The Visible-UV Absorption and Circular Dichroism Studies of Poly(α-L-glutamic acid)–Copper(II) Complexes with Emphasis on the Extrinsic Cotton Effect†

Kiwamu Yamaoka* and Tsutomu Masujima

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

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The visible-UV absorption and circular dichroism, CD, of poly(α-L-glutamic acid)-Cu(II) solutions have been measured at 25 °C in the pH range 7 to 3.5 and at the mixing ratio, R, (Glu residues-to-Cu^{2+}) 32 to 4. Complex formation between poly(Glu) and Cu^{2+} was evident from the three absorption bands of bound Cu(II) around 700, 370, and 250 nm. The molar absorption coefficients, ε, varied with pH and R in a complicated but systematic manner. The pH dependence of ε, however, was not in parallel with the helix-coil transition of poly(Glu)–Cu(II) complexes. The extrinsic CD bands of bound Cu(II) have been detected in the three absorption band regions, and found to depend on both pH and R. The optical dissymmetry factor indicated that the 700 and 250 nm absorption bands of bound Cu(II) are associated with multiple CD bands of opposite signs. The intrinsic CD showed that the Cu(II)- and pH-induced helices of the poly(Glu)–Cu(II) complex are alike regarding the polymer backbone.

In order to understand the catalytic function of polypeptide–metal ion complexes, the detailed study of the binding of the transition metal ion to the poly (amino acid) appears to be indispensable. For this purpose, poly(α-L-glutamic acid), poly(Glu), and Cu^{2+} have been extensively utilized as a model system.[1–8] The conformation of the macromolecular complex, *i.e.*, the long-range or overall structure of poly(Glu) bound by Cu(II), and complex formation between Cu(II) and ligands, *i.e.*, the species of the Cu(II)–Glu residue complex, however, remain unclarified. Potentiometric titration is almost powerless from this viewpoint,[1] but optical spectroscopic studies should reveal changes in the configuration of Cu(II) bound by ligands and the conformational transition of poly(Glu) induced by bound Cu(II).

Optical absorption studies on complex formation between Cu(II) and simple polyelectrolytes such as polyacrylate (denoted as poly(Acr)) and polymethacrylate (denoted as poly(Methacr)) are numerous. In the presence of such polyanions, the absorption spectra of Cu(II) show three bands around 700, 360, and 250 nm.[9–15] The interaction between Cu(II) and poly(amino acids) has also been extensively studied, *i.e.*, poly(Glu),[2–4,8] poly(Lys),[16–18] poly(Arg),[19] poly(His),[20] poly(Gly),[21] and poly(Ala).[22,23] The circular dichroism, CD, method is valuable for studies of both complex formation and conformational transition, provided that the intrinsic CD of the chromophore of poly(Glu) and the extrinsic CD of the complex formed between the optically inactive metal ion and the active Glu residue appear separately but simultaneously in the respective wavelength regions. Hence, the conformational change of the macromolecular poly(Glu)–Cu(II) complex can be related to the formation of the ligand complex between Cu(II) and Glu residues. CD studies on the complex formation with Cu(II) have been reported for poly(Glu),[2] poly(Lys),[16–18] poly(Orn),[18] poly(Dbu),[18] poly(Arg),[19] poly(His),[20] poly(Ala),[23] and some Schiff-base derivatives of poly(Lys) and poly(Orn).[24]

In the present work, both the absorption and CD spectra of poly(Glu)–Cu(II) solutions have been studied by varying the pH between 7 and 3.5 and the mixing ratio (Glu residues to Cu^{2+}) between 32 and 4. The poly(Glu)–Cu(II) complex exhibited three absorption bands in the 700, 370, and 250 nm regions. The pH dependence of the intensity of each band has been found to be more complicated than previously reported.[2] The extrinsic CD of bound Cu(II) has been observed in all three absorption bands, again contrary to a previous report.[2] The pH dependence of the CD of Cu(II) is not, however, completely in parallel, although closely related, to the helix-coil transition of the macromolecular poly(Glu)–Cu(II) complex. The present absorption and CD data suggest that the variation in the absorption intensity of the bound Cu(II) with pH results not only from the change in amount and species of Cu(II)–Glu ligand complexes but also from the conformational change. Moreover, another conformational transition, possibly helix-to-*aggregate*, has been shown to exist for the poly(Glu)–Cu(II) complex at low pH where the helix is already completed. The present data are discussed in terms of the feedback of the poly(Glu) conformation to the complex formation between Glu ligands and Cu(II), and *vice versa*.

Experimental

Materials. Sodium poly(α-L-glutamate), abbreviated simply as poly(Glu), was kindly supplied from Ajinomoto Co., Ltd. (Tokyo) and purified by dialyzing against redistilled water for 24 h, freeze-drying, and vacuum-drying at 56 °C for 7 h. The weight-average degree of polymerization DP_w was determined to be *ca.* 700 from the intrinsic viscosity in 2 M NaCl solution. The stock poly(Glu) solution was prepared by weighing the anhydrous sample with a mean residue weight of 151. Sodium polyacrylate, abbreviated simply as poly(Acr), was purchased from Nakarai Chemicals Co. (Kyoto) and purified by fractional precipitation,[7] (DP_w 7700). In the CD study the poly(Glu) sample with a DP_w of 610, purchased from Pilot Chemicals, Inc., was exhaustively dialyzed against distilled water and freeze-dried. Reagent grade copper(II) chloride dihydrate was dissolved in distilled water prior to use.

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† Macromolecule–Metal Ion Complexes. IV. For the preceding papers, see Refs. 5—7.

Preparation of the Polymer-Cu(II) Solutions. Typically, a sample solution at the appropriate pH, mixing ratio, *R*, and ionic strength was prepared by adding appropriate amounts of NaCl, $CuCl_2$, and HCl solutions, in this order, to either poly(Glu) or poly(Acr) solutions. The value of *R*, defined as the ratio of the molar residue concentration of polymer to the total concentration of added Cu^{2+}, was varied by adjusting the amount of Cu^{2+}. The final concentration of the polymer residue was maintained constant at 8 mM throughout. The salt concentration in the polymer-Cu(II) solution was maintained constant at 7.5 mM in terms of the sum of HCl and NaCl by adjusting either of them. The effect of the mixing order was tested by interchanging the order of addition between $CuCl_2$ and HCl at room temperature. No difference was observed in the absorption and CD spectra, unless the pH of the solution was very low. However, the temperature effect on the absorption spectra of poly(Glu)-Cu(II) solutions was remarkable at low pH in that the temperature cycle between 4 and 70 °C showed irreversible changes. Therefore, the solutions were all prepared and stored at 25 °C.

Measurements of Absorption Spectra and pH. The absorption spectra were measured on a Hitachi Model EPS-3T recording spectrophotometer in the wavelength region 1200—210 nm at 25 °C, temperature-controlled water being circulated through the cell holder. The slit width was set at 0.18 mm at 700 nm. An integrated-sphere attachment was used to minimize the effect of scattered light from poly(Glu)-Cu(II) solutions at low pH (<3.8). The absorption was expressed in terms of the apparent molar absorption coefficient, ε ($cm^{-1}\cdot mol^{-1}\cdot dm^3$), of the total Cu^{2+} added. Matched pairs of quartz cells, 1—50 mm in length were used. The pH of the solutions used for absorption measurements was determined on a Hitachi-Horiba Model N-5 pH meter. A one minute wait preceded by two minutes' stirring was necessary before the reading of the pH, because of a slight time dependence.

