¹³C Nuclear Magnetic Relaxation of Poly(D-glutamic acid) in Aqueous Solution

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ABSTRACT: The molecular conformation and dynamics of poly(D-glutamic acid) in aqueous solution were studied by ¹³C nuclear magnetic resonance spectroscopy. Chemical shift, spin—lattice relaxation time (T_1), spin—spin relaxation time (T_2), and the nuclear Overhauser enhancement (NOE) were measured as functions of pH at 300 K. Increasing pH resulted in upfield shifts of C_α and peptide C' carbons, reflecting the helix-to-coil transition and downfield shifts of C_β , C_γ , and C_δ carbons, reflecting the ionization of the side-chain carboxyl group. T_1 , T_2 , and NOE increased with increasing pH. The effective reorientational correlation time (τ_{eff}) of C_α obtained from the combination of T_1 and NOE was 2.8 nsec in the helix region. This indicates that τ_{eff} of C_α is determined by not only the overall motion of the molecule but also by appreciable local segmental motions of the backbone. When going to the coil, τ_{eff} of C_α decreases by a factor of 4.3 as a result of the onset of rapid segmental motion. There is a progressive increase in T_1 values of the side-chain carbons as going away from the backbone, suggesting that the end of the side-chain undergoes more a rapid internal reorientation than the others even in the helix state. The peptide carbonyl carbons relaxes more slowly in D₂O than in H₂O, suggesting that the relaxation is contributed appreciably from the amide proton which is exchanged with deuterium in D₂O solution.

KEY WORDS Poly(D-glutamic acid) / Helix—Coil Transition / ¹³C NMR / Chemical Shift / Spin—Lattice Relaxation Time / Spin—Spin Relaxation Time / Nuclear Overhauser Enhancement / Correlation Time /

The conformational transition of a polypeptide in solution has been extensively studied using many physical techniques,¹ including nuclear magnetic resonance (NMR) spectroscopy² which can monitor individual atoms in a molecule. So far, the approach to the conformational study of polypeptide by NMR was to observe the change in the chemical shift caused by the conformational transition. The observed change in the chemical shift was interpreted empirically by reference to the results of other measurements, *i.e.*, optical rotatory dispersion, circular dichroism, intrinsic viscosity, etc.

In addition to chemical shifts, nuclear magnetic relaxation times provide useful information about the conformational transition of a molecule. Spin—lattice relaxation time (T_1), spin—spin

relaxation time (T_2), and the nuclear Overhauser enhancement (NOE) are sensitive functions of the correlation time of reorientational motion of internuclear vector.^{3–6} Thus, these NMR parameters are useful to estimate the correlation time of molecular motion.

There are a few ¹³C nuclear magnetic relaxation studies on homopolypeptides; poly(γ -benzyl L-glutamate),^{7,8} poly(L-lysine),⁹ poly(L-proline),¹⁰ and poly(L-hydroxyproline).¹⁰ It is well known that poly(D-glutamic acid) undergoes the coil-to-helix transition in an aqueous solution when the pH is lowered. In the present work, we carried out ¹³C NMR studies of poly(D-glutamic acid) in aqueous solution in order to obtain information about the relationship between molecular motions and the conformations. The chemical shift, T_1 , T_2 ,

and NOE of the backbone and side-chain carbons were measured as functions of pH.

EXPERIMENTAL

The sodium salt of poly(D-glutamic acid)(PGA) used in this work was prepared by alkaline hydrolysis of poly (γ -methyl D-glutamate) provided by the Ajinomoto Co. After exhaustive dialysis against distilled water and passing through a Chelex-100 (Bio-Rad) column to remove paramagnetic impurities, PGA was lyophilized to a powder. The degree of polymerization was determined to be 260 from the intrinsic viscosity measured in 0.2-M NaCl solution at pH 7.1 and at 298 K.¹¹

PGA solutions were prepared at a residual concentration of 0.67 M in 99.8-% D₂O obtained from Commissariat à l'Énergie Atomique (CEA) and in distilled and deionized H₂O. Adjustments of pH were made with 1-N NaOD and DCl obtained from Merck. The pH was measured on a Hitachi-Horiba M-7 pH meter equipped with a combination micro-electrode. The pH values reported here are direct meter readings without correction for any deuterium isotope effect. Since the measured values of T_1 of all the carbons were less than about 2 s, no attempt was made to remove oxygen gas dissolved in the solution.

