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The Effect of Chain Length on the Formation of the Intermolecular β-Structure of Poly(*S*-carboxymethyl-L-cysteine)

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ABSTRACT: The formation of the β-structure of well-fractionated low molecular weight samples of poly(*S*-carboxymethyl-L-cysteine) was examined. In solutions with no added salt and a polymer concentration of 1×10^{-3} residue mol dm^{-3} (N), the β-structure was formed at a pH lower than about 3.5 for one sample ($DP_n = 15.6$), while it was not formed throughout the entire soluble range of pH for another sample ($DP \simeq 12$). The concentration dependent β-disordered conversion of a sample ($DP_n = 15.6$) was not reversible. In 20 mM $NaClO_4$ solutions, two samples, $DP \simeq 10$ and $DP_n = 15.6$, remained essentially in a disordered state throughout the entire soluble range of pH when examined at a polymer concentration of 1×10^{-3} N. The chain length dependent formation of the β-structure was compared with available data on oligo(L-glutamic acid) [M. Rinaudo and A. Domard, *J. Am. Chem. Soc.*, **98**, 6360 (1975)]. An uncharged β-structure consisting of *S*-carboxymethyl-L-cysteine was found to be less stable than that of L-glutamic acid residue, relative to the disordered states of the respective residues.

KEY WORDS Stability of the β-Structure / Poly(*S*-carboxymethyl-L-cysteine) / Oligopeptides / Intermolecular β-Structure / Circular Dichroism /

The β-structure of oligopeptides is formed by intermolecular association. For monodisperse samples in organic solvents, the formation of the β-structure is characterized by the presence of a critical concentration. The stability of the β-structure formed by various amino acid residues can be quantitatively compared in terms of this concentration under the condition that chain length, end groups, and solvent conditions are the same.[1]

The β-structure in aqueous solutions has been less explored. Monodisperse oligopeptides of L-glutamic acid was prepared[2] and the effect of chain length on their conformation was examined.[3] Other studies using monodisperse oligopeptides have also been carried out. The usefulness of these substances for characterizing the intermolecular β-structure is evident.[4-10] The intermolecular β-structure of poly[*S*-carboxymethyl-L-cysteine] ([Cys-$(CH_2COOH)]_n$) was studied using a sample with an average degree of polymerization (DP) of about 20.[11,12] A value of about −250 cal (−1.1 kJ) was obtained for the standard free energy of association per mole of residues (on a molarity basis) from the reversible concentration dependence[12] assuming a linear association model (aggregates are all in register). To separate the contribution of the propagation step from this over-all free energy change, a similar study on samples of different chain lengths will have to be made.

Recently, we succeded in preparing well fractionated low-molecular-weight samples of $[Cys(CH_2COOH)]_n$.[13] In the present study, the formation of the β-structure in aqueous solutions was examined using these samples with chain lengths ranging from 10 to about 16.

EXPERIMENTAL

The samples used has the composition: C_2H_5NH-[-$COH(CH_2SCH_2COOH)NH$-]$_n$ $COCH_3$. Both synthetic and fractionation procedures as well as molecular weight distribution are reported elsewhere.[13] The number-average degree of polymerization DP_n was determined by proton NMR and ion-exchange chromatography. The following results were obtained: IM-1 (—, 15.6), IM-2 (13.5, 14.2), IM-3 (12.7, 12.3), and IM-4 (9.4, 10.0), where the first figure in each parenthesis represents the result from NMR. Samples IM-2, IM-3 and IM-4 consisted mainly of chains of DP = 14, 12, and 10, respectively, while sample IM-1 had a considerably broad distribution characterized by an average degree of polymerization DP_n of 15.6. The overlap fractions of the chain length distributions of two adjacent samples did not significantly interfere with the observation of chain length effect, as will be shown below.

Polymer solutions were prepared by suspending protonated samples in water or 20 mM aqueous $NaClO_4$ followed by neutralization with NaOH. After complete dissolution at neutral pH, the solutions were adjusted to different pH by the addition of HCl. Each solution was kept at 24 ± 2°C for 12—24 h before the measurements of circular dichroism (CD) and pH. The measured results were the same during 18—48 h after the preparation of the solutions. The polymer concentration was expressed in the residue molarity C_p (N). Circular dichroism (CD) was measured at 25 ± 0.1°C on a Jasco J-40 A circular dichrograph with cells of 0.2, 0.5, 1, 2, 5, and 10 mm light paths. In most cases, four scans were averaged. The CD data were expressed in terms of the residue ellipticity $[\theta]$.