Measurements of the CD Spectra and pH. The CD spectra of poly(Glu)-Cu(II) solutions were measured on a Cary Model 60 spectropolarimeter with a Model 6001 CD accessary at 25 °C. The visible and UV CD spectra at 750—240 nm have been expressed in terms of the molar ellipticity, $[\theta]$ ($deg\cdot cm^2\cdot dmol^{-1}$), of the total Cu^{2+} added, the far UV CD spectra at 250—185 nm being expressed in terms of $[\theta]$ of the mean residue concentration of poly(Glu). Quartz cells of 5 cm (700—300 nm), 0.5 and 0.2 cm (340—240 nm), and 0.05 cm (below 250 nm) have been used. The slit width was set manually at 0.3 mm between 750 and 600 nm (the dispersion being 15 nm at 750 nm and 8.5 nm at 600 nm). The slit width was automatically varied at a preset dispersion of 1.7 nm in the visible and UV regions, and was set at 2.4 mm in the far UV region. The pH of the solutions used for CD measurements was determined on either a Radiometer TTT-1 or a Corning Digital Research pH meter in a manner similar to above.

Results

Absorption Spectra of Cu^{2+} in the Presence of Poly(Glu). Figures 1(a—c) and 2(a—c) illustrate the absorption spectra of poly(Glu)-Cu(II) solutions at three values of *R*. In the presence of poly(Glu), three absorption maxima appear in the spectra of Cu^{2+}: a broad band (ε=40—150) around 700 nm, a weak band (ε=10—50) around 370 nm, and a strong band (ε=3000—5500) around 250 nm. The formation of the complex between poly(Glu) and Cu^{2+} is thus evident. The spectrum of poly(Glu) exhibits only an ascending lobe below 250 nm (Fig. 2(f)). The spectrum of $CuCl_2$ shows a weak and broad peak (ε=10—20) around 800 nm and an ascending lobe below 250 nm (Figs. 1(a) and 2(f)).

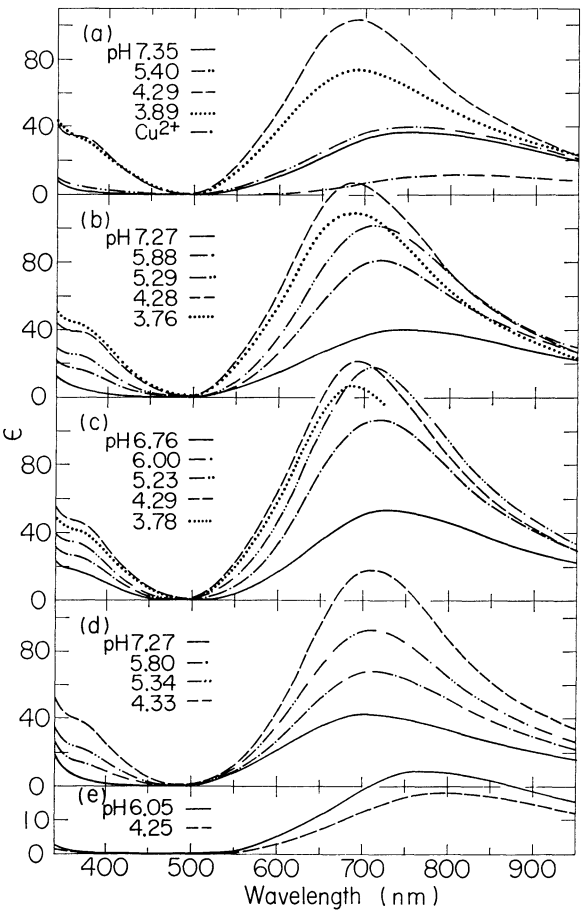

Fig. 1. The visible absorption spectra of Cu^{2+} in the presence of poly(Glu) ((a) $R=32$, (b) $R=16$, and (c) $R=8$), poly(Acr) ((d) $R=16$), and butyrate ((e) $R=16$) at various pH. The spectrum of Cu^{2+} at pH=3.81 in the absence of polymer is also shown in (a) for comparison. The pH=3.78 spectrum in (c) was taken with the integrated-sphere attachment which limits the wavelength to 800 nm. The concentration of added HCl plus NaCl is 7.5 mM in terms of the total Cl^- throughout. The molar absorption coefficient, ε, is expressed in terms of the total concentration of Cu^{2+} present in solution.

The 700, 370, and 250 nm bands, in the spectra of poly(Glu)-Cu(II) solutions at $R=32$ essentially remain unchanged in the pH range 7—5 but become most intense at pH 4.2—4.3 (Figs. 1(a) and 2(a)). In contrast, all the three bands of the $R=16$ and 8 solutions increase in intensity steadily with decreasing pH (Figs. 1(b—c) and 2(b—c)). The shape of the 700 nm band at each value of *R* is skewed, tailing towards the long wavelength side. The family of spectra showed no isosbestic point in any of the three absorption bands in the entire pH range 7—3.5.

Absorption Spectra of Cu^{2+} in the Presence of Poly(Acr) and Butyric Acid. The absorption spectra of Cu^{2+} were measured in the presence of either poly(Acr) (polymeric model lacking in the amide group),

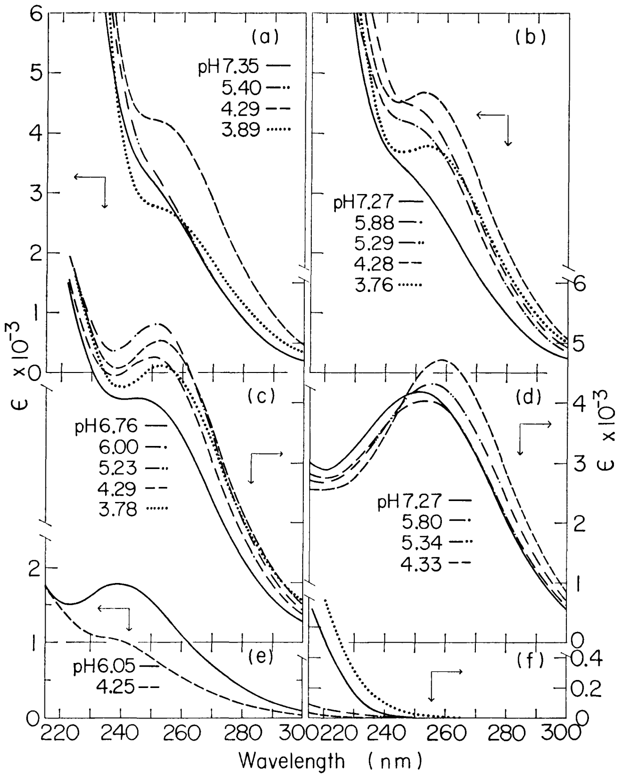

Fig. 2. The UV absorption spectra of Cu^{2+} in the presence of poly(Glu) ((a) $R=32$, (b) $R=16$, and (c) $R=8$), poly(Acr) ((d) $R=16$), and butyrate ((e) $R=16$) at various pH. The spectra of poly(Glu) at pH 7.09 (——), poly(Acr) at pH 5.39 (—·—), butyrate at pH 5.25 (----), and $CuCl_2$ at pH 3.81 (······) are shown in (f) for comparison.

