Biophysical Chemistry 16 (1982) 67–77
Elsevier Biomedical Press

REVERSIBLE AND IRREVERSIBLE CONVERSION BETWEEN THE INTERMOLECULAR β-STRUCTURE AND THE DISORDERED STATE OF POLY(*S*-CARBOXYMETHYL-L-CYSTEINE) IN AQUEOUS MEDIA

Kaoru SAITO, Hiroshi MAEDA * and Shoichi IKEDA
Department of Chemistry, Faculty of Science, Nagoya University, Nagoya 464, Japan

Received 15th October 1981
Revised manuscript received 4th May 1982
Accepted 4th May 1982

Key words: *Poly(S-carboxymethyl-L-cysteine; Isodichroic point; Circular dichroism; Irreversible association; Intermolecular β-structure*

Conversion between the intermolecular β-structure and the disordered state of a fractionated low molecular weight sample of poly(*S*-carboxymethyl-L-cysteine) was examined mainly by the measurements of circular dichroism in the absence of salt as well as in the presence of 20 mM $NaClO_4$ or NaCl. In 20 mM $NaClO_4$ or NaCl solutions, the conversion was reversible. Under this condition, it was confirmed by direct and unambiguous evidence provided from the viscosity and the reduced scattering intensity that the β-structure was formed by intermolecular association. At low degrees of neutralization, the pH increased on dilution while it remained constant over a wide range of concentration at a high degree of neutralization. In the absence of salt, the conversion was often irreversible with respect to a concentration change at a constant degree of neutralization or to a change in the degree of neutralization at a constant concentration. The extent of the irreversible conversion decreased with the amount of β-structure in the solution. The dissociation of aggregates was very slow at low ionic strengths. It was inferred that the irreversible nature of the conversion arose from this slow dissociation of aggregates.

1. Introduction

The β-structure of oligopeptides is formed by intermolecular association. Since the pioneering work of Goodman et all [1], a considerable amount of work has been carried out on the β-structure of monodisperse oligopeptides in nonaqueous media. A critical concentration for the formation of the β-structure has been found in monodisperse oligopeptides of a given chain length [2]. The existence of a critical chain length (usually 6–7) for the β-formation has been also confirmed for a given concentration [3]. Relative stabilities of various β-structures formed by different kinds of oligopeptides can be estimated based on their critical concentrations [2]. Recently, monodisperse hexa(γ-benzyl-L-glutamate) was synthesized in our laboratory [4] and its association phenomena in nonaqueous media were investigated by means of infrared spectra [5], circular dichroism (CD) [5] and molecular weight determination by light-scattering measurements [6].

In aqueous media, however, only a few studies have been done on the β-structure formed by association. Poly(ethylene glycol)-bound monodisperse oligopeptides of L-alanine [7] and L-methionine [8] were found to form the β-structure in aqueous media. Monodisperse oligopeptides of L-lysine [9] and of L-glutamic acid [10,11] were prepared by ion-exchange chromatography. In the case of oligo(L-glutamic acid), Rinaudo and Domard [12] found that the β-structure was formed when the degree of polymerization (DP) was 8–12. When DP exceeded about 14, however, coexistence of the α-helix and the β-structure was noticed.

We have been studying the β-structure of poly(*S*-carboxymethyl-L-cysteine) in aqueous media [13–19]. In this polypeptide, contributions

---

* To whom correspondence should be addressed.

from the α-helix can be safely neglected. Although in the polypeptides of high molecular weights the β-structure is expected to be formed by intramolecular chain folding, the β-structure of a fractionated polypeptide of a low molecular weight ($\overline{DP}_w = 46$) is formed only by intermolecular association [19]. In the present study, the association-dissociation of another fractionated low molecular weight sample of the polypeptide has been investigated.

In the study of irreversible potentiometric titration of poly(methacrylic acid), Leyte et al. [20] have suggested a possible role of the irreversible conversion in a molecular mechanism of information storage. In this context, the irreversible association studied here may have biological significance, since this phenomenon is likely to occur in many polypeptides, which are more closely related to biological phenomena than is poly(methacrylic acid).

2. Experimental section

A sample (sample code SMA) of poly(*S*-carboxymethyl-L-cysteine) used in the present study was prepared in a similar but slightly different way to another sample (sample code KM) used in a previous study [19]. Sample SMA was obtained by collecting the fractions corresponding to an ionic strength range between 0.6 and 0.7 M eluted from a DEAE-cellulose column.

The degree of polymerization (DP) of sample SMA has not been determined at present but a reasonable estimate for this can be made. Light-scattering measurements in 0.2 M NaCl solutions at pH 7 gave a value of about 17, when combined with the refractive index increment determined on the nondialyzed solution. Accordingly, the DP may be larger than 17 when the effects of dialysis are taken into account. On the other hand, the polymers involved in sample SMA are suggested to have DP values larger than 17 from the study on ion-exchange chromatography (H. Maeda, to be published). All these results suggest that the average DP of sample SMA most likely lies between 20 and 30.

CD was recorded on a Jasco circular dichrograph J-40 at 25 ± 1°C, using cells of path lengths, 10, 5, 2, 1, 0.5 and 0.2 mm.

Measurements of pH were carried out with a Hitachi-Horiba pH meter Model F-7 SS under a nitrogen atmosphere.

For solutions of low concentrations (below about 5×10^{-4} N), care was taken to purge off carbon dioxide in solutions before the measurements of pH and CD by prolonged passage (30–90 min) of nitrogen saturated with moisture.

