Electron Spin Resonance Study of the Correlation Times and Rotational Motions of Spin-Labeled Poly(α-L-glutamic acid) in Aqueous Solutions with Reference to the Optical Rotatory Dispersion Data of the pH-Induced Helix–Coil Transition

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ABSTRACT: Electron spin resonance (ESR) spectra and the optical rotatory dispersion (ORD) of poly(α-L-glutamic acid) spin labeled at the side chain [poly(slGlu)] were measured in aqueous 0.008, 0.15, and 2 M NaCl solutions at 15–17 °C over the pH range 4–7. The helical content (f_h) was estimated from the ORD, while the rotational diffusion coefficient (R) of the nitroxide spin label was evaluated from ESR on the basis of a line-shape theory which assumes axially symmetric rotational diffusion. The result shows that the piperidine ring rotates anisotropically about an axis parallel to the N–O radical bond ($R_{\parallel} = (5–7) \times 10^8\ s^{-1}$) and about an axis perpendicular to it ($R_{\perp} \simeq 1.5 \times 10^8\ s^{-1}$) in randomly coiled poly(slGlu), whereas the rotation becomes almost isotropic with an isotropic correlation time (τ_0) of 1.6 ns in completely helical poly(slGlu). This τ_0 value was interpreted in terms of multiple motions of the side chain. The experimental value agreed with the τ_0 calculated with a free and independent diffusion model under the assumptions that the C^{α}–C^{β} and C^{β}–C^{γ} bonds rotate with R values of $(0.1–0.8) \times 10^8$ and $(0.5–1.0) \times 10^8\ s^{-1}$, respectively, and that the N–C^4 and C^{γ}–C^{δ} bonds both rotate with an R of $1 \times 10^8\ s^{-1}$. In the helix–coil transition region, the rotation parallel to the N–O radical bond slows down remarkably as f_h exceeds ca. 0.5, whereas the perpendicular rotation persists with $R = (1–1.5) \times 10^8\ s^{-1}$. Hence, an extended helical segment may be a prerequisite for the restricted internal rotations of the side chain. A slow interconversion of the spin label (rate constant $\ll$ ca. $10^8\ s^{-1}$) between helical and coiled states was examined.

UV absorption, optical rotatory dispersion, and circular dichroism techniques have been extensively used to study polypeptide conformation, but they are not effective in studying the dynamics of conformational changes in the side chain. The multiple internal rotations of the side chain of several homopolypeptides[1-3] and proteins[4-6] were recently investigated by means of NMR. The spin-labeling ESR method should also be useful for studying the rotational motion of the side chain of macromolecules.[7] This method should be able to detect dynamical behavior of the polymer side chains that is not compatible with the NMR time scale since the ESR time scale is roughly 10^3 times shorter than the NMR time scale.

In most ESR studies,[7] both dynamics and local environment of the spin-labeled molecules have been deduced from the mobility of the nitroxide ring moiety. A nitroxide radical in the five- or six-membered ring (usually pyrroline, pyrrolidine, or piperidine) is attached to a target site of a macromolecule via several covalent bonds as joints. Since the joints are mostly flexible, their internal rotations affect the mobility of the ring considerably. Therefore, it is indispensable to clarify the effect of the joints on the mobility of the ring and to relate this with the molecular motion of the spin-labeled polymers.

For this purpose an appropriate system is a spin-labeled homopolypeptide which undergoes a helix–coil transition. The rotational mobility of the spin-label ring attached to the end of the side chain of the polypeptide may be closely related both to multiple internal rotations of the side chain and to changes in backbone conformation. The pH dependence of the mobility of the spin label attached to poly(lysine)[8] and poly(glutamic acid)[1,9] has been shown in the helix–coil transition region. In these systems, however, the correlation between the rotations of the nitroxide ring, the side-chain bonds, and the whole molecule is yet to be established.

In the present work the ESR and ORD of spin-labeled poly(glutamic acid) [poly(slGlu)] were measured in aqueous solutions under identical conditions of temperature, concentration, pH, and added NaCl concentration. The helical fraction (f_h) of the backbone was estimated from the ORD, while the rotational correlation time (τ) of the label was evaluated from ESR on the basis of a line-shape theory for a label which undergoes axially symmetric rotational diffusion.[10] For completely helical poly(slGlu), the observed τ value was compared with the calculated τ value on the basis of a free diffusion model for multiple internal rotations of the side chain. From these data the rotational diffusion coefficients were estimated for joints between the nitroxide ring and the backbone. The relation between τ and f_h in the helix–coil transition region is discussed from two alternative views regarding the rate process: (A) the spin label undergoes a rapid interconversion between the helical, coiled, and intermediate states, giving rise to a single-component ESR spectrum or (B) the spin label undergoes a slow interconversion resulting in a multicomponent spectrum.

Experimental Section

Materials. Poly(sodium α-L-glutamate) [poly(Glu)] was a gift of Dr. Hiroshi Sato of Mitsubishi Rayon Co. and was purified by fractional precipitation. 4-Amino-2,2,6,6-tetramethylpiperidine 1-oxyl (Aldrich) and dicyclohexylcarbodiimide (DCC) were purified by sublimation. 1-Hydroxybenzotriazole (HOBt) (Nakarai Chemical Co. Ltd., Kyoto) and all other chemicals of reagent grade were used without further purification.