or butyric acid (model of the side-chain carboxylate of poly(Glu)). Figures 1(d) and 2(d) show that the three absorption bands appear again around 710 nm (a broad band $\varepsilon=40$—150), 370 nm (a weak band $\varepsilon=10$—40), and 250 nm (a strong band $\varepsilon=2000$—4500) in the spectra of poly(Acr)–Cu(II) solutions. The 250 nm peak is distinct because of the weaker background absorption of poly(Acr) (Fig. 2(f)). There is no clear isosbestic point in the 250 nm or 700 nm band in the entire pH range 7.3—3.8. The spectrum of Cu^{2+} in the presence of butyric acid shows two bands: one in the region 800—750 nm ($\varepsilon=12$—25) and one around 240 nm ($\varepsilon=1000$—2000) as indicated in Figs. 1(e) and 2(e). In contrast with the two polymer–Cu(II) systems, the spectrum of butyric acid–Cu(II) decreased steadily with decrease in pH, becoming close to that of the Cu(II) aquo-complex. No measurement was carried out at a pH higher than 6.5 because of the secondary reaction of precipitation.

pH *Dependence of the Absorption of Poly(Glu)–Cu(II).* Each spectrum of poly(Glu)–Cu(II) complex may be characterized by three absorption bands, and the variations in ε with pH at four selected wavelengths are shown in Figs. 3(a—d). At $R=32$ the ε at 680 nm, ε_{680}, is approximately constant in the pH range 7—5.5, but increases sharply attaining a maximum at pH=4.3 and decreasing with a further lowering of pH. At $R=16$, however, the ε_{680} begins to increase at pH=7 reaching a maximum at pH=4.1 after a marked shoulder near pH=5. At $R=8$, the ε_{680} becomes still larger between pH=7 and 5 showing a shoulder around pH 5.6, maxima at pH=4.8 and between 4.1—4.3. These peculiar changes in ε have been found for the first time. The intermediate shoulder may have been previously overlooked or mistaken for maxima.[2] It is now clear that the absorption of poly(Glu)–Cu(II) complexes depends on both pH and R in a complicated but systematic manner hitherto unreported.

The pH dependence of ε has also been determined at 880 nm (Fig. 3(b)), because the 700 nm band exhibits a marked skewness. The ε_{880} values generally behave in parallel with the ε_{680} values at $R=32$ but not at $R=16$ and 8. This difference may be attributed to the development of a long-wavelength component band around 900 nm with a decrease in R. The ε_{370} *vs.* pH curve at $R=8$ shows a maximum at pH=4.1 but none at pH=4.8, as illustrated in the ε_{680} *vs.* pH curve. This peculiarity offers two possible explanations, *viz.*, the origin of the 370 nm band differs from that of the remainder, or the maxima in the ε *vs.* pH curves at $R=8$ are only apparent and actually result from a delicate interplay of two or more processes involved in the formation of Cu(II)–Glu ligand complexes.

Contour Diagram of the Absorption of Poly(Glu)–Cu(II). A typical contour diagram is shown in Fig. 4, where the ε_{710} values for a number of poly(Glu)–Cu(II) solutions are related to pH and R. Similar diagrams may also be drawn for other wavelengths. Figure 4 clearly shows that there are two distinct ridges which lead to two separate summits indicated by X and X′ respectively. The first (AX) is along the R path at a slightly varying value of pH and the second (BX′) is along the pH path at an almost constant R value of approximately 4. In addition, a third, broader and less distinct ridge (CX′) leading to the summit X′ exists. This path depends on both pH and R in clear contrast with the two other paths and represents the marked hump observed in the ε *vs.* pH curves for the $R=16$ and 8 poly(Glu)–Cu(II) solutions (Figs. 3(a—d)). The slope stretching out between $R=50$ and 4 and below pH 4.4—4.2 on the left side of the first ridge (AX) is only apparent and should be a plateau with an approximate height of 150 in terms of the ε of the bound Cu(II).[25] The appearance of this slope has been attributed to the dissociation of the bound Cu(II) from the polymer site at low pH.[25]

Extrinsic CD of Bound Cu(II) in the 750—300 nm Region. The appearance of the extrinsic CD of Cu(II) should attest to the formation of Cu(II)–Glu ligand complexes. Two positive extrinsic CD bands are shown in Figs. 5(a—d): the long-wavelength CD band between 750 and 500 nm (designated as the visible CD band) and the short-wavelength band between 500 and 300 nm (designated as the near UV CD band). These CD bands correspond to the broad 700 nm and weak 370 nm absorption bands shown in Figs. 1(a—c) and have not hitherto been reported.

At a high value of R of 32, the peaks of the visible

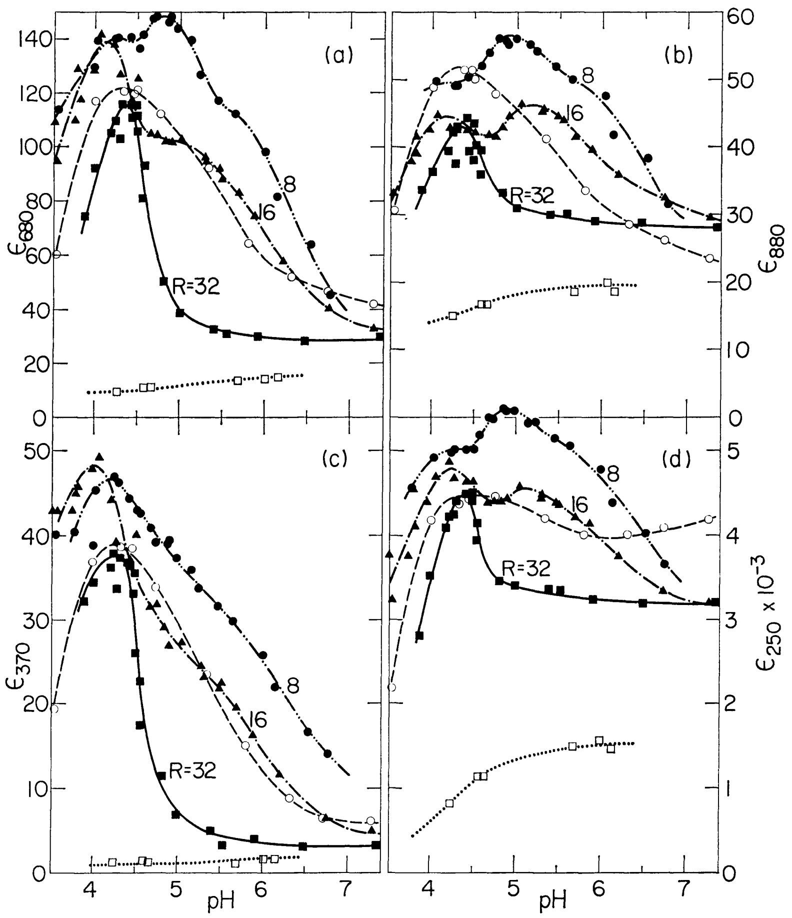

Fig. 3. The variation in the absorption coefficients of Cu^{2+} with pH in the presence of poly(Glu), poly(Acr), and butyrate at various R and at selected wavelengths of (a) 680 nm, (b) 880 nm, (c) 370 nm, and (d) 250 nm. Poly(Glu) ($R=32$ —■—, 16 —·▲—·, and 8 —··●—··), poly(Acr) ($R=16$ ——○——), and butylate ($R=16$ ······□······).

