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The comprehensive acid–base characterization of glutathione

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ABSTRACT

Glutathione in its thiol (GSH) and disulfide (GSSG) forms, and 4 related compounds were studied by ^{1}H NMR-pH titrations and a case-tailored evaluation method. The resulting acid–base properties are quantified in terms of 128 microscopic protonation constants; the first complete set of such parameters for this vitally important pair of compounds. The concomitant 12 interactivity parameters were also determined. Since biological redox systems are regularly compared to the GSH–GSSG pair, the eight microscopic thiolate basicities determined this way are exclusive means for assessing subtle redox parameters in a wide pH range.

1. Introduction

Glutathione (γ-L-glutamyl-L-cysteinyl-glycine, GSH) is the single most important non-enzymatic antioxidant in biological systems. In addition to its major role in oxidative stress and related pathophysiological conditions [1], glutathione is also the preeminent means of detoxification [2]. The ubiquitous and effective GSH–GSSG redox system is the most thoroughly studied one [3], does not require enzyme catalysis to function, can be applied in aqueous media under a wide range of conditions. These advantages make the GSH-GSSG system a 'gold standard' in thiol–disulfide biochemistry; hence, every thiol-containing antioxidant is compared to glutathione [4]. It is a key feature of any thiol–disulfide redox system, however, that only the deprotonated thiol species are reactive in the redox process, i.e. only the anionic thiolate can be oxidized directly [5]. Since the deprotonated fraction of thiols depends on the solution pH (typically strong dependence in the 6–11 pH range), the oxidation–reduction potential of thiol-containing biomolecules is also pH-dependent. These interwoven acid–base and redox processes are usually further influenced by overlapping protonation processes of multiple basic centers in the molecule in question. Therefore, macroscopic physico-chemical parameters will not characterize the thiolate moiety specifically. For an exact/detailed elaboration, the site-specific, so-called microscopic parameters, are needed [6,7].

The acid–base properties of GSH have long been in the focus of scientific interest [8–11], and even the site-specific protonation constants of GSH [12] and GSSG [13] have been studied. Nevertheless, these values are only those of the major pathways. The ongoing work in our research group regarding species-specific redox properties of thiol-containing biomolecules (to be published) requires the site-specific protonation constants, including the minor microspecies; otherwise, redox processes could only be characterized at the level of phenomenon, especially for acidic media. The microscopic protonation constants of GSH in the present study have the added benefit of being determined in consistent conditions of 95 (v/v%) H_2O and 0.15 mol/L ionic strength.

2. Materials and methods

2.1. Materials

All chemicals were purchased from Sigma, and used without further purification.

2.2. NMR spectroscopy measurements

NMR spectra were recorded on a Varian 600 MHz spectrometer at 25 °C. The solvent in every case was an aqueous solution with H_2O:D_2O, 95:5 (v/v) (0.15 mol/L ionic strength), using DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) as the reference compound. The sample volume was 600 μL. In proton NMR experiments, pH values were determined by internal indicator molecules optimized for NMR [14,15], and the water resonance was diminished by double pulsed field gradient spin-echo

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(number of transients = 16, number of points = 65 536, acquisition time = 3.407 s, relaxation delay = 1.5 s).

2.3. Data analysis

For the analysis of NMR titration curves of proton chemical shifts versus pH, the software Origin Pro 8 (OriginLab Corp., Northampton, MA, USA) was used. The standard deviations of protonation constant values obtained from the regression analyses were used to calculate the uncertainty of other equilibrium constant values, according to Gaussian propogation of errors principle, see Section 3.

2.4. TOF MS measurements

The exact masses of the synthesized and isolated compounds were determined with an Agilent 6230 time-of-flight mass spectrometer equipped with a JetStream electrospray ion source in positive ion mode. JetStream parameters: drying gas (N_2) flow and temperature: 10.0 L/min and 325 °C; nebulizer gas (N_2) pressure: 10 psi; capillary voltage: 4000 V; sheath gas flow and temperature: 7.5 L/min and 325 °C. TOF MS parameters: fragmentor voltage: 170 V; skimmer potential: 170 V; OCT 1 RF Vpp: 750 V. Samples (0.1–0.3 μL) were introduced by the Agilent 1260 Infinity HPLC system (flow rate = 0.5 mL/min, 0.1% formic acid in 70 (v/v%) methanol–water mixture). Reference masses of *m*/*z* 121.050873 and 922.009798 were used to calibrate the mass axis during analysis. Mass spectra were acquired over the *m*/*z* range 100–1000 at an acquisition rate of 250 ms/spectrum and processed using Agilent MassHunter B.02.00 software.

2.5. Synthetic protocols

*Glutathione methyl ester (Gly) hydrochloride (**1**)* was synthesized by dissolving 0.114 g (0.37 mmol) glutathione in 5 mL of methanol and cooling down to 0 °C. Next, 0.6 eqv (18 μL) oxalyl chloride was added and the solution was stirred at ambient temperature for 24 h. The reaction mixture was then evaporated in vacuo to yield a clear glassy solid (0.132 g, 92%). ^{1}H NMR (600 MHz, H_2O:D_2O, 95:5, v/v) δ (ppm) 1.91 (2H, m, βCH_2(Glu)), 2.34 (2H, m, γCH_2(Glu)), 2.75 (2H, m, βCH_2(Cys)), 3.44 (1H, t, J = 6 Hz, αCH(Glu)), 3.61 (3H, s, OCH_3(Gly)), 3.89 (2H, dd, J = 5, 18 Hz, αCH(Gly)), 4.22 (1H, dd, J = 2.5, 5 Hz, αCH(Cys)); HRMS *m*/*z* [M+H]$^+$ Calc 322.1073 Found 322.1012.

