^{13}C- and Water Proton-Nuclear Magnetic Relaxation of Cu(II)–Poly(D-glutamic acid) Complex in Aqueous Solution

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ABSTRACT: ^{13}C- and water proton-nuclear magnetic relaxation times were measured as functions of temperature and pH to study the interaction of Cu(II) with poly(D-glutamic acid) (PGA) in aqueous solution. In the pH region from 4.5 to 8, the addition of Cu(II) significantly influences the relaxation times of C_γ and C_δ carbons, while those of C_β, C_α, and peptide C' are not influenced. The relaxation times of C_γ and C_δ increase with increasing temperature, indicating that a fast exchange between the complexed and uncomplexed states occurs. The scalar relaxation is dominant to line broadening, suggesting that a significant amount of electron spin density is transferred from Cu(II) to C_γ. Above pH 9, no paramagnetic effects of Cu(II) upon ^{13}C spectra of PGA were observed. The water proton relaxation of Cu(II) aqueous solution is markedly enhanced by the addition of PGA at an acidic pH, but suppressed at an alkaline pH. These results show that carboxylate groups of PGA and water molecules are bound to Cu(II) at pH ≤ 8, but excluded from Cu(II) at pH ≥ 9.
    KEY WORDS   Poly(D-glutamic acid) / Paramagnetic Effects / Cu(II) Complex / NMR / Spin-Lattice Relaxation Time / Spin-Spin Relaxation Time / Correlation Time /

The interaction of metal ions with poly(α-amino acid) has been extensively studied.[1] Such complexes can be considered as useful models for understanding the behavior of metalloproteins. Particularly, copper complexes of poly(α-amino acid) have been investigated in detail from the points of view of structural properties and catalytic activities.[1-9] Poly(L-glutamic acid) (PGA) forms a complex with Cu(II), the nature of which has been examined by a variety of physical techniques.[7-9] It has been suggested for the Cu(II)–PGA complex that ligands, coordinated to Cu(II), are the carboxylate groups and the peptide nitrogens in the acidic solution, and the nitrogen atoms in the alkaline solution.

Little nuclear magnetic resonance (NMR) study has been made of the interaction of poly(α-amino acid) with the metal ion.[10,11] Our previous ^{1}H NMR study showed that the paramagnetic metal ions of Co(II) and Ni(II) interact with the carboxyl groups of the side chain of PGA as well as with the nitrogen atoms of the backbone.[10] The presence of paramagnetic centers in a metal complex provides useful information for elucidating the structure and dynamics of the complex in solution, if spin-lattice and spin-spin relaxation times (T_1 and T_2) and chemical shift of the complex are measured.[12-14]

In the present work, we have measured the ^{13}C-nuclear magnetic relaxation times of the Cu(II)–PGA complex in an aqueous solution as functions of temperature and pH in order to obtain further information concerning the relationship between the structure and the dynamics of the exchange process between the complexed and uncomplexed states of the complex. Water proton relaxation times were also measured.

EXPERIMENTAL

The sodium salt of poly(D-glutamic acid) (PGA) was prepared from poly(γ-methyl L-glutamate) as described previously.[15] Analytical grade anhydrous copper(II) chloride was used. Solutions for ^{13}C- and ^{1}H-NMR measurements were prepared with D$_2$O (99.8%) obtained from Commissariat à l'Énergie Atomique and with redistilled and deionized H$_2$O, respectively. Aliquots of CuCl$_2$ aqueous solution

were added to the PGA solution to prepare the complex, and the resulting solution was stirred for a sufficiently long time. Adjustments of pH were made with NaOH and HCl solutions. The pH was measured on a Hitachi-Horiba M-7 pH meter equipped with a combination micro-electrode (Nisshin Rika Co.). The pH values reported here are direct meter readings without correction for the deuterium isotope effect in the D_2O solution.

