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^{14}N NMR relaxation times of several protein amino acids in aqueous solution—comparison with ^{17}O NMR data and estimation of the relative hydration numbers in the cationic and zwitterionic forms

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Abstract

The ^{14}N nuclear magnetic resonance (NMR) linewidths of the α-amino groups of several protein amino acids were measured in aqueous solution, with and without composite proton decoupling, to estimate the effect of proton exchange and molecular weight on the linewidths. It is shown that, contrary to earlier claims, the increase in the linewidth at low pH is not exclusively due to the effect of proton exchange broadening. The ^{14}N linewidths, under composite proton decoupling, increase with the bulk of the amino acid, and increase at low pH. Statistical treatment of the experimental ^{14}N and literature ^{17}O NMR data was performed assuming two models: (i) an isotropic molecular reorientation of a rigid sphere in a medium of viscosity η, (ii) a stochastic diffusion of the amino and carboxyl groups comprising contributions from internal (τ_{int}) and overall (τ_{mol}) motions. Assuming a single correlation time from overall molecular reorientation (τ_{mol}), then, a linear correlation was found between the linewidths and the molecular weights of the protein amino acids at the pH values 0.5 and 6.0, which are characteristic of the cationic and zwitterionic forms, respectively. The slopes of the straight-lines were found to be dependent of pH for ^{14}N, contrary to the ^{17}O linear correlations whose slopes were found to be independent of pH. Assuming effective correlation times of the amino and carboxyl groups, which comprise contributions from the internal (τ_{int}) and overall (τ_{mol}) motions, then, a significant improvement of the statistics of the regression analysis was observed. The ^{14}N relaxation data, in conjunction with ^{17}O NMR linewidths, can be interpreted by assuming that the ^{14}N quadrupole coupling constants (NQCCs) are influenced by the protonation state of the carboxyl group, the ^{17}O NQCCs remain constant, and the cationic form of the amino acids is hydrated by an excess of 1–3 molecules of water relative to the zwitterionic state.

Keywords: ^{14}N NMR; ^{17}O NMR; Quadrupolar relaxation; Amino acids; Hydration

1. Introduction

The hydration of amino acids and peptides is a problem of fundamental significance and it is a prerequisite to the understanding of protein–water interactions [1–6]. It is therefore reasonable that the hydration of amino acids has been extensively studied with a variety of physicochemical techniques including volumetric studies by density measurements [7–10], multinuclear NMR [6,11,12], infrared spectroscopy [13], ion cyclotron resonance (ICR) mass spectroscopy [14], and theoretical calculations [15–18].

Early ^{13}C relaxation times of glycine were reported to be independent on the pH [19]. The ^{17}O linewidths of several amino acids were observed to increase at low pH with respect to neutral pH [20–22]. A similar, though smaller effect was observed for the ^{13}C and ^{15}N relaxation rates of glycine [23,24] and later for the ^{2}H relaxation rates of glycine and alanine [25]. Fiat and collaborators [26] attempted to estimate the number of specific hydration sites of the α-carboxyl group of amino acids in the different ionization states on the basis of ^{17}O shielding. These authors suggested hydration numbers

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of 3 and 2 at acid and neutral pH, respectively. Lauterwein et al. [27], provided a detailed investigation of the ^{17}O linewidths of the α-carboxyl groups of several protein amino acids. A linear correlation was found between the linewidths and the molecular weights of the amino acids at the pH values 0.5, 6.0, and 12.5, which are characteristic of the three ionization states of the amino acids: the cationic form prevails at pH 0.5, the anionic at pH 12.5, and the zwitterionic form at pH 6. The slopes of the straight-lines were found to be independent of pH. However, the linewidths were found to increase relative to those of neutral or basic pH. This was interpreted by assuming that the α-carboxylic group is hydrated by an excess of two molecules of water relative to the α-carboxylate group.

^{14}N NMR relaxation parameters can be considered as an excellent means to investigate solution interactions and molecular dynamics, but the general utility of the ^{14}N probe has not yet approached that of the ^{13}C and ^{15}N nuclei [28]. It is however of interest to use the nitrogen-14 nucleus that is located at strategic molecular sites and it is directly involved in solute–solvent interactions [29–31]. Furthermore, since the ^{14}N nucleus has a spin quantum number of 1, it relaxes essentially by quadrupolar interaction. ^{14}N spin relaxation data can therefore be translated into dynamic information, provided that the nuclear quadrupole coupling constant (NQCC) of the amino acids can be obtained by independent means.

In this paper, we contribute to a further understanding of the ^{14}N relaxation of several protein amino acids in aqueous solution. The effect of proton transfer on the linewidths was measured as a function of pH. We show that the ^{14}N linewidths depend upon the molecular weight of the amino acid and that a change in the linewidths under composite proton decoupling at pH 0.5 and 6.0 is mostly indicative of a change in the number of hydration molecules of water and in the NQCC values. Comparison also was made with literature ^{17}O relaxation data [27] in order to get a coherent picture of the hydration state of the amino acids.

2. Experimental

2.1. Materials

The amino acids were purchased from Sigma and used without further purification.

