The 225–240-nm Circular Dichroism Band in Disordered and Charged Polypeptides

W. B. Rippon* and W. A. Hiltner

Division of Macromolecular Science, Case Western Reserve University, Cleveland, Ohio 44106.
Received July 12, 1972

ABSTRACT: The interpretation of the circular dichroism spectra obtained from charged polypeptides such as poly(glutamic acid) and poly(Lys) has, in the past, involved two opposing views of the conformation of such polyelectrolytes. This paper presents spectra obtained from a sequential polypeptide with one charged residue per dipeptide. The effects of heat and added salt on the spectra along with comparisons with spectra from a number of other synthetic and natural polypeptides suggest that the charged form, in the absence of salt and at low temperatures, approximates the extended helix proposed for poly(glutamic acid). Further, this helix may be disrupted by heat or added salt and this disruption results in a deepening, and a blue shift, of the 238-nm trough to give a spectrum similar to that reported for gelatin. Evidence is presented which suggests that this trough is not due to α-helical content as has been suggested and, further, the trough is absent or very weak for disordered poly(imino acids).

The interpretation of circular dichroism curves obtained from native protein materials has relied on the use of synthetic homopolypeptides to establish the positions and strengths of the optical bands in the ultraviolet region.[1-5] Much of this information has come from studies using poly(L-glutamic acid) and poly(L-lysine),[6,7] due to their solubility in aqueous solvent systems. In addition these polymers undergo conformational changes associated with ionization of the side-chain carboxylic acid and primary amine functions, respectively. In the ionized form these polymers were considered to behave as random polypeptides and the three-banded spectrum observed (small trough, medium peak, large trough) was used as a model for the random regions of proteins.[8,9] Recently there has been some controversy as to the nature of the spectrum, *i.e.*, the existence or absence of the 238-nm trough,[3] and the suitability of the model.[10] The 238-nm band has now been found to arise if small amounts of salt are present in the solution and can be removed by dialyses and reappears upon addition of salt. It also shows a similar sensitivity to pH.[10,11] Simple conformational calculations based on electrostatic interactions[12] and, more recently, complete calculations, considering all the major contributions to potential energy,[13] have suggested that highly charged polypeptides have local segments in an extended form which approximates the 3_1 helix described for poly(L-proline) and poly(Gly). Tiffany and Krimm,[14] thus proposed that the circular dichroism spectrum for random polypeptides was a simple trough at ~200 nm with variable magnitude depending on the protein considered and the two-banded spectrum observed for charged polypeptides, in the absence of salt, arose from the extended helix. Addition of salt, or neutralization of some charges by adjustment of pH, resulted, according to their view, in an intermediate structural state.

Myer,[11] however, suggests that the charged polypeptides are still to be considered random and concludes that the 238-nm trough is due to some 2–5% α-helical structure which arises from either partial neutralization of the charged groups or shielding of the electrostatic repulsions due to formation of salt shells around the ionized groups. This interpretation is based on the significantly different band positions observed for ionized polymers compared to those for the main optically active 3_1 helix, poly(proline) II, and the successful computer simulation of the three-banded curves as a summation of the two-banded curve and 2–5% of the curve established for the α-helical form.

In a previous communication it has been shown that the band positions observed for poly(proline) II and the charged poly(α-amino acids) can be rationalized on the basis of the difference between the electronic transitions of imino acids (proline) and amino acids.[15]

In this paper we present evidence, obtained from studies on a number of synthetic and natural polypeptides, which suggests that the 238-nm trough is likely to be due to a discrete electronic transition, *i.e.*, has a molecular origin, and is not due merely to the presence of a small amount of helix as had been suggested by earlier work.[11]

Experimental Section

Poly(Glu-Ala) was synthesized by Dr. Kovacs of St. John University and sedimentation equilibrium measurements indicated a molecular weight of ~5000. Poly(Glu(OEt)-Gly-Gly) and poly(Ala-Gly-Gly) were synthesized by Dr. J. M. Anderson of this Division and have been described elsewhere.[16] Poly(Gly-Pro-Pro) with mol wt ~8000 was fractionated from a sample obtained from Miles Research Laboratories, poly(L-glutamic acid) was obtained from Mann Research Laboratories. The salt soluble collagen was extracted by Dr. L. J. Gathercole from rat skin according to established procedures.

