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The species- and site-specific acid–base properties of penicillamine and its homodisulfide

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ABSTRACT

Penicillamine, penicillamine disulfide and 4 related compounds were studied by ^{1}H NMR-pH titrations and case-tailored evaluation methods. The resulting acid–base properties are quantified in terms of 14 macroscopic and 28 microscopic protonation constants and the concomitant 7 interactivity parameters. The species- and site-specific basicities are interpreted by means of inductive and shielding effects through various intra- and intermolecular comparisons. The thiolate basicities determined this way are key parameters and exclusive means for the prediction of thiolate oxidizabilities and chelate forming properties in order to understand and influence chelation therapy and oxidative stress at the molecular level.

1. Introduction

(2*S*)-2-Amino-3-methyl-3-sulfanylbutanoic acid, or penicillamine (also known as 3-mercapto-D-valine, 3,3-dimethyl-D-cysteine) is a nonproteinogenic amino acid containing a thiol group. The three polar functional groups in penicillamine undergo characteristic chemical reactions and differ in their ability to participate in various chemical and biochemical reactions [1]. Penicillamine was at first solely of interest as a key substance in the structural elucidation of penicillins and as a central building block in their total synthesis [2]. Notwithstanding that its L-isomer is toxic; D-penicillamine is widely used in medicine to treat Wilson's disease [3], heavy metal poisoning [4], cystinuria [5] and rheumatoid arthritis [6]. The thiol group as a typical weak acid occurs mainly in neutral form in physiologic conditions, while the ammonium and carboxylate groups deliver a zwitterionic structure. However, it is the thiolate form that is directly engaged in thiol–disulfide redox equilibria [7]. It has also been reported that thiolate basicities are in correlation with the half-cell redox potentials [8]. Since most biomolecules bear multiple protonation sites which protonate in an overlapping fashion, site-specific characterization of acid–base equilibria helps untangle the interactions between the basic moieties. Such thiolate basicities are indirect indicators of the thiolate oxidizability in the particular protonation stage of the neighboring groups [9]. Penicillamine primarily metabolizes *via* its disulfide form, and the pharmacological and therapeutic actions are largely explained by its ability to take part in thiol–disulfide exchange reactions. For example the formation of the mixed disulfide from penicillamine and cysteine is decisive for the treatment of cystinuria as penicillamine disulfide and penicillamine-cysteine disulfide are much more soluble than cystine and are thus eliminated [2,10].

Although the acid–base properties of penicillamine have been reported in a few papers [11–15], no data appeared on the site-specific protonation constants of D-penicillamine and D-penicillamine disulfide. Here we report the determination of all the site-specific basicities for D-penicillamine, and its homodisulfide using ^{1}H NMR-pH titrations on the parent molecules and 4 synthesized auxiliary compounds. This is the first complete microspeciation of these molecules, providing *sine qua non* constituents for a comprehensive species- and site-specific characterization of the complex, codependent acid–base and thiol–disulfide equilibrium system, that is of crucial importance in maintaining the intracellular redox homeostasis.

2. Materials and methods

2.1. Materials

All chemicals (including D-penicillamine) were purchased from Sigma, and were used without further purification.

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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 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 [16,17], and the water resonance was diminished by double pulsed field gradient spin echo (nt = 16, np = 64 000, acquisition time = 3.33 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. In all regression analyses the non-linear curve fitting option was used with the following function [18]:

$$\delta_{obs}(pH) = \frac{\delta_L + \sum_{i=1}^{n} \delta_{H_iL} \times 10^{\log\beta_i - i\times pH}}{1 + \sum_{i=1}^{n} 10^{\log\beta_i - i\times pH}} \quad (1)$$

where δ_L is the chemical shift of the unprotonated ligand (L), δ_{H_iL} values stand for the chemical shifts of successively protonated ligands, where n is the maximum number of protons that can bind to L, and β is the cumulative protonation macroconstant, as exemplified in Eq. (1). The standard deviations of log β values from the regression analyses were used to calculate the Gaussian propagation of uncertainty to the other equilibrium constants derived in Section 3.

2.4. TOF MS measurements

The exact mass of the synthesized and isolated compounds was 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: 325 °C and 7.5 l/min. TOF MS parameters: fragmentor voltage: 170 V; skimmer potential: 170 V; OCT 1 RF Vpp: 750 V. Samples were introduced (0.1–0.3 μl) by the Agilent 1260 Infinity HPLC system (flow rate = 0.5 ml/min, 70% methanol–water mixture 0.1% formic acid). 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

D-*Penicillamine methyl ester hydrochloride* (**2**) was synthesized by dissolving 0.10 g (0.67 mmol) D-penicillamine (**1**) in 5 ml methanol and bubbling dry HCl gas into the solution for 15 min at room temperature [19]. After stirring overnight at room temperature the reaction mixture was evaporated *in vacuo* to yield a white solid (0.13 g, 98%). Mp: 187–188 °C; ^{1}H NMR (600 MHz, H_2O:D_2O, 95:5, v/v) δ (ppm) 1.42 (3H, s, βCH_3), 1.45 (3H, s, βCH_3), 3.68 (1H, s, αH), 3.79 (3H, s, OCH_3); HRMS *m/z* $[M+H]^+$ Calc 164.0745 Found 164.0724.

