nature of solvent. Furthermore it seems that there is little influence on bond stability due to neighboring CH_3 substituents, for if methyl group effects were present these should be modified by a transfer from an aqueous to an apolar solvent. The intrinsic interaction between hydrogen-donor and hydrogen-acceptor groups is thus not sensitive to its immediate (noncovalent) environment.

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Structure of Macromolecular Aggregates. I. Aggregation-Induced Conformational Changes in Polypeptides*

Gordon G. Hammes and Stephen E. Schullery

ABSTRACT: The interactions between the polyacids poly-α-L-glutamic acid and poly-α-L-aspartic acid and the polybases poly-L-lysine and poly-L-ornithine in a variety of solvents have been studied with optical rotatory dispersion and circular dichroism. The data obtained suggest that poly-α-L-glutamic acid and poly-L-lysine form a β-pleated-sheet structure with 1:1 stoichiometry at pH 4 and 7 in aqueous 0.01 M NaF, although poly-L-lysine by itself is in a random coil conformation under the same conditions and poly-α-L-glutamic acid by itself is in a random coil conformation at pH 7 and an α-helix conformation at pH 4. No interaction was detected in the poly-α-L-glutamic acid–poly-L-lysine mixture at pH 11 where poly-L-lysine is helical and poly-α-L-glutamic acid is a random coil. The rotatory spectra of the other polyacid–polybase mixtures, poly-α-L-glutamic acid–poly-L-ornithine, poly-α-L-aspartic acid–poly-L-ornithine, and poly-α-L-aspartic acid–poly-L-lysine, at pH 7 are considerably reduced in amplitude, but cannot be identified with any specific geometric structure. Since β structure is observed only with the polyacids and polybases having the longest hydrocarbon side chains, hydrophobic interactions are apparently of importance in the stabilization of the aggregate. Rotatory spectra characteristic of α-helical structure are observed in methanol–water–0.01 M NaF (pH 7) solutions of mixtures of poly-α-L-aspartic acid–poly-L-ornithine and poly-α-L-aspartic acid–poly-L-lysine at methanol concentrations where none of these polypeptides are helical by themselves. An investigation of the stoichiometry suggests that primarily the polybase is being converted into an α helix. The nature of the poly-α-L-glutamic acid–poly-L-ornithine and poly-α-L-glutamic acid–poly-L-lysine interactions in methanol–water solutions could not be determined, apparently because of very extensive aggregation. These results indicate extreme conformational changes can occur upon aggregation of macromolecules. The nature of the aggregate is a sensitive function of the polypeptide side chains as well as of solvent composition. The effects of aggregation on rotatory spectra are also considered.

Many of the functional properties of biological systems are dependent upon the molecules in the system existing in a specific state of aggregation, for example, protein coats of viruses, the quaternary structure of enzymes and membranes. In order to better understand the factors influencing the structure of protein aggregates and the properties of such aggregates, we have investigated the structure of polypeptide aggregates formed under a variety of conditions. Optical rotatory dispersion and circular dichroism measurements were made on solutions of mixtures of the polyacids and polybases PAA, PGA, PO, and PL.[1] In aqueous solution (0.01 M NaF), PGA and PL appear to interact to form a β-pleated sheet when both polypeptides are initially random coils (pH 7) and when PGA is initially in an α-he-

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: PAA, poly-α-L-aspartic acid; PGA, poly-α-L-glutamic acid; PO, poly-L-ornithine; PL, poly-L-lysine.

lical conformation and PL is a random coil (pH 4). Aggregation of PAA with PL or PO in 60% (v/v) methanol–water–0.01 M NaF produces optical rotatory dispersion and circular dichroism spectra characteristic of an α helix, even though both polypeptides are initially in a random coil configuration. Thus aggregation can cause extreme changes in macromolecular conformation. Whether or not a specific geometric structure is produced and what the structure is, is a sensitive function of the length and ionization state of the polypeptide side chains, as well as of the solvent composition.