Spin Labeling of Poly(Glu). Spin concentrations determined by ESR in a spin-labeled poly(Glu) prepared by Nordio's method were consistently fivefold lower than those determined potentiometrically. This is an indication that a considerable amount of nonspin fragments was attached to the side-chain carboxylates so as to suppress the ionization. This is probably due to an N-acylurea formed by the side reaction between a carboxyl group and DCC.[11] In order to avoid the side reaction, we modified Nordio's method and used HOBt:[12] A solution of 0.49 mM DCC and 0.45 mM HOBt in N,N'-dimethylformamide (DMF) was added dropwise to a stirred mixture of 1.24 g (8.2 mM) of poly(Glu) (acid form) and 0.41 mM of the nitroxide radical in 30 mL of DMF at 0 °C. The mixture was then stirred for 1 h at 0 °C and for another 0.5 h at room temperature. The bulk of the DMF was evaporated under reduced pressure and a 5% $NaHCO_3$ aqueous solution was added to the residue. The white precipitate

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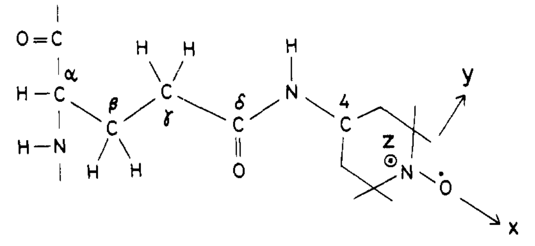

Figure 1. Spin label containing a Glu residue of poly(slGlu). *x*, *y*, and *z* are the molecular axes of the radical moiety.

(DCC-urea) was filtered off under suction. The filtrate was acidified with HCl to precipitate spin-labeled poly(Glu) [poly(slGlu)] which was then repeatedly washed with dilute HCl until no ESR signal could be detected in the concentrated washings. (The unreacted radical could not be removed completely by dialysis alone.) The poly(slGlu) was dissolved in 50 mL of water (pH 10) and this solution was dialyzed against twice-distilled water for 24 h. The dialyzed solution was freeze-dried to give 0.82 g (66%) of poly(slGlu).

The content of the radicals bound to the poly(Glu) was determined to be 1.3 ± 0.1 (ESR) and 2 ± 1% (potentiometric titration) of the total residue concentration of the poly(slGlu). This sample is denoted as poly(slGlu I). Its weight-average degree of polymerization (DP_w) was determined to be ca. 220 from the intrinsic viscosity in the 2 M NaCl solution. Another poly(slGlu) was prepared with the molar mixing ratio of radicals to Glu residue initially set at 0.17 in the reaction mixture. The content of the bound radicals in this sample, denoted as poly(slGlu II) (DP_w = ca. 220), was determined to be 12.5 ± 0.5 (ESR) and 14 ± 1% (potentiometric titration). The chemical structure of the spin-labeled residue of poly(slGlu) is shown in Figure 1.

Preparation of the Sample Solution. Stock solutions (ca. 16 mM) of poly(slGlu I) or poly(slGlu II) and poly(Glu) were prepared by dissolving the polymer (vacuum dried at 56 °C) in distilled water. The NaCl concentrations in the ca. 8 mM poly(slGlu) solution were 0.008, 0.15, and 2 M. The pH of the sample solution was adjusted by addition of HCl.

Measurements. ESR measurements were carried out with a JEOL Model JES-ME-3X X-band spectrometer with 100-kHz modulation (modulation width of 0.63 G, unless otherwise stated) at 15–17 °C. The microwave power was kept at 5 mW to avoid a power saturation effect. The parallel hyperfine coupling constant ($A_\parallel$) was determined to be 38.1 G in the frozen aqueous solution at 77 K. The perpendicular hyperfine coupling constant ($A_\perp$) was estimated to be 6.6 G from the $A_\parallel$ value and an isotropic hyperfine coupling constant (A_0) of 17.1 G. Optical rotatory dispersion (ORD) curves were measured with a JASCO Model ORD/UV-5 spectropolarimeter with a 0.3-cm-long quartz cell in the same temperature range as for the ESR measurements. The mean residue rotation of Glu at 233 nm ($[m]_{233}$) was calculated with the mean residue weights of 151.1, 152.8, and 167.5 for poly(Glu), poly(slGlu I), and poly(slGlu II), respectively. Values of $[m]_{233}$ are expressed in 10(deg cm^2 mol^{-1}).

Results and Discussion

pH Dependence of the ORD of Spin-Labeled Poly(Glu). In order to determine the helical fraction (f_h), we measured the ORD of poly(slGlu I) and poly(slGlu II) at various pH values in aqueous 0.008, 0.15, and 2 M NaCl solutions. The values of $[m]_{233}$ are plotted against pH in Figure 2. The sharp changes in $[m]_{233}$ show that both polymers undergo a helix–coil transition. The further increase in $-[m]_{233}$ at low pH (<4.5) corresponds to the so-called "aggregation" of poly(Glu).[13] The values of $[m]_{233}$ are -1.93×10^4 and -1.94×10^4 for the completely helical poly(slGlu I) and poly(slGlu II) ($[m]_{233}{}^h$), respectively, and -0.19×10^4 and -0.10×10^4 for the randomly coiled poly(slGlu I) and poly(slGlu II) ($[m]_{233}{}^c$), respectively. The $[m]_{233}{}^h$ values are in good agreement with -1.94×10^4 for the α-helical poly(Glu),[13] but the $[m]_{233}{}^c$ values are slightly smaller than -0.31×10^4 for the random-coil poly(Glu).

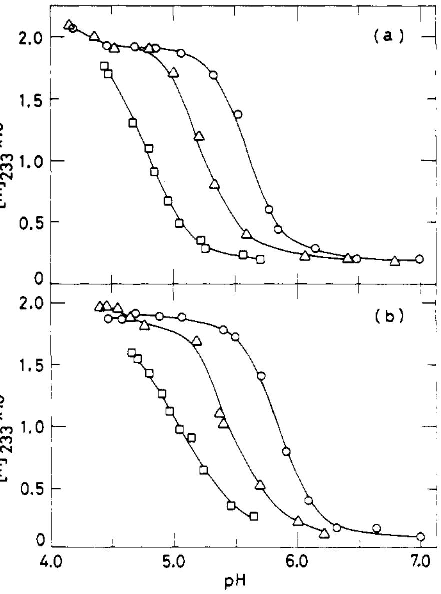

Figure 2. pH dependence of the mean residue rotation at 233 nm ($[m]_{233}$) of (a) poly(slGlu I) and (b) poly(slGlu II) in the presence of 0.008 (○), 0.15 (△), and 2 M (□) NaCl.