S-Methyl D-*penicillamine* (**3**) was synthesized from 0.10 g (0.67 mmol) D-penicillamine (**1**) using 54 μl (1.3 equiv.) methyl iodide and 0.02 g (1.3 equiv.) sodium hydride in 5 ml methanol under N_2 atmosphere [20]. After stirring overnight at room temperature the reaction mixture was evaporated *in vacuo*. The residual oil was purified by column chromatography on silica gel (ethyl acetate–hexane, 1:5, v/v) to afford compound **3** as a white solid (0.11 g, 99%). Mp: 253–260 °C. ^{1}H NMR (600 MHz, H_2O:D_2O, 95:5, v/v) δ (ppm) 1.31 (3H, s, βCH_3), 1.52 (3H, s, βCH_3), 2.07 (3H, s, SCH_3), 3.67 (1H, s, αH); HRMS *m/z* $[M+H]^+$ Calc 164.0745 Found 164.0687.

S-Methyl D-*penicillamine methyl ester hydrochloride* (**4**) was synthesized in a similar fashion to compound **2** from 0.05 g (0.31 mmol) *S*-methyl D-penicillamine (**3**) to yield 0.06 g (98%) yellow oil. ^{1}H NMR (600 MHz, H_2O:D_2O, 95:5, v/v) δ (ppm) 1.48 (3H, s, βCH_3), 1.56 (3H, s, βCH_3), 1.92 (3H, s, SCH_3), 3.71 (3H, s, OCH_3), 3.88 (1H, s, αH); HRMS *m/z* $[M+H]^+$ Calc 178.0902 Found 178.0930.

D-*Penicillamine disulfide* (**5**) was synthesized *in situ* by dissolving 0.10 g (0.67 mmol) D-penicillamine (**1**) into a 5% H_2O_2 aqueous solution and adjusting the pH to 8. After stirring overnight the aqueous solution was used directly for NMR measurements. ^{1}H NMR (600 MHz, H_2O:D_2O, 95:5, v/v) δ (ppm) 1.44 (3H, s, βCH_3), 1.55 (3H, s, βCH_3), 4.01 (1H, s, αH); HRMS *m/z* $[M+H]^+$ Calc 297.0943 Found 297.1017.

D-*Penicillamine disulfide dimethyl ester* (***6***) was synthesized in a similar fashion to compound **5** from 0.05 g (0.25 mmol) D-penicillamine methyl ester hydrochloride (**2**). ^{1}H NMR (600 MHz, H_2O:D_2O, 95:5, v/v) δ (ppm) 1.50 (3H, s, βCH_3), 1.57 (3H, s, βCH_3), 3.66 (3H, s, OCH_3), 4.19 (1H, s, αH); HRMS *m/z* $[M+H]^+$ Calc 325.1256 Found 325.1296.

3. Results

Figure 1 shows the formulae of the molecules studied. Figure 2 represents the microscopic protonation schemes of penicillamine (A) and penicillamine disulfide (B). Macroequilibria (top lines) indicate the stoichiometry of the successively protonated ligand and the stepwise macroscopic protonation constants. In the microspeciation schemes the 8 and 16 microspecies with their one-letter symbols (a, b, . . ., h for penicillamine, and *a*, *b*, . . ., *p* [in italics] for penicillamine disulfide), and the 12 and 32 microscopic protonation constants are depicted (k^O, $k_O{}^N$, $k_{ON}{}^S$, . . .), for sake of consistency penicillamine disulfide protonation constants are depicted in italics. The superscript of k indicates the protonating group while the subscript (if any) shows the site(s) already protonated. N, S and O symbolize the amino, thiolate and carboxylate sites, respectively. Some examples of macro- and microconstants of penicillamine are:

$$K_1 = \frac{[HL^-]}{[L^{2-}][H^+]} \quad K_2 = \frac{[H_2L]}{[HL^-][H^+]} \quad K_1K_2 = \beta_2 = \frac{[H_2L]}{[L^{2-}][H^+]^2} \quad (2)$$

$$k^O = \frac{[d]}{[a][H^+]} \quad k_O^N = \frac{[f]}{[d][H^+]} \quad k_{ON}^S = \frac{[h]}{[f][H^+]} \quad (3)$$

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

$$[HL^-] = [b] + [c] + [d] \quad (4)$$

$$[H_2L] = [e] + [f] + [g] \quad (5)$$

The following equations show the relationships between the micro- and macroconstants of penicillamine [21]:

$$K_1 = k^N + k^S + k^O \quad (6)$$

$$K_1K_2 = k^Nk_N^S + k^Nk_N^O + k^Sk_S^O = k^Sk_S^N + k^Ok_O^N + k^Ok_O^S = \ldots \quad (7)$$

$$K_1K_2K_3 = k^Nk_N^Sk_{SN}^O = k^Sk_S^Nk_{SN}^O = \ldots \quad (8)$$

Eqs. (7) and (8) can be written in 2 and 6 different, equivalent ways depending on the path of protonation. To characterize all of the microscopic bacisities, the introduction and utilization of auxiliary compounds are necessary.