RESULTS AND DISCUSSION

First, three samples of $[Cys(CH_2COOH)]_n$ were examined in solutions with no added salt.

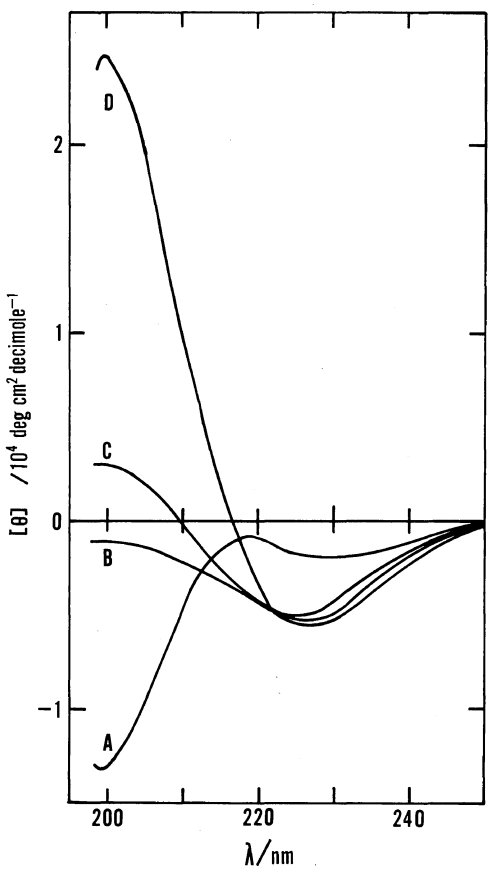

Figure 1. Circular dichroism spectra in solutions with no added salt at various values of pH. Sample: DP_n = 15.6 (IM-1); polymer concentration, 5.0×10^{-3} N. pH: (A) 6.84; (B) 3.65; (C) 3.52; (D) 3.40.

Although a small amount of salt (NaCl) was produced during the neutralization–acidification cycle, its effect was negligible.[11,14]

The CD spectra of a sample (DP_n = 15.6) at various pH at a concentration of about 5×10^{-3} N are shown in Figure 1. The spectrum at pH 6.84 characterizes the charged disordered state of $[Cys(CH_2COOH)]_n$.[11] When the pH decreased to about 3.65, significant changes occurred in the negative bands around 200 and 225 nm. However, a large part of the change at 225 nm may be attributed to factors other than the disordered state–β conversion,[15] such as either a charge effect on the chromophore or a certain change in the distri-

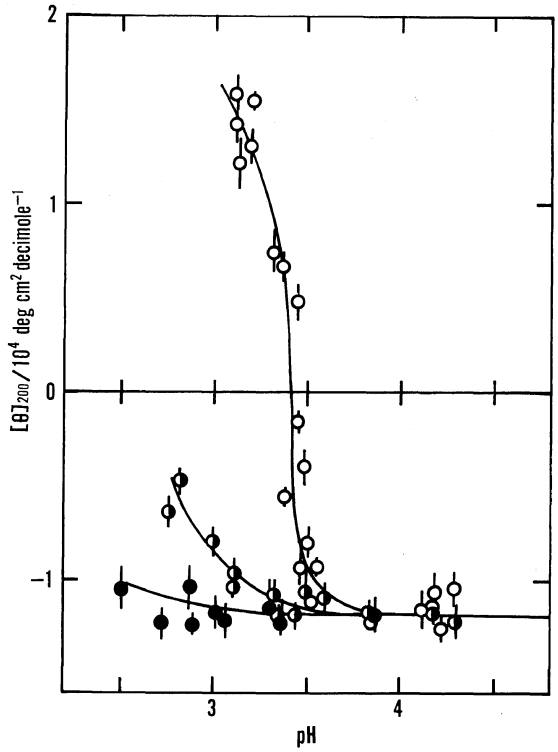

Figure 2. pH dependence of the residue ellipticity at 200 nm, $[\theta]_{200}$, in solutions with no added salt. Polymer concentration, 1.0×10^{-3} N. Samples: (○) $DP_n = 15.6$ (IM-1); (◑) $DP \simeq 14$ (IM-2); (●) $DP \simeq 12$ (IM-3).

bution of local conformations on the Ramachandran map. When the pH was further reduced from 3.65 to 3.40, the negative band around 200 nm became positive, indicating the formation of the β-structure, while the band at 225 nm changed only slightly at this pH change.

