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^{15}N NMR Studies of Amino Acids and their Reaction Products with Formaldehyde

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Natural abundance ^{15}N NMR spectroscopy has been used to investigate the effect of pH on the ^{15}N chemical shifts of lysine and of ε-hydroxymethyllysine. A computer calculation which fits the chemical shifts of both α- and ε-nitrogen atoms versus pH has been used to predict the pK_a values. ^{15}N chemical shifts and some $^1J(^{15}NH)$ values of some other amino acids and of their reaction products with formaldehyde are also reported.

Modification of the properties of proteins by treatment with formaldehyde is of importance in a number of problems, including protection of proteins from degradation by rumen microorganisms. It is known that formaldehyde reacts with labile hydrogen atoms on the side-chains of amino acids to give, as a first step, addition reactions which lead to the formation of hydroxymethyl derivatives[1] and, as a second step, cross-linking between peptide chains which results from dehydration reactions. In earlier work we studied by ^{13}C NMR the reaction of formaldehyde with amino acids having a functional side-chain,[2] concentrating mainly on lysine,[3] which is more generally concerned in the cross-linking reactions between amino acids.

In this paper we report the values of the ^{15}N chemical shifts of some amino acids and hydroxymethylamino acids, and discuss the titration curves for L-lysine and ε-*N*-hydroxymethyl-L-lysine determined from a study of the pH dependence of the ^{15}N chemical shifts.

EXPERIMENTAL

Amino acids were purchased from Sigma Chemical Company and were used without further purification, since paramagnetic impurities which are frequently found in commercial samples[4,5] do not induce significant variations in δ^{15}N. A 10 M solution of formaldehyde was used, prepared by depolymerization of pure paraformaldehyde in distilled water at 85 °C. Saturated solutions of amino acids in water at a convenient pH were normally used, and pH adjustments were made with concentrated HCl and NaOH. To avoid gelification of highly basic solutions during preparation, less basic solutions of lysine–formaldehyde were prepared and, after a few minutes, concentrated NaOH was added to obtain the convenient pH. ^{15}N NMR spectra were obtained at 25.35 MHz using a Bruker WM 250 spectrometer. The field-frequency lock and the chemical shift reference (a low-frequency shift from the reference is negative) were provided by a mixture of CD_3NO_2 and $CH_3{}^{15}NO_2$ contained in a coaxial capillary tube inside the 15 mm diameter tube. All spectra were recorded at approximately 298 K. Typical running conditions were: data points 16 K, sweep width 10 000 Hz, acquisition time 0.82 s, pulse width 45 μs (35° flip angle), quadratic detection without pulse delay.

RESULTS AND DISCUSSION

The pH dependences of the ^{15}N chemical shifts of both nitrogen resonances of lysine are shown in Fig. 1. The 8 ppm high-frequency shift observed for both the α- and ε-nitrogen atoms as the pH is lowered from 13–14 to 3–7 is similar to that observed on amine protonation,[6,7] and also to that of the α-nitrogen of arginine.[8] As previously mentioned,[8,9] the 1 ppm low-frequency shift of the α-nitrogen when the carboxyl group is protonated may be due to the change in interactions between this group and the α-NH_3^+. The reverse effect observed on the ε-nitrogen when the carboxyl group is protonated (0.9 ppm high-frequency shift) may be due to a stronger interaction of the carboxyl anion COO^- with the ε-nitrogen than with the α-nitrogen, which is less basic.

Formaldehyde reacts with lysine according to an equilibrated reaction to give ε-hydroxymethyllysine.[2] The pH dependence of the ^{15}N chemical shifts of each

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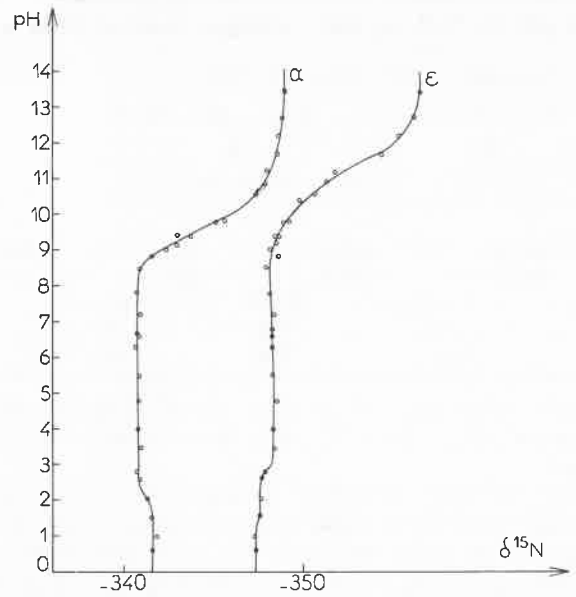


Figure 1. pH dependence of the ^{15}N chemical shifts of lysine in water solution.

of the resonances is shown in Fig. 2. For a given pH, no difference is observed between the chemical shifts of the α-nitrogen atoms of lysine and of ε-hydroxymethyllysine. The ε-nitrogen resonance of residual lysine was not observed at high pH since only a small proportion of lysine at equilibrium is expected in this pH range.[3] The 11 ppm high-frequency shift of the ε-nitrogen atom in hydroxymethyllysine observed when the pH is changed from 7 to 5 can be compared with that caused by secondary amine protonation, while the high-frequency shift observed for this nucleus when the carboxyl group is protonated has the same order of magnitude as in lysine.