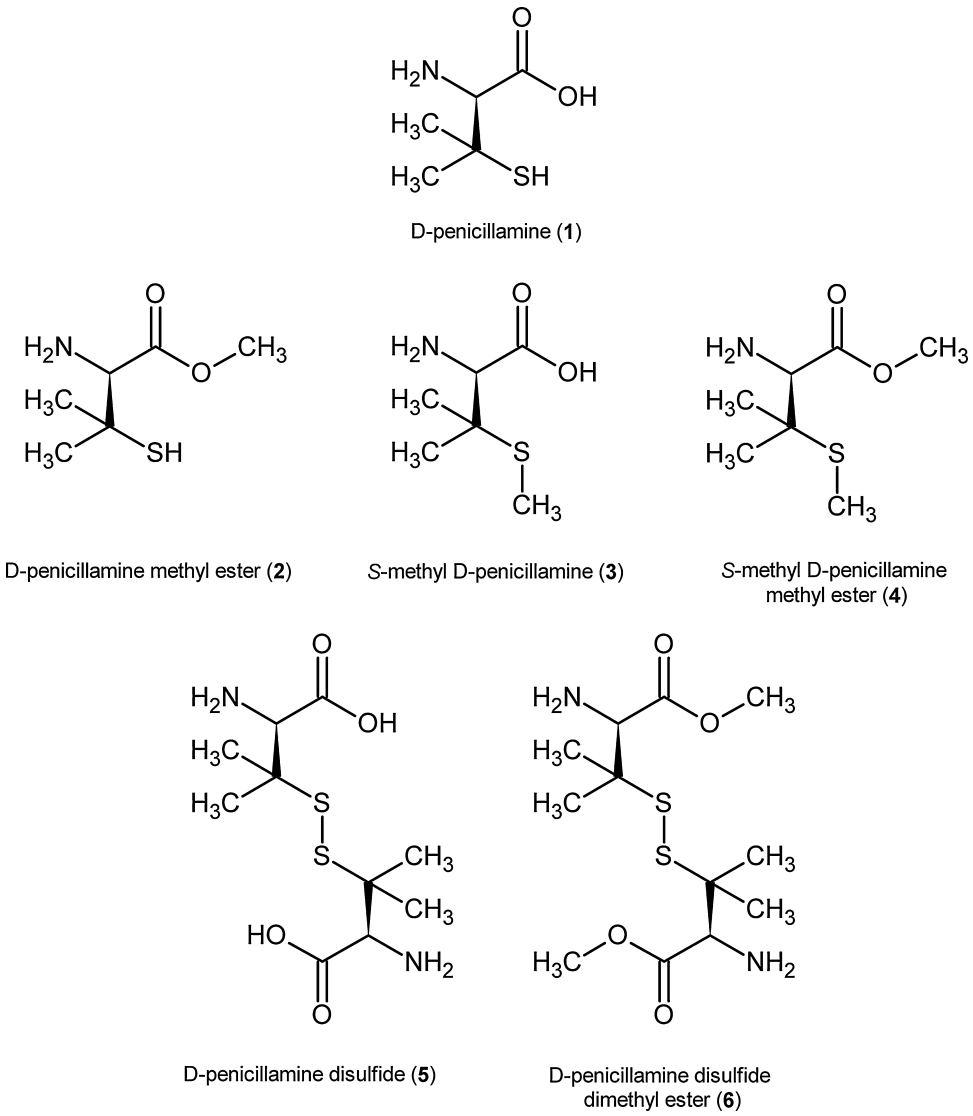

Figure 1. The formulae of the compounds studied.

Table 1
Table of protonation constants.

Penicillamine				Penicillamine disulfide			
Penicillamine		Penicillamine methyl ester		Penicillamine disulfide		Penicillamine disulfide dimethyl ester	
Macroscopic protonation constants							
$\log K_1$	10.80 ± 0.01	$\log K_1$	8.99 ± 0.01	$\log K_1$	9.48 ± 0.03	$\log K_1$	7.23 ± 0.02
$\log K_2$	8.07 ± 0.01	$\log K_2$	6.90 ± 0.01	$\log K_2$	8.57 ± 0.03	$\log K_2$	6.32 ± 0.01
$\log K_3$	2.04 ± 0.01	*S*-methyl penicillamine		$\log K_3$	2.34 ± 0.04		
S-Methyl penicillamine methyl ester		$\log K_1$	9.53 ± 0.01	$\log K_4$	1.61 ± 0.04		
$\log K$	7.65 ± 0.01	$\log K_2$	2.04 ± 0.01				
Microscopic Protonation Constants							
$\log k^{N}$	10.78 ± 0.01	$\log k_S{}^{O}$	3.92 ± 0.01	$\log k^{N}$	9.18 ± 0.03	$\log k^{O}_{NN'}$	2.04 ± 0.04
$\log k^{S}$	9.34 ± 0.01	$\log k_O{}^{N}$	8.90 ± 0.01	$\log k^{O}$	4.29 ± 0.05	$\log k^{N'}_{O'N}$	6.99 ± 0.03
$\log k^{O}$	5.02 ± 0.01	$\log k_O{}^{S}$	8.24 ± 0.01	$\log k^{N'}_{N}$	8.87 ± 0.03	$\log k^{O}_{O'N}$	2.28 ± 0.07
$\log k_N{}^{S}$	8.09 ± 0.01	$\log k_{SN}{}^{O}$	2.04 ± 0.01	$\log k^{O'}_{N}$	3.92 ± 0.04	$\log k^{N'}_{ON}$	8.50 ± 0.01
$\log k_N{}^{O}$	3.14 ± 0.01	$\log k_{ON}{}^{S}$	6.99 ± 0.01	$\log k_N{}^{O}$	2.41 ± 0.05	$\log k^{O'}_{ON}$	3.79 ± 0.04
$\log k_S{}^{N}$	9.53 ± 0.01	$\log k_{OS}{}^{N}$	7.65 ± 0.01	$\log k^{N'}_{O}$	8.81 ± 0.02	$\log k^{N}_{OO'}$	6.93 ± 0.02
				$\log k_O{}^{N}$	7.30 ± 0.05	$\log k^{O'}_{ONN'}$	1.91 ± 0.04
				$\log k^{O'}_{O}$	4.16 ± 0.07	$\log k^{N'}_{OO'N}$	6.62 ± 0.01
Interactivity parameters							
$\log \Delta E_{N/S}$	1.25 ± 0.01	$\log \Delta E_{S/O}$	1.10 ± 0.01	$\log \Delta E_{N/O}$	1.88 ± 0.01	$\log \Delta E_{N/N'}$	0.31 ± 0.04
$\log \Delta E_{N/O}$	1.88 ± 0.01			$\log \Delta E_{N/O'}$	0.37 ± 0.04	$\log \Delta E_{O/O'}$	0.13 ± 0.06

