Circular Dichroism Studies on α-L-Glutamic Acid Oligomers in Solution

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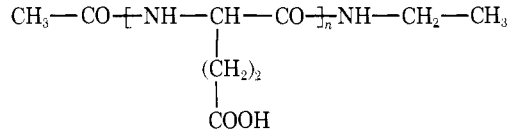
Abstract: A series of oligomers **1** with $1 \leq DP < 13$ and polymers of low polydispersity with $13 < \overline{DP}_n \leq 50$ has been prepared and studied to show the influence of polymerization degree, nature of the counterions, and ionic strength on their conformations in solution. For this purpose, the optical properties (CD) have been systematically investigated. In this work, one proves the existence of four specific conformations which are well characterized and shows that the spectra have to be considered in each case as a combination of only two limiting forms (α helix + random, β sheet + random, or extended form + random).

In a previous paper,[1] the synthesis and potentiometric behavior in salt-free aqueous solutions of α-L-glutamic acid oligomers have been discussed. The general formula of these derivatives is



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in which the end groups have been deliberately blocked to specifically test the behavior of the ionic side chains. The evolution of the electrostatic potential as a function of the polymerization degree (DP) and of the neutralization degree (α) has been discussed. Nevertheless, a complete interpretation of the electrostatic phenomena needs a correlation between the properties observed (pH or pK) and the conformations. In fact, the charge density is determined as the ratio of the net charge over the mean length of the molecule and the conformational transition is directly related to the repulsive interactions strictly given by the charge density. So, it seems very important to correlate the pontentiometric behavior to the conformation for each system.

In this work, the circular dichroism (CD) measurements are presented and discussed on oligomers up to $\overline{DP}_n = 50$ and compared to those obtained with a polymer of $\overline{DP} = 400$. The experimental spectra are interpreted in terms of a combination of limiting spectra corresponding to the four possible conformations in aqueous solutions: α helix, β sheet, unordered random form, and extended structure.

Experimental Section

CD spectra were recorded on a dicrograph D.C. III from Jobin Yvon (France) by use of a 1-mm quartz cell at room temperature (20 °C). The CD has been tested in the region of characteristic electronic absorption bands for amide groups between 190 and 250 nm. The experimental data are reported in Figures 1-6 as the difference in the extinction coefficients for left- and right-handed circularly polarized light, $\Delta\epsilon$, for solutions of equal concentration expressed in equiv $l.^{-1}$ The ellipticity per monomer residue is then calculated by

$$\theta = \Delta\epsilon \times 3300 \text{ (deg cm}^2\text{)/dmol}$$

To test the influence of the DP on the ellipticity (Figure 7), $\Delta\epsilon$ values are corrected by the factor DP/DP + 1 in order to express $\Delta\epsilon$ by peptidic bond.

The sample of polyglutamic acid is from Pilot; its $\overline{DP} = 400$ is given by viscosimetric measurements. The α-L-glutamic acid from Fluka had been used to prepare the oligomers with $\overline{DP}$ up to 50. The oligomers up to 12 are perfectly homogeneous and separated by ion-exchange chromatography. The oligomers with higher DP are low polydispersity fractions.[1]

Each sample is dissolved in water in the presence of NaOH to obtain a pH ~8 and then passed through ion-exchanger IR 120 H^+. The concentration is determined by conductimetric titration and adjusted to 10^{-3} equiv $l.^{-1}$. The neutralization degree varies from 0 to 1 by successive additions of NaOH and $Ca(OH)_2$ (0.04 N). The variation of the ionic strength is obtained by addition of NaCl and $CaCl_2$ solutions. Eventually the solutions under their acidic form are left for 24 h in order to test aging or adjusted at pH 3 with 0.1 N HCl to repress the autodissociation of the polycarboxylic acid.