¹³C NMR spectra were obtained at 15.04 MHz using a JEOL FX-60Q Fourier transform spectrometer with a quadrature phase detector. All measurements were done at a temperature of 300 K. The chemical shifts reported here were measured

relative to the resonance of internal dioxane and corrected to tetramethylsilane (TMS) by the relation of $\delta_{\text{TMS}} = \delta_{\text{dioxane}} + 67.86$ ppm.¹² Spectral widths of 3002 Hz with 8 K data points and 1500 Hz with 4 K data points were used. The T_1 measurements were performed by the inversion recovery method^{13,14} for protonated carbons and by the saturation recovery method^{15,16} for nonprotonated carbons. T_2 was estimated from the measured line width $\Delta\nu$ corrected for digital broadening, using the relation $1/T_2 = \pi\Delta\nu$. The NOE was determined for protonated carbons by the comparison with the intensities of fully decoupled and gated decoupled spectra.^{17,18}

RESULTS AND DISCUSSION

Chemical Shift

The proton-decoupled ¹³C NMR spectra of PGA at pH 4.9 and 7.5 are shown in Figure 1. The assignment of all peaks follows from that by Lyrerla, *et al.*¹⁹ It is apparent that the line widths of all resonances are broader at pH 4.9 than at pH 7.5.

Figure 2 shows the effect of pH on the chemical shifts of PGA in the pH range of 4.8 to 10.1. Since PGA strongly aggregates in the lower pH region at concentrations studied here,²⁰ measurements were made only above pH 4.8, at which pH PGA will be a partial helix in consideration of deuterium effect on the pH measurements.²¹ Below pH 4.8 where white precipitates are formed in the solution, it is very difficult to observe

Figure 1. Comparison of PGA spectra at two pH's at 300 K: a, pH 4.9, 14,000 scans; b, pH 7.5, 10,000 scans. Repetition time of 90° pulses is 1.5 s. Chemical shift scale in ppm from TMS.

Figure 2. pH Dependence of chemical shifts of PGA at 300 K.

resonances.

When pH increases, the α -carbon (C_α) and the peptide carbonyl carbon (C') resonances move upfield by about 2 ppm, while the β -carbon (C_β), the γ -carbon (C_γ), and the side-chain carboxyl carbon (C_δ) resonances move downfield by comparable amounts. Keim, *et al.*, have shown that all ^{13}C resonances of glutamic acid incorporated as central residue of a linear pentapeptide Gly-Gly-Glu-Gly-Gly, not forming a helix, move downfield by 1–3 ppm with increasing pH.²² ^{13}C NMR studies on some homopolypeptides in aqueous and non-aqueous solutions indicated that upfield shifts of C_α and C' resonances are accompanied by the transition from helix to coil.^{7,9,19,23,24} Our results therefore indicate that the shifts of C_α and C' resonances are due mainly to the opening of the hydrogen bonding of PGA, and that the behavior of C_β , C_γ , and C_δ resonances reflect primarily the ionization of the carboxyl group of the side chain. These results are in good agreement with those of Lyster, *et al.*,¹⁹ except for the difference between D and L enantiomers.

Figure 3. Inversion-recovery ^{13}C spectra of C_α , C_β , and C_δ carbons of PGA at pH 7.5 and at 300 K. Each spectrum is the result of 2,500 scans with a waiting time of 1.5 s. The delay time is shown at the right of each spectrum in milli-seconds.