^{13}C-NMR spectra were obtained at 15.04 MHz using a JEOL FX-60Q spectrometer with a quadrature phase detector. All measurements were carried out at controlled temperatures using a JEOL temperature control unit. Temperatures were calibrated with a thermometer before and after each measurement. Dioxane was used as the internal standard because of the unlikelihood of its competition with PGA residues and D_2O in entering the first coordination sphere of the metal ion. Chemical shifts were corrected to tetramethylsilane (TMS) by the relation cited in the literature.[16] The spin-lattice relaxation time (T_1) was measured by two methods. The inversion recovery method[17,18] was adopted for protonated carbons in metal-free solutions and all carbons in complex solutions. The saturation recovery method[19,20] was applied to nonprotonated carbons in metal-free solutions. The spin-spin relaxation time (T_2) was estimated from the measured line width (Δv) corrected for digital broadening using the relation of $1/T_2 = \pi\Delta v$. The spin-lattice relaxation time of water proton was measured on the JEOL FX-60Q spectrometer at 60 MHz by the inversion recovery method.[17,18]

THEORETICAL

Before presenting and discussing the experimental results, it will be useful to present briefly the theory of NMR relaxation times pertinent to this study. The spin-lattice relaxation time (T_1) and the spin-spin relaxation time (T_2) of nuclei bound near a paramagnetic site, describing by T_{1M} and T_{2M}, respectively, are represented by the Solomon–Bloembergen equations.[21,22] In the case of Cu(II), whose electron spin relaxation time is relatively long ($\sim 10^{-8}$ s),[23] the equations may be simplified as follows

$$\frac{1}{T_{1M}} = \frac{\gamma_I^2 \, g^2 \, \beta^2}{10 \, r^6}\left(\frac{3 \, \tau_c}{1 + \omega_I^2 \, \tau_c^2}\right) \qquad (1)$$

$$\frac{1}{T_{2M}} = \frac{\gamma_I^2 \, g^2 \, \beta^2}{20 \, r^6}\left(4 \, \tau_c + \frac{3 \, \tau_c}{1 + \omega_I^2 \, \tau_c^2}\right) + \frac{1}{4}\left(\frac{A}{\hbar}\right)^2 \tau_e \qquad (2)$$

where γ_I is the nuclear gyromagnetic ratio, g the electronic g factor, β the Bohr magneton, ω_I the angular frequency of the nucleus of I, r the nucleus-metal distance, A the hyperfine coupling constant, and τ_c and τ_e the correlation times for the dipolar and scalar relaxations, respectively. T_{1M} is contributed from the dipolar relaxation and T_{2M} from both the dipolar and scalar relaxations.

The correlation times are defined by

$$\tau_c^{-1} = \tau_R^{-1} + \tau_M^{-1} + \tau_s^{-1} \qquad (3)$$

$$\tau_e^{-1} = \tau_M^{-1} + \tau_s^{-1} \qquad (4)$$

where τ_R is the correlation time of the tumbling motion of the complex, τ_M the life time of a nucleus in the bound site, and τ_s the electron-spin relaxation time. For Cu(II) in aqueous solution at room temperature, $\tau_e \simeq \tau_s \leq \tau_M$.[23]

If a chemical exchange is present, the observed values of T_1 and T_2 are remarkably affected. Neglecting the chemical shift difference between bound and unbound resonances, in the presence of a small concentration of paramagnetic ion, the contribution of the paramagnetic ion to the relaxation times will be given by[23,24]

$$T_{ip}^{-1} = T_i^{-1} - T_{io}^{-1} = fq/(T_{iM} + \tau_M) \quad (i = 1, 2) \qquad (5)$$

where T_i and T_{io} are the observed relaxation times in the presence and absence of paramagnetic ion, respectively, f, the molar ratio of paramagnetic ion to observing ligand, and q, the number of observing ligand in the first coordination sphere of the metal ion.