Experimental Section

The polypeptides obtained from Pilot Chemicals were reported to have the following molecular weights: PGA (sodium salt, lot G-80), 86,000; PAA (lot A-38), 20,000; PO (hydrobromide, lot O-19), 90,000; and PL (hydrobromide, lot L-77), 100,000. The polypeptides were put into solution and exhaustively dialyzed against water in order to get rid of species with molecular weight less than about 10,000. In the case of PO and PL the solutions were first dialyzed against aqueous 0.5 M NaF and then against water. The resultant polymer solutions were then lyopholized and stock solutions, about 10^{-3} M in monomer, were prepared as needed. In the case of PAA, the recovery after dialysis was only about 3% as compared with 60–70% recovery for the other polymers; also a lyophilized polymer could not be obtained so that the concentrated polymer solution was used to prepare a stock solution. The polypeptide concentration was determined from optical density measurements in the ultraviolet area using the known extinction coefficients for PGA (Rosenheck and Doty, 1961; Imahori and Tanaka, 1959), PAA (McDiarmid and Doty, 1966), and PL (Rosenheck and Doty, 1961). A micro-Kjeldahl analysis was used to determine the concentration in the case of PO. The spectroscopic measurement and Kjeldahl analysis were found to give identical results for PAA solutions. Demineralized, distilled water and analytical reagent grade NaF were used to prepare all solutions. NaF was selected as an inert salt because of its low extinction coefficient in the far-ultraviolet region. Preliminary experiments with NaCl in place of NaF gave identical results in the aqueous PGA–PL system.

Aqueous mixtures of polypeptides were prepared by diluting each polypeptide solution to the desired concentration (usually 1.5×10^{-4} M in monomer) and adjusting the pH to the desired value with concentrated NaOH or HCl. The solutions were mixed by rapidly pipetting one solution into the other, and the pH was rechecked. In no case was more than a slight adjustment of pH necessary. In preparing the water–methanol solutions, the stock polypeptide solutions were first adjusted to pH 7.0 and then diluted with the appropriate methanol–water–NaF solutions. The solutions were then mixed as previously described. For the 60% (v/v) methanol–water–0.01 M NaF solution, the apparent pH, measured with a glass electrode which had been soaked in the solvent for 24 hr prior to use, was identical before and after mixing.

Absorbancy measurements were made with a Beckmann DU or a Zeiss PMQ II spectrophotometer. The pH measurements were made with a Radiometer Model 26 pH meter. Optical rotatory dispersion and circular dichrosim measurements of the mixtures were carried out with a Cary 60 spectropolarimeter with circular dichroism attachment using 1-cm or 1-mm path-length cells. All measurements were made at room temperature (~25°).

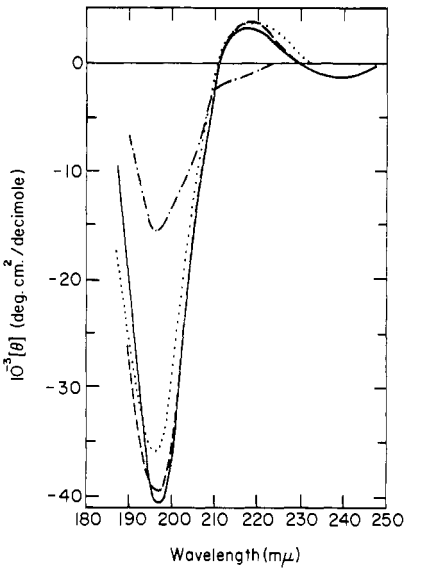

FIGURE 1: Plot of the mean residue ellipticity *vs.* wavelength for polypeptide solutions at pH 7, 0.01 M NaF: PGA (—); PO (– – –); PI (· · · ·); PAA (–·–·).

Results and Interpretation of Data

Both optical rotatory dispersion and circular dichroism measurements were made on a large number of polypeptide solutions encompassing a wide range of polypeptide compositions and concentrations, salt concentrations, and solvent compositions. Only data for cases where a well-defined structure of the aggregate was evident are presented here. In most cases, only circular dichroism results are presented, and the wavelength range is restricted to the regions of the ultraviolet absorption maxima. The mean residue rotation, $[m]$, and ellipticity, $[\theta]$, were calculated in the usual manner (*cf.* Yang, 1967; Scanu and Hirz, 1968).