T_1 , T_2 (Line Width), and NOE

Figure 3 shows the inversion-recovery Fourier transform spectra of protonated carbons of PGA. We show the pH dependence of T_1 for each carbon in Figure 4. For C_α , C_β , and C_γ carbons, NT_1 values are plotted, where N is the number of protons directly bound to the carbon, and for C_δ and C' carbons, simply the T_1 values are plotted. In the region above pH 6, T_1 values of all carbons do not vary with pH, while with decreasing pH below 6 T_1 values decrease sharply. The pH dependence of T_1 values is very similar to that of the chemical shift as shown in Figure 2. NOE and the line width are plotted against pH in Figures 5 and 6, respectively. These results

Figure 4. pH Dependence of NT_1 value for C_α , C_β , and C_γ carbons, and T_1 value for C' and C_δ carbons at 300 K.

Figure 5. pH Dependence of NOE for protonated carbons at 300 K: $\circ$, C_α ; $\bullet$, C_β ; $\times$, C_γ . I means error bar.

Figure 6. pH Dependence of line widths of PGA at 300 K: $\circ$, C_α ; $\bullet$, C_β ; $\times$, C_γ ; $\ominus$, C_δ ; $\bullet$, C' . I means error bar.

suggest that the correlation time of tumbling motion becomes longer in conjunction with the coil-to-helix transition.

One of the dominant mechanisms for nuclear relaxation of ^{13}C nuclei is the dipole-dipole interaction with neighboring protons, modulated by reorientational motions of macromolecules.^{5,6} If the details of reorientation of ^{13}C - ^1H internuclear vectors are known, it is possible to present expressions of T_1 , T_2 , and NOE in terms of correlation times of reorientational motions. Here, we assume that a simplified model of isotropic motion with a single effective correlation time (τ_{eff}) can describe the main feature of the nuclear relaxation. Then, T_1 , T_2 , and NOE are given by^{3,5}

$$\frac{1}{T_1} = \frac{N}{20} \cdot \frac{\hbar^2 \gamma_C^2 \gamma_H^2}{r^6} \{ J(\omega_H - \omega_C) + 3J(\omega_C) + 6J(\omega_C + \omega_H) \} \quad (1)$$

$$\frac{1}{T_2} = \frac{N}{40} \cdot \frac{\hbar^2 \gamma_C^2 \gamma_H^2}{r^6} \{ J(\omega_H - \omega_C) + 3J(\omega_C) + 6J(\omega_C + \omega_H) + 4J(0) + 6J(\omega_H) \} \quad (2)$$

$$\text{NOE} = 1 + \frac{\gamma_H}{\gamma_C} \cdot \frac{6J(\omega_H + \omega_C) - J(\omega_H - \omega_C)}{J(\omega_H - \omega_C) + 3J(\omega_C) + 6J(\omega_H + \omega_C)} \quad (3)$$

and the spectral density is given by

$$J(\omega) = 2\tau_{\text{eff}} / (1 + \omega^2 \tau_{\text{eff}}^2) \quad (4)$$

where $\hbar$ is Planck's constant divided by 2π , γ_C and γ_H are the gyromagnetic ratios of ^{13}C and ^1H , respectively, r is the distance between ^{13}C and ^1H

nuclei, and ω_C and ω_H are the angular resonance frequencies of ¹³C and ¹H nuclei, respectively.

C_α Carbon

At pH 7.5 and at 300 K, a measured NOE of 2.7 ± 0.2 is maximal as shown in Figure 4, and is in the limit where NOE is no longer a sensitive function of the correlation time. This leads to two important consequences: the nuclear relaxation of C_α is determined only by ¹³C—¹H dipole—dipole interaction, and the extreme narrowing conditions are valid. Using eq 1 under extreme narrowing conditions, the T₁ value of 73 ms measured at pH 7.5 yields $\tau_{\text{eff}} = 0.65$ nsec, assuming the C—H distance, $r = 1.08$ Å.

