Temperature-dependent β structure–random coil conversion of poly(S-carboxymethyl-L-cysteine)

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The effect of temperature on the β structure–random coil conversion of poly(S-carboxymethyl-L-cysteine) has been examined on the following well defined systems: folded-chain β and extended-chain β structures. The conversion was reversible but the rate of β formation was very slow for both types of β structure. For folded-chain β structure, the rate of β formation decreased with decreasing polymer concentration. Optical activity associated with the nπ* transition of peptide chromophore scarcely changed with the temperature-induced β–coil conversion.

(Keywords: temperature-induced conformational change; β structure-random coil conversion; poly(S-carboxymethyl-L-cysteine))

INTRODUCTION

Studies on the β structure–random coil conversion of synthetic polypeptides in aqueous media have been performed mainly on three kinds of polypeptides: poly(L-lysine) (PLL)[1–6], poly(L-tyrosine) (PLT)[7–14] and poly(S-carboxymethyl-L-cysteine)[15–22]. The effect of temperature on the β–coil conversion on PLL[4] and PLT[11] has been examined with potentiometric titrations. However, reported values of the enthalpy of β formation were negative for PLT[11] but positive for PLL[4]. Implicit problems involved in the potentiometric titration of β-PLL have recently been discussed[23]. Coil–β conversion with temperature at constant charge has not been examined on PLT and in the case of PLL it will be highly limited since the β structure is stable only at high temperature and low charge densities.

The β–random coil conversion of poly(S-carboxymethyl-L-cysteine) (poly(Cys(CH_2COOH))) in aqueous solutions has been studied mostly by changing the pH of the medium[15–21] or changing polypeptide concentration in the case of intermolecular β structure of short extended chains[18–20]. Temperature dependence of the conversion was examined only briefly[15–17]. Furthermore, these studies were done with solutions containing various types of β structure: intramolecularly folded, intermolecularly associated and aggregates consisting of both types. In the present study, temperature dependence of the random coil (or disordered state)–β structure conversion of poly(Cys(CH_2COOH)) is examined on two well defined systems: folded-chain β structure of long chains and intermolecular β structure of short extended chains.

EXPERIMENTAL

Average degrees of polymerization (*DP*) of the two samples of poly(S-carboxymethyl-L-cysteine) (poly(Cys(CH_2COOH))) used in the present study are 630 and 20. The long-chain sample is the same as that used in previous studies[21,22]. The short-chain sample was obtained by fractionation with ion-exchange chromatography[19]. Circular dichroism (c.d.) spectra were obtained with a Jasco J 40 A circular dichrograph, using cells of light path 1 and 10 mm. Four scans were averaged in most cases.

Polymer solutions were prepared first at neutral pH where polypeptides were molecularly dispersed as fully ionized random coils. Then the pH of the solution was adjusted by the addition of HCl or $HClO_4$. The solutions were kept at least 15 h at room temperature (24 ± 2°C) to attain equilibrium. Then solutions were heated to prescribed temperature in the cells for c.d. measurements.

The β content f_β was calculated from residue ellipticity $[\theta]$ at a fixed wavelength from:

$$f_\beta = ([\theta] - [\theta]_c)/([\theta]_\beta - [\theta]_c) \qquad (1)$$

where $[\theta]_\beta$ and $[\theta]_c$ represent the values for 100% β structure and random coil. A value of 3.6×10^4 determined at 25°C in the previous study[21] was tentatively used for $[\theta]_\beta$ at 200 nm at any temperature, ignoring its temperature dependence. On the other hand, values for $[\theta]_c$ were taken from the c.d. spectra of fully charged random coils at a given temperature as given by *Figure 2*.

RESULTS

Folded-chain β structure of the high-molecular-weight sample ($DP_w = 630$)

According to a recent characterization of the sample in 50 mM NaCl solutions[21], the β structure is formed by chain folding. This intramolecular β structure exists in a molecularly dispersed state with a negligible extent of aggregation at a very low concentration (C_p) of about 1×10^{-4} M (~17 mg l^{-1}). However, aggregation of

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folded-chain β structures is dominant at high concentrations such as $C_p = 1 \times 10^{-3}$ M.