Results and Discussion

Different works have been published about small oligopeptides[2-5] and show the influence of the DP on physico-

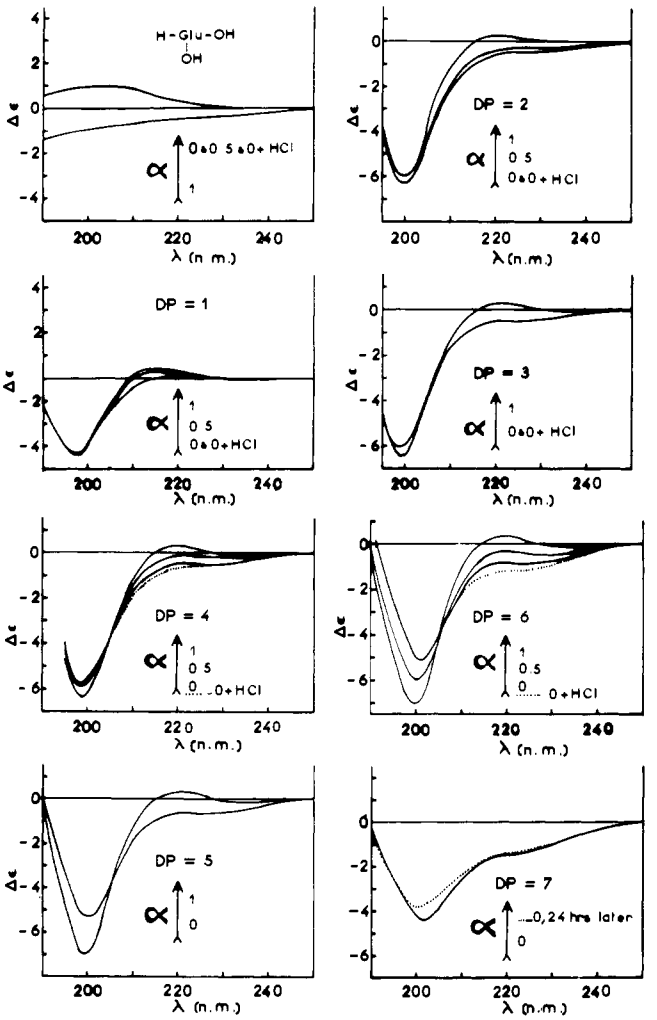

Figure 1. Circular dichroism spectra of the α-L-glutamic acid oligomers (DP = 1, 2, 3, 4, 5, 6, and 7) as a function of the neutralization degree: (—) initial readings; (· · ·) readings after addition of HCl or after ageing of 24 h.

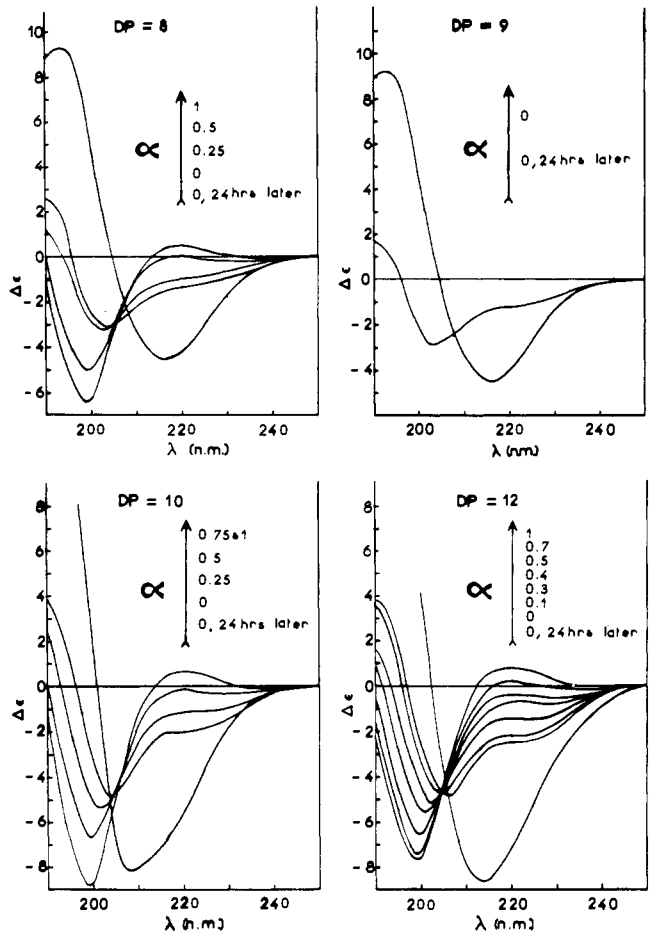

Figure 2. Circular dichroism spectra of the α-L-glutamic acid oligomers (DP = 8, 9, 10, and 12) as a function of the neutralization degree and 24 h later for $\alpha = 0$.

