA were 30.5% (85 mg) for γECA, 18.7% (54 mg) for γECS, 25.3% for γECE (80 mg), and 54.7% (138 mg) for γECGam. The final purification was done using HPLC (Hewlett Packard) on an Alltech Econsil C18 10U preparative column (22 × 250 mm, 5 μm grain), in a 0–100% 0.1% TFA–water to 0.1% TFA–acetonitrile gradient, detected at 200 nm, controlled with a Hypersil BDC C18 analytical column (4.6 × 250 mm) from Merck. The purity and identity of the peptides was finally confirmed using mass spectrometry, utilizing a Finnigan MAT TSQ 700 instrument. The *m*/*z* values found (calculated) for (M + H) were: γECG: 308.2 (308.3), γECGam: 307.1 (307.3), γECGOEt: 336.1 (336.4), γECA: 322.5 (322.4), γECS: 338.0 (338.4), and γECE: 380.0 (380.4).

Potentiometry

Potentiometric titrations of the peptides and Glu in the presence of 0.1 M KNO_3 or 2.0 M KNO_3 were performed at 298 K, over the pH range 2.2–10.5, using a Molspin automatic titrator, with 0.1 M NaOH as titrant. Changes of pH were monitored with a combined glass–Ag/AgCl electrode, calibrated daily in H^+ concentrations by HNO_3 titrations.[15] Sample volumes of 1.5–2 ml and compound concentrations of 1 mM were used. The experimental data were analysed using the SUPERQUAD program.[16] Standard deviations computed by SUPERQUAD refer to random errors.

Electronic absorption (UV-Vis)

Spectrophotometric acid–base titrations of all peptides were performed in 1 cm cuvettes at 25 °C, on a Cary 50 Bio spectrophotometer, using the reporter wavelength of 232 nm. The accurate concentrations of thiol samples were determined at 412 nm, in 1 cm cuvettes, using the colorimetric reaction with DTNB in 50 mM phosphate buffer at pH 9.0. The value of ε_{412} of $DTNB_{red}$ (thiolate product of DTNB reduction) of 13600 M^{-1} cm^{-1} was used.[17] Redox potentials were determined indirectly, by following the equilibrium of the thiol–disulfide exchange reaction between the thiol studied and DTNB, whose redox potential was determined in a reaction with DTT.[10] The initial concentrations of thiols were of the order of 10^{-5} M and that of DTNB was $2{\cdot}10^{-4}$ M. These experiments were performed at T = 25 °C, at pH 6.3, 7.0, 7.4 and 7.9, controlled by 50 mM phosphate buffers.

NMR

^{1}H NMR spectra of 5 mM samples of thiol peptides in D_2O were recorded at 298 K, on a Bruker AMX-300 spectrometer, at 300 MHz. TSP (sodium (3-trimethylsilyl)-2,2,3,3-tetradeuteriopropionate) was used as an internal ^{1}H standard. The pH of samples was controlled using small volumes of concentrated DCl or NaOD.

Results

Acid–base properties

Potentiometric titrations of GSH and its five analogues at I = 0.1 M revealed a very strong linear correlation between the logarithms of the values of two most basic protonation macroconstants, pK_1 and pK_2 as defined by the following reactions (charges of peptides omitted for simplicity):

$$H_2L \overset{pK_2}{\longleftrightarrow} HL; \quad HL \overset{pK_1}{\longleftrightarrow} L$$

These constants correspond to the thiol and amine groups (see below). This correlation is presented in Fig. 1, and is described quantitatively by eqn. 1 (standard errors on the last significant digits of all values yielded by experiments are given in parentheses, absolute values of the linear correlation coefficient R are also provided).

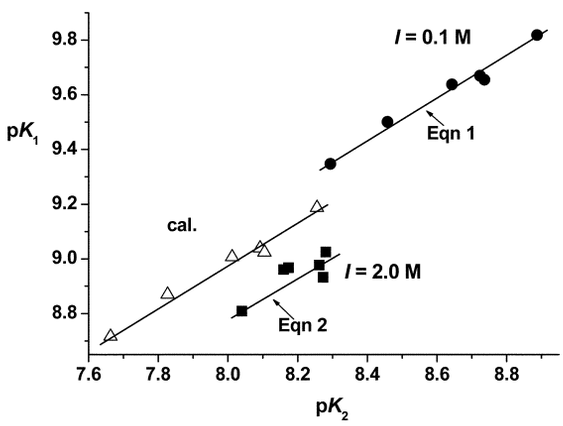


Fig. 1 Empirical linear correlations between logarithmic values of macroconstants pK_1 and pK_2 in γECX peptides, obtained by potentiometry: (●), values at I = 0.1 M; (■), values at I = 2.0 M; (Δ), values at I = 0.1 M, extrapolated to I = 2.0 M, with Debye–Hückel formula.[18] References to equations for individual correlation lines are indicated by their numbers.