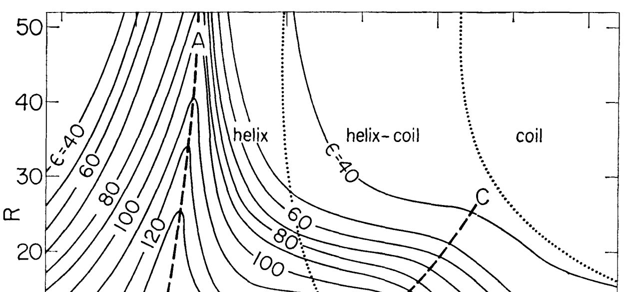

Fig. 4. The contour diagram of absorption intensity of Cu^{2+} at 710 nm in the presence of poly(Glu) at various pH and R. The numerals indicate the apparent molar extinction coefficients of Cu^{2+}. See text for other details.

and near UV CD bands center at 660 and 375 nm (Fig. 5(a)). The intensity increases with a decrease in pH becoming highest at pH 4.60, below which it decreases largely due to dissociation of the bound Cu(II).[25] At $R=8$, the CD bands have already appeared in the high pH range 6—7. The peak position and intensity depend on pH (Fig. 5(b)). At a value of $R=4$, CD bands with peaks at 670 nm and *ca.* 365 nm appear in the high pH range (Fig. 5(c)). The effect of R on the extrinsic CD band is shown in Fig. 5(d) at pH 5.75±0.05, which corresponds approximately to the midpoint of the helix-coil transition of poly(Glu) in the absence of Cu^{2+}. The highest value of $[\theta]$ for the visible CD band is approximately 430, when the Glu residues greatly exceed the Cu(II), *i.e.*, $R=32$ rather than 4, and when the pH of poly(Glu)-Cu(II) solution is low such that the helical structure of poly(Glu) is completed. Hence, the optical activity induced in the Cu(II) bound to poly(Glu) depends on both the backbone conformation of the macromolecular poly(Glu)-Cu(II) complex and the species of the Cu(II)-Glu ligand complex.

Extrinsic CD of Bound Cu(II) in the 310—240 nm Region. Corresponding to the UV absorption band of Cu(II) bound to poly(Glu) (Fig. 2), an extrinsic CD band appears in the 290—250 nm region (designated as the UV CD band) as shown in Fig. 6. The R 32 poly(Glu)-Cu(II) complex is in a helical conformation at pH 5.10 (*vide post*); yet, the negative $[\theta]$ increases only steadily. A shallow minimum and

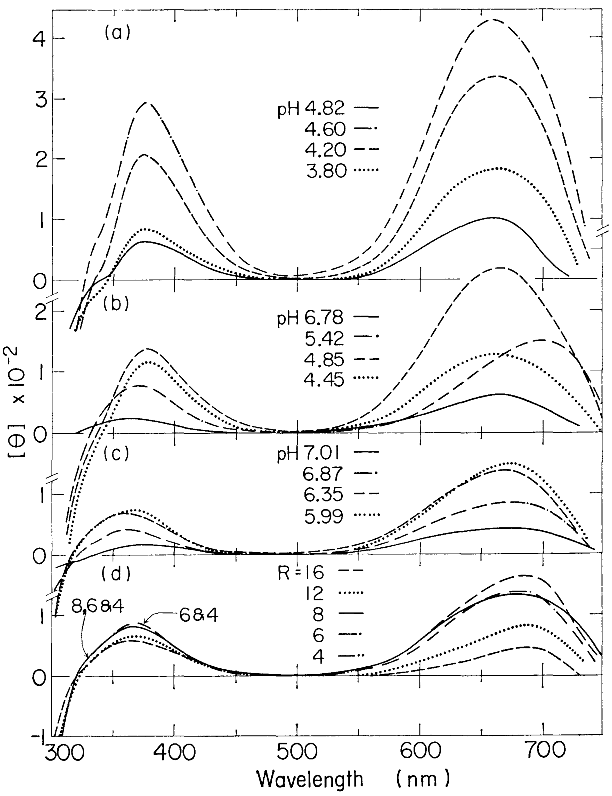

Fig. 5. The visible and near UV extrinsic CD spectra of Cu(II) bound to poly(Glu) at various R and pH. (a) $R=32$, (b) $R=8$, (c) $R=4$, and (d) pH=5.75. The molar ellipticity $[\theta]$ is expressed in terms of the total Cu^{2+} concentration in each poly(Glu)-Cu(II) solution.

maximum appear at 280 and 258 nm with the decrease in pH, respectively, and fully develope at pH 4.60 (Fig. 6(a)). The UV CD band represents a double extrinsic Cotton effect (*cf.* Fig. 10). The UV CD band at R 8 and 4 is shown in Figs. 6(b) and (c). This CD band is not seen at higher pH where the overall conformation is mostly in the random-coil, it however gradually appears as the pH of the solution is lowered. At pH 5.75, where the formation of the helical structure is half completed, the UV CD band becomes discernible as the value of R is decreased (Fig. 6(d)). This is an indication that the helical structure is fostered by the binding of Cu^{2+} to poly(Glu).

Intrinsic CD of the Glu Residue of Poly(Glu)-Cu(II) in the 250—185 nm Region. The conformation of macromolecular poly(Glu)-Cu(II) complexes is reflected in the intrinsic Cotton effect of the peptide chromophore in the far UV CD spectra as shown in Fig. 7. The CD spectra of poly(Glu) are also given as a reference (Fig. 7(a)). An isoelliptic point appears at 203.5 nm in the pH range 7.5—5.1 but deviation occurs at lower pH (<3.8). The intrinsic CD spectra behave essentially the same as the reference poly(Glu) when the Glu residues are in excess ($R=32$ in Fig. 7(b)). The general shapes of the CD spectra indicate that the helical conformation of poly(Glu)-Cu(II) is not altered by the formation of the Cu(II)-Glu ligand complex.

The intrinsic CD spectra exhibit a pH dependence when the bound Cu(II) is more densely populated on poly(Glu) ($R=8$ in Fig. 7(c)), indicating the existence of helix-coil transition. The $[\theta]$ values at 192 and 222 nm of a solution containing four peptide residues per Cu^{2+} manifest partial formation of the helix at pH 7 (R 4 in Fig. 7(d)). The R 4 CD spectrum is the largest at pH 5.75 where the reference poly(Glu) is still partly in a random-coil (a further lowering of pH promoted fibrous floats). Deviation from the isoelliptic point at 203.7 nm occurs, however, and does not develop as high as the CD spectra for the complete helical conformation (R ∞ and 32). Induction of the helix by bound Cu(II) is evident from the CD spectra of poly(Glu)-Cu(II) solutions (R 8 at pH 7.10 and R 4 at pH 6.87 in Figs. 7(c) and (d)) to which no proton was added.

