Multinuclear NMR and Potentiometric Study on Tautomerism during Protonation and Zinc(II) Complex Formation of Some Imidazole-containing Peptide Derivatives

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The acid–base properties and zinc(II) complexes of glycylhistamine, sarcosylhistamine, carcinine and carnosine have been studied by potentiometric, ^{13}C and ^{14}N NMR methods. Macroscopic species for the three states of protonation (LH_2^{2+}, LH^+, L) and the corresponding microspecies involving three protonation sites (terminal amino, N-1 and -3 imidazole nitrogens) are quantitatively estimated for the metal-free ligands. Zinc(II) complexation is shown to reverse the tautomeric preference between 1- and 3-H tautomeric forms of the imidazole ring (in LH^+ and L), as compared to the free ligands where the 1-H tautomer is predominant.

Histamine has long been recognized as an important chemical messenger and mediator in inflammation, allergy, and in neuronal processes.[1] The liberation of histamine from its derivatives (for example from its peptides) is an important means to regulate the processes mentioned above. In the last two decades a number of naturally occurring C-terminal histamine-containing pseudo-peptides (called peptidoamines) have been identified. Carcinine (β-alanylhistamine) was discovered in cardiac tissue of the crustacean *Carcinus maenas*[2] in 1973 and has since been identified in several tissues of the rat, guinea-pig, mouse and human, in levels as high as those reported for the related imidazole compounds, carnosine (β-alanylhistidine) histidine and histamine.[3] Radioisotopic studies have shown that carcinine is metabolically linked to both histamine and carnosine.[3] It was also found that carcinine, unlike carnosine, exerted a dose-dependent positive ionotropic effect in guinea-pig hearts.[4] Furthermore, carcinine was assumed to take part both in the mammalian cardiac physiology and cardiovascular response to stress. The formation of several histamine containing peptidoamines has been shown in pig hypothalamic tissue,[5] in rat brain,[6] and they were proposed as transmitter peptides in the central nervous system.[7] Histamine-containing peptidoamines have also been found in bee venom[8] and their simple dipeptide derivative (glycylhistamine) was shown to be a radio protective agent.[9]

The tautomerism of the imidazole ring in these compounds is an important factor in view of their biological action. In this respect, there is particular interest in the tautomerism of histidine, using ^{13}C NMR[10,11] and ^{15}N NMR[12,13] spectroscopies. Microscopic protonation equilibrium studies have been reported in a number of papers on histidine and histidine-containing peptides.[14–16] The ^1H NMR–pH profile of histidine and other related compounds was used to determine the macroscopic pK values of individual tautomers of histidine.[17]

Imidazole rings are involved in the coordination sphere of several zinc(II)-bound metalloproteins. Thus the formation of the complex between zinc(II) and some histidine-containing peptides used as model systems has been widely studied.[18–21] NMR techniques were used to gain structural information about the environment of the zinc-binding sites in the case of histidine[22] and histidine-containing peptides.[19–21] In the case of Ala-His, Gly-His and Gly-His-Lys complexes, it was found that Zn^{II} was able to promote the deprotonation of the amide group. Recently, several papers have reported different kinds of pharmacological uses of zinc(II)–carnosine[23–25] complex. By means of a comparative ^{13}C CP/MAS NMR study, Matsukura *et al.*[23] proposed a dimeric or oligomeric structure with the coordination of N^1 in the imidazole ring for the water-insoluble zinc(II)-carnosine complex. More recently we reported the Cu^{II}-, Ni^{II}- and Co^{II}-coordination properties of some histamine-containing peptidoamines.[26–27]

The present paper reports the protonation and zinc(II)-complex formation of glycylhistamine (Gly-Hist), sarcosylhistamine (Sar-Hist), carcinine and carnosine, with special regard to microscopic protonation and tautomerism. ^{14}N NMR spectroscopy techniques, in spite of their low sensitivity and quadrupolar relaxation, were found useful to study the tautomerism of the imidazole ring.

Experimental

Materials.—Glycylhistamine, sarcosylhistamine and carcinine were synthesized in our laboratory from *N-tert*-butoxycarbonyl-glycine, or -sarcosine, or -β-alanine (respectively), and histamine hydrochloride (Sigma products). BOP [benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate, Neosystem product] was used as coupling agent. The final products were then obtained by cleavage of the protecting *tert*-butoxycarbonyl group using hydrochloric acid dissolved in ether. The structure and purity of these compounds were checked by ^1H and ^{13}C NMR spectroscopy, elemental analysis (C, H, N, Cl) and acid–base titration. Carnosine and $Zn(ClO_4)_2$ were Fluka products. Stock solution of zinc(II) perchlorate was standardized complexometrically.

Potentiometric Measurements.—The dissociation and stability constant determinations by pH titrations were made at 298 ± 0.1 K, under a nitrogen atmosphere at an ionic strength I of 0.1 mol dm^{-3} $NaClO_4$. Changes in pH were followed by using an Orion (cat. no. 91–03) combined glass electrode and an Orion 901 pH-meter. For the quantitative evaluation of data, the correlation shown between the experimental electromotrice force values (E) and the equilibrium hydrogen-ion concentrations [H^+] was used; j_H and j_{OH} are fitting parameters in acidic and alkaline media for the correction of experimental errors, mainly due to the liquid-junction potential and to the possible alkaline and acids errors of the glass electrode, and K_w is the autoprotolysis constant of water ($10^{-13.75}$). The protonation and complex formation constants were calculated as the

$$E = E_0 + \frac{RT}{F}\log[H^+] + j_H[H^+] + j_{OH}[H^+]^{-1}K_W$$

average over six and ten independent titrations, respectively using concentrations of 0.01–0.025 mol dm^{-3} and metal to ligand ratios varying from 1:1 to 1:6. For other pH determinations, the pH-meter was calibrated using standard buffer solutions (Merck). Each run used a 5 cm^3 sample titrated with a 0.03 mol dm^{-3} NaOH solution.

