838 © 1983 The Chemical Society of Japan Bull. Chem. Soc. Jpn., 56, 838—845 (1983) [Vol. 56, No. 3

Conformational Changes of the Poly(α-L-glutamic acid)–Cu(II) Macromolecular Complexes in the pH Range of 4—7. A Light Scattering Study with Emphasis on Aggregation and Helix-coil Transitions

Tsutomu Masujima[†]

Faculty of Science, Hiroshima University, Higashisenda-machi, Naka-ku, Hiroshima 730

(Received April 15, 1982)

The overall conformational changes of the macromolecular complex between poly(α-L-glutamic acid), poly(Glu), and Cu(II) in 0.15 M NaCl were investigated by means of the light scattering method at three mixing ratios of Glu residues to Cu^{2+}, R, (32, 16, and 8) in the pH range of 4—7. The root-mean-square radius of gyration and the weight-averaged molecular weight of poly(Glu) were determined by means of the Zimm plot, and those pH dependences show that the poly(Glu) shrinks in the course of the coil-to-helix transition and that it forms aggregates at pH values lower than 4.7, where helix formation is almost completed. In the presence of Cu^{2+} ions, the bound Cu(II) accelerates the shrinking of the poly(Glu) conformation and induces the aggregation in a high pH region, where the helix is imperfect. With the aid of theoretical treatments in the text, the aggregation scheme in the helix region was disclosed to be side-by-side, in which a 70—95% length of a helix overlaps one another. All these results and the pH dependence of the radius of gyration per poly(Glu) molecule were discussed in relation to the complex formation between Cu(II) and poly(Glu).

The poly(α-L-glutamic acid), poly(Glu), has been used extensively as a model substance of the proteins. Thus, the systems including both poly(Glu) and the transition-metal ions are interesting as models of the metallo-proteins or of the materials of the functional metal-polymer complex, and so they have been studied extensively.[1–19] In these transition-metal ions, the Cu(II) was shown to be rather unique in the induction of the conformational changes in poly(Glu),[2,11,14,16,17,19] the strength of the binding,[13,15–17] and the complicated complex formations.[12–18] Our recent work has clarified that three classes of complexes predominate either in the helix or extended-coil region, in the helix-coil intermediate transition region, or in the low-pH region, where the helix formation has already been completed.[15] The characteristic formation of the complex should be correlated with the conformational changes of poly(Glu). Since no definite conformation, however, was observed in the helix-coil intermediate region, a study of the conformational changes from other points of veiw is needed. One important point of view is that of the overall conformations of poly(Glu). The light scattering method is useful for this purpose, because it can discriminate the aggregation and the change in the scale of poly(Glu) by the use of the weight-averaged molecular weight, M_w, and the z-averaged root-mean-square radius of gyration, $\langle s^2\rangle_z^{1/2}$, of poly(Glu) respectively.

Aqueous solutions of poly(Glu) or poly(Glu)–Cu(II) have been studied slightly by means of the light scattering method.[18,20,21] These studies have, though, been related to the aggregation[18,20] or have been only of the random coil of poly(Glu).[21] In the present study, poly(Glu)–Cu(II) macromolecular complexes at various pHs and various mixing ratios of Glu residues to Cu^{2+}, R, in 0.15 M (1 M=1 mol dm^{-3}) NaCl were investigated. With the observed values of M_w and $\langle s^2\rangle_z^{1/2}$, the pH dependence of the overall conformational changes in poly(Glu) and the effect of Cu(II) binding on this changes were determined from the points of view of aggregation and the compactness of the poly(Glu) molecule. Furthermore, with the aid of some theoretical treatments developed (Theoretical), the equilibrium and the scheme (*e.g.*, side-by-side or head-to-tail) of aggregation, and the participation of H^+ ions in the aggregation were analyzed. Finally, the pH dependence of the extension per poly(Glu) molecule in the presence of Cu(II) was discussed in relation to the characteristic complex formations between poly(Glu) and Cu(II).

Experimental

Materials and Preparations of Poly(Glu) and Poly(Glu)–Cu(II) Solutions. Sodium poly(Glu), hereafter denoted simply as poly(Glu), was the same sample as has been described elsewhere.[11] The other chemicals were all of a reagent grade and were used without further purification. The preparation of the poly(Glu) and poly(Glu)–Cu(II) solutions prior to dialysis was the same as has been described previously.[15] The mixing ratio, R, defined as the molar residue concentration of poly(Glu) to the total concentration of Cu^{2+} ions, was adjusted by changing the amount of Cu^{2+} ions. Freshly redistilled, dust-free water was used for all preparations of solutions. The sample solutions were dialyzed against 2 l of a 0.15 M NaCl solution for 48 h. For a Cu^{2+} containing sample, an adequate amount of Cu^{2+} ions was also added to the outer-side solution prior to dialysis in order to diminish the change in the R of the inner-side solution in the course of dialysis. In this case, the concentration of the unbound Cu^{2+} was calculated on the basis of the data of equilibrium dialysis.[15] For the pH adjustment of a sample at a low pH (<4.5), H^+ ions were added indirectly from the outer-side solution in order to prevent the precipitation of poly(Glu). The dialyzed sample (inner side) was diluted with the outer-side dialysis solution when the concentration dependence of light scattering and the refractive index were measured. All the sample solutions were clarified by a careful and slow flow rate (1—3 ml/min) filtration[22] through a pair of 23 mmϕ Fuji micro filters FM-22 (0.22 μm pore) (Fuji Photo Film Co., Ltd., Japan), which adsorbed slight proteins (less than 1 $\mu g/cm^2$). The concentrations of poly(Glu) in all the samples after the light-scattering and refractive-index measurements were deter-

---

[†] Present address: Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine, Kasumi 1-2-3, Minami-ku, Hiroshima 734.

mined by means of the micro-Biuret method with a precision of ±1% or less.[23] The value of 151.1 g/mol was used as the Glu residue mass throughout the experiments.