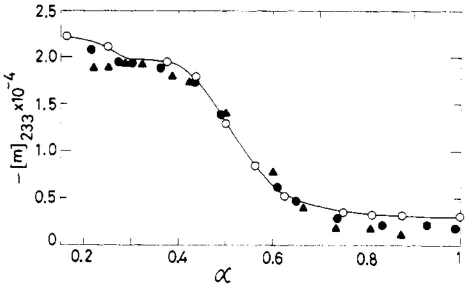

Figure 3. Dependence of the mean residue rotation at 233 nm ($[m]_{233}$) of poly(slGlu I) (●), poly(slGlu II) (▲), and unlabeled poly(Glu) (○) in the presence of 8 mM NaCl on the degree of neutralization of α (see eq 2).

With these $[m]_{233}{}^h$ and $[m]_{233}{}^c$, the f_h of poly(slGlu) was calculated from

$$f_h = \frac{[m]_{233} - [m]_{233}{}^c}{[m]_{233}{}^h - [m]_{233}{}^c} \qquad (1)$$

No precipitate of poly(slGlu) was observed in the pH range covered in this work.

Effect of Labeling on the Helix–Coil Transition of Poly(Glu). The effect of labeling on the helix–coil transition of poly(Glu) is shown in Figure 3, where $[m]_{233}$ values are plotted against the degree of neutralization (α). Since the spin-labeled side chain of a Glu residue does not dissociate, α is defined as

$$\alpha = \{(C_p - C_r) - [\mathrm{HCl}]_{\mathrm{add}}\}/C_p \qquad (2)$$

where C_p is the total residue concentration, C_r is the concentration of the spin-labeled residue, and $[\mathrm{HCl}]_{\mathrm{add}}$ is the concentration of HCl added to a sample solution. The transition curves of poly(slGlu I) and poly(slGlu II) almost coincide with the curve of unlabeled poly(Glu). This result indicates that the labeled residue plays almost the same role as the undissociated residue (COOH) in the helix–coil transition and that no other effect of labeling on the transition exists when the content of the labels is less than 12%.

pH Dependence of the ESR Spectra of Poly(slGlu). Figure 4 shows some typical ESR spectra of the poly(slGlu I) at four pH values. The ESR spectrum of poly(slGlu) in the random-coil state at pH 6.98 exhibits a sharp three-line signal, indicating a very rapid rotation of the label. The ESR spectrum becomes broader as the pH is decreased but still retains the three-line signal even at pH 4.86, where the poly(slGlu) is in a helix conformation. At the lowest pH (pH 3.58), where the poly(slGlu) forms aggregates, as judged from the ORD data, the ESR spectrum becomes much broader and asymmetric, indicating that the labels rotate slowly (ca. 20–70 ns).[14] The ESR spectra of poly(slGlu II) (Figure 9) were broader than those of poly(slGlu I) over the entire pH range because of a spin–spin interaction.

Rotational Correlation Time of the Spin Label. In order to estimate the rotational correlation time (τ) of the spin label, we analyzed the ESR spectra of poly(slGlu) in the manner proposed by Freed et al.[10] The line width (T_2^{-1}) of a motionally narrowed ESR spectrum is expressed as

$$T_2^{-1}(\tilde{M}) = A + B\cdot\tilde{M} + C\cdot\tilde{M}^2 \qquad (3)$$

where $\tilde{M}$ is the spectral index number of the hyperfine lines due to a nitrogen atom[10] and A, B, and C are the line-width parameters. When the secular and pseudosecular terms are comparable but the nonsecular terms are negligible, both B and C in eq 3 can be derived under the assumption that the rotational diffusion tensor (**R**) is axially symmetric:[10]

$$B = \frac{8\pi}{15}\omega_0\{g^{(0)}D^{(0)}\tau(0) + 2g^{(2)}D^{(2)}\tau(2)\} \qquad (4)$$

$$C = \frac{4\pi^2}{3}\{(D^{(0)})^2\tau(0) + 2(D^{(2)})^2\tau(2)\} \qquad (5)$$

$$\tau(0) = (6R_{x'})^{-1} = (6R_{y'})^{-1} \qquad (6)$$

$$\tau(2) = (2R_{x'} + 4R_{z'})^{-1} \qquad (7)$$

$$g^{(0)} = \frac{1}{6^{1/2}}\{2g_{z'} - (g_{x'} + g_{y'})\} \qquad (8)$$

$$g^{(2)} = \tfrac{1}{2}(g_{x'} - g_{y'}) \qquad (9)$$

$$D^{(0)} = \frac{1}{2(6^{1/2})}\{2A_{z'} - (A_{x'} + A_{y'})\} \quad \text{in Hz} \qquad (10)$$

$$D^{(2)} = \tfrac{1}{4}(A_{x'} - A_{y'}) \quad \text{in Hz} \qquad (11)$$

x', y', and z' are the principal axes of **R** (the symmetry axis is z'), ω_0 is the resonance magnetic field in angular frequencies, $g^{(0)}$, $g^{(2)}$, $D^{(0)}$, and $D^{(2)}$ are the irreducible tensor components of **g** and **A**, and R_i ($i = x', y', z'$) is the rotational diffusion coefficient along the i axis. With a degree of anisotropy N ($\equiv R_{\parallel}/R_{\perp}$, where $R_{\parallel} \equiv R_{z'}$ and $R_{\perp} \equiv R_{x'} = R_{y'}$), the ratio C/B becomes from eq 4 and 5

$$\frac{C}{B} = \frac{5\pi\{(D^{(0)})^2(1 + 2N)/3 + 2(D^{(2)})^2\}}{2\omega_0\{g^{(0)}D^{(0)}(1 + 2N)/3 + 2g^{(2)}D^{(2)}\}} \qquad (12)$$

C/B does not depend on the magnitudes of R and τ but should vary with N. Theoretical C/B values at various values of N are given in Table I. They were calculated under the condition that the z' axis of **R** is parallel to any one of the molecular axes (x, y, or z) of the spin label (see Figure 1). When the radical rotates isotropically, i.e., when $N = 1$, C/B becomes constant at 1.18 for poly(slGlu). The correlation time estimated from C (eq 5), assuming $N = 1$, was denoted as the isotropic correlation time (τ_0) as in previous papers.[1,7–9] The line-width parameters B and C were calculated from the peak-to-peak line width of the observed ESR spectrum by Labský's method B,[15] which neglects the broadening due to the nitroxide ring proton–electron interaction, since good agreement was obtained on the correlation times calculated by methods A and B, the former of which takes into account the proton hyperfine interaction.[15]

