Nuclear Magnetic Resonance Study of Poly(γ -glutamic acid)–Cu(II) and Mn(II) Complexes

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(Received August 5, 1989)

ABSTRACT: The interactions of poly(γ -glutamic acid) with Cu(II) and Mn(II) ions in aqueous solution were studied by ^1H and ^{13}C nuclear magnetic resonances. The addition of Cu(II) ions to poly(γ -glutamic acid) solution selectively broadens the α -proton signal, the side-chain carboxyl carbon signal, and the α -carbon signal. The addition of Mn(II) selectively broadens the α -proton signal, the side-chain carboxyl carbon signal, and the main chain carbonyl carbon signal. It is concluded that the oxygen atom of the side-chain carboxyl group of poly(γ -glutamic acid) binds to Cu(II) and Mn(II) ions in aqueous solution at neutral pH and at room temperature. It is also probable that the nitrogen atom of the amide group binds to Cu(II) and that the oxygen atom of the carbonyl group binds to Mn(II). These groups bound to Cu(II) and Mn(II) ions undergo rapid exchange between the bound and free states, and the life time of the bound state is short, less than 10^{-4} s for Mn(II) complex.

KEY WORDS NMR / ^1H / ^{13}C / Poly(γ -glutamic acid) / Metal Complexes / Cu(II) / Mn(II) /

Poly(γ -glutamic acid) (P γ GA) is a major component of a viscous materials produced by *Bacillus subtilis*. The physicochemical properties of P γ GA have been studied by many workers.^{1–5} They do not, however, seem to have been made as extensively as studies on poly(α -amino acid)s. We studied the interaction of water-soluble poly(α -amino acid)s such as poly(glutamic acid) and poly(lysine) with paramagnetic metal ions by means of nuclear magnetic resonance (NMR).^{6–10} It is a natural extension to study the interactions of P γ GA with metal ions.

NMR provides detailed information about the structure and dynamics of molecules in solution. Paramagnetic metal ions such as Cu(II) and Mn(II) have large electronic mag-

netic moments, which are 660 times or more larger than nuclear magnetic moments of ^1H and ^{13}C . When these paramagnetic metal ions are located in the neighborhood of nuclei, they broaden NMR lines and decrease spin–lattice and spin–spin relaxation times T_1 and T_2 of the nuclei. By observing such effects, we can obtain structural and dynamic information about polymer–metal complex. In this work, we study the interactions of Cu(II) and Mn(II) ions with P γ GA in aqueous solution by means of NMR methods.

EXPERIMENTAL

The sample of sodium salt of P γ GA was provided by Takeda Chemical Industries Ltd.,

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Japan. For ^1H NMR measurements P γ GA was dissolved in D_2O and lyophilized twice in order to replace exchangeable proton with deuterium. The concentration of P γ GA sample for ^1H NMR measurements was 5 mM. ^{13}C NMR measurements were made for a 400 mM P γ GA sample.

NMR measurements were carried out on a JEOL JNM-GX270 spectrometer (^1H resonance frequency of 270 MHz and ^{13}C resonance frequency of 67.5 MHz). The longitudinal relaxation time T_1 was measured by the inversion recovery method. The chemical shift of ^1H spectra was measured relative to the 3-(trimethylsilyl)propionic acid- d_4 sodium salt (TSP) signal as 0 ppm and ^{13}C chemical shift relative to the dioxane signal as 76.8 ppm. The temperature was changed from 30 to 80°C.

pH of samples was adjusted by adding aliquots of concentrated DCl or NaOD solutions. The values of pH here are direct meter-readings of a Hitachi-Horiba pH-meter without correction of deuterium effects.

Aliquots of 5 mM $\text{Cu}(\text{NO}_3)_2$ or $\text{Mn}(\text{NO}_3)_2$ deuterium oxide solution were added suc-

cessively to P γ GA samples. The concentration of metal ions is expressed as the molar ratio of metal ions to P γ GA residues $f = [\text{M}]/[\text{P}\gamma\text{GA}]$.

RESULTS AND DISCUSSION

Figure 1 shows the 270 MHz ^1H NMR spectrum of P γ GA in D_2O in the absence of paramagnetic metal ions at neutral pH and at a temperature of 80°C. Assignments of resonances indicated in the figure were made using two-dimensional NMR spectra (data are not shown here), and are in agreement with previous results.² Impurity peaks marked by X are due to methanol used for the sample preparation. Chemical shifts of resonances at room temperature are plotted in Figure 2 as a function of pH. All resonances undergo up-field shifts with increasing pH from 3 to 5, where the carboxyl group is ionized. The change of the α -proton signal is most significant, reflecting proximity to the ionizable carboxyl group. It was previously suggested that such changes in chemical shift are indicative of conformational differences at various pH.²

Figure 1. 270 MHz ^1H NMR spectrum of poly(γ -glutamic acid) in D_2O in the absence of paramagnetic metal ions at neutral pH and at a temperature of 80°C. X marks indicate impurity peaks. Chemical shift was measured in ppm units from the internal standard TSP.

It should be, however, noted that these changes in chemical shift can be also interpreted in terms of direct effects of ionization of the carboxyl group.