The correlation described by eqn. 1 was largely lost at I = 2.0 M (Fig. 1, eqn. 2):

$$pK_1 = 0.75(5) \times pK_2 + 3.1(4) \; (R = 0.99) \qquad (1)$$

$$pK_1 = 0.6(2) \times pK_2 + 4(2) \; (R = 0.82) \qquad (2)$$

This loss of correlation was accompanied by more than two-fold reduction of the spread of pK_a values. On the other hand, the shifts of the averages of the pK_a values for all six peptides between I = 0.1 M and I = 2.0 M, −0.65 and −0.42, for pK_1 and pK_2, respectively, were in a good agreement with the value of −0.63 log units, prognosed by the Debye–Hückel formula.[18] The values of all the constants, determined by potentiometry, are presented in Table 1.

For the sake of comparison, protonation constants of Glu were measured at I = 0.1 M. The values obtained were: pK_1, 9.588(7); pK_2, 4.202(7); pK_3, 2.21(2).

Further experiments were subsequently performed to identify the source of such a strong correlation of pK_a values in γECX peptides, specifically at a lower ionic strength. First, NMR titrations were performed for all peptides in D_2O in order to identify possible intramolecular interactions, which would be reflected in specific sensitivities of particular non-dissociable protons to deprotonations of acidic and/or basic moieties in these peptides. The titration curves thus obtained for individual protons were fitted to obtain approximate pK_a* values (uncorrected for the H/D isotope effect), which are presented in Table 2. Examples of such spectra and titrations, for GSH, are shown in Fig. 2.

Table 1 Dissociation constants (pK_i) of γECX peptides, obtained from potentiometric experiments at 25 °C and $I = 0.1$ M or 2.0 M (KNO_3) – the latter in italics. Statistical errors of determinations on the last digits are given in parentheses

Peptide	pK_1	pK_2	pK_3	pK_4	pK_5
γECG	9.655(2)	8.736(2)	3.512(3)	2.126(7)	
	8.932(3)	*8.273(2)*	*3.469(4)*	*2.184(7)*	
γECA	9.670(4)	8.723(4)	3.637(6)	2.16(1)	
	8.977(4)	*8.262(4)*	*3.560(6)*	*2.479(8)*	
γECS	9.638(4)	8.643(4)	3.425(6)	2.07(1)	
	8.961(7)	*8.160(6)*	*3.33(1)*	*2.45(1)*	
γECE	9.818(4)	8.887(4)	4.760(5)	3.455(6)	2.29(1)
	9.025(7)	*8.281(6)*	*4.851(9)*	*3.454(9)*	*2.57(1)*
γECGOEt	9.501(6)	8.458(7)	2.33(2)		
	8.967(9)	*8.174(8)*	*2.47(2)*		
γECGam	9.347(5)	8.294(5)	2.25(1)		
	8.809(9)	*8.039(8)*	*2.51(2)*		

Table 2 Logarithmic values of protonation group constants (pK_i) at the thiol, amine and C-terminal functions of γECX peptides, calculated from ^{1}H NMR data.[a] Differences of chemical shifts ($\Delta\delta$, ppm) between the protonated and deprotonated forms are given in italics

Peptide	p$K_{\alpha Glu}$ [b] $\Delta\delta_{\alpha Glu}$	p$K_{\beta Cys}$ [c] $\Delta\delta_{\beta Cys}$	p$K_{\alpha X}$ [d] $\Delta\delta_{\alpha X}$	p$K_{\beta X}$ $\Delta\delta_{\beta X}$
γECG	9.65(3)	9.09(4)	*8.85(2)*	
	0.513	*0.108*	*0.007*	
γECA	9.73(3)	9.25(2)	9.24(7)	9.00(5)
	0.512	*0.124*	*0.032*	*0.010*
γECS	9.67(2)	9.04(4)	8.7(1)	8.85(8)
	0.516	*0.105*	*0.015*	*0.007*
γECE	10.00(7)	9.33(6)	9.69(2)	
	0.510	*0.122*	*0.015*	
γECGOEt	9.25(3)	8.74(1)	8.88(4)	
	0.437	*0.111*	*0.025*	
γECGam	9.47(2)	8.52(2)	8.57(1)	
	0.532	*0.088*	*0.022*	

[a] Calculated using pH*, uncorrected readings of pH in D_2O solutions. [b] Group constant specific to the amine function. [c] Group constant specific to the thiol function. [d] Apparent group constant exhibited on the protons of the C-terminal residue.