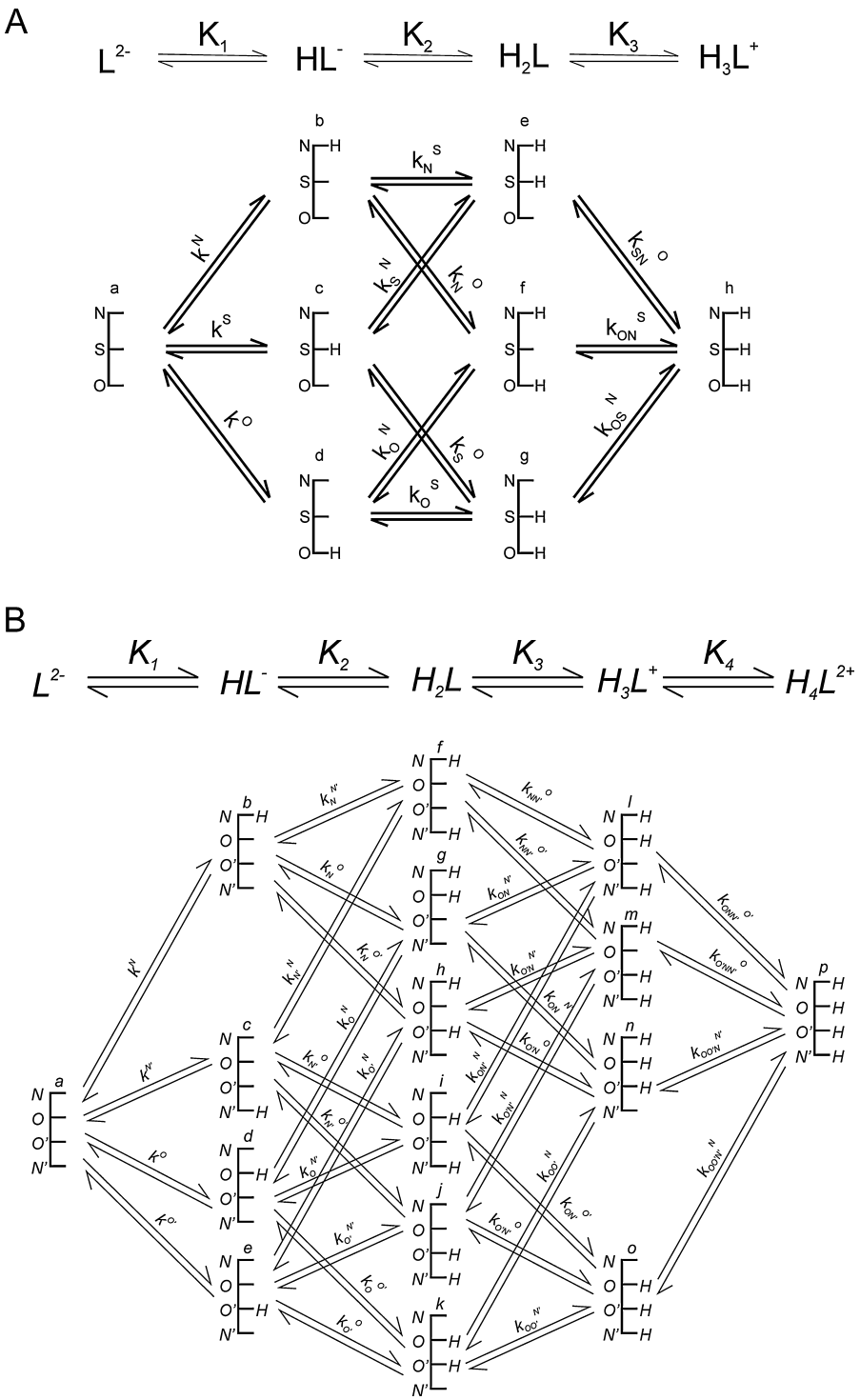

Figure 2. The protonation macro- and microequilibrium schemes of penicillamine (A) and penicillamine disulfide (B). N, S, O labels denote the amino, thiolate, and carboxylate groups, respectively.