RESULTS AND DISCUSSION

Effects of Cu(II)

The effects of adding Cu(II) on the ^{13}C-NMR spectrum of PGA are shown in Figure 1 at pH 7.4 and at 300 K. Assignments of peaks follow from the literature of Lyerla, et al.[25] This figure illustrates the selective broadening of ^{13}C resonances. With further addition of Cu(II), the carboxyl carbon(C_δ) and the γ-carbon(C_γ) resonances of the side chain particularly show broadenings, while the β-carbon(C_β),
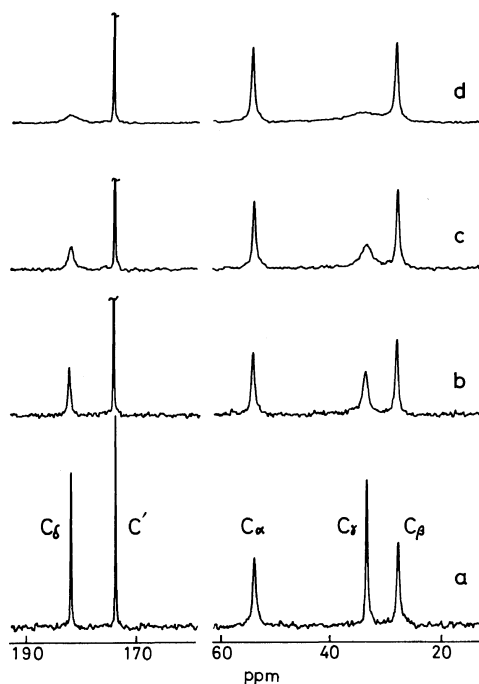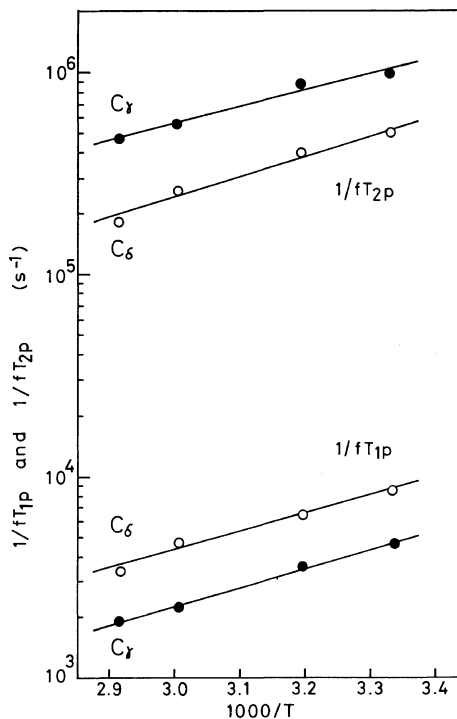
Figure 1. Effects of Cu(II) on ^{13}C-NMR spectra of PGA (0.93 M in monomer units) at pH 7.4 and 300 K: (a), $f=$[Cu(II)]/[PGA]$=0$, 3072 scans; (b), $f=4.39 \times 10^{-5}$, 3072 scans; (c), $f=9.25 \times 10^{-5}$, 6000 scans; (d), $f=2.41 \times 10^{-4}$, 13,000 scans. Repetition time of 45° pulses is 1.5 s. Chemical shift scale in ppm from TMS.

Figure 2. Temperature dependence of $1/fT_{1p}$ and $1/fT_{2p}$: (●), C_γ; (○), C_δ. $f=$[Cu(II)]/[PGA]$=9.25 \times 10^{-5}$.

the α-carbon (C_α), and the peptide carbonyl carbon(C') resonances were not affected at the Cu(II) concentrations studied. These observations indicate that Cu(II) specifically interacts with the carboxylate group of the side chain of PGA. Paramagnetic shift, however, could not be observed for any resonances beyond the broadening.