Circular dichroism measurements were made on a Jasco J20 spectropolarimeter and temperature studies employed a Nestlab TE3 circulating heating bath. This was coupled to a refrigerated

(1) S. N. Timasheff, H. Susi, R. Townsend, L. Steven, M. J. Gorbonoff, and T. F. Kumosinski in "Conformation of Biopolymers," Vol. 1, G. N. Ramachandran, Ed., Academic Press, New York, N. Y., 1967, p 173.
(2) J. T. Yang in "Poly-α-amino Acids," G. D. Fasman, Ed., Marcel Dekker, Inc., New York, N. Y., 1967, p 239.
(3) S. Beychok in "Poly-α-amino Acids," G. D. Fasman, Ed., Marcel Dekker, Inc., New York, N. Y., 1967, p 293.
(4) J. D. Carver, E. Schechter, and E. R. Blout, *J. Amer. Chem. Soc.*, 88, 2550 (1966).
(5) A. J. Adler, R. Hoving, J. Potter, M. Wells, and G. D. Fasman, *J. Amer. Chem. Soc.*, 90, 36, 47 (1968).
(6) P. Doty, A. Wada, J. T. Yang, and E. R. Blout, *J. Polym. Sci., Part A*, 23, 851 (1957).
(7) G. Holzworth and P. Doty, *J. Amer. Chem. Soc.*, 87, 218 (1965).
(8) N. Greenfield and G. D. Fasman, *Biochemistry*, 8, 4108 (1967).
(9) A. Warashina and A. I. Kegami, *Biopolymers*, 11, 529 (1972).
(10) M. L. Tiffany and S. Krimm, *Biopolymers*, 6, 1379 (1968).
(11) Y. P. Meyer, *Macromolecules*, 2, 624 (1969).
(12) S. Krimm and J. E. Mark, *Proc. Nat. Acad. Sci. U. S.*, 60, 1122 (1968).
(13) W. Hiltner and A. J. Hopfinger, and A. G. Walton, *J. Amer. Chem. Soc.*, 94, 4324 (1972).
(14) M. L. Tiffany and S. Krimm, *Biopolymers*, 8, 347 (1969).
(15) W. B. Rippon and A. G. Walton, *Biopolymers*, 10, 1207 (1971).
(16) J. M. Anderson, W. B. Rippon, and A. G. Walton, *Biochem. Biophys. Res. Commun.*, 39, 802 (1970).

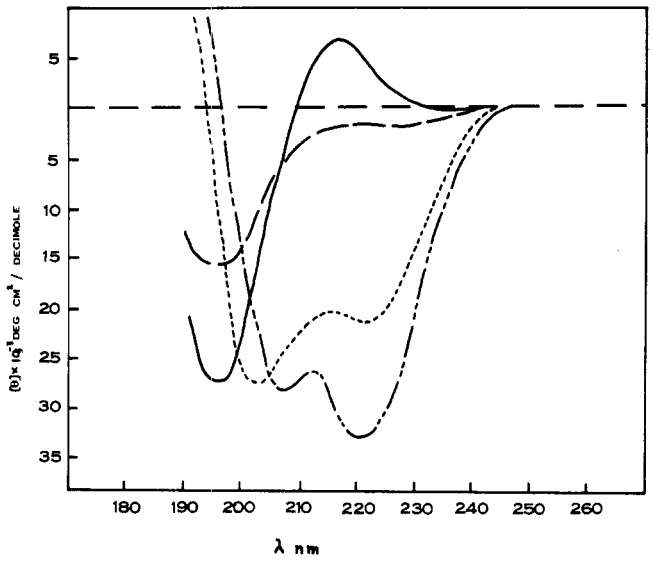

Figure 1. Circular dichroism spectra for poly(Glu-Ala) at pH 3.5 [at 25° (- - - - -), 90° (— —), and 50% dioxane (— - - —)] and at pH 10.5 [at 25° (——), and 90° (— —)].

bath at −25° for temperature less than 10° above ambient temperature. pH measurements were taken with a Sargents Model L3 pH meter and the ionizable polymers were dialyzed against distilled, deionized water adjusted to the appropriate pH and salt concentration with reagent grade NaOH, HCl, and NaCl. The dialysis involved three changes and was carried out at 1°.