Light Scattering and Differential Refractive Index Measurements. The light scattering (L.S.) photometer was a Shimadzu type PG-21 apparatus, with a Takeda Riken digital multimeter, type TR-6855. The incident-light beam of the 436 nm wavelength of the Hg lamp was used. The cross section of the beam was 4×12 mm^2. The calibration of the instrument was achieved by the use of the freshly distilled benzene and standard polyethylene oxide of molecular weights of 8×10^4 and 15×10^4 (Toyo Soda SE-8 and SE-15 respectively; Toyo Soda Co., Ltd., Japan). The reference value of the R_{90} of benzene was 46.5×10^{-6} cm^{-1} (25 °C).[24] The intensities of the vertical and horizontal components of the scattered light at a given angle, θ, with a vertically polarized incident beam, $V_v(\theta)$ and $H_v(\theta)$ respectively, were calculated by means of the scattered-light intensities (without an analysing polarizer) with vertically (θ=30—135°), $U_v(\theta)$, and horizontally (90° only), $U_h(90)$, polarized incident beams as follows:[25,26]

$$V_v(\theta) = U_v(\theta) - H_v(\theta) \tag{1}$$

$$H_v(\theta) = U_h(90)/2. \tag{2}$$

Tomimatsu's method was followed for the correction of the back reflection of the cylindrical cell (38 mmϕ) (in a water jacket).[27] All L.S. measurements were carried out at 25±0.1 °C. The contributions of Cu^{2+} ions and the optical absorption of poly(Glu)-Cu(II) solutions ($\varepsilon \simeq 20$ at most at 436 nm) to the scattered-light intensity were negligible.[28]

The differential refractive index, dn/dc, of a sample solution at 436 nm and at 25±0.1 °C was determined by the use of a Shimadzu differential refractometer (the Debye type) which has been modified in some respects. The method of detecting the slit image was changed to that with the photomultiplier. The Debye cell was capped to prevent the evaporation of the solvent, and the optical bench below the photomultiplier box was thermostated. In the measurements of both L.S. and dn/dc, the outer-side dialysis solution was used as a blank solution. The pH variation in the sample solution in the course of dilution was less than ±0.02 at pH≤5.5 and less than ±0.04 at pH>5.5.

The pH values of the sample solutions were determined on a Hitachi-Horiba Model N-5 pH meter after the L.S. and dn/dc measurements.

Theoretical

General Aggregation Models and the Variation in M_w. Two typical models can be assumed, called; a) open association;

$$\begin{aligned} M_1 + M_1 &\overset{K_1}{\rightleftharpoons} M_2 \\ M_2 + M_1 &\overset{K_2}{\rightleftharpoons} M_3 \\ &\cdots\cdots \\ M_{n-1} + M_1 &\overset{K_{n-1}}{\rightleftharpoons} M_n \\ &\cdots\cdots \end{aligned} \tag{3}$$

and b) closed association;

$$nM_1 \overset{K'}{\rightleftharpoons} M_n, \tag{4}$$

where M_n is the n-mer of poly(Glu) and where K or K' is the equilibrium constant of association.[29] Since the precipitation of poly(Glu) is observed at pH values lower than 3.5, the aggregation process of poly(Glu) molecules seems to be of the former type, in which the value of n increases indefinitely. Since no definite correlating function has been found among the K values, all the steps in the open association are assumed to be equivalent, *i.e.*, identical K values, for a mathematically rigorous model;[29]

$$K_1 = K_2 = \cdots = K_n = \cdots = K. \tag{5}$$

This model implies an independence of the equilibrium constants from the degree of association.[29] Thus,

$$(K_1\cdot K_2\cdot \cdots \cdot K_{n-1} =)K^{n-1} = \frac{\gamma_n[M_n]}{(\gamma_1[M_1])^n}, \tag{6}$$

where γ_n is the activity coefficient of n-mer. As Ogston and Winzor showed in their theoretical study, $\gamma_n/\gamma_1{}^n$ in Eq. 6 can be regarded as 1 because of the self-cancellation of the activity coefficients in the equilibrium expression for the association of charged spherical or rod-shaped polymers.[30] In this case, Eq. 6 can be treated simply as;[31]

$$[M_n] = K^{n-1}[M_1]^n = [M_1]Y^{n-1}, \tag{7}$$

where

$$Y = K[M_1]. \tag{8}$$

The variation in $[M_n]/[M_1]$ with n at various parameters of Y is shown in Fig. 1. The figure shows that the weight-averaged molecular weight can be determined by the use of Y. The total molar concentration of unimers, C_0, included in unimers and multimers is;

$$C_0 = [M_1] + 2[M_2] + \cdots + n[M_n] + \cdots. \tag{9}$$

By combining Eqs. 7 and 9, we obtain;

$$C_0 = \frac{[M_1]}{(1-K[M_1])^2} = \frac{[M_1]}{(1-Y)^2}, \tag{10}$$

when $|Y|<1$. The weight-averaged molecular weight, M_w, which can be determined experimentally is;

$$M_w = \frac{M_1^2[M_1]\sum n^2(K[M_1])^{n-1}}{M_1C_0}, \tag{11}$$

when combined with Eq. 10, Eq. 11 was reduced;[29]

$$\frac{M_w}{M_1} = \frac{1+K[M_1]}{1-K[M_1]} = \frac{1+Y}{1-Y}. \tag{12}$$

These equations show that the observed values of M_w

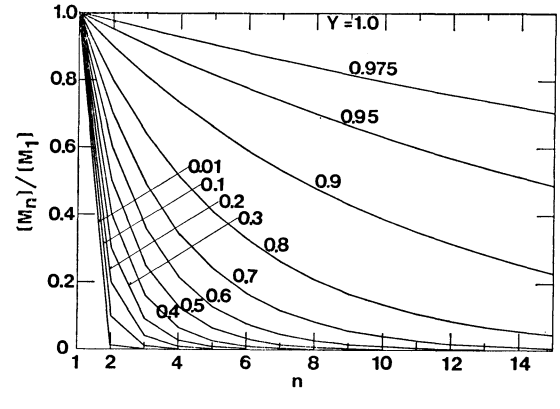

Fig. 1. The distribution curve of the molar concentration ratio of n-mer to unimer *vs.* n in the aggregated system at various assumed values of $Y(=K[M_1])$.