Light scattering was measured on a Chromatix low-angle light-scattering photometer KMX-6 at room temperature (24 ± 2°C) at a wavelength of 633 nm. For the present work a 6–7° annulus was chosen to observe the scattered light, which collected the light of about 4–5° scattering angle for the solutions. A 0.2 mm field stop was selected which defined the scattering volume. Solutions were filtered through a Millipore membrane filter of 0.22 μm pore size. For the solutions of low pH containing a considerable amount of aggregates (β-structure), membranes of pore size smaller than 0.45 μm did not work well and these solutions were filtered through a membrane of 1.2 μm pore size.

Viscosity was measured at 25.0 ± 0.1°C using an Ubbelohde-type viscometer. The flow time of solvent (20 mM $NaClO_4$) was 173 s.

Polymer concentration is expressed either in normality (residue molarity) C_p (N) or in weight c_p (g/dl) referred to the protonated species. Salt concentration (C_s), either added or produced by a neutralization-protonation cycle, is expressed in molarity (M). β denotes the degree of neutralization of side chain carboxyl groups. In low ionic strength media, an irreversible conversion was often observed. The solution must be specified by its composition (C_p, C_s, β) and the way of preparation.

Solutions were prepared mostly by the following two ways, unless otherwise stated. Solutions of given compositions (C_p, C_s, β) were prepared from solutions of a sodium salt (C_p, C_s^o, $\beta \simeq 1$) by the addition of HCl. Here, C_s differs from C_s^o by the amount of NaCl produced by the addition of HCl. This way of preparation is referred to as 'standard' throughout this paper. Alternatively, solutions (C_p, C_s, β) were prepared by dilution with water from corresponding solutions (C_p^o, C_s^o, β), where $C_p/C_p^o = C_s/C_s^o$ or by dilution with a salt solution (C_s) from corresponding solutions (C_p^o, C_s, β). Solutions were kept at room temperature (23–25°C) for 1–3 days before measurements. Values of residue ellipticities were constant and did not depend on the measuring time during this period.

3. Results

3.1. Dependence of the CD spectra on the β-structure-disorder conversion

The conversion between the β-structure and the disordered state was monitored by CD spectra. An example for a C_p of 7.5×10^{-4} N is given in fig. 1. At neutral pH, the CD spectrum (curve a) was characterized by two negative bands at 226–230 and 200 nm. When the pH was reduced to 4.6 by the addition of HCl, the spectrum depicted by curve b was different from curve a. However, this spectral change has been suggested to be associated with a change of optical properties of the chromophores involved (hereafter simply referred to as 'charge effect') rather than with a conformational change of polypeptide chains [18]. When the pH was lowered further, a band around 200 nm became positive, while a band around 225 nm became slightly more negative. This change arose from conversion to the β-structure. At pH 3.2, the CD spectrum (curve g) showed the presence of a considerable amount of the β-structure. The conversion was characterized by an isodichroic point near 218 nm. It occurred within a narrow pH region from curve b to curve g. According to a previous study [18], the changes of the residue ellipticity around 200 nm, $[\theta]_{200}$, are mostly contributed by the β-coil change, while one-third of the corresponding changes around 225 nm arises from the charge effect. In the present study, the residue ellipticities around 200–205 nm were used to represent the extent of conversion. When the pH was 2.9 (curve h), however, the isodichroic point disappeared, a positive peak around 200 nm decreased, and a negative peak around 225 nm became less negative. This change of CD spectra (from curve g to curve h) is supposed to originate from the stacking of the pleated sheets [18].

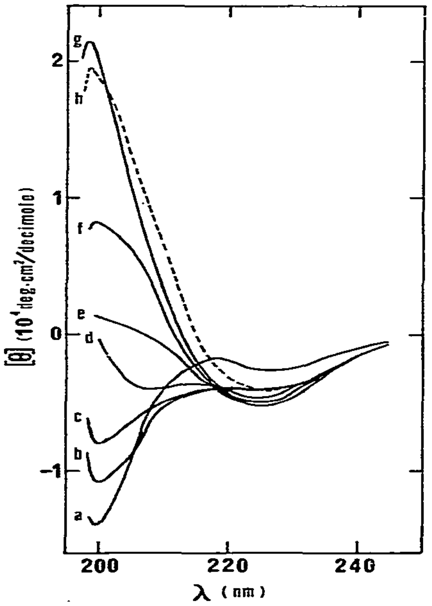

Fig. 1. CD spectra of poly(S-carboxymethyl-L-cysteine) at various pH values. $C_P = 7.5 \times 10^{-4}$ N. pH: (a) 6.64, (b) 4.57, (c) 4.00, (d) 3.70, (e) 3.49, (f) 3.32, (g) 3.22 and (h) 2.85.

3.2. The β-structure-disorder conversion in the presence of salts

Quantitative analyses of the association of polyions will be made easier if the ionic strength is higher. On the other hand, the solubility of the β-structure decreases with increasing ionic strength, as shown in the present study. Taking such situations into account, we have chosen an ionic strength of 20 mM as a condition of excess salt in this section, $NaClO_4$ was used throughout the experiments unless otherwise stated.