2.2. ^{14}N NMR measurements

^{14}N NMR spectra were obtained on a Bruker AMX-400 instrument equipped with a multinuclear high resolution 5 mm probe at 28.91 MHz. No field/frequency lock was used. Chemical shifts were reported relative to pure nitromethane in an external reference in a sample replacement technique. The spectrometer parameters were as follows: 90° pulse 12.5 μs, spectral width 8.77 kHz, pulse repetition time ~40 ms, number of scans 4000–5000. NMR spectra were obtained at 40 °C. Temperature was controlled to within ± 1 K. pH was adjusted by adding solution of NaOH or HCl and determined with a pH meter. In aqueous solutions, 10^{-5} M ethylenediaminetetraacetic acid (EDTA) was added to prevent line broadening by paramagnetic impurities.

Proton decoupling experiments were performed with power-gated decoupling with a WALTZ-16 composite pulse sequence [32,33]. The power of the decoupler was set to ~1 W during acquisition and practically zero during the relaxation delay of 0.1 s, giving an average power of 200 mW.

The single ^{14}N resonances were fitted to a Lorentzian lineshape function (Bruker UXNMR program). After correction for eventual line broadening factors applied to the FIDs before Fourier transform, the linewidths at half height, $\Delta\nu_{1/2}$, were transformed to give the transverse relaxation times according to $T_2 = 1/(\pi\Delta\nu_{1/2})$. The contribution of the magnetic field inhomogeneity to the linewidth was estimated to be <1 Hz.

^{14}N longitudinal relaxation times (T_1) were measured by the inversion-recovery method. For the pulse sequence $(180\text{–}\tau\text{–}90°\text{–}T_d)_n$ approximately 10–12 values of τ were selected, T_d was $>5T_1$ of the amino acids, and $n = 5000$. For the evaluation of the T_1 the peak intensities were used for a three-parameter nonlinear least-squares procedure.

2.3. Viscosity measurements

The viscosities of the solutions were measured with an Ubbelohde viscometer. Kinetic energy correction was made using the table of Hagenbach corrections supplied by the manufacturers. Flow times were measured to an accuracy of ±0.01 s. A constant temperature bath, controlled within ±0.1 °C was used at 40 °C. At least four measurements were made for each solution.

3. Results and discussion

In diamagnetic solutions, the ^{14}N nuclei are mainly relaxed by the quadrupolar mechanism. In the motional narrowing limit $\omega_0^2\tau_c^2 \ll 1$, which is expected to be valid for dilute solutions of amino acids, and assuming isotropic molecular reorientation, the expression for the longitudinal (T_{1Q}) and transverse (T_{2Q}) relaxation times is given by

$$\pi\Delta\nu_{1/2} = \frac{1}{T_{1Q}} = \frac{1}{T_{2Q}} = \frac{3\pi^2}{10}\frac{(2I+3)}{I^2(2I-1)}\chi^2\left(1+\frac{\varepsilon^2}{3}\right)\tau_c, \quad (1)$$

where I is the spin quantum number ($I = 1$ for ^{14}N), ω_0 is the Larmor frequency in hertz (Hz), τ_c is the effective correlation times for reorientation at the site of the amino group in seconds, $\Delta\nu_{1/2}$ is the resonance linewidth at half height in Hz, ε is the asymmetry parameter, and χ the nuclear quadrupole coupling constant in Hz. Therefore, the ^{14}N relaxation data can, in principle, be interpreted into dynamic information provided that the χ can be obtained by independent means (variation in the asymmetry parameter is generally ignored since the contribution from this source to the linewidth would be less than 15%, except for exceptional cases with $\varepsilon > 0.7$). It should be emphasized, however, that although the ^{14}N χ values and asymmetry parameters have been measured for several amino acids in the zwitterionic form [34–38], there is a very limited information on the $^{14}N\chi$ values of the amino groups in the different ionization states [37].

3.1. Effects of proton transfer on ^{14}N linewidths

The ^{14}N linewidths of Gly at pH < 7, without composite broadband decoupling, do not follow a simple one-proton titration equilibrium in rapid exchange (Fig. 1a). The one proton titration curve is given by

$$\Delta\nu_{1/2}(\mathrm{pH}) = \frac{\Delta\nu_{1/2}(X_1) + \Delta\nu_{1/2}(X_2)10^{X_3}}{1 + 10^{X_3}}, \qquad (2)$$

where the symbols X_1–X_3 have the following meaning: (a) for acids, $X_1 = \mathrm{AH}$, $X_2 = \mathrm{A}^-$, $X_3 = \mathrm{pH} - \mathrm{p}K_a$ and (b) for bases, $X_1 = \mathrm{BH}^+, X_2 = \mathrm{B}, X_3 = \mathrm{pH} + \mathrm{p}K_b - \mathrm{p}K_w$. A nonlinear least-squares fit of the experimental points (pH 0.7–5.0), gave a $\mathrm{p}K_a = 3.3 \pm 0.4$ for the carboxyl group, which is not in agreement with the literature value of $\mathrm{p}K_a = 2.3$ [39], with $\Delta\nu_{1/2}(\mathrm{AH}) = 131.5 \pm 1.5$ Hz and $\Delta\nu_{1/2}(\mathrm{A}^-) = 113.3 \pm 1.8$ Hz (Fig. 1b (ii)). Furthermore, using t test and the Hessian matrix for the calculation of the confidence intervals of the calculated curve, 36% of the experimental points were found to be outside the confidence interval region. This discrepancy from simple one-proton titration equilibrium has already been noticed in ^{14}N NMR [40,41] lineshape analysis of several amino acids, and was attributed to line broadening due to an exchange of protons between the amino group and the solvent [41]. Similar effects have been observed in ^{15}N NMR [42]. The use of power-gated decoupling with the WALTZ-16 composite pulse sequence [32,33] resulted in a sufficient elimination of proton exchange broadening without heating the sample. Under these conditions, the linewidth (pH 0.5–6.0) shows an inflection point around the $\mathrm{p}K_a = 1.8 \pm 0.2$ of the carboxyl group, which is in reasonable agreement with the literature value [39], following a simple one-proton titration equilibrium (Fig. 1b (i)). The data fit very well to a sigmoid function, with $\Delta\nu_{1/2}(\mathrm{AH}) = 72.1 \pm 2.3$ Hz and $\Delta\nu_{1/2}(\mathrm{A}^-) = 47.7 \pm 1.2$ Hz. Using t test and the Hessian matrix for the calculation of the confidence intervals of the calculated curve, only 10% of the experimental points were found to be outside the confidence interval region. Since the two lines of the sigmoid function are parallel to the pH-axis, this is an indication that no other phenomenon contributes to the whole procedure. If, for example, there was a contribution from a phenomenon following a first-order function, the two lines would be parallel one to the other, but not parallel to the pH-axis.

