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Studies of pH and Anion Complexation Effects on L-Arginine by Natural Abundance ^{15}N Nuclear Magnetic Resonance Spectroscopy[1]

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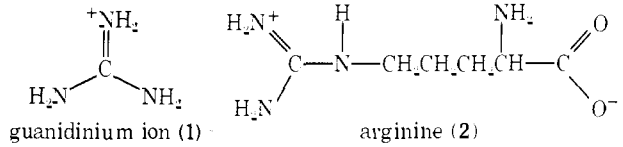
Abstract: Natural-abundance nitrogen-15 nuclear magnetic resonance (NMR) spectroscopy has been used to investigate (1) the effect of pH on the ^{15}N chemical shifts of L-arginine and (2) the possible effects on ^{15}N chemical shifts and T_1 values of the complexation of L-arginine with chloride ions, phosphate ions, and adenosine triphosphate (ATP) in aqueous solutions. Guanidine carbonate solutions and protamine sulfate solutions containing some of these ions have also been examined. On protonation of the guanidinium group of L-arginine, the guanidino nitrogens and the -NH- nitrogen show upfield shifts of 15 and 3 ppm, respectively, as a result of change in the second-order paramagnetic effect, while the α-amino nitrogen shows an 8-ppm downfield shift on protonation as a result of decreased shielding. Complexation with equimolar phosphate ions produces a small downfield shift of approximately 0.8 ppm in the chemical shift of the guanidino nitrogens of L-arginine. No significant changes in ^{15}N shifts or T_1 values were observed on complexation with chloride ions or adenosine triphosphate.

Several enzymes have been found to contain arginyl residues which appear to interact with negatively charged phosphate or carboxylate groups of substrates or cofactors.[2] Chemical modification of positively charged arginyl residues (and the resulting loss of charge) has been shown to result in deactivation of the enzymes.[2] Riordan and co-workers have further suggested that enzymes which interact with anionic substances or cofactors will probably contain arginyl residues at the active site.[3] The binding of DNA with histone and protamine is considered to involve electrostatic interactions between the phosphate anions of DNA and the positively charged arginyl and lysyl residues of these nucleoproteins. The guanidinium group of methylguanidine has been shown by x-ray crystallography to complex with a phosphate anion through multiple hydrogen bonds.[4] The complexation of L-arginine with phosphate and chloride ions in aqueous solution has been studied by ^{31}P and ^{35}Cl nuclear magnetic resonance spectroscopy. Katz and co-workers[5] have reported a downfield shift of 0.5 ppm in ^{31}P chemical shift of methyl phosphate on addition of L-arginine in D_2O solution at pH 6.15. Jönsson and co-workers[6] have measured the pH dependence of ^{35}Cl chemical shift in an aqueous mixture of NaCl and L-arginine and observed a distinct change in ^{35}Cl shift on deprotonation of the guanidinium group. The present study was designed (1) to determine a complete ^{15}N chemical shift–pH titration curve for L-arginine, and (2) to investigate the utility of ^{15}N NMR for detection and characterization of complexations of guanidinium ion (**1**) and L-arginine (**2**) with fluoroborate ions, chloride ions, phosphate ions, and ATP in water solutions.



guanidinium ion (1) arginine (2)

Experimental Section

Natural-abundance ^{15}N NMR spectra were recorded using the pulse Fourier-transform technique with a Bruker WH-180 spectrometer operating at 18.25 MHz. An external 1 M $H^{15}NO_3/H_2O$ capillary reference was used in 25-mm spinning sample tubes. Normal operating conditions for guanidine carbonate and arginine solutions (for spectra other than T_1 measurements) were 55-μs pulses (70° flip angle) with a 1.5-s pulse delay, and for protamine solutions were 60-μs pulses with a 0.58-s delay. With full proton decoupling, the sample temperatures were about 55° C. Spectra were obtained of 2 M aqueous solutions of guanidine carbonate with internal D_2O field-frequency locks, and the L-arginine spectra were similarly taken of 1 M aqueous solutions. Protamine sulfate spectra were of 0.0315 M aqueous solutions. L-Arginine was purchased from Sigma Chemical Co., and guanidine carbonate from Matheson Coleman and Bell.

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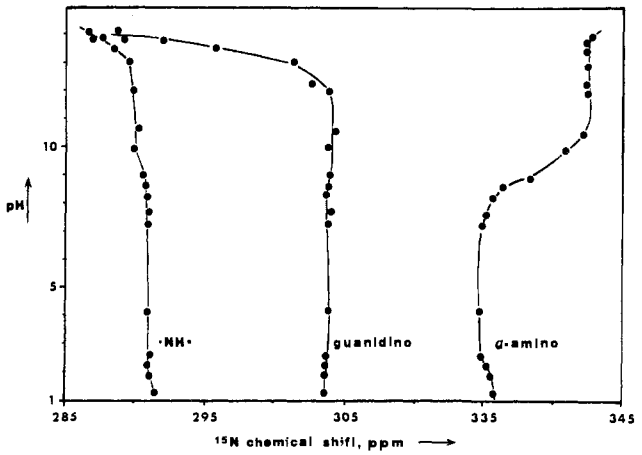

Figure 1. The pH dependence of ^{15}N chemical shifts of L-arginine at 25 °C. The chemical shifts are in parts per million upfield of $H^{15}NO_3$. The accuracy of pH values is ±0.05 pH unit for pH ≤13.5. For pH >13.5, the error may be as much as 0.1–0.3 pH unit because of the high potassium ion concentrations.