As increasing amounts of Cu(II) ions are successively added to P γ GA solution, ^1H

Figure 2. Chemical shifts of ^1H resonances of poly(γ -glutamic acid) in D_2O at room temperature as a function of pH: $\circ$, α -proton; $\bullet$ and $\odot$, β -protons; $\bullet$, γ -protons.

spectrum changes as shown in Figure 3. All resonances broaden with increasing Cu(II) concentration, indicating that P γ GA residues exist either in the bulk water or bound to Cu(II), and that chemical exchange occurs between these two states. In particular, the α -proton resonance shows a remarkable broadening, indicating that α -proton is closer to Cu(II) than other protons.

Figure 4 shows line widths of α - and γ -proton resonances as a function of temperature at neutral pH and at a $[\text{Cu(II)}]/[\text{P}\gamma\text{GA}]$ molar ratio of 1×10^{-3} . Line widths for all resonances decrease with increasing temperature, suggesting that the chemical exchange is rapid at neutral pH.

Figure 5 shows 67.5 MHz ^{13}C NMR spectra of P γ GA in D_2O at various Cu(II) concentrations at a temperature of 50°C and at neutral pH. ^{13}C spectral assignments indicated in spectrum a in the absence of Cu(II) were made by selective ^1H decoupling. On adding Cu(II) ions, the selective line-broadening is clearly seen. The carboxyl carbon and α -car-

Figure 3. 270 MHz proton NMR spectra of poly(γ -glutamic acid) in D_2O at various Cu(II) concentrations at room temperature and at neutral pH: (a), $f = [\text{Cu(II)}]/[\text{P}\gamma\text{GA}] = 0$; (b), $f = 5 \times 10^{-3}$; (c), $f = 1 \times 10^{-2}$; (d), $f = 2 \times 10^{-2}$; (e), $f = 5 \times 10^{-2}$. Chemical shift was measured in ppm units from the internal standard TSP.

Figure 4. Line widths of α - (○) and γ -proton (●) resonances of poly(γ -glutamic acid) as a function of temperature at neutral pH at $f = [\text{Cu(II)}]/[\text{P}\gamma\text{GA}] = 1 \times 10^{-3}$.

bon resonances broaden remarkably as Cu(II) concentration increases.

The results obtained here suggest that there exists a Cu(II)-P γ GA complex in which the oxygen atom of the side-chain carboxyl group and probably also the nitrogen atom of amide group of P γ GA are bound to Cu(II). The life time of the bound state is short, and the carboxyl and amide groups undergo rapid exchange at room temperature and at neutral pH between the bound and free states.

Figure 6 shows 67.5 MHz ^{13}C NMR spectra

Figure 5. 67.5 MHz ^{13}C NMR spectra of poly(γ -glutamic acid) in D_2O at various Cu(II) concentrations at room temperature and at neutral pH: (a), $f = [\text{Cu(II)}]/[\text{P}\gamma\text{GA}] = 0$; (b), $f = 8 \times 10^{-5}$; (c), $f = 1 \times 10^{-4}$; (d), $f = 2 \times 10^{-4}$; (e), $f = 3 \times 10^{-5}$. Chemical shift was measured in ppm units relative to the internal dioxane signal as 76.8 ppm.

Figure 6. 67.5 MHz ^{13}C NMR spectra of poly(γ -glutamic acid) in D_2O at various Mn(II) concentrations at room temperature and at neutral pH: (a), $f = [\text{Mn(II)}]/[\text{PyGA}] = 0$; (b), $f = 1 \times 10^{-5}$; (c), $f = 2 \times 10^{-5}$; (d), $f = 4 \times 10^{-5}$; (e), $f = 6 \times 10^{-5}$; (f), $f = 1 \times 10^{-4}$. Chemical shift was measured in ppm units relative to the internal dioxane signal as 76.8 ppm.

at various Mn(II) concentrations at room temperature and at neutral pH. As is the case of Cu(II) , upon the addition of Mn(II) the side-chain carboxyl carbon resonance increases in line width. Furthermore, the carbonyl carbon resonance also increases in line width, while

the α -carbon resonance remains unchanged in contrast to the system of Cu(II) . These results indicate that the side-chain carboxyl group as well as the main-chain carbonyl group are bound to Mn(II) at neutral pH, and that rapid chemical exchange occurs between bound and

Figure 7. The paramagnetic contribution of Mn(II) to the spin-lattice relaxation rate for the carboxyl carbon (○) and carbonyl carbon (●) resonances of poly(γ -glutamic acid) at neutral pH as a function of reciprocal temperature at $f = [\text{Mn(II)}]/[\text{P}\gamma\text{GA}] = 1 \times 10^{-5}$.

free states.