If we assume the polypeptide in the coil state as an equivalent rigid sphere, the correlation time of overall tumbling motion (τ_0) is given by

$$\tau_0 = 4\pi\eta r_0^3 / 3kT \quad (5)$$

where η is the viscosity of the solvent and r_0 is the radius of the sphere. The value of r_0 may be approximated by the radius of gyration of a freely jointed chain of n unit of length l , $l\sqrt{n/6}$. Using the degree of polymerization of 260 as n , l of 3.8 Å,²⁵ and η of 10.5 mP for D₂O at 300 K,²⁶ we obtain a correlation time of 17 nsec from eq 5. It should be noted that this calculated value is a lower limit, since the excluded volume effect is not taken into account for r_0 and polymer chains are subject to entanglements. The actual value of the correlation time for overall tumbling motion would be therefore greater than the calculated value of 17 nsec. The effective correlation time measured at pH 7.5 where the polypeptide is in the coil state is significantly smaller than the calculated value for overall tumbling motion. This suggests that the correlation time of C_α is determined by local segmental motions of the backbone rather than by overall motion of the molecule. It is found that the effective correlation time obtained here is in good agreement with those of poly(γ -benzyl L-glutamate)⁸ and of poly(L-lysine)⁹ in the coil state at room temperature.

Using the effective correlation time obtained from T₁, we calculate NOE and T₂ from eq 2 and 3. The results are shown in Table I. The calculated NOE is consistent with the measured one. On the other hand, the calculated T₂ is two times greater than the one estimated from the line

Table I. Comparison of measured NOE and T₂ values of C_α carbon in the helix and the coil states with the values calculated using τ_{eff} estimated from measured T₁ at 300 K

	pH ^a	
	4.9	7.5
Measured T ₁ , ms	38	73
Measured τ_{eff} , ns	2.8 ^b	0.65
Measured NOE	1.8	2.7
Calculated NOE	2.1	2.9
Measured T ₂ , ms	16	37
Calculated T ₂ , ms	13	72

^a Direct meter reading in D₂O solution.

^b This value was obtained from the combination of T₁ and NOE values.

width.

At pH 4.9 and at 300 K the observed value of T₁ is 38 msec and the NOE is 1.8. The combination of T₁ and NOE values yields $\tau_{\text{eff}} = 2.8$ nsec. It turns out that under these conditions, the approximation of the extreme narrowing is not valid for C_α. At this pH, PGA is thought to be in the helix region. Thus, it is reasonable to consider that the effective correlation time, longer at pH 4.9 than at pH 7.5, is the consequence of slower segmental motions of the molecules in the helix region.

It has been supposed that a helical PGA molecule in solution behaves like a rigid prolate ellipsoid²⁷ undergoing rotational diffusion characterized by two rotational diffusion constants. These are designated as D_a and D_b for rotation about major axis and rotation about an axis perpendicular to the major axis, respectively. The dimensions of the ellipsoid, a (the major semi-axis) and b (the minor semi-axis), were each assumed to be half the length of the helix and to have an average radius in solution. Here, we assume the diameter of the solvated helix to be 15 Å,²⁷ and the length per peptide residue to be 1.5 Å. For a highly prolate ellipsoid with an axial ratio ($\rho = b/a$) much less than unity, D_a and D_b are given by²⁸

$$D_a = \frac{3kT}{16\pi\eta ab^2}$$

$$D_b = \frac{3kT}{16\pi\eta a^3} \left\{ 2 \ln \left(\frac{2}{\rho} \right) - 1 \right\} \quad (6)$$

And the spectral density is given by⁴

$$J(\omega) = \frac{2A\tau_A}{1 + \omega^2\tau_A^2} + \frac{2B\tau_B}{1 + \omega^2\tau_B^2} + \frac{2C\tau_C}{1 + \omega^2\tau_C^2} \quad (7)$$

where $A = (3 \cos^2 \theta - 1)^2/4$, $\tau_A^{-1} = 6D_b$, $B = 3 \sin^2 \theta \cdot \cos^2 \theta$, $\tau_B^{-1} = D_a + 5D_b$, $C = 3 \sin^4 \theta/4$, and $\tau_C^{-1} = 4D_a + 2D_b$, where θ is the angle between the C_α —H internuclear vector and the major axis of ellipsoid.