Clupeine, a protamine isolated from herring, was obtained as protamine sulfate from the Sigma Chemical Co.

The ^{15}N T_1 values were obtained using the fast inversion recovery (FIRFT) technique.[7] Before measurement of the T_1 values, the solutions were treated with Chelex-100, using the method of Irving and Lapidot,[8] and purged with nitrogen. The pH of each solution was determined with a Radiometer PHM-26C pH meter using a combined glass reference electrode and adjusted with hydrochloric acid and potassium hydroxide. Viscosity measurements were made in an Ostwald viscosimeter immersed in a constant-temperature bath.

Results and Discussion

Aqueous 1 M solutions of L-arginine show three ^{15}N absorptions. Of these, the resonance at 304 ppm can be assigned to the two equivalent guanidino nitrogens through comparison with the resonance in guanidine carbonate (301.7 ppm); the α-amino nitrogen resonance is surely the one at 342 ppm through comparison with α-amino chemical shifts for other amino acids, and the -NH- absorption is, by elimination, the one at 289 ppm. (A compatible, more rigorous, assignment of peaks has been reported by Pregosin and co-workers.[9])

The pH dependence of the chemical shift of each of the three resonances is shown in Figure 1. The 8-ppm downfield shift observed for the α-amino nitrogen at 342 ppm as the pH is lowered from 10.6 to 7.3 (pK_a = 9.0 for the α-amino group of L-arginine)[10] is about as expected from decreased shielding of a saturated amine nitrogen on protonation. The 15-ppm upfield shift of the guanidino nitrogens and the 3-ppm upfield shift of the -NH- nitrogen, which result when pH is lowered from 14.1 to 12.0 and protonation occurs, are probably due to a change in the second-order paramagnetic effect of the neutral guanidino nitrogens. Previous studies[11] of the pH dependence of the ^{15}N chemical shift of L-arginine found a similar shift in the highly basic region. However, it was also reported that above pH 13.5 the guanidino nitrogen resonance separates into two resonances, one at approximately 285 ppm and the other at 342 ppm, while the α-amino resonance could not be observed above pH 5.8. The present work and a recent paper by Blomberg and co-workers[12] show clearly that the single resonance at 288 ppm observed in the highly basic region represents the neutral guanidino nitrogens, and that the 342-ppm resonance represents the α-amino nitrogen; the quenching of this nitrogen resonance near its pK_a was almost certainly the result of enhanced relaxation arising from traces of paramagnetic ions, as has been observed for glycine.[13]

On protonation of the carboxyl group (pK_a = 2.2), the α-amino nitrogen resonance shifts 0.6 ppm upfield. This may be due to increased shielding of the nitrogen on loss of hydrogen bonding between the $-NH_3^+$ group and the $-COO^-$ groups as has been suggested by Blomberg and co-workers[12] for glycine. The 0.7-ppm upfield shift of the -NH- group on protonation of the α-amino group (pK_a = 9.0) may be similarly ascribed to increased shielding resulting from the loss of interactions, such as hydrogen bonding, with the neutral α-amino group.

Table I. ^{15}N NMR Shifts[a] of Guanidine Carbonate, L-Arginine, and Protamine Sulfate in Presence of Anions

sample (mole ratio)	pH	2 M guanidine carbonate	1 M arginine -NH-	1 M arginine guanidino	1 M arginine α-NH_2
alone	11.5	301.7	290.3	304.4	342.7
NaCl (1:1)	11.1	301.0	289.7	304.0	342.7
HPO_4^{2-} (1:1)	10.6	300.6	289.6	303.3	341.4
HBF_4 (1:1)	8.9	302.7	291.2	304.2	336.1[b]
HPO_4^{2-} and $H_2PO_4^-$[c] (1:1)	7.3		290.5	303.1	334.4[b]
HCl (1:1)	6.0	301.7	290.8	303.8	335.1[b]
ATP (1:1)	4.4		291.0	303.7	334.8[b]
HBF_4	6.3[d]		290.7	304.0[e]	
HPO_4^{2-} and $H_2PO_4^-$ (1:1)	6.3[d]		290.4	303.2[e]	334.4[b]
HPO_4^{2-} and $H_2PO_4^-$ (1:2)	6.3[d]		290.4	303.0[e]	334.3[b]
HPO_4^{2-} and $H_2PO_4^-$ (1:3)	6.3[d]		290.6	303.1[e]	334.5[b]
protamine alone[f]	2.8		290-.2[g]	303.5[h]	
ATP (1:1)[i]	2.8		290-.4[g]	303.4[h]	

[a] Chemical shifts are in parts per million upfield from nitric acid. [b] This shift, compared to those above, results from the change in pH from 11.5 on adding acid (see Figure 1). [c] HCl was added to adjust pH to 7.3. [d] pH adjusted by NaOH. [e] Precision is about ±0.1 ppm. [f] Protamine sulfate at 0.03 M. This substance was too insoluble in H_2O to take its ^{15}N spectrum at higher pH values. [g] Chemical shift of arginyl -NH- resonances in protamine sulfate. [h] Chemical shift of arginyl guanidino nitrogens of protamine sulfate. [i] Arginyl residue: ATP = 1:1.