We first consider the results obtained in aqueous solution. In Figure 1 the circular dichroism spectra of PGA, PAA, PO, and PL at pH 7.0, 0.01 M NaF are presented. At this pH and ionic strength the side chains of all four polypeptides are essentially completely charged (McDiarmid and Doty, 1966; Appelquist and Doty, 1962; Blauer and Alfassi, 1967). All except PAA exhibit the characteristic spectrum of a random coil (Velluz and Legrand, 1965; Holzworth and Doty, 1964): a large trough at ~198 mμ, a crossingover the zero axis at ~211 mμ, and a small peak at ~217 mμ. Nevertheless, PAA is probably also in the random coil form under these conditions (McDiarmid and Doty, 1966).

The circular dichroism spectra of equimolar mixtures of the four possible combinations of polyacid and poly-

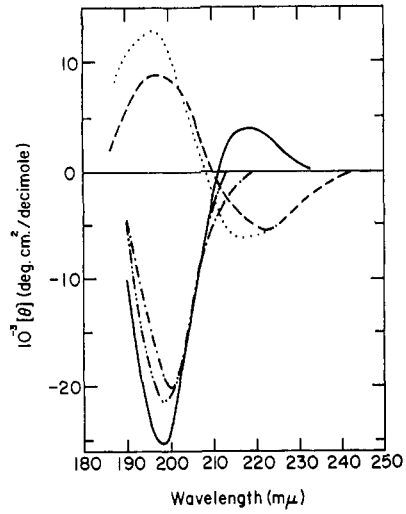

FIGURE 2: Plot of the mean residue ellipticity *vs.* wavelength for polyacid–polybase mixtures at pH 7, 0.01 M NaF: 1:1 mole ratio PGA–PL (– – –); 41:59 mole ratio PGA–PL (· · · ·); 1:1 mole ratio PGA–PO (——); 1:1 mole ratio PAA–PL (–·–·); 1:1 PAA–PO (–··–··).

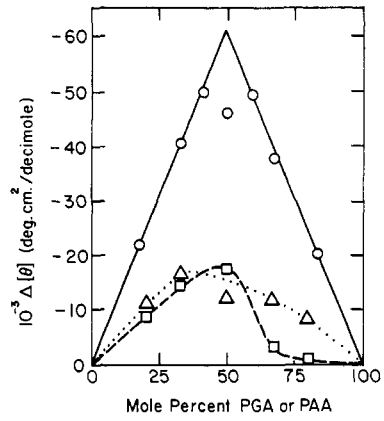

FIGURE 3: Continuous variation plot of mean residue difference ellipticities of polyacid–polybase mixtures: $\Delta[\theta]$ at 195 mμ for PGA–PL at pH 7.0, 0.01 M NaF (——) (○), $\Delta[\theta]$ at 222 mμ for PAA–PO in neutral 60% methanol, 0.01 M NaF (– – –) (□); $\Delta[\theta]$ at 222 mμ PAA–PL in neutral 60% methanol, 0.01 M NaF (· · · ·) (Δ).

base are given in Figure 2. The pH, ionic strength, and total molar residue concentration are the same as employed to obtain the data in Figure 1. The spectra of the PGA–PO, PAA–PO, and PAA–PL mixtures are characteristic of polypeptide random coil configurations, although the mean residue ellipticity is decreased 20–45% relative to that anticipated from the properties of the individual polypeptides. No turbidity was visible. Further investigation of the PAA–PO system indicated that reducing the NaF concentration from 0.01 to 0.001 M produced neither visible turbidity nor a change in the mean residue ellipticity. An increase in the concentrations of both components by a factor of 3 or 10 caused considerable turbidity, but the mean residue ellipticity was unchanged.

