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The complete microspeciation of arginine and citrulline

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ABSTRACT

^{1}H NMR–pH titrations of arginine, the most basic natural amino acid and citrulline, its neutral counterpart were carried out. Two other closely related auxiliary compounds were also studied. The 8 macroscopic protonation constants were determined. Combining the four datasets in a deductive method, all the 12 microconstants of arginine, the 4 microconstants of citrulline and arginine amide were calculated. An error-propagation analysis and the pH-dependent distribution of the 8 arginine microspecies are provided.

1. Introduction

Arginine is the most basic natural amino acid, and it is conditionally essential for humans. It plays an important role in blood pressure regulation and NO production [1,2], while it is converted into ornithine, the precursor of polyamines and urea. Arginine is a precursor of creatine, which is essential in the energy metabolism in muscle. Arginine is also capable to increase growth hormone secretion [3]. Moreover, systemic administration of Arg has been shown to improve cardiovascular function in patients with coronary artery disease [4,5].

The most basic form of arginine has three proton-binding sites: guanidino (G), primary amino (A) and carboxylate (C) moiety, which is the order if basicities, too. Such markedly different proton affinities have long been known to preset distinctively different major and minor microspecies. In the highly specific biochemical processes, however, the major species are not necessarily the reactive ones [6,7]. As a triprotic molecule, the protonation scheme consists of 8 microspecies and 12 microconstants [8]. There are 3 monoprotonated and 3 diprotonated isomers (Fig. 1), which differ only in the site(s) of protonation [9].

Citrulline is another ubiquitous amino acid in mammals either as a sovereign molecule or as a part of citrullinated proteins. Its metabolism involves NO synthase, argininosuccinate synthetase and ornithine carbamoyltransferase enzymes. Latter produces citrulline in the body.

Thus citrulline is an important molecule in its own right, but also as the closest relative of arginine. The two compounds are isoelectronic and their deprotonated forms are as much isosteric as possible. The guanidino–carbamido difference causes, however, significant dissimilarity in the side-chain basicity, enabling citrulline an ultimate model of deprotonated arginine.

Despite the enormous biological significance, the microspeciation of arginine has only been attempted by pH-potentiometry, in which high pH values are admittedly distorted and the minor microspecies are neglected [10]. No report appeared on the microspeciation of citrulline.

In order to mimic those microspecies too, in which the carboxylate is protonated while the other basic sites can change their acid–base status, two further compounds were used: arginine amide ($ArgNH_2$) and citrulline methyl ester (CitOMe). The compounds investigated are shown in Fig. 2.

The extreme difficulties of arginine microspeciation lie in the following facts:

- The first protonation step takes places at high pH, where an undistorted pH cannot be measured by glass electrode.
- Some of the microspecies occur in very low concentration; their contribution to the analytical sign is negligible. Therefore, auxiliary compounds have to be used to model the minor species and to obtain reliable microconstants.

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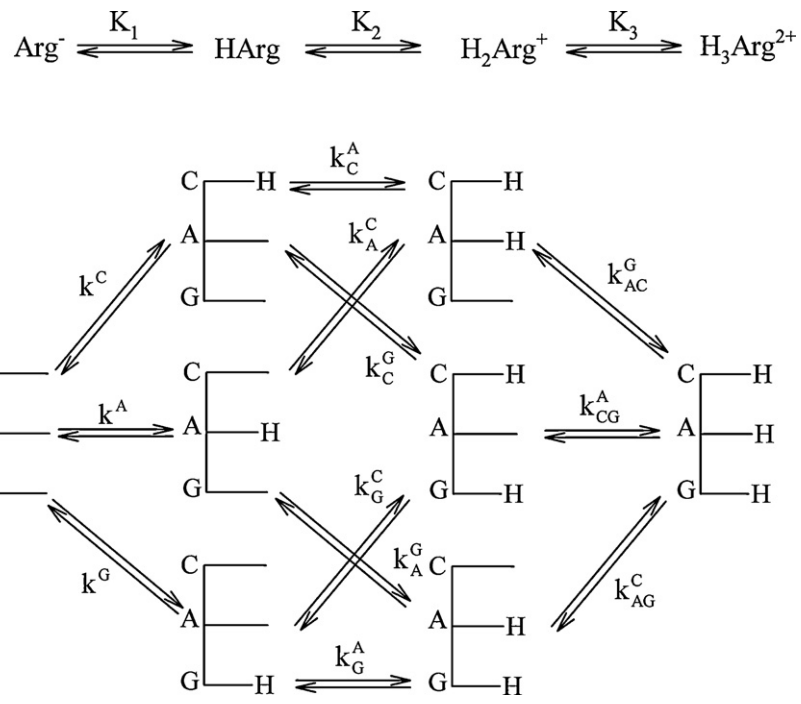