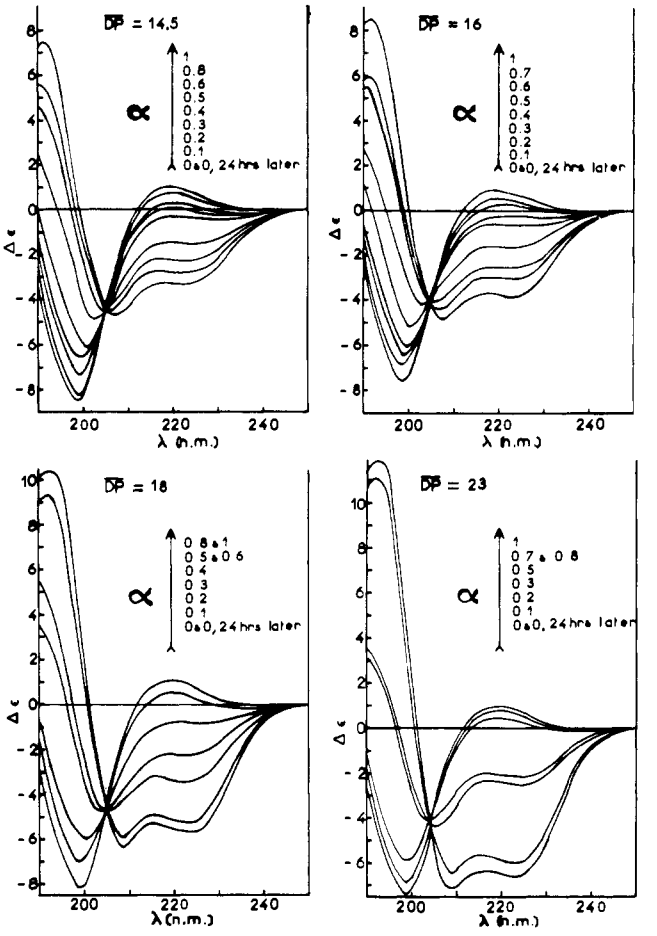

Figure 3. Circular dichroism spectra of the α-L-glutamic acid oligomers ($\overline{DP}_n$ = 14.5, 16, 18, and 23) as a function of the neutralization degree and 24 h later for $\alpha = 0$.

chemical properties, especially on their conformation. Parameters such as solvent composition, temperature, and aging have been studied on the polymers.[6,7] Nevertheless, only a few results are available regarding oligomers with DP over 10.

In Figures 1–5, α-L-glutamic acid oligomers CD spectra with DP from 1 to 50 are given; the behavior of the oligomers is compared to those of the polymer ($\overline{DP}$ = 400). On each oligomer and on the polymer, the neutralization degree varies from 0 to 1 in order to test the influence of the charge density on the conformations in solution; the influence of the DP on the stability of each conformation may also be deduced; aging is at last investigated on each oligomer (Figures 1–5) and is by another way a contribution to the study of the conformational stability.

From Figure 1, the importance of obtaining the end-blocked monomer as a reference for the series is clearly shown.

Figures 1–5 are characterized by an isodichroic point corresponding to $\lambda = 205 \pm 1$ nm, $\Delta\epsilon = -4 \pm 0.5$ as soon as DP is equal or larger than 3; an exception occurs when DP = 8 and 9 for which this point is not clear and corresponds to $\Delta\epsilon_{205} = -3$ and $\Delta\epsilon_{205} = -2.8$, respectively.

Finally, the comparison of CD spectra with monovalent and divalent counterions on the fully ionized solutes is presented on Figure 6. The maximum $\Delta\epsilon_{220}$ decreases and the minimum $\Delta\epsilon_{198}$ increases with divalent counterions. The effect may correspond to a lowering of the extension degree of the molecule when Ca^{2+} intramolecular bridges appear and when the charge density diminishes, due to the well-known strong binding of divalent counterions. If the ionic strength increases on the $\alpha = 1$ Na forms, the observed effect is of the same order; the screening salt effect produces a decrease of interionic repulsions and, following, a lowering of the molecular extension. This effect has been previously confirmed by hydrodynamic data.[8]

Influence of the Chain Length. The low DP CD spectra are characterized by one minimum ($\lambda \sim 200$ nm) slightly depending on the neutralization degree and on the DP. When the latter increases, new CD spectra appear with one maximum ($\lambda \sim 192$ nm) and two minima ($\lambda = 210$ and 224 nm) generally attributed to the $\pi\pi^*_\perp$, $\pi\pi^*_\parallel$, and $n\pi^*$ electronic transitions, respectively. Simultaneously, a great dependence of the CD spectrum with the neutralization degree occurs.