In *Figure 1*, the c.d. spectra of a solution ($C_p = 1 \times 10^{-4}$ M, 50 mM $NaClO_4$, pH = 4.81) at two different temperatures are shown. Spectra at 25°C are represented by full and chain curves and those at 63°C are indicated by a broken curve. It is seen that folded-chain β structure is destroyed as the temperature increases. Although the conversion is reversible with respect to temperature change, the formation of the β structure is very slow. The spectrum represented by the chain curve was obtained about 20 h after the solution was cooled down to 25°C from 63°C. It is to be noted that the change of the c.d. spectra occurs in the range of 200 nm but it is negligible in the range of 225 nm. The β content f_β changes from about 65% at 25°C to about 0% at 63°C, as estimated from the change of residue ellipticity at 200 nm, $[\theta]_{200}$.

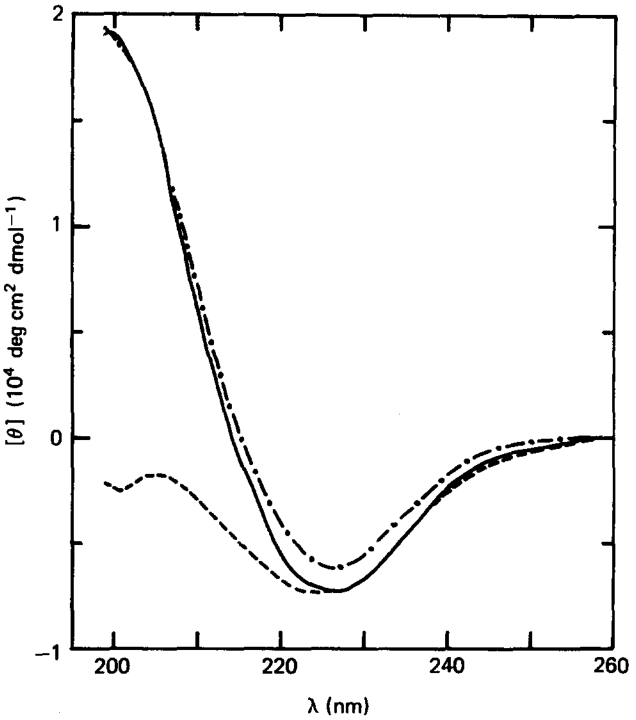

Figure 1 C.d. spectra of folded-chain β structure of poly(Cys(CH_2COOH)) ($DP = 630$) as a function of temperature. Temperature: 25°C (———), 63°C (-----) and 25°C (–·–·–) about 25 h after cooling down from 63°C. $C_p = 1.0 \times 10^{-4}$ M; 50 mM $NaClO_4$; pH = 4.81 (at 25°C)

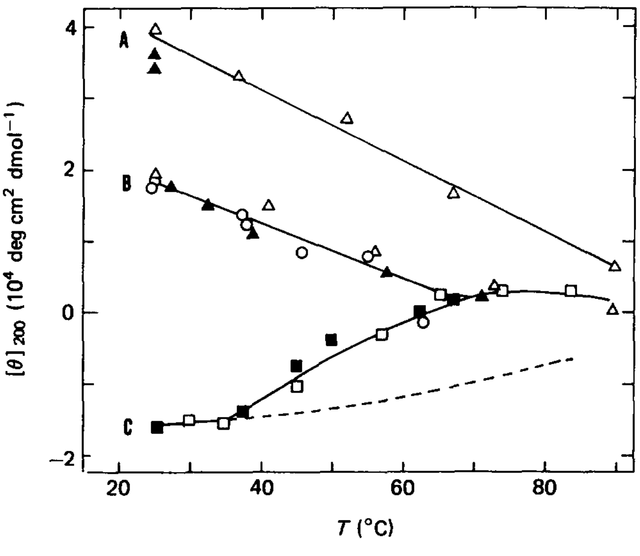

Figure 2 Temperature dependence of the residue ellipticities at 200 nm of poly(Cys(CH_2COOH)) ($DP = 630$) in 50 mM NaCl. Curve A, $C_p = 8.6 \times 10^{-4}$ M, pH 4.8; curve B, $C_p = 1.0 \times 10^{-3}$ M, pH 4.99 (triangles) and $C_p = 1.0 \times 10^{-4}$ M, pH 4.83 (circles); and curve C, $C_p = 1.0 \times 10^{-4}$ M, pH 6.67 (squares)—the broken curve represents the residue ellipticity at band positions. Open (filled) symbols refer to heating (cooling) process