NMR Measurements.—All NMR spectra were measured in H_2O at 298 K (except some ^{14}N NMR spectra). For ^1H NMR (and partly for ^{14}N NMR studies) a ligand concentration of 0.025 mol dm^{-3} and a 0.1 mol dm^{-3} ionic strength ($NaClO_4$) were used for the sake of comparison with pH measurements. For ^{13}C and ^{14}N NMR spectra, a 0.2 mol dm^{-3} ligand concentration was used. The spectra were recorded on a Bruker AM-400 spectrometer equipped with a high-resolution probe working at 400, 100.62 and 28.91 MHz frequencies for ^1H, ^{13}C and ^{14}N nuclei, respectively. The chemical shifts are referred to the signal of dioxane (3.7 ppm for ^1H and 67.4 ppm for ^{13}C NMR) and KNO_3 (0 ppm for ^{14}N NMR). The spectrometer parameters for ^{14}N NMR measurements were 90° pulses of 64 μs, spectral widths of 15 kHz and pulse repetition times of 0.5 s. For ^{13}C and ^{14}N NMR experiments wide-band proton decoupling was used.

Results and Discussion

Ligand Protonation.—Macroscopic (K) and microscopic (k) constants for the deprotonation equilibria of the amino (a) and imidazole (i) groups in the ligands studied are shown in Schemes 1 and 2. Starting from the fully protonated ligand LH_2 (charges omitted), two pathways are possible (Scheme 1). The first step involves deprotonation of either the amino group (k^a) or the imidazole ring (k^i) to yield the tautomeric forms LH_a and LH_i. The latter species is itself a mixture of two tautomeric forms LH_{N^1H} and LH_{N^3H} according to whether deprotonation of the imidazolium cation occurs on nitrogens N-3 or N-1, respectively, as shown in Scheme 2. This further distinction will be examined in subsequent paragraphs, but is actually immaterial at the present stage of the discussion. Coming back to the pathways described in Scheme 1, a second deprotonation from either LH_a (k_a^i) or LH_i (k_i^a) results into the neutral ligand L (in fact a mixture of tautomeric species L_{N^1H} and L_{N^3H}, see further).

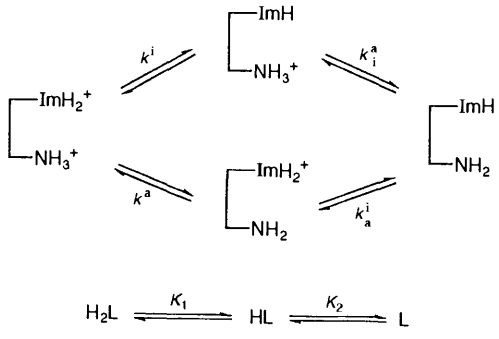
The microconstants shown in Scheme 1 are related[14] to macroconstants K_1 and K_2 as follows:

$$K_1 = k^a + k^i \quad (1)$$

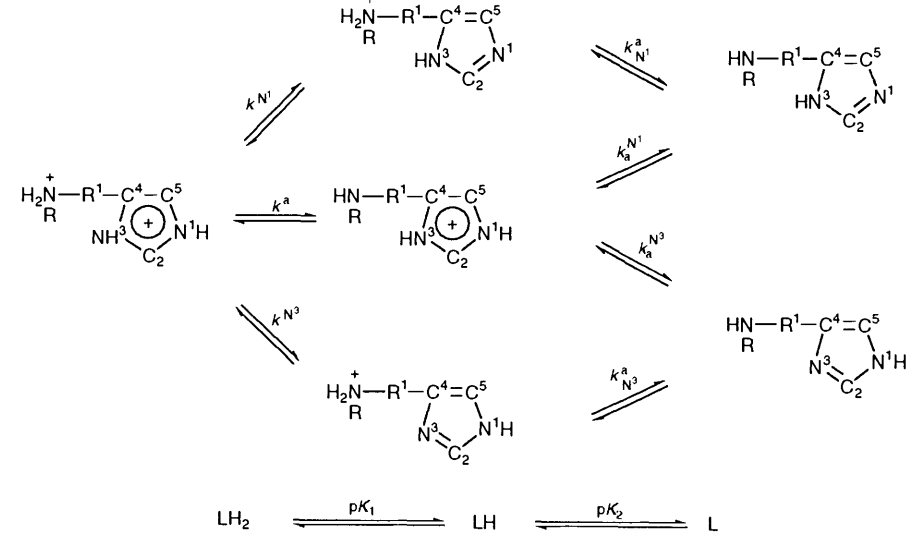
$$K_1K_2 = k^ak_a^i = k^ik_i^a \quad (2)$$

The potentiometrically determined macroconstants K_1 and K_2 are listed in Table 1. In the case of carcinine and carnosine, the two deprotonation steps are well apart from each other on the pK-scale (by more than two units), as a consequence of the reduced electron-withdrawing effect of the carbonyl group on the terminal amino-group in a β-alanyl residue (*i.e.* one carbon further than in Gly-Hist and Sar-Hist). This ensures a nearly 100% predominance of LH_i in the tautomeric monocation LH, with $K_1 \cong k^i$ and $K_2 \cong k_i^a$. In the case of ligands containing α-amino acid residues (Gly-Hist and Sar-Hist), the terminal amino-groups have less basic character, this accounts for the simultaneous occurrence of both deprotonation pathways and for the necessity of considering the full set of eqns. (1) and (2). These equations point out the existence of three independent microconstants. The knowledge of K_1, K_2 from pH measurements is insufficient by itself to find out the three microconstants mentioned above.

Additional information required for this purpose may be conveniently found by ^1H or ^{13}C NMR, since the pH-



Scheme 1 Schematic macro- and micro-deprotonation equilibria for amino (NH_2) and imidazole (ImH) deprotonation of the ligands studied



Carcinine: R = H, R^1 = $-C^{10}H_2-C^9H_2-C^8(O)-NH-C^7H_2-C^6H_2-$
Carnosine: R = H, R^1 = $-C^{10}H_2-C^9H_2-C^8(O)-NH-C^7H(CO_2)-C^6H_2-$
Gly-Hist: R = H, R^1 = $-C^9H_2-C^8(O)-NH-C^7H_2-C^6H_2$
Sar-Hist: R = $C^{10}H_3$, R^1 = $-C^9H_2-C^8(O)-NH-C^7H_2-C^6H_2$

Scheme 2 Full set of microspecies and microconstants involved in the deprotonation of imidazole-containing peptide derivatives

Table 1 Macro- and micro-dissociation constants of the ligands studied, I = 0.1 mol dm^{-3} $NaClO_4$, T = 298 K, estimated errors in parentheses (last two digits)