For the PGA helix used here, a value of a is estimated to be 195 Å from the degree of polymerization and that of b to be 7.5 Å from a radius of the solvated helix.²⁷ The angle between the C_α —H vector and the axis of the helix is calculated to be 65° from the coordinates of the α -helix.²⁹ Using these parameters, we obtained $\tau_A = 766$ nsec, $\tau_B = 44.3$ nsec, and $\tau_C = 11.6$ nsec at 300 K. Equations 1, 3, and 7 with these correlation times yield a value of T_1 of 33 msec and a value of NOE of 1.2. It is inferred from the combination of the values of T_1 and NOE that the effective correlation time of the C_α —H vector fixed to the ellipsoid is 23 nsec, which is greater than the correlation time of T_1 minimum.

The correlation times calculated on the assumption of a rigid ellipsoid, determined by overall motion of the molecule, are apparently longer than the measured correlation time. This suggests that the tumbling motion of the C_α —H vector is not only overall tumbling motion but also involves appreciable local segmental motion of the backbone. This is clear from the fact that at pH 4.9, PGA is a partial helix as mentioned previously. Using the effective correlation time of 2.8 nsec estimated from T_1 and NOE, we can calculate the values of NOE and T_2 , which are found to be in reasonable agreement with measured values as shown in Table I.

When going from helix to coil with increasing pH, T_1 and NOE increase, and the line width decreases. All these observations are consistent with a decrease in τ_{eff} , as a result of the onset of the fast segmental motion of the polymer backbone.

C_β and C_γ Carbons

In all pH regions studied, as one goes away from the α -carbon of the backbone to the γ -carbon of the side chain, there is a progressive increase in NT_1 values and also in NOE, as shown in Figures 4 and 5. This increase in NT_1 and NOE is due to the decrease in the correlation time of motions in the sequence of C_α , C_β , and C_γ . These results

indicate that C_β and C_γ carbons of the side chain undergo internal reorientation. Such internal reorientation will be possible even in the helix state, since the side chains of PGA are located at the surface of the helix. The internal reorientation of the side chain has been reported for esters of poly(glutamic acid) in non-aqueous solution.^{7,8,30}

Under the extreme narrowing conditions, the spin-lattice relaxation time of a protonated carbon, undergoing internal reorientation as well as isotropic overall reorientation, is given⁴

$$\frac{1}{NT_1} = \frac{\hbar^2 \gamma_C^2 \gamma_H^2}{r^6} \tau_{c1} \left(A + \frac{B\tau_{\text{int}}}{\tau_{\text{int}} + \tau_{c1}} + \frac{C\tau_{\text{int}}}{\tau_{\text{int}} + 4\tau_{c1}} \right)$$

$$A = (3 \cos^2 \phi - 1)^2/4, \quad B = 3 \sin^2 \phi \cos^2 \phi,$$

$$C = 3 \sin^4 \phi/4 \quad (8)$$

where τ_{int} is the correlation time of internal reorientation, τ_{c1} is the correlation time for reorientation of the axis of internal motion, and ϕ is the angle between the C—H internuclear vector and the axis of internal reorientation. For the β -carbon, the internal rotation about C_α — C_β bond is allowed, and ϕ is a tetrahedral angle. If the correlation time of the axis is assumed to be equal to the effective correlation time for C_α , τ_{int} for C_β can be calculated from eq 8. The correlation time for internal reorientation for the γ -carbon was calculated by assuming that τ_{c1} for C_γ is the effective correlation time for C_β which is estimated from eq 1 under extreme narrowing conditions. The results are summarized in Table II at pH 5.1 and 7.5. It is found that the effective correlation time progressively decreases in the order of C_α , C_β , and C_γ . The correlation time of internal reorientation also shows a similar trend. It is seen that the internal motion of C_γ carbon is about three times faster than that of C_β carbon. These results suggest that the end of the