3.2.1. Evidence for association accompanying formation of the β-structure

When salt is present in excess ($C_s \gg C_p$), it is expected that the number of charges of a polyion is determined only by the pH. Accordingly, there should be no concentration dependence of the fraction of β-structure at a given pH, if association does not take place. Values of $[\theta]_{200}$ at various pH

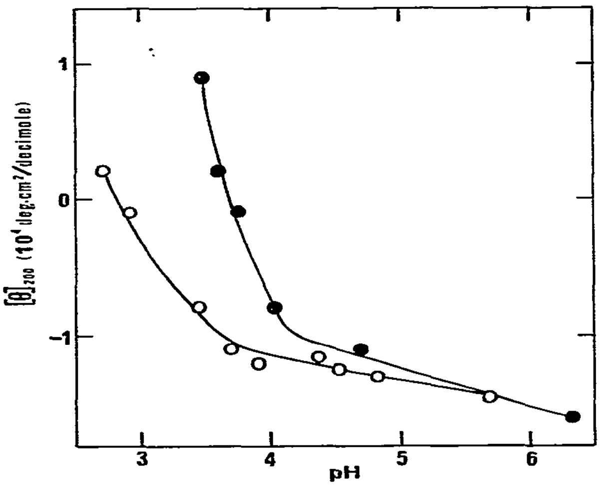

Fig. 2. Dependence of residue ellipticities at 200 nm on pH in 20 mM NaClO$_4$ solutions. $C_p = 6.0 \times 10^{-3}$ (●) and 1.0×10^{-4} N (○).

values are shown in fig. 2 for two concentrations, 6.0×10^{-3} and 1.0×10^{-4} N. The fraction of β-structure was larger for a higher concentration. This concentration dependence at high ionic strength indicated that association was coupled with formation of the β-structure. At low ionic strengths, where the pH depends on the polyion concentration [21–23], the dependence of the fraction of β-structure on the concentration cannot be related immediately to the effects of association.

Reduced viscosities, η_{sp}/c_p, at 0.096 g/dl (6.0×10^{-3} N) were measured at different pH values. When the association was absent, the reduced viscosity of the sample was only 0.06 dl/g, as shown in fig. 3. However, the reduced viscosity increased as the pH decreased below 3.75 and reached a very high value of 2.3 dl/g at pH 3.60. This marked increase in viscosity indicated an extensive association at low pH.

Light-scattering intensities were also measured at a concentration of 0.096 g/dl (6.0×10^{-3} N) for various pH values. In fig. 3, values of ΔR_θ are plotted against pH, where ΔR_θ is the reduced scattering intensity at an average scattering angle of 4–5° *. In parallel with the increase in reduced

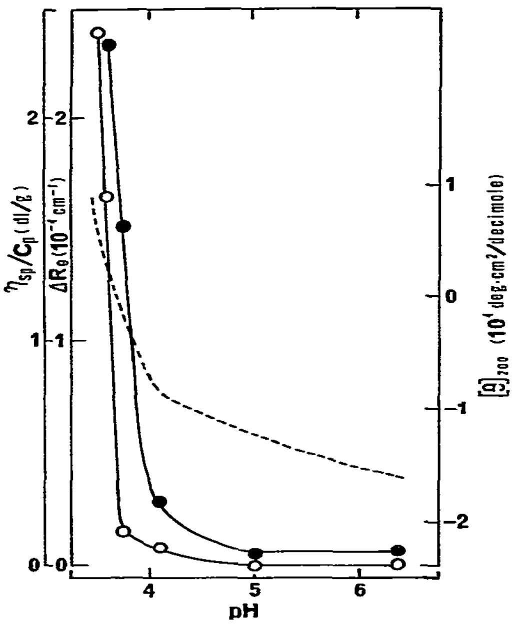

Fig. 3. Dependence of the reduced viscosities and reduced scattering intensities on pH in 20 mM NaClO$_4$ solutions. $C_p = 6.0 \times 10^{-3}$ N (0.096 g/dl). (●) Reduced viscosities (η_{sp}/c_p) and (○) reduced scattering intensities (ΔR_θ) for a scattering angle of 4–5°. $[\theta]_{200}$ in fig. 2 is also shown for comparison (dashed curve).

viscosity, the light-scattering intensity also increased sharply at low pH. The scattering intensity is related to the weight average molecular weight $\overline{M}_w$ by $Kc_p/\Delta R_\theta = \overline{M}_w^{-1}$, if the second virial coefficient is neglected and ΔR_θ is approximated as R_0. If a value of 0.19 ml/g is used for the refractive index increment **, then a value of 2.6×10^{-7} is assigned to the constant K*. Values of 0.6, 6.5 and 9.4×10^5 are obtained for $\overline{M}_w$ at pH values of 3.75, 3.60 and 3.51, respectively. From these values, the number of polymers associated is estimated to be larger than 10^2 at these low pH values.

The three independent measurements described above provide evidence for the coupling of association-dissociation with the β-structure-disorder

---

* Here, both K and ΔR_0 are defined as being twice as large as those usually adopted, since the factor $(1+\cos^2\theta)$, set equal to 2 in the present study, is included in K rather than ΔR_0.

** A value of 0.191 ml/g was obtained in 0.2 M NaCl solutions of poly[Cys(CH$_2$CO$_2$Na)] at 633 nm for the refractive index increment (25.0°C) at constant salt concentration.

change monitored by CD. It is noticeable in fig. 3 that the increase in residue ellipticity began to occur within the pH range where both reduced viscosity and scattering intensity remained unchanged. Small aggregates such as dimers might not be detected by viscosity or light scattering. Alternatively, the increase in residue ellipticity within this pH range might be partly due to the charge effect [18] as explained in fig. 1.