Compound	pK_{CO_2H}	pK_1	pK_2	pk^a	pk^i	pk^a_i	pk^i_a
Gly-Hist	—	6.76 (01)	8.04 (01)	7.65 (02)	6.82 (02)	7.98 (02)	7.15 (02)
Sar-Hist	—	6.77 (01)	8.31 (01)	7.94 (02)	6.80 (02)	8.28 (02)	7.13 (02)
Carcinine	—	6.84 (01)	9.21 (01)	—	—	—	—
Carnosine	2.63 (02)	6.74 (01)	9.35 (01)	—	—	—	—

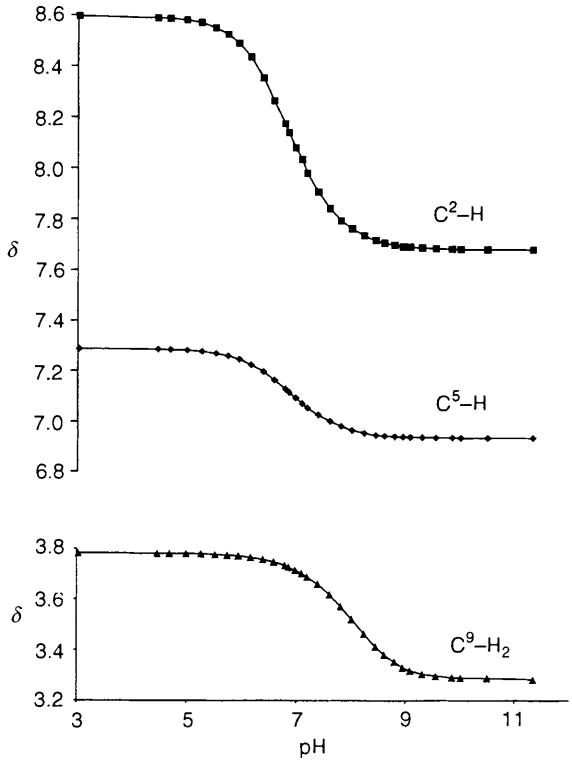


Fig. 1 pH Dependence of the chemical shifts of imidazole 2-, 5-H and glycyl 9-H_2 protons of Gly-Hist (0.025 mol dm^{-3} aqueous solution; T = 298 K; I = 0.1 mol dm^{-3} $NaClO_4$)

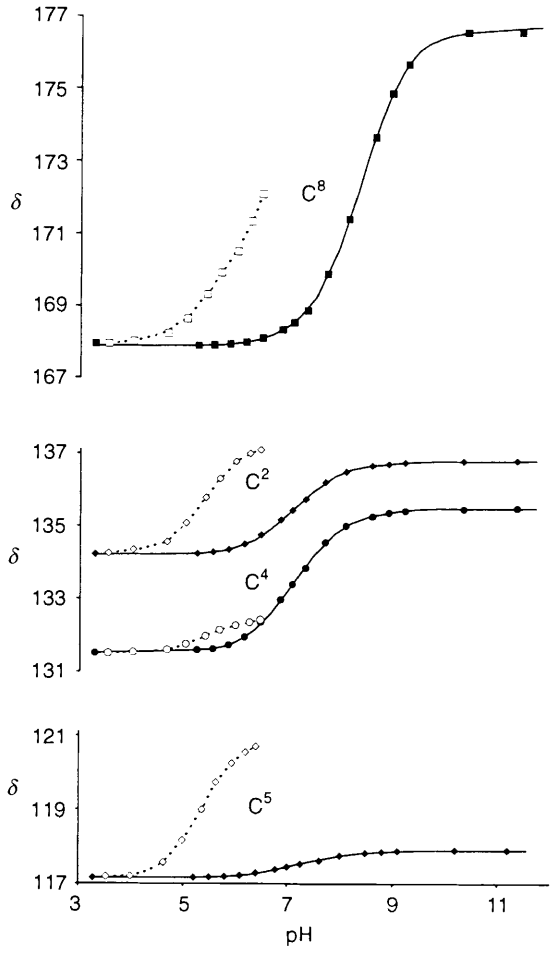


Fig. 2 ^{13}C NMR–pH profiles of C-2, -4, -5 and -8 of Gly-Hist (0.2 mol dm^{-3} aqueous solution) with (empty symbols) and without (full symbols) added $Zn(ClO_4)_2$ (0.08 mol dm^{-3})

dependent chemical shifts of nuclei in the vicinity of the proton-binding sites reflect their protonation states. If δ_{LH_2}, δ_{LH} and δ_L are the chemical shifts of a given nucleus in species LH_2, LH and L, then the observed chemical shift is given by eqn. (3),

$$\delta_{obs} = p_{LH_2}\delta_{LH_2} + p_{LH}\delta_{LH} + p_L\delta_L \quad (3)$$

where p_{LH_2}, p_{LH} and p_L are the molar fractions of the above species (with respect to the total ligand) to be computed as a function of the pH, *e.g.* eqn. (4), where δ_{LH_2} and δ_L are

$$p_{LH_2} = (1 + K_1/[H^+] + K_1K_2/[H^+]^2)^{-1} \quad (4)$$

obtained as the limiting values of δ_{obs} on either the acidic or the basic sides of titration curves representing δ_{obs} *vs.* the pH (Figs. 1 and 2). The chemical shift of the monocation δ_{LH} is itself a weighted mean over those (δ_{LH_i}, δ_{LH_a}) of the two microspecies LH_i and LH_a according to eqn. (5). The molar fractions p_{LH_i}, p_{LH_a} of the two microspecies (still referred to the total ligand) are proportional to microconstants k^i and k^a [eqns. (6) and (7)], respectively, and their molar ratio LH_i:LH_a is constant all along the titration curve, being equal to the ratio of microconstants k^i/k^a.