Results

Figure 1 shows the circular dichroism spectra obtained at 25 and 90° for solutions of poly(Glu-Ala) dialyzed against deionized water adjusted to pH 10.0 and 3.5. The high pH, low temperature curve is similar to that reported for poly(L-glutamic acid) under these conditions, however, unlike poly(L-glutamic acid), dialysis of the solutions did not result in an elimination of the 238-nm trough. The low pH, low temperature curve is also reminiscent of that obtained for poly(L-glutamic acid) under similar conditions although the magnitudes of the ellipticities are somewhat less. Addition of methanol to this solution results in a deepening of the 225- and 207-nm troughs consistent with an increase in α helicity. Heating either the low pH, α-helical form, or the high pH extended form results in a collapse of the circular dichroism bands to give rise to a simple two-banded spectrum with a small trough ($[\theta] \simeq -2000$ (deg cm^2)/dmol) centered at 225 nm and a larger trough ($[\theta] \simeq -16{,}000$ (deg cm^2)/dmol) centered at ~198 nm.

Figure 2 shows the effect of added sodium chloride on the ~238- and ~215-nm bands seen at high pH. These effects are similar to those already reported for poly(L-glutamic acid).[11] These curves also mirror those obtained for solutions at pH 10.0 and temperatures between the 25 and 90° shown in Figure 1. Further, spectra obtained for solutions at pH's between 10 and 3.5 showed a deepening and blue shift of the 238-nm trough as the pH was lowered. Thus it is possible to induce gradual changes in the circular dichroism spectra either by variations of the pH or the temperature. These changes are completely reversible.

Previous workers have interpreted similar data for the pH dependence of various physical measurements on poly(L-glutamic acid) in terms of a random-coil to α-helix conformational transition.[17] We, however, have indica-

(17) D. Poland and H. A. Scheraga, "Theory of Helix-Coil Transition in Biopolymers," Academic Press, New York, N. Y., 1970.

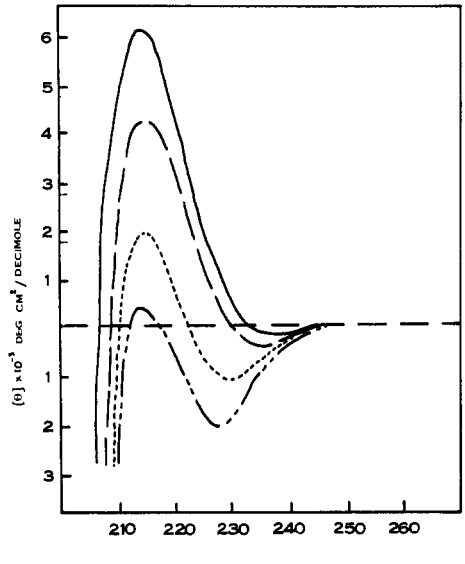

Figure 2. Circular dichroism spectra of poly(Glu-Ala) at pH 10.5 in 0.0 M (——), 0.1 M (— —), 0.50 M (- - - -), and 1.0 M (— - - —) NaCl.

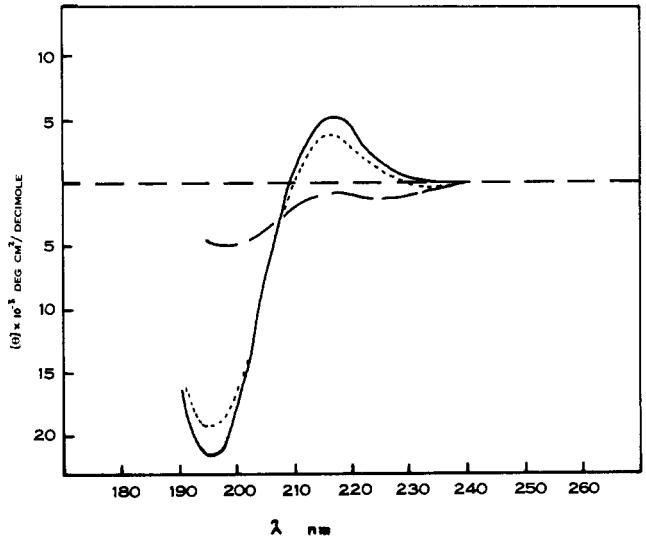

Figure 3. Circular dichroism spectra for poly(Ala-Gly-Gly) at 25° (——), and 60° (— —), and poly(Glu(OEt)-Gly-Gly) at 25° (- - - -), in aqueous solution.