Fig. 1. Protonation scheme of arginine, in terms of K_1, K_2, K_3 stepwise macroconstants and $k^C, \ldots, k^C_{AG}$ microconstants. Superscripts of the microconstants indicate the site of protonation, whereas subscript(s) (if any) show the site(s) already protonated. *C*, *A* and *G* stand for carboxylate, amino, guanidino, respectively.

- Because of the vicinity of the protonation sites, none of the spectroscopic signals is site-specific.

^{1}H NMR–pH titrations were done to monitor the protonation processes. To improve the accuracy of pH measurement in highly basic solutions, a new set of *in situ* NMR–pH indicator molecules were introduced and a least-distorted pH-determination method was elaborated [11].

2. Experimental

2.1. Materials

Arginine, arginine amide dihydrochloride, citrulline, the indicator molecules (TRIS, sarcosine, *tert*-butylamine, acetone oxime and 1-methylguanidine), D_2O (99 atom% D) and chlorotrimethylsilane were purchased from Sigma–Aldrich Co. Other chemicals of analytical grade were obtained from commercial suppliers and used without further purification. Bidistilled water was used for all solutions.

2.2. Synthesis of citrulline methyl ester

The methyl ester of citrulline was synthesized according to the procedure published by Li and Sha [12]. Citrulline was taken into a round bottomed flask, and 2 equiv. chlorotrimethylsilane was added slowly during stirring. Then methanol was added, and the resulting solution was stirred for 24 h at room temperature. The solvents were removed *in vacuo*, and the product was isolated as the hydrochloride salt of citrulline methyl ester. ^{1}H NMR (D_2O): δ

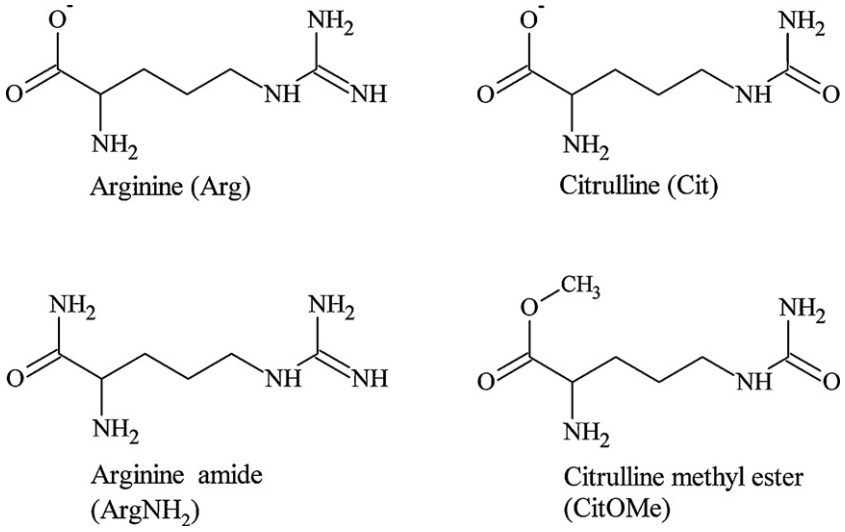

Fig. 2. Constitutional formulae of the compounds studied, in their most basic form.

Table 1
The protonation constants and the limiting chemical shifts of the indicators.

	log *K*	δ_L (ppm)	δ_{HL} (ppm)
Sarcosine	10.15	3.106	3.613
		2.281	2.737
tert-Butylamine	10.99	1.099	1.369
Acetone oxime	12.08	1.828	1.896
		1.769	1.897
1-Methylguanidine	13.43	2.691	2.825

4.20 (t, 1H), 3.85 (s, 3H), 3.19 (t, 2H), 1.98 (m, 2H), 1.64 (m, 2H); ^{13}C NMR: δ 173.5, 164.1, 56.7, 55.6, 42.3, 30.0, 27.6.