$$\delta_{LH} = (p_{LH_i}\delta_{LH_i} + p_{LH_a}\delta_{LH_a})/(p_{LH_i} + p_{LH_a}) \quad (5)$$

$$p_{LH_i} = p_{LH}k^i/K_1 = p_{LH_2}k^i/[H^+] \quad (6)$$

$$p_{LH_a} = p_{LH}k^a/K_1 = p_{LH_2}k^a/[H^+] \quad (7)$$

The above treatment shows that NMR titration curves can only yield the averaged value δ_{LH}, however the computation of k^i and k^a requires the knowledge of δ_{LH_i} and δ_{LH_a}. The chemical shifts δ_{LH_i}, δ_{LH_a} in the two microspecies may be estimated on assuming that chemical shifts δ^i, δ^a on each moiety of the molecules investigated—namely the imidazole ring (i) on the one hand, the glycyl (or sarcosyl) residue (a) on the other hand—are independent of whether the other group is protonated or deprotonated. This amounts to the expressions [eqns. (8) and (9)].

$$\delta^i_{LH_i} = \delta^i_L \text{ and } \delta^i_{LH_a} = \delta^i_{LH_2} \quad (8)$$

$$\delta^a_{LH_i} = \delta^a_{LH_2} \text{ and } \delta^a_{LH_a} = \delta^a_L \quad (9)$$

In the ligands studied the two proton-binding sites are well

separated, which supports the validity of the latter estimation. Indeed, the effect of other groups on the monitored chemical shift can be neglected in the case of glycylhistidine,[16] a closely related compound. A variant of the above two-step procedure is to recast eqn. (3) for nuclei of type i or a separately, giving eqns. (10) or (11) respectively.

$$\delta^{i}_{obs} = (p_{LH_2} + p_{LH_a})\delta^{i}_{LH_2} + (p_{LH_i} + p_L)\delta^{i}_{L} \quad (10)$$

$$\delta^{a}_{obs} = (p_{LH_2} + p_{LH_i})\delta^{a}_{LH_2} + (p_{LH_a} + p_L)\delta^{a}_{L} \quad (11)$$

Introducing eqns. (4), (6) and (7) into eqns. (10) and (11) allows us to obtain the operational expressions (12) and (13) (with either subscripts i or a, respectively).

$$\frac{k^{i,a}[H^+] + K_1K_2}{[H^+]^2 + K_1[H^+] + K_1K_2} = \frac{\delta^{i,a}_{LH_2} - \delta^{i,a}_{obs}}{\delta^{i,a}_{LH_2} - \delta^{i,a}_{L}} \quad (12, 13)$$

Using the NMR data relative to the protons born on carbons C-2 (type i) and C-9 (type a) and K_1, K_2 values measured by potentiometry, fits of $(\delta^{i,a}_{LH_2} - \delta^{i,a}_{obs})/(\delta^{i,a}_{LH_2} - \delta^{i,a}_{L})$ as a function of the pH yield k^i and k^a, respectively, and therefore k^i_i and k^i_a by using eqn. (2). The values of the microconstants thus obtained are reported in Table 1.

Similar computations were carried out using ^{13}C data and the titration curves of Fig. 2. However, the concentration of the ligand is clearly higher in ^{13}C NMR experiments than in potentiometric measurements. Under these conditions, the apparent ionization constants K_1, K_2 may have slightly different values from those obtained by potentiometry. K_1, K_2 were consequently adjusted in a first step by fitting ^{13}C NMR data according to eqn. (14) [obtained as the sum of eqns. (12) and (13)].

$$\frac{K_1[H^+] + K_1K_2}{[H^+]^2 + K_1[H^+] + K_1K_2} = \sum_{i,a} \frac{\delta^{i,a}_{LH_2} - \delta^{i,a}_{obs}}{\delta^{i,a}_{LH_2} - \delta^{i,a}_{L}} \quad (14)$$

Microconstants k^i and k^a were then obtained as above in a second step of computation, using eqns. (12) and (13) separately, with the K_1, K_2 values determined in the first step. Carbons C-2, C-4 and C-8 and C-9 were used as nuclei of types i and a. The results obtained are as follows: pK_1 = 6.96, 7.00, 7.02 and 6.90 and pK_2 = 8.25, 8.55, 9.36 and 9.55 for Gly-Hist, Sar-Hist, carcinine, carnosine respectively. The microconstants computed from ^{13}C NMR experiments are: pK^a = 7.95 and 8.36, pK^i = 7.02 and 7.03, pk^a_i = 8.16 and 8.55, pk^i_a = 7.23 and 7.22 for Gly-Hist and Sar-Hist, respectively.

As expected[28] for acid–base pairs of the electrical type $LH_2^{2+}/LH^+/L$, all the pKs are raised, by *ca.* 0.2 to 0.4 units, on increasing the overall ionic strength from 0.1 (pH-metric and ^1H NMR experiments) to *ca.* 0.6 mol dm^{-3} (^{13}C NMR). Deprotonation constants from ^{13}C NMR data result from the adjustment of four parameters (K_1, K_2, k^i, k^a) instead of two only from ^1H NMR data (k^i, k^a). Thus, pk values obtained by ^1H NMR (Table 1) are expected to be more reliable (uncertainty deduced from the fitting procedure: ± 0.02 log unit) than those obtained by ^{13}C NMR, and will be preferably used in the discussion below.

These values require further explanation. (i) The pH-independent concentration ratio of the two tautomers LH_a : LH_i (or k^a/k^i) is 0.148 and 0.071 for Gly-Hist and Sar-Hist respectively, which corresponds to relative amounts of the minor tautomer of 12.9 and 6.6%, respectively. This result is particularly interesting as far as the metal ion (M) binding site in MLH complexes (charges omitted) with the mono-protonated ligand is concerned, in line with the view that, besides the usually proposed imidazole coordination, the alternative amino-coordinated structure can also form.[27]

(ii) The basicity of one binding site is modified by the state of protonation of the second site present in the same molecule. This influence may be characterized in the present case by the pk variation of each group upon the deprotonation of the other one, the so-called *interactivity parameter* $\Delta = pk^i_a - pk^i = pk^a_i - pk^a$. Δ Values of 0.32 and 0.34 found in the cases of Gly-Hist and Sar-Hist are indeed clearly smaller than that in histamine (Δ = 1.02), due to the closer vicinity of the two interacting sites and to stronger hydrogen bonding between N-3 and the terminal ammonio-group in the latter compound. Δ Values in the present compounds are still important if one considers that the two protonation sites are separated by six connecting atoms, suggesting that this influence has to act through space, presumably favoured by weak hydrogen bonding between the two sites, eventually mediated by one (or several) bridging water molecule(s). This view is somewhat supported by the fact that these interactivity parameters are closely similar to that of glycylhistidine[16] (in fact also equal to 0.32) for which direct evidence for hydrogen bonding was independently brought by spectroscopic investigations.[29] This comparison also shows that in glycylhistidine, the carboxylate group attached along the chain of connecting atoms has little influence on the microprotonation of the nitrogen atoms at the two ends of the chain.