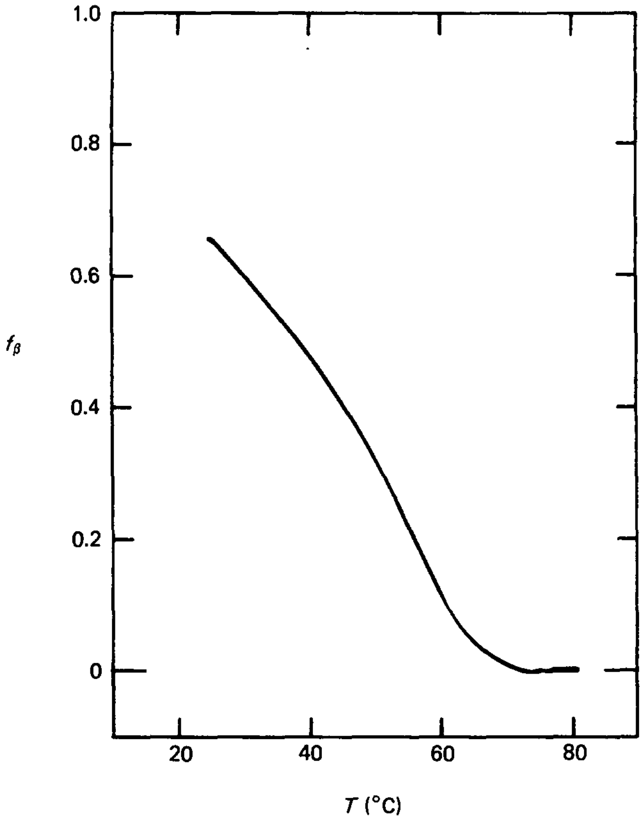

Figure 3 Temperature dependence of β content f_β of approximately unimolecular conversion. $C_p = 1.0 \times 10^{-4}$ M; pH = 4.83 (25°C); 50 mM NaCl

In *Figure 2*, values of $[\theta]_{200}$ are shown for different temperatures. Curves A and B refer to the solutions of different β contents at 25°C. Data represented by triangles were obtained at high concentrations ($\sim 1 \times 10^{-3}$ M). A solution was first heated from 25°C to specified temperatures successively, as shown by open symbols. Then, the solution at 90°C was cooled down to specified temperatures successively as shown by filled symbols. For curves A and B, data represented by filled triangles were obtained at about 2–3 h and 30 min, respectively, after each prescribed temperature was attained. The results indicate that β formation is much faster at high concentrations than at 1×10^{-4} M shown in *Figure 1*.

In *Figure 2*, results at $C_p = 1 \times 10^{-4}$ M are also shown with circles on curve B. Since the reaction at this concentration can be approximated as unimolecular conversion[21], it is pertinent to define an equilibrium constant K as

$$K = f_\beta / (1 - f_\beta) \qquad (2)$$

The dependence of f_β on temperature is given in *Figure 3*. It is interesting to note that sharpness of the conversion in *Figure 3* is comparable to that of thermally induced α-helix–coil conversion of poly(L-glutamic acid) (PGA)[24] and poly(N^5-(4-hydroxybutyl)-L-glutamine) (PHBG)[25].