2.3. ^{1}H NMR titrations

All measurements were carried out on a Varian Inova spectrometer (600 MHz for ^{1}H). Spectra were recorded at 25 °C, and referenced to internal DSS. The titrations were carried out in a medium of 95% (v/v) H_2O and 5% (v/v) D_2O. Such a small amount of D_2O shifts the pH scale only by 0.02 unit, according to the Gross–Butler–Purlee theory [13,14]. The water signal was suppressed by double pulse field gradient spin echo pulse sequence [15]. Spectra were processed with VNMRJ 2.2C software.

For pH measurement a Metrohm 6.0234.110 combined glass electrode was also used, which was calibrated with four NIST standard buffer solutions. To avoid the uncertainty of the glass electrode at pH > 12, the pH was determined by indicator molecules (Table 1). Concept and details of the accurate, high pH determinations are described in a separate paper [11]. Below pH 9 the pH meter readings were used. Briefly, the protonation constant of sarcosine was determined by nonlinear fitting of chemical shift versus glass electrode pH readings. (Eq. (1))

$$\delta^{obs} = \frac{\delta_L + \delta_{HL} K[H^+]}{1 + K[H^+]} \quad (1)$$

where δ^{obs} is the observed chemical shift, δ_L and δ_{HL} are the chemical shifts of the deprotonated and protonated species, and K is the protonation constant.

Since the accuracy and precision of pH-meter readings dramatically decreases at pH > 12.5, the difference of the protonation constants was determined, by fitting to δ_2^{obs} versus δ_1^{obs} datasets.

$$\delta_{Ind_2}^{obs} = \frac{\delta_{HInd_2}(\delta_{Ind_1}^{obs} - \delta_{Ind_1}) + 10^{\Delta \log K}\delta_{Ind_2}\left(\delta_{HInd_1} - \delta_{Ind_1}^{obs}\right)}{10^{\Delta \log K}\left(\delta_{HInd_1} - \delta_{Ind_1}^{obs}\right) + \left(\delta_{Ind_1}^{obs} - \delta_{Ind_1}\right)} \quad (2)$$

where Ind_1 and Ind_2 refer to the first and second indicator molecule, $\Delta \log K = \log K_{Ind_2} - \log K_{Ind_1}$.

The pH of the samples were calculated by a modified Henderson–Hasselbach equation (Eq. (3)), where K_{Ind} is the protonation constant, δ_{Ind} and δ_{HInd} are the limiting chemical shifts of the deprotonated and protonated forms, respectively and δ_{Ind}^{obs} is the observed chemical shift.

$$pH_{Ind} = \log K_{Ind} + \log \frac{\delta_{Ind}^{obs} - \delta_{HInd}}{\delta_{Ind} - \delta_{Ind}^{obs}} \quad (3)$$

Solution concentration of the ligands was 2 mM, whereas those of the indicator molecules were 0.5–2 mM. The spectra were referenced to DSS (sodium-(3-trimethylsilyl)-1-propanesulfonate). Acidic and basic stock solutions were prepared with a concentration of 1 M HCl/NaOH. The ionic strength was kept constant at 1 M by adding a calculated volume of 2 M KCl to each sample. As the ester (CitOMe) rapidly hydrolyzes in basic solution, it was dissolved in 1 M KCl solution, and was then rapidly NMR–pH titrated without using the glass electrode by 0.1 M NaOH, 0.9 M KCl solution.

3. Results and discussion

The microspeciation of arginine was done by the following processes:

- NMR–pH titrations of Arg, Cit, $ArgNH_2$, CitOMe.
- Determination of the respective 3, 2, 2, 1 pieces of macroconstants.
- Calculation of the microconstants and the concentrations of the microspecies.
- Validation of the results by comparing the adequate microconstant k_C^A with the macroconstant of CitOMe.

