

The Effect of Polyelectrolyte Complex Formation on pH-Induced Conformational Changes of Polypeptides for Systems of Poly(L-glutamic acid)—Poly(ethyleneimine) and Poly(L-lysine)—Carboxymethyl Cellulose

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ABSTRACT: The conformational changes of polypeptides associated with the formation of polyelectrolyte complexes were studied as a function of pH for the systems of poly(L-glutamic acid)—poly(ethyleneimine) and poly(L-lysine)—carboxymethyl cellulose, by means of optical rotatory dispersion and circular dichroism spectroscopies, and potentiometric and turbidimetric measurements. With the poly(L-glutamic acid)—poly(ethyleneimine) system, the pH-induced helix formation of the polypeptide was promoted by the presence of poly(ethyleneimine), while with the poly(L-lysine)—carboxymethyl cellulose system, the pH-induced coil-to-helix transition was scarcely affected by the presence of carboxymethyl cellulose. These results are interpreted in terms of chain flexibility of the partner polymer of the polypeptide.

KEY WORDS Polyelectrolyte Complex / Poly(L-glutamic Acid) / Poly(L-lysine) / Polyethyleneimine / Carboxymethyl Cellulose / Coil-to-Helix Transition / Chain Conformations /

The reaction between oppositely charged, naturally occurring polymers has been studied by using biopolymers such as nucleic acids, proteins, and polysaccharides mainly on the standpoint of biochemical interests.¹⁻⁶ More recently, studies of the formation and properties of polyelectrolyte complexes composed of synthetic polycations and polyanions have been reported with respect to physicochemical aspects,⁷⁻¹¹ industrial applications,¹² and conformation.¹³⁻¹⁶

The molecular chains of an ionizable polypeptide exist in a random coil form when the degree of ionization is high but in a helical form as the degree of ionization becomes low. When an ionizable polypeptide reacts with an oppositely charged polyelectrolyte and forms a polyelectrolyte complex, the electrostatic potential of the polypeptide and the degree of ionization should both decrease. Therefore, the pH value at which the polypeptide starts to form an α -helix is expected to be affected by the

complex formation unless there is hindrance to the formation of intramolecular hydrogen bonds in the polypeptide.

The molecular chains of poly(L-lysine) are known to take a random coil form at pH 6.8 in 0.1-*M* sodium chloride solution. Gratzner and McPhi⁷ have observed a Cotton effect characteristic of α -helix at the same pH when this polymer was reacted with an excess of poly(acrylic acid). Further, Zezin, *et al.*,⁹ have reported that the coil-to-helix transition region (pH=8.5—9) of poly(L-lysine hydrobromide) in a mixed solvent of water and ethanol at 20°C shifted to pH 3.5—4 by complex formation with poly(acrylic acid). On the other hand, Zezin, *et al.*, have reported that the transition region, pH=6.0—6.5, of poly(L-glutamic acid) is shifted to pH=3—5 by complex formation with poly(ethyleneimine). Consequently they have suggested disintegration of the α -helix in poly(L-glutamic acid) systems and stabilization of the α -helix in poly(L-lysine) systems associated with complex forma-

tion with a flexible partner polyelectrolyte.

The experimental results cited above may be summarized as follows. Complex formation of an ionizable polypeptide with an oppositely charged polyelectrolyte causes the transition region of poly(L-lysine) to shift to the lower pH region, with the result that there is a larger charge density on poly(L-lysine) itself. However the transition region of poly(L-glutamic acid) is also shifted to the lower pH region even though in this case this results in a smaller charge density on the polypeptide itself. The latter result seems to be contradictory to the suggested explanation.

The purpose of this study was to clarify the effects of a partner polyelectrolyte on the pH-induced coil-to-helix transition of an ionizable polypeptide mainly from the conformational aspect of the partner polymer chain. The polyelectrolyte complexes investigated were poly(L-glutamic acid) (PLGA)—polyethylenimine (PEI), and poly(L-lysine) (PLL)—carboxymethyl cellulose (CMC). PEI is a weakly basic polyelectrolyte and its backbone chain is regarded as flexible. CMC is a weakly acidic polysaccharide and pyranose rings in the chain are rigid and linked by β -1,4-glucoside linkages. Thus CMC molecules behave as semiflexible chains in solution.^{17,18}