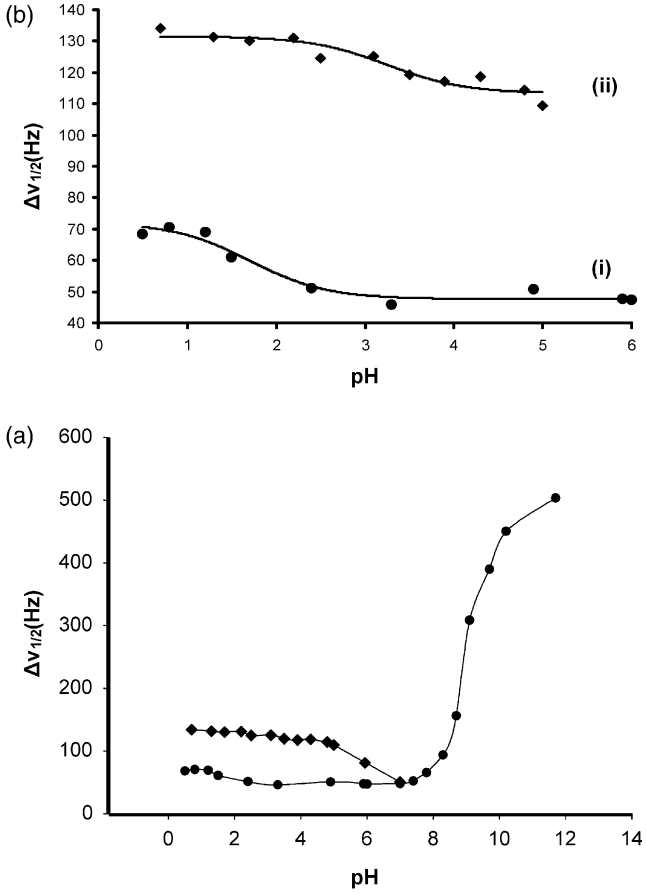

Fig. 1. The pH dependence of the ^{14}N NMR linewidths of Gly (◆, without decoupling; ●, with decoupling using the WALTZ-16 composite pulse sequence). (a) The overall titration curve, (b) expansion of the titration curve from pH 0.5 to 6.0. The solid lines between pH 0.5 and 6.0, correspond to a nonlinear least squares fit of the experimental points to one-proton titration curve of Eq. (2).

From the above it is evident that, contrary to earlier claims [40,41], the proton exchange broadening does not seem to be the exclusive factor affecting the ^{14}N linewidth at low pH. The extra broadening at low pH of the curve of Fig. 1b (ii), compared to that of Fig. 1b (i) under proton decoupling experiments, arises from residual ^1H–^{14}N J-coupling, which is averaged due to the exchange between amine protons and solvent (H_2O). It should be recalled that the increase of the ^{14}N linewidth of Gly at low pH in the titration curve under decoupling conditions (Fig. 1b, (i)), could be due to an increase in

the ^{14}N nuclear quadrupole coupling constant and/or to an increase in the rotation correlation time and, therefore, the hydration state (see Section 3.3).

The influence of the residual ^{1}H–^{14}N *J*-coupling above pH 7 is negligible due to faster exchange between amine protons and solvent. The marked increase therefore, of the linewidth under composite proton decoupling, is due to an increase of the electric field gradient of the $-NH_2$ group compared to that of the $-NH_3^+$. The data between pH 6–11.7 correspond to a $pK_b = 4.9 \pm 0.1$ ($pK_a = 9.1$), which is in agreement with the literature value of $pK_a = 9.8$ (39), with $\Delta\nu_{1/2}(BH^+) = 44.3 \pm 6.0$ Hz and $\Delta\nu_{1/2}(B) = 492.6 \pm 11.6$ Hz. Assuming approximately the same correlation time for the anionic and the zwitterionic forms, the relative magnitude of the χ values in the two forms is about 3.4.