tions of three conformations when the temperature transition is included and favor the interpretation involving an extended helix, first introduced by Tiffany and Krimm,[10] and assigning the high temperature forms to randomized chains. Figure 3 shows the circular dichroism spectra obtained for poly(Ala-Gly-Gly) and poly(Glu(OEt)-Gly-Gly) and the similarity between the high temperature forms of these polymers and the 90° curves shown in Figure 1 should be noted. We have previously presented evidence for the room temperature form of poly(Ala-Gly-Gly) being a 3_1 helix[15,18,19] and the greater sensitivity of current instrumentation reveals a very weak band at 225 nm for the heated polymers.

Previous reports have identified the circular dichroism spectrum of gelatin with that of a random polypeptide.

(18) W. B. Rippon and A. G. Walton, *J. Amer. Chem. Soc.*, **94**, 4319 (1972).

(19) J. C. Andries, J. M. Anderson, and A. G. Walton, *Biopolymers*, **10**, 1049 (1971).

Table I
Summary of the Circular Dichroism Ellipticities for the Two Negative Troughs for a Series of Polypetides in the Random Form

Polypeptide	% Gly	% Pro	(deg cm²)/d mol $[\theta] \sim 225$	(deg cm²)/d mol $[\theta] \sim 200$
$(Glu\text{-}Ala)_n$	0	0	2000	16,000
$(Ala\text{-}Gly\text{-}Gly)_n$	66	0	800	5,000
Collagen	33	22	1200	11,300
α1CB2	33	33	700	16,900
$(Gly\text{-}Pro\text{-}Pro)_n$	33	66	100	25,000

Table I summarizes the data we obtained for collagen along with similar results reported by Piez and Sherman for the heat-denatured α1-CB2 fragment of collagen.[20] It is evident that the 225-nm band is present in both cases however is reduced in magnitude for the α1-CB2 fragment. This reduction appears to be correlated with the proline and glycine content and suggests that the equivalent of the 225-nm transition for imino acids is very weak. Poly(L-proline) heat precipitates[21] and hence is unsuitable for temperature studies of this type but poly(Gly-Pro-Pro) undergoes a temperature-sensitive transition to a randomized form. The circular dichroism spectrum summarized in Table I for this polytripeptide at 60° shows a very weak 225-nm trough which is two orders of magnitude less than the main band centered at 202 nm.

Discussion

The spectra reported in this paper suggest that there are at least three forms of poly(Glu-Ala), of these, the low pH, low temperature, form is clearly predominantly α helical and comparison between the heated forms and the spectrum of heat-denatured collagen suggests that the high temperature form is random. The third form, obtained at high pH, is thus considered to be related to the extended helix described for charged homopolypeptides. However, the persistence of the 238-nm trough after dialysis and the observations regarding heat and salt on this trough suggests that the extended helix for the polydipeptide is more flexible than that found for the charged homopolypeptide. The α helix also appears to be less stable for poly(Glu-Ala) than poly(L-glutamic acid) since dioxane is required for maximum helicity of the former but not the latter polypeptides. These differences in apparent stability are also reflected in the sensitivity to heat. We have shown essential identity between the low and high pH curves at 90° for poly(Glu-Ala) whereas for poly(L-glutamic acid), although they move together, they never coalesce.

A theoretical conformational analysis of poly(Glu-Ala) has been conducted in this laboratory.[22] A single minimum of energy was found, corresponding to an extended helix with 2.6 residues/turn. This conformation is similar to that reported earlier for poly(Glu).[13] The two differ in that the configurational entropy calculated for poly(Glu-Ala) is ~10 eu, whereas that for poly(Glu) is ~7 eu. Thus, the calculations indicate that the residues of poly(Glu-Ala) experience lower potential gradients and can occupy larger regions of conformational hyperspace than can those of poly(Glu), *i.e.*, the former extended helix is the more flexible, in agreement with the experimental observations presented here.