3.2.2. Dependence of the β-structure-disorder conversion on polymer concentration

The effects of concentration on the conversion between the β-structure and disordered state were examined at constant degrees of neutralization β. Results are shown in fig. 4a and b for three different values of β: 0.25, 0.15 and 0. Dilution was carried out from solutions of 4.0×10^{-3}, 2.0×10^{-3} and 1.44×10^{-3} N for the case of $\beta = 0.25$, 0.15 and 0, respectively. By using two series of preparation, standard and dilution, complete reversibility was confirmed at this ionic strength (20 mM) for all degrees of neutralization examined. In fig. 4b, results are shown by filled circules when NaCl was used in place of $NaClO_4$. No difference is seen between the data on these two series of solutions. In these figures, the residue ellipticity tends to that of the disordered state as C_p approaches 10^{-4} N or below. This important behavior provides further confirmation that the β-structure is formed only by association.

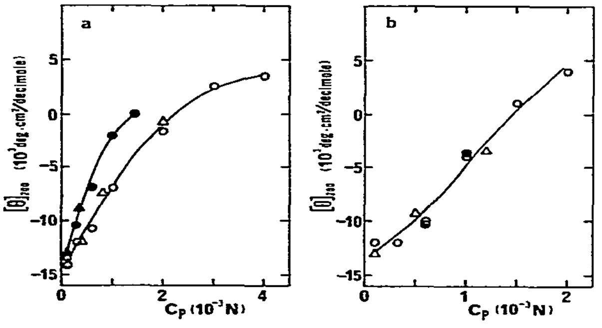

Fig. 4. Concentration dependence of residue ellipticities at 200 nm for different degrees of neutralization in 20 mM $NaClO_4$ solutions. Degrees of neutralization: (a) 0 (●, ▲), 0.25 (○, △); and (b) 0.15. Circles refer to the solutions prepared in the standard way (see text). Triangles refer to the solutions prepared by dilution from the solutions of 4.0×10^{-3} ($\beta=0.25$), 2.0×10^{-3} ($\beta=0.15$), and 1.44×10^{-3} N ($\beta=0$). Filled circles in b represent the results obtained in 20 mM NaCl solutions.

The concentration dependence of the residue ellipticity [19] or sedimentation constant [24] was previously reported also for the case of the intramolecularly folded β-structure where the residue ellipticity as 200 nm at low concentrations was kept at a constant value of about 2×10^4, which was interpreted as the value characterizing the intramolecular β-structure without aggregation [19].

In figs. 2 and 4, the maximum values of $[\theta]_{200}$ attained without precipitation are 10^4 or smaller in the presence of 20 mM $NaClO_4$. In the absence of added salt, on the other hand, the maximum value exceeds 2×10^4 as shown in fig. 1. Therefore, the solubility of the β-structure is clearly enhanced at low ionic strengths.

3.2.3. The dependence of pH on polymer concentration in the presence of excess salt

In the present study, the association of charged polymers is considered. Quantitative analyses of the association will be largely facilitated if the

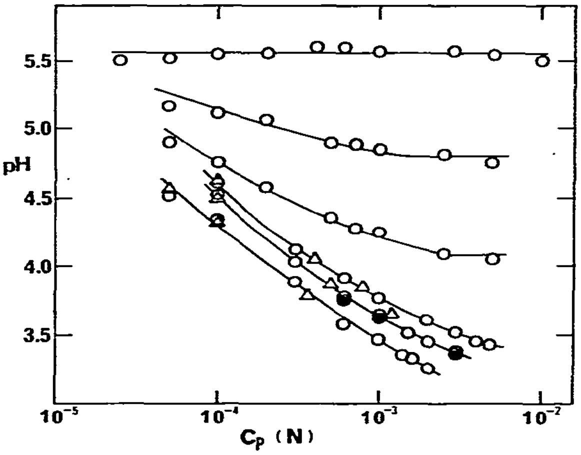

Fig. 5. Concentration dependence of pH at different degrees of neutralization in 20 mM $NaClO_4$ solutions. Degrees of neutralization (from top to bottom): 0.89, 0.70, 0.50, 0.25, 0.15 and 0. Results in 20 mM NaCl solutions are shown by filled circles at $\beta=0.15$. Circles and triangles have the same meanings as in fig. 4.

activity coefficient (due to electrostatic interactions) and the number of charges on each species are both kept constant irrespective of the polymer concentration. For a polyelectrolyte in the presence of excess salt, these conditions are supposed to hold and the pH does not increase significantly on dilution.

In fig. 5, values of pH are shown plotted against the logarithm of the polymer concentration at different degrees of neutralization β. At a high degree of neutralization ($\beta = 0.89$), the pH was constant (5.55 ± 0.05) over a wide concentration range of more than two orders (2.6×10^{-5}–1.0×10^{-2} N). This constancy of pH was just the behavior expected of polyelectrolytes and confirmed that electrostatic interactions among charged species were negligible. This in turn confirmed that both activity coefficient and charges were kept constant irrespective of the concentration.

It is reasonable to assume that the electrostatic activity coefficient was independent of the polymer concentration at any other degree of neutralization, since it was confirmed at a high degree of neutralization of 0.89.

It is also reasonable to assume that another requirement of constant charges was satisfied under the condition of excess salt, unless polyelectrolyte nature was violated.

At low degrees of neutralization, however, a considerable increase in pH occurred on dilution, as seen in fig. 5. The observed direction of the pH change was opposite to what was expected from the effects of association of polymers. Further, the pH change was found at $\beta = 0.5$, where the association did not occur appreciably. A similar pH dependence was also found for a fractionated oligomer, which did not undergo the association. From these results, it is clear that the association was not the cause of the observed pH change.

As is well known, the pH of a solution of a simple weak acid generally depends on its concentration in the presence of excess salt. In the present study, polymers of 20–30 repeating units are used. At a degree of neutralization of 0.1, for example, each polymer carries only two or three charges. The electrostatic properties of such a solution may resemble those of a mixture of simple electrolytes, such as partially neutralized weak acid with $NaClO_4$. Therefore, the observed pH changes at low degrees of neutralization in fig. 5 are well understood if the low molecular weight nature of the present sample is taken into consideration.