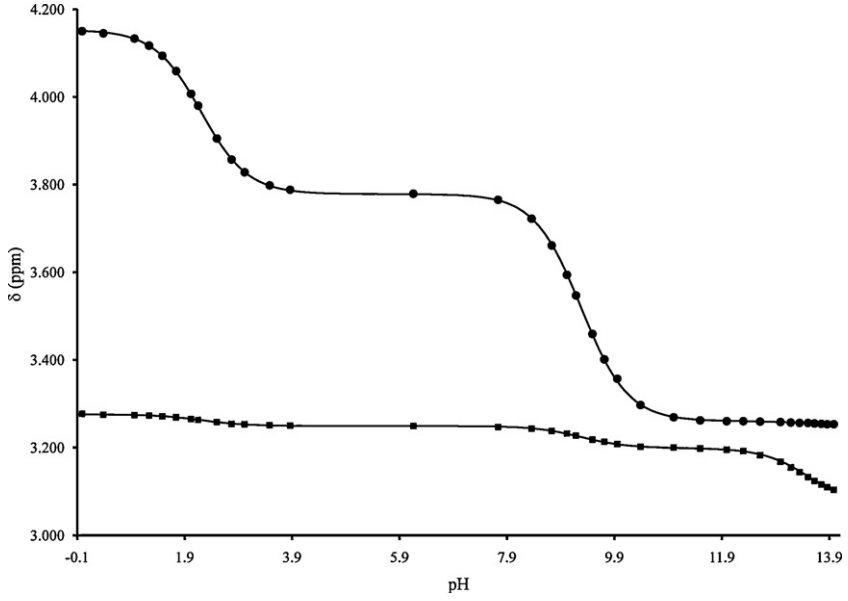

Fig. 3. The titration curves of arginine. Dots and squares are the experimental points of α and δ protons, respectively; solid lines are the computer fits.

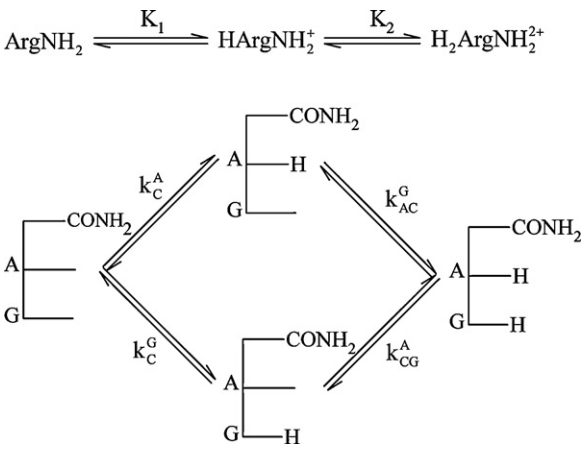

Fig. 4. The microspeciation scheme of $ArgNH_2$.

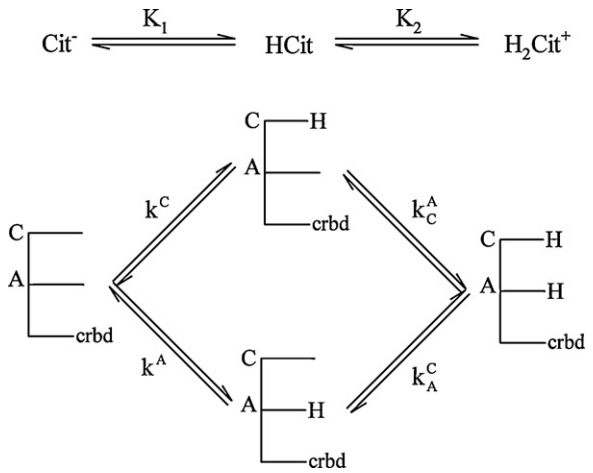

Fig. 5. The microspeciation scheme of citrulline, where crbd stands for the carbamido moiety.

3.1. NMR analysis of the compounds

Arginine, citrulline and arginine amide contain four nonexchanging protons, on the α, β, γ and δ carbons. The β and γ protons are overlapping. Moreover, their splitting pattern is complicated, we therefore observed the triplets of the α and δ protons. The ester has an additional signal of the O-methyl group.

3.2. Determination of the macroconstants

The measured chemical shift – pH datasets were evaluated with nonlinear parameter fitting, using the OriginPro 8 software.