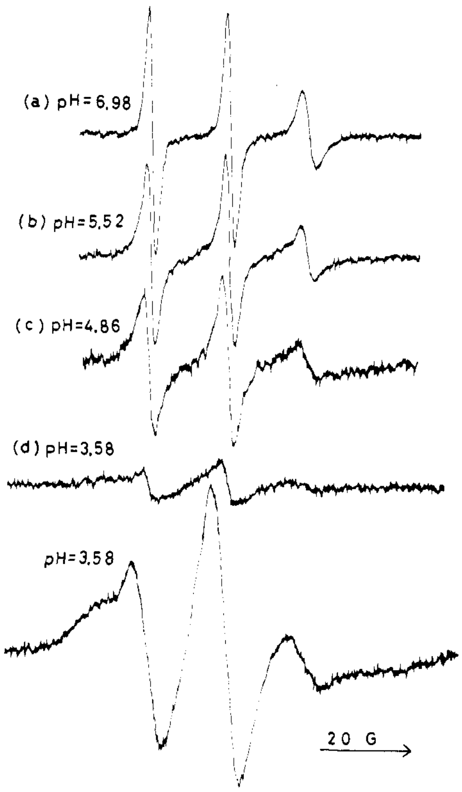

Figure 4. ESR spectra of poly(slGlu I) at (a) pH 6.98 (coil), (b) pH 5.52 (helix–coil), (c) pH 4.86 (helix), and (d) pH 3.58 (aggregate) in the presence of 8 mM NaCl at 15 °C. The ESR spectra of d were measured with modulation widths of 0.63 (upper) and 6.3 G (lower).

Table I
Theoretical Values of C/B

	C/B^a		
N	$z' \parallel x$	$z' \parallel y$	$z' \parallel z$
6	0.84	1.98	1.18
3	0.97	1.52	1.18
1	1.18	1.18	1.18
$^1/_2$	1.28	1.09	1.18

[a] C/B values are calculated with the following: $A_{\perp} = A_x = A_y = 6.6$ G, $A_{\parallel} = A_z = 38.1$ G, $g_x = 2.0096$, $g_y = 2.0064$, $g_z = 2.0027$, and a resonance magnetic field of 3179 G. The g values of 2,2,6,6-tetramethyl-4-piperidinol 1-oxyl determined in a single crystal were utilized.[34]

Isotropic Correlation Time of the Spin Label. As the first step for a critical comparison of the ESR and ORD data, the isotropic correlation time (τ_0) was calculated with eq 5 at various pHs and was compared with the $[m]_{233}$ values in Figure 5. The curves of τ_0 clearly deviate from those of $[m]_{233}$, although both transition curves shift toward low pH with an increase in added NaCl concentration. These results imply that the pH dependence of τ_0 is closely related to the conformational change of the polymer backbone but no simple linear relationship exists between τ_0 and $[m]_{233}$. Hence the ESR line width was analyzed more cautiously by estimating C/B from the peak-to-peak line heights of the observed ESR spectra.[10] Experimental values of C/B are listed in Table II. They are close to the theoretical value for the isotropically ro-

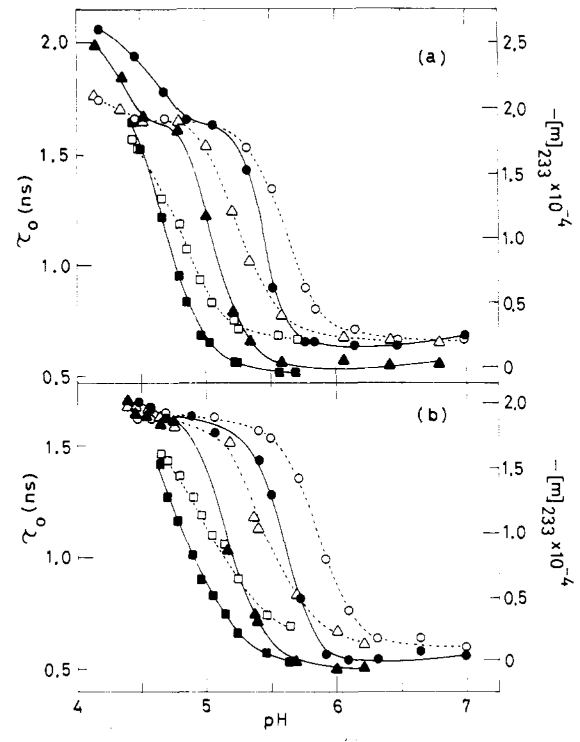

Figure 5. pH dependence of τ_0 of (a) poly(slGlu I) and (b) poly(slGlu II) in the presence of 0.008 (●), 0.15 (▲), and 2 M (■) NaCl. The corresponding changes in $[m]_{233}$ are shown by open symbols for comparison.

Table II
Experimental Values of C/B

state	[NaCl], mM	poly(slGlu I) pH	poly(slGlu I) C/B	poly(slGlu II) pH	poly(slGlu II) C/B
coil	8	6.98	0.92	6.99	0.96
	150	6.79	0.87	6.21	0.97
	2000	5.70	0.88	5.64	0.95
helix-coil	8	5.52	1.10	5.71	1.08
	150	5.20	1.03	5.37	1.02
	2000	4.84	1.02	5.05	1.08
helix	8	5.05	1.16	4.88	1.22
	150	4.80	1.13	4.65	1.18

tating label (C/B = 1.18) only in the completely helical state. In both coiled and intermediate helix–coil states, the observed C/B values are much less than 1.18. This result strongly indicates that the label rotates anisotropically.[10]

Anisotropic Rotational Correlation Times of the Spin Label. Since the observed ESR spectra of the poly(slGlu) are motionally narrowed even in the completely helical state, the linkages between a label and the C^α of a spin-labeled Glu evidently undergo a multiple internal rotation.[16] The internal rotations of the side chain may induce a complicated multiple rotational motion of the piperidine ring. However, such motion may be described, in the first approximation, by an axially symmetric rotational-diffusion tensor, as has been shown by Pilař et al.[17] in the study of anisotropic rotational reorientation of the piperidine ring attached to the side chain of poly(methacrylamide)-type copolymer (HPMA). On the basis of the idea of Pilař et al.,[17] the preferential symmetry axis or the z' axis of **R** in poly(slGlu) was similarly assumed parallel to the direction of the molecular x axis of the N–O radical bond, i.e., $z' \| x$ (cf. Figure 1), in order to calculate the values of $R_\|$ and $R_\perp$. This assumption seems to be justified by the experimental C/B values in the coiled and intermediate states (cf. Tables I and II) because they are well explained with the case of $z' \| x$.