This finding is consistent with the previous observation[21] that pH width is also similar for two pH-induced conversions: helix–coil conversion of PGA and β–coil conversion of poly(Cys(CH$_2$COOH)). The enthalpy of β formation ΔH_β is given as

$$\Delta H_\beta = [RT^2/f_\beta(1-f_\beta)](\partial f_\beta/\partial T) \qquad (3)$$

A value of about −43 kJ (10 kcal) was obtained for ΔH_β, when evaluated at $f_\beta = 0.5$. On the other hand, a value of −7.1 kJ (1.7 kcal) was recently determined for ΔH_β per uncharged residue from the temperature dependence of the solubilities of monodisperse oligopeptides of poly(Cys(CH$_2$COOH))[26]. Hence, a cooperative unit consisting of about six residues is suggested for temperature-induced conversion. When the data on the thermal helix–coil conversion[24,25] are analysed in the same way, enthalpies of α-helix formation ΔH_α are about −40 kJ (9.6 kcal) and −30 kJ (7.2 kcal) for PGA[24] and PHBG[25], respectively. In the case of helix–coil conversion, however, the enthalpy change for an uncharged residue has been evaluated to be about −200 to −100 cal[25,27]. Hence, a large cooperative unit of about 10–40 residues is calculated. On the other hand, the quoted value of −7.1 kJ (1.7 kcal) for the β structure refers to the uncharged residue in three-dimensional precipitates, much more stabilized than the unaggregated folded-chain β structure in solution. Further, since ΔH_β obtained in the present study contains an electrostatic contribution, which is positive, the value of ΔH_β for uncharged polymers should be more negative. Therefore, the estimated cooperative length here should be taken as the minimum value.

In *Figure 4*, typical kinetic data on β formation are indicated at two different concentrations. The curves represent the time course of the extent of reaction *f*, defined by equation (4), after the temperature was cooled to 25°C from 85°C:

$$f = \{[\theta](\infty) - [\theta](t)\}/\{[\theta](\infty) - [\theta](0)\} \qquad (4)$$

Here, $[\theta](t)$ denotes the residue ellipticity at time *t* for a chosen wavelength (usually 205–208 nm). Final values ($[\theta](\infty)$) were determined at 21–72 h and 11–50 h after cooling down to 25°C for $C_p = 1\times10^{-4}$ M (a) and 1×10^{-3} M (b), respectively. The cooling time was different for different concentrations since light paths of the cells used differed by a factor of 10. Final temperatures were attained within about 3 min for the 1 mm cell and 10 min for the 10 mm cell. Accordingly, the time course within cooling time was not indicated. Since the conversion takes place in a very narrow pH region, especially at $C_p = 1\times10^{-3}$ M[21], the β content f_β or $[\theta]_{200}(\infty)$ works better as a pH indicator than the measured pH value itself. At low pH where the β structure carries a small number of charges, the rate of β formation scarcely depends on the polymer concentration, as shown by curves labelled A. This suggests that aggregation occurs rapidly at this low pH even at low concentration. At a higher pH range where both β structure and random coils are present to a significant amount (curves B, C and D), the rate of β formation becomes slow irrespective of concentration. Furthermore, the rate is much smaller at a low concentration (a) than at a high concentration (b). This may be seen when two curves labelled B are compared. The present kinetic behaviour at 1×10^{-4} is consistent with equilibrium study on pH-dependent conversion at the same concentration[21]. In the conversion region, aggregation is largely suppressed at this low concentration. The effect of polymer concentration was also examined under conditions corresponding to the final β content of about 53–60%, as shown in *Figure 5*. Some of the time courses of β formation in *Figures 4* and *5* reach constant values within about 60 min, which are, however, appreciably greater than zero, completion of the reaction. This behaviour clearly indicates the presence of a very slow process in the formation of the β structure, extending over several tens of hours. The kinetic data in the present study cannot be described in terms of a single-exponential term nor the superposition of two such terms. This behaviour is in contrast with the reported kinetics on PLL[5] or PLT[10,14], but is consistent with the behaviour for a long-chain molecule, where many relaxation times are generally expected, just like the helix–coil transition[28].