and M_1 give the value of Y by means of Eq. 12; then, with the values of Y and C_0, the values of $[\mathrm{M}_1]$ and subsequently K can be determined by the use of Eqs. 8 and 10.

pH-Dependent Aggregation Models and the Variation in M_w. As will be shown later, the aggregation of poly(Glu) depends on the pH. This phenomenon shows that the binding of H^+ ions contributes to the aggregation. If the binding of $m\mathrm{H}^+$ ions advances the one step of the open association, the following association process can be assumed as a first approximation;

$$\begin{array}{l} \mathrm{M}_1' + \mathrm{M}_1' + m\mathrm{H}^+ \xrightleftharpoons{K''} \mathrm{M}_2' \\ \mathrm{M}_2' + \mathrm{M}_1' + m\mathrm{H}^+ \xrightleftharpoons{K''} \mathrm{M}_3' \\ \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots \\ \mathrm{M}_{n-1}' + \mathrm{M}_1' + m\mathrm{H}^+ \xrightleftharpoons{K''} \mathrm{M}_n' \\ \cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots. \end{array} \tag{13}$$

Then, just as in the preceding section,

$$[\mathrm{M}_n'] = K''^{n-1}[\mathrm{M}_1']^n((\gamma_{\mathrm{H}^+}[\mathrm{H}^+])^m)^{n-1}, \tag{14}$$

where γ_{H^+} is the activity coefficient of the H^+ ion. In this case, the following treatment is the same as in the previous open-association model when we regard Y as;

$$Y = K''[\mathrm{M}_1'](\gamma_{\mathrm{H}^+}[\mathrm{H}^+])^m(=Y_\mathrm{H}). \tag{15}$$

By combining Eqs. 14 and 10, we obtain;

$$\frac{Y_\mathrm{H}}{(1-Y_\mathrm{H})^2} = C_0K''(\gamma_{\mathrm{H}^+}[\mathrm{H}^+])^m. \tag{16}$$

On electrochemical detection, $\mathrm{pH} = -\log(\gamma_{\mathrm{H}^+}[\mathrm{H}^+])$. Thus,

$$\log\left(\frac{Y_\mathrm{H}}{(1-Y_\mathrm{H})^2}\right) = \log(C_0K'') - m\cdot\mathrm{pH}. \tag{17}$$

When we plot the pH dependence of the $\log(Y_\mathrm{H}/(1-Y_\mathrm{H})^2)$, which can be determined by the data of M_w and M_1, the straight line gives the value of m from the slope and that of K'' from the intercept. When the curved plot is obtained, this model is unsuitable. Both K'' and m can be determined by the use of the two sets of data of M_w and pH by Eq. 17, but they should be nominal values in the curved plot.

Variation in the Mean-square Radius of Gyration in the Aggregate System. The variation in the mean-square radius of gyration, $\langle s^2\rangle_\mathrm{z}$, with aggregation is more complicated than that of the M_w, because the variation in $\langle s^2\rangle_\mathrm{z}$ is affected by the conformations of the polymer and $\langle s^2\rangle$ is z-averaged. In general,

$$\langle s^2\rangle_\mathrm{z} = \frac{\sum n^2M_1{}^2Y^{n-1}\langle s_n^2\rangle}{\sum n^2M_1{}^2Y^{n-1}}, \tag{18}$$

where the open association is assumed and $\langle s_n^2\rangle$ is the mean-square radius of the gyration of the n-mer with the molecular weight of M_n. In the following, the conformation of the polymer should be assumed prior to the analysis.

1) Aggregation of Helices. In the association of helices, when the σ part (in length) of a helix overlaps with the unimer or multimer of the helix in a side-by-side or head-to-tail scheme, the length of the multimer, L, can be related with that of the unimer helix, L_1, by;

$$L = L_1(1+(n-1)(1-\sigma)). \tag{19}$$

Since the $\langle s_1^2\rangle$ of a helix equals $L_1^2/12$,[32] the $\langle s^2\rangle$ of aggregated n-helices, $\langle s_n^2\rangle$, is;

$$\langle s_n^2\rangle = \frac{L_1^2(1+(n-1)(1-\sigma))^2}{12} \tag{20}$$

$$= \langle s_1^2\rangle(1+(n-1)(1-\sigma))^2. \tag{21}$$

At $\sigma=0$, the head-to-tail aggregation scheme is assumed; on the other hand, at $\sigma=1$, the perfect side-by-side scheme is assumed. By combining Eqs. 18 and 21, we obtain;

$$\frac{\langle s^2\rangle_\mathrm{z}}{\langle s_1^2\rangle} = \frac{\sum n^2Y^{n-1}(1+(n-1)(1-\sigma))^2}{\sum n^2Y^{n-1}}. \tag{22}$$

For the various σ values, the $(\langle s^2\rangle_\mathrm{z}/\langle s_1^2\rangle)^{1/2}$ was calculated; it is plotted against Y and M_w/M_1 in Fig. 2 (a and b), which shows that the relation between $(\langle s^2\rangle_\mathrm{z}/\langle s_1^2\rangle)^{1/2}$ and M_w/M_1 is almost linear.