In Figure 7, the values of $\Delta\epsilon$ corresponding to $\lambda = 224$ nm, $\alpha = 0$; $\lambda = 210$ nm, $\alpha = 0$; $\lambda = 192$ nm, $\alpha = 0$; $\lambda = 220$ nm, $\alpha = 1$; and $\lambda = 198$ nm, $\alpha = 1$ are plotted as a function of DP. Both sets of wavelengths are choosen as characteristic of the helical and extended structure, respectively. Finally, the evolution of the lowest wavelength minimum is plotted as λ min (DP). The wavelength increases up to 210 nm and in the same time, the corresponding $\Delta\epsilon_{min}$ grows through a maximum for $\lambda = 205$ nm and then decreases.

In each representation, a small variation with the DP is observed as long as the DP is lower than 6 due to the influence of the chain length on the absorption spectra as previously discussed for small helical polymers by Tinoco et al.[9] Then it follows a transition and a stabilization of well-defined conformations: when $\alpha = 0$, the helical structure is obtained for DP = 25 characterized by the position of the minimum at 210 nm; the corresponding $\Delta\epsilon_{224}$ get a constant value when DP = 50 and is attributed to the 100% of helicity. When $\alpha = 1$, the extended conformation is stabilized for DP ~ 20 followed by a slightly increasing $\Delta\epsilon$ with DP. The corresponding spectrum

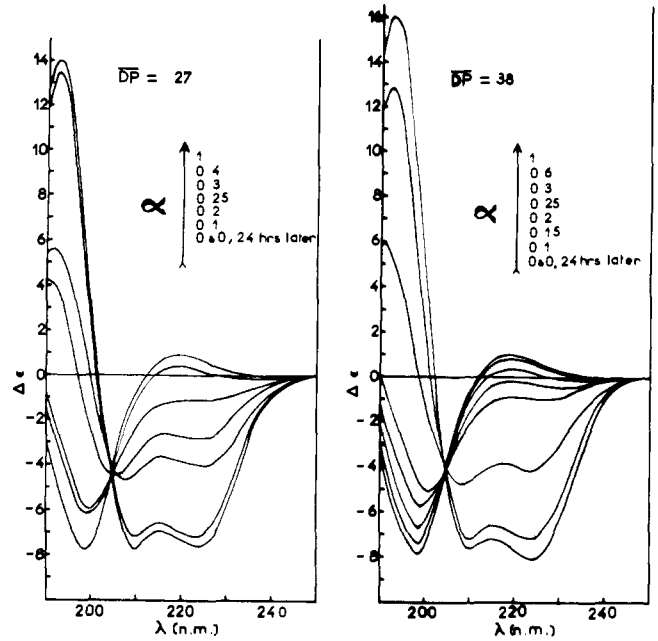

Figure 4. Circular dichroism spectra of the α-L-glutamic acid oligomers ($\overline{DP}_n$ = 27 and 38) as a function of the neutralization degree and 24 h later for α = 0.