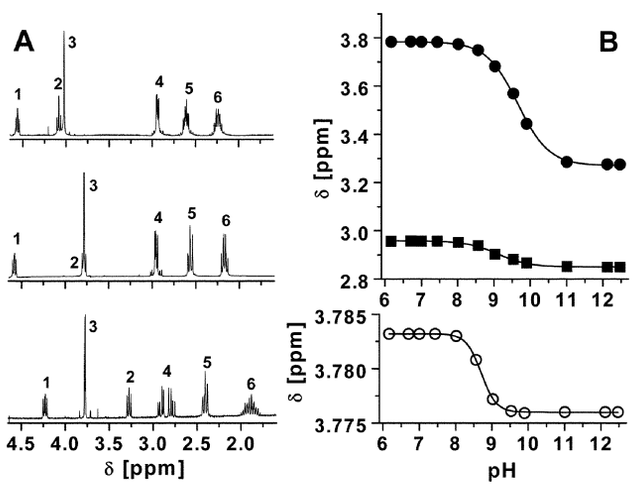


Fig. 2 A. Examples of ^{1}H NMR spectra of GSH at pH 1.8 (top), 6.4 (middle) and 10.8 (bottom). Signal assignments: 1, α-Cys; 2, α-Glu; 3, Gly; 4, β-Cys; 5, γ-Glu; 6, β-Glu. B. Titration curves derived from chemical shifts in ^{1}H NMR spectra of GSH: (●), α-Glu; (■), average of β-Cys; (○), Gly.

The pK_a values of the carboxylic groups in GSH and its analogues are within the range 2–4, which is 4–6 pH units lower than those of pK_2 and pK_1, respectively (Table 1). Therefore, there is no overlap of protonation processes between the carboxyls on one hand and the basic groups on another. At physiological pH, the carboxyl functions are fully deprotonated in all analogues studied and their acid/base equilibria do not influence the physiological properties of GSH directly. All further analysis was therefore limited to the thiol and amine functions.

Table 3 Values of ε_{232} and redox potentials of γECX peptides

Peptide	ε_{232}	$E^{0'}$ [a]/V *vs.* NHE	$E^{0'}$ [b]/V *vs.* NHE
γECG	5967(29)	−0.210(3)	−0.240(3)
γECA	6041(42)	−0.210(2)	−0.240(2)
γECS	6481(41)	−0.206(3)	−0.236(3)
γECE	5622(102)	−0.213(2)	−0.244(2)
γECGOEt	7480(125)	−0.202(2)	−0.233(2)
γECGam	8523(62)	−0.198(3)	−0.229(3)

[a] Experimental values at peptide concentrations of 10^{-5} M. [b] Calculated values at peptide concentrations of 10^{-3} M, recalculated according to ref. 12.

Electronic spectra

The deprotonation of GSH and its derivatives at alkaline pH was also followed with UV spectroscopy. Much lower concentrations of peptides were used, compared to NMR experiments (10–20 μM). The difference spectra revealed the presence of a typical thiolate absorption band centred at 232 nm for all analogues, which gained intensity in the course of titration with alkali.[19] The fitting of the titration curves, generated using ε_{232} values, yielded apparent pK_a values which were intermediate between those determined by NMR and by potentiometry for the thiol and amine functions. The low slopes of these titration curves indicated that the thiolate absorption was sensitive to the deprotonation of the amine. The maximum values of ε_{232} for fully deprotonated peptides (at thiolate and amine functions) were obtained from these titrations and are provided in Table 3. Fig. 3A presents the correlation between the values of pK_2 and ε_{232}, according to eqn. 3:

$$pK_2 = -0.00019(2) \times \varepsilon_{232} + 9.9(1)\ (R = 0.97) \qquad (3)$$

Redox potentials

The redox potentials of GSH and its analogues were determined indirectly, by monitoring the reduction of DTNB disulfide, at a thiol concentration of 10^{-5} M. The potential of DTNB under analogous conditions, −94.3(7) mV, was established in its reaction with DTT, using the published value for $E^{o'}_{DTT}$, −327 mV.[10] The redox potentials were then calculated from values of A_{412} of reaction mixtures, using the published value of ε_{412} for DTNB[17] and the Nernst equation for the reaction monitored (eqn. 4).

$$E^{0'}_{RSH_{red/ox}} = E^{0'}_{DTNB_{red/ox}} - \frac{[RSSR] \times [DTNB_{red}]^2}{[RSH]^2 \times [DTNB_{ox}]} \qquad (4)$$

This method takes advantage of the reaction equilibria which are pH-independent, as indicated by the reaction stoichiometry