(iii) Protonation shifts are directed towards higher or lower fields for ^{13}C and ^1H nuclei respectively. Protonation shifts in the imidazole ring seem relatively insensitive to the nature of the chain attached to C-4, in line with the assumed lack of interplay between both moieties of the molecule. As expected, protonation shifts are larger in position 2, in direct vicinity with either of the two possibly protonated nitrogens N-1 and -3. The relative magnitudes of the protonation shifts observed for the adjacent carbons C-8, -9 and -10 in the second moiety of the ligands are in an unexpected sequence with respect to the distance between the observed nuclei and the amino end. The greatest shift is measured for carbons in a β-position with respect to the amino nitrogen, *i.e.* for the carbonyl carbon atoms C-8 in the case of terminal α-amino acids (in Gly-Hist and Sar-Hist) and for the methylenic carbon C-9 in the case of terminal β-amino acids (in carcinine and carnosine). These facts agree with earlier findings and can only be explained in terms of electrical field theory.[30]

N-3 *and* N-1 *Tautomerism in the Imidazole Ring.*—The neutral imidazole ring can exist in two tautomeric structures, illustrated in Scheme 2 for both the neutral ligand L and the monocation LH_i, namely either the 1-H (L_{N^1H} and LH_{N^1H}) or the 3-H (L_{N^3H} and LH_{N^3H}) tautomers. On the basis of pH-dependent ^{13}C chemical shifts for L-histidine, Reynolds *et al.*[10a] concluded that the N^1H tautomeric form was preferred over the N^3H tautomeric in neutral histidine by about 4:1. This conclusion was drawn from a comparison of the protonation shifts of C-4 and -5 carbons in L-histidine (−4.8 and +0.7 ppm, respectively) with those of model compounds, the 1-CH_3 (+2.3 and −7.1 ppm) and 3-CH_3 (−6.8 and +2.1 ppm) derivatives. Protonation shifts measured in the present compounds, *e.g.* −4.0 and −0.8 ppm for carbons C-4 and -5 of Gly-Hist are also intermediate between those of the above two model molecules. A similar estimation, assuming that C-4 and -5 proton shifts in the two tautomeric ligands L_{N^1H} and L_{N^3H} are equal to those in 1- and 3-methylhistidine respectively, and that the protonation shifts in Gly-Hist are a weighted mean over the values mentioned above, allows us to compute the presence of approximately 69 ± 2% 1-H tautomeric form. Values for the other ligands are reported in Table 2.

However ^{13}C chemical shifts are sensitive to many structural influences which could vitiate this estimation (*e.g.* the small

Table 2 Chemical shift variations $\Delta\delta$ (ppm) of carbons C-4 and C-5 induced by the protonation of the imidazole ring, and the corresponding fraction p of the 1-H tautomeric form

Compound	C-4		C-5	
	$\Delta\delta$	p	$\Delta\delta$	p
Model molecules				
N^3-Methylhistidine[a]	−2.3	0.0	7.1	0.0
N^1-Methylhistidine[a]	6.8	1.0	−2.1	1.0
Ligands				
Histidine[a]	4.8	0.78	−0.7	0.85
Gly-Hist	3.9	0.68	0.7	0.70
Sar-Hist	3.85	0.68	0.8	0.68
Carcinine	3.8	0.64	0.9	0.67
Carnosine	3.7	0.66	0.8	0.68

[a] Ref. 10(*a*).

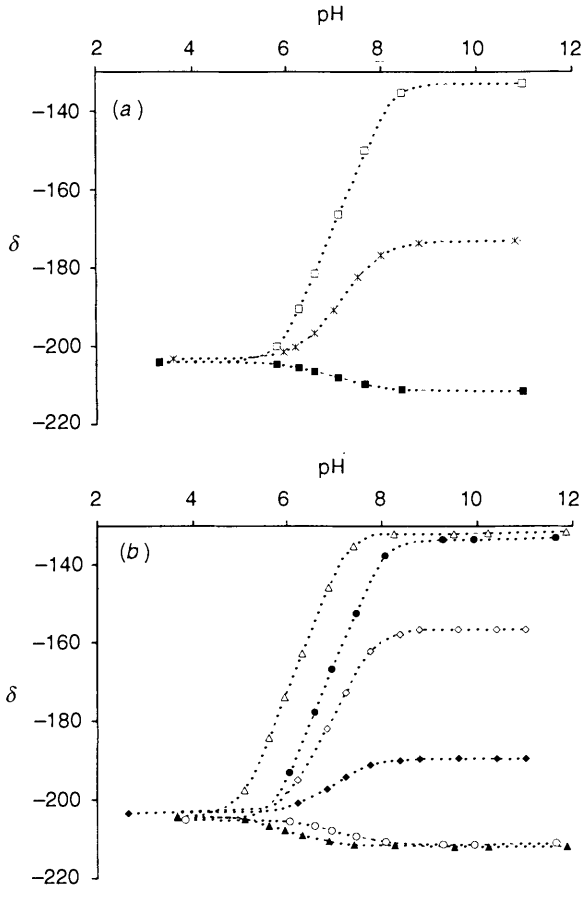


Fig. 3 pH Dependence of ^{14}N chemical shifts in some imidazole derivatives (N-1 and -3 only, represented by full and empty symbols, respectively). (*a*) Imidazole (*) and *N*-methylimidazole (□). (*b*) 1-Methylhistamine (△), 3-methylhistamine (○) and Gly-Hist (◇).

chemical shifts induced by the protonation of the amino group between couples of species such as L_{N^1H} and LH_{N^1H}). It thus appears desirable to confirm these conclusions and improve these estimations by observing the protonated nuclei themselves, namely N-1 and -3. Such studies may use ^{15}N NMR, as already described with ^{15}N-enriched imidazole, 1-methylimidazole[13] and histidine,[12] as well as ^{14}N NMR. We decided initially to use ^{14}N NMR which does not require the preparation of ^{15}N-enriched bioligands. Very few ^{14}N NMR studies of amino acids and peptides have been published,[31–34] and, to our knowledge, there is no systematic study on tautomerism.