From this low molecular weight nature, it is also expected that the number of ionized carboxyl groups increases at extremely low concentrations even at a constant degree of neutralization. Some data suggesting enhanced ionization are provided from those concerning the isodichroic point. In fig. 6, the residue ellipticity at 218 nm, $[\theta]_{218}$, is plotted against polymer concentration for three degrees of neutralization, 0.25, 0.15 and 0. For a concentration range larger than 3–5×10^{-4} N, $[\theta]_{218}$ remained constant irrespective of the concentration for all the degrees of neutralization examined. When the concentration was lower than 3–5×10^{-4} N, however, $[\theta]_{218}$ deviated from this constant level and became less negative on dilution. As seen in fig. 1 and as described previously [18], the residue ellipticity around 218 nm becomes less negative as the contribution from charge effect becomes significant. The observed deviation of $[\theta]_{218}$ at low concentrations was consistent with the charge effect with respect to both direction and magnitude.

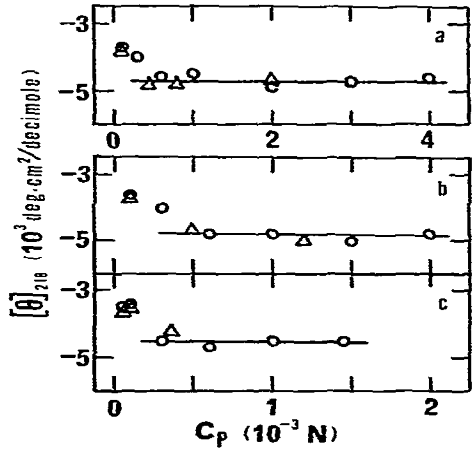

Fig. 6. Dependence of the residue ellipticities at 218 nm on the polymer concentration C_p in 20 mM $NaClO_4$ solutions. Degrees of neutralization: (a) 0.25, (b) 0.15 and (c) 0. Deviations at low concentrations are discussed in the text. Circles and triangles have the same meanings as in fig. 4.

In fig. 4a and b, the residue ellipticity $[\theta]_{200}$ tends to a value characterizing the disordered state (-10^4 or more negative) at concentrations where the charge effect is not yet noticeable. Therefore, the reasoning in the preceding section need not be altered even when the effect of the inferred self-ionization at low concentrations is taken into account.

The data on solutions containing NaCl are also shown for $\beta = 0.15$ by the filled circles in Fig. 5. As expected, no difference exists between the points indicated by filled and open circles. The reversibility with respect to pH was also examined at low degrees of neutralization where the association occurred to a considerable extent. As shown in fig. 5, there was no difference between the two series of solutions: prepared by the standard way (circles) and by dilution (triangles).

3.3. The β-structure-disordered state conversion at low ionic strengths

As pointed out in the preceding section, the solubility of the β-structure is greatly reduced on addition of salt. Hence, studies in media of low ionic strengths are favorable for qualitative characterizations including spectroscopic studies. In this section, we will examine the conversion between the disordered state and the intermolecular β-structure in the absence of added salt. NaCl is produced by neutralization-protonation reactions but its concentration is lower than or, at most, of the same order as the polymer concentration, $C_s \lesssim C_p$.

3.3.1. Dependence of the β-structure-disorder conversion on polymer concentration at constant degrees of neutralization

In fig. 7a and b, the residue ellipticities at 205 nm are plotted against C_p for two different values of β, 0.25 and 0, respectively. In these figures, data on the solutions prepared in the standard way are represented by circles, while those on the solutions prepared by dilution are given by triangles. As shown in fig. 7a where $\beta = 0.25$, the solutions prepared by dilution of a solution of $C_p = 2.5 \times 10^{-3}$ N showed similar values of $[\theta]_{205}$ to the solutions prepared in the standard way. The conversion was reversible in this case. On the other hand, the solutions prepared by dilution of a solution of $C_p = 4 \times 10^{-3}$ N showed different values of $[\theta]_{205}$ from those prepared in the standard way, i.e., these two kinds of solutions contained different amounts of the β-structure. The association-dissociation was thus irreversible. The extent of irreversible conversion was small if dilution was carried out from a solution of a low concentration. When fig. 7a ($\beta = 0.25$) is compared with 7b ($\beta = 0$), the irreversibility is reduced as the degree of neutralization increases. The dependence of the residue ellipicity at 218 nm on polymer concentration is given in fig. 8a and b for $\beta = 0$ and 0.25. The presence of an isodichroic point should be noted except at low concentrations ($C_p \lesssim 2 \times 10^{-4}$ N), where the charge effect modifies the CD spec-

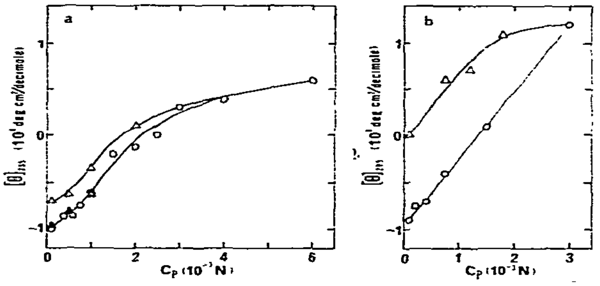

Fig. 7. Dependence of residue ellipticities at 205 nm on the polymer concentration in salt-free solutions. Degrees of neutralization: (a) 0.25 and (b) 0. Circles refer to solutions prepared in the standard way (see text). Triangles refer to solutions prepared by dilution from solutions of 4.0×10^{-3} (a, open), 2.5×10^{-3} (a, filled), and 3.0×10^{-3} N (b).