Although the family of circular dichroism spectra obtained with titration from high pH to low pH suggest that the 238-nm trough seen at pH 10 might be due to persistence of a small fraction of the low pH form, the increase in this trough seen when the high pHl form is heated compared to the decrease when the α-helical form is heated seems to argue against a common conformational origin. In addition a similar band is found in heated collagen, with 20% proline and 33% glycine, poly(Ala-Gly-Gly) and poly(Glu(OEt)-Gly-Gly) and it is unlikely that the α helix would be found in these polymers. Thus it seems reasonable to assign this band to a specific electronic transition which is strongly dichroic in the α helix and weaker for the less rigid more extended forms. Since the 225-nm band in the α helix is accepted as arising from the $n\text{-}\pi^*$ transition it is suggested that this transition also gives rise to the weak 225-nm trough in the random form.

The apparent dependence of the magnitude of the 225-nm trough on proline content and its almost complete absence from denatured poly(Gly-Pro-Pro) may be interpreted either as a consequence of the stereochemical restrictions imposed by the proline ring structure or to a inherent weakness of the $n\text{-}\pi^*$ transition for imino acids. The absorption spectra of models for the prolyl residue show a very weak $n\text{-}\pi^*$ transition around 225-235 nm[22-24] and Schellman[25] suggests that the rotational strength of the $n\text{-}\pi^*$ transition for poly(L-proline) II should be much less than that calculated for the α helix. Either way it apparently is a property of the imino acids and may provide a means of monitoring effects in the nonproline regions of collagen independent of effects from proline rich regions.

Analysis of the curves obtained at temperatures between the two extremes reported indicates that at least part of the increase seen in the high wavelength trough is due to the reduction in the 215-nm peak which overlaps with the troughs on either side of it. Thus although no trough is apparent in the native form of collagen or the 3_1 helical form of poly(Ala-Gly-Gly) it is most likely present at least to a small extent but the larger positive contribution from the neighboring band prevents observation of the trough. Should this be the case then there are at least three dichroic bands observed for the 3_1 helix. The high-wavelength band has been assigned to the $n\text{-}\pi^*$ transition leaving the remaining two to have arisen from the $\pi\text{-}\pi^*$ transition by exciton interaction. These observations are consistent with the early exciton calculations on poly(L-proline) II.[23] The shift in position of minimum from 238 nm for the extended coil to 225, for the α-helix and disordered forms is another consequence of the large positive band in the former which is absent in the latter two forms.

Conclusion

The studies reported in this paper have presented further evidence for an extended helical conformation for charged polypeptides. This conformation appears to be more stable, and have less freedom, for a totally charged polypeptide whereas, dilution of charge, by pH adjustments, addition of salt, or insertion of neutral residues causes a loosening of the extended helix. The extent of

(20) K. A. Piez and M. R. Sherman, *Biochemistry*, **9**, 4129 (1970).
(21) W. L. Mattice and L. Mandelkern, *Macromolecules*, **4**, 271 (1971).
(22) W. A. Hiltner, A. J. Hopfinger, and A. G. Walton, *Biopolymers*, in press.
(23) A. N. Glazer and K. Rosenheck, *J. Biol. Chem.*, **237**, 3674 (1962).
(24) W. G. Gratzer, W. Rhodes, and G. D. Fasman, *Biopolymers*, **1**, 319 (1963).
(25) J. A. Schellman and C. Schellman, *Proteins*, **2**, 1 (1964).

this increase in conformational freedom can be ascertained from the magnitude of a circular dichroism band in the 225–240-nm region. Since we have observed this band in the spectrum of polymers with high proline and glycine contents and at elevated temperatures, it is unlikely that it is unique to the α-helical polypeptides. The very weak band seen in the spectrum of poly(Gly-Pro-Pro) has been rationalized either because of the restricted conformational space available to this residue or the inherent weakness of the n–π^* transition in poly(imides).