3.2. Correlation of the ^{14}N and ^{17}O linewidths with molecular weight at pH 6—the case of isotropic motion

The ^{14}N NMR spectra of the α-amino groups of the protein amino acids were recorded in aqueous solution at pH ~ 6.0, at 40 °C, to enable direct comparison with the literature ^{17}O relaxation time data [27]. The ^{14}N and ^{17}O linewidths of the resonances are reported in Table 1. It can be seen that both ^{14}N and ^{17}O linewidths increase with the bulk of the amino acid side chains and appear to be independent of the α-carbon substitution.

If τ_c in Eq. (1) can be identified with a single correlation time from overall molecular reorientation (τ_{mol}), then, the expression for an isotropically tumbling rigid sphere in a medium of viscosity η can be applied [43]

$$\tau_{mol} = V_m \eta f_r / KT, \tag{3}$$

where V_m is the molecular volume and f_r is the microviscosity factor <1 depending on the relative sizes of solute and solvent and on stick and slip conditions [44]. V_m can be estimated as

$$V_m = 0.74\,MW/N_0\rho, \tag{4}$$

where N_0 is the Avogadro's number and MW and ρ are the molecular weight and the density of the solute, respectively. Eq. (4) implies that the amino acids in the crystalline state adopt a hexagonal compact structure occupying 74% of the available space in the lattice as

Table 1
^{14}N and ^{17}O linewidths of protein amino acids in different ionization states[a]

Amino acid	MW[f]	$\Delta\nu_{1/2}$, Hz[b,c]			
		pH 0.5		pH 6.0	
		^{14}N	^{17}O	^{14}N	^{17}O
Gly	75.07	55 (67)	205 (233)	36 (40)	132 (131)
Ala	89.10	71 (79)	276 (274)	45 (49)	194 (172)
Sar	89.10	83 (80)	272 (274)	60 (49)	170 (172)
N,*N*-Dimethyl-Gly	103.10		329 (314)		198 (212)
Ser	105.09	97 (94)	359 (320)	57 (58)	249 (218)
Pro	115.13	87 (104)	286 (349)	60 (64)	197 (247)
Val	117.15	102 (105)	376 (355)	72 (65)	280 (253)
Thr	119.12	118 (107)	410 (360)	71 (66)	300 (259)
Cys	121.26	104 (109)	[e]	59 (67)	254 (265)
4-Hydroxy-Pro	131.13		366 (395)		275 (293)
Ile	131.18	115 (118)	453 (395)	65 (73)	335 (294)
Leu	131.18	124 (118)	397 (395)	71 (72)	298 (294)
Asn	132.12	[d]	[e]	[d]	274 (296)
Asp	133.11	140 (120)	[d]	65 (74)	285 (299)
Gln	146.15	[d]	[e]	[d]	330 (337)
Lys	146.19	[d]	443 (439)	[d]	354 (337)
Glu	147.13	146 (133)	[d]	88 (82)	316 (340)
Met	149.21	133 (135)	397 (448)	78 (83)	297 (346)
His	154.16	169 (140)	438 (465)	99 (86)	359 (363)
Phe	165.19	143 (150)	485 (494)	88 (93)	376 (392)
Arg	174.20	165 (159)	482 (520)	111 (98)	394 (418)
Tyr	181.19	134 (164)	558 (540)	102 (102)	457 (438)
O-Methyl-Tyr	195.19		622 (581)		525 (479)
Trp	203.23	180 (186)	602 (607)	108 (115)	508 (505)

[a] The 0.1 M solutions in H_2O containing 10^{-5}M EDTA; $T = 40$ °C.
[b] Linewidths at half-height, estimated errors < ±5%.
[c] Values in parentheses correspond to linewidths resulting from regression analysis assuming isotropic molecular motion and linear approximation.
[d] Overlapping resonances.
[e] Not measured because of degradation.
[f] MW of the zwitterionic form.

Table 2
Statistics for a least-squares linear regression analysis of Eq. (6)[a]

Statistics	pH 0.5		pH 6.0	
	^{14}N	^{17}O	^{14}N	^{17}O
Slope	0.92 ± 0.10[b]	2.89 ± 0.22	0.58 ± 0.05	2.91 ± 0.17
Intercept	-3.30 ± 13.7	12.1 ± 31.2	-3.16 ± 7.4	-88.4 ± 23.9
Sum of squares	3.1×10^3	2.1×10^4	9.3×10^2	1.6×10^4
Correlation coefficients	0.85	0.91	0.88	0.93
No. of points	18	19	18	24

[a] Fitting of the linewidth data was performed via a linear equation of the type $y = ax + b$, where $x =$ molecular weight of the amino acids.
[b] $\pm$ standard deviations.

spheres [27]. In the case of ^{14}N, inclusion of Eq. (1) results in a relationship between the linewidths and the molecular weights of the form

$$\Delta\nu_{1/2} = \frac{1}{T_2} = \frac{3\pi}{2}\frac{0.74\chi^2\eta f_r}{N_0\rho KT}\,\mathrm{MW}, \tag{5}$$

where the asymmetry correction has been ignored. For roughly spherical molecules, Eq. (5) predicts that the ^{14}N linewidths are proportional to the effective MW for small molecules, if the other parameters do not change significantly within the series of the amino acids studied. Indeed the viscosity of the 0.1 M solutions was found to be independent of both the amino acid and the ionization state ($\eta = 0.89 \pm 0.02$ cP at pH 0.5 and 6.0). Formal evaluation of the micro viscosity factors from the van der Waals radii and applying the Gierer–Wirtz formula gave, for example, a value of $f_r = 0.22$ for glycine [27]. Edward [45], from experimental diffusion coefficients of several amino acids, evaluated values of $f_r = 0.79$ and 0.88 for glycine and alanine, respectively. Since the hydration of the amino acids is expected to be rather extensive, the stick boundary condition $f_r \sim 1$ seems to be a reasonable approximation for the amino acids in aqueous solution [27].