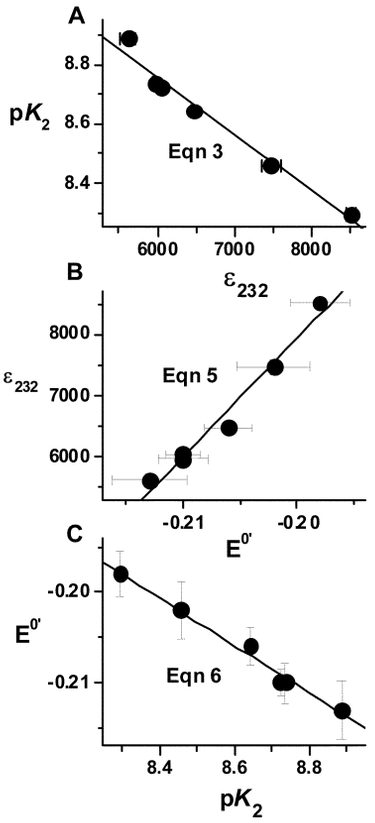


Fig. 3 Linear correlations of the thiol-related properties: A. Correlation of values of thiol macroconstants with thiol-characteristic molar absorption coefficients at 232 nm; B. Correlation of molar absorption coefficients at 232 nm with redox potentials, obtained at [thiol] = 10^{-5} M; C. Correlation of redox potentials with values of thiol macroconstants. References to equations for individual correlation lines are indicated by their numbers. Error bars are indicated unless they are smaller than symbol sizes.

and confirmed by identical results, obtained by us at pH 6.3, 7.0, 7.4 and 7.9. The redox potentials determined experimentally are presented in Table 3. Fig. 3B demonstrates a linear correlation of $E^{0'}$ values with ε_{232} (eqn. 5):

$$\varepsilon_{232} = 1.93(17)\ 10^5 \times E^{0'} + 0.47(4)\ 10^5\ (R = 0.99) \quad (5)$$

The correlation between the redox potential and the thiol-related pK_2 (see below) is also very strong. Redox potentials of monothiols are concentration-dependent, due to the bimolecular stoichiometry of disulfide formation.[10–12] Therefore, Table 3 also contains the corresponding values, recalculated for millimolar concentrations.[10] The differences are 30 to 31 mV. The results for GSH are in an excellent agreement with the data published previously.[10–12] The correlation, presented in Fig. 3C, is described by eqn. 6, where $A = -0.02(1)$ for [thiol] = 10^{-3} M and 0.02(1) for [thiol] = 10^{-5} M.

$$E^{0'} = -0.026(1) \times \mathrm{p}K_2 + A\ (R = 0.99) \quad (6)$$

Fig. 4 presents the correlations of protonation constants, electronic absorption coefficients and redox potentials with the stoichiometric electronic charges at the C-terminal residue of GSH and its analogues at pH 7 and above, z_C (eqns 7–10).

$$\mathrm{p}K_1 = -0.167(9) \times z_C + 9.49(1)\ (R = 0.99) \quad (7)$$

$$\mathrm{p}K_2 = -0.27(5) \times z_C + 8.40(5)\ (R = 0.94) \quad (8)$$

$$\varepsilon_{232} = 1200(300) \times z_C + 7700(400)\ (R = 0.88) \quad (9)$$

$$\mathrm{E}^{0'} = -0.007(1) \times z_C - 0.201(2)\ (R = 0.92) \quad (10)$$

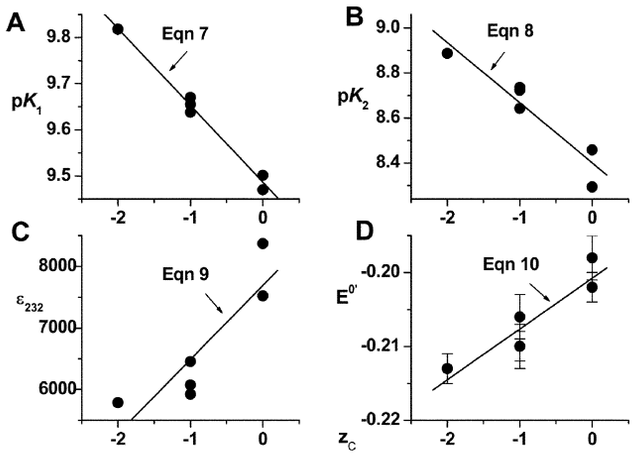


Fig. 4 Linear correlations between the physicochemical parameters of GSH analogues and z_C, stoichiometric electronic charge on the C-terminal residue in species H_2L, HL and L; A. Correlation with the amine macroconstant; B, correlation with the thiol macroconstant; C, correlation with the thiol-characteristic molar absorption coefficient at 232 nm; D, correlation with the standard redox potential at [thiol] = 10^{-5} M. References to equations for individual correlation lines are indicated by their numbers. Error bars are indicated only in part D, because they are smaller than symbol sizes in other cases.