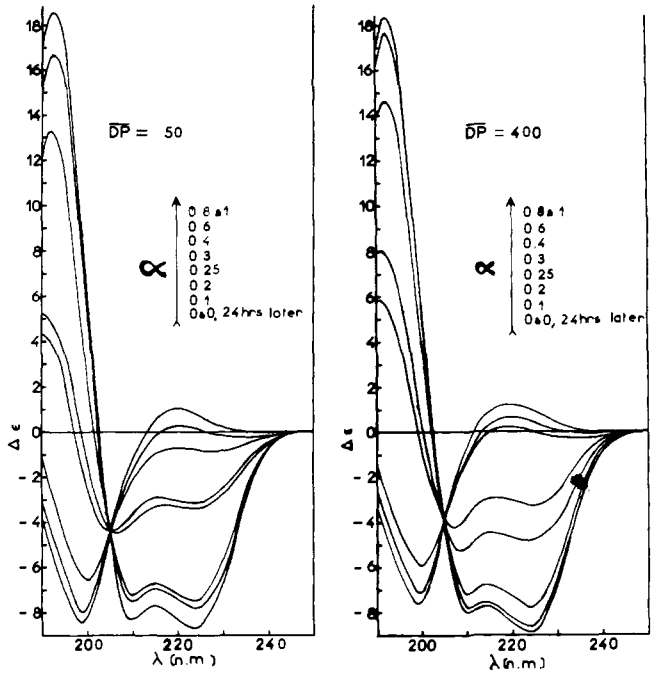

Figure 5. Circular dichroism spectra of the α-L-glutamic acid oligomers ($\overline{DP}_n$ = 50 and 400) as a function of the neutralization degree and 24 h later for α = 0.

is analogous to that of polyproline II; nevertheless, this conformation presents nearly the same extension degree as a fully extended peptide chain and nothing can be definitively concluded about the exact characteristics of the regular structure obtained.

In conclusion, it is clearly established that the α helix is obtained as soon as $\overline{DP}_n$ = 25 with 100% helicity when $\overline{DP}_n$ = 50. When the neutralization degree is equal to 1, the conformation is extended and stabilized when $\overline{DP}_n$ is over 20.

Stability of the Different Structures. The stability of the structures corresponding to α = 0 has been tested by addition of HCl to the solution or by 24 h of aging at room temperature. From Figures 1–5, the conclusion is that the structures adopted with DP < 4 and DP > 12 are very stable.

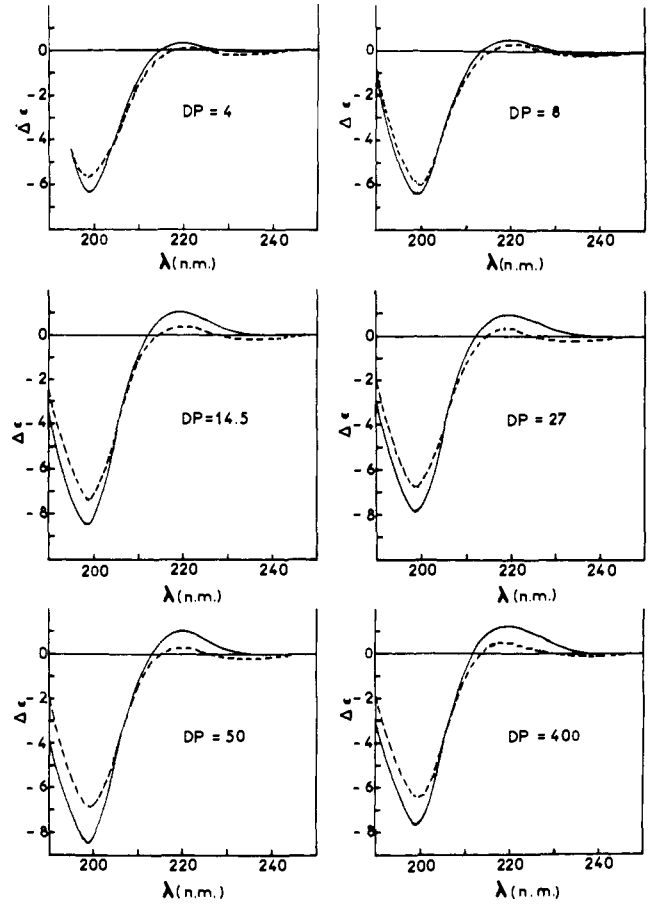

Figure 6. Circular dichroism spectra of α-L-glutamic acid oligomers for α = 1 and DP = 4, 8, 14.5, 27, 50, and 400. Comparison between (—) Na^+ and (- - -) Ca^{2+}.

When the values of DP are 4–7 a precipitation following aggregation of small unordered chains occurs without large modification of the spectra (Figure 1).

