CIRCULAR DICHROISM OF PUTATIVE UNORDERED POLYPEPTIDES AND PROTEINS

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SUMMARY. The CD spectrum of fully protonated poly-L-lysine is altered by various salts. These altered CD spectra can be quantitatively accounted for by conversion of a fraction of the "unordered" form to the "helical" form. We find no evidence for an "extended-helical" chain conformation. We confirm that presumably unordered proteins have a qualitatively different CD spectrum from that of unordered polylysine. The CD spectrum of presumably unordered proteins cannot be accounted for by any mixture of unordered and helical polylysine forms. This result requires revision of some current analyses of protein CD spectra.

Synthetic polypeptides have been extensively employed as reference materials in the analysis of protein structure. Polylysine in particular has been an attractive choice because of its water solubility and its ready intercomvertibility between structural states. Greenfield and Fasman (1) have recently presented an attractive analysis of the CD of proteins, based on polylysine reference states. However, on examining several proteins in denaturing solvents, we found that their CD spectra differ significantly from the CD spectrum of the so-called "unordered" or "random" conformation of polylysine. This disagreement had already been noted by Tiffany and Krimm (2), who also interpret the CD spectrum of poly-L-lysine (pLL) at low pH and low ionic strength as representing an "extended-helix" polypeptide structure, stabilized by electrostatic interactions. In further studies (3) they show removal of the 218nm peak from both poly-L-glutamic acid and pLL in strong salt solutions, and interpret the CD changes as conversion of "extended-helical" polypeptide to a normal random conformation. Since Tiffany and Krimm's results and interpretations challenge the validity of the Greenfield-Fasman analysis, we have

re-examined electrolyte effects on the CD spectra of pLL.[1]

MATERIALS AND METHODS. Poly-L-lysine was obtained from Pilot Chemicals: Lot #L-7A, M=115,000 and Lot #L-90, M=130,000 (the latter sample generously supplied by Prof. W. G. Miller). A third sample of pLL was obtained from Mann Laboratories, Lot #P2115, M=50,000. All three samples of pLL showed the same qualitative and quantitative dichroic behavior in the several solvent systems examined. Sperm whale myoglobin was a gift of Prof. E. S. Benson.

Measurements of circular dichroism were made on a Durrum-Jasco CD/SP-5 circular dichrometer, calibrated according to Cassim and Yang (6). Silica cells of 0.50mm and 0.10mm optical path were used for most of the measurements. Frequent baseline recordings were made with the cells and solvents. CD spectra were taken on samples at ambient temperature (23-25°C).

RESULTS. The CD spectra of pLL in its familiar helical (pH 11.4) and U/EH (pH 3.7) forms is shown in Figure 1. The results agree well with published data(1). Also shown are the spectra in 0.50M $Ca(ClO_4)_2$ (apparently helical) and in 3M $Ca(ClO_4)_2$ (apparent mixture of helical and U/EH).

Titration of pLL from pH 11.2 with $HClO_4$ first produces a transition from helix to U/EH (218nm peak present). With further addition of $HClO_4$ (0.04 to 1.0M) a transition back to helix occurs (half-conversion at about 0.2M (ClO_4^-)). Finally, at higher concentrations (1.0M to 6.0M) of ClO_4^- a gradual transition to the U/EH is seen. The titration curve ($[\theta]_{218}$ vs. (ClO_4^-)) of pLL with ClO_4^- was essentially the same with $Ca(ClO_4)_2$ as with $HClO_4$ over the range: $10^{-2}M < (ClO_4^-) < 1M$. At $(HClO_4) > 1M$ some turbidity develops with pLL, complicating comparisons.

Several other salts were examined to explore anion specificity. NaCl and $CaCl_2$ at pH 3-4 yield superposable plots of $[\theta]_{218}$ vs. (Cl^-) up to the solubility limits of NaCl, showing as with the perchlorates that the trans-

[1] Inasmuch as two structures (unordered, and extended-helix) have been proposed for pLL in its low-pH, low ionic strength form, we will identify this form as U/EH until we complete presentation of evidence leading to a clear preference for one structure.