T_{1p} and T_{2p} of C_γ and C_δ Carbons

It was found that the values of $1/T_{1p}$ and $1/T_{2p}$ for C_γ and C_δ carbons increased by increasing the Cu(II) concentration. On the other hand, the T_1 and T_2 values of other carbons, except for C_γ and C_δ did not vary on addition of Cu(II).

Figure 2 shows $1/fT_{1p}$ and $1/fT_{2p}$ for C_γ and C_δ carbons as a function of the reciprocal of temperature. It is apparent that both $1/fT_{1p}$ and $1/fT_{2p}$ decrease with increasing temperature. These results indicate that T_{1p} and T_{2p} are controlled by T_{1M} and T_{2M}, respectively, rather than by τ_M. That is, T_{1M},

$T_{2M} \gg \tau_M$ in eq 5 (fast exchange). The relation of $T_{1p}/T_{2p} \gg 1$ holds in all temperature ranges studied, indicating that scalar relaxation is dominant in T_{2M}, while T_{1M} is dominated by dipolar relaxation. Paramagnetic broadening in the present case does not show the dependence on r^{-6} in eq 2.

Because q and τ_e are not known in eq 2, it is not possible to estimate the value of the hyperfine coupling constant A. However, the ratio of A for each carbon can be evaluated from the following equation. Under the conditions of fast exchange, the combination of eq 1, 2, and 5 yields

$$\frac{1}{T_{2p}} = \frac{7}{6}\frac{1}{T_{1p}} + \frac{fq}{4}\left(\frac{A}{\hbar}\right)\tau_e \qquad (6)$$

We obtained $|A(C_\gamma)/A(C_\delta)| = 1.7$ and $|A(C_\beta)/A(C_\delta)| = 0$ at pH 7.4 and 300 K. These results suggest that there is a significant amount of electron spin density transferred from Cu(II) to the C_γ carbon. The presence of such considerable scalar interaction demonstrates that Cu(II) is bound di-

rectly to carboxylate groups.

Under the conditions of fast exchange, T_{1p} can be written as

$$\frac{1}{T_{1p}} = \frac{3fq}{10} \frac{\gamma_I^2 \, g^2 \, \beta^2}{r^6} \tau_c \qquad (7)$$

Although Cu(II) has anisotropic g values, as the first apporoximation, we used the average g value obtained by Takesada, et al.[7] If τ_c and q are known, the distance between carbon and copper can be obtained from eq 7. It has been reported that τ_s is of the order of 10^{-8} s and $\tau_s \simeq \tau_e \leq \tau_M$ in aqueous solution at room temperature.[23] It was found to be $\tau_M \ll T_{2M} \simeq 10^{-6}$ s from Figure 2. If we assume that the rotational correlation time of the metal–carbon bond, τ_R, is of the same order of magnitude as the rotational correlation time of the C–H bond in metal-free PGA, τ_R^{-1} is $5 \times 10^9 \, s^{-1}$ for the C_γ carbon.[15] This yields $\tau_c^{-1} \simeq 5 \times 10^9 \, s^{-1}$. Furthermore we assume that all Cu(II) ions added are bound to carboxylate groups and $q = 2$.[26] On these assumptions, the distance between C_γ carbon and copper is estimated to be 3.0 ± 1.0 Å from eq 7 at pH 7.4 and at 300 K. Since the rotational correlation time for C_δ carbon is not known, we postulate that a possible upper limit of τ_c for C_δ is the same as τ_c for C_γ carbon. The same calculation as for C_δ carbon yields the distance between C_δ carbon and copper to be 2.5 ± 1.0 Å, which should be noted as the maximum distance.