Eq. (5) can be rewritten in a more familiar form, as:

$$\Delta\nu_{1/2} = \alpha_0 + \alpha_1\,\mathrm{MW}, \tag{6}$$

where α_1 represents the contribution to the linewidth of the quadrupolar coupling constant, density and temperature, and α_0 represents solvent viscosity independent contributions to the linewidth due, possibly, to the hydration of the amino acids.

Table 2 collects the results of the linear least-squares analysis showing good correlation coefficients. Surprisingly, the intercept α_0, which is expected to be zero or a small positive number, is negative in three out of the four of the cases. If we test the null hypothesis that α_0 is statistically equal to zero in all cases, then, according to the theory: $-(\mathrm{var}\,\alpha_0)^{1/2} \cdot t_{(\beta/2,m-n)} < 0 - \alpha_0 < (\mathrm{var}\,\alpha_0)^{1/2} \cdot t_{(\beta/2,m-n)}$, where $\mathrm{var}\,\alpha_0$ is the variance of the intercept and $t_{(\beta/2,m-n)}$ is the $100\beta/2$ percentage point of the t distribution with $m-n$ degrees of freedom. In all cases, except for ^{17}O at pH 6.0, the null hypothesis is fulfilled, which means that statistically the intercepts are equal to zero. The straight-line correlations are not highly significant, even if correlation coefficient values (R^2) are above 0.8, since almost 50% of the experimental points lie outside of the 95% confidence intervals of the least-squares lines.

The linear correlation between $\Delta\nu_{1/2}$ and MW at pH 6 for both ^{14}N and ^{17}O nuclei is in support of the hydrodynamic model of Eq. (5). This suggests that the nitrogen and oxygen NQCCs of the amino acids change little from one compound to another. Assuming that the standard deviation of the slope for ^{14}N at pH 6 (Table 2), which is $\pm 8\%$, is due only to the scatter of the square of the NQCCs, then, we can easily obtain that the scatter of the NQCCs themselves is $\pm 2.8\%$. This is in agreement with the NQCCs values obtained by nuclear quadrupole resonance (NQR) [37,38] for several amino acids in the zwitterionic form (Table 3). Except for the Pro and Sar residues, the mean NQCC value of the rest of the amino acids is 1230 kHz, with a scatter of $\pm 12\%$. The higher NQCCs for Pro and Sar can be explained by the lower symmetry of the $>NH_2^+$ group, in comparison with the $-NH_3^+$ group in the solid state. We should mention, however, that in solution no significant change

Table 3
Literature ^{14}N nuclear quadrupole coupling constants [37,38]

Amino acid	MW[a]	Zwitterionic χ (kHz)	Cationic χ (kHz)
Gly	75.1	1249	
Ala	89.1	1205	
Sar	89.1	1505	
Ser	105.1	1205	
Pro	115.1	1623	
Val	117.1	1215	
Thr	119.1	1158	
Cys	121.3	1273	
Asn	132.1	1299	
Asp	133.1	1287	
Glu	147.1	1115	1193
His	154.2	1251	1256
Phe	165.2	1363	1069
Tyr	181.2	1078	977
Trp	203.2	1270	

[a] MW of the zwitterionic form.

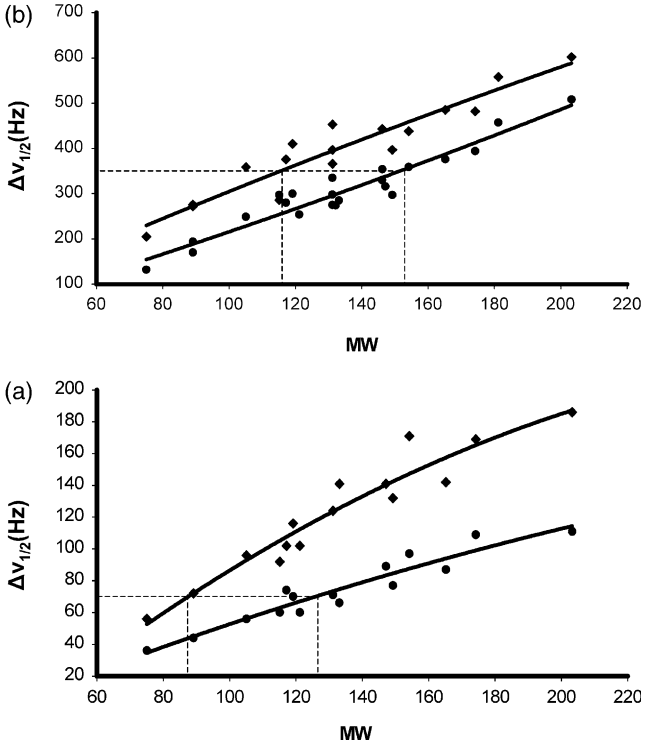

Fig. 2. Plot of the ^{14}N (a) and ^{17}O (b) linewidths, $\Delta\nu_{1/2}$, of the protein amino acids versus their molecular weights, MW: (◆) pH 0.5; (●) pH 6.0. All lines correspond to a nonlinear least squares fit of the experimental points according to Eq. (11). Dotted lines indicate the difference in MW for the same $\Delta\nu_{1/2}$ values.

in the NQCC was observed for the Pro residue (Table 1, Fig. 2).