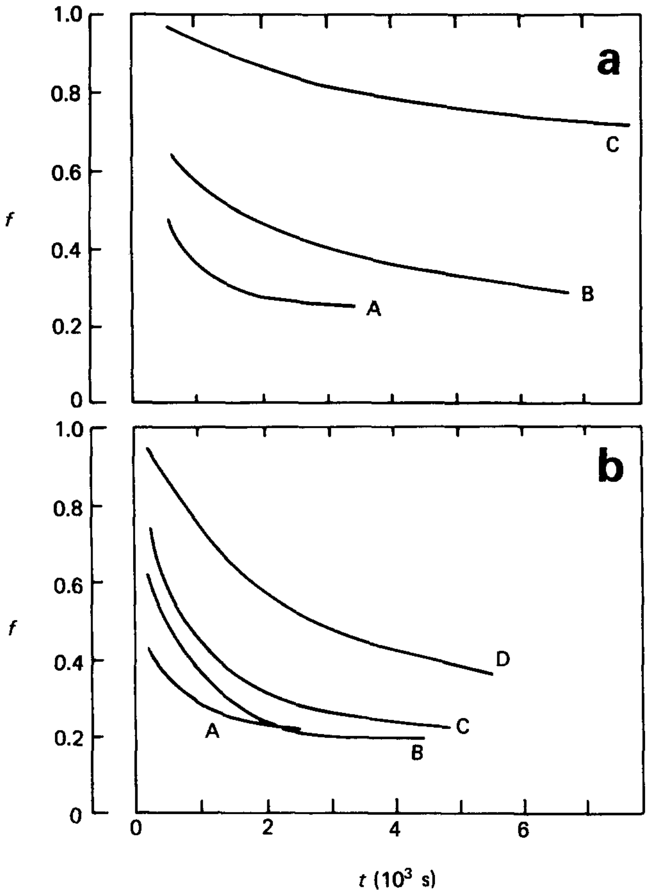

Figure 4 Kinetics of β formation of poly(Cys(CH$_2$COOH)) ($DP = 630$) in 50 mM NaCl at 25°C cooled from 85°C. The extent of reaction *f* is defined in the text (equation (4)). (a) $C_p = 10\times10^{-4}$ M; $[\theta]_{200}(\infty)$ (10^4 deg cm^2 dmol^{-1}) = 3.9 (A), 2.0 (B) and 1.5 (C); pH = 4.59 (A), 4.82 (B) and 4.92 (C). (b) $C_p = 1.0\times10^{-3}$ M; $[\theta]_{200}(\infty)$ (10^4 deg cm^2 dmol^{-1} = 3.8 (A), 2.2(B), 1.8 (C) and 0.6 (D); pH = 4.74 (A), 4.97 (B), 4.98 (C) and 4.98 (D)

In most experiments, the values of $[\theta]_{200}(\infty)$ coincided with those before heating. At $C_p = 1\times10^{-4}$ M, however, it was found sometimes that the values of $[\theta]_{200}(\infty)$ taken at 2–3 days after cooling down were smaller than those before heating. At $C_p = 1\times10^{-3}$ M, this type of irreversible behaviour was scarcely observed. This irreversible behaviour was considered to arise from a slight pH change accompanying a heating–cooling process (within a measurement precision ±0.02) or from any degradation at high temperature. However, the use of