An approximate determination of the pK_a values is possible from the curves representing the variation of δ^{15}N versus pH. However, in order to determine these values more precisely, a calculation which takes into account all the resonances for each product has been performed. The Simplex program used requires initial estimations of the chemical shifts of the four chemical species as well as estimations of the pK_a values. These initial estimations allow calculations of the chemical

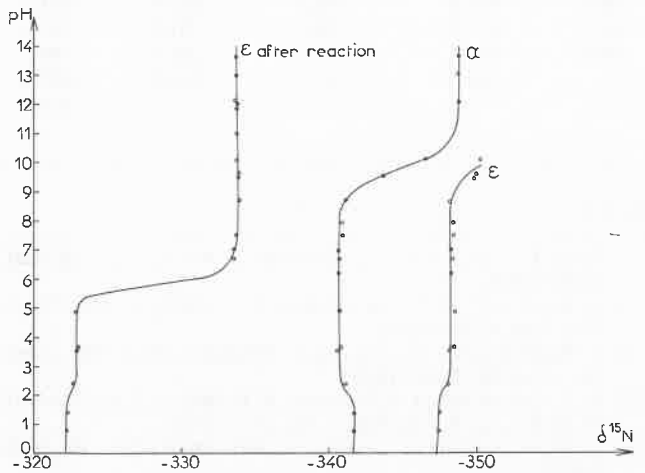
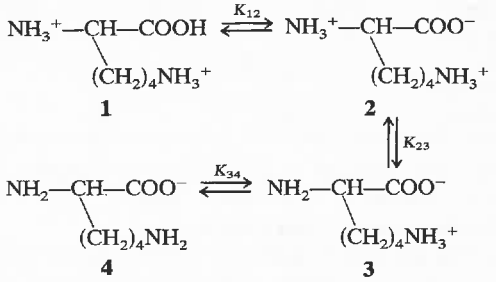


Figure 2. pH dependence of the ^{15}N chemical shifts of lysine in water–formaldehyde solution.

shifts for each nitrogen resonance at various pH values using the equation

$$\delta_{calc} = \sum_{i=1}^{4} P_i \delta_i$$

where P_i is the population of each species present in solution and δ_i is the chemical shift of these species. The following scheme is used to characterize the chemical pathway;



where

$$K_{12} = \frac{[H^+]P_2}{P_1}; \qquad K_{23} = \frac{[H^+]P_3}{P_2}; \qquad K_{34} = \frac{[H^+]P_4}{P_3}$$

If the small difference in the pK values of α-NH_2 and ε-NH_2 groups is considered, a more complete representation would have taken into account the protonation of the ε-NH_2 group, as the step before that of the α-NH_2 group, when the pH is lowered. In fact, the pH dependence of the chemical shifts and, also, that of the pK_a values is very similar for the α-NH_2 group in lysine and in hydroxymethyllysine, and we have neglected the contribution from the protonation step of the ε-NH_2 group. Moreover, the precision of the fit which involves 7 parameters and 30 observations—although reasonably good—is not high enough for the distinction between the two schemes to be significant. A change of less than 0.05 pK_a unit does not induce a significant change in the computed chemical shifts.

The population of each species can be computed from the pH and the pK_a values as shown in the following equations:

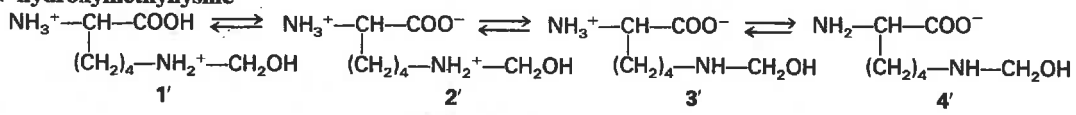
$$P_2 = \frac{P_1 K_{12}}{[H^+]}; \qquad P_3 = \frac{P_2 K_{23}}{[H^+]}; \qquad P_4 = \frac{P_3 K_{34}}{[H^+]}$$

$$P_1 = \frac{1}{1 + \frac{K_{12}}{[H^+]} + \frac{K_{12}K_{23}}{[H^+]^2} + \frac{K_{12}K_{23}K_{34}}{[H^+]^3}}$$

The calculated chemical shifts are compared with those observed to give the best pK_a values by an iterative procedure. The calculated pK_a values for lysine and for ε-hydroxymethyllysine are reported in Table 1, as well as those of the chemical shifts of each protonated species of the lysine and its derivative.