3.3. Increase of the ^{14}N and ^{17}O linewidths at pH 0.5

Upon protonation of the α-carboxyl groups of the amino acids, the linear correlation of the ^{14}N linewidth with molecular weight was preserved, however, the slopes of the two straight-lines at pH 0.5 and 6 are different (Table 2). A linear correlation was also observed between the ^{17}O linewidths and the molecular weights of the amino acids at pH values 0.5 and 6; however, the slopes of the straight-lines were found to be independent of pH. Four explanations for the increase of the ^{14}N and ^{17}O linewidths at low pH are possible: (a) chemical exchange, (b) intermolecular associations, (c) a change in the hydration state and, therefore, of the effective molecular weight of the amino acids at low pH, and/or (d) a change in the ^{14}N and ^{17}O NQCC.

The possibility of line broadening due to chemical exchange should be excluded since the measure of the ^{14}N and ^{17}O T_1 and T_2 values of some representative amino acids at both pH 0.5 and 6, did not result in a difference in the two relaxation modes. Furthermore, proton exchange broadening is effectively removed by composite decoupling. The pH-dependent self-association of the amino acids should be excluded since no concentration dependence of the ^{14}N and ^{17}O linewidths is observed at different pH values, for molar concentrations ranging from 20 to 100 mM. The almost identical slopes obtained at pH 0.5 and 6.0 for ^{17}O indicate that the oxygen NQCCs, within experimental error, are independent of pH. Bagno and Scorrano [46] calculated a value of 10.3 MHz for the carboxyl group of the formic acid and 8.9 MHz for the formate anion, compared to 7.3 MHz for the experimental value of sodium formate [47]. Furthermore, Gready [48,49] studied the influence of protonation and hydrogen bonding on the ^{17}O NQCCs using ab initio calculations. Dimerization decreased the NQCC of the C=O group by −1.7 MHz and increased that of the C–OH group by +0.9 MHz; thus, the mean effect would be smaller because of a partial cancellation of the NQCC changes. The effect of solvation on NQCC value has been theoretically investigated within a supermolecule approach, which showed that hydrogen-bonding interactions cause a 10–30% decrease of NQCC (with respect to the isolated molecule) at the heteroatom participating in the bonding [46]. Very recently, Wu and Dong [50] determined by the use of two-dimensional ^{17}O multiple quantum magic-angle spinning NMR in the solid, the NQCC value of D-alanine in the zwitterionic state and D,L-glutamic acid. HCl in the free acid form. They reported NQCCs of 7.60 and 6.40 MHz for the C=O and $-O^-$ oxygen of alanine and 7.20 and 6.80 for the corresponding oxygen of the α-COOH group of glutamic acid. It appears, that the ^{17}O NQCC of the carboxyl group is practically independent upon the degree of ionization. However, it should be emphasized that it is very difficult to transfer the results for a static system to complex situation in solution. Therefore, small changes of the NQCC of the carboxyl group as a result of differences in ionization state, hydration and different side chain substitution effects for the individual amino acids cannot be excluded.

Under the hypothesis that the ^{17}O NQCC of the amino acid is independent of both the ionization and the degree of hydration of the carboxyl group, then, the increase in the ^{17}O linewidths at acidic pH ($\sim 100 \pm 31$ Hz), relative to those at neutral pH, can be explained by a change in the rotational correlation time of the amino acids, Eq. (1), and hence in their effective molecular weights. The observed ^{17}O linewidths, therefore, imply that the cationic form of the amino acids is more hydrated by an average of 1.3–2.5 molecules of water than the zwitterionic form [27]. These hydrated complexes must have lifetimes that are smaller than the NMR chemical shift time scale but presumably larger than the rotational correlation time scale (5–30 ps at 40 °C) to reorient as proper units.

The difference in the ^{14}N linewidths at the two ionization states is dependent upon the MW and the

intercept of the plot of the linewidths versus MW is, statistically, equal to zero. Differences, therefore, in $\Delta\nu_{1/2}$ are not only due to differences in the correlation times, but also due to a decrease in the ^{14}N NQCC on deprotonation of the carboxyl group. Differences in the correlation times are also supported from the results of Perrin and Yang [51] who found, using NMR spectroscopy, that the correlation times for $-NH_3^+$ rotation are 4.6 and 5.5 ps for the zwitterionic and cationic form, respectively. The deprotonation of the carboxyl group can exert its influence on the amino group in two ways; first, due to a through-bond inductive effect, and second, by an interaction of its associated electric field with the amino group. Deprotonation of the carboxyl group would affect both factors, but only the latter is expected to change the local asymmetry around the nitrogen and therefore the NQCC. This influence appears feasible when the average through-space intramolecular N$\cdots$O distance of 2.4–2.7 Å of the amino acids in the zwitterionic state is considered. Interestingly, the experimental work of Godfrey and Brown [52] showed that the neutral form of Gly in the gas phase exists primarily in two forms, a transoid form in which the OH and $-NH_2$ groups are not in mutual proximity, and a cinoid form in which the OH is conceptually in a bonding distance to the electron pair of the $-NH_2$ moiety. In water, theoretical calculations indicated that the zwitterionic *cis* conformation of Gly is stabilized with regard to the neutral *cis* form [53,54]; however, intramolecular hydrogen-bond interactions should be excluded. This is, probably, due to massive solvation that breaks up any intramolecular H-bond structure [18,54]. An additional variation, therefore, of the NQCC might also result from solvation changes upon deprotonation of the carboxyl group. Unfortunately, the NQCC values of only four amino acids in the cationic form have so far been reported in the literature [37] (Table 3). The NQCC value of His seems to remain constant in the zwitterionic and cationic form, for Glu there is a slight increase in the cationic form, but for Phe and Tyr there is significant reduction of the NQCC value in the cationic form compared to that in the zwitterionic. However, the values obtained by NQR are not necessarily the same as in the liquid state, since in the solid state new forms of inter- and intra-molecular interactions are to be expected.