In Figure 2, the residue ellipticities of three samples at 200 nm, $[\theta]_{200}$, are plotted against the pH of the solutions with no added salt. The polymer concentration is 1×10^{-3} N. It is seen that the sample with $DP_n = 15.6$ forms the β-structure at pH below about 3.5. The sample with $DP \simeq 14$ begins to form the β-structure at a pH below 3.1, while the sample with $DP \simeq 12$ does not form the β-structure in a pH range higher than about 2.5. Hence, the stability of the β-structure of the present polypeptide markedly depends on chain length in the range from 12 to 16. The polymers precipitated when the pH was lowered below about 3.1, 2.7, and 2.5 for samples of $DP_n = 15.6$, $DP \simeq 14$, and $DP \simeq 12$, respectively. The range of pH where the polymers remained soluble was thus wider for a shorter chain. The formation of the β-structure of the sample with $DP \simeq 14$ was examined also at 3×10^{-3} N. The residue ellipticity $[\theta]_{200}$ reached about -7×10^3 at pH 3.45 and precipitation occurred in a pH rage lower than 3.4.

In the previous study,[11] the association–dissociation accompanying the intermolecular β-structure was found to occur reversibly only in the presence of an added salt (higher than 10^{-2} M). Therefore, in the present study, the conformation was examined in 20 mM $NaClO_4$ solutions with two samples of $DP \simeq 10$ and $DP_n = 15.6$. The results are shown in

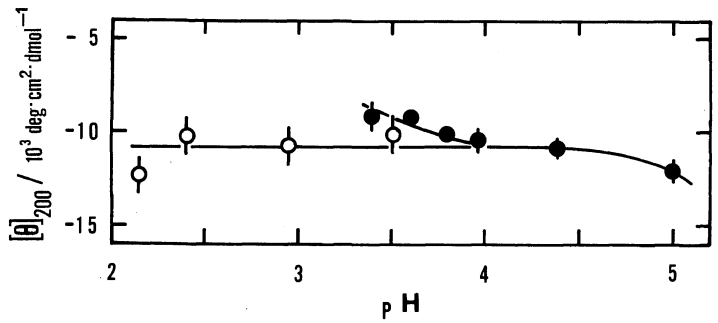

Figure 3. pH dependence of the residue ellipticity at 200 nm, $[\theta]_{200}$ in 20 mM $NaClO_4$ solutions. Polymer concentration, 1.0×10^{-3} N. Samples: (●) $DP_n = 15.6$ (IM-1); (○) $DP \simeq 10$ (IM-4).

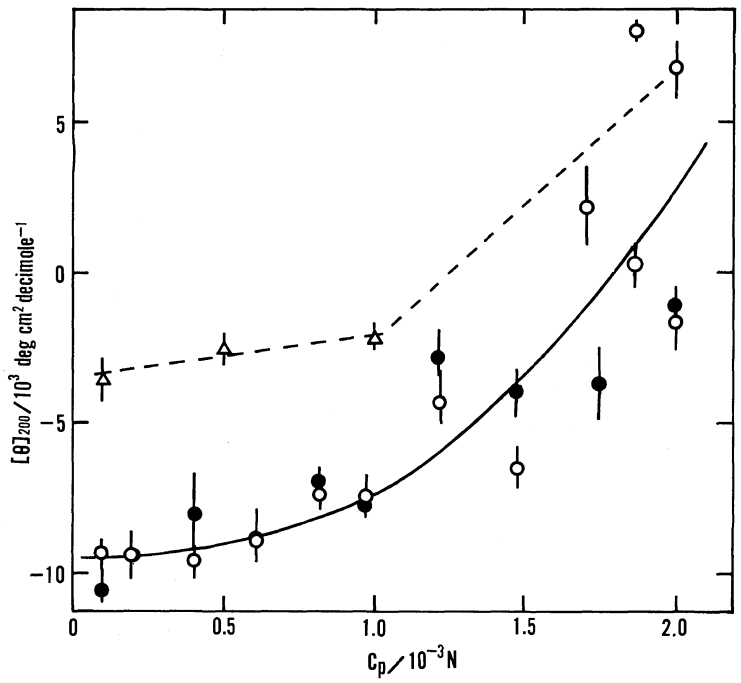

Figure 4. Dependence of the residue ellipticity at 200 nm, $[\theta]_{200}$, on the polymer concentration C_p at a fixed pH of 3.42 ± 0.05 in solutions with no added salt. Sample: $DP_n = 15.6$ (IM-1). Circles represent data on solutions prepared by the addition of HCl to neutral pH solutions of different concentrations. Triangles represent data on solutions prepared by dilution of the solution of the same pH at 2.0×10^{-3} N. Unfilled and filled circles refer to different series of experiments. The solid curve is drawn according to the model described in the text with $\sigma = 0.02$ and $K = 550\,N^{-1}$.