3.1. *The microscopic protonation constants of* D*-penicillamine*

D-Penicillamine was titrated (titration curve in Fig. 3) using ^{1}H NMR under near physiological conditions to afford its macroscopic protonation constants (Table 1). For the calculation of the 12 protonation microconstants through a multivariable equation system, pieces of additional information are necessary. The quality and quantity of the additional information depend on the acid–base properties of the substance. It is chemically evident that basicities of the sites decrease in the amino, thiolate, carboxylate order. Basicity of the thiolate is "comparably lower" while basicity of the carboxylate is "orders of magnitude lower" than that of the amino group. Therefore, those microspecies, in which the carboxylate is protonated but the amino is not protonated are "orders of magnitude minor" ones. Similarly, those microspecies in which the thiolate is protonated but the amino is not are the "relative minor" ones. The "orders of magnitude minor" substances cannot be detected with analytical methods like NMR or UV, but they may have an important role in highly specific biochemical processes [22]. To elucidate the complete microspeciation, auxiliary compounds that mimic the

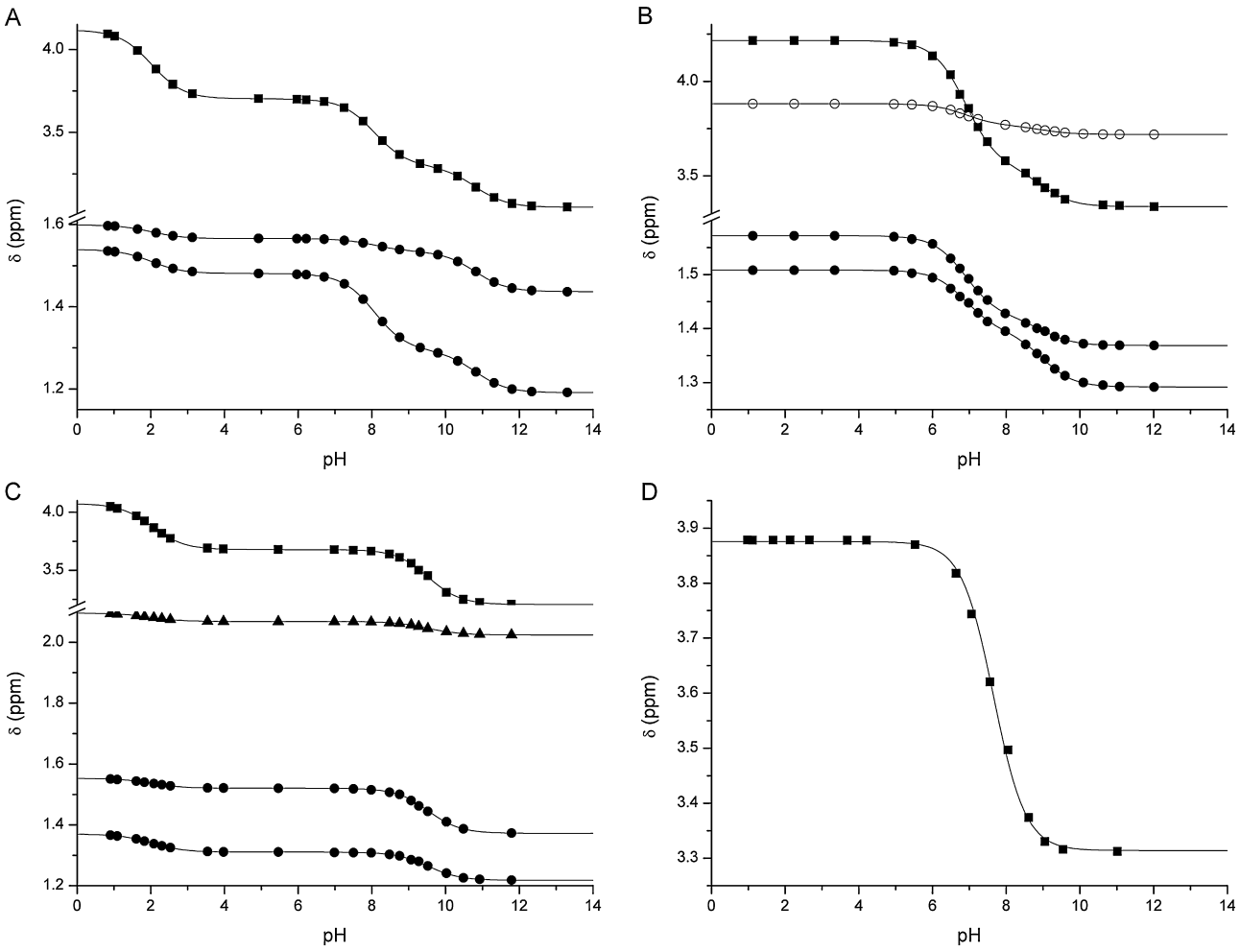

Figure 3. The plot of ^{1}H chemical shifts *versus* pH; (■) α proton, (•) CH_3 protons, (○) *O*-methyl protons, (▲) *S*-methyl protons. (A) Penicillamine; (B) penicillamine methyl ester; (C) *S*-methyl penicillamine; (D) *S*-methyl penicillamine methyl ester.

order of magnitude minor or relative minor substances, have to be used. In this case, model compounds of the c, d and g microspecies were synthesized: *S*-methyl penicillamine, penicillamine methyl ester and *S*-methyl penicillamine methyl ester. The site in which the methyl group is connected remains in neutral state, with practically identical electronic effects on the rest of the molecule as —COOH and —SH groups [23].