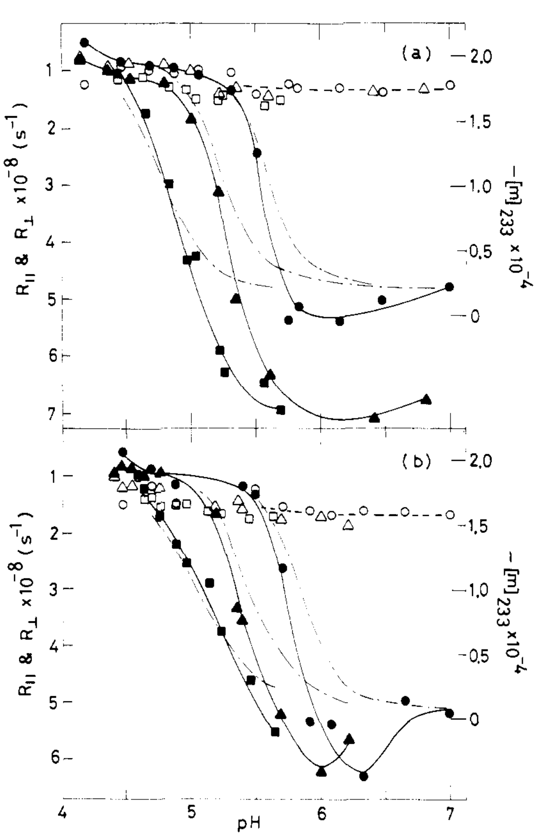

Figure 6. pH dependence of $R_\perp$ and $R_\|$ for (a) poly(slGlu I) and (b) poly(slGlu II) in the presence of 0.008 (circles), 0.15 (triangles), and 2 M (squares) NaCl. Open symbols are for $R_\perp$, while closed ones are for $R_\|$. The corresponding changes in $[m]_{233}$ (–·–) are shown for comparison.

Components of the tensor **R**, $R_\|$ and $R_\perp$, were calculated from the observed ESR spectra with the aid of eq 4 and 5 under the assumption of $z' \| x$. The values of $R_\|$ and $R_\perp$ are plotted against pH in Figure 6, together with the $[m]_{233}$ values for comparison. The helix formation results in a marked change in $R_\|$ but not in $R_\perp$. The $R_\|$ vs. pH curve generally shows a minimum at the onset of the coil-to-helix transition. This indicates that poly(slGlu) passes through an intermediate compact-coiled state.[1,18]

The values of N, $R_\|$, and $R_\perp$ of the random-coiled poly(slGlu) were estimated to be ca. 3.3–4.7, (5–7) × 10^8 s^{-1} ($\tau_\| = {}^1/_6 R_\|$ = 0.2–0.3 ns), and ca. 1.5 × 10^8 s^{-1} ($\tau(0) = {}^1/_6 R_\perp$ = 1.1 ns), respectively. It has been suggested that, in the case of random-coiled copolymers, the mode of rotational reorientation about the z' axis is determined by the properties of the side chain;[17] the $R_\|$ value may be regarded as a measure of flexibility of the side chain. From the comparison of the above values for poly(slGlu) with those estimated for various HPMA's (cf. Figure 13 in ref 17), the side chain of the random-coiled poly(slGlu) is supposed to be as flexible as that of HPMA-IV$_5$ or HPMA-IV$_8$. The macromolecular rotational diffusion coefficient (R_p) of the coiled poly(slGlu) was estimated to be 0.5 × 10^6 s^{-1} (${}^1/_6 R_p$ = 320 ns) on the basis of the Stokes–Einstein relation with a radius of gyration of 6.4 nm.[1] Since this R_p value is much smaller than the $R_\perp$ value of 1.5 × 10^8 s^{-1}, the $R_\perp$ rotation probably results from segmental motion of the backbone.[17]

In the helical poly(slGlu), the $R_\|$ value becomes nearly equal to the $R_\perp$ value (Figure 6). This may be due to the increase in the rigidity of the side chain of the helical poly(slGlu). The high rigidity of the side chain in the helical state as contrasted with the flexibility in the coiled state retards the rotational reorientation about the symmetry axis of **R**, resulting in an apparent isotropic motion, as pointed out by Pilař et al.[17] In a recent study of poly(slGlu),[1] the τ_0 values estimated from both *C* and *B* terms indicated that the label undergoes an anisotropic rotation in the helical rather than in the coiled state. The difference in molecular weights of the labeled poly(Glu) may be responsible for this reversed result.[19]