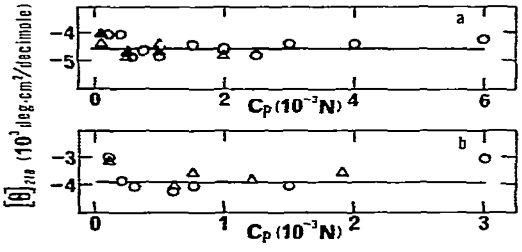

Fig. 8. Dependence of the residue ellipticities at 218 nm on the polymer concentration C_p in salt-free solutions. Degrees of neutralization: (a) 0.25 and (b) 0. Symbols have the same meanings as described in fig. 7a and b.

tra. Since an isodichroic point was found in both series of solutions, the difference between two solutions was the fraction of the β-structure, f_β, in spite of the identical composition (C_p, C_s, β). Solutions prepared by dilution contained a larger amount of the β-structure than those prepared in the standard way. It is likely that these two solutions correspond to a state of free energy minimum under a given set of state variables (C_p, C_s, β) plus a given value of the parameter f_β or f_β'. Here, a quantity with (without) prime refers to the solutions prepared by dilution (the standard way). Based on this assumption, it is expected that pH′ (C_p, C_s, β: f_β') is higher than pH (C_p, C_s, β: f_β), since $f_\beta' > f_\beta$. As shown later, this is found to be the case.

3.3.2. Dependence of the β-structure-disorder conversion on the degree of neutralization at constant polymer concentrations

The dependence of the β-structure-disorder conversion on the degree of neutralization was examined in detail in a previous study using another sample, KM [19]. Here, the results are summarized only schematically in fig. 9. In this type of experiment, another way of preparation was also employed. Solutions (C_p, $C_s = 0$, β) were prepared from solutions of zero degree of neutralization (C_p, $C_s = 0$, $\beta = 0$) by the addition of NaOH. At a high concentration (4×10^{-3} N), the conversion was reversible as judged from CD, as shown by curve a. When solutions of a low concentration (8×10^{-5} N) were prepared first by dilution at $\beta = 0$ followed by the addition of NaOH, they gave a definite conversion curve from β-structure to disorder (curve b). In contrast, when solutions (8×10^{-5} N) were prepared by the standard way, i.e., by the addition of HCl to the solutions at $\beta = 0.7$, they did not show conversion to the β-structure (curve c). Thus, the conversion was highly irreversible at a low concentration (8×10^{-5} N). Apparently, intermolecular association was essential for formation of the β-structure. When solutions ($C_p = 4 \times 10^{-3}$ N) of various degrees of neutralization were diluted to 8×10^{-5} N, then the residue ellipticities of the diluted solutions fell on curve b. Accordingly, curve b was realized by at least two different pathways.

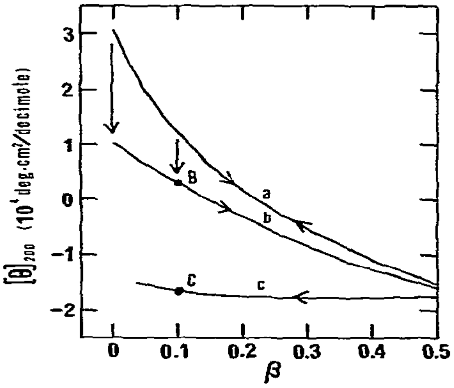

Fig. 9. Dependence of residue ellipticities at 200 nm on the degree of neutralization β. Curve a: $C_p = 4 \times 10^{-3}$ N. Curve b: $C_p = 8 \times 10^{-5}$ N, obtained on solutions prepared from a solution ($\beta = 0$) by the addition of NaOH. Curve c: $C_p = 8 \times 10^{-5}$ N, obtained on solutions prepared from a solution ($\beta = 0.7$) by the addition of HCl. Two states B and C of the same degree of neutralization are referred to in section 4.

3.3.3. Mixing of two solutions of identical composition

Two solutions of identical composition ($C_p = 6.0 \times 10^{-4}$ N, $C_s = 6.0 \times 10^{-4}$ N, $\beta = 0$) were prepared; one (solution a) by the addition of HCl to a solution ($C_p = 6.1 \times 10^{-4}$ N, $C_s = 0$, $\beta = 1$) and the other (solution b) by dilution from a solution ($C_p = C_s = 3.0 \times 10^{-3}$ N, $\beta = 0$). The corresponding CD spectra are shown in fig. 10. Solution b contained a larger amount of the β-structure than

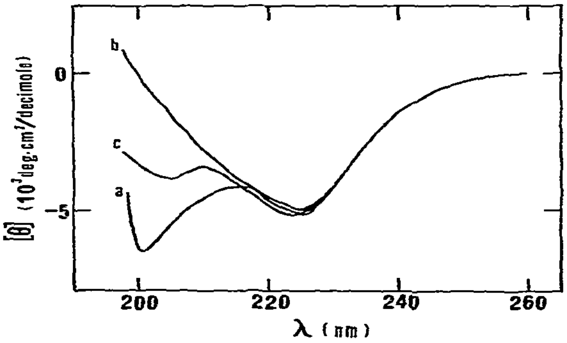

Fig. 10. CD spectra for three solutions of the same composition. $C_p = 6.0 \times 10^{-4}$ N, $C_s = 6.0 \times 10^{-4}$ M, $\beta = 0$. For details see text.

did solution a. Solution a had a pH of 3.74 while solution b had a pH of 3.84. By mixing these two solutions of equal volume, we prepared another, solution c, of the same composition. Even after a few days, the CD spectrum of solution c was approximately given as an average of spectra a and b. Solutions a and b were stable and the observed irreversibility was not due to the difficulty of nucleation in inducing intermolecular association for the β-structure.