2) Aggregation in the Helix-Coil Transition Region. Since the conformation of poly(Glu) changes in the helix-coil region, a general estimation of $\langle s^2\rangle_\mathrm{z}$ is needed.

$$\langle s^2\rangle = \mathrm{const}\cdot M^q, \tag{23}$$

where $q=2/3$, 1, and 2 for spheres, a Gaussian coil, and a thin rod respectively;[31] thus,

$$\frac{\langle s_n^2\rangle}{\langle s_1^2\rangle} = \left(\frac{M_n}{M_1}\right)^q(=n^q). \tag{24}$$

By combining Eqs. 18 and 24, we obtain;

$$\frac{\langle s^2\rangle_\mathrm{z}}{\langle s_1^2\rangle} = \frac{\sum n^{2+q}Y^{n-1}}{\sum n^2Y^{n-1}}. \tag{25}$$

In Fig. 3, the value of $(\langle s^2\rangle_\mathrm{z}/\langle s_1^2\rangle)^{1/2}$ is plotted against M_w/M_1 at the various q values assumed. In this case, it should be noted that there is an implicit assumption that $\langle s_n^2\rangle$ increases additively with n, just like the increment in the $\langle s^2\rangle$ of the unimers at various molecular-weights. When the association and the conformational changes occur simultaneously, the $(\langle s^2\rangle_\mathrm{z}/$

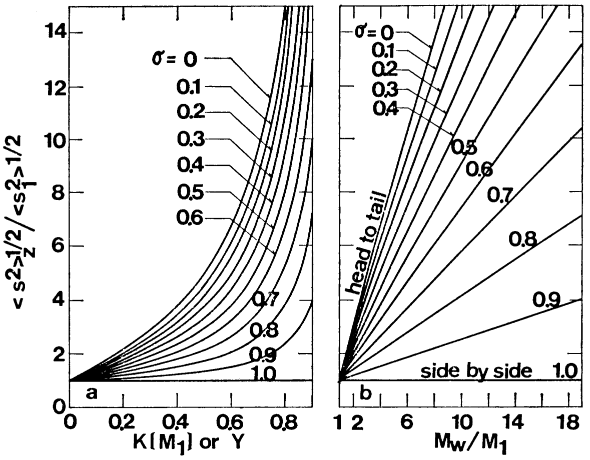

Fig. 2. The dependence of $\langle s^2\rangle_\mathrm{z}^{1/2}/\langle s_1^2\rangle^{1/2}$ on (a) Y $(=K[\mathrm{M}_1])$, and (b) M_w/M_1, at various overlapping parameters of σ, when the aggregation of helices is assumed to be the scheme of Eq. 22. The summation was carried out from $n=1$ to 100.

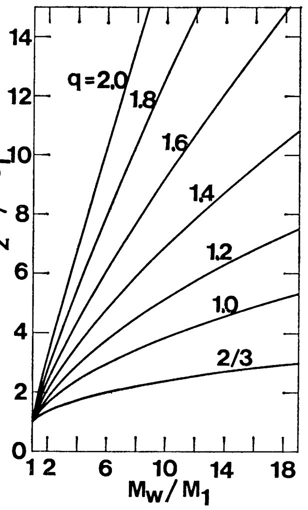

Fig. 3. The dependence of $\langle s^2\rangle_z^{1/2}/\langle s_1^2\rangle^{1/2}$ on M_w/M_1 at various conformational parameters of q, when the poly(Glu) is assumed to aggregate in the helix-coil transition region according to the scheme of Eq. 25. The summation was carried out from $n=1$ to 100.

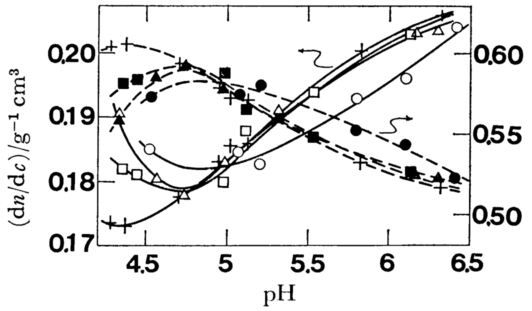

Fig. 4. The pH dependence of dn/dc (solid lines) and the partial specific volume (dashed lines) of poly(Glu) in 0.15 M NaCl solutions at various R of ∞ (poly(Glu) only) (crosses), 32 (squares), 16 (triangles), and 8 (circles).

$\langle s_1^2\rangle)^{1/2}$ *vs.* M_w/M_1 curves will be very complicated.

Results and Discussion

pH Dependence of d*n*/d*c and the Partial Specific Volume.* Figure 4 shows the pH dependence of dn/dc and the partial specific volume, $\bar{v}$, of poly(Glu) in a 0.15 M NaCl solution at various R values. The value of $\bar{v}$ was calculated by means of Heller's equation using dn/dc;[33)]

$$\bar{v} = 1.069 - 2.679\ (dn/dc), \tag{26}$$

as will be detailed in a subsequent paper.[19)] The increment in $\bar{v}$ with the helix formation was interpreted as showing voids formed in the helix conformation and/or a change in the hydration of water accompanied by the transition.[34)] In every case of R, almost all (more than 95%) the Cu^{2+} ions bind with poly(Glu) at pH values higher than 5, and then dissociate with a lowering of the pH.[15)] The binding of the Cu^{2+} ion instead of two Na^+ ions should increase the n value; however, the value of dn/dc in the presence of Cu^{2+} ions decreases at a low R. Thus the decrease in dn/dc (increment of $\bar{v}$) in pH 5—6.5 at $R=8$ should be due to the helix formation of poly(Glu) induced by Cu(II).[15)] The decrease in $\bar{v}$ at a low pH may show the exclusion of hydrated water on the aggregate formation. With the value of dn/dc and the Rayleigh ratio, the M_w and $\langle s^2\rangle_z^{1/2}$ of poly(Glu) were determined according to Zimm's method.[35)]

Zimm Plots of Poly(Glu) at Various R and pH Values. The Rayleigh ratio, which is corrected for the optical anisotropy, $R'(\theta)$, was determined as follows:[25,26)]

$$R'(\theta) = R_{V_v}(\theta) - (4/3)R_{V_h}(\theta). \tag{27}$$

Then,

$$K^*c/R'(\theta) = (1/M_w)(1+(16\pi^2/3\lambda^2)\langle s^2\rangle_z \times \sin^2(\theta/2))+2A_2c, \tag{28}$$

where K^* is the optical constant, c is the concentration of poly(Glu) (g/ml), λ is the wavelength of the incident beam (cm), and A_2 is the second virial coefficient.