Multiple Internal Rotation of the Spin-Labeled Side Chain of Helical Poly(slGlu). The piperidine ring of the completely helical poly(slGlu) rotates almost isotropically ($R_{\parallel} = R_{\perp}$ = ca. 1×10^8 s^{-1} and τ_0 = ca. 1.6 ns), as shown in Figure 6. Wittebort and Szabo[2] found that information on the multiple internal rotations of a side chain of a rodlike polymer can be obtained from NMR data. In order to estimate the rotational diffusion coefficients of the internal rotations of the side chain of the completely helical poly(slGlu), we apply one of their NMR theories to our ESR system. Under the assumption that all internal rotations are diffusional and mutually independent, an isotropic correlation time (τ_0*) of the spin label may be written as

$$\tau_0{}^* = \sum_{ab_1b_2\cdots b_n} |d_{ab_1}{}^{(2)}(\beta_{\mathrm{D}1})|^2 |d_{b_1b_2}{}^{(2)}(\beta_{12})|^2 \cdots |d_{b_{n-1}b_n}{}^{(2)}(\beta_{N-1N})|^2 |d_{b_n0}{}^{(2)}(\beta_{\mathrm{NF}})|^2 [6R_{\mathrm{p},\perp} + (R_{\mathrm{p},\parallel} - R_{\mathrm{p},\perp})a^2 + b_1{}^2R_1 + \cdots + b_n{}^2R_n]^{-1} \quad (13)$$

where $d_{mm'}{}^{(2)}$ is a Wigner rotation matrix element of second rank, β_{ij} is the Euler angle between the *i*th and *j*th bonds, R_i is the rotational diffusion coefficient about the *i*th bond, and $R_{\mathrm{p},\parallel}$ and $R_{\mathrm{p},\perp}$ are the rotational diffusion coefficients about the long and short axes of a rodlike polymer, respectively.

In order to calculate the τ_0* value in eq 13, we have used values from the literature for the angles of β_{ij} in the Wigner matrix elements: $\beta_{\mathrm{D}1}$ between the long axis of the polymer and the C$^\alpha$–C$^\beta$ bond is 63.6°;[20] β_{12} between the C$^\alpha$–C$^\beta$ and C$^\beta$–C$^\gamma$ bonds and β_{23} between the C$^\beta$–C$^\gamma$ and C$^\gamma$–C$^\delta$ bonds are both 109.5°.[20] The trans configuration of the amide bond and the forbidden rotation of the C$^\delta$–N bond are assumed because of double-bond character;[21] therefore, β_{34} between the C$^\gamma$–C$^\delta$ and N–C^4 bonds becomes 9°. The angle $\beta_{4\mathrm{F}}$ between the N–C^4 bond and the molecular *z* axis of the spin label is 77° from an X-ray result of the radical 2,2,6,6-tetramethyl-4-piperidinol 1-oxyl in a chair conformation[22] or 42° from ESR simulation results of spin-labeled poly(γ-benzyl glutamate).[23,24] With the assumption that the poly(slGlu) of DP$_\mathrm{w}$ = 220 is the α helix with short and long axes of 1 and 33 nm,[25] both $R_{\mathrm{p},\perp}$ and $R_{\mathrm{p},\parallel}$ of poly(slGlu) are estimated to be 3.4×10^5 s^{-1} ($\tau_{\mathrm{p},\perp}$ = 490 ns) and 2.5×10^7 s^{-1} ($\tau_{\mathrm{p},\parallel}$ = 6.7 ns), respectively, according to Perrin's equation.[26] The observed value of $R_{\parallel} = 1 \times 10^8$ s^{-1} is taken for R_4, the rotation about the terminal N–C^4 bond, because the label ring rotates isotropically in the helical state. The remaining R_1, R_2, and R_3 are considered as parameters and adjusted to fit the experimental value of the isotropic correlation time ($\tau_0 \simeq$ 1.6 ns). R_1 is assumed to be smaller than R_2 because the rotation about the C$^\alpha$–C$^\beta$ bond should be more restricted than R_2 rotation by the backbone.[2,3] The orientation of the label was not affected appreciably by the rotation about the C$^\gamma$–C$^\delta$ bond with R_3 because of a small angle β_{34} of 9°; thus, R_3 is assumed to be equal to R_4. The values of R_1 and R_2 are mostly responsible for the observed value of τ_0.

The theoretical values of τ_0* were calculated with $R_3 = R_4 = 1 \times 10^8$ s^{-1} and are plotted in Figure 7. The results indicate that plausible R_1 and R_2 values are in the ranges of (0.1–0.5) × 10^8 and (0.5–1.0) × 10^8 s^{-1}, respectively, for $\beta_{4\mathrm{F}}$ = 77°. In the case of $\beta_{4\mathrm{F}}$ = 42°, plausible R_1 and R_2 values were estimated to be in the ranges of (0.7–0.8) × 10^8 and (0.9–1.0) × 10^8 s^{-1}, respectively. As a result, there appears to be no remarkable dependence of both R_1 and R_2 values on $\beta_{4\mathrm{F}}$. If the multiple internal rotations of the side chain are independent of the DP$_\mathrm{w}$ of poly(Glu), these R_1–R_4 values are applicable to any poly(Glu). For a poly(Glu) of DP$_\mathrm{w}$ 100, which gives 58 and 3 ns to the overall correlation times of $\tau_{\mathrm{p},\perp}$ and $\tau_{\mathrm{p},\parallel}$, respectively, for example, the τ_0* value is calculated, by using $\beta_{4\mathrm{F}}$ = 77°, to be 1.26 ns with $R_1 = 0.2 \times 10^8$, $R_2 = 0.8 \times 10^8$, and $R_3 = R_4 = 1 \times 10^8$ s^{-1}. This value of 1.26 ns predicted from our data is in good agreement with the value of 1.0–1.2 ns experimentally estimated for a poly(slGlu) by Tsutsumi et al.[1] By the method of NMR, the correlation times of the internal rotations about the C$^\alpha$–C$^\beta$ and C$^\beta$–C$^\gamma$ bonds have been estimated to be 2.7 and 0.94 ns in the helical poly(Glu), respectively.[3] They are comparable to the present ESR results of $(6R_1)^{-1}$ = 3.3–17 ns and $(6R_2)^{-1}$ = 1.7–3.3 ns for $\beta_{4\mathrm{F}}$ = 77° and $(6R_1)^{-1}$ = 2.1–2.4 ns and $(6R_2)^{-1}$ = 1.7–1.9 ns for $\beta_{4\mathrm{F}}$ = 42°.