On the assumptions of $\tau_e \simeq \tau_s \simeq 10^{-8} \, s^{23}$ and $q = 2$,[26] the absolute value of A can be estimated from eq 6. The values of $|A/h|$ obtained are 2×10^8 Hz and 1×10^8 Hz for C_γ and C_δ carbons, respectively.

pH Dependence of ^{13}C Relaxation Rate

Figure 3 shows the pH dependence of ^{13}C spectra in the presence of Cu(II) at 300 K. In the lower pH region, it has been observed for the metal-free PGA that all ^{13}C signals broaden due to increasing the correlation time in going from coil to helix.[15] The addition of Cu(II) results in further broadening of only the C_γ and C_δ resonances. This fact suggests that carboxylate groups are bound to Cu(II) even in the helix region. Cu(II) is known to induce the coil-to-helix transition.[7,8,26] However, the concentrations of Cu(II) used in this work are too low to observe this effect.

ESR studies of the complex at about pH 5 have shown that Cu(II) is bound to nitrogen atoms of the backbones and to carboxylate groups of the side chains.[7,26] If nitrogens are bound to Cu(II), the relaxation times of the C_α and C' carbons of the backbone would be influenced. However, the present study shows that the relaxation times of the two carbons are the same as those of metal-free PGA in the Cu(II) concentrations studied here, indicating that Cu(II) is not bound to nitrogen atoms at these concentrations. This disagreement between the ESR results and our NMR results may arise from the difference in Cu(II) concentrations; the ESR measurements have been made at Cu(II) concentrations about 10^3 times greater' than our NMR measurements. Thus, both results may suggest that at small Cu(II) concentrations, Cu(II) is bound only to carboxylate groups.

As shown in Figure 3, with increasing pH, the peak intensities of C_γ and C_δ carbons increase. At pH 11.1 the spectrum is apparently the same as that of the metal-free PGA in Figure 1(a).

Figure 4 shows the pH dependence of $1/fT_{2p}$ for the C_γ and C_δ carbon of the complex at 300 K. With increasing pH, $1/fT_{2p}$ for C_γ and C_δ carbons begins to decrease in the vicinity of pH 8 from constant values on the lower pH region and vanish above pH 9. Values of $1/fT_{2p}$ for other carbons except for C_γ and C_δ were zero for all pH's studied. These results indicate that carboxylate groups are released from Cu(II) at pH 7–8, and that the binding of Cu(II) to carboxylate groups does not occur in the alkaline pH region.
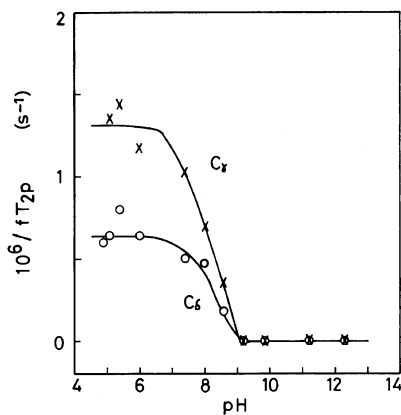
T_1 of Water Proton

To obtain further information on the properties of the complex, we measured the spin-lattice relaxation time of the water proton of the complex in a 0.2-M NaCl solution. Table I shows the results at pH 6.8 and at 296 K. The T_1 value of the water proton in Cu(II) aqueous solution is five times shorter than that in PGA aqueous solution. When PGA is added to Cu(II) aqueous solution, the T_1 value of the water proton becomes two times shorter again than that of the Cu(II) aqueous solution. This is due to the correlation time of tumbling motion of water molecules bound to Cu(II) being longer in the presence of PGA than in the absence of PGA because of the binding of Cu(II) to PGA. These results indicate that Cu(II) bound to PGA contains water molecules in the first coordination sphere at this pH.