The macroconstants were calculated using Eq. (4)

$$\delta^{obs} = \frac{\delta_L + \delta_{HL}\beta_1[H^+] + \delta_{H_2L}\beta_2[H^+]^2 + \delta_{H_3L}\beta_3[H^+]^3}{1 + \beta_1[H^+] + \beta_2[H^+]^2 + \beta_3[H^+]^3} \quad (4)$$

where δ^{obs} is the observed chemical shift, δ_L, δ_{HL}, δ_{H_2L}, δ_{H_3L} are the chemical shifts of the non-protonated, mono-, di-, and triprotonated species, respectively, while β_1, β_2, β_3 are the cumulative protonation constants. By the simultaneous fitting of the observed α and δ ^{1}H NMR chemical shift – pH datasets, the macroconstants were calculated. The protonation steps are well-separated in all compounds (Fig. 3), indicating that the determination of the minor microspecies certainly needs the auxiliary compounds.

The results are listed in Table 2

3.3. Calculation of the microconstants

Relationships between the cumulative (β) macroconstants and the microconstants for arginine are as follows:

$$\beta_1 = k^C + k^A + k^G \quad (5)$$

$$\beta_2 = k^A k_A^C + k^A k_A^G + k^C k_C^G = k^C k_C^A + k^G k_G^A + k^C k_C^G \quad (6)$$

$$\beta_3 = k^G k_G^A k_{AG}^C = k^A k_A^G k_{AG}^C = k^C k_C^A k_{AC}^G \quad (7)$$

Comparing the microspeciation schemes (Figs. 1, 4 and 5), some of the microconstants can be calculated from appropriate macroconstants:

$$k^C = \frac{\beta_3^{Arg}}{\beta_2^{ArgNH_2}} = \frac{k^C k_C^A k_{AC}^G}{k_C^A k_{AC}^G} \quad (8)$$

$$k^G = \beta_1^{Arg} - \beta_1^{Cit} = (k^G + k^A + k^C) - (k^A + k^C) \quad (9)$$

By these microconstants and the seven macroconstants, all the constants could be calculated using Eqs. (5)–(7), as follows:

$$k^A = \beta_1^{Cit} - k^C = (k^A + k^C) - k^C \quad (10)$$

$$k_A^C = \frac{\beta_2^{Cit}}{k^A} = \frac{k^A k_A^C}{k^C} \quad (11)$$

$$k_C^A = \frac{\beta_2^{Cit}}{k^C} = \frac{k^C k_C^A}{k^C} \quad (12)$$

$$k_C^G = \beta_1^{ArgNH_2} - k_C^A = \left(k_C^G + k_C^A\right) - k_C^A \quad (13)$$

$$k_A^G = \frac{\beta_2^{Arg} - k^A \cdot k_A^C - k^C \cdot k_C^G}{k^A} = \frac{\left(k^A \cdot k_A^G + k^A \cdot k_A^C + k^C \cdot k_C^G\right) - k^A \cdot k_A^C - k^C \cdot k_C^G}{k^A} \quad (14)$$

The formation of the microspecies protonated on *A* and *G* sites can be considered as a Hessian cycle, therefore:

$$k_G^A = \frac{k_A^G k^A}{k^G} \quad (15)$$

For the microspecies protonated on *C* and *G* sites, the same is applied:

$$k_G^C = \frac{k^C k_C^G}{k^G} \quad (16)$$

$$k_{AG}^C = \frac{\beta_3^{Arg}}{k^A k_A^G} = \frac{k^A k_A^G k_{AG}^C}{k^A k_A^G} \quad (17)$$

$$k_{CG}^A = \frac{\beta_3^{Arg}}{k^C k_C^G} = \frac{k^C k_C^G k_{CG}^A}{k^c k_C^G} \quad (18)$$

$$k_{AC}^G = \frac{\beta_3^{Arg}}{k^A k_A^C} = \frac{k^A k_A^C k_{AC}^G}{k^A k_A^C} \quad (19)$$

Table 2
The macroscopic protonation constants of the compounds.

	Arginine	Arginine amide	Citrulline
$\log \beta_1$	13.54 ± 0.01	13.41 ± 0.03	9.50 ± 0.01
$\log \beta_2$	22.82 ± 0.01	20.94 ± 0.04	11.83 ± 0.02
$\log \beta_3$	25.03 ± 0.01		

Table 3
The microconstants of arginine.