Figure 5 shows the Zimm plots of poly(Glu) solutions at various R values and at pH 6.1—6.3. The almost linear plots seem to show that the molecular-weight distribution of the sample is rather narrow,[32)] which is consistent with the results of electric birefringence ($M_w/M_n=1.13$).[36)] The values of M_w obtained were 1.06×10^5, 1.22×10^5, and 1.79×10^5, the $\langle s^2\rangle^{1/2}$ values were 290, 250, and 210 Å, and the $A_2\times10^3$ values were 2.9, 1.5, and 0.93 at $R=\infty$, 16, and 8 respectively. The increment of M_w with the addition of Cu(II) shows the participation of Cu(II) in the formation of the aggregate at a high pH, while the decrease in $\langle s^2\rangle_z^{1/2}$ shows that the bound Cu(II) induces the shrinking of the poly(Glu) conformation.

Figure 6 shows the Zimm plot for poly(Glu) at various R values and at low pH (4.28—4.52), where the formation of the helix is completed.[15)] The curvilinear plots should show that the molecular-weight distribution is dispersive or that the unimer and multimer of poly(Glu) coexist under these conditions.[32)] However, the negative A_2 which is frequently seen in the aggregate systems was not observed, and the plot was almost linear in the experimented-on concentration

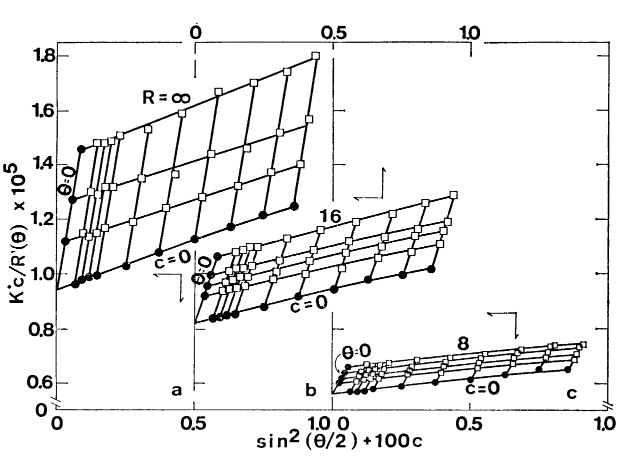

Fig. 5. The Zimm plots for poly(Glu) in 0.15 M NaCl solutions at high pH regions in (a) the absence ($R=\infty$) (pH 6.32) and (b,c) the presence of Cu^{2+} ions ((b) $R=16$ (pH 6.16) and (c) 8 (pH 6.09)).

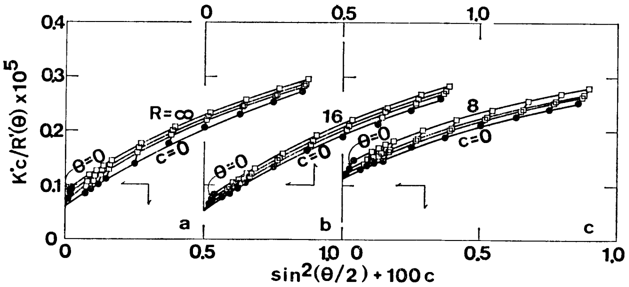

Fig. 6. The Zimm plots for poly(Glu) in 0.15 M NaCl solutions at low pH regions in (a) the absence (pH 4.28) and (b,c) the presence of Cu^{2+} ions ((b) $R=16$ (pH 4.34), and (c) 8 (pH 4.52)).

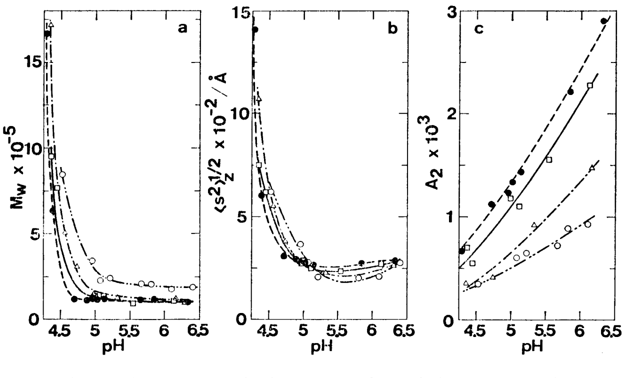

Fig. 7. The pH variation of (a) weight-averaged molecular weight, M_w, (b) z-averaged root-mean square of radius of gyration, $\langle s^2\rangle_z^{1/2}$, and (c) second virial coefficient, A_2, of poly(Glu) in the absence (--●--) and the presence ($R=32$ (—□—), 16 (—·△—·), and 8 (—··○—··)) of Cu^{2+} ions.

range. This may be mainly due to the large equilibrium constant of association[29] (noted later) of this system, in which the associated polymers hardly dissociate from each other with the decrease in the concentration. The value of A_2 was then determined by the slope of the linear region of the $\theta=0$ curve from the high concentration in the Zimm plot. The obtained values of M_w were 1.67×10^6, 1.72×10^6, and 8.47×10^5, while those of $\langle s^2\rangle_z^{1/2}$ were 1410, 1070, and 621 Å and those of $A_2\times10^3$ were 0.67, 0.36, and 0.35, at $R=\infty$, 16, and 8 respectively. In this pH range, the increment of M_w at any R shows that the aggregation of helices is induced by bound H^+ and Cu(II) ions.

pH Dependence of M_w, $\langle s^2\rangle_z^{1/2}$, and A_2. The values of M_w, $\langle s^2\rangle_z^{1/2}$, and A_2 thus determined at various R and pH values are plotted against the pH in Fig. 7. When the A_2, was not determined, the values of M_w and $\langle s^2\rangle_z^{1/2}$ were determined according to Eq. 27 with the light scattering data at one concentration and the interpolated A_2 value. Figure 7(a) shows the M_w *vs.* pH curves. The value of M_w is almost constant at pH values higher than 5.5 and at $R=\infty$ and 32. The M_w data in this region were averaged to 1.07×10^5, which was assumed to be the M_w of the unimer of poly(Glu), *i.e.*, M_1. The increment in M_w from this value means the aggregate formation. In the absence of Cu(II), the aggregation is formed rather drastically at pH values lower than 4.7, and the value of M_w increases to 1.67×10^6 at pH 4.28. With the addition of Cu(II) in a poly(Glu) solution, the starting pH of aggregate formation shifted to the high pH range at about 5, 6, and higher than 6.5 (not determined) at $R=32$, 16, and 8 respectively. The bound Cu(II) induces the aggregate formation of poly(Glu) at high pH values where the helix formation is imperfect. The M_w *vs.* pH curves at various R values seem finally to overlap with each other at some low pH value, because the Cu(II) dissociates from poly(Glu) with a lowering of the pH.[15]