Two circular dichroism spectra of PGA–PL mixtures are included in Figure 2. They are strikingly different from the spectra of the other polypeptide mixtures. The spectrum of the 41:59 mole ratio PGA–PL mixture, in which no turbidity was visible, is essentially identical with that expected for a β structure: a ~197-mμ peak, a ~208-mμ crossingover the zero axis, and a ~218-mμ trough have been observed for the β form of silk fibroin (Iizuka and Yang, 1966) and a 218-mμ trough has been reported for the β conformation of PL (Sarkar and Doty, 1966). The amplitude of the ellipticity at 218 mμ is about 30% of that observed for the β conformation of PL. The 1:1 molar residue mixture of PGA and PL was slightly turbid. The spectrum appears to be that of a red-shifted β structure, with a reduction in the mean residue ellipticities. The crossover has shifted ~2 mμ and the 218-mμ trough has shifted ~5 mμ toward longer wavelengths. The infrared data of Blout and Idelson (1958) for a 1:1 mole ratio PGA–PL mixture in D_2O are also consistent with the formation of β structure. The stoichiometry of the PGA–PL interaction was investigated by determining the circular dichroism and optical rotatory dispersion spectra at various molar ratios while holding the total molar concentration constant. A continuous variation plot of the difference in the mean residue ellipticity of the mixture and that calculated for the pure polypeptide components at 195 mμ is shown in Figure 3. These results indicate the stoichiometry of the aggregate is 1:1. The fact that the difference ellipticity is less for the 1:1 mole ratio mixture than for the 41:59 and 59:41 mole ratio PGA–PL mixtures may be due to the turbidity of the 1:1 mole ratio mixture.

The 1:1 mole ratio PGA–PL mixture was also investigated at pH 4 and 11. At pH 4, most of the carboxyl side chains of PGA are protonated and the polymer is helical (Wada, 1960), while PL is a random coil with protonated side chains (Rosenheck and Doty, 1961). The optical rotatory dispersion and circular dichroism spectra of the pure polypeptides are shown in Figures 4 and 5, together with the spectra of the 1:1 mixtures. No turbidity of the mixtures was visible. The results indicate that the helical and random coil structures are converted into a β structure on aggregation. The relative magnitudes of the rotatory spectra are greater at pH 4 than at pH 7. This can be attributed either to more extensive β-structure formation at pH 4 or to a difference in the nature of the aggregate at the two pH values (see the discussion below for further consideration of this point). At pH 11, PL is largely uncharged and helical, while PGA is a negatively charged random coil. The circular dichroism spectrum of the 1:1 mixture could be quantitatively calculated from the spectra of the pure polypeptides, which strongly suggests no appreciable interaction between PGA and PL occurs.

The aggregation of the polyacids and polybases was also studied in methanol–water–0.01 M NaF solvents. All solutions were prepared from stock solutions at pH 7 as described in the Experimental Section. The circular dichroism spectra of the individual polypeptides were determined over the entire range of methanol–water compositions. In the cases of PL, PO, and PGA the conformation changed from a random coil in pure water to a helix in 90% (v/v) methanol. The spectrum of the helix, which has two negative extrema at ~208 and

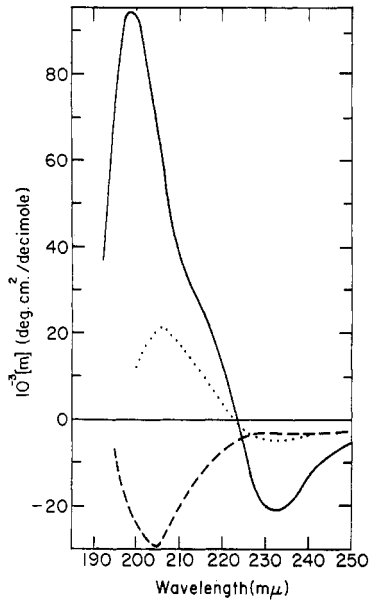

FIGURE 4: Mean residue rotation *vs.* wavelength for aqueous PGA–PL solutions at pH 4.0, 0.01 M NaF: PGA (——); PL (– – –); 1:1 mole ratio PGA–PL (· · · ·).

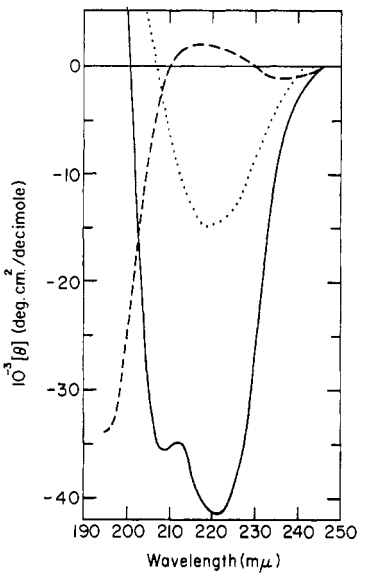

FIGURE 5: Mean residue ellipticity *vs.* wavelength for aqueous PGA–PL solutions at pH 4.0, 0.01 M NaF: PGA (——); PL (– – –); 1:1 mole ratio PGA–PL (· · · ·).