Mixing-ratio Dependence of the Far UV CD of Poly(Glu)-Cu(II) and the Visible Absorption of Bound Cu(II). In order to clarify the effect of Cu^{2+} on the conformational changes of poly(Glu)-Cu(II) complexes at various values of R, the far UV CD spectra were measured at a fixed pH of 5.75±0.05 which corresponds approximately halfway to the helix-coil transition of poly(Glu). The results are shown in Fig. 8, where the CD spectra show a typical helix-coil transition pattern. All CD spectra cross at 204 nm with the exception of the R 4 spectrum. The pattern of the spectral change is similar to that of the proton-induced helix-coil transition of poly(Glu) (Fig. 7(a)), indicating that the Cu(II)-induced helix is approximately the same as the proton-induced helix. Hence, the Cu(II) bound to the random-coil or partially helical poly(Glu) probably promotes, but does not alter, the original helical structure in the macromolecular complex assuming the ionizable carboxyls are not completely un-ionized by the protons. This condition is fulfilled even at R 4 and pH 5.75 where approximately 70% of the carboxyls are uncharged.[26]

Corresponding to the increase in CD intensity, the visible absorption band of Cu(II) also steadily develops (inset Fig. 8). The correlation between CD and absorption is, however, not linear as shown in Fig. 9, where the values of $[\theta]$ at the positive and negative maxima (192 and 223 nm) are plotted against R, together with the change in the ε_{710} of the visible band. Both $[\theta]_{192}$ and $-[\theta]_{223}$ run in parallel approaching the limiting values of the complete helix as R becomes less than 4, as indicated by the horizontal line. The curve shows a sharp increment when R is around and below 10, suggesting that the induction of helical structure by the bound Cu(II) is cooperative in nature, *i.e.*, chelation and charge neutralization being responsible.[25,26] The ε_{710} values change almost linearly with R between 25 and 5. Since the amount of unbound Cu^{2+} is very small,[25,26] the ε_{710} *vs.* R curve indicates that complex formation between Cu(II) and the ligands is less cooperative than in helix formation. Thus, the latter may be essentially an overall phenomenon, while the former may be the sum of various local phenomena regarding complex species and binding modes.

Comparison between Electronic Absorption, Extrinsic CD

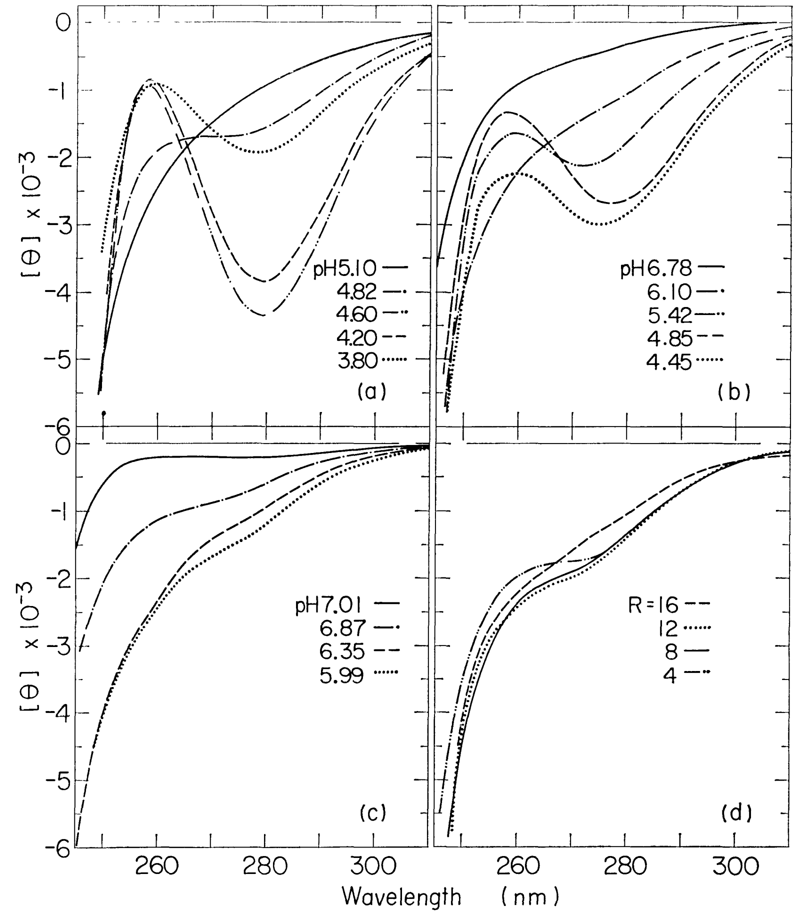

Fig. 6. The UV extrinsic CD spectra of Cu(II) bound to poly(Glu) at various *R* and pH. Notations are all the same as those in Fig. 5.

and Optical Dissymmetry Factor of Bound Cu(II). In order to realize the spectroscopic property of Cu(II) bound to Glu residues, a typical CD spectrum of Cu(II) bound to the complete helix (*R* 32 and pH 4.6) is compared with the corresponding absorption spectrum in Fig. 10. The optical dissymmetry factor, *g*,[27] is expressed as the ratio of the molar CD, $\Delta\varepsilon$, to the molar absorption coefficient, ε. The peak of the visible CD band is at 660 nm, while the absorption band is located at 690 nm, tailing toward the red. This unparalleled situation is reflected on the *g*-factor which is constant only in the 650—550 nm region descending toward zero around 740 nm. This indicates that the visible absorption band of bound Cu(II) is a composite of two or more optically active component bands. The *g*-factors of the visible Cu(II) band are generally larger for the *R* 32 complex than for the *R* 8 or 4 complex, indicating that optical dissymmetry for the visible band is more pronounced when the bound Cu(II) is less densely populated on the polymer.

Nozawa and Hatano[16] have observed the double CD band for a poly(Lys)-Cu(II) complex in the visible region. Garnier and Tosi[19] have shown that the extrinsic CD of poly(Arg)-Cu(II) is either double or separable into two components in the visible region. The sign, position, and magnitude of the visible CD of Cu(II) bound to poly(Glu) differ from those data previously reported. The difference probably results from the specificity of the ligands participating in complex formation, *i.e.*, γ-carboxylate *vs.* ε-amino or 4-guanidinium, and the stereoselectivity of a particular poly(amino acid) for chelation, but not from the ability of forming helical structures.[19]

It is not self-evident that the near UV CD band is a double type, because the absorption of bound Cu(II) is weak and discernible only as a shoulder so that the wavelength dependence of the *g*-factor is only apparent. The greater *g*-value for the 370 nm band may indicate that the contribution of the magnetic transition moment to the rotatory power is greater in the near UV CD band (450—350 nm) than in the visible CD band (700—550 nm). A similar trend has been observed at all values of *R* and pH. Thus, the near UV CD band is more optically dissymmetric.