Parallel with the M_w change, the variation in $\langle s^2\rangle_z^{1/2}$ with the pH is shown in Fig. 7(b). At $R=\infty$, the value of $\langle s^2\rangle_z^{1/2}$ decreases in the course of the transition from random coil to helix and then increases with the formation of helix and aggregation. In the presence of Cu^{2+} ions, $\langle s^2\rangle_z^{1/2}$ further decreases in the intermediate helix-coil transition region. The bound Cu(II) seems to induce the shrinking of the poly(Glu) conformation in the helix-coil region. In the low pH range, the value of $\langle s^2\rangle_z^{1/2}$ increases with further aggregate formation. The difference between the pH dependence of M_w and $\langle s^2\rangle_z^{1/2}$ at low pH values (in Figs. 7(a) and (b)) will be discussed later in relation to the aggregation scheme.

For the sample which has been dialyzed in equilibrium, the expression for A_2 is;

$$A_2 = \frac{10^3}{2M_p^2}\left(\frac{Z^{*2}}{2m'} + \frac{\partial \ln \gamma_p^*}{\partial c}\right), \tag{29}$$

where Z^* is the number of charges on the polymer, γ_p^* is the activity coefficient of the polymer, and m' is the concentration of the salt (mol dm^{-3}) at equilibrium.[37] Since the A_2 is identical for the unimer and the multimer,[38,39] M_p in Eq. 29 can be regarded as M_1. When we assume that the first Donnan term in Eq. 29 mainly contributes to the A_2 value, the number of effective charges on a poly(Glu) unimer can be estimated to be 140—69, 130—61, 100—47, and 80—49, at $R=\infty(\alpha\cdot=0.94$—$0.24)$, 32 $(\alpha\cdot=0.80$—$0.21)$, 16 $(\alpha\cdot=0.75$—$0.15)$, and 8 $(\alpha\cdot=0.73$—$0.15)$ respectively, where $\alpha\cdot$ is the degree of dissociation of Glu residues unbound by H^+ and Cu^{2+}.[15] The values of Z^* show a tendency to decrease with a lowering of the pH and are parallel with the $\alpha\cdot$. However, the Z^* values are 2 to 5 times smaller than the number of charges on a polymer, which were estimated by the degree of dissociation. This should show qualitatively that the charge-suppression effect by the salt-ion binding is included in the A_2 value and that the decrease in A_2 in Fig. 7(c) with a lowering of the pH and R should be a charge-neutralization effect caused by the binding of the Cu^{2+} and H^+ ions. However, the second term of Eq. 29 could not be determined by this study. Since it has been reported that the activity coefficient of poly(Glu) increases with the polymer concentration (positive second term in Eq. 29),[40] the real values of Z^* should be smaller than the values noted above.

The Equilibrium of the Aggregate Formation of Poly(Glu). With the data shown above, a more detailed discussion of the equilibrium of aggregation and its scheme can be given in combination with the theoretical treatment.

Since the M_w varies depending on the pH, Eq. 13 can be adopted for the analysis of the aggregation equilibrium. Since only two data of M_w at $R=\infty$ were available in the aggregation region, two simultaneous equations according to Eq. 17 were solved to determine the values of m and K''. The data of M_w at pH 4.28 and 4.38 give the number of H^+ ions, *i.e.*, m, to be 18.8 which should take part in the one-step association. With the aid of the value of C_0 ($=1.97\times10^{-6}$ mol of poly(Glu) unimer/dm^3), the log K'' can be estimated to be 87.9. This K'' value may be quite large but still reasonable, because the term of $((\gamma_{H^+}[H^+])^m)^{-1}$ contributes to this value, as may be seen in Eqs. 15 and 16. Since more than 62% of the Glu residues are discharged by H^+ binding at this pH,[15] the number of H^+ unbound Glu residues resting on a helix is less than 269 ($=(1-0.62)\times107000/151.1$). Thus, more than 3.5% ($=(18.8/2/269)\times100$) of the H^+ unbound Glu residues on a helix should be discharged by H^+ for the advance of the one-step association at pH 4.28—4.38. The contribution of Cu^{2+} ions to the aggregation, however, can not be estimated by this analysis, because the competitive binding between H^+ and Cu^{2+} ions to poly(Glu)[15] makes this analysis complicated. The attractive forces for the association of poly(Glu) may be hydrogen bonding between protonized carboxyl groups as in polymethacrylate,[41] intermolecular chelation by Cu(II), and hydrophobic interaction.

Aggregation Scheme, Side-by-side or Head-to-tail. Such an induction of aggregation by H^+ and Cu(II) binding suggests that helices associate in a side-by-side scheme, judging from the binding region of H^+ and Cu(II) on a helix. In order to determine whether the aggregation scheme is side-by-side or head-to-tail, $\langle s^2\rangle_z^{1/2}$ and M_w are correlated and the plots are fitted to the theoretical curves as shown in Fig. 8. Prior to this analysis, $\langle s_1^2\rangle^{1/2}$ should be assumed. The results of the viscosity study showed that the helix of poly(Glu) in 0.15 M NaCl is a slightly fat α-helix, with its long axis 640—1200 Å long (minor axis 10—18 Å),[19] in which the length of the α-helix of 1063 Å is included. In Fig. 8, the plots of $\langle s^2\rangle_z^{1/2}/\langle s_1^2\rangle^{1/2}$ *vs.* M_w/M_1, which were determined by means of the data at a helical content over 90%, are dispersed around the theoretical curves at $\sigma=0.7$—0.8 and $\sigma=0.9$—0.95, depending on the assumed helix types, with long axes of 640 (the upper group of points) and 1200 Å (the lower group of points) respectively, but irrespective of R. This shows that each helix in the multimer overlaps to each other in side-by-side, attached with a 70—95% length of a helix. This means that almost all of the bound Cu(II) is surrounded by helices of poly(Glu) in this case. Thus, it can form the characteristic complex with poly(Glu) ligands, as will be discussed later.