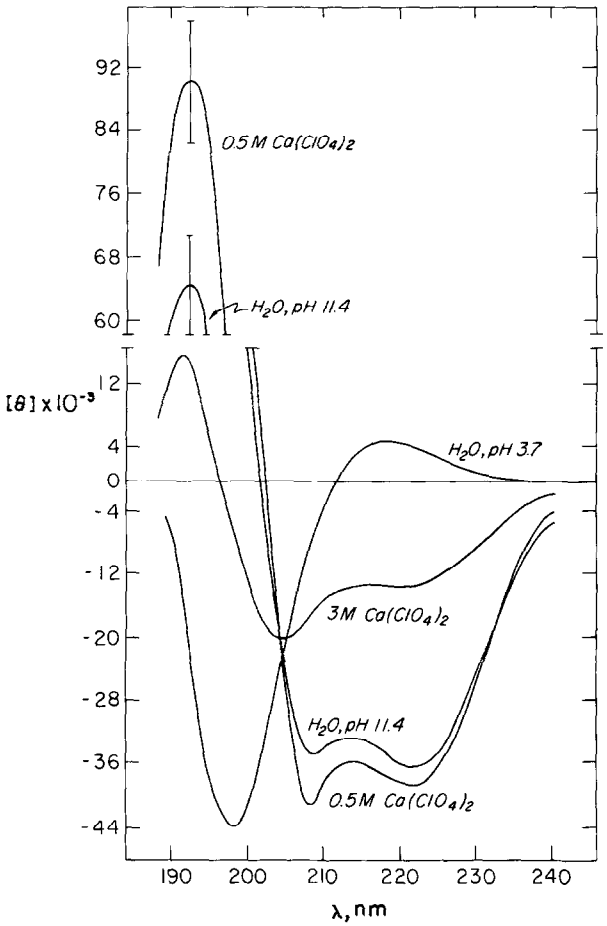

Fig. 1. CD spectra of poly-L-lysine in various aqueous solvents. The spectrum labelled H_2O, pH 3.7 was obtained by dilution with water of a stock solution exhaustively dialyzed vs. 10^{-3} M HCl. This solution was then used as a stock solution to prepare solutions for observation. The concentration of pLL was determined by the colorimetric ninhydrin method of Stein and Moore (4) as previously calibrated for pLL (5). The pH 11.4 solution was obtained by addition of 1M NaOH to the water-diluted stock solution of pLL. The two $Ca(ClO_4)_2$ solutions were pH 3-4.

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formation is indifferent to the cation. At 12N $CaCl_2$ the CD spectrum represents about 30% helical content. NaH_2PO_4 added to pLL at pH 3-4 shows a substantially larger effect than do the chlorides (Table 1).

Dichroic spectra intermediate between the "helical" and U/EH extremes were tested for fit to a two-state model, i.e., we attempted to account for the observed spectra as a mixture of helical and U/EH species. We took pLL

at pH 11.4 as 100% helix and pLL at pH 3.7 as 100% U/EH, and employing the relation:

$$[\theta]_\lambda = a[\theta]_{\lambda,h} + (1-a)[\theta]_{\lambda,u} \qquad \text{Eq. (1)}$$

where $[\theta]$ is the observed molar ellipticity at wavelength λ, a is the fraction of peptide bonds in helical conformation, and subscripts h and u designate the helical and U/EH forms. The above equation was cast in the form:

$$\frac{[\theta]_\lambda - [\theta]_{\lambda,u}}{[\theta]_{\lambda,h} - [\theta]_{\lambda,u}} \times 100 = \% \text{ helix} \qquad \text{Eq. (2)}$$

We evaluated Eq. (2) at 2nm intervals over the range λ = 228 to 212nm. This range appears to satisfy the criteria of good discrimination between the two states, and low "noise" in the experimental signal. The results are summarized in Table I.

Five proteins (myoglobin, ribonuclease, β-lactoglobulin, chymotrypsinogen, and lysozyme) have been studied in their fully-reduced forms (via excess dithiothreitol) in 6.0M guanidinium chloride (GuCl). These all show a CD spectrum similar to that of myoglobin, shown in Fig. 2. A positive dichroic band was not seen at or near 218nm for any of these denatured proteins. Our attempts to fit several mixtures of helical and U/EH pLL spectra are also drawn in Fig. 2 for comparison. We take it as self-evident that the CD of denatured proteins cannot be accounted for with the two-state polylysine model of Eq. (2).

We also examined pLL in 6M GuCl. At pH 6 the CD of pLL is the same in 6M GuCl as in 0.01M NaCl. However, at pH 11 in 6M GuCl, pLL shows a CD spectrum

TABLE I

Analysis of CD Spectra of pLL as Mixtures of "Helical" and "Random" Forms

	3M $Ca(ClO_4)_2$	6M $CaCl_2$	6M NaH_2PO_4	5M NaCl
Mean % helix*	26.0	32.2	68.0	14.6
Std. Dev., %	3.4	6.8	1.7	2.0
	0.10M $HClO_4$	2M $CaCl_2$	3M $CaCl_2$	
Mean % helix*	14.7	10.4	14.7	
Std. Dev., %	1.6	2.0	2.5	

*The arithmetic mean of estimates at 9 wavelengths at 2nm intervals from 228 to 212nm, computed with Equation 2.