The correlation time of the tumbling motion of the aqua Cu(II) complex was estimated as 5×10^{-11} s
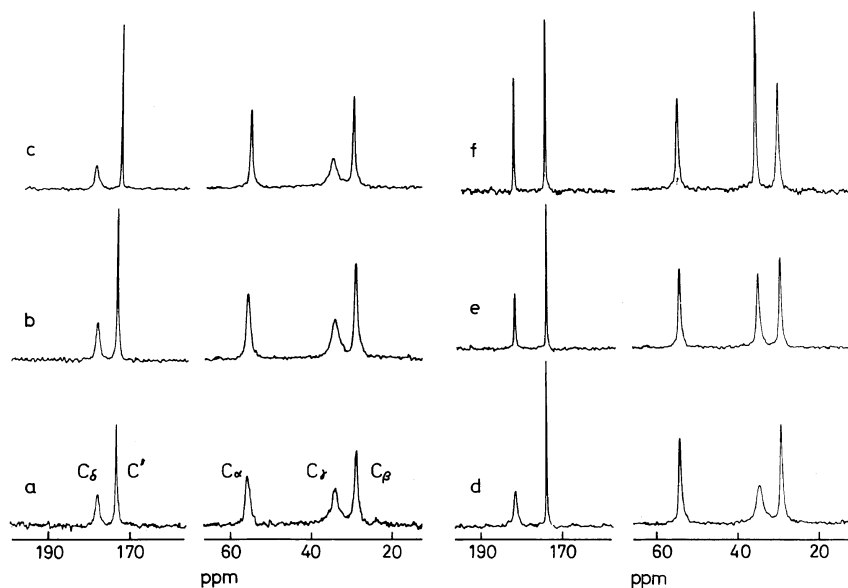
Figure 3. pH Dependence of ^{13}C spectra of Cu(II)–PGA complex at 300 K: (a), pH 4.9, $f = [\text{Cu(II)}]/[\text{PGA}] = 6.91 \times 10^{-5}$, 30,000 scans; (b), pH 5.1, $f = 5.36 \times 10^{-5}$, 40,000 scans; (c), pH 5.4, $f = 5.12 \times 10^{-5}$, 20,000 scans; (d), pH 7.4, $f = 9.25 \times 10^{-5}$, 6,000 scans; (e), pH 8.6, $f = 6.92 \times 10^{-5}$, 6,000 scans; (f), pH 11.1, $f = 6.92 \times 10^{-5}$, 3,000 scans. [PGA] = 0.7–0.6 M. Repetition time of 90° pulses is 1.5 s. Chemical shift scale in ppm from TMS.

**Figure 4.** pH Dependence of $1/fT_{2\text{p}}$ at 300 K: ($\times$), C_γ; ($\bigcirc$), C_δ. [PGA] = 0.7–0.6 M.
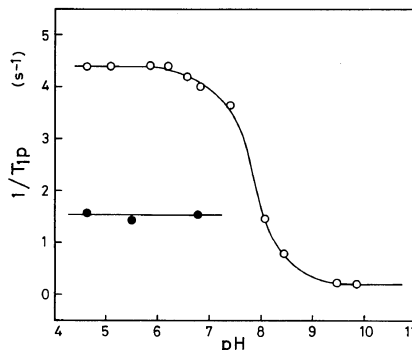
Figure 5. pH Dependence of $1/T_{1\text{p}}$ of water proton in 0.2 M NaCl solution at 296 K. [Cu(II)] = 2.1 mM: ($\bigcirc$), [PGA] = 69 mM; ($\bullet$), [PGA] = 0 mM.

from eq 7, on the assumption that the number of water molecules coordinated to Cu(II) is four, the distance between copper and water proton is 2.77 Å, and $\tau_M \ll T_{1M}$.[27] The correlation time in the PGA–Cu(II)–H$_2$O system was also estimated in a similar way as above except for the assumption of the coordination number. In the presence of PGA, the