On the other hand, it is difficult to analyze the aggregation scheme in the helix-coil transition region with Eq. 24, because the conformation of the poly(Glu) unimer is not identical; it changes from a helix to not only a rather extended coil, but also to a compact coil, especially in the presence of Cu^{2+} ions, as has been shown above or in an subsequent viscosity study.[19]

However, in the helix-coil region, the increase in M_w is only 2—3 fold at most. Thus, the curve of $\langle s^2\rangle_z^{1/2}/\langle s_1^2\rangle^{1/2}$ *vs.* M_w/M_1 can be regarded as almost linear in the M_w/M_1 range of 1—3 as may be seen in Fig. 3. Since the curves of $\langle s^2\rangle_z^{1/2}/\langle s_1^2\rangle^{1/2}$ *vs.* M_w/M_1 for the aggregation of helices are also linear, as is shown in Fig. 2, the $\langle s^2\rangle_z^{1/2}/n$ value ($n=M_w/M_1$) was obtained; it should be a measure of the extension per poly(Glu) molecule.

The pH Dependence of $\langle s^2\rangle_z^{1/2}/n$ *and the Complex Formation.* Figure 9(a) shows the pH dependence of $\langle s^2\rangle_z^{1/2}/n$ at various values of R. In the absence of the Cu^{2+} ion and at around pH 5.5, poly(Glu) molecules shrink in the course of transition from a random coil to a helix, and at pH values lower than 4.7, they form the aggregate. Since the aggregate is of the side-by-side form, the $\langle s^2\rangle_z^{1/2}/n$ value does not increase additively with n; thus, $\langle s^2\rangle_z^{1/2}/n$ decreases with the low-

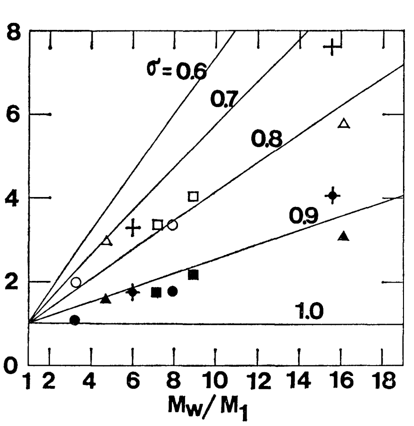

Fig. 8. The dependence of the observed data of $\langle s^2\rangle_z^{1/2}/\langle s_1^2\rangle^{1/2}$ on the values of M_w/M_1 of poly(Glu) at low pH region and at various R(∞ (crosses), 32 (squares), 16 (triangles), and 8 (circles)).
The $\langle s_1^2\rangle^{1/2}$ was assumed to be $640/\sqrt{12}$ Å (open symbols) or $1200/\sqrt{12}$ (filled symbols) Å. The solid lines are theoretical curves which were shown in Fig. 2(b).

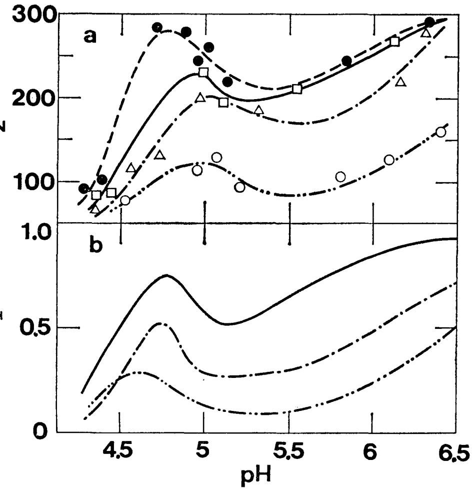

Fig. 9. The comparison of pH dependence of (a) root-mean-square radius of gyration divided by n, $\langle s^2\rangle_z^{1/2}/n$, and (b) the fraction of Complex I, f_I, from the Ref. 15: $R=\infty$ (--, ●), 32 (——, □), 16 (—·, △), and 8 (—··, ○).

ering of the pH because of one-sided increase in *n*. In the presence of Cu(II), $\langle s^2\rangle_z^{1/2}/n$ further decreases over the entire pH range. It seems that the bound Cu(II) induces the further shrinking of poly(Glu) and/or changes its conformation to a near-spherical type, whose model shows the small slope of the $\langle s^2\rangle_z^{1/2}/\langle s_1^2\rangle^{1/2}$ *vs.* *n* curve shown in Fig. 3.

It is interesting to compare the pH variation in $\langle s^2\rangle_z^{1/2}/n$ with that in the fraction of one of the three classes of poly(Glu)–Cu(II) complexes, *i.e.*, f_1 (the fraction of Complex I in Ref. 15), which was clarified by the spectroscopic method.[15] The curves in both figures are almost parallel to each other. In the region where f_1 decreases from 1.0 in Fig. 9(b), Complexes II and III are predominant instead of I in the pH ranges at around 5—5.5 and below 5 respectively.[15] This should show that Complex II appears when the poly(Glu) shrinks in the helix-coil intermediate transition region, while Complex III appears in parallel with the aggregate formation. The Cu(II) which is surrounded by the ligands of poly(Glu) in the intra- and inter-molecular fashions should form the chelates characteristic of its coordination field, *i.e.*, the species, number, and symmetry of the ligand field, like the complexes proposed in Ref. 15. The chelation is different from that of Complex I, in which Cu(II) seems to bind the extended coil or helix of poly(Glu). This interpretation is quite consistent with all our previously reported results.[11,13–15]

Conclusion

It was concluded that, in addition to the helix-coil transition, the conformation of poly(Glu) shrinks in the course of the helix-coil transition and forms an aggregate of helices in the low-pH range. The shrinking and the aggregate formation of poly(Glu) induce the chelation of Cu(II) to poly(Glu) residues in intra- and inter-molecular fashions, and *vice versa*. It is quite consistent that, in Complexes II and III, the Cu(II) binds to the side-chain carboxylate groups which are remote from, and independent of, each other on a single polymer strand or on different ones, as is shown by stoichiometric analysis.[15] Thus, this work further confirms our conclusions that the Cu(II) binds to the nearest-neighbor ligands on the extended coil or the helix portion of the polymer (Complex I), or that it binds to the ligands in the compact coil of poly(Glu)–Cu(II) (Complex II) while, in the low-pH range, Cu(II) binds to the ligands of the helical poly(Glu) in the multistranded aggregate (Complex III).[11,13–15]

The author would like to express his deep gratitude to Associate Professor Kiwamu Yamaoka of Hiroshima University for his helpful discussions and continuous encouragement in the course of this study.