From the square root of the slopes of Table 2, an increase by a factor of 26% of the NQCC in the cationic form compared to that in the zwitterionic form can be calculated, since $\chi_{0.5}/\chi_{6.0} = (0.92/0.58)^{1/2} = 1.26$. In order to treat the experimental results, we have to take into account the influence of both the change in the NQCCs and the hydration state. Changes in hydration state will shift the whole curve across the pH- and the $\Delta\nu_{1/2}$-axis, but the slopes of the two curves will remain the same. Changes in NQCCs would change the slope of the curve and would shift further the curve across the $\Delta\nu_{1/2}$-axis. It is obvious that the influence of variations of ^{14}N NQCCs in $\Delta\nu_{1/2}$ values is less for small molecular weights. The differences in the hydration, therefore, should be estimated at the minimum $\Delta\nu_{1/2}$-value. At $\Delta\nu_{1/2} = 70$ Hz, the difference in MW is 46.4 ± 14.0, which corresponds to an excess of 1.8–3.3 water molecules in the cationic form, compared to that in the zwitterionic form, in reasonable agreement with the results obtained from ^{17}O data.

The ^{14}N experimental data can also be treated with the hypothesis that the hydration number is a function of the MW [10]. Then, Eq. (6) will become

$$\Delta\nu_{1/2} = \alpha_0 + \alpha_1(\mathrm{MW} + 18f(\mathrm{MW})). \tag{7}$$

Taking into account a linear correlation between the hydration number and the MW of the amino acids in the zwitterionic state of the form $f(\mathrm{MW}) = -0.73 + 3.35\times 10^{-2}\,\mathrm{MW}$, as it was calculated from [10], then, the general expression of the $f(\mathrm{MW})$ function will be: $f(\mathrm{MW}) = \beta_0 + \beta_1\,\mathrm{MW}$. In this case, Eq. (7) reduces to Eq. (6). Such a treatment could possibly explain the ^{14}N data, but not the ^{17}O data; therefore, no attempt to minimize such a function took place.

3.4. The case of internal motion

Nery et al. [55,56], from ^{13}C longitudinal relaxation studies of uniformly enriched glycine, evaluated a larger correlation time for the C_α–C_0 bond ($\tau_{CC} = 17 \pm 4$ ps) than for the C_α–H bond ($\tau_{CH} = 5.5 \pm 0.3$ ps) since the later undergoes rapid internal rotation around the C–C-axis. A reexamination, therefore, of the assumption of isotropic molecular motion seemed to be necessary and it would be of interest to investigate the case of an effective correlation time comprising contributions from internal (τ_{int}) and overall motions (τ_{mol}). In order to check the hypothesis of internal motion, a polynomial (up to 4th degree) was fitted to our experimental data, and two more statistical parameters, except of R^2, were examined; the sum of squares (SS) and the F ratio (Table 4). Both SS and F values indicate that a second-degree polynomial is a better approximation for data treatment.

If the quadrupolar nucleus, e.g., ^{14}N or ^{17}O, is located within a portion of the molecule that is capable of internal rotation, then, the effective motion of the nucleus, τ_c, is a superposition of the internal rotation and the overall molecular motion. Assuming stochastic diffusion of the amino and carboxyl groups and extreme narrowing condition, τ_c is given by [57–59]

$$\tau_c = \tau_{mol}\left[A + (B + C)\frac{(12/r)\tau_{int}}{\tau_{mol} + (12/r)\tau_{int}}\right], \tag{8}$$

with

Table 4
Statistics for a least-squares polynomial regression analysis

D.P.	pH 0.5				pH 6.0			
	^{14}N		^{17}O		^{14}N		^{17}O	
	SS[a]	*F* Ratio	SS	*F* Ratio	SS	*F* Ratio	SS	*F* Ratio
0	2.0×10^4	1579.07	2.3×10^5	2625.65	7.6×10^3	1542.42	2.2×10^5	3074.64
1	3.1×10^3	122.79	2.1×10^4	169.89	9.3×10^2	115.30	1.6×10^4	284.68
2	2.6×10^3	1.79	2.0×10^4	0.02	9.3×10^2	0.44	1.5×10^4	1.59
3	2.6×10^3	0.25	1.8×10^4	2.27	8.8×10^2	0.46	1.4×10^4	0.95
4	2.4×10^3	0.23	1.7×10^4	0.67	7.5×10^2	0.63	1.4×10^4	0.44
		(4.49)[b]		(4.45)		(4.49)		(4.30)

[a] SS, sum of squares.
[b] Theoretical *F* ratio values obtained from Student's test (*t* test).