The macroconstants of the auxiliary compounds (Table 1) were also attained by ^{1}H NMR titrations (titration curves in Fig. 3). The microscopic protonation constants of penicillamine were calculated using equations similar to (6) and (8) bearing in mind that the macroscopic protonation constants of the model compounds are components of the penicillamine microspeciation scheme. For example the protonation constant of *S*-methyl penicillamine methyl ester amino group is equivalent to $k_{OS}{}^{N}$. The equations utilized to calculate the microscopic constants are as follows:

$$\log k^{S} = \log \beta_3^{\text{penicillamine}} - \log \beta_2^{S\text{-methyl penicillamine}} \tag{9}$$

$$\log k^{O} = \log \beta_3^{\text{penicillamine}} - \log \beta_2^{\text{penicillamine methyl ester}} \tag{10}$$

$$\log k_S^{O} = \log \beta_2^{S\text{-methyl penicillamine}} - \log K^{S\text{-methyl penicillamine methyl ester}} \tag{11}$$

$$\log k_O^{S} = \log \beta_2^{\text{penicillamine methyl ester}} - \log K^{S\text{-methyl penicillamine methyl ester}} \tag{12}$$

$$k_S^{N} + k_S^{O} = K_1^{S\text{-methyl penicillamine}} \tag{13}$$

$$k_O^{N} + k_O^{S} = K_1^{\text{penicillamine methyl ester}} \tag{14}$$

$$\log k_S^{N} + \log k_{SN}^{O} = \log \beta_2^{S\text{-methyl penicillamine}} \tag{15}$$

$$\log k_O^{N} + \log k_{ON}^{S} = \log \beta_2^{\text{penicillamine methyl ester}} \tag{16}$$

The remaining microconstants ($\log k^{N}$, $\log k_N{}^{S}$, and $\log k_N{}^{O}$) were calculated using the amino/thiolate interactivity parameter that could be obtained from the relationships in Eq. (8) and microconstants involved:

$$\log \Delta E_{N/S} = \log k_O^{N} - \log K^{S\text{-methyl penicillamine methyl ester}} = \log k^{N} - \log k_S^{N} \tag{17}$$

$$\log \beta_3^{\text{penicillamine}} = \log k^{N} + \log k_N^{S} + \log k_{SN}^{O} = \log k^{N} + \log k_N^{O} + \log k_{ON}^{S} \tag{18}$$

The interactivity parameter shows to what extent the protonation of site A reduces the basicity of site B, and *vice versa*. The interactivity parameter is generally considered to be the most invariant quantity in analogous moieties of different compounds and also in various protonation states of the neighboring moiety in the same molecule [24]. The protonation constants of penicillamine with the interactivity parameters are compiled in Table 1.

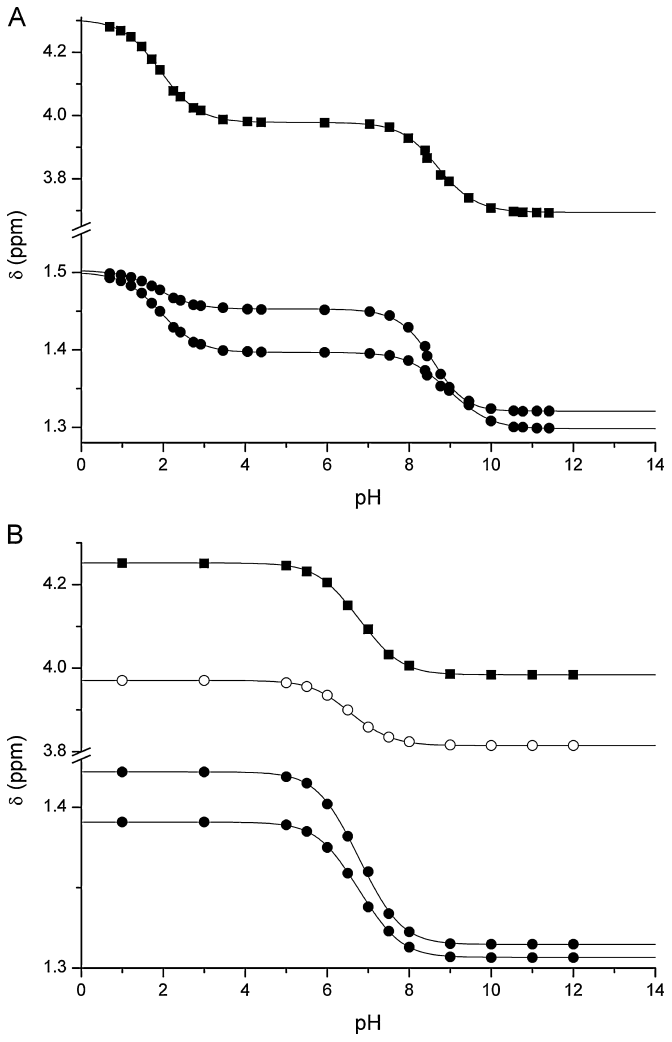

Figure 4. The plot of ^{1}H chemical shifts *versus* pH; (■) α proton, (•) CH_3 protons, (○) *O*-methyl protons. (A) Penicillamine disulfide; (B) penicillamine disulfide dimethyl ester.