*Glutathione dimethyl ester hydrochloride (**2**)* was synthesized by dissolving 0.102 g (0.33 mmol) glutathione in 5 mL of methanol and cooling down to 0 °C. Next, 1.1 eqv (30 μL) oxalyl chloride was added and the solution was stirred at ambient temperature for 24 h. The reaction mixture was then evaporated in vacuo to yield a clear oil (0.118 g, 96%). ^{1}H NMR (600 MHz, H_2O:D_2O, 95:5, v/v) δ (ppm) 2.21 (2H, m, βCH_2(Glu)), 2.58 (2H, m, γCH_2(Glu)), 2.94 (2H, m, βCH_2(Cys)), 3.75 (3H, s, OCH_3(Gly)), 3.91 (3H, s, OCH_3(Glu)), 3.97 (1H, t, J = 6 Hz, αCH(Glu)), 4.04 (2H, d, J = 6 Hz, αCH(Gly)), 4.55 (1H, t, J = 6 Hz, αCH(Cys)); HRMS *m*/*z* [M+H]$^+$ Calc 336.1229 Found 336.1574.

*Glutathione disulfide tetramethyl ester (**3**)* was synthesized in situ before NMR measurements by dissolving (**1**) in 2% $H_2O_{2(aq)}$ solution and adjusting pH to 8.5. After 10 min ^{1}H NMR revealed that the oxidation had commenced and no starting material was detectable, neither was any bubbling observed. Ester hydrolysis was determined to be ~2%, however this did not disturb the NMR titration. This aqueous solution was directly used in subsequent ^{1}H NMR-pH titration experiments.

*Glutathione disulfide dimethyl ester (Gly) (**4**)* was synthesized in a similar fashion as (**3**) using (**2**) as starting material.

3. Results

Figure 1 represents the microscopic protonation schemes of GSH (A) and GSSG (B). Macroequilibria (top lines) indicate the stoichiometry of the successively protonated ligand and the stepwise macroscopic protonation constants. In the microspeciation schemes, 16 and 32 chemically different microspecies with their one-letter symbols (a, b, . . ., p for GSH, and a, b, c, d, e [as examples] for GSSG), and 32 and 96 microscopic protonation constants are depicted (k^N, k^S_N, k^G_{NS}, k^E_{NSG}, . . .). The superscript at k for any microconstant indicates the protonating group while the subscript (if any) shows the site(s) already protonated. N, S, G and E symbolize the amino, thiolate, glycinyl carboxylate and glutamyl carboxylate (after their one-letter symbol) sites, respectively. Some examples of macro- and microconstants of GSH are (note that all symbols used below pertain to GSH):

$$K_1 = \frac{[HL^{2-}]}{[L^{3-}][H^+]} \quad K_2 = \frac{[H_2L^-]}{[HL^{2-}][H^+]} \quad K_1K_2 = \frac{[H_2L^-]}{[L^{3-}][H^+]^2} \tag{1}$$

$$k^N = \frac{[b]}{[a][H^+]} \quad k^S_N = \frac{[f]}{[b][H^+]} \quad k^G_{NS} = \frac{[l]}{[f][H^+]} \tag{2}$$

Concentrations of the various macrospecies comprise the sum of the concentration of those microspecies that contain the same number of protons. For example in GSH:

$$[HL^{2-}] = [b] + [c] + [d] + [e] \tag{3}$$

$$[H_2L^-] = [f] + [g] + [h] + [i] + [j] + [k] \tag{4}$$

The following equations show the relationships between the micro- and macroconstants of GSH [16]:

$$K_1 = k^N + k^S + k^G + k^E \tag{5}$$

$$K_1K_2 = k^Nk^S_N + k^Nk^G_N + k^Nk^E_N + k^Sk^G_S + k^Sk^E_S + k^Gk^E_G = \cdots \tag{6}$$

$$K_1K_2K_3 = k^Nk^S_Nk^G_{NS} + k^Nk^S_Nk^E_{NS} + k^Gk^E_Gk^N_{GE} + k^Gk^E_Gk^S_{GE} = \cdots \tag{7}$$

$$K_1K_2K_3K_4 = k^Nk^S_Nk^G_{NS}k^E_{NSG} = \cdots \tag{8}$$

Eqs. (6)–(8) can be written in 64, 1296 and 24 different, equivalent ways depending on the path of protonation. To characterize all of the microscopic basicities, the introduction and utilization of auxiliary compounds are necessary [17].

3.1. The microscopic protonation constants of GSH

In GSH the glutamyl, glycinyl, and cysteinyl α-protons were monitored throughout the pH range with high resolution ^{1}H NMR (pH titration curves in Figure 2). Due to inherent basicities of the four basic centers in GSH, at basic pH predominantly the amino and thiolate moieties protonate in an overlapping manner. Upon lowering the pH, the separate, yet again simultaneous protonation of the two carboxylates takes place. These protonation pathways comprise the major pathway of the GSH microspeciation scheme. Furthermore, it is apparent from the chemical shift-pH profiles that each α proton is selectively perturbed only by the basic moiety in its immediate proximity; i.e. the glycinyl α proton is selective for the glycinyl carboxylate, the cysteinyl α proton is selective for the thiolate, and the glutamyl α proton is selective for the adjacent amino and glutamyl carboxylate protonations. Therefore, by fitting sigmoid curves that specifically express the protonation fraction of each site, one can attain the microscopic protonation constants of the major pathway [13]. For the pH range 6–14 the following function was fitted to the data of the glutamyl and cysteinyl α protons:

$$\delta_{obs(pH)} = \frac{(\delta_{HL} - \delta_L) \times (k^X[H^+] + K_1K_2[H^+]^2)}{1 + (k^N + k^S)[H^+] + K_1K_2[H^+]^2} + \delta_L \tag{9}$$