EXPERIMENTAL

Materials

The monomers, *N*-carboxy- γ -benzyl-L-glutamate anhydride and *N*-carboxy- ϵ -*N*-carbobenzyl-L-lysine anhydride were prepared by the method proposed by Blout and Karlson,¹⁹ and were polymerized after purification according to our previous paper.^{20,21} The degrees of polymerization of poly(γ -benzyl-L-glutamate) and poly(ϵ -*N*-carbobenzyl-L-lysine), estimated from the intrinsic viscosities,^{22,23} were 1760 and 1480, respectively. Hydrolysis of poly(γ -benzyl-L-glutamate) (PBLG) was carried out basically according to the method of Wada.²⁴ That is, PBLG was dissolved in a 1:1 (v/v) mixture of benzene and dioxane at 50°C and anhydrous hydrogen bromide was bubbled into the mixture. The reaction mixture was allowed to stand for 8 hr. The precipitate, a syring gel, was washed several times with mix-

tures of acetone and ethyl ether, dissolved in 5% NaHCO₃ aqueous solution, and then dialysed against water adjusted to pH 7. The degree of debenzylation was confirmed to be higher than 99 mol% from the ultraviolet absorption spectra. The degree of polymerization of poly(sodium L-glutamate) was estimated to be 420 by using the viscosity equation proposed by Wada.²⁵

Poly(ϵ -*N*-carbobenzyl-L-lysine) was hydrolyzed according to a modification of the method of Fasman,²⁶ by dissolving it in a 1:1 (v/v) mixture of chloroform and dioxane. After bubbling anhydrous hydrogen bromide through the solution for 1 hr, and then passing nitrogen for 2 hr, the precipitate formed was dialysed against water at pH 3.

CMC and PEI used in this work were the same as those reported in the preceding paper.²⁷ The carboxyl content of CMC was 0.75 per glucose residue. The molecular weight of PEI was 9000. Aqueous solutions of CMC and PEI were deionized with ion-exchange resins to prepare the stock solutions. Conductivity water was used throughout the experiment. A complex solution was prepared in the following way: First a stock solution of the polypeptide in its charged coil conformation was diluted to a given concentration; a solution of the partner polyelectrolyte was added, and then the pH of the system was varied.

Apparatus

The polymer concentration in aqueous solution was determined by drying the solution to constant weight *in vacuo*, or by measuring the conductivity of the solution by means of a Yanagimoto Conductivity Outfit Model MY-8. The pH values of aqueous solutions of polyelectrolytes were measured by a Yanagimoto pH meter Type 7 at 25±0.01°C, under nitrogen atmosphere if necessary.

The optical rotatory dispersion (ORD) was measured at 25°C with a Yanagimoto OR-100 Type Spectropolarimeter using a tungsten lamp as the light source. The wave lengths used ranged from 325 to 610 nm and the path length of the glass cell was 50 mm. The circular dichroism (CD) spectra were measured at 25±0.5°C with a JASCO J-20 CD/ORD Spectropolarimeter equipped with a quartz cell of path

length 0.5 or 1 mm. The total polymer concentrations in the aqueous solutions were measured in the range from 0.002 to 0.003-*M* with respect to amino acid residues by CD.

RESULTS AND DISCUSSION

PLGA, PLL, CMC, and PEI are all weak polyelectrolytes, and hence their ionization depends on pH and plays an important role in the formation of polyelectrolyte complexes.²⁸ Relations between the degree of ionization α and the pH are shown in Figure 1a for PLGA and PEI, and in Figure 1b for PLL and CMC, where α is evaluated by assuming that the presence of polyelectrolytes had no effects on the activities of H^+ and OH^- . From Figures 2a and 2b, in which the apparent dissociation constant pK_a is plotted against α , it is apparent that the chain conformations of PLGA and PLL change with the pH.

The formation of polyelectrolyte complexes in the present systems was accompanied by turbidity, *e.g.*, an equimolar mixture of polycation and polyanion gave a white precipitate at high polymer concentration, and was opalescent at low polymer concentration. Such turbidity in solutions of equimolar complexes disturbed the

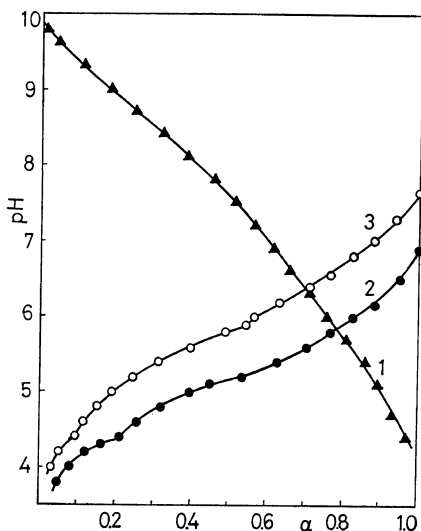


Figure 1a. Titration curves of 0.00492-*N* PEI in 0.05-*M* NaCl (1), 0.00325-*N* PLGA-Na in 0.1-*M* NaCl (2), and 0.00325-*N* PLGA-Na in 20-% propanol aqueous solution (3).