To determine the effects of anions on the resonance of guanidine and L-arginine nitrogens, equivalent amounts of salts or acids were added and the shifts compared to those produced by fluoroborate, which was not expected to complex significantly in aqueous solution. As can be seen from Table I, no very significant changes in shifts were observed on addition of chloride ions. For phosphate-arginine mixtures, the ^{15}N shifts were measured at pH 6.3 at arginine-phosphate mole ratios of 1:1, 1:2, and 1:3 and compared to those produced by fluoroborate. The guanidino nitrogens show a small downfield shift of 0.8 ppm on addition of equimolar phosphate, which may be due to a change in the second-order paramagnetic effect of the guanidino nitrogens on hydrogen bonding with phosphate oxygens. Increasing the phosphate-arginine mole ratio, however, caused no further increase in guanidino nitrogen shifts. Possible effect of phosphate anions on the arginyl residues of protamines was investigated by comparing the nitrogen shifts of arginyl residues in clupeine, a protamine isolated from herring, in the presence and absence of ATP.[14] This protamine has a molecular weight of approximately 5000 and contains 20 arginyl residues per molecule. As can be seen in Table I, no significant changes in shifts were observed on addition of ATP.

If complexation of anions with guanidinium nitrogens does in fact occur in water, the T_1 relaxation times would be expected to be shortened. The T_1 data obtained in the presence

Table II. T_1 Values for L-Arginine Nitrogens in the Presence of Anions

sample	concn,[a] M	pH	temp, °C	viscosity, cP	T_1, s guanidino	T_1, s -NH-	T_1, s α-amino
no addends	1.0	10.1	56	0.66	8.3	14.0	[b]
L-arginine with NaCl	1.0	9.4	58	0.80	7.7	13.5	[b]
L-arginine with HCl	1.0	6.0	51	0.82	4.8	9.7	7.1
L-arginine with HCl	1.0	2.7	53	0.85	6.6	9.7	6.9
no addends[c]	2.5	10.1	51	1.33	2.9	5.7	4.5
L-arginine with glycerol[c]	1.0	10.1	51	1.35	3.6	6.6	4.2
L-arginine with ATP[c]	0.6 each	10.1	49	1.32	2.5	5.9	2.3

[a] Concentration of L-arginine unless otherwise specified. [b] No resonance observed. [c] 10 mM EDTA added.

and absence of various anions are summarized in Table II. It appears that the pH effect on the nitrogen T_1 values is much more significant than the effects arising from complexation with chloride anions. Protonation changes are not expected to occur in the pH range studied here for guanidino and -NH- nitrogens which have $pK_a = 12.48$,[10] but possible conformation changes resulting from the deprotonation of the α-amino nitrogen ($pK_a = 9.0$) and its interaction with the guanidinium group may contribute to the observed change in T_1 of guanidino and -NH- nitrogens between pH 6.0 and 9.4. The T_1 changes may also be due to traces of heavy-metal ions not removed by the Chelex treatment, and such changes in amino acid relaxation times with pH associated with heavy metal ions are well documented.[8,13] Trace metal ions were in fact responsible for the quenching of the α-amino resonance at high pH; after a second treatment with Chelex and addition of 10 mM EDTA, the α-amino resonance was clearly observed at pH 10.1.

On addition of ATP to the arginine solutions, the viscosity increased substantially. In view of the well-known effects of viscosity on T_1, the T_1's of the arginine nitrogens in the presence of ATP were compared at pH 10.1 with those in arginine solutions made isoviscous with (1) increasing arginine concentration and (2) addition of glycerol. The results provide no evidence for significant changes in T_1 due to complexation with ATP, as can be seen in Table II.

The low sensitivity of guanidino and arginyl nitrogen chemical-shift and T_1 data for aqueous solution toward anionic substances, which have been postulated to complex with such residues of enzymes, do not necessarily mean that ^{15}N NMR cannot be useful for such studies. Arginine in water may well have reduced tendency for complexation compared to arginyl residues in enzymes and, in fact, Borders and co-workers[15] have shown that free arginine reacts with 2,3-butanedione about 15 times more slowly than the essential arginyl residue of creatin kinase. Hydrophobic environments near the active site of the enzyme could very well enhance the binding capabilities of arginyl residues toward anions, and this idea will be investigated further.

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