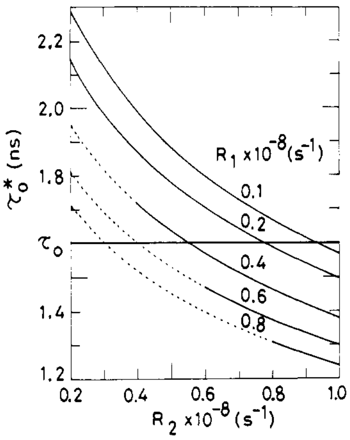

Figure 7. Theoretical isotropic correlation time (τ_0*) calculated with eq 13 as a function of R_2 at various values of R_1 with $R_3 = R_4 = 1 \times 10^8$ s^{-1} and $\beta_{4\mathrm{F}}$ = 77° (see text). The condition that $R_1 < R_2$ is indicated by the solid curves. The observed isotropic correlation time of τ_0 (1.6 ns) of the spin label in the completely helical poly(slGlu) is marked by a horizontal solid line.

Dependence of Correlation Time on Helical Fraction. The isotropic correlation time τ_0 and the anisotropic parallel correlation time $\tau_{\parallel}$ (=$^1/_6R_{\parallel}$) are plotted against the f_h in Figure 8. Both τ_0 and $\tau_{\parallel}$ of poly(slGlu) become slow in 8 and 150 mM NaCl solutions as f_h exceeds about 0.5. The dependence of τ_0 and $\tau_{\parallel}$ in 2 M NaCl solution differs slightly from that in 8 and 150 mM NaCl solutions. This is probably because some aggregates coexist with single-strand helices at lower f_h.[1] An ESR spectrum observed in the helix–coil transition region is known theoretically to depend on the rate constant of the interconversion between helical, coiled, and intermediate states.[27,28] If the interconversion rate is much faster than the reciprocal of the transverse relaxation time ($T_2{}^{-1}$), an observed ESR spectrum should be a single-component type. The correlation time estimated from its line width is a mean value averaged over all possible states.[28] This is designated as case A.

On the contrary, if the rate is much slower than $T_2{}^{-1}$, an observed spectrum should be a composite of two component spectra or more according to the number of states involved. The change in correlation times estimated from the line width with f_h is only formal and should be considered as a measure of the variation in the concentrations of spin labels in different states.[28] This is designated as case B. The spectrum of case A would gradually transform

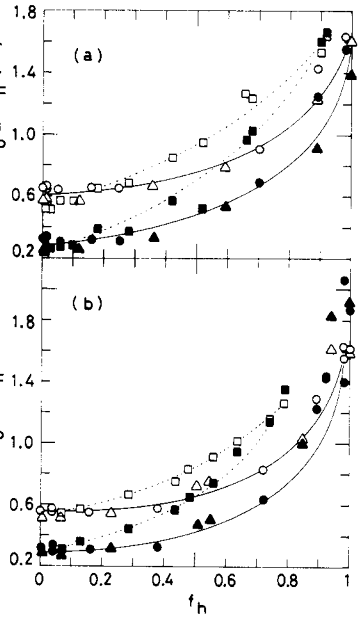

Figure 8. Dependence of τ_0 and $\tau_\parallel$ (=$^1/_6R_\parallel$) on the helical fraction f_h from ORD for (a) poly(slGlu I) and (b) poly(slGlu II) in the presence of 0.008 (circles), 0.15 (triangles), and 2 M (squares) NaCl. Open symbols are for τ_0, while closed ones are for $\tau_\parallel$. The dependencies of $\tau(0)$ (=$^1/_6R_\perp$) are shown in Figure 10.

to the spectrum of case B as the interconversion rate becomes slow. If the spin label can take only two states (completely helical or random-coil state), i.e., if poly(slGlu) undergoes the helix–coil transition by an all-or-none process, the interconversion rate could be estimated from the variation of the line width of the ESR spectrum with f_h obtained from the ORD.[28] However, the all-or-none transition of poly(slGlu) in aqueous solution is unlikely considering its DP_w of 220 because an all-or-none model is theoretically known to be valid for a DP of 10–20.[29] The following two alternative interpretations will be provided for the τ vs. f_h plots in Figure 8.

Case A (Fast Interconversion). The assignment of the $R_\parallel$ ($\tau_\parallel = {}^1/_6R_\parallel$) or $R_\perp$ rotation to the individual internal rotations of the side chain is difficult in the helix–coil transition region; yet, the change in $\tau_\parallel$ should reflect a change in local environments of the side chain. Thus, the $\tau_\parallel$ vs. f_h plot may be interpreted as indicating that formation of an extended helical segment is a prerequisite for the side-chain rotation to be restricted by the helical backbone. In this connection, it is of interest to note that the mean-square radius of gyration of a homopolypeptide is known both theoretically and experimentally to depend on the helical fraction in almost the same manner as the correlation time of the spin label (Figure 8).[30,31] This is conceivable because the increase in the radius of gyration parallels the increase of rodlike portion in the polymer chain.

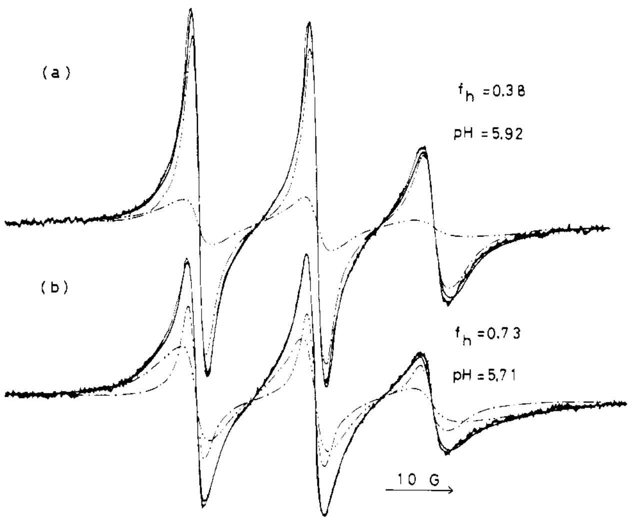

Figure 9. The synthesized ($S_{syn}(f)$) and observed ($S_{h-c}{}^{obsd}$) ESR spectra at (a) f_h = 0.38 and (b) f_h = 0.73 for poly(slGlu II) in the presence of 8 mM NaCl. $S_{syn}(f_h)$ is drawn with the smooth solid curve and $S_{h-c}{}^{obsd}$ is drawn with the noisy thick curve. The component spectra observed in the helical and coiled states are shown by (–···–) and (–··–), respectively.