When DP is equal to 8 and 9, a kinetic process is observed with modification of the conformation to a well-defined structure analogous to the β structure; this one is characterized by a CD spectrum with a maximum at λ = 194 nm and a minimum at 217 nm in concordance with the literature.[3] The conversion is accelerated with addition of HCl.

Both processes of instability and precipitation under β conformation seem to appear when DP is equal to 10 and 12.

It can be concluded that there exists a critical length (DP = 8–12) for which the β structure is the most stable conformation in solution.

Computed CD Spectra. Generally, the different CD spectra obtained on peptides or proteins are interpreted as a combination of three limited spectra corresponding to the α, β, and random structures as previously investigated by Myer[10] and Fasman et al.[11] Nevertheless, any solution based on a combination of three structures is unadapted to explain experimental results.

For interpretation, one must conclude that the β structure cannot be used in combination with α and random structure, if an isodichroic point at λ = 205 nm exists. Then, some hypotheses on homooligomers can be introduced concerning the possible transitions: when α varies from 1 to 0, we propose to introduce the evolution from the extended form (called ES) through an unordered form (or random structure called RC) to that of the α helix or to the β form. As proposed by Krimm,[6] we adopt the existence of the unordered form; the model with DP 5, α = 0 just before the transitions discussed in Figure 7

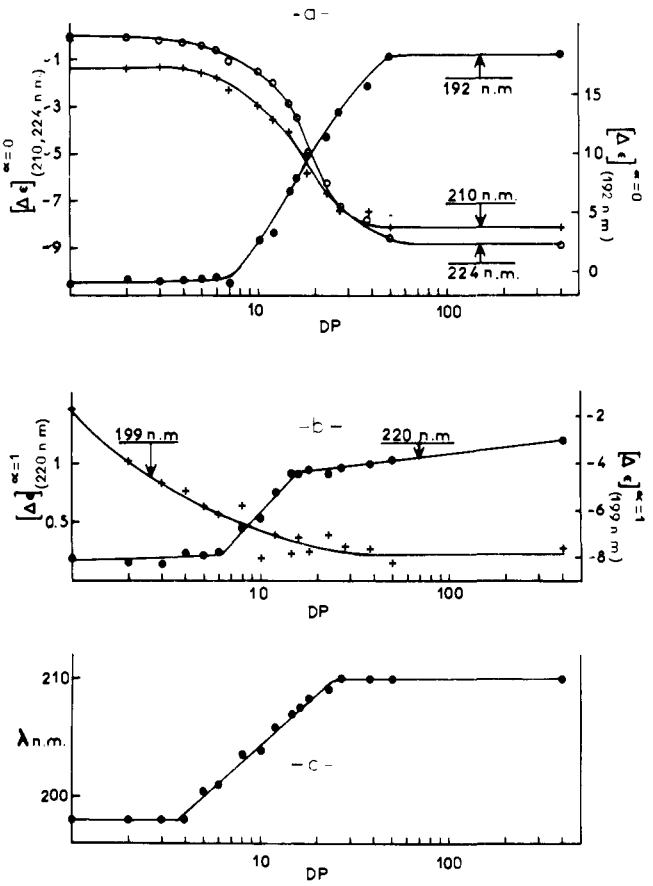

Figure 7. (a) Variation of $\Delta\epsilon$ for $\alpha = 0$ at 192, 210, and 224 nm as a function of DP. (b) Variation of $\Delta\epsilon$ for $\alpha = 1$ at 199 and 222 nm as a function of DP. (c) Variation of the wavelength of the first minimum as a function of DP.