Figure 7 shows the paramagnetic contribution of Mn(II) to the spin-lattice relaxation rate $1/T_{1p}$ for the carboxyl carbon and the carbonyl carbon resonances at neutral pH as a function of reciprocal temperature. $1/T_{1p}$ is defined by

$$1/T_{1p} = 1/T_{1\text{obs}} - 1/T_{10}, \quad (1)$$

where $T_{1\text{obs}}$ and T_{10} are spin lattice relaxation times in the presence and absence of Mn(II), respectively. When the nuclei undergo chemical exchange between the bound and free states with a life time of the bound state τ_M much greater than τ_c , the correlation time of the electron- ^{13}C dipolar interaction of the complex, $1/T_{1p}$ is described as follows^{11,12}

$$1/T_{1p} = fq[(T_{1M} + \tau_M)]. \quad (2)$$

Here, f is the molar ratio of Mn(II) and P γ GA residues, q the number of equivalent carbon nuclei coordinated to Mn(II), and T_{1M} the spin-lattice relaxation time of the complex. This is given by

$$1/T_{1M} = 7\tau_c\gamma^2g^2\beta^2/2r^6, \quad (3)$$

where β is the Bohr magneton, g the g -factor, γ the magnetogyric ratio of ^{13}C nucleus, and r

the distance between Mn(II) and an observing nucleus of the complex.

As seen in Figure 7, for carboxyl and carbonyl carbon resonances, the value of $1/T_{1p}$ decreases with increasing temperature, suggesting that the life time of the bound state τ_M is shorter than T_{1M} , *i.e.*, $T_{1M} \gg \tau_M$. Thus, eq 1 combined with eq 3 is reduced to

$$1/T_{1p} = 7fq\tau_c\gamma^2g^2\beta^2/2r^6. \quad (4)$$

We assume $q = 1-6$, because the side chains of P γ GA occupy any one of 6 coordination sites of octahedral Mn(II). We also assume that τ_c in eq 4 is equal to the correlation time of tumbling motion of the vector of the α -carbon-proton distance in the absence of Mn(II) at room temperature $1.2 \times 10^{-10}\text{s}$, which is estimated from the T_{10} value of α -carbon. Using these values, eq 4 gives approximate distances of Mn(II) to the main-chain carbonyl carbon, α -carbon, and the side-chain carboxyl carbon of the complex. These carbons are found to be located within $4 \text{ \AA}$ from Mn(II); the carboxyl carbon is at the most closest distance to Mn(II).

Using $\tau_c = 1.2 \times 10^{-10}$ and $r = 4 \text{ \AA}$, T_{1M} of eq 3 can be estimated to be 10^{-4}s . As judged from $T_{1M} \gg \tau_M$ at room temperature and at neutral pH, the life time of the bound state is shorter than 10^{-4}s , but much longer the dipolar correlation time 10^{-10}s .

In conclusion, in aqueous solution at neutral pH and at room temperature there exists weak Cu(II)-P γ GA complex in which oxygen atom of the side-chain carboxyl group and probably the nitrogen atom of amide group of P γ GA are bound to Cu(II). These groups bound to Cu(II) undergo rapid chemical exchange with water molecules. For Mn(II)-P γ GA complex oxygen atoms of the carboxyl and carbonyl groups are coordinated to Mn(II) and also undergo rapid exchange between the bound and free states. The life time of the bound state is shorter than 10^{-4}s , but much longer than 10^{-10}s .

Acknowledgments. The authors wish to thank Mr. Mamoru Terasaki, Director of Foos Reserach Laboratory, Takeda Chemical Industries Ltd. for providing the sample. This work was supported by the Grant-in-Aid for Scientific Research on Priority Area of "Dynamic Interactions and Electronic Processes of Macromolecular Complexes" from the Ministry of Education, Science, and Culture of Japan (No. 62612501, No. 63612501, No. 01612004).

REFERENCES

1. T. Mineshita, S. Matsumura, H. Ono, T. Watanabe, and S. Ono, *Bull. Chem. Soc. Jpn.*, **42**, 594 (1969).
2. D. Balasubramanian, C. C. Kalita, and J. Kovacs, *Biopolymers*, **12**, 1089 (1973).
3. S. Sawa, T. Murakawa, T. Watanabe, S. Murao, and S. Omata, *J. Agri. Chem. Soc. Jpn.*, **47**, 167 (1973) (in Japanese).
4. D. I. Marlborough, *Biopolymers*, **12**, 1083 (1973).
5. T. Hara, Y. Fujino, and S. Ueda, *J. Appl. Biochem.*, **4**, 112 (1982).
6. O. Iwaki, K. Hikichi, M. Kaneko, S. Shimizu, and T. Maruyama, *Polym. J.*, **4**, 623 (1973).
7. T. Hiraoki, M. Kaneko, and K. Hikichi, *Polym. J.*, **11**, 397 (1979).
8. T. Hiraoki, M. Kaneko, and K. Hikichi, *Polym. J.*, **11**, 591 (1979).
9. N. Higuchi, K. Kakiuchi, Y. Kyogoku, and K. Hikichi, *Macromolecules*, **13**, 79 (1980).
10. N. Higuchi, T. Hiraoki, and K. Hikichi, *Macromolecules*, **13**, 81 (1980).
11. T. J. Swift and R. E. Connick, *J. Chem. Phys.*, **37**, 307 (1962).
12. Z. Luz and S. Meiboom, *J. Chem. Phys.*, **40**, 2686 (1964).