Synthesis and Conformational Studies in Solution of Sequential Copolypeptides: Poly(L-prolyl-L-α-phenylglycyl-L-proline)†

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ABSTRACT: High molecular weight poly(L-prolyl-L-α-phenylglycyl-L-proline) was synthesized by condensation of L-prolyl-L-α-phenylglycyl-L-proline pentachlorophenyl ester in *N,N*-dimethylformamide. The solution properties of this material have been investigated by circular dichroism and 220-MHz nuclear magnetic resonance spectroscopy in solvents such as trifluoroethanol (F_3EtOH), chloroform, trifluoroacetic acid (F_3CCOOH), chloroform–F_3CCOOH and F_3EtOH–F_3CCOOH mixtures. Ordered structures can be formed in F_3EtOH and chloroform, which we believe to be similar to the poly-L-proline II form. The ordered form easily undergoes a conformational transition to an unordered form upon addition of small amounts of F_3CCOOH to the chloroform solution. The ordered structure is not disrupted upon addition of 12% F_3CCOOH to a F_3EtOH solution of the polypeptide.

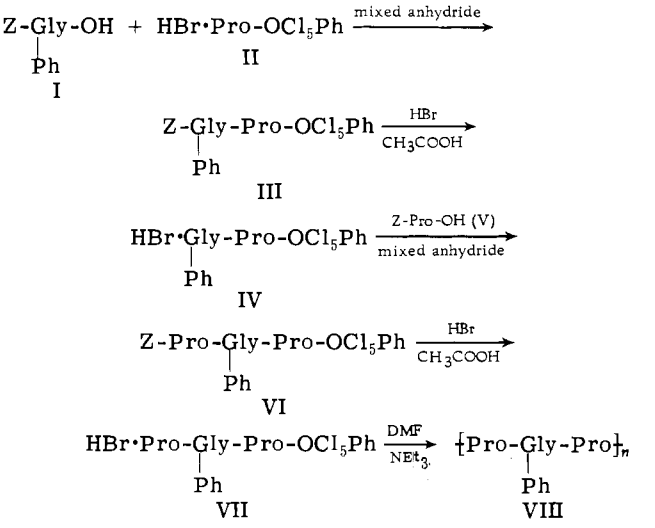
The synthesis and conformational properties of several sequential copolypeptides containing L-proline and glycine have been extensively reported in the literature (Harrington *et al.*, 1966; Ramachandran, 1967; Andreeva *et al.*, 1967; Venkatachalam and Ramachandran, 1969; Carver and Blout, 1967; Mattice and Mandelkern, 1970, 1971a). Many authors studied sequential copolymers containing glycine at the third residue because this periodicity occurs in collagen (Bensusan, 1969; Kang and Gross, 1970; Kang *et al.*, 1967; Butler, 1970) and it is also necessary to stabilize the triple helix arrangement in the ordered portions of collagen (Rich and Crick, 1955, 1961; Ramachandran and Sasisekharan, 1965; Ramachandran, 1967). Poly(L-prolylglycyl-L-proline) shows a collagen-like structure in the solid state (Traub and Yonath, 1966; Yonath and Traub, 1969) and several copolypeptides with glycine at every third residue exhibit a similar conformation (Segal *et al.*, 1969; Andrias and Walton, 