Discussion

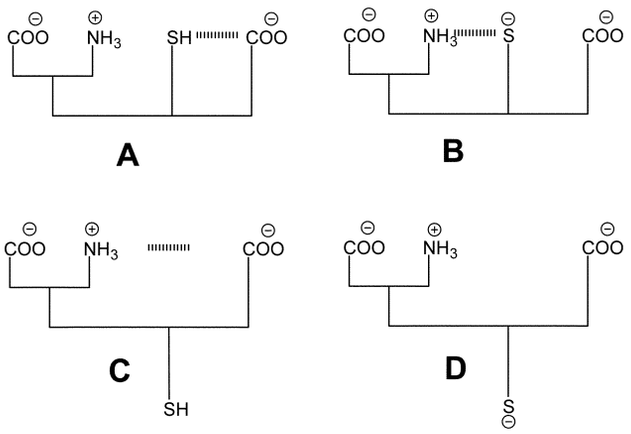
Acid–base properties

The values of protonation macroconstants for GSH are in a good agreement with those obtained before on many occasions by Rabenstein *et al.* and also by some other authors;[20–27] the small differences stem from differences in ionic strengths employed. Our constants for $I = 0.1$ are virtually identical to those obtained a long time ago at $I = 0.15$[28] and $I = 0.16$,[29] and are very close (within 0.1 log units for each pK_a) with those published recently for $I = 0.1$.[30] In contrast, our results for $I = 2.0$ differ significantly from those published previously for $I = 1$[31] or $I = 3$.[32] However, as demonstrated in Fig. 1, our results correspond to the trend expected on the basis of the Debye–Hückel theory, consistently for six peptides, while the previous studies, on GSH alone, did not. This confirms the correctness of our results.

The issue of microprotonation and microspeciation of GSH was studied in detail by Rabenstein.[20] The conclusion of that study, crucial for the results presented here is that the K_1 and K_2 can be roughly assigned to the amine and thiol group, respectively, and therefore, the major one of the two microspecies within the HL stoichiometry (*ca.* 85%) includes the charged residues: the deprotonated thiolate and the protonated amine, as presented in Scheme 2, structure B. The comparison of values of protonation constants of other GSH-like peptides (Table 1) with those of group constants, obtained by NMR and presented in Table 2, indicates that the similar populations of microforms are present for HL species of all peptides studied here.

Structural consequences of correlations

The existence of a strong linear correlation between pK_1 and pK_2 at $I = 0.1$ M, which is largely lost at the high ionic strength of 2.0 M (Fig. 1), indicates the presence of ionic interactions, through space, within the molecules of GSH and its analogues. These interactions manifest themselves directly in the NMR spectra. The NMR titrations of C-terminal protons in all peptides studied indicated that their chemical shifts are specifically sensitive to the protonation state of the thiol (Fig. 2). The apparent pK_a values, calculated for the C-terminal α protons in these peptides (and also β protons in γECA and γECS, Table 2) are numerically very close to the thiol-specific group constants, calculated using chemical shifts of Cys β-protons. The only



Scheme 2 Sketch of major electrostatic interactions proposed in various species of GSH (hashed lines): A. C-terminal carboxylate with thiol in H_2L, this work; B. thiol with protonated amine in HL, this work; C. C-terminal carboxylate with protonated amine in H_2L;[43] D. lack of intramolecular interactions in HL in extended conformation, bound to glutathione S-transferases.[8,45–47] The structure analogous to Case D is also valid for the neutral, solid state H_3L in the X-ray structure[37,38] and the fully deprotonated L at high pH.

exception is γECE, in which the combined effects of the thiol and amine groups manifest themselves in the apparent pK_a value exhibited by the α proton of the C-terminal Glu residue, which is an average of the thiol and amine group constants. The magnitude of the chemical shift change on C-terminal α protons upon the thiol deprotonation ($\Delta\delta$), presented in Table 2, is quite high – up to 25% of the effect seen on Cys β protons. The effects on β protons in γECA and γECS are lower, which suggests that the interaction with the thiol occurs through the α carboxyl/carbonyl moiety. The transfer of such an effect through bonds can be safely excluded, because of a long distance of six chemical bonds (including a peptide moiety) separating the sulfur from the C-terminal α proton. Also, this effect, if transferred through bonds, should have also been manifested on γGlu protons, which are separated from the sulfur by a very similar path, also including a peptide moiety (Scheme 1). This was not the case, the chemical shifts of γ protons of the γGlu residue were sensitive only to deprotonations within the γGlu residue. Therefore, the NMR titrations provided experimental evidence for the location of the SH group close to the C-terminal amino acid in the H_2L species of GSH and the corresponding species of other peptides studied. The further argument for this concept is provided by strong correlations between the stoichiometric electronic charges at the C-terminal residue of GSH and its analogues at pH 7 and above, z_C, and the crucial properties of the thiol group – its pK_a and $E^{0'}$ (Fig. 4). Even the intensity of its absorption band in the deprotonated form L correlates with z_C, although the strength of this correlation, expressed by the value of R, is lower.