Figure 3, which indicates these two polymers to be in a disordered state throughout the entire soluble range of pH in 20 mM $NaClO_4$ solutions.

The β-structure in solution has stability against dissociation (or disordered state) and also against precipitation (or the solid state β-structure). The observed effects of ionic strength and polymer concentration on the stability of our polypeptide indicate that the precipitates are more stable than the β-structure in solution.

Association systems can be characterized by examining the effect of solute concentration. For the association of polyions, it is necessary to change the concentration keeping the activity coefficient and ionization degree constant. Practically, the concentration change at constant pH in a medium of sufficiently high ionic strength meets this requirement, as was demonstrated in a previous study.[12] In the present study, however, the chain lengths of the polymers were not large. Further, since pK_0 of the carboxyl group of the present amino acid residue is 3.2,[16] the charge densities of the polymers were very low in the pH range where the β-structure was formed appreciably. Consequently, it was considered that the condition of no added salt does not matter in examining the concentration effect on the formation of the β-structure.

In Figure 4, the 200 nm residue ellipticities at a pH of 3.42 ± 0.05 in solutions with no added salt are shown as a function of polymer concentration. The solutions represented by the triangles, which were prepared by dilution of a solution of 2.0×10^{-3} N, contained much more β-structure than the other solutions represented by the circles, which were prepared by acidification of the respective solutions of the same solute concentration at neutral pH. Thus, no reversible conversion was obtained in the present study, as anticipated from the results of previous work.[11,14] According to the previous results,[11] it is very likely that the circles in Figure 4, rather than the triangles, represent the equilibrium concentration dependence. The data were tentatively analyzed on the basis of the association model used previously, which is concisely described by

$$\begin{aligned} A_1 + A_1 &\overset{\sigma K}{=} A_2 \\ A_{i-1} + A_1 &\overset{K}{=} A_i \qquad (i \geqq 3) \end{aligned} \tag{1}$$

Here, A_i represents an aggregate consisting of i chains. The data were found to fit with $\sigma = 0.02$ and $K = 550\ \mathrm{N}^{-1}$, as shown by the solid curve in Figure 4. This is in contrast with the previous result in which the best fit of the data was found with $\sigma = 1$ and $K = 1.82 \times 10^2\ \mathrm{N}^{-1}$.[12] A cooperativity appears in the present system ($\sigma = 0.02$), though it was negligible in the previous study ($\sigma = 1$). Hence, we see that the cooperativity depends on molecular weight distribution and/or on chain length.

The data points in Figure 4 are scattered more widely in the concentration range where the β-structure is present ($C_p > 1 \times 10^3$ N) than the previous data for a sample of larger chain length (see Figure 7 of ref 11). In preparing the solutions, a small volume of HCl solution was added in several drops with a micropipette to a solution of neutral pH. Different places in the solution transiently had different pH from the average or the final value. The formation of β-aggregates occurred so fast that more β-structure was present in the region of lower pH than in the rest of the same solution. After the pH of the solution became uniform and reached the final value, the amount of the β-structure of the solution is larger than that is expected for the solution of the same composition subject to no transiently inhomogeneous pH during its preparation, because the dissociation of β-aggregates was very slow. The scattering of data points was thus related to the irreversible nature of the conversion. The present result suggests that the dissociation of β-aggregates becomes slower as the chain becomes shorter. The β-structure formed by the association of short chains with narrow molecular distribution should be in register more perfectly, and hence the aggregates should dissociate more slowly than otherwise.

When all the results given in Figures 2, 3, and 4 are combined, it becomes evident that the present study on the intermolecular β-structure in aqueous solutions was unfortunately limited by the available ranges of both chain length and solvent conditions.