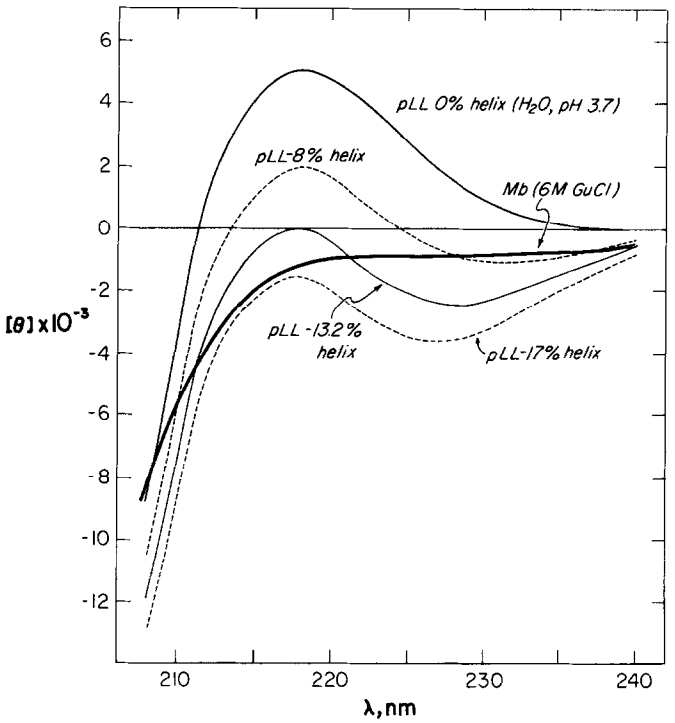

Fig. 2. CD spectra of disordered myoglobin, and poly-L-lysine containing largely the "unordered" form mixed with small fractions of the helical form. Myoglobin was dissolved in 6M GuCl, containing 10^{-3} M phosphate buffer, pH 6.2. The "0% helix" pLL spectrum shows the same experimental data as the curve labelled "H_2O, pH 3.7" in Fig. 1. The other three pLL curves are computed by means of Eq. (2) as detailed in the text.

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containing about 10% helix as analyzed by Eq. (2). This unexpected result was confirmed with all three samples of pLL. Similarly, a small fraction of helical form was found in pLL at pH 11 in 8M urea.

DISCUSSION. Can the experimental CD spectra be accounted for as a mixture of U/EH and helix? The results in Table I show that it can for pLL.

In Table I we show the mean % helix and the standard deviation for pLL in seven different solvent systems. The low standard deviations demonstrate the presence of only U/EH and helical conformations in these systems. We deliberately chose systems displaying less than 1/3 loss of random CD characteristics on the assumption that a putative "unordered structure" (similar to myoglobin in GuCl) could show itself most easily in this range of conversion.

Since the CD of fully charged pLL is the same in 6M GuCl as in very dilute electrolyte solutions, this structural form (U/EH) cannot be electrostatically stabilized. Taken with the success of a two-state model in fitting the dichroic spectra of pLL in several electrolyte systems, the foregoing strongly argues against the existence of an "extended-helical" form of pLL. We conclude that the U/EH form is "unordered". We believe that much or all of the CD data of Tiffany and Krimm (3) may be interpreted as mixtures of helical and "unordered" forms, and will not require invocation of a third structural form.

Myer (7) has applied a successful two-state analysis to the pH-controlled (pk~10) helix-coil transition of pLL. His studies appear to have been made at very low ionic strength, and therefore do not permit a definite decision on Tiffany and Krimm's proposed electrostatically-stabilized "extended-helix".

The mechanism of ClO_4^- stabilization of pLL helix probably involves anion-binding by the helical form of the poly-cation. A similarly strong binding of SCN^- by pLL has been inferred by Ciferri, et al. (8) from H^+-titration studies The helix-disrupting behavior of high ClO_4^- concentrations is consistent with the general solvent-mediated "denaturing" effects of ClO_4^- (9). Helix-stabilization by Cl^- and $H_2PO_4^-$ occurs at much higher concentrations than for ClO_4^-, and may proceed by electrostatic screening as well as by ion-binding. We note in Table I that the highest standard deviations occur in the solutions with $(Ca^{++}) > 3M$. This may reflect a small extent of specific interactions of Ca^{++} with the polypeptide (9). Protonated pLL appears to offer an attractive model ion-binding system.

We are in full accord with Tiffany and Krimm (2,3) that "random" proteins and "random" homopolyamino acids show substantially different CD spectra. We do not at present have an explanation for this difference. It no longer appears justifiable to use the "random" or "unordered" form of pLL as a reference state in the Greenfield-Fasman analysis. We are presently carrying out quantitative comparisons of several proteins in randomizing solvents with

a view toward establishing a more reliable reference state for unordered proteins.

Tanford, et al. (10) have provided molecular-kinetic evidence that 6M GuCl converts many globular proteins to statistically-random chain conformations. Contrary to our expectations and those to be expected from Tanford's studies, we find a small but real fraction of pLL helix persisting in this solvent. This finding necessarily raises doubts about the generality of strong GuCl as a randomizing solvent for proteins.

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