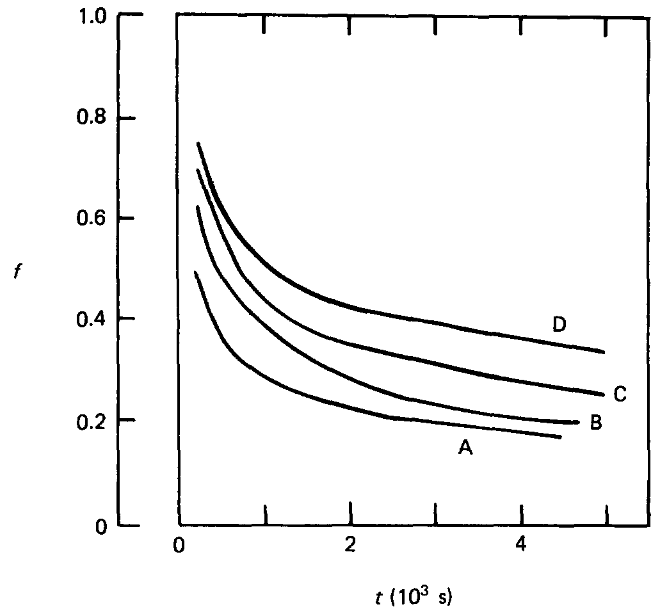

Figure 5 Kinetics of β formation at different polymer concentrations in 50 mM NaCl at 25°C cooled from 85°C. The extent of reaction f is defined in the text (equation (4)). $[\theta]_{200}(\infty)$ (10^4 deg cm^2 dmol^{-1}) = 1.4–1.8; pH = 4.96–4.99; C_p (1.0×10^{-3} M) = 5.0 (A), 4.0 (B), 3.0 (C) and 1.5 (D)

acetate buffer (20 mM) revealed unfavourable results; the irreversible behaviour became significant even at $C_p = 1 \times 10^{-3}$ M. This was one of the reasons why buffer solutions were not employed in the present study. No chemical modifications occurred at high temperatures such as chain scission or partial degradation. This was confirmed by the following experiments on the solutions once heated up to 90°C for 1 h. Molecular weight distribution revealed on a Sephadex G-150 column was identical to that of the original sample. The pH-induced β–coil conversion curve of the solution was superimposable on that obtained on the original sample.

Kinetics of disintegration of the β structure were also examined at 82–86°C for two concentrations, 1×10^{-4} and 1×10^{-3} M. The reaction was nearly completed at both concentrations after the heating time was over. Therefore, disintegration of the β structure seems to occur much faster than its formation. It is to be noted, however, that the rate of disintegration of the β structure was studied at much higher temperature than that for the β formation (25°C).

The temperature dependence of c.d. spectra of charged random coils is shown in *Figure 6* and also in *Figure 2* (curve C and broken curve). The c.d. spectra of random coils vary significantly with temperature and the change is reversible. At 83.6°C (broken curve), the band around 200 nm becomes much less negative and the band position is red-shifted. These changes resemble the temperature dependence of the c.d. spectra of charged random coil PGA[29]. Changes of c.d. spectra were fast in the case of random coils for both increasing and decreasing temperature. It is to be noted in *Figure 6* that the negative band around 225 nm is scarcely affected by a temperature change. The broken curve in *Figure 2* represents the residue ellipticities at band positions.

Intermolecular β structure of the low-molecular-weight sample (DP = 20)

This sample consists of short chains and cannot form the β structure by chain folding. The β structure of the sample is formed thoroughly by intermolecular association of extended chains[18–20]. Therefore, at a given pH, the β structure is formed at high concentrations but only disordered chains exist at low concentrations[18–20].

Figure 7 shows the c.d. spectra at three temperatures of a solution, $C_p = 1.2 \times 10^{-3}$ M, 20 mM NaClO$_4$, degree of neutralization 0.15. Changes of the c.d. spectra accompanying a temperature change occur only around 200 nm. It has been found in the case of intermolecular β structure that c.d. spectra around 225 nm change only little when it is induced by a concentration change[19,20] or by pH change[19]. *Figure 7* clearly indicates the conversion to the disordered state from the intermolecular β structure with increasing temperature. A heating curve (the full curve through the open circles) in *Figure 8* started from 25°C reaches a random-coil region above 60°C, since in this region $[\theta]_{200}$ becomes less negative as