Case B (Slow Interconversion). The observed ESR spectra seem to be of a single-component type in the helix–coil transition region, as shown in Figure 4. This does not necessarily indicate that the label undergoes a rapid interconversion. If the line widths of individual component spectra are not very different from one another, the envelope of a multicomponent spectrum cannot be distinguished from a single-component spectrum. This can be illustrated by synthesizing an ESR spectrum from only two-component spectra.[32] Under the assumption that the intensity of the ESR spectrum of poly(slGlu) observed at an intermediate pH ($S_{h-c}{}^{obsd}$) consists of two spectra of the spin label (one observed in the complete helix state ($S_h{}^{obsd}$) and the other observed in the complete coil state ($S_c{}^{obsd}$)), expression 14 is derivable, where k_h, k_c, and k_{h-c} are the

$$S_{h-c}{}^{obsd} = \left\{\frac{C_h}{C_t}\frac{S_h{}^{obsd}}{k_h} + \frac{C_c}{C_t}\frac{S_c{}^{obsd}}{k_c}\right\}k_{h-c} \qquad (14)$$

amplification factors of the signals at the respective pH values and are obtained by the double integration of the observed spectra and C_t is the sum of the concentrations of the spin labels in the helix (C_h) and coil (C_c) states. The synthesized spectrum [$S_{syn}(f_h)$] at the same pH may be expressed as

$$S_{syn}(f_h) = \left\{f_h\frac{S_h{}^{obsd}}{k_h} + (1 - f_h)\frac{S_c{}^{obsd}}{k_c}\right\}k_{h-c} \qquad (15)$$

where the f_h value estimated from the ORD data at this pH (Figure 2) is utilized.

The $S_{syn}(f_h)$ at f_h = 0.38 and f_h = 0.73 are compared with $S_{h-c}{}^{obsd}$ at the corresponding pH values in Figure 9. Although the all-or-none process is assumed, their agreement is excellent. In a like manner, the ESR spectra were synthesized at several f_h values with eq 15. From the line width of these synthesized spectra, the apparent values of $\tau(0)$ and $\tau(2)$ were calculated (cf. eq 6 and 7). The variation of $\tau(0)$ and $\tau(2)$ with f_h (solid curves) is very closely related to the dependence of the experimental values of poly(slGlu), as shown in Figure 10. These results indicate that the possibility that the spin label undergoes a slow interconversion with a rate constant much smaller than $T_2{}^{-1}$, which is approximately 10^8 s^{-1}, cannot be excluded.

Closing Remarks

The present ESR study has clarified how the conformational change of the poly(slGlu) backbone affects the rotational mobility of the nitroxide piperidine ring attached to the side-chain carboxyl group. The interdependence of the motions between the side chain and the backbone was shown by comparing ESR with ORD data. The piperidine ring of slGlu in the random-coil state rotates rapidly and anisotropically about an axis parallel to the N–O radical bond with an $R_\parallel$ of (5–7) × 10^8 s^{-1} ($\tau_\parallel$ in 0.2–0.3 ns) and about the axis perpendicular to this bond with an $R_\perp$ of ca. 1.5 × 10^8 s^{-1} ($\tau_\perp$ nearly 1.1 ns). The ring in the helix state undergoes an almost isotropic rotation

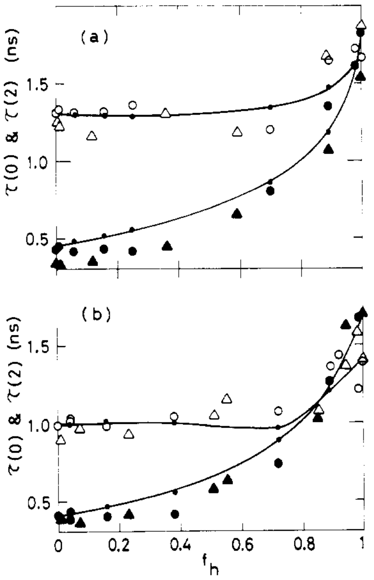

Figure 10. Variation of $\tau(0)$ and $\tau(2)$ with f_h for (a) poly(slGlu I) and (b) poly(slGlu II) in the presence of 8 (circles) and 150 mM (triangles) NaCl. Open symbols are for $\tau(0)$, while closed ones are for $\tau(2)$. The f_h dependence of $\tau(0)$ and $\tau(2)$ calculated from the synthesized spectra (Figure 9) as a measure of the line-width variation is shown by solid lines (–•–).

with $R_\parallel$ and $R_\perp$ of ca. 1×10^8 s^{-1} (τ_0 nearly 1.6 ns). This τ_0 was explained by assuming that the C$^\alpha$–C$^\beta$ and C$^\beta$–C$^\gamma$ bonds rotate with rotational diffusion coefficients of (0.1–0.8) × 10^8 and (0.5–1.0) × 10^8 s^{-1}, respectively, and that the terminal N–C^4 and C$^\gamma$–C$^\delta$ bonds both rotate with a diffusion coefficient of 1×10^8 s^{-1}. These results are obtained with the assumption of the free and independent rotation of the side chain. Detailed analysis with a restricted diffusion or jump model for a multiple internal rotation[2,33] may be needed in order to obtain more realistic information on dynamics of polypeptides with long side chains.

In the helix–coil region, an overall rotation of the piperidine ring becomes slow as extended helical segments are formed. Comparison with the ORD data clearly show that the change of the correlation time of the label is not linearly proportional to the helical fraction of the backbone. If the piperidine ring undergoes a rapid interconversion between helical, coiled, and intermediate states (its rate constant $\gg T_2^{-1}$), the formation of an extended helical segment is required for the side-chain rotation to be restricted by the backbone. However, simulation of the observed spectrum in terms of two component spectra of the helix and coil states equally suggests the alternative possibility that the spin label interconverts slowly between two conformational states. ESR study alone is difficult to interpret in such a way as to shed light on the kinetic aspect of the interconversion process.

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