The latter effect, and the apparent strong dependency of the pK_1 values on pK_2 and on z_C (Fig. 4) indicate that the structuring of the GSH and analogues is still present in the HL species, in which the major microspecies is deprotonated at the thiol, as noted above. Our data do not allow the assignment of these correlations unequivocally to the interactions of the protonated amine with the thiolate or the C-terminal carboxylate. It should, however, be noted that these interactions are mutually exclusive, as indicated by an analysis of a molecular model, and are confirmed by the values of pK_1 in γECX analogues with $z_C = -1$. These values are very close to that for the free glutamic acid, in which the charge analogous to that of z_C is brought about by the γ carboxylate (determined here as 9.588, in a good agreement with literature values[33–35]).

The value of the slope in eqn. 1 is 0.75, in line with the decreased control of the C-terminal residue on the N-terminal one, which is further away than the thiol. The same effect is presented more directly by the comparison of the slopes of eqn. 7 (−0.27 for the thiol) and eqn. 8 (−0.167 for the amine). The value of the slope in eqn. 2, at high ionic strength, which shields electrostatic interactions, is not only low, but also contains a much higher statistical error, reflected in a low R value.

The involvement of the thiolate in the control of pK_1 is, however, indicated by the fact that the amine deprotonation has no effect on the C-terminal residue in five of the six peptides. Furthermore, the striking susceptibility of the HL species in GSH to air oxidation, compared to that of the L species, can be rationalised in terms of a direct interaction between the deprotonated thiolate and the protonated amine.[36]

The importance of a direct interaction between the amine and the C-terminal residue is clearly higher in the case of γECE, where the side-chain carboxylate in the C-terminal Glu residue provides an additional site of ionic interactions.

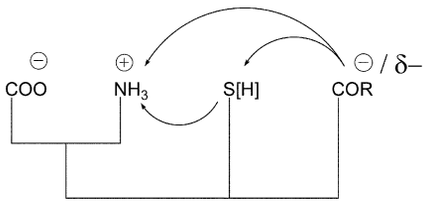
Comparison with previous results

The conformational preferences in the molecule of GSH, emerging from our results, are sketched in Scheme 2, together with some of the results of previous studies. The X-ray structures of GSH exhibit no intra-, and little intermolecular interactions, with a GSH molecule in an extended, S-shaped conformation.[37,38] One reason for the lack of intramolecular interactions in the solid state is in the acidic crystallisation conditions, yielding a peculiar neutral H_3L form, with protonated carboxyls and a deprotonated amine, which is never formed in solution.

The previous NMR-based studies of GSH conformation led to disparate conclusions, regarding conformational preferences of GSH, such as a structuring of the Glu moiety, or a presence of various side-chain–backbone interactions.[39–41] The most comprehensive of those studies led to the conclusion that at pH 7 the Gly residue is particularly mobile, and the Glu part of the GSH molecule is the most rigid.[41] Three theoretical studies of GSH conformation are known to the authors. In two of these a salt bridge between the protonated amine and the Gly carboxylate was proposed, while interactions involving the thiol were excluded.[42,43] The third one suggested an interaction between the protonated thiol sulfur and the Cys amide moiety in the H_2L species of GSH.[44] Our results are in a qualitative agreement with the latter view, and with the ^{13}C relaxation data,[41] because a high mobility of the C-terminus does not exclude ionic interactions, which are not directional.

The structures of GSH complexed with its major target proteins, GSH transferases, present GSH in the extended conformation.[8,45–47] An analogous extended conformation was observed for GSH bound to GSH synthetase.[48] These structures are dictated by multiple hydrogen bonds between GSH and protein residues, which easily overcome any intramolecular interactions in GSH, and therefore cannot provide a guide for GSH conformation in solution.

The demonstration that the charge on the C-terminal residue of GSH or its analogue controls properties of its thiol group through space quantitatively is the most important result of this study. Eqns 7 and 9 predict that the effect is equal to 0.27 pH units and 7 mV per unit charge on the C-terminus, respectively. The interactions within the GSH molecule, indicated by our results, are summarised in Scheme 3. Our results correspond interestingly, in a qualitative way, to results of recent works regarding the thiol activation in GSH transferases. The thiolate pK_a of the enzyme-bonded GSH is lowered to *ca.* 6.0, with the proton released to the solution.[49] After a long debate on the relevance of particular interactions between the thiol(ate) and the binding site residues,[49–51] it was concluded that the crucial effect is exerted by an arginine residue, at the distance of 3–4 Å from the thiolate.[51] This finding demonstrates the susceptibility of the thiol group in GSH to electrostatic influence, albeit in a different conformational milieu.



Scheme 3 Summary of intramolecular interactions in GSH and its analogues, proposed in this work. R denotes O^-, OEt or NH_2.