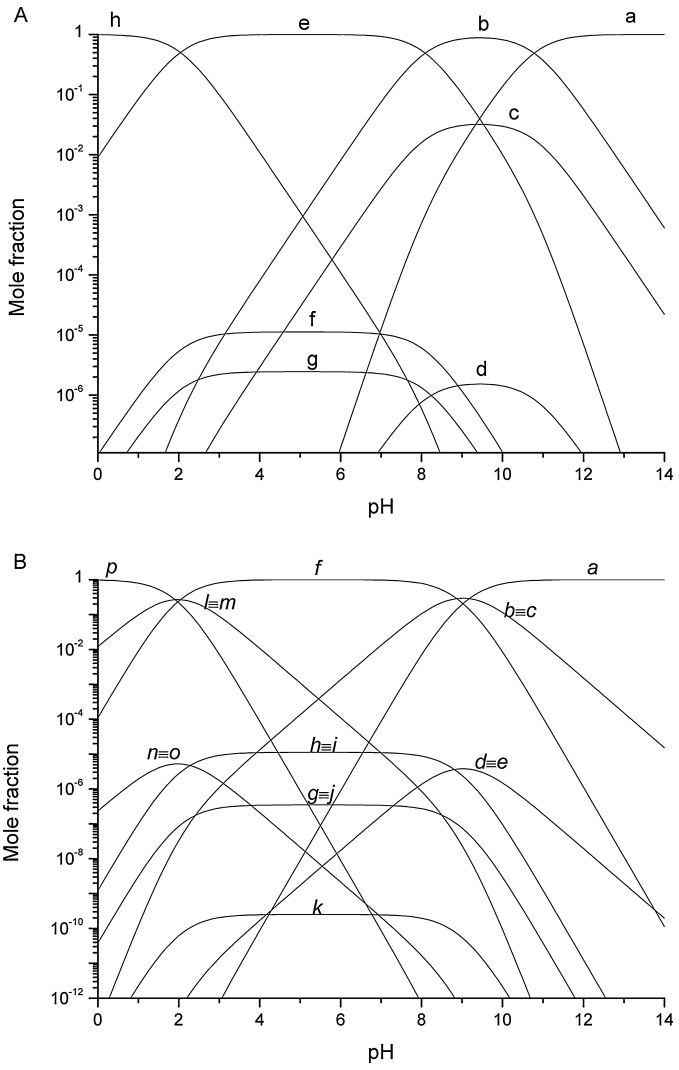

Figure 5. The relative concentrations in logarithmic scale of the various microspecies of penicillamine (A) and penicillamine disulfide (B) as a function of pH. Letters a–h and *a*–*p* represent the microspecies of penicillamine and penicillamine disulfide as defined in Figure 2.

The pH-dependent mole fraction diagram of the compounds is in Figure 5.

3.2. The microscopic protonation constants of penicillamine disulfide

The microspeciation of penicillamine disulfide is simplified by two facts: (1) the symmetry of the molecular structure (the opposite amino and carboxylate groups are chemically identical), and (2) the well-separated protonation pH range of the amino and carboxylate groups. Fact (1) results in the equality of certain microscopic protonation constants: $k^{N} = k^{N'}$, $k^{O} = k^{O'}$, $k^{N'}_{N} = k^{N}_{N'}$, $k^{O'}_{N} = k^{O}_{N'}$, $k^{O}_{N} = k^{O'}_{N'}$, $k^{N'}_{O} = k^{N}_{O'}$, $k^{N}_{O} = k^{N'}_{O'}$, $k^{O'}_{O} = k^{O}_{O'}$, $k^{O}_{NN'} = k^{O'}_{NN'}$, $k^{N'}_{O'N} = k^{N}_{ON'}$, $k^{O}_{O'N} = k^{O'}_{ON'}$, $k^{N'}_{ON} = k^{N}_{O'N'}$, $k^{O'}_{ON} = k^{O}_{O'N'}$, $k^{N}_{OO'} = k^{N'}_{OO'}$, $k^{O'}_{ONN'} = k^{O}_{O'NN'}$, $k^{N'}_{OO'N} = k^{N}_{OO'N'}$. Since the first and last two protonation steps in penicillamine disulfide are practically separated (titration curve in Fig. 4), they appear as independent systems. The first two protonation steps are overwhelmingly those of the amino groups, since the mono- and biprotonated ligands in which any of the two carboxylate groups is protonated are "orders of magnitude minor". Therefore the microscopic protonation constants of the major pathway can be calculated using the following equations:

$$\log k^{N} = \log K_1^{\text{penicillamine disulfide}} - \log 2 \tag{19}$$

$$\log k^{N'}_{N} = \log K_2^{\text{penicillamine disulfide}} + \log 2 \tag{20}$$

$$\log k^{O}_{NN'} = \log K_3^{\text{penicillamine disulfide}} - \log 2 \tag{21}$$

$$\log k^{O'}_{ONN'} = \log K_4^{\text{penicillamine disulfide}} + \log 2 \tag{22}$$

In order to elucidate the minor pathway, an auxiliary compound was synthesized and characterized. Penicillamine disulfide dimethyl ester was used to model the microspecies where both carboxylates are protonated. Using the principles as above, additional microscopic protonation constants of penicillamine disulfide were attained:

$$\log k^{N}_{OO'} = \log K_1^{\text{penicillamine disulfide dimethyl ester}} - \log 2 \tag{23}$$

$$\log k^{N'}_{OO'N} = \log K_2^{\text{penicillamine disulfide dimethyl ester}} - \log 2 \tag{24}$$

The missing microconstants were calculated using the amino/carboxylate interactivity parameter of penicillamine (regarded as "same-side" amino/carboxylate interactivity parameter in penicillamine disulfide), and the other interactivity parameters of penicillamine disulfide listed in Table 1:

$$\log \Delta E_{N/O} = \log k_S^N - \log K^{S\text{-methyl penicillamine methyl ester}} = \log k^N - \log k_O^N \quad (25)$$

All of the macroscopic and microscopic protonation constants of penicillamine disulfide with the interactivity parameters are listed in Table 1. The pH-dependent mole fraction diagram of the various microspecies is in Figure 5.