Table II. Effective correlation time (τ_{eff}) and correlation time of internal reorientation (τ_{int}) at 300 K

pH ^a	C_α	C_β		C_γ	
	τ_{eff} , ns	τ_{eff} ns	τ_{int} ns	τ_{eff} ns	τ_{int} ns
5.1	0.96	0.53	2.7	0.26	0.94
7.5	0.65	0.35	1.5	0.16	0.55

^a Direct meter reading in D₂O solution.

side chain undergoes more rapid motions than others even in the helix state. This added degree of motional freedom is also convinced from the fact of the larger NOE observed for C_γ carbon as compared with the C_α carbon. It is also seen that the internal motion of the C_β and C_γ carbons are about two times slower at pH 5.1 than at pH 7.5, suggesting that the internal motions of the side-chain carbons in the helix state are more hindered than they are in the coil state.

C' Carbon

Figure 4 indicates that the *T*₁ value of the peptide C' carbon is an order of magnitude greater than that of C_α carbon. This is due to the fact that there is no proton directly bonded to the C' carbon. The same situation is found for C_δ carbon.

Table III. Comparison of *T*₁ value of C' carbon in H₂O and D₂O in coil state at 300 K

	pH 7.5 in H ₂ O	pH 7.5 ^a in D ₂ O
<i>T</i> ₁ , s	0.88	1.28

^a Direct meter reading in D₂O solution.

We measured the spin-lattice relaxation time of C' carbon in both D₂O and H₂O at neutral pH to identify neighboring magnetic nuclei which greatly contribute to the relaxation of C' carbon. It was found that C' carbon relaxes more rapidly in H₂O than in D₂O. Results are shown in Table III. The measured *T*₁ value is longer in D₂O than in H₂O by 45%. It is reasonable to assume that the main contribution to the *T*₁ value of C' carbon is the dipole-dipole interaction with a directly bonded nitrogen (¹⁴N), α-proton which is two bonds removed, and an amide proton, also two bonds removed. The amide proton is readily exchanged with deuterium in a D₂O solution. Therefore, the longer relaxation time in D₂O is due to the exchange of a proton for deuterium of the amide group. Because the gyromagnetic ratio of deuterium (γ_D) is smaller than that of proton (γ_H), the ¹³C-²D dipole-dipole relaxation is less effective compared to the ¹³C-¹H dipole-dipole relaxation. In order to confirm this consideration we calculated the distance between C' carbon and the amide proton. Under the extreme narrowing conditions, the distance *r* will be given by

$$\left(\frac{1}{T_1}\right)_{\text{H}_2\text{O}} - \left(\frac{1}{T_1}\right)_{\text{D}_2\text{O}} = \frac{\hbar^2 \gamma_D^2 \gamma_H^2}{r^6} \tau \left\{ 1 - \frac{I_D(I_D+1)\gamma_D^2}{I_H(I_H+1)\gamma_H^2} \right\} \quad (9)$$

where τ is the correlation time of C' carbon, *I*_D and *I*_H are the spin quantum numbers of deuterium and proton, respectively, and other symbols have been mentioned previously. Assuming that τ of C' carbon is identical to the correlation time of C_α carbon, and using the measured *T*₁ value in H₂O and D₂O, the resulting distance is 1.98 Å. This value is in good agreement with value of 2.04 Å calculated from bond distance and angle³¹ in spite of rough assumption.

CONCLUSION

The ¹³C chemical shifts and relaxation times provided a reasonably detailed picture of the conformation and motion of PGA in aqueous solution. C_α and C' resonances move upfield in conjunction with the helix-to-coil transition, while C_β, C_γ, and C_δ resonances move downfield in conjunction with the ionization of the carboxyl group. These results are in good agreement with those of Lyerla, *et al.*¹⁹ The spin-lattice relaxation times and NOEs of protonated carbons increase when pH increases from 4.9. The *T*₁ values of C_α carbon indicate that even in the helix region at pH 4.9, PGA undergoes local segmental motions. As moving away from the backbone, the correlation times of the side-chain carbons progressively decrease, showing the presence of internal reorientation. It is found that the spin-lattice relaxation of C' carbon is greatly affected by the dipole-dipole interaction with the amide proton which is two bonds removed.

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