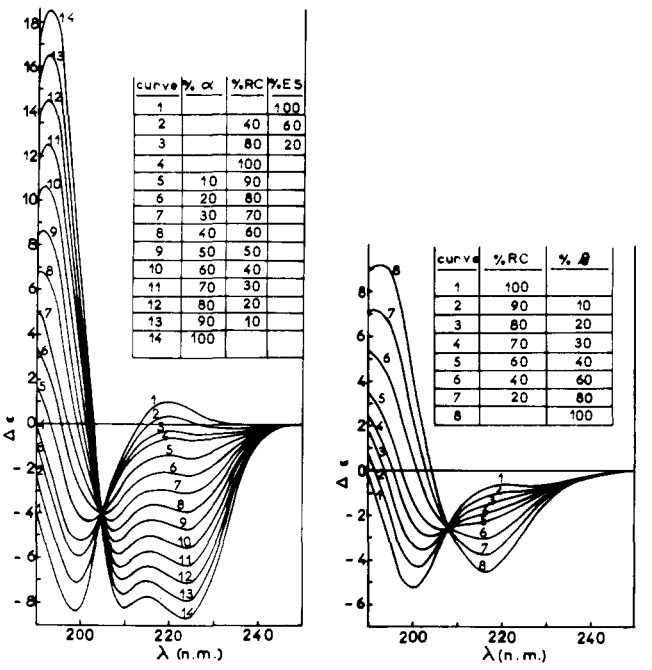

Figure 8. Calculated circular dichroism of $(Glu)_n$ varying percentages of α helix, random conformation, extended structure, and β structure as indicated.

Table I. Characteristics of the Four Limiting Spectra Used as References

Structures		Spectrum characteristics		
		Max (+)	Min (−)	Min (−)
α helix	λ, nm	192	210	224
	$\Delta\epsilon$	+18.4	−8	−8.9
β sheet	λ, nm	193	217	
	$\Delta\epsilon$	+9.2	−4.5	
Random	λ, nm		200	
	$\Delta\epsilon$		−5.3	
Extended structure	λ, nm	220	199	
	$\Delta\epsilon$	+1.2	−8	

Table II. Estimated Percentage of α Helix, β Structure, and Random Structure at $\alpha = 0$ from Computed CD Curves (Figure 8)

DP	Structure content, %		
	α helix	β structure	Random
5[a]			100
8		20	80
10	15		85
12	25		75
14.5	35		65
18	59		41
23	75		25
27	88		12
38	92		8
50[a]	100		0
400	100		0

[a] References.

has been adopted to represent this structure and can be found with the different oligomers when α is 0.4 with $\overline{DP}_n$ 16, 0.25 with $\overline{DP}_n$ 38, ... From these hypotheses, two families of CD spectra are drawn in Figure 8 which can be used to interpret every experimental spectrum. The construction of the computed spectra needs the knowledge of the four limiting spectra whose characteristics are given in Table I. The evaluation of structure content of the oligomers, obtained by means of calculated spectra (Figure 8) when $\alpha = 0$, is given in Table II.

The question is about the nature of the α-helix ordered form with low DP oligomers. The good concordance between experimental CD spectra and the calculated ones by introduction of the characteristics of the α-helix structure obtained with $\overline{DP}_n = 50$, $\alpha = 0$ seems to be enough to conclude that, as soon as DP = 10, a small fraction of the chain adopts an α-helical structure following the model of Zimm and Bragg[12] and Lifson and Roig.[13]

It is interesting to confirm that the β structure appears, as often previously found, when DP = 7 or 8. When DP = 10–12, the degree of helicity is low and, with aging, the β structure is established. With $\overline{DP}_n = 14.5$, the helicity grows to 40% and becomes stable without contribution of the β form.

Concerning the possibility of induction of the different structures, the stability of a given mixture of oligomers under β form (DP = 8, $\alpha = 0$) with oligomers under random structure DP = 5, $\alpha = 0$ or with α-helical oligomers ($\overline{DP}_n = 50$, $\alpha = 0$) has been proved; the resulting CD spectra correspond strictly to the calculated spectra given by Fasman[11] for α, β mixtures or by Figure 8 for random, β mixtures. The conclusion is that there is no cooperative effect between the different forms or induction of structures using one of them as a matrix.

Conclusion

In this work, the circular dichroism of a series of α-L-glutamic acid oligomers has been discussed. The influence of the polymerization degree has been considered to relate the most

probable conformation in solution to the neutralization degree, to the nature of the counterions, and to the ionic strength.

The four limiting spectra corresponding to the α helix, β, unordered, and extended structures have been obtained and used to propose an interpretation of each of the experimental CD spectra. The transitions are considered by introduction of only two contributions depending on the neutralization degree and on the DP: α-unordered, unordered-extended, or β-unordered forms. This treatment is original and gives very good concordance between experimental and calculated spectra.

The limiting forms can be used when discussing the structure of other peptides and proteins. This work proves that there is characteristic DP for the existence of each ordered form and implies that over DP = 10, there is practically no variation in the CD characteristics of limiting structures. These results can be used to get a better resolution of CD spectra on proteins by taking into account the influence of the length of each sequence.