~222 mμ and crosses the zero axis at ~202 mμ (Iizuka and Yang, 1966; Sarkar and Doty, 1966), can be easily identified. The transition from coil to helix in various mixed solvents is well documented (*cf.* Fasman, 1967). We have found that at pH 7 the change from coil to helix occurs in 50–65% (v/v) methanol for PGA, in 60–75% methanol for PL, and in 75–90% methanol for PO. The change observed in the mean residue ellipticity of PAA at 222 mμ in 90% methanol is less than 5% of those found with the other polypeptides, and probably represents solvent effects on the chromophore rather than a change in polymer conformation. Optical rotatory dispersion measurements in aqueous solution indicate that PAA does not have the characteristic spectrum of an α helix even at pH 3.5.

All of the 1:1 polyacid–polybase mixtures in the methanol–water solutions showed turbidity which usually resulted in precipitation within 24 hr. Often the turbidity was slow to develop, requiring about 15 min before becoming apparent. After development of the turbidity, which required no longer than 1 hr, optical rotatory dispersion and circular dichroism spectra were reproducible for many hours and all reported spectra were taken within this latter time period. In one of the most turbid solutions, the mean residue ellipticity was found to be larger in the early stages of aggregation, but the form of the wavelength dependence of the ellipticity was the same at all times. The insensitivity of the Cary 60 spectropolarimeter to scattered light is well documented (Steim and Fleischer, 1967; Wallach and Zahler, 1966; Lenard and Singer, 1966). Turbidity *per se* does not necessarily reduce the circular dichroism signal; for example, we found that a clear 1:1 solution of PAA and PO at 1.5×10^{-4} M total monomer concentration in a 1-cm cell had the same circular dichroism spectrum as a very turbid 1:1 solution at 1.5×10^{-3} M total monomer concentration in a 1-mm cell. Also an increase in optical rotation accompanies the aggregation of PGA at low pH prior to precipitation (Cassim and Yang, 1967; Tomimatsu *et al.*, 1966).

Although the polyacids and polybases all appear to interact in methanol–water solutions, the circular dichroism spectra are not always readily interpretable. The mean residue ellipticities for the PGA–PO system were very small and virtually unchanged over a range of methanol concentration of 50–90% by volume. A small trough occurred at approximately 222 mμ ($[\theta] \approx 3 \times 10^3$ (deg cm^2)/dmole) with a crossingover the zero axis at ~211 mμ. The circular dichroism spectra for 1:1 mole ratio PGA–PL mixtures in 50, 60, and 75% methanol were similar to those found for the PGA–PO mixture. However, in 90% methanol, the magnitude of the ellipticity was greater ($[\theta]_{222} \approx -10 \times 10^3$ (deg cm^2)/dmole) and the doublet trough characteristic of the α helix could be resolved suggesting aggregation of preexistent helices. The apparent low value of the molar ellipticity for the above systems is probably due to large scale aggregation. This explanation is supported by the fact that the 222-mμ trough of the 1:1 PGA–PL–50% methanol system had a magnitude of $[\theta] = -7 \times 10^3$ (deg cm^2)/dmole 10 min after mixing, but this value fell to -2×10^3 (deg cm^2)/dmole within 1 hr.

The 1:1 mixtures of PAA–PO and PAA–PL displayed dramatic changes in their circular dichroism spectra relative to those of the individual polypeptides in 60% methanol solutions. The results are summarized in Figures 6 and 7. All of the polypeptides are in the random coil conformation in 60% methanol, but the circular dichroism spectra of the two mixtures possess the characteristic doublet of the helix, red-shifted 2–3 mμ from that usually observed. Similar results were obtained for the PAA–PL mixture in 50% methanol. The magnitude of the ellipticity is about 40% of that observed for solutions of helical PGA, PL, and PO. Continuous variation plots of the difference in mean residue ellipticity at 222