Site: Carboxylate		Site: Amino		Site: Guanidino	
Microconstant	Value	Microconstant	Value	Microconstant	Value
$\log k^{C}$	4.09 ± 0.04	$\log k^{A}$	9.49 ± 0.01	$\log k^{G}$	13.54 ± 0.01
$\log k^{C}_{A}$	2.33 ± 0.02	$\log k^{A}_{C}$	7.75 ± 0.04	$\log k^{G}_{C}$	13.41 ± 0.04
$\log k^{C}_{G}$	3.96 ± 0.05	$\log k^{A}_{G}$	9.28 ± 0.02	$\log k^{G}_{A}$	13.32 ± 0.04
$\log k^{C}_{AG}$	2.21 ± 0.02	$\log k^{A}_{CG}$	7.53 ± 0.05	$\log k^{C}_{AG}$	13.20 ± 0.06

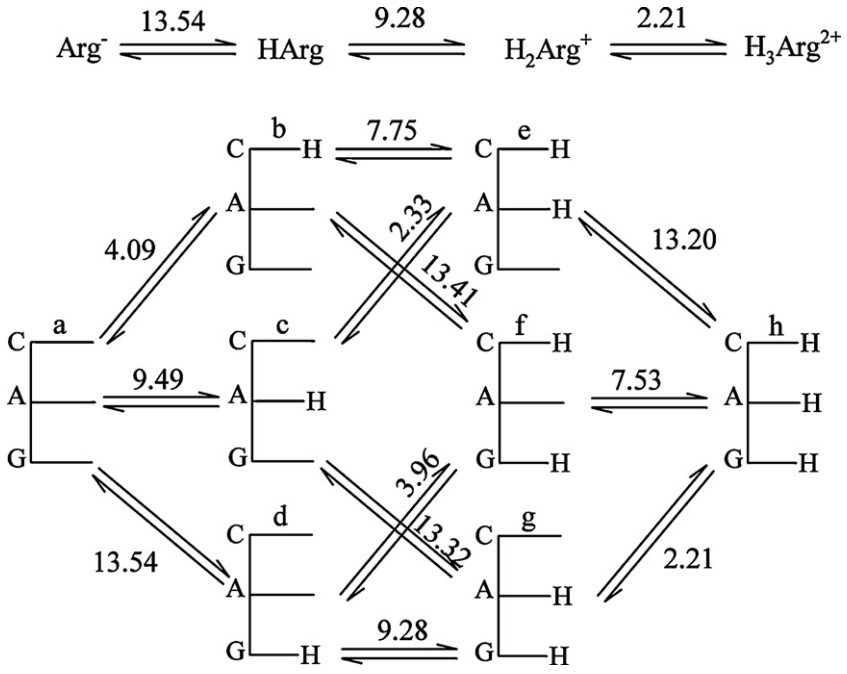

Fig. 6. The microscopic protonation constants of arginine.