Fig. 3 shows the pH-dependence of ^{14}N spectra of Gly-Hist. The spectra exhibit four signals corresponding to the four different nitrogens found in Gly-Hist. The observation of the amide nitrogen around −260 ppm is unusual, the signal being generally too broad to be detected due to very large nuclear quadrupole coupling constant[35] (NQCC). The single peak at about −340 ppm can be assigned to the terminal amino-group, and the remaining two peaks to imidazole nitrogens. A downfield shift by *ca.* 20 ppm is observed during the deprotonation of the amino group, following a single one-proton titration curve, accompanied by a drastic increase of linewidth attributed to the loss of near-tetrahedral symmetry (increase of NQCC) around the amino nitrogen.

Assignments of N-1 and -3 resonances are based on the change in their chemical shifts during the deprotonation of the imidazole ring. At acidic pH, the chemical shifts of the two nitrogens are nearly identical. In the course of deprotonation at higher pH, the two nitrogens are shifted to lower fields at different extents. At pH 9 the two signals are well separated by *ca.* 30 ppm. Their shifts as a function of pH (Fig. 4) follow titration curves corresponding to pK values close to that of 6.96 determined from ^{13}C NMR pH-profile. This separation mainly arises from a contribution of n–π transition in the paramagnetic shielding tensor of nitrogen.[36] Since the deprotonation occurs mostly at N-3, the corresponding signal is that appearing at lower field. Deprotonation of the imidazole nitrogens is accompanied by line broadening, as in the case of the amino nitrogen and for similar reasons, however to a lesser extent.

The observed chemical shift of each nitrogen is the average over the two tautomeric forms standing in fast exchange. Using the same procedure as that of Reynolds *et al.*[10] mentioned above in the case of ^{13}C NMR, we can estimate the proportions p and $(1 - p)$ of 1- and 3-H tautomers by comparing the ^{14}N shifts presently observed with those in model compounds having pure tautomeric forms, namely the *N*-methyl derivatives of histamine. The NMR–pH profiles of nitrogens N-1 and -3 in 1- and 3-methylhistamines and in *N*-methylimidazole (Fig. 4) proved to be nearly identical, especially on either the acidic (pH < 4) or the basic (pH > 8) ends of the pH range. This strongly supports the assumption made above of the independence of imidazole chemical shifts from structural details in 4-substituents, such as the protonation state of the terminal amino-group. These curves correspond to pK values of *ca.* 6.10, 6.88 and 7.02 for 1- or 3-methylhistamine and *N*-methylimidazole, respectively. The significant pK difference between the two imidazole nitrogens of histamine *N*-methyl derivatives is similar to that found between 1- or 3-methylhistidines.[17] If we turn now to the ^{14}N NMR–pH profiles of Gly-Hist (Fig. 4), we can observe that the chemical shifts of nitrogens N-1 and -3 are nearly constant at pH > 8, in spite of a conversion of the monoprotonated ligand LH (mostly under the species LH_i) into the neutral molecule L from pH 7 to *ca.* 10. This is clear evidence for identical ratios [p and $(1 - p)$] of 1- and 3-H tautomers in both the neutral ligand L and the amino-protonated species LH_i; contrary to what is observed in the case of histamine.[34] These observations permitted in turn an easy calculation of tautomeric fractions p and $(1 - p)$ as follows. The deprotonation of a given nitrogen in *N*-methyl derivatives results into a +71.5 ppm shift at that nitrogen and a −7.2 ppm shift (mean values) at the nitrogen bound to a methyl group. Under the above mentioned assumptions, the observed deprotonation shifts of imidazole nitrogens, *e.g.* Δ_{N^3} for N-3, may be expressed as weighted means according to eqn. (15). The ^{14}N NMR–pH profile of

$$\Delta_{N^3} = 71.5\,p - 7.2\,(1 - p) \qquad (15)$$

imidazole was used to test the validity of the method (Fig. 4), in

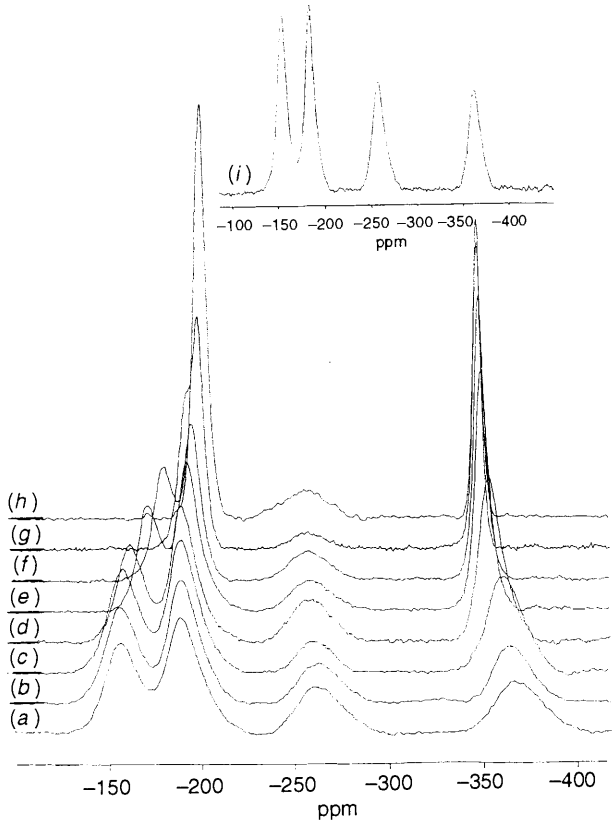


Fig. 4 ^{14}N NMR spectra of Gly-Hist in 0.2 mol dm^{-3} aqueous solution {T = 298 K and pH = 10.4, 8.79, 8.38, 7.75, 7.24, 6.84, 6.23 and 2.66 [(*a*) to (*h*), respectively] and T = 323 K and pH = 10.6 (*i*)}

Table 3 Chemical shift variations $\Delta\delta$ (ppm) of nitrogens N-1 and -3 induced by the deprotonation of the imidazole ring, and the corresponding fraction p of the 1-H tautomeric form (T = 298 K; concentration of ligand: 0.2 mol dm^{-3}; estimated error: p ± 0.03)

Compound	$\Delta\delta_{N^1}$	$\Delta\delta_{N^3}$	p
Model molecules			
1-Methylhistidine	−8.0	71.6	1.00
3-Methylhistamine	71.7	−6.3	0.00
N-Methylimidazole	−7.3	71.2	1.00
Ligands			
Imidazole	31.5	31.5	0.49_2
Gly-Hist	14.6	46.5	0.68
Sar-Hist	15.0	47.8	0.70
Carcinine	15.9	48.9	0.71
Carnosine	15.8	46.0	0.68

which case equal proportions (at ± 1%) of each tautomer were effectively computed from eqn. (15).