The influence of the local electrostatic environment on the properties of the thiol group was also studied with the respect to the kinetics of disulfide formation. The effect of ionic neighbours of the Cys residue on the formation of mixed disulfides of peptides with organic thiols was studied at various ionic strengths.[52] It was concluded that at $I \leq 20$ mM the direct neighbours in a random coil peptide could affect the rate constant of disulfide formation by a factor of up to 10^6. Another study, on a structured peptide, demonstrated that longer range interactions are less predictable.[53] The effects detected by us in the series of GSH analogues certainly fall in the first of these two cases.

Relevance of electrostatic control for GSH reactivity

The correlation of all properties of the thiol group with the value of z_C means that any charged residue in the vicinity of the Gly residue in GSH, *e.g.* in the docking pocket in a protein, will exhibit the same kind of influence. In this way the environment of the protein cysteine residue can contribute to the control of the equilibrium and kinetics of such processes as Cys glutathionylation, one of central concepts in the emerging field of cellular redox signalling.[54] Our findings may also help interpret the processes of GSH activation in various GSH transferase-like enzymes, turning the attention into possible interactions with anionic, as well as cationic side chains. It is also likely, although purely speculative at this stage of research, that the same kind of control may be exerted upon nitrosoglutathione, the intracellular nitric oxide carrier and transnitrosation agent.[54] Summarising, we may state that, contrary to the conclusion presented in a relatively recent review on the crystallography of peptides,[55] there is a relationship between structure and function of GSH, but it can be clearly demonstrated only within a series of related molecules.

Acknowledgements

This work was sponsored by the Polish State Committee for Scientific Research (KBN), grant No 7 T09A 079 20 and grant No 4 T09A 030 22. We thank Dr Jaroslaw Poznanski and Dr Jacek Wójcik for helpful discussions.