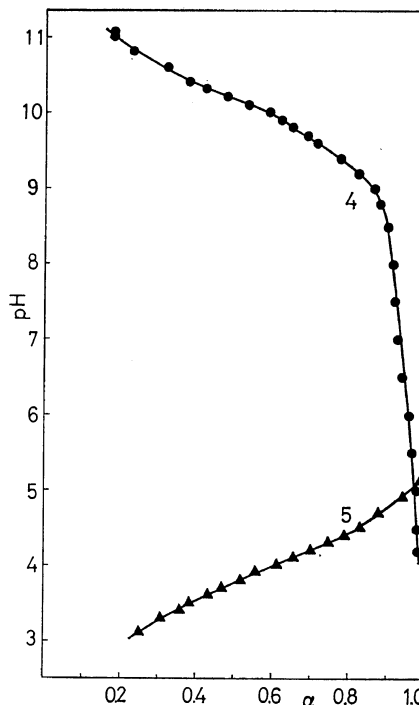


Figure 1b. Titration curves of 0.00729-*N* PLL-HBr in 0.1-*M* NaCl (4) and 0.00362-*N* CMC in 0.05-*M* NaCl (5).

ORD measurements in the region 325 to 610 nm. Therefore ORD measurements were performed for complex solutions containing a small amount of the partner polyelectrolyte with respect to the polypeptide whose concentration was *ca.* 0.01-*M*. Even then, it was difficult to compare all the experimental results by using b_0 of the Moffitt equation,²⁹ so that the effective residue rotation $[m']_{350}$ at 350 nm is used when discussing the results.

The ORD data for PLGA and PLGA-PEI complexes are shown in Figure 3. Measurements were performed by lowering the pH of solution stepwise. The addition of propanol to an opalescent complex solution led to a transparent one. Such opalescence in a complex solution scarcely influences the ORD measurements as shown by curve 2 in Figure 3. With respect to the transition pH of PLGA, it is evident from Figures 3 and 2a that our PLGA begins to form α -helix at pH 5.5 and $\alpha=0.69$ in 0.1-*M* NaCl.

Figure 4 shows CD spectra of PLGA—PEI complexes for three different mixing ratios. In the case of the PLGA—PEI (1 : 0.724) complex, a red shift of the 222 nm band assigned to $n \rightarrow \pi^*$ is observed in the figure. Therefore the residue ellipticity $[\theta]$ at 225 nm for this complex and at 222 nm for the other complexes and for PLGA

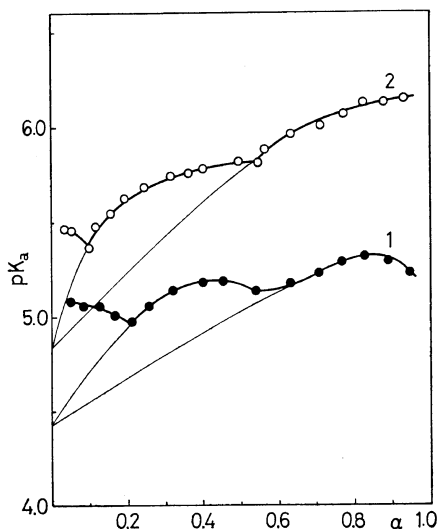


Figure 2a. Apparent dissociation constants plotted against α of 0.00325-N PLGA—Na in 0.1-M NaCl (1) and in 20-% propanol aqueous solution (2).

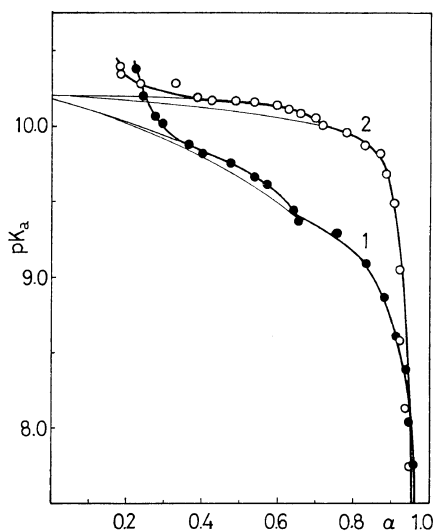


Figure 2b. Apparent dissociation constants plotted against α of 0.0073-N PLL—HBr in water (1) and 0.0058-N PLL—HBr in 0.1-M NaCl solution (2).