The macroscopic and microscopic protonation constants (25 °C, 0.15 mol/l ionic strength), and interactivity parameters of penicillamine, penicillamine disulfide and their model compounds in log units ±s.d.

4. Discussion

A profound submolecular acid–base evaluation of penicillamine and penicillamine disulfide allows deeper insight compared to the conventional macroscopic pK_a values, especially when minor microspecies are considered. Also, several remarkable peculiarities can be observed when penicillamine microconstants are compared to those of cysteine, the related molecule of analogous skeleton.

The first and second protonation constants of penicillamine are predominated overlappingly by the amino and thiolate sites, the amino being typically more favored. The carboxylate group protonates at much lower pH region. In fact, the monoprotonated microspecies are mainly b and c in decreasing order of abundance, while some 4 orders of magnitude smaller amount of d is present. The biprotonated microspecies occurs mainly in the form of e. Among the minor biprotonated species, the one protonated at the amino and carboxylate groups is favored over the other minor microspecies. When the carboxylate is protonated, and the molecule takes on a second proton, the amino group is favored over the negatively charged thiolate (as opposed to cysteine [9]). Due to the electron-donating properties of the methyl groups of penicillamine, the amino bacisity is higher in penicillamine compared to that of cysteine. On the other hand, the bacisity of the thiolate is lower in penicillamine, presumably due to steric hinderance of the two methyl moieties. The basicity of the carboxylate is only marginally higher in penicillamine.

In penicillamine disulfide the N and N′ amino groups protonate parallel, followed by the also parallel protonation of the two carboxylates. It is noteworthy that in the instance of biprotonated disulfide (major f microspecies: both amino groups protonated) the most favored minor microspecies is the one with opposite amino and carboxylate groups protonated (h = i), due to the Coulombic repulsion between hydronium ions and the ammonium ion, hence the opposite carboxylate will be slightly more basic. By far the least favored biprotonated species (k) is the one with both carboxylates protonated. The bacisities of the amino and carboxylate groups are also higher in penicillamine disulfide than in cystine due to the electron-donating methyl groups.

Concerning NMR-pH properties, it is also noteworthy that in penicillamine the protonation shifts of the α proton are 0.25, 0.4, 0.42 ppm, due to the protonation of the amino, thiolate, and carboxylate groups, respectively. Surprisingly, the smallest protonation shift on the α proton belongs to the amino site, which is closest to the observed α proton. Yet, these values are much larger than those of the two methyl groups: 0.09, 0.04, and 0.03 ppm; 0.1, 0.19, and 0.06 ppm. The amino and carboxylate groups being closer to the α proton naturally produce greater protonation shifts on the α proton. Since the thiolate is equidistant from the α and CH_3 protons, the difference in their thiolate protonation shifts might be caused by a shielding effect on the methyl groups. Furthermore, there is considerable difference between the two thiolate protonation shifts of the methyl groups, so much so that one of the methyl groups is almost insensitive to the adjacent thiolate protonation. This is another indication of the unique steric properties of the two chemically distinct methyl groups.

The interactivity parameter is a good indicator of the basicity-reducing effect on one of the sites when the other site protonates. For example, the esterification of the carboxyl moiety decreases the basicity of the amino and thiolate groups, while the *S*-methylation has no effect on the carboxylate site, because the thiolate is practically completely protonated when the carboxylate protonates ($K_2^{S\text{-methyl penicillamine}} = k_{SN}^{O\text{ penicillamine}}$). In penicillamine, the interactivity parameters are in decreasing order as follows: amino/carboxylate, amino/thiolate and thiolate/carboxylate. Because of the steric effects of the methyl groups, the thiolate-containing interactivity parameters are smaller in penicillamine than in cysteine, while no difference can be observed between their amino/carboxylate interactivity parameters. Relative to the long covalent distance, the interactivity parameter between the opposing amino and carboxylate groups is higher than expected. This phenomenon might be caused by the flexible molecular structure enabling Coulombic attraction between the protonated ammonium and the opposing carboxylate. On the other hand, these opposing side interactivities are smaller than in cystine. Because of the two shielding methyl groups, penicillamine disulfide is presumably less flexible than cystine, which can explain the lower interactivity parameters in penicillamine disulfide.

5. Conclusions

The 28 site-specific basicities determined for penicillamine, penicillamine disulfide and 4 related compounds is a significant dataset to interpret the behavior of these therapeutically important compounds in their biochemical, synthetic and analytical reactions. Since thiolate basicities are related to their redox and chelating properties, the 10 different thiolate protonation constants provide sound means at the molecular level to predict thiolate oxidizabilities, a key parameter to understand and influence oxidative stress and heavy metal chelation and to produce custom-tailored penicillamine-derived drug candidates.

Conflict of interest

The authors report no conflict of interest.

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