The calculated p values are 0.69, 0.70, 0.72 and 0.68 for Gly-Hist, Sar-Hist, carcinine and carnosine, respectively (Table 3), in good agreement with those determined from ^{13}C NMR data, *e.g.* p = 0.69 ± 0.02 for Gly-Hist (Table 2). These values are identical to those found for C-terminal histidine-peptides[10a] and for fully deprotonated histidine[17] or histamine (computed value using recent NMR data from the literature).[34] The ^{14}N NMR–pH profiles were found to be independent of the ligand concentration (0.025 to 0.2 mol dm^{-3}), thus validating the use of pH-determined values for K_1, K_2 in the following computation. Microconstants k^{N^3} and k^{N^1} for the deprotonation of the fully protonated ligand (see Scheme 2) can be computed from the macroconstants K_1, K_2, the microconstant k^i given above [eqn. (1) and Table 1] and the tautomeric ratio p, using eqns. (16) and (17).

$$k^i = k^{N^1} + k^{N^3} \quad (16)$$

$$k^{N^3}/k^{N^1} = p/(1 - p) \quad (17)$$

Finally the last set of microconstants $k^a_{N^3}$, $k^a_{N^1}$, $k^{N^1}_a$, $k^{N^3}_a$ for the deprotonation of the monocation LH into the neutral ligand L (Scheme 2) can be deduced using eqns. (18) to (20).

$$k^a_i = k^a_{N^3} = k^a_{N^1} \quad (18)$$

$$k^i_a = k^{N^1}_a + k^{N^3}_a \quad (19)$$

$$k^{N^3}_a/k^{N^1}_a = p/(1 - p) \quad (20)$$

The values calculated for Gly-Hist and Sar-Hist are reported in Table 4. Distribution curves of all the tautomeric species computed on the basis of the formation constants reported in Tables 1 and 5 are represented in Fig. 5. Similar computations can be performed in the more simple case of carcinine and carnosine, where $K_1 \cong k^i$, $K_2 \cong k^a_i$ and $p_{LH_i} \cong 1$ (see above). Closely similar values of pK^{N^3} and pK^{N^1} are found for carcinine (6.98 and 7.39) and carnosine (6.91 and 7.23), reconfirming the pK independence of the imidazole ring towards the exact nature of substituents on carbon C-4. The knowledge of the basicity of N-1 and -3 nitrogens at the molecular level may be of importance, concerning, for example, the action of histamine or his potential agonist especially on the H_2-receptor[37] or the mechanism of peptide hydrolysis promoted by α-chymotrypsin.[38] In these latter examples, the proton transfer is mediated by the imidazole ring in a concerted mechanism where the proton-acceptor and proton-donor sites are the two nitrogens.

Some indications may also be given concerning the effect of temperature. At 50 °C, ^{14}N lines are sharper than at 25 °C [Fig. 3(i)] due to faster molecular reorientation with an activation energy of 13.7 kJ mol^{-1} for Gly-Hist at pH = 6.97, a typical value for the ligands studied. The proportion of the more abundant tautomer was found p = 0.68 for Gly-Hist at 50 °C.

Zinc(II)*-complexes.*—Investigations are limited to pH < ~7.5 because of precipitation phenomena occurring at higher pH, even for high ligand to metal ion ratios. pH-Metric titration shows the formation of species ZnLH and ZnL (charges omitted) for all the ligands studied. However the bis complex ZnL_2 was also observed in detectable amounts in the case of Gly-Hist and Sar-Hist only, probably due to their much lower pK_2 values. The stability constants for these complexes are listed in Table 5. Values for the Zn(II)-carnosine system had already been reported in the literature,[18] in good agreement with our results.

The analysis of the precipitates obtained at pH > 7.5 shows a ligand-to-metal ion ratio of 1:1 for all the ligands, in accordance with results previously reported for the Zn(II)-carnosine system.[23] Precipitation is complete at pH ~8.5 after the consumption of two base equivalents per zinc(II) ion (values in excess with respect to the same titration performed with the metal-free ligands). One base equivalent can be attributed to the deprotonation of the terminal amino-group, and the second one to the metal ion promoted deprotonation of the peptide nitrogen, which can occur only at high pH (> 7.5). The zinc(II) ion was effectively shown to be able to promote the deprotonation of the amido-group in the case of some histidine-containing peptides.[18–20] An alternative possibility for base consumption is the deprotonation of a coordinated water molecule.

In order to gain structural information about these complexes, ^{13}C and ^{14}N NMR measurements were carried out in the pH range 3 to 7.5. ^{13}C NMR–pH profiles, such as those represented for the Zn^{II}/Gly-Hist system in Fig. 2, show that

Table 4 Microconstants for the deprotonation of N-1 and -3 tautomeric microspecies (T = 298 K; I = 0.1 mol dm^{-3} $NaClO_4$; estimated errors: ± 0.03)

Compound	pk^{N^1}	pk^{N^3}	$pk_a^{N^1}$	$pk_a^{N^3}$	$pk_{N^1}^a = pk_{N^3}^a$
Gly-Hist	7.33	6.98	7.66	7.31	7.98
Sar-Hist	7.32	6.95	7.56	7.28	8.28

Table 5 Stability constants of Zn^{II} complexes (β_{pqr} = $[M_pL_qH_r]/([M]^p[L]^q[H]^r)$; T = 298 K; I = 0.1 mol dm^{-3} $NaClO_4$; estimated errors in parentheses (last two digits)