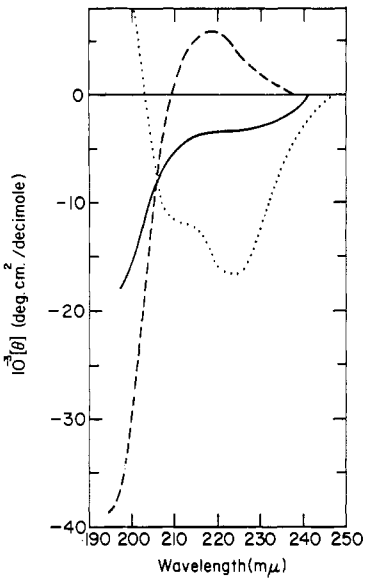

FIGURE 6: Mean residue ellipticity *vs.* wavelength for PAA–PO in neutral 60% methanol, 0.01 M NaF: PAA (——); PO (– – –); 1:1 mole ratio PAA–PO (· · · ·).

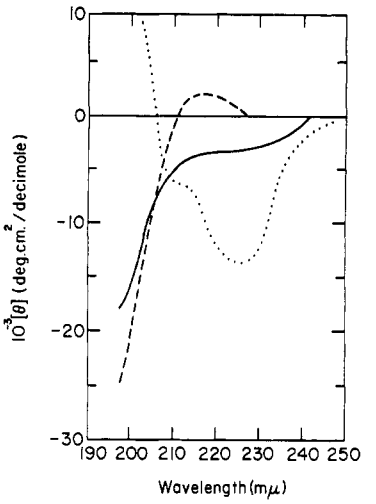

FIGURE 7: Mean residue ellipticity *vs.* wavelength for PAA–PL in neutral 60% methanol, 0.01 M NaF: PAA (——); PL (– – –); 1:1 mole ratio PAA–PL (· · · ·).

mμ of the mixture and the sum of the ellipticities which would have resulted from having both components separate *vs.* molar composition of the mixture is included in Figure 3. These plots are quite unsymmetrical: the mixtures containing higher percentages of polybase show more helix formation than those containing mostly PAA. The 1:1 mole ratio mixtures were the only ones with visible turbidity, and the magnitudes of the mean residue rotatory properties of these solutions may not be strictly comparable with those of the other solutions.

Discussion

The results presented indicate that very specific structures can be found in polyacid–polybase aggregates which can be quite different from the structures of the unaggregated polypeptides under identical conditions. A similar observation has been reported by Gratzer and McPhie (1966) for the interaction of PL and polyacrylic acid in neutral aqueous and dioxane–water solvents. The general form of the optical rotatory dispersion and circular dichroism spectra in the mixtures studied in our work are quite similar to those reported for solutions of various polypeptides and proteins by themselves, but the magnitudes of the mean residue ellipticity and rotation are considerably less than anticipated for complete conversion from random coil to α-helical or β-structure conformation. This may be due to incomplete conversion of the polypeptides into the specific structure or may be due to a change in the rotatory properties of the polypeptides in the environment of the aggregate. Similar uncertainties in the quantitative interpretaion of circular dichroism spectra were encountered by Iizuka and Yang (1966) who found that the 218-mμ circular dichroism trough for silk fibroin decreased under conditions where infrared spectra suggested an increase in β structure. Also Sarkar and Doty (1966) reported that when PL β structure is induced by adding sodium dodecyl sulfate to a neutral PL solution or to an alkaline solution already in the β conformation, the mean residue ellipticity at 218 mμ of the resulting β structure is only about half that found for β structure produced by heating alkaline PL to 50° for 10 min. In view of these findings, estimates of the extent of helical and β-structure formation from optical rotatory dispersion and circular dichroism measurements of aggregates of polypeptides and proteins must be viewed with considerable caution. Nevertheless, the qualitative interpretation of the form of the optical rotatory dispersion and circular dichroism spectra of polypeptide aggregates in this work appears to be reasonable and self-consistent.

Many of the rotatory spectra are slightly red shifted with respect to those encountered in unaggregated solutions. This appears to be a frequent characteristic of aggregated polypeptides (Cassim and Yang, 1967) and has also been observed with membranes (Lenard and Singer, 1966) and aggregates of mitochondrial structural protein (Steim and Fleischer, 1967). The aggregation, at least in the PGA–PL β structure, must be quite extensive since filtration of both the turbid 1:1 mole ratio PGA–PL mixture and the clear 41 mole % PGA mixture with a Millipore filter (0.4 μ) completely eliminates the optical rotatory dispersion and circular dichroism spectra.