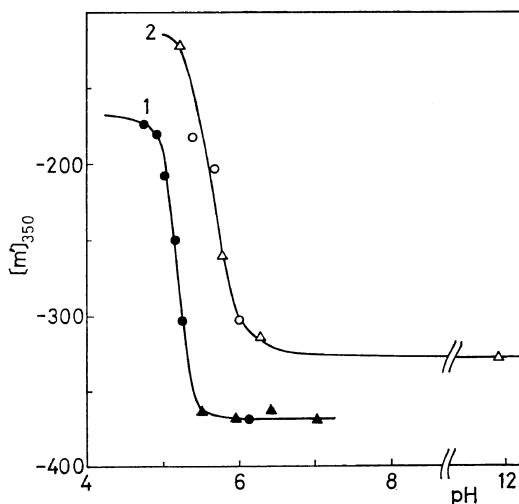


Figure 3. Relations between $[m]_{350}$ and pH for PLGA and PLGA—PEI complexes: ●, 0.0107-M PLGA in 0.1-M NaCl; ▲, PLGA—PEI (1 : 0.012) complex in 0.1-M NaCl; ○, PLGA—PEI (1 : 0.030) complex in 0.1-M NaCl; △, the latter system where propanol is added to 20% (v/v).

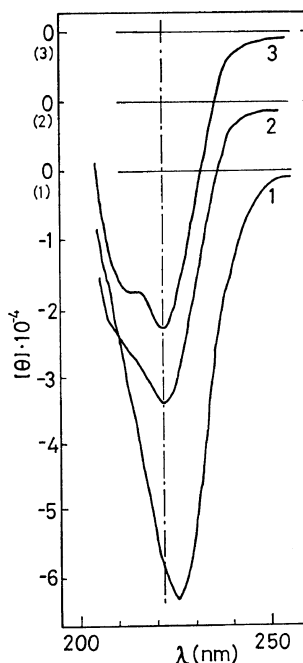


Figure 4. CD spectra of PLGA—PEI (1 : .724) complex at pH 4.88 (1), PLGA—PEI (1 : 0.333) complex at pH 4.91 (2), and PLGA—PEI (1 : 0.099) complex at pH 4.37 (3) in aqueous solution.

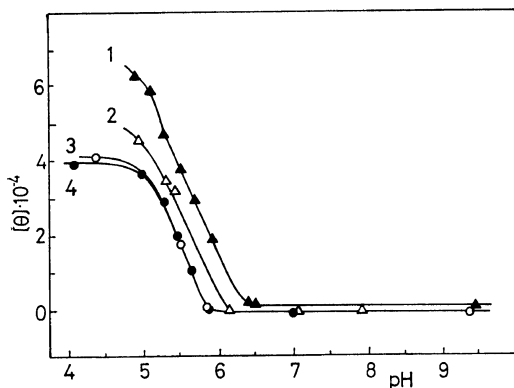


Figure 5. The pH induced coil-to-helix transition of PLGA (4), PLGA—PEI (1 : 0.724) complex (1), PLGA—PEI (1 : 0.333) complex (2), and PLGA—PEI (1 : 0.099) complex (3) in aqueous solution.

has been plotted against pH in Figure 5. According to Figure 5, PLGA in an aqueous solution begins to form α -helix at pH 5.8 and $\alpha = 0.71$.¹³ Cassim and Taylor³⁰ have reported that PLGA began to undergo transition at pH = ca. 5.2 in 0.2-*M* NaCl at 20°C, and Zimm and Rice³¹ have observed that PLGA began to undergo transition at $\alpha = 0.75$ in 0.1-*M* KCl at 25°C.

From the data mentioned above, the pH value at the beginning of the transition reported by Zezin, *et al.*,⁹ seems to be somewhat larger. The result of our ORD and CD measurements suggests that PLGA begins the coil-to-helix transition at a higher pH value due to complex formation with some of the PEI. With respect to the stability of α -helix in ionizable polypeptides, Tanaka and Nakajima³² have reported in a theoretical study on chain conformations of polypeptides containing ionizable side chains that the number of ionizable groups at the starting point of the transition is larger for an acidic polypeptide than for a basic polypeptide if the lengths of both side chains are the same. This conclusion seems to be rather incompatible with the result of Zezin, *et al.* The pH, the ionic strength, the dielectric constant of solvent, the degree of polymerization, and the form of ionic linkage in the complex such as scrambled salt- and ladder-forms¹² are factors which need to be considered.