3.3.4. Disappearance of irreversibility by the addition of salt

Effects of the addition of a neutral salt, $NaClO_4$, were examined on the two solutions which were used in the mixing experiments described in the preceding section. The results are shown in fig. 11. In solution a, the effects were negligible up to 10 mM. In solution b, effects appeared above a few mM. At 5 mM, the CD spectrum resembled spectrum c in fig. 10, suggesting dissociation of the β-structure. At 10 mM, the CD spectrum coincided with spectrum a in fig. 10 and the excess amount of β-structure in solution b over solution a completely disappeared and the two solutions became identical. Solution a was in an equilibrium state at any $NaClO_4$ concentration examined. Solution b was in a metastable state, which reached an equilibrium state when 10 mM $NaClO_4$ was added. At 5 mM $NaClO_4$, change of the residue ellipticity occurred within 1 day and the value remained constant afterwards for another 3 days. Accordingly, in the intermediate concentrations of $NaClO_4$, solution b was in another metastable state.

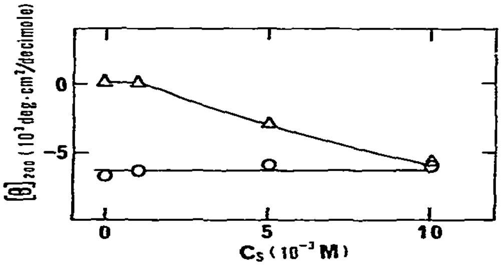

Fig. 11. Dependence of residue ellipticities on the concentration C_s of $NaClO_4$. $C_p = 6 \times 10^{-4}$ N, $\beta = 0$. Circles and triangles refer to solutions prepared from solutions a and b in fig. 10 (see text).

4. Discussion

4.1. The irreversible association-dissociation

In the absence of salt, an irreversible association-dissociation was found with respect to a concentration change at a constant degree of neutralization β (fig. 7) or with respect to β at a constant C_p (fig. 9). A summary of these two types of irreversibility can be stated as follows; a solution prepared from a solution of a higher β-structure content always contains a larger amount of the β-structure than the other solution of identical composition, prepared from a solution of the disordered state. Obviously, these two types of irreversibility arise from a common process, either a slow dissociation of aggregates or a slow association of polymers in the disordered state.

A solution corresponding to a point on curve b in fig. 9 can be prepared by at least two different pathways. This is a requirement for a given state to be an equilibrium state and any state defined by curve b corresponds to at least a metastable state. On the other hand, an equilibrium state should be established by fluctuations around it. However, the fluctuations around a state B, arbitrarily chosen on curve b, are largely suppressed, as shown by the following reasoning. A fluctuation toward a state of larger β-structure content is extremely suppressed, since the concentration of unassociated polymer in state B is lower than that of a state C. The fluctuation toward state C should also be suppressed, otherwise state B would change into state C. The idea of the difficulty of nucleation for association was explicitly shown as invalid by a mixing experiment (fig. 10). Finally, the addition of a simple salt provided a kinetic route for the dissociation of aggregates, which was otherwise difficult (fig. 11). From all these findings, it is now clear that the dissociation of aggregates is extremely difficult at low ionic strengths.

A possible model for the process of dissociation of aggregates will be derived from these results. When various interactions responsible for the β-structure (association)-disordered state (dissociation) conversion are considered, it is likely that nonelectrostatic interactions always favor the β-structure. This associative condition of a medium is counteracted by electrostatic repulsion, which favors the disordered state. These considerations lead us to a model in which electrostatic repulsive force is required for a single polymer chain to dissociate from either end of an aggregate. This repulsive force will be supplied by a transient increase in charge density at either end of an aggregate, produced by fluctuation. This charge fluctuation requires a work which depends on ionic strength. At low ionic strengths, large charge fluctuations do not occur easily and the dissociation is kinetically suppressed. If this model is valid, the result at 5 mM $NaClO_4$ in fig. 11 can be reasonably understood as follows. The aggregates have a size distribution and the aggregates of different sizes have different extents of charge fluctuation at a given ionic strength. Accordingly, aggregates smaller than a certain size can dissociate, while other aggregates remain unchanged at 5 mM ionic strength.

The aggregation of α-helical poly(L-glutamic acid) (PGA) in aqueous media has been observed and its irreversible nature has been studied [27]. In the case of PGA, the aggregation of α-helices occurred separately from the helix-coil transition. In contrast, the β-structure-disordered conversion is always coupled with association in the present case. For the β-structure, there is another aggregation process, i.e., stacking of the pleated sheets. This stacking process rather than the formation of pleated sheets is comparable with the aggregation of α-helices in the sense that is occurs, in essence, independently of the conformational change of polypeptide chains. In the case of PGA, the irreversible aggregation was examined with respect to temperature change, while irreversibilities with respect to both concentration and the degree of neutralization have been examined in the present study. A hysteresis loop was also observed for a temperature-dependent β-structure-disordered conversion of the present sample (K. Saito and H. Maeda, unpublished results).