The net CD band of bound Cu(II) in the 320—240 nm region may be obtained by subtracting the background intrinsic CD of the Glu residue. The far UV intrinsic CD spectrum of the *R* 32 poly(Glu)-Cu(II) at pH 4.6 differs from that of the same at pH 5.1 by a constant factor of 1.25±0.05 between 250 and 200 nm. Therefore, the background ellipticity of the pH 4.6 complex is considered to be 1.25 times larger than that of the pH 5.1 complex which shows no extrinsic UV CD band (Fig. 6(a)). The net CD band of the bound Cu(II) thus cleared of background

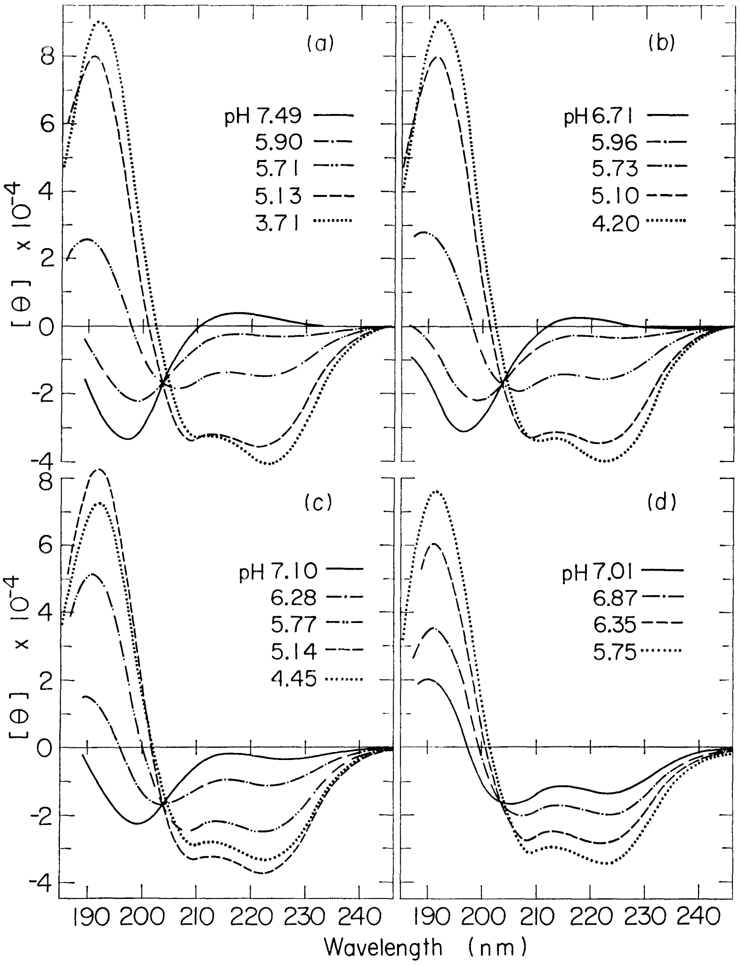

Fig. 7. The far UV intrinsic CD spectra of the peptide chromophore of poly(Glu)-Cu(II) solutions at various R and pH. (a) $R=\infty$ (no Cu^{2+} present), (b) $R=32$, (c) $R=8$, and (d) $R=4$. The molar ellipticity $[\theta]$ is expressed in terms of the mean molar residue concentration of poly(Glu) in the presence and absence of Cu^{2+}.

is double : a negative component with a peak at 280 nm followed by a positive peak around 256 nm. This doublet feature appears to be retained in most of the UV extrinsic CD spectra in Fig. 6. It has also been observed in the poly(Lys)- and poly(Arg)-Cu(II) complexes,[16-18] although the respective signs are probably reversed relative to the poly(Glu)-Cu(II) complex.

Discussion

Conformational Transitions of Poly(Glu)-Cu(II) Complex and the Formation of Cu(II)-Glu Ligand Complex. In order to relate the pH- and Cu(II)-induced conformational transitions of poly(Glu)-Cu(II) to the formation of Cu(II)-Glu ligand complex, the extrinsic CD and absorption data are compared with the far UV intrinsic CD in Fig. 11. The ε_{680}, $[\theta]_{680}$, and $[\theta]_{370}$ values of bound Cu(II) are also plotted against $[\theta]_{223}$ in Fig. 12, which directly shows the dependence of the optical properties of bound Cu(II) on the conformation of macromolecular complexes. For the R 32 complex, both the visible CD ($[\theta]_{680}\approx 0$) and electronic absorption ($\varepsilon_{680}\approx 30$) of bound Cu(II) are weak and remain constant over a wide pH range 7—5 (Fig. 11). The helix-coil transition of the macromolecular complex does occur in the narrow pH range 5.1 and 6.5; however, the bound Cu(II) hardly affects the transition, as clearly seen in Fig. 12. Thus, the feed-back-effect of the macromolecular complex on the Cu(II)-Glu ligand complex, and *vice versa*, is weak when the bound Cu(II) is populated sparsely on poly(Glu). This effect however becomes pronounced for the macromolecular complex in which Cu(II) is distributed in a high proportion (R 8 or 4), since both the extrinsic CD and absorption of the bound Cu(II) and the $[\theta]_{223}$ of the Glu residue are already large around pH 7 where the poly(Glu) without Cu(II) is in the extended-coil form (Fig. 11).

As Figs. 11 and 12 indicate, the optical properties of bound Cu(II) for the R 8 and 4 complexes vary together in parallel, but *never* change linearly with respect to the helix content of the poly(Glu) backbone. (It is assumed that $[\theta]_{223}$ is equal to -35400 for a 100% helix.) In fact, the optical properties change with pH (Fig. 11) and helix content (Fig. 12), in a manner which is unexpectedly complicated, in the helix-coil transition, contrary to a previous report.[2] For example, the distinct hump in the ε_{680} curve around

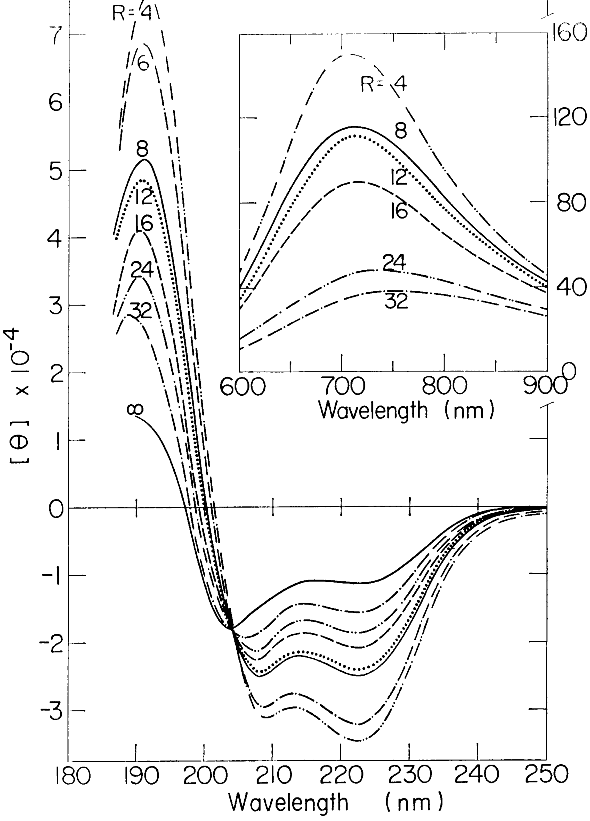

Fig. 8. The variation in the far UV intrinsic CD spectra of poly(Glu)-Cu(II) solutions with R at a pH of 5.75±0.05. The molar ellipticity is expressed as in Fig. 7. The corresponding visible absorption spectra are given in the inset.