Table I. Chain length dependence of the secondary structure of oligopeptides[a]

Oligopeptides	Chain length 6 8 10 12 14 16	
Et-$[Glu]_n$-Ac[c]	Disordered — β — $\alpha+\beta$	$\alpha'=0$ ($pK_0=4.5$) 20°C
Et-$[SCMC]_n$-Ac[b]	Disordered — β	$pH<pK_0$ (−3.2) 24±1°C

[a] Measured in solutions with no added salt at a concentration of 1×10^{-3} N.
[b] SCMC = *S*-carboxymethyl-L-cysteine.
[c] Data taken from ref 3. α' denotes the degree of neutralization.

Comparison of the Stability of the β-Structure between Glutamic Acid and S-Carboxymethyl-L-Cysteine Residues

In our study, chain length effect on the formation of the β-structure was examined under the conditions nearly identical with those in studying the monodisperse oligopeptides of L-glutamic acid ($[Glu]_n$).[3] Hence, the stability of the β-structure of *S*-carboxymethyl-L-cysteine ($[Cys(CH_2COOH)]_n$) can be compared with that of $[Glu]_n$ at least qualitatively. In Table I, relevant data to facilitate the comparison are summarized.

The intrinsic dissociation constants of the carboxyl groups in these two residues are considerably different: 4.5 for L-glutamic acid[17] and 3.2 for *S*-carboxymethyl-L-cysteine.[16] Because of this difference, the two residues cannot be investigated under exactly the same conditions. For example, the degree of self-ionization is larger for $[Cys(CH_2COOH)]_n$ than for $[Glu]_n$ if compared at the zero degree of neutralization α'. The comparison is best carried out at the same degree of ionization α. However, this is impossible since the available data on $[Glu]_n$ refer to $\alpha'=0$ instead of a specified value of α. For long chain poly(carboxylic acid) in high ionic strength media (more than 0.1 M), α is about 0.05—0.1 at $\alpha'=0$. Therefore, it is likely that α was larger than 0.1 in the study on $[Glu]_n$. For $[Cys(CH_2COOH)]_n$ in the range of pH lower than pK_0, α was smaller than that of the solutions at $\alpha'=0$. Therefore, it can be seen from Table I that the β-structure formed by L-glutamic acid residues carrying residual charges is more stable than that formed by nearly uncharged $[Cys(CH_2COOH)]_n$, when referred to the disordered states of the respective residues. Consequently, it may be concluded that the β-structure of uncharged $[Glu]_n$ is more stable than that of uncharged $[Cys(CH_2COOH)]_n$.

It has been established that the glutamic acid residue is α-forming,[17-23] while the derivatives of cysteine are β-forming.[21-26] This was found not only from studies on synthetic polypeptides[17-20,24-26] but also from conformational parameters calculated from proteins.[21-23]

The parameter P_β for β-sheet formation, calculated from proteins, was 0.26, 1.23, and 1.30 for negatively charged glutamate, glutamine, and cysteine residues, respectively.[22,23] When the present result is compared with these data, it follows that the β-forming power of uncharged glutamic acid is greater than that of negatively charged glutamate and probably comparable to that of glutamine and that carboxymethylation of cysteine slightly destabilizes the β-sheet.

The free energy change $\Delta G^\circ_{c\to\beta}$ for the β-coil conversion of uncharged polypeptides was evaluated from the potentiometric titration,[16,27-31] and a value of about −800 cal mol^{-1} was obtained for $[Cys(CH_2COOH)]_n$.

However, this value refers to the β-structure in large aggregates, *i.e.*, in the solid state.[16] For $\Delta G^{\circ}_{c\to\beta}$ corresponding to no significant aggregation, a value of -300 cal mol^{-1} was obtained for poly(L-tyrosine),[27,28] and a value less negative than -500 cal mol^{-1} for $[Cys(CH_2COOH)]_n$.[32] At any rate, it is likely that $\Delta G^{\circ}_{c\to\beta}$ for $[Cys(CH_2COOH)]_n$ is more negative than -300 cal mol^{-1}. It is clear that the value of $\Delta G^{\circ}_{c\to\beta}$ for poly(L-glutamic acid) (PGA) is less negative than $\Delta G^{\circ}_{c\to\alpha}$, which is about -180 cal mol^{-1},[20] since α-helix is more stable than β-structure for PGA. Therefore, $\Delta G^{\circ}_{c\to\beta}$ for $[Cys(CH_2COOH)]_n$ is more negative than that for PGA in the case of long chain polymers. This conclusion is opposed to the present suggestion as to the stability of the β-structure of oligopeptides formed by these two residues. To reconcile these incompatible results, one of the following two assumptions must be made.