Finally, the stability of each oligomer is discussed and the DP is shown to have a large influence on the most stable conformation for a given solvent condition.

References and Notes

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Ionic Strength and pH Effects on the Rates of Reduction of Blue Copper Proteins by $Fe(EDTA)^{2-}$. Comparison of the Reactivities of *Pseudomonas aeruginosa* Azurin and Bean Plastocyanin with Various Redox Agents

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Abstract: The rates of the anaerobic reduction of *Pseudomonas aeruginosa* azurin, bean plastocyanin, and *Rhus vernicifera* laccase and stellacyanin by $Fe(EDTA)^{2-}$ have been measured in the pH range 5.6–7.8. The results in each case have been analyzed in terms of electron transfer to protonated and deprotonated protein species in rapid equilibrium. Rate constants for the protonated (k_a) and deprotonated (k_b) forms and pK values are as follows: azurin, $k_a = 2.4\ (10^3)$, $k_b = 1.0\ (10^3)\ M^{-1}\ s^{-1}$, p$K$ = 6.4; plastocyanin, $k_a = 5.5\ (10^4)$, $k_b = 3.1\ (10^4)\ M^{-1}\ s^{-1}$, p$K$ = 6.1; laccase, $k_a = 4.6\ (10^2)$, $k_b = 1.6\ (10^2)\ M^{-1}\ s^{-1}$, p$K$ = 6.8; stellacyanin, $k_a = 5.7\ (10^5)$, $k_b = 5.1\ (10^5)\ M^{-1}\ s^{-1}$, p$K$ = 6.4. The ionic strength dependences for the reduction of azurin and plastocyanin have also been measured. Marcus theory has been employed to analyze the ionic strength data, and protein self-exchange rate constants (k_{11}) that include correction for electrostatic effects have been calculated. The electrostatics-corrected k_{11} values ($M^{-1}\ s^{-1}$) for azurin are as follows: 7 (10^{-3}) [$Fe(EDTA)^{2-}$], 2 (10^4) (cytochrome *c*), and 8 (10^7) (cytochrome c_{551}). For plastocyanin, k_{11} values are 10 [$Fe(EDTA)^{2-}$], 2 (10^6) (cytochrome *c*), and approximately 4 (10^{10}) (cytochrome *f*); all rate constants except the one based on cytochrome *f* are corrected for electrostatic effects. It is proposed that $Fe(EDTA)^{2-}$ cannot penetrate the hydrophobic region surrounding the blue copper center in azurin and is forced to transfer an electron over a relatively long distance (>3 Å). The redox centers in cytochromes apparently can approach the plastocyanin and azurin blue copper centers, resulting in efficient electron transfer. For both blue copper proteins, electron-transfer reactivity is greatest when a physiological partner is involved.

We have previously reported[1] second-order rate constants and activation parameters for the reduction of the blue (or type 1)[2,3] copper in *Pseudomonas aeruginosa* azurin, bean plastocyanin, and *Rhus vernicifera* laccase and stellacyanin by $Fe(EDTA)^{2-}$. In order to compensate for the different potentials of the type 1 copper in the four proteins, relative Cu(II)/Cu(I) self-exchange rates were calculated from Marcus theory and found to vary over ten orders of magnitude, according to 2 (10^{10}) (stellacyanin) > 1 (10^5) (plastocyanin) > 7 (10^2) (azurin) > 1 (laccase).[1] As our previous studies did not give careful consideration to medium effects on the rate constants, we report in this paper the nature of the pH and ionic strength dependences for the $Fe(EDTA)^{2-}$ reductions. Employing Marcus theory with inclusion of electrostatic effects, we have analyzed in detail the ionic strength dependences of azurin and plastocyanin electron-transfer reactions. This analysis has allowed us to calculate electrostatics-corrected, blue-protein self-exchange rate constants (k_{11}) from cross reaction kinetic data. The k_{11} values for azurin and plastocyanin based on cross reactions with $Fe(EDTA)^{2-}$ and horse heart cytochrome *c* are compared with those estimated for probable physiological substrates, and the results are discussed in terms of the accessibility of the copper site to each redox agent.