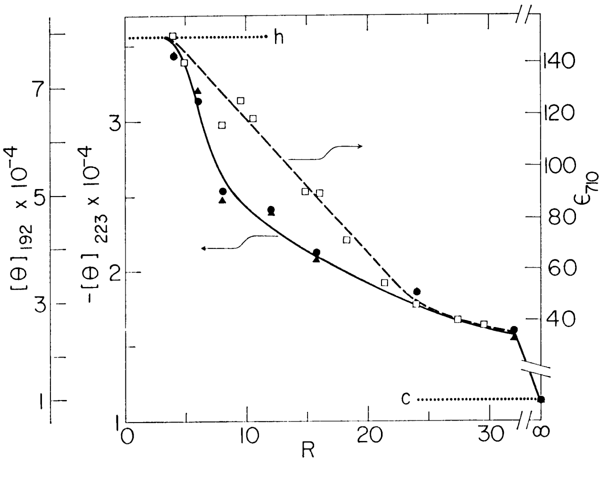

Fig. 9. The effect of added Cu^{2+} on the induction of helical structure in poly(Glu) and the formation of Cu(II)-Glu ligand complex at a constant pH of 5.75. The two horizontal lines (······h and c······) indicate the values of $[\theta]_{192}$ (—●—) and $[\theta]_{223}$ (—▲—) for the poly(Glu) solutions without Cu^{2+} ions at pH 5.10 and 7.49, respectively. The molar absorption coefficient ε_{710} (--□--) is the same as plotted in Fig. 4.

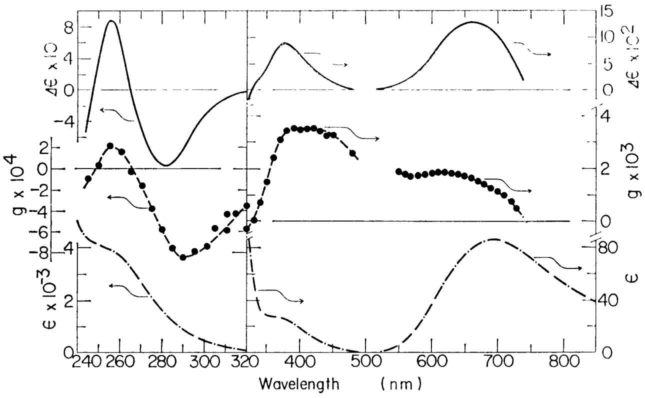

Fig. 10. Comparison between extrinsic CD spectrum, $\Delta\varepsilon$, electronic absorption spectrum, ε, and optical dissymmetry factor, g, of a poly(Glu)-Cu(II) solution at R=32 and pH=4.60. The extrinsic CD spectrum is expressed in terms of the molar circular dichroism $\Delta\varepsilon$ (=$[\theta]$/3305).

pH 5.3—6.0 (Figs. 3, 4, and 11(b)) probably corresponds to the second stage of the helix-coil transition after the midpoint, but not to the completion of the helix or to the midpoint as previously concluded.[2)]

The most prominent change in the optical properties of the bound Cu(II) probably occurs after completion of the intramolecular helix of poly(Glu)-Cu(II) complex regardless of the amount of the Cu(II) bound to the Glu residues (Figs. 11 and 12). The ascending trend in the visible CD and absorption of Cu(II) is closely related to the second transition in the $[\theta]_{223}$ *vs.* pH curve (Fig. 11). Evidently, the backbone of the macromolecular complex undergoes a further conformational transition, probably, helix to *aggregates*, at low pH 5—4.4.[26,28—31)] It is however undeterminable from optical measurements alone whether or not the changes in optical properties arise from the transition of the intra- or interhelical conformation. In summary, it is probably the dissymmetric conformation of the Cu(II)-Glu ligand complex that is primarily responsible for the induced optical activity. Such a dissymmetry may be conferred on Cu(II) by stereospecific binding (or chelation) to the side-chain carboxylates and backbone amides bi- or tridentately. The overall conformation of a macromolecular complex would promote such binding. These conclusions are new and important.

The processes which are most responsible for the change in optical properties of the Cu(II)-Glu ligand complexes probably belong to two regions: the first half of the helix-coil transition region where the backbone of poly(Glu)-Cu(II) attains a compact-coil form due to the formation of fragmental helices, and the second transition region where the backbone undergoes a further conformational transition, possibly, from helix to *aggregates*. Thus, the species of Cu(II)-Glu ligand complexes are multiple, probably three or more altogether. It is a delicate interplay of these processes and the concentrations of the complex species involved that are responsible for the very involved dependence of the optical properties of poly(Glu)-Cu(II) on both pH and R.

The Cu(II)-Glu Ligand Complex and Its Absorption and CD Spectra. Complex formation between Cu(II) and Glu residues is evident from the absorption

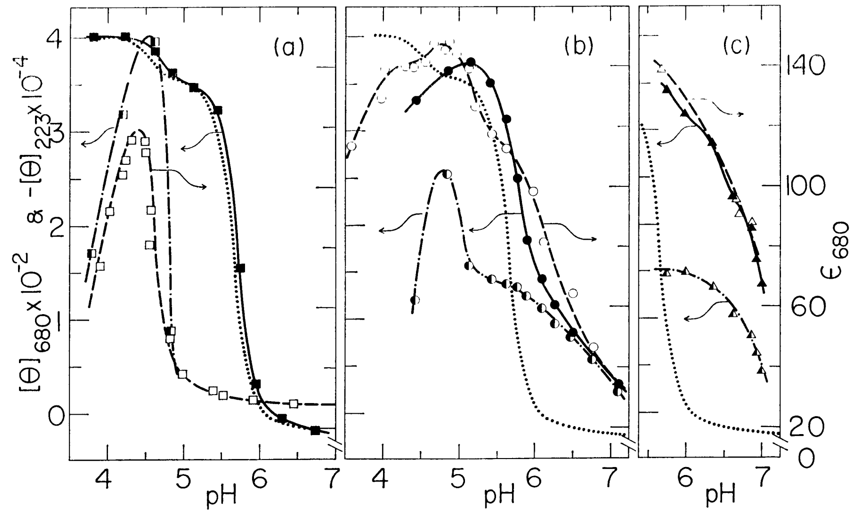

Fig. 11. Comparison of the pH-induced helix-coil transition curves of poly(Glu)–Cu(II) at three values of R (a) 32 (squares), (b) 8 (circles), and (c) 4 (triangles) with the pH variation of the corresponding visible CD and absorption of Cu(II). ε_{680} (——), $[\theta]_{680}$ (—·—), $[\theta]_{223}$ (——), and $[\theta]_{223}$ at $R=\infty$ (······).