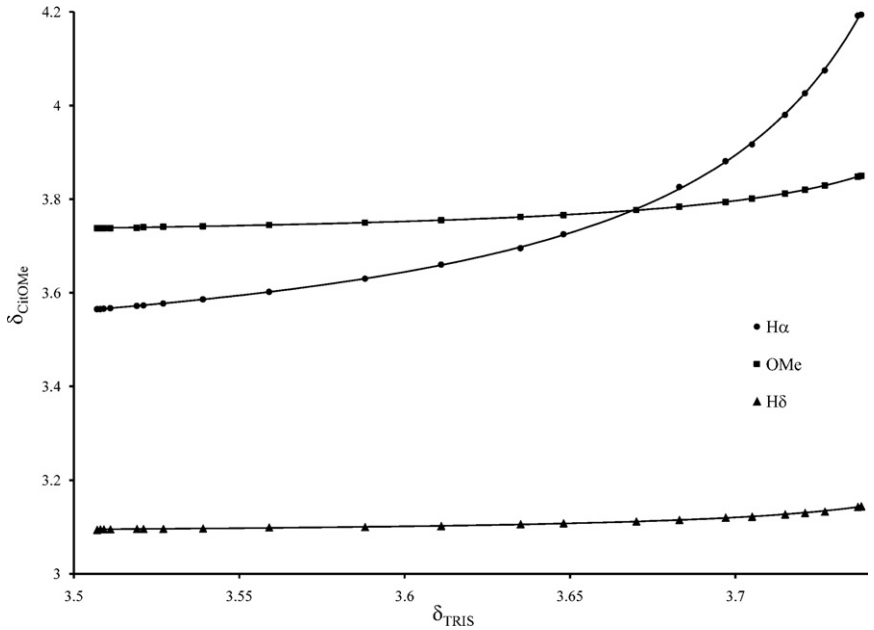

Fig. 7. Plot of ^{1}H chemical shifts CitOMe protons as a function of the CH_2 chemical shift of TRIS during the electrodeless titration. The computer fits are the solid lines.

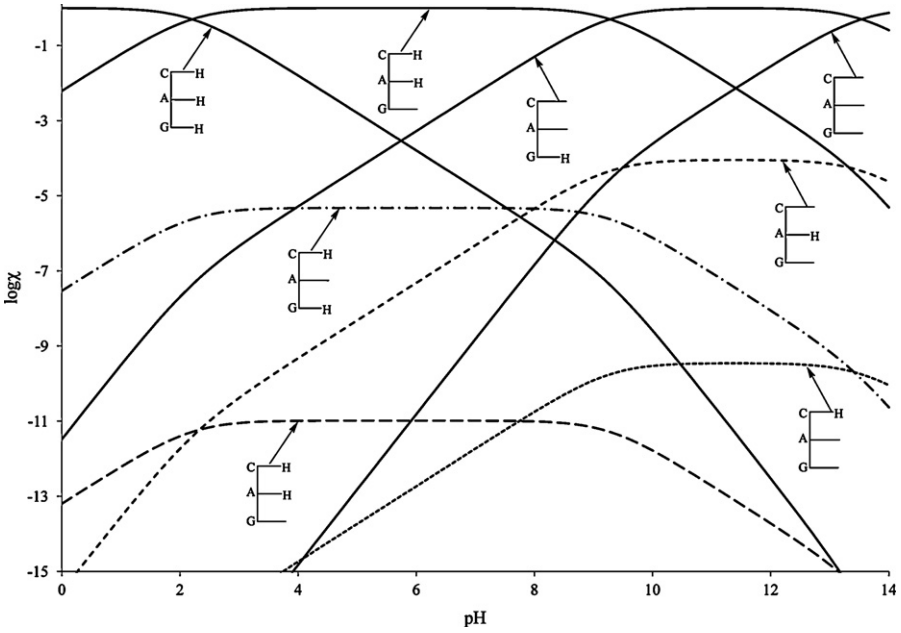

Fig. 8. Distribution of the microspecies of arginine as a function of pH.

The errors of the microconstants were calculated with the quadratic error propagation rule of Gauss [16]. For instance the error of k^G is:

$$\sigma^2_{k^G} = \left(\frac{\partial k^G}{\partial \log \beta_1^{\mathrm{Arg}}}\right)^2 \sigma^2_{\log \beta_1^{\mathrm{Arg}}} + \left(\frac{\partial k^G}{\partial \log \beta_1^{\mathrm{Cit}}}\right)^2 \sigma^2_{\log \beta_1^{\mathrm{Cit}}} \quad (20)$$

By algebraic transformations Eq. (20) can be rearranged:

$$\sigma_{\log k^G} = \sqrt{\left(\frac{10^{\log \beta_1^{\mathrm{Arg}}}}{10^{\log k^G}}\right)^2 \sigma^2_{\log \beta_1^{\mathrm{Arg}}} + \left(\frac{10^{\log \beta_1^{\mathrm{Cit}}}}{10^{\log k^G}}\right)^2 \sigma^2_{\log \beta_1^{\mathrm{Cit}}}} \quad (21)$$

The microconstants are listed in Table 3, and shown in Fig. 6.