The random form of a number of polypeptides has been shown to give rise to a two-banded circular dichroism. A weak band at ~225 in addition to the stronger band centered at ~200 nm. The intensity of the former band appears to depend on the glycine and proline content of the polypeptide with higher proportions of these residues decreasing the circular dichroism. On the other hand, the presence of glycine tends to reduce the 200-nm band whilst the presence of proline increases this band. In addition Piez and Sherman[20] have already noted a dependence between the magnitude of the ~200-nm trough and imino acid content which is further substantiated by the results reported here for poly(Gly-Pro-Pro). The effects have been interpreted in terms of the symmetry of the glycine residue and the stereochemical restrictions imposed by the proline residue.

Acknowledgments

The authors express appreciation to Dr. Kovacs for providing the poly(Glu-Ala) used in this study and to Miss A. Ferszt for her technical assistance. We acknowledge support through the National Institute of Dental Research Program Project No. DE 02587 and a postdoctoral fellowship.

Probability Distributions for the Radii of Gyration of Short, Branched, Random-Flight Chains

Santosh K. Gupta and W. C. Forsman*

School of Chemical Engineering and Laboratory for Research on the Structure of Matter, University of Pennsylvania, Philadelphia, Pennsylvania 19174. *Received October 19, 1972*

ABSTRACT: We have computed the probability distributions for the dimensionless radii of gyration, ξ_3, of some typical, short, branched random-flight chains using a formulation published previously. As expected, the results show that chains with branches located nearer to the central segment are more compact than those with branches near the ends. We also found that the probability of observing chains with values of ξ_3 between about 1.0 and 1.5 increased markedly with branching, at the expense of the distribution at higher values of ξ_3, while the probability of observing chains with values of ξ_3 below about 1.0 was relatively unchanged.

In a recent publication,[1] we presented a general treatment of random-flight statistics both in the presence and absence of intramolecular interactions, and indicated how it could account for the effects of branching. We have since obtained numerical solutions for the one-, two-, and three-dimensional distribution functions of the unperturbed radius of gyration for some typical, short, branched chains and have observed that these results are consistent with our present intuitive notions on how the distributions would differ from those of linear chains of an equal number of statistical segments.

The notation is the same as that used previously.[1,2] We define the dimensionless radii of gyration and its components as[3]

$$\begin{aligned} \xi_1^2 &= \pi^2\beta^2 S_x^2 \\ \xi_2^2 &= \pi^2\beta^2(S_x^2 + S_y^2) \\ \xi_3^2 &= \pi^2\beta^2 S^2 \end{aligned} \tag{1}$$

where S_x, $(S_x^2 + S_y^2)^{1/2}$, and S represent the one-, two-, and three-dimensional radii of gyration respectively, $\beta^2 = (3/2nl^2)$, l^2 being the mean-square length of a statistical segment and n, the number of statistical segments per chain. The normalized distribution functions can then be written as

$$W^0(\xi_j) = \frac{n(n+1)}{\pi^3}\left|\mathbf{D}(0)\right|^{j/2}\xi_j\int_{-\infty}^{\infty}\frac{\exp\left[-\frac{in(n+1)}{\pi^2}\xi_j^2\phi\right]d\phi}{\left|\mathbf{D}(\phi)\right|^{j/2}} \tag{2}$$

where $j = 1, 2, 3$, and where the method for generating the matrix $\mathbf{D}(\phi)$ for linear and branched chains has been described previously,[1] and $i = (-1)^{1/2}$. Equation 2 may be simplified in terms of real and imaginary parts, the latter being zero due to symmetry about $\phi = 0$. The simplified equation is analogous to eq 3 of reference 2 and can be integrated numerically.

Results for the linear chain and five typical branched chains, shown in Figure 1, each having ten statistical segments, are shown in Figures 2 and 3. The integrations

(1) S. K. Gupta and W. C. Forsman, *Macromolecules*, **5**, 779 (1972).
(2) S. K. Gupta and W. C. Forsman, *J. Chem. Phys.*, **55**, 2594 (1971).
(3) R. F. Hoffman and W. C. Forsman, *J. Chem. Phys.*, **50**, 2316 (1969).