References

1 H. Sies, *Free Radical Biol. Med.*, 1999, **27**, 916.
2 A combined Chemical Abstracts and Medline search.
3 C. Jacob, W. Maret and B. Vallee, *Proc. Natl. Acad. Sci. USA*, 1998, **95**, 3489.
4 S. Casagrande, V. Bonetto, M. Fratelli, E. Gianazza, I. Eberini, T. Massignan, M. Salmona, G. Chang, A. Holmgren and P. Ghezzi, *Proc. Natl. Acad. Sci. USA*, 2002, **99**, 9745.
5 M. Lo Bello, M. Nuccetelli, A. M. Caccuri, L. Stella, M. W. Parker, J. Rossjohn, W. J. McKinstry, A. F. Mozzi, G. Federici, F. Polizio, J. Z. Pedersen and G. Ricci, *J. Biol. Chem.*, 2001, **276**, 42138.
6 A. Krężel and W. Bal, *Acta Biochim. Polon.*, 1999, **46**, 567.
7 M. Gochfeld, *Environ. Health Perspect.*, 1997, **105**(Suppl. 4), 817.
8 M. Nicotra, M. Paci, A. J. Oakley, M. W. Parker, M. Lo Bello, A. M. Caccuri, G. Federeci and G. Ricci, *Biochemistry*, 1998, **37**, 3020.
9 IUPAC and Academic Software, Stability Constants Database, 2001, v. 4.11.
10 W. J. Lees and G. M. Whitesides, *J. Org. Chem.*, 1993, **58**, 642.
11 K. K. Millis, K. H. Weaver and D. L. Rabenstein, *J. Org. Chem.*, 1993, **58**, 4144.
12 F. Q. Schafer and G. R. Buettner, *Free Radical Biol. Med.*, 2001, **30**, 1191.
13 W. C. Chan and P. D. White (eds.) Fmoc Solid Phase Peptide Synthesis. A Practical Approach., Oxford Univesity Press, 2000, New York.
14 Solid Phase Synthesis, *Methods Enzymol.*, ed. G. B. Fields, Academic Press, New York, 1997, p. 289.
15 H. Irving, M. G. Miles and L. D. Pettit, *Anal. Chim. Acta*, 1967, **38**, 475.
16 P. Gans, A. Sabatini and A. Vacca, *J. Chem. Soc., Dalton Trans.*, 1985, 1195.
17 G. L. Ellman, *Arch. Biochem. Biophys.*, 1958, **74**, 443.
18 P. W. Atkins, *Physical Chemistry,* sixth edn., Oxford University Press, Oxford, 1998.
19 K. J. Voycechowsky, K. D. Wittrup and R. T. Rains, *Chem. Biol.*, 1999, **6**, 871.
20 D. L. Rabenstein, *J. Am. Chem. Soc.*, 1973, **95**, 2797.
21 L. Osterberg, R. Ligaarden and D. Persson, *J. Inorg. Biochem.*, 1979, **10**, 341.
22 B. Harman and I. Sovago, *Inorg. Chim. Acta*, 1983, **80**, 75.
23 Y. Theriault, B. V. Cheesman, A. P. Arnold and D. L. Rabenstein, *Can. J. Chem.*, 1984, **62**, 1312.
24 M. J. Hynes and M. O'Dowd, *J. Chem. Soc., Dalton Trans.*, 1987, 563.
25 B. Cheesman, A. Arnold and D. L. Rabenstein, *J. Am. Chem. Soc.*, 1988, **110**, 6359.
26 W. Kadima and D. L. Rabenstein, *J. Inorg. Biochem.*, 1990, **38**, 277.
27 H. Kozlowski, J. Urbanska, I. Sovago, K. Varnagy, T. Kiss, J. Spychala and K. Cherifi, *Polyhedron*, 1990, **9**, 831.
28 N. C. Li and R. A. Manning, *J. Am. Chem. Soc.*, 1955, **77**, 5225.
29 J. T. Edsall and B. R. Martin, *Proc. Natl. Acad. Sci. USA*, 1958, **44**, 505.
30 P. D. Oram, X. Fang, Q. Fernando, P. Letkeman and D. Letkeman, *Chem. Res. Toxicol.*, 1996, **9**, 709.
31 A. M. Corrie, M. D. Walker and D. R. Williams, *J. Chem. Soc., Dalton Trans.*, 1976, 1012.
32 A. P Arnold and A. J. Canty, *Can. J. Chem.*, 1983, **61**, 1428.
33 G. J. M. Heijne and W. E. van der Linden, *Talanta*, 1975, **22**, 923.
34 T. Sakurai, O. Yamauchi and A. Nakahara, *Bull. Chem. Soc. Jpn.*, 1978, **51**, 3203.
35 P. G. Daniele, P. Amico and G. Ostacoli, *Ann. Chim. (Rome)*, 1984, **74**, 105.
36 A. Krężel, W. Szczepanik, M. Sokolowska, M. Jezowska-Bojczuk and W. Bal, *Chem. Res. Toxicol.*, 2003, **16**, 855.
37 W. B. Wright, *Acta Crystallogr.*, 1958, **11**, 632.
38 C. H. Görbitz, *Acta Chem. Scand.*, 1987, **B41**, 362.
39 S. V. Zenin, G. I. Chuprina and A. Y. Krylova, *Zh. Obshch. Khim.*, 1975, **45**, 1337.
40 S. Fujiwara, G. Formicka-Kozlowska and H. Kozlowski, *Bull. Chem. Soc. Jpn.*, 1977, **50**, 3131.
41 M. York, G. R. Beilharz and P. W. Kuchel, *Int. J. Pept. Protein Res.*, 1987, **29**, 638.
42 T. P. Sandalova and P. I. Belobrov, *Bioorg. Khim.*, 1983, **9**, 1013.
43 J. Berges, J. Caillet, J. Langlet and Z. Abedinzadeh, *Theor. Chim. Acta*, 1993, **85**, 87.
44 P. R. Laurence and C. Thomson, *Theor. Chim. Acta*, 1980, **57**, 25.
45 X. Ji, P. Zhang, R. Armstrong and G. Gilliland, *Biochemistry*, 1992, **31**, 10169.
46 P. Reinemer, H. W. Dirr, R. Ladenstein, R. Huber, M. Lo Bello, G. Federici and M. W. Parker, *J. Mol. Biol.*, 1992, **227**, 214.
47 L. Bousset, H. Belrhali, R. Melki and S. Morera, *Biochemistry*, 2001, **40**, 13564.
48 T. Hara, H. Kato, Y. Katsube and J. Oda, *Biochemistry*, 1996, **35**, 11967.
49 A. M. Caccuri, M. Lo Bello, M. Nuccetelli, M. Nicotra, P. Rossi, G. Antonini, G. Federeci and G. Ricci, *Biochemistry*, 1998, **37**, 3028.
50 G. Xiao, S. Liu, X. Ji, W. W. Johnson, J. Chen, J. F. Parsons, W. J. Stevens, G. L. Gilliland and R. N. Armstrong, *Biochemistry*, 1996, **35**, 4753.
51 Y. V. Patskovsky, L. N. Patskovska and I. Listowsky, *J. Biol. Chem.*, 2000, **275**, 3296.
52 G. H. Snyder, M. J. Cennerazzo, A. J. Karalis and D. Field, *Biochemistry*, 1981, **20**, 6509.
53 A. Jacobi, M. Huber-Wunderlich, J. Hennecke and R. Glockshuber, *J. Biol. Chem.*, 1997, **272**, 21692.
54 S. O. Kim, K. Merchant, R. Nudelman, W. F. Beyer Jr., T. Keng, J. DeAngelo, A. Hausladen and J. S. Stamler, *Cell*, 2002, **109**, 383.
55 M. Marraud and A. Aubry, *Biopolymers*, 1996, **40**, 45.