4.2. Dependence of pH on polymer concentration at low degrees of neutralization

The dependence of pH on polyelectrolyte concentration is considered here based on the two-phase model proposed by Oosawa [26]. According to this model, the distribution of small ions around a polyion is given by eq. 1 in terms of a phase equilibrium between the inner phase (C_k', volume v) and the outer phase (C_k, volume $V-v$)

$$C_k' = C_k \exp[z_k e_0 \Delta\psi_0/kT] \equiv C_k \exp[z_k \Delta\phi_0] \quad (1)$$

Here, z_k is the valence of the k-th ion, e_0 the protonic charge and $\Delta\psi_0$ the electric potential difference between the two phases. The total number of k-th ions per polyion, N_k^t, is given by

$$N_k^t = C_k(V-v) + C_k' v \quad (2)$$

Approximately, the pH of the solution is given by $-\log C_H$ and the free volume per polyion, V, is inversely proportional to the polymer concentration C_p. The concentration dependence of pH is given by eq. 3, which is obtained when eqs. 1 and 2 are combined.

$$C_H = N_H^t[V - v + ve^{\Delta\phi_0}]^{-1} \quad (3)$$

For polyelectrolytes, the term $ve^{\Delta\phi_0}$ is much larger than $V-v$. Accordingly,

$$C_H = (N_H^t/v)e^{-\Delta\phi_0} \quad (4)$$

In the presence of excess salt, $\Delta\phi_0$ scarcely depends on C_p and the pH becomes independent of C_p. If, on the other hand, $e^{\Delta\phi_0}$ is close to unity, as found in the present study at low degrees of neutralization or in the case of simple weak acids, then

$$C_H = N_H^t/V \quad (5)$$

For strong acids, such as HCl, the total number of H^+ is kept constant and hence the pH varies linearly with log V. For partially neutralized simple weak acids, however, the dissociation equilibrium constant K_0 exists, which gives the following relationship between C_H and the degree of ionization α.

$$K_0 = C_H\alpha/(1-\alpha) \quad (6)$$

Accordingly, the increase in pH on dilution is not as large as what is expected from the above logarithmic dependence and α increases on dilution at a constant degree of neutralization β. Under the situation that eqs. 5 and 6 hold, v loses its significance and the two-phase model breaks down.

In the present study, we can expect the above two distinct dependences for the employed low molecular weight sample depending on the degree of neutralization. At a high degree of neutralization, $\Delta\phi_0$ is still large. Eq. 4 applies in this case and the pH does not depend on C_p. At low degrees of neutralization, $\Delta\phi_0$ is negligibly small and eqs. 5 and 6 hold. We can expect in this case that the pH increases considerably on dilution and that the self-ionization takes place appreciably at extremely low concentrations.

Acknowledgement

This work is done with partial financial support from the Ministry of Education, Science and Culture Grant No. 56540256.

References

1 M. Goodman, E.E. Schmitt and D.A. Yphantis, J. Am. Chem. Soc. 84 (1962) 1288.
2 C. Toniolo and G.M. Bonora, in Peptides: chemistry, structure and biology, eds. R. Walter and J. Meienhofer (Ann Arbor Science, Ann Arbor, MI (1975) p. 145.
3 C. Toniolo and G.M. Bonora, Makromol. Chem. 175 (1974) 2203.
4 K. Okahashi and S. Ikeda, Int. J. Peptide Protein Res. 13 (1979) 462.
5 K. Okahashi and S. Ikeda, Biopolymers 18 (1979) 2105.
6 S. Ikeda and K. Okahashi, Biopolymers 18 (1979) 2115.
7 M. Mutter, H. Mutter, R. Uhmann and E. Bayer, Biopolymers 15 (1976) 917.
8 C. Toniolo, G.M. Bonora, S. Salardi and M. Mutter, Macromolecules 12 (1979) 620.
9 A. Yaron, M.C. Otey, H.A. Sober, E. Katchalski, S. Ehrlich-Rogozinskia and A. Berger, Biopolymers 11 (1972) 607.
10 R.L. Snipp, W.G. Miller and R.E. Nylund, J. Am. Chem. Soc. 87 (1965) 3547.
11 M. Rinaudo and A. Domard, Biopolymers 14 (1975) 2035.
12 M. Rinaudo and A. Domard, J. Am. Chem. Soc. 98 (1976) 6360.
13 S. Ikeda, Biopolymers 5 (1967) 359.
14 S. Ikeda and G.D. Fasman, J. Mol. Biol. 30 (1967) 491.
15 H. Maeda and S. Ikeda, Biopolymers 14 (1975) 1623.
16 S. Ikeda, A. Fukutome, T. Imae and T. Yoshida, Biopolymers 18 (1979) 335.
17 K. Ooi, H. Maeda and S. Ikeda, Biopolymers 19 (1980) 1743.
18 H. Maeda and K. Ooi, Biopolymers 20 (1981) 1549.
19 H. Maeda, K. Kadono and S. Ikeda, Macromolecules (1982) in the press.
20 J.C. Leyte, H.M.R. Arbouw-van der Veen and L.H. Zuiderweg, J. Phys. Chem. 76 (1972) 2559.
21 H. Maeda and F. Oosawa, J. Phys. Chem. 76 (1972) 3445.
22 G.S. Manning and A. Holtzer, J. Phys. Chem. 77 (1973) 2206.
23 K. Nitta and S. Sugai, J. Phys. Chem. 78 (1974) 1189.
24 E. Patton and H.E. Auer, Biopolymers 14 (1975) 849.
25 B.R. Jennings, G. Spach and T.M. Schuster, Biopolymers 6 (1968) 635
26 F. Oosawa, Polyelectrolytes (Marcel Dekker, New York, 1971) Ch. 2, 6 and 8.