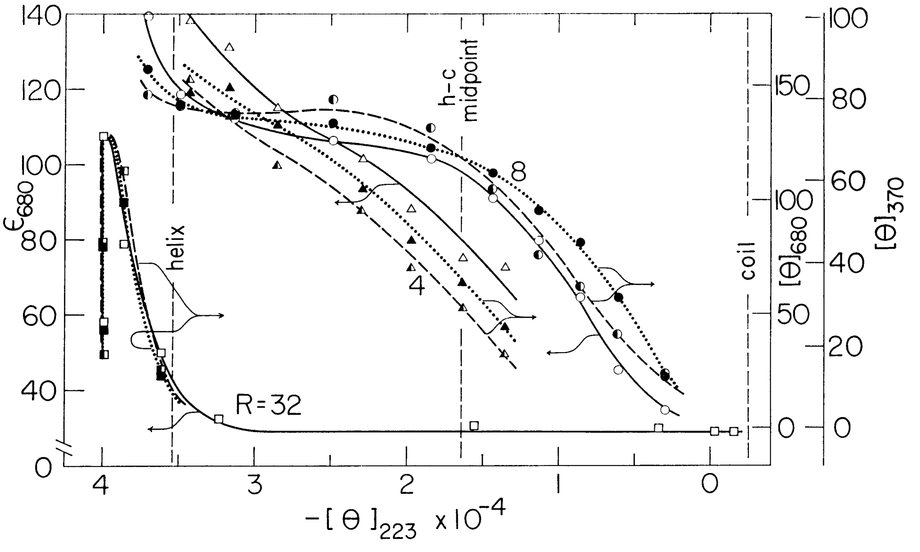

Fig. 12. Correlation between the intrinsic CD of poly(Glu)–Cu(II), $[\theta]_{223}$, and the absorption and extrinsic CD of the Cu(II)–Glu residue complex at three R values of 32 (squares), 8 (circles), and 4 (triangles). ε_{680} (——), $[\theta]_{370}$ (——), and $[\theta]_{680}$ (······). The actual values of $[\theta]_{370}$ and $[\theta]_{680}$ at $R=32$ should be quadrupled. Regardless of the mixing ratios between Glu residues and Cu^{2+}, the $[\theta]_{223}$ values of +2500, −16500, and −35400 are assumed for the extended-coil, the midpoint of helix-coil transition, and the complete helix, respectively.

and extrinsic CD spectra of Cu(II) (Figs. 1, 2, 5, and 6). The present data manifest that previous reports have unraveled only a part of the whole process involved in the complex formation.[2,3] The absorption band at 700 nm results from the binding of Cu^{2+} by the ionized carboxylate (and possibly amide) groups, as the data of poly(Acr)- and butyrate–Cu(II) solutions (Fig. 1) and poly(carboxylic acid)–Cu(II) complexes have indicated.[9–15] The number of the ligand carboxylates participating in complex formation may range from one to four, most likely two ionized carboxylates.[26] An absorption study is insufficient to ascertain the mechanism of carboxylate neutralization and chelation and the role of the amide group of the Glu residue in chelation. Detailed studies of equilibrium dialysis and electron spin resonance are needed.

The skewed 700 nm band of the poly(Glu)–Cu(II) complex (Figs. 1 and 5) is not simple but composite revealing another component band on the long-wavelength side around 900 nm (Figs. 1 and 3), as reported for the poly(Methacr)–Cu(II) complex.[13] The multiple band structure has also been supported by CD data (Figs. 5 and 10). Hirasawa and Kon[32] analyzed the apparent 700 nm band of Cu(II) doped in L-histidine into three component bands centering at 714, 781, and 901 nm; these component bands may or may not correspond to the 685 and *ca.* 900 nm bands of the Cu(II)–Glu ligand complex. The CD spectrum of a poly(Glu)–Cu(II) solution at R 32 was scanned to 800 nm with a red-sensitive Hamamatsu R446 photomultiplier. At the longest attainable wavelength, the *negative* CD component was only steadily increasing. Hence, the possibility still exists that the 700 nm absorption band of the bound Cu(II) is a composite of two, instead of three, out of four component bands. The actual situation may be very complicated, since the chemical species of the Cu(II)–Glu ligand complexes would be numerous in the poly(Glu)–Cu(II) macro-complex as indicated by the pH and R dependence of ε (Figs. 3 and 11). For instance, two side-chain carboxylates can chelate to a Cu(II) either in the *cis* or *trans* position. The absorption peaks for these isomers are known to be slightly different in amino acid–Cu(II) complexes.[33)]

The origin of the near UV band of the Cu(II)–carboxylic acid complex remains a point of controversy[34)] and, therefore, the 370 nm band of the poly(Glu)–Cu(II) complex deserves comment. Leyte *et al.*[13)] suggested the presence of binuclear Cu(II) complexes of Cu(II)–acetate type dimeric structure in the poly(Methacr)–Cu(II) complex on the basis of the appearance of the 380 nm band. The optical absorption studies alone however can not resolve the question whether or not the 370 nm band reflects the presence of the binuclear Cu(II) complex in which direct Cu(II)–Cu(II) interaction exists.[34)] The 350—380 nm shoulder appears in the absorption spectra of the Cu(II) bound to poly(Lys),[17)] poly(Arg),[19)] and poly(His),[20)] all of which lack the carboxyl side-chain group. It should also be noted that the 370 nm band is associated with Cu(II)–β-diketone complexes which form a monomeric structure in solution.[35)] Model buildings with CPK atomic models indicate that the dimer structure of the Cu(II)–acetate type is impossible with four side-chain carboxylates in a single-stranded poly(Glu) which may exist as the α-helix at low pH. The dimer is quite likely to occur if Cu(II) bridges two poly(Glu) strands by binding to two carboxylates of each polymer-chain. Comparative work by ESR and other magnetic methods is necessary here to identify the individual chemical species of Cu(II)–Glu ligand complexes.

The 370 nm CD band shows a slight hump in the shorter wavelength region suggesting a doublet structure (Fig. 5), although the absorption reveals no such feature (Fig. 1). Dubicki[36)] reported that the 370 nm band of the Cu(II)–acetate complex is a doublet, assigning it to the charge-transfer transition of ligand-to-metal nature. Graddon[35)] considered the 370 nm band of Cu(II)–ethyl acetoacetate complex as originating from the higher energy d-d transition (d_{xz}, $d_{yz}\rightarrow d_{x^2-y^2}$ type). His term-scheme is compatible with the present CD data of poly(Glu)–Cu(II) complexes assuming a weakening of degeneracy of the near UV d-d transition and that the low energy 700 nm band consists of two of the four d-d components. The final assignment of the d-d transition of Cu(II) and, consequently, the most likely coordination of the Cu(II)–Glu ligand complex remains unclear. The detailed CD study of a number of polypeptide–Cu(II) complexes in the near IR region is necessary for solving these problems.

The 250 nm band has been studied because of the high absorption intensity and existence in the spectra of Cu(II) in the presence of polyanions such as poly(Glu),[2,3,8)] poly(Methacr)[11—13)] and poly(Lys).[17)] The band has been assigned to the allowed charge-transfer transition between carboxyl ligands and the metal ion.[2)] The 250 nm charge-transfer band is composite, as indicated by the positive and negative CD component bands (Fig. 10). The unambiguous determination of the number and polarization of the optical transition moments involved in the poly(Glu)–Cu(II) complex are important for the understanding of the origin of induced optical activity associated with bound Cu(II). Such studies may be conducted by means of film dichroism.[37)]

Conclusion

The Cu^{2+} ion bound to poly(Glu) exhibits the extrinsic Cotton effects of multiple type in three characteristic absorption bands in the visible, near UV, and UV regions. The effect of bound Cu(II) on the polymer conformation induces a helical structure but reduces the cooperative nature of the helix-coil transition. The absorption and CD spectra of bound Cu(II) in this transition region are related but the behavior is not in parallel with the backbone conformation. In addition to the ordinary helix-coil transition, a second conformational transition is involved in the macromolecular poly(Glu)–Cu(II) complex in the pH range <5.3—5.2. Complex formation between Cu(II) and Glu residues is strongly affected by this transition. The pH dependence of absorption and CD intensities results largely from the interplay of the two conformational transitions of the macrocomplex which affect the number of species and stereospecific configurations of the Cu(II)–Glu ligand complexes populated on the poly(Glu).

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