As is apparent from Figure 3, the pH shifts of the coil-to-helix transition do not occur for

the PLGA—PEI (1 : 0.012) complex but are observed for the PLGA—PEI (1 : 0.030) complex. Assuming that PLGA reacts with PEI stoichiometrically and forms a polyelectrolyte complex, the mean degree of ionization, α'_{PLGA} , in the PLGA—PEI complex solution is expressed by the following equation

$$\alpha'_{\text{PLGA}} = \alpha_{\text{PLGA}} - \alpha_{\text{PEI}}(C_{\text{PEI}}/C_{\text{PLGA}})$$

where *C* is the concentration of ionizable groups in polyelectrolytes. α'_{PLGA} 's in the PLGA—PEI (1 : 0.012) complex and the PLGA—PEI (1 : 0.030) complex at pH 6 were 0.82 and 0.81, respectively, calculated by using Figure 1a. These numerical values do not differ significantly, so the PEI molecules in the latter system do not appear to be dispersed homogeneously throughout the complex solution but appear to react only with certain of the PLGA molecules, and to participate in their conformational change.

The solution of the PLGA—PEI (1 : 0.030) complex in 0.1-*M* NaCl turned from opalescent to transparent when 20% (v/v) propanol was added. The addition of propanol can decrease the dielectric constant of the complex solution and may weaken hydrophobic interactions among complexes. Considering these phenomena from another standpoint, the pairing between oppositely charged ionic groups often seems to proceed, though partially, in a ladder form between two polyelectrolyte molecules. Thus the PLGA—PEI (1 : 0.030) complex in propanol solution below pH 6 may be partly in a double helical structure similar to the PLGA—PLL complex as suggested by Noguchi.³³

In connection with the α -helical structure of PLGA in a double helical structure, it is noted for the PLGA—PEI (1 : 0.0724) complex, as shown in Figures 4 and 5, that there is a red shift for the $n \rightarrow \pi^*$ transition and a larger residue ellipticity than the value ($-\theta = 40,000$ degree $\text{cm}^2/\text{dicimole}$) assigned to a perfect α -helix. These characteristic features in the complex formation of PLGA with PEI may be ascribed to: a) the distortion of α -helical structure of PLGA; b) the stabilization of PLGA side chains by ionic bonds; c) the promotion of intramolecular hydrogen bonding in the α -helix due to the dielectric change.

Finally the flexibility of the partner poly-

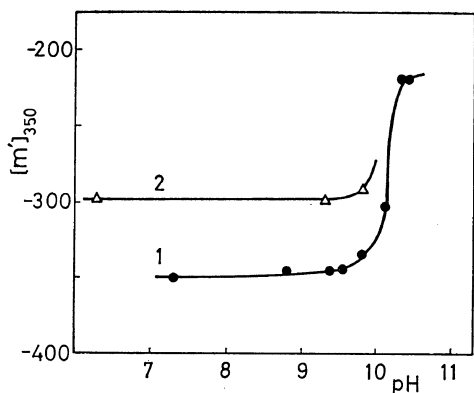


Figure 6. Relations between $[m']_{350}$ and pH for PLL and PLL—CMC complexes: ●, 0.0157-*M* PLL; △, PLL—CMC (1 : 0.091) complex.

electrolyte will be discussed. The ORD data for PLL and PLL—CMC complexes are shown in Figure 6, where measurements are carried out by increasing the pH of the solution stepwise. At pH higher than *ca.* 10, the increase of the faint turbidity in a complex solution disturbed the ORD measurements. The results from Figures 6 and 2b indicate that the coil-to-helix transition of PLL begins at pH 9.7 and $\alpha=0.69$. The data are in good agreement with those reported by Chou and Scheraga,³⁴ *i.e.*, pH=9.75 and $\alpha=0.6$ in 0.1-*M* KCl at 20°C.

As is apparent from Figure 6, the PLL—CMC (1 : 0.091) complex starts the conformational transition at almost the same pH as that of PLL; in this case a shift of the transition region due to complex formation is not observed. On the other hand, it has been reported⁷ that PLL exists in an α -helical conformation in PLL—poly(acrylic acid) (1 : 1.2) complex at pH 6.8. Such a difference between (polyacrylic acid) and CMC in the helix formation may be attributed to the difference in backbone chains; the former molecules are flexible, while the latter are semi-flexible. For this reason, CMC may not be able to take part in the formation of a α -helical structure with PLL, even partially.

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