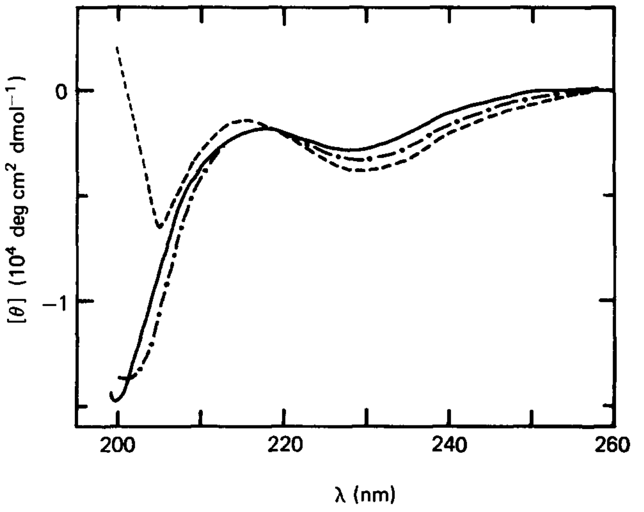

Figure 6 C.d. spectra of charged random coils of poly(Cys(CH$_2$COOH)) ($DP = 630$) as a function of temperature in 50 mM NaCl. Temperature: 25°C (———), 83.6°C (-----) 25°C (-·-·-) after cooling down from 83.6°C. $C_p = 1.0 \times 10^{-4}$ M; pH = 6.67

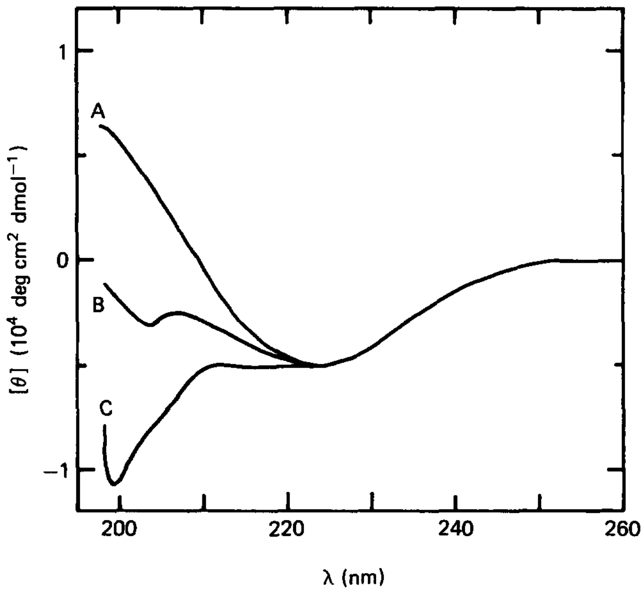

Figure 7 C.d. spectra of intermolecular β structure of poly(Cys(CH$_2$COOH)) ($DP = 20$) as a function of temperature in 20 mM NaClO$_4$. $C_p = 1.2 \times 10^{-3}$ M; degree of neutralization 0.15. Temperature = 2°C (A), 25°C (B) and 60°C (C)

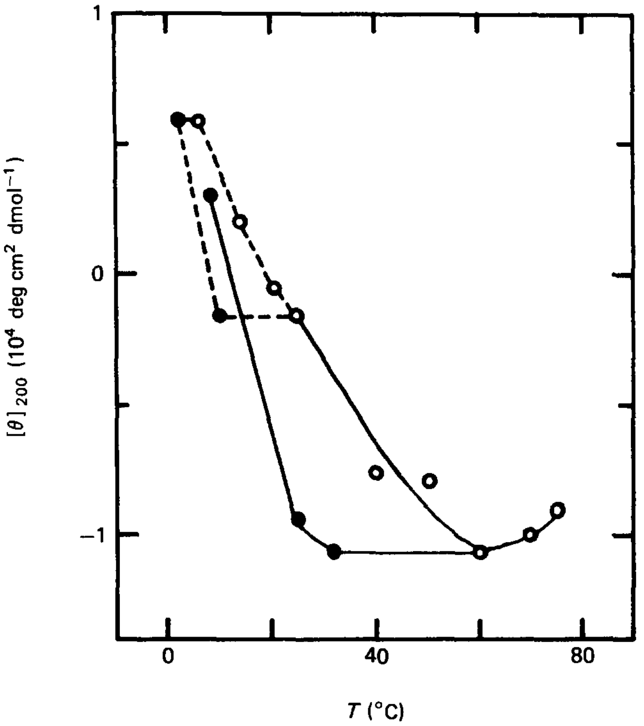

Figure 8 Temperature dependence of the residue ellipticities at 200 nm $[\theta]_{200}$ of short-chain poly(Cys(CH_2COOH)) ($DP=20$) in 20 mM $NaClO_4$. Degree of neutralization 0.15. Open (filled) circles refer to heating (cooling) process