	Gly-Hist	Sar-Hist	Carcinine	Carnosine
$\log\beta_{111}$ (ZnLH)	9.91 (10)	10.01 (10)	10.75 (10)	11.51 (10)
$\log\beta_{110}$ (ZnL)	3.58 (05)	3.08 (06)	3.53 (06)	4.05 (05)
$\log\beta_{120}$ (ZnL_2)	6.80 (08)	6.31 (09)	—	—

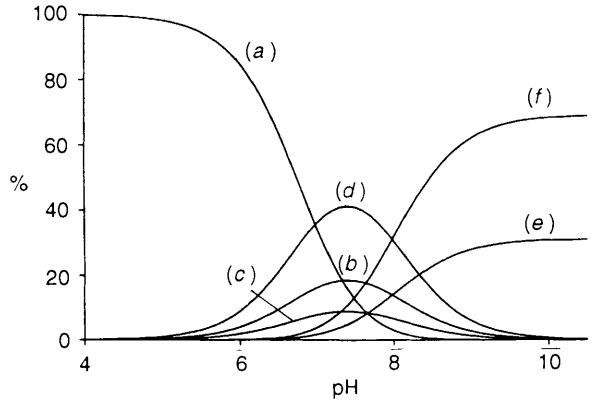


Fig. 5 Concentration distribution of Gly-Hist species and microspecies: LH_2, (*a*); LH_{N^3H}, (*b*); LH_a, (*c*); LH_{N^1H}, (*d*); L_{N^3H}, (*e*); and L_{N^1H} (*f*) as a function of pH (0.025 mol dm^{-3}; T = 298 K; I = 0.1 mol dm^{-3} $NaClO_4$)

coordination to the metal ion induces large chemical shifts in both moieties of the ligand molecule, thus pointing to the terminal amino-group and the imidazole ring as binding sites. This fact was also confirmed by ^{14}N NMR measurements, where line broadenings of amino and imidazole nitrogens occur at much lower pH and at a higher extent than was found in the case of metal-free ligands. Relatively important line broadenings (by *ca.* 35 Hz) were also found for the carbonyl ^{13}C signal in the case of Gly-Hist and Sar-Hist, probably reflecting an additional weak coordination of the carbonyl group to the metal ion.

An important feature in the systems studied presently is a large upfield shift of C-5 and small upfield shift of C-4 (as compared to the metal-free ligand, see Fig. 2), while there is a nearly equivalent upfield shift of C-2 carbon in the complex and in the free ligand. A similar observation has been reported for the Zn^{II}-Asp-Ala-His-*N*-methylamide system.[39] It should be stressed that these measurements were carried out with an excess of ligand (ligand to metal ion ratio of 2.5 : 1) and a 75–85% presence (at pH ~ 7.5) of neutral imidazole ring (coordinated, or not, to Zn^{II} ion) as deduced from potentiometric measurements performed in the same conditions. These shifts may arise either from electronic effects of the coordinated Zn^{II} atom over the observed ^{13}C nuclei, or from a change in the tautomeric preference (between 1- and 3-H tautomers) during complexation. In fact ^{13}C chemical shifts induced by Zn^{II} coordination appear to be of small magnitude only,[22,40] so that the shifts actually observed for carbon C-5 and -4 in the imidazole ring can presumably be accounted by a predominance of 3-H tautomeric imidazole rings in the complex. This proposal would favour N-1 coordination of the metal ion at the expense of N-3-coordination, in apparent contradiction with X-ray structures of Zn^{II} complexes with histidine and many histidine-containing peptides. However in all these conflicting examples, the close proximity of the second binding site (besides the imidazole ring) allows the formation of a six-membered chelate in the case of N-3-coordination which is more stable than a seven-membered chelate which would form in the case of N-1-coordination.[13]C NMR effectively shows large and small shifts of carbons C-4 and -5, respectively, in the Zn^{II}-histidine system,[22] *i.e.* in an opposite sequence to that observed with our compounds. This reversal can be assigned to the absence of strong chelate effects when the two binding sites in the ligand are far from each other, by eight bonds at least in our compounds. This view is supported by the fact that the few X-ray structures of metal complexes of monodentately coordinated 4-substituted imidazole derivatives[41] all reveal N-1-coordination.

One may also wonder how it is possible that the ratio of N-3- and 1-coordinated complexes does not reflect the available binding sites in the metal-free ligand. A similar situation has been described for solutions of $Co^{III}(NH_3)_5$–4-methylimidazole[42] and $Fe^{III}(CN)_5$–histidine[43] complexes. In freshly prepared solutions, approximately 35 and 10% N-1-coordinated form was found for Co^{III} and Fe^{III} complexes, as expected from the tautomeric 3-H/1-H ratios in the metal-free ligands.[10a,11] The ratio of the N-3- and 1-coordinated isomeric complexes however changes slowly in the course of time so as to obtain a 100% predominance of the energetically favoured N-1-coordinated isomer. The reported driving force is mostly steric in origin and could also account for the change of tautomeric preference observed for the Zn^{II} complexation of the ligands presently studied.

A last question concerns the second binding site (amino-group) involved in complexation. Due to the large number of connecting atoms between the terminal amino-group and the N-1 nitrogen in the imidazole ring, two types of joint coordination are possible, involving the formation of either a macrochelate—as it was in the case of Zn^{II}-Asp-Ala-His-*N*-methylamide[39]—or a polynuclear species with ligand molecules bridging two metallic centres. In principle, these two possibilities may be distinguished by studies at variable ligand to metal ion ratios. Precipitation phenomena prevented such investigations, so that there is no evidence to prefer either of these two possibilities on the basis of the present experiments.

Conclusions

A combined use of pH, ^1H, ^{13}C and ^{14}N NMR measurements, has allowed us to give for the first time a complete picture of all the microspecies and microconstants in protonation equilibria of a few molecules used as models for imidazole-containing peptides, which may be of importance in view of the proton transfer reactions mediated by imidazole ring in biological systems. Zn^{II} complexation studies show that, even if the 1-H tautomeric form of the imidazole ring is preferred in the metal-free ligands, the tautomeric preference can be completely upset after metal ion coordination. The relative populations of the N-1, -3 available binding sites in the free ligand are thus not the sole factor determining their relative coordinating ability. The present investigations using model imidazole-containing peptides may help to understand the relatively frequent occurrence of N-1-coordination of histidyl residues in Zn^{II}-metalloproteins.[44]

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