The specific structure of the aggregate is extremely sensitive to the length of the polypeptide side chains: PAA has one methylene group in the side chain *vs.* two for PGA, and PO has three methylene groups *vs.* four for PL, but only the PGA–PL combination in water results in the stabilization of β structure. Moreover, even when PGA is uncharged, its α-helical structure is disrupted and a β structure is formed with PL. The symmetry of the continuous variation plot (Figure 3) suggests both PGA and PL are entering into the β conformation. Since a stable β structure is observed only in the polyacid–polybase mixture containing the longest side chains, hydrophobic interactions are apparently of considerable importance. The fact that β structure is formed at pH 4 and 7, but not at pH 11 may be due to

the ability of the PL-amino groups to hydrogen bond with the PGA carboxyl groups at the lower pH values, but not at the highest pH, or may be due to the relative stability of the PL α-helical structure. An antiparallel β structure with alternate polyacid–polybase backbones was constructed using CPK space-filling models, and it can be seen that interpolypeptide side-chain hydrogen bonds are sterically possible for all four possible combinations: PGA–PL, PGA–PO, PAA–PL, and PAA–PO. Thus, although such hydrogen bonding may be an important stabilizing factor, it is not sufficient in itself. Turbidity was only visible in the pH 7 solution. This is probably due to extensive stacking of the β-pleated sheets through electrostatic interactions which are not present at pH 4 where the PGA is essentially uncharged. Copolymers of *l*-lysine and *l*-glutamic acid form some α-helical structure rather than β structure in solution, although β structure appears in films cast from the solutions (Doty *et al.*, 1958; Blout and Idelson, 1958).

No β structures were formed in methanol–water solutions. This is not unexpected if hydrophobic interactions are of importance in stabilizing the PGA–PL β structure. However, stabilization of β structure through hydrophobic interactions is not a general property of β structures since the β structure of silk fibroin is actually stabilized in methanol (Iizuka and Yang, 1966). For the PAA–PO and PAA–PL mixtures in neutral methanol–water–0.01 M NaF the helix conformation is stabilized whereas under the same conditions the individual components are mostly random coils. This can be mainly attributed to charge neutralization of the side chains which promotes helix formation, although other types of side-chain interactions may also play a role. Since PL is converted into an α-helix configuration at lower methanol concentrations than PO, the finding of an α-helical aggregate for PAA–PL at a lower methanol concentration (50%) than for PAA–PO (60%) is as expected. The previously discussed reluctance of PAA to become helical and the asymmetry of the continuous variation plots (Figure 3) indicate the polybase becomes helical in the aggregate rather than PAA. A higher ratio of PAA to polybase is required before helix formation is induced for PO relative to PL. This again indicates the PL helix is more stable than that of PO (Chaudhuri and Yang, 1968). A possible structure of the aggregates is one of aggregated helices of PO or PL held together by strands of PAA. Perhaps similar structures are of relevance in membranes where similar circular dichroism spectra are observed (Lenard and Singer, 1966; Stem and Fleischer, 1967).

The results obtained cannot exclude the possibility that PGA–PO and PGA–PL mixtures in methanol consist of aggregated helices. The circular dichroism spectrum of PGA–PL in 90% methanol is very likely due to aggregated helices since both PGA and PL are helical by themselves in 90% methanol and a visible interaction occurs. Under conditions where PGA, PO, and PL are in random coil configurations, the low intensity of the circular dichroism spectra of the mixtures may be due either to the lack of specific structure in the aggregate or to very extensively aggregated specific structures.

The results presented here indicate the specific structure of polypeptide aggregates depends very critically upon the nature of the side chains and significant conformational changes can accompany aggregation. The estimation of the amount of a particular structure from the magnitude of the mean residue rotation or ellipticity in aggregated systems appears to be very tenuous and uncertain. These findings may be of considerable relevance in trying to understand the structure of biologically important aggregates such as membranes.

Acknowledgment

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