$$A = \frac{1}{4}(3\cos^2\theta - 1)^2, \quad B = 3\sin^2\theta\cos^2\theta,$$
$$C = \frac{3}{4}\sin^4\theta,$$

where θ is the angle between the rotation axis and the main field gradient and r is the r-fold jump mechanism. If we define for simplicity $\tau_i = (12/r)\tau_{\text{int}}$ and since the sum of A, B, and C is equal to 1, then we can rewrite Eq. (8) as

$$\tau_c = \tau_{\text{mol}} \frac{A\tau_{\text{mol}} + \tau_i}{\tau_{\text{mol}} + \tau_i}. \tag{9}$$

After some well-known mathematical transformations, Eq. (9) will get the form

$$\tau_c = (1 - A)\tau_i + A\tau_{\text{mol}} - \frac{(1 - A)\tau_i^2}{\tau_{\text{mol}} + \tau_i}, \tag{10}$$

which is more convenient for nonlinear minimization. From Eqs (1), (3), (4), and (10) and since A and τ_i can be assumed to be constant for all the amino acids, we get

$$\Delta\nu_{1/2} = \alpha_0 + \alpha_1 \text{MW} + \frac{\alpha_2}{\text{MW} + \alpha_3}, \tag{11}$$

where α_0–α_3 are constants, which are calculated using a constrained minimization process (Table 5). The only constrain used during the minimization process, was the value of α_3. Since α_3 arises from a fast internal motion, a value less than 20% of the minimum MW can be accepted (in this case τ_i should be less than 20% of τ_{mol} value for Gly). Eq. (11) is more general and describes also the case of anisotropic overall reorientation with only fast internal motion, if the relevant tensors are axially symmetric [60,61]. From Eq. (11) it is obvious that a second or a third-degree polynomial approximation would give better results than the linear approximation model. The type of the curve strongly depends upon the $\tau_{\text{mol}}/\tau_{\text{int}}$ ratio. Since Eq. (11) is a nonlinear approximation, only SS can be used for comparison with the results from polynomial approximation. The SS values obtained using Eq. (11) are smaller than those from Eq. (6), and from any other polynomial approach.

In the case of ^{17}O experimental data, the minimization of Eq. (11) gave a mean difference of 35.8 ± 17.3 in MW between pH 0.5 and 6.0 for three different $\Delta\nu_{1/2}$ values: 250, 350 (Fig. 2b) and 500 Hz. This can be interpreted by an excess of 1–3 water molecules at pH = 0.5. This is in agreement with the results obtained from Eq. (6). However, as mentioned before, it was impossible to make the same treatment in the case of ^{14}N. Since the nitrogen NQCCs are different in the two examined pH values, it is obvious from Eq. (5) that the overall slopes of the two curves at the two pHs will be different. According to the discussion made for the linear model, the influence of variations of ^{14}N NQCCs in

Table 5
Statistics for a least-squares nonlinear regression analysis of Eq. (11)[a]

Statistics	pH 0.5		pH 6.0	
	^{14}N	^{17}O	^{14}N	^{17}O
α_0	-6.77 ± 12.97[b]	19.98 ± 30.08	0.33 ± 7.59	-84.72 ± 23.30
α_1	0.93 ± 0.09	2.85 ± 0.21	0.55 ± 0.06	2.88 ± 0.16
α_2	-17.85 ± 14.03	12.97 ± 10.35	3.55 ± 12.73	12.33 ± 6.88
α_3	15.84 ± 2.04	11.67 ± 0.55	17.40 ± 2.56	11.59 ± 0.50
Sum of squares	2.0×10^3	1.5×10^4	7.4×10^2	1.3×10^4
No. of points	18	19	18	24

[a] Fitting of the linewidth data was performed via a nonlinear equation of the type $y = \alpha_0 + \alpha_1 * x + \alpha_2/(x + \alpha_3)$, where x = molecular weight of the amino acids.
[b] ± Standard deviations.

$\Delta\nu_{1/2}$ values is less for small molecular weights. Therefore, for $\Delta\nu_{1/2} = 70$ Hz (Fig. 2a), the difference in MW will be a good approximation of the difference in hydration in the two states. The calculated value was found to be 45.2 ± 7.4, which corresponds to an excess of 2–3 water molecules in the cationic form compared to that in the zwitterionic form. This is in reasonable agreement with the results obtained from ^{17}O NMR data.

4. Conclusions

We have demonstrated that the ^{14}N linewidths of several protein amino acids under composite proton decoupling increase with the bulk of the amino acids and that the linewidths at acidic pH (~0.5) were increased relative to those at neutral pH (~6.0). Statistical treatment of the experimental data assuming an isotropic molecular reorientation of a rigid sphere indicates a linear correlation between linewidths, under proton composite decoupling, and the molecular weights of the amino acids at the pH values 0.5 and 6.0. Assuming an effective correlation time of the amino group, which comprises contributions from the internal and overall motions, then, a significant improvement of the statistics of the regression analysis was observed. The ^{14}N relaxation data in conjunction with ^{17}O NMR linewidths can be interpreted by assuming that the ^{14}N NQCCs are influenced by the protonation state of the carboxyl group, the ^{17}O NQCCs remain constant within experimental error, and the cationic form of the amino acids is hydrated by an excess of one to three molecules of water relative to the zwitterionic state.

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