It is worth noting that ^{13}C (Ref. 10) and ^{15}N NMR give consistent pK_a for lysine. As expected, the ionization state of the carboxyl group and of the α-nitrogen atom are not affected by the hydroxymethylation of the ε-amino group ($\Delta pK_{COOH} = 0.06$ and $\Delta pK_{\alpha\text{-}NH_2} = 0.17$). As only one molecule of formaldehyde is able to add to the ε-nitrogen atom to yield the monohydroxymethyl derivative,[2] this correlates well with the low pK_a value calculated for the ε-hydroxymethyl nitrogen. The 16–25 ppm high-frequency effect observed between the ε-nitrogens of lysine and its de-

Table 1. Estimated values of δ_i and estimated and refined values of pK_a for both α- and ε-nitrogen atoms of lysine and ε-*N*-hydroxymethyllysine



	Lysine				ε-*N*-Hydroxymethyllysine			
	1	2	3	4	1′	2′	3′	4′
$\delta_i\alpha$-N (ppm)	−341.8	−340.8	−347.3	−348.8	−341.8	−340.8	−341.0	−348.8
$\delta_i\varepsilon$-N (ppm)	−347.4	−348.3	−350.4	−356.4	−322.2	−322.9	−333.8	−333.8
Reaction	1⇄2	2⇄3	3⇄4		1′⇄2′	2′⇄3′	3′⇄4′	
Estimated pK_a	2.2	9.7	11.2		2.2	6.0	9.7	
Refined pK_a	2.2_5	9.6	11.5_5		2.3	5.8	9.7_5	

rivative also reflects a large loss of basicity of the ε-amine function between lysine and its ε-monohydroxymethyl derivative.

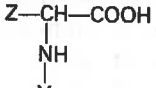
The ^{15}N chemical shifts of some amino acids and their reaction products with formaldehyde are reported in Table 2. The chemical shifts were obtained only in mildly acidic conditions (pH range 4–6) and the influence of pH on these chemical shifts was not studied. However, it is interesting that the high-frequency shift produced by hydroxymethylation is significant in every case (21.7–26.1 ppm), which means that the loss of basicity is the same as that observed for lysine. In the case of cysteine hydroxymethylation takes place on the thiol group, and simultaneous dehydration between the CH_2OH and the α-NH_2 yields a cyclic product; these trends are confirmed by the use of α-*N*-acetyl derivatives of the amino acids concerned.

It is interesting that a CH_2OH group substituted on an ω-NH_2 group induces a high-frequency shift comparable with that of a relatively long-chain alkyl group, C_nH_{2n+1} ($n>3$), in a disubstituted aliphatic amine.

The $^1J(^{15}NH)$ coupling constants for lysine and arginine and their hydroxymethyl derivatives were measured in acidic medium (pH = 1). The hydroxymethylation of the ε-nitrogen atom of lysine and of the guanidino group of arginine does not modify $^1J(^{15}NH)$, showing no modification of the hybridization of the nitrogen atom. It should be interesting to measure these coupling constants at a different state of protonation of the nitrogen atom, but these values are not obtainable at high pH, probably owing to the critical exchange rate of the protons.[5,11]

The coupling constants measured for lysine and its hydroxymethyl derivative lie between 74 and 75 Hz for both nitrogens. For arginine and its derivative they are between 92 and 94 Hz for the NH and the guanidino group, while $^1J(^{15}NH)$ of the α-nitrogen is not obtainable, even at low pH values.

Table 2. ^{15}N chemical shifts of unreacted and reacted amino acids in water or in water–formaldehyde solutions at pH 4.0–6.0



			α-Amino acid (Y = H)			α-N-Acetylamino acid (Y = CH_3CO)		
			Water solution	Water–Formaldehyde solution		Water solution	Water–formaldehyde solution	
Parent amino acid	Z	Nitrogen atom	Unreacted amino acid	Unreacted amino acid	Reacted amino acid	unreacted amino acid	Unreacted amino acid	Reacted amino acid
Cysteine	CH_2SH	α-N	−342.8	−343.3	−319.1[a]	−257.6	−256.3	−256.3
Ornithine	$(CH_2)_3NH_2$	α-N	−340.9	−341.2	−341.2			
		δ-N	−346.6	−347.0	−323.0			
Lysine	$(CH_2)_4NH_2$	α-N	−340.8	−340.8	−340.8	−250.1	−251.2	−251.2
		ε-N	−348.4	−348.5	−322.9	−349.3	−349.4	−323.3
Arginine	$(CH_2)_3NHC(=NH)NH_2$	α-N	−340.5	−341.2	−341.2	−250.2	−251.0	−251.0
		δ-N	−296.6	−296.8	−296.3	−296.8	−297.0	−296.3
		ε-N	−308.5	−308.7	−286.1	−310.5	−310.5	−287.3
								−309.6

[a] Cyclic derivative.

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