A preliminary work (Light Scattering of Poly(Glu)–Cu(II) Solutions without Equilibrium Dialysis) was presented at the Annual Meeting of the Chemical Society of Japan in the Chugoku and Shikoku Areas (at Ube) in 1979.

References

1) A. L. Jacobson, *Biopolymers*, **1**, 269 (1963).
2) H. Takesada, H. Yamazaki, and A. Wada, *Biopolymers*, **4**, 713 (1966).
3) Y. Hibino and S. Sugai, *Rep. Prog. Polym. Phys. Jpn.*, **11**, 513 (1968).
4) H. Sigel and G. Blauer, *Helv. Chim. Acta*, **51**, 1246 (1968).
5) S. Yamashoji, H. Yoshida, and G. Kajimoto, *Yukagaku*, **25**, 128 (1976).
6) J. A. Marinsky, *Coord. Chem. Rev.*, **19**, 125 (1976).
7) O. Iwaki, K. Hikichi, M. Kaneko, S. Shimizu, and T. Maruyama, *Polym. J.*, **4**, 623 (1973).
8) M. Branca, M. E. Marini, and B. Pispisa, *Biopolymers*, **15**, 2219 (1976).
9) T. Hiraoki, A. Tsutsumi, K. Hikichi, and M. Kaneko, *Rep. Prog. Polym. Phys. Jpn.*, **19**, 527 (1976).
10) T. Hiraoki, M. Kaneko, and K. Hikichi, *Rep. Prog. Polym. Phys. Jpn.*, **21**, 525 (1978).
11) K. Yamaoka and T. Masujima, *Bull. Chem. Soc. Jpn.*, **52**, 1286 (1979).
12) T. Hiraoki, M. Kaneko, and K. Hikichi, *Polym. J.*, **11**, 397 (1979).
13) K. Yamaoka and T. Masujima, *Polym. J.*, **11**, 889 (1979).
14) S. Noji and K. Yamaoka, *Macromolecules*, **12**, 1110 (1979).
15) T. Masujima and K. Yamaoka, *Biopolymers*, **19**, 477 (1980).
16) N. Imai and J. A. Marinsky, *Macromolecules*, **13**, 275 (1980).
17) M. Koide and E. Tsuchida, *Makromol. Chem.*, **182**, 359 (1981).
18) M. Koide and E. Tsuchida, *Makromol. Chem.*, **182**, 367 (1981).
19) T. Masujima, K. Yamaoka, and J. Hori, *Bull. Chem. Soc. Jpn.*, **56**, (1983) in press.
20) Y. Tomimatsu, L. Vitello, and W. Gaffield, *Biopolymers*, **4**, 653 (1966).
21) R. B. Hawkins and A. Holzer, *Macromolecules*, **5**, 294, (1972).
22) W. H. Kulicke and R. Kniewske, *Macromol. Chem., Rapid Commun.*, **1**, 719 (1980).
23) R. F. Itzhaki and D. M. Gill, *Anal. Biochem.*, **9**, 401 (1964).
24) J. P. Kratohvil, G. Dezelic, M. Kerker, and E. Matijevic, *J. Polym. Sci.*, **57**, 59 (1962).
25) H. Utiyama and M. Kurata, *Bull. Inst. Chem. Res. Kyoto Univ.*, **42**, 128 (1964).
26) H. Utiyama, "Light Scattering From Polymer Solutions," ed by M. B. Huglin, Academic Press, London (1972), Chap. 4.
27) Y. Tomimatsu and K. J. Palmer, *J. Phys. Chem.*, **67**, 1720 (1963).
28) B. A. Brice, G. C. Nutting, and M. Halmer, *J. Am. Chem. Soc.*, **75**, 824 (1953).
29) H. G. Elias, "Light Scattering From Polymer Solutions," ed by M. B. Huglin, Academic Press, London (1972), Chap. 9.
30) A. G. Ogston and D. J. Winzor, *J. Phys. Chem.*, **79**, 2496 (1975).
31) E. T. Adams, Jr., *Biochemistry*, **4**, 1646 (1965).
32) P. Kratochvil, "Light Scattering From Polymer Solutions," ed by M. B. Huglin, Academic Press, London (1972), Chap. 7.
33) W. Heller, *J. Polym. Sci., Part A-2*, **4**, 209 (1966).

34) H. Noguchi and J. T. Yang, *Biopolymers*, **1**, 359 (1963).
35) B. H. Zimm, *J. Chem. Phys.*, **16**, 1099 (1948).
36) K. Yamaoka and K. Ueda, *J. Phys. Chem.*, **86**, 406 (1982).
37) M. Nakagaki and K. Ikeda, "Hikari Sanran Jikkenho," ed by M. Nakagaki and H. Inagaki, Nankodo, Tokyo (1965), Chap. 10.
38) H. Edelhoch, E. Katchalski, R. H. Mayburv, W. L. Hughes, Jr., and J. T. Edsall, *J. Am. Chem. Soc.*, **75**, 5058 (1953).
39) R. Townend and S. N. Timasheff, *J. Am. Chem. Soc.*, **82**, 3168 (1960).
40) M. Sugimura, T. Okubo, and N. Ise, *Polymer Preprints, Japan*, **31**, 654 (1982).
41) N. A. Kuznetsov, Z. A. Roganova, and A. L. Smolyanski, *Vysokomol. Soedin., Ser. A*, **20**, 791 (1978).