(a) A specific interaction destabilizing the β-sheet of $[Cys(CH_2COOH)]_n$ works between the side chains and the blocking group(s).

(b) The stability of the intermolecular β-structure in the present study differs from that of the intramolecular β-structure from previous potentiometric titrations.

REFERENCES

1. C. Toniolo and G. M. Bonora, "Peptides: Chemistry, Structure and Biology," R. Walter and J. Meienhofer, Ed., Ann Arbor Science, Ann Arbor, Michigan, 1975, p 145.
2. M. Rinaudo and A. Domard, *Biopolymers*, **14**, 2035 (1975).
3. M. Rinaudo and A. Domard, *J. Am. Chem. Soc.*, **98**, 6360 (1976).
4. A. Yaron, M. C. Otey, H. A. Sober, E. Katchalski, S. Ehrlich-Rogozinski, and A. Berger, *Biopolymers*, **11**, 607 (1972).
5. R. L. Snipp, W. G. Miller, and R. E. Nylund, *J. Am. Chem. Soc.*, **87**, 3547 (1965).
6. M. Mutter, *Macromolecules*, **10**, 1413 (1977).
7. M. Mutter, H. Mutter, R. Uhman, and E. Bayer, *Biopolymers*, **15**, 917 (1976).
8. C. Toniolo, G. M. Bonora, and M. Mutter, *J. Am. Chem. Soc.*, **101**, 450 (1979).
9. C. Toniolo, G. M. Bonora, S. Salardi, and M. Mutter, *Macromolecules*, **12**, 620 (1979).
10. W. L. Mattice and W. H. Harrison, *Biopolymers*, **15**, 559 (1976).
11. K. Saito, H. Maeda, and S. Ikeda, *Biophys. Chem.*, **16**, 67 (1982).
12. H. Maeda, K. Saito, and S. Ikeda, *Bull. Chem. Soc. Jpn.*, **56**, 602 (1983).
13. H. Maeda, T. Ito, H. Suzuki, S. Hirata, I. Kako, M. Yoshino, S. Ikeda, and Y. Kobayashi, *Biopolymers*, **22**, 2173 (1983).
14. H. Maeda, K. Kadono, and S. Ikeda, *Macromolecules*, **15**, 822 (1982).
15. H. Maeda and K. Ooi, *Biopolymers*, **20**, 1549 (1981).
16. H. Maeda and S. Ikeda, *Biopolymers*, **14**, 1623 (1975).
17. M. Nagasawa and A. Holtzer, *J. Am. Chem. Soc.*, **86**, 538 (1964).
18. P. Doty, A. Wada, J. T. Yang, and E. R. Blout, *J. Polym. Sci.*, **23**, 851 (1957).
19. W. G. Miller and R. E. Nylund, *J. Am. Chem. Soc.*, **87**, 3542 (1965).
20. D. S. Olander and A. Holtzer, *J. Am. Chem. Soc.*, **90**, 4549 (1968).
21. P. Y. Chou and G. D. Fasman, *Biochemistry*, **13**, 211 (1974).
22. P. Y. Chou and G. D. Fasman, *Biochemistry*, **13**, 222 (1974).
23. P. Y. Chou and G. D. Fasman, *Adv. Enzymol.*, **47**, 45 (1978).
24. S. Ikeda, *Biopolymers*, **5**, 359 (1967).
25. H. Maeda and S.Ikeda, *Biopolymers*, **10**, 1635 (1971).
26. T. Tomiyama and S. Ikeda, *Macromolecules*, **12**, 165 (1979).
27. R. P. McKnight and H. E. Auer, *Macromolecules*, **9**, 939 (1976).
28. H. E. Auer and R. P. McKnight, *Biochemistry*, **17**, 2798 (1978).
29. D. Pederson, D. Gabriel, and J. Hermans, Jr., *Biopolymers*, **10**, 2133 (1971).
30. R. Mandel and G. D. Fasman, *Biopolymers*, **14**, 1633 (1975).
31. B. Walter and G. D. Fasman, *Biopolymers*, **16**, 17 (1977).
32. H. Maeda, Y. Gatto, and S. Ikeda, *Macromolecules*, **17**, in press (1984).