number of water molecules coordinated to Cu(II) was assumed to be 2, since two of four coordination sites of Cu(II) are occupied by carboxyl groups of PGA.[26] The estimation yields a value of 2×10^{-10} s which is larger than that in the absence of PGA. It is of interest to note that the correlation time thus obtained is approximated to be the correlation time
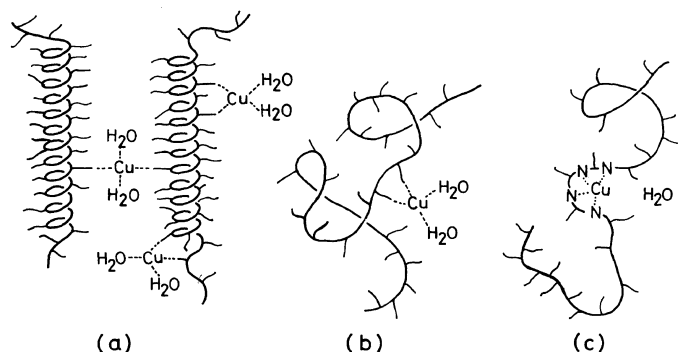
Figure 6. Schematic representation of Cu(II)–PGA complexes: (a), in the acid pH region; (b), in the neutral pH region; (c), in the alkaline pH region.

Table I. Spin-lattice relaxation times and correlation times of water proton[a]

	T_1, s	τ_c, s
$H_2O + PGA$	2.88	—
$H_2O + Cu(II)$	0.54	5.3×10^{-11}
$H_2O + Cu(II) + PGA$	0.23	2.1×10^{-10}

[a] 0.2 M NaCl; Cu(II), 2.1 mM; PGA, 69 mM; pH, 6.8; temp, 296 K.

of the tumbling motion of the C_γ carbon of PGA, τ_R, since water molecules are bound to Cu(II) which is bound to PGA. The correlation time of the tumbling motion of the complex estimated from the water proton relaxation is in good agreement with the results obtained from ^{13}C-nuclear relaxation of PGA.

Figure 5 shows the pH dependence of $1/T_{1p}$ of the water proton for the 2.1-mM Cu(II) aqueous solution in both the presence and absence of PGA at 296 K. The change of pH through the helix–coil transition region does not have any effect on T_{1p} in the presence of PGA. This indicates that Cu(II) remains bound to PGA even in the helix region, in agreement with the results of ^{13}C NMR described above. The independence of $1/T_{1p}$ with pH means that the product of q *and* τ_c in eq 7 remains unchanged with pH. On the other hand, with increasing pH from neutral to alkaline, the value of $1/T_{1p}$ begins to decrease to a value below that of the aqua Cu(II) complex in a pH region of 7 to 8 and vanishes in an alkaline pH region. This decrease in $1/T_{1p}$ arises from the onset of water molecules

released from the first hydration sphere of Cu(II).

Complex Structure

In figure 6, we depict schematic representations of the Cu(II)–PGA complex in the presence of a very small Cu(II) concentration. In the neutral pH region (pH 6–8) where PGA is in the coil state, two carboxylate groups and two water molecules are coordinated to Cu(II) (b). In the acidic pH region (pH 4.5–5.5) where PGA is in the helix state, carboxyl groups and water molecules remain in the first coordination sphere of Cu(II). The complex may be formed within a molecule, between molecules, or with the coil parts of the molecule (a).

Hojo and his coworkers showed from measurements of pH titration, visible spectra, and viscosity for Cu(II) complexes with PGA, poly(L-alanine), poly(DL-alanine) that in the alkaline pH region four nitrogen atoms of the backbone are bound to Cu(II).[28,29] This study shows that water molecules and carboxylate groups are not bound to Cu(II) in the alkaline pH region (pH ≥ 9). Figure 6 (c) shows a speculative structure of the complex at alkaline pH; only four nitrogen atoms are bound to Cu(II), and water molecules and carboxylate groups are not in the first coordination sphere of the metal ion. If nitrogen atoms are bound to Cu(II), the effect of paramagnetic Cu(II) should appear on the ^{13}C spectrum, especially C_α and C' carbons of the backbone. However, no effect upon ^{13}C spectra could be observed. Possibly the chemical exchange between the complexed and uncomplexed states is very slow, and spectra in the complexed state are too broad to be observed. Therefore, ^{13}C spectra at

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