3.4. The microconstants of citrulline

As Fig. 5 shows, citrulline has four microconstants, which are: k^A, k^C, k_A^C and k_C^A, and are identical with the appropriate microconstants of arginine. The k^C microconstant can be calculated according to Eq. (8), and the others are:

$$\log k^A = \log\left(\beta_1^{\mathrm{Cit}} - k^C\right) = 9.49 \pm 0.01 \quad (22)$$

$$\log k_A^C = \log \beta_2^{\mathrm{Cit}} - \log k^A = 2.33 \pm 0.02 \quad (23)$$

$$\log k_C^A = \log \beta_2^{\mathrm{Cit}} - \log k^C = 7.75 \pm 0.04 \quad (24)$$

3.5. Validation of the microconstants

The protonation constant of CitOMe was determined by an "electrodeless" titration, using the method of Perrin and Fabian [17]. By this method the difference of the protonation constants can be calculated, if two compounds are titrated together. The solution pH is the same, therefore, according Eq. (3):

$$\log K^{\mathrm{CitOMe}} + \log \frac{\delta^{obs}_{\mathrm{CitOMe}} - \delta_{H\mathrm{CitOMe}^+}}{\delta_{\mathrm{CitOMe}} - \delta^{obs}_{\mathrm{CitOMe}}} = \log K^{Ind} + \log \frac{\delta^{obs}_{Ind} - \delta_{HInd}}{\delta_{Ind} - \delta^{obs}_{Ind}} \quad (25)$$

Thus the pH of the solution does not have to be known, the $\log K^{\mathrm{CitOMe}}$ value can be easily calculated after a simple rearrangement. To take into account several experimental points, the following curve-fitting formula can be applied:

$$\delta^{obs}_{\mathrm{CitOMe}} = \delta_L^{\mathrm{CitOMe}} + \frac{\left(\delta_{HL}^{\mathrm{CitOMe}} - \delta_L^{\mathrm{CitOMe}}\right)\left(\delta^{obs}_{Ind} - \delta_{Ind}\right)}{\left(1 - 10^{\Delta \log K}\right)\left(\delta^{obs}_{Ind} - \delta_{Ind}\right) + 10^{\Delta \log K}\left(\delta_{HInd} - \delta_{Ind}\right)} \quad (26)$$

The $\log K$ value of the indicator has to be certainly close to the protonation constant of the molecule in question, preferably within less than 1 $\log K$ units. Therefore TRIS (tris(hydroxymethyl)aminomethane) was used as an indicator. The indicator parameters were determined by calibrating NMR–pH titrations, under the same ionic strength and temperature. The protonation constant is 8.36 ± 0.01; the limiting chemical shifts are 3.508 ± 0.001 ppm and 3.738 ± 0.001 ppm for the deprotonated and protonated forms, respectively.

During the evaluation three observed proton signals of CitOMe (α, δ and OCH_3) were used (Fig. 7). The nonlinear curve fitting yielded $\Delta \log K = -0.669 \pm 0.002$, consequently the protonation constant of CitOMe is 7.69 ± 0.02, which is in excellent agreement with the previously, deductively calculated arginine microconstant $k_C^A = 7.75 \pm 0.04$. The small difference (0.06 $\log K$ units) can be caused by using different types of model compounds: an ester and an amide. Since the –COOH and $–CONH_2$ moieties are isoelectronic, and their solvatation properties, electron-withdrawing, etc. effects are closer than those in the $–COOH–COOCH_3$ pair of moieties.

The calculation is nicely verified in this complicated system by the independently obtained value.

4. Conclusions

Protonation constants of the guanidino site, the most basic moiety in biological systems were quantified by an unbiased, undistorted NMR–pH method.

The complete microspeciation of arginine, including 8 microspecies and 12 microconstants and also the microspeciation of citrulline were achieved for the first time.

Using the determined microconstants, the pH-dependent distribution of the eight microspecies could be calculated (Fig. 8).

Concerning the protonation isomers one predominates over the others by 5 and 10 orders of magnitude. In the pH range 2.2–9.2 the monocationic form is overwhelming. The isoelectric point of arginine, where the overall charge of the molecule is zero is pH 11.41, indicating that arginine occurs in a monocationic form in human tissues.

The isoelectric point of citrulline is pH 5.91, a great difference for two isoelectronic compounds, making also obvious by great differences in reactivity.

Acknowledgement

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