temperature increases, just as seen in *Figure 2* for long-chain random coils. When cooled down to 25°C, however, formation of the β structure did not occur readily and further cooling to 8°C accelerated β formation. These changes are represented by the full curve through the filled circles. Similar hysteresis was observed when a solution at 25°C was first cooled down to 2°C (the broken curve through the filled circles) and then heated to 25°C (the broken curve through the open circles).

DISCUSSION

Rate of formation of the β structure

In the present study, it was found that while disintegration of the β structure occurred readily at high temperatures, its formation at 25°C was very slow for both intramolecular and intermolecular β structures.

For folded-chain β structure, the rate depends on the polymer concentation except at low pH. The rate becomes smaller as the concentration decreases. At high concentrations, the formation occurs rather fast, yet there remains a slow process as shown in *Figures 4* and *5*. In the case of β formation of poly(L-lysine) (PLL), it has been suggested that intramolecular chain folding is difficult for an isolated chain but it readily occurs for a chain adsorbed on a β aggregate[6].

For intermolecular β structure of short extended chains, where the problem of difficult nucleation for chain folding is absent, the rate is also small even when the initial state contains a significant amount of the β structure (*Figure 8*, broken curves). This result suggests that association of two strands is the rate-limiting step. Therefore, enhancement of the formation rate at high concentrations in the case of the long-chain sample suggests that intermolecular association facilitates not only the nucleation step but also the growth step of the folded-chain β structure.

Several studies on other systems should be mentioned here. The rate of conversion of the α-helix to β structure of PLL was measured at 44–55°C[3,5], which has been known to be considerably irreversible. A pH-induced conversion of coil to β of PLL was also measured[6] at a high temperature of 55°C. The typical time domain in these two types of kinetics is 40–80 min around 50°C, which is not much different from that found in the present study. In the case of poly(N^{ε}-methyl-L-lysine) and poly(N^{δ}-ethyl-L-ornithine)[30], the kinetics of α to β conversion is similar to that of PLL. However, little has been proved about the presence of a slow process in these studies. In the case of poly(L-tyrosine), the rate of β formation induced by a pH change was measured[10,14], and two typical time domains observed are 10 ms and 1 s[14].

Ellipticity around 225 nm as a tool to characterize the β structure of poly(Cys(CH_2COOH))

The optical activity around 225 nm associated with the $n\pi^*$ transition of poly(Cys(CH_2COOH)) has at least three contributions: charge effect[31], environmental effect and chain configuration. Changes of c.d. spectra accompanying a pH-induced β–coil conversion generally involve all these three contributions.

In the case of a short-chain intermolecular β structure, it has been shown that $[\theta]_{225}$ remains constant when the conversion is induced by a concentration change at low pH. For a pH-induced conversion, $[\theta]_{225}$ varied at the initial stage of the conversion due to the charge effect but it remained constant for the rest of the conversion region, where $[\theta]_{200}$ changed with pH[19]. Contrary to the intermolecular β structure, almost all data to date show that the negative c.d. band around 225 nm develops with the formation of folded-chain β structure. In the present study, $[\theta]_{225}$ did not change appreciably accompanying the conformational change from the β structure to random coils for both folded-chain and extended-chain β structures. Therefore, the present result from the temperature-dependent conversion is consistent with previous results[19,20] in the case of short chains but it is in contrast with them in the case of long chains. Recently, it has been found that $[\theta]_{225}$ remains constant when the β structure is induced by a pH change in 3 M guanidine hydrochloride but it becomes more negative, as usual, in other media containing a denaturant such as urea, LiCl and betaine, as the β structure is formed[22]. It is hoped, therefore, that $[\theta]_{225}$ provides some information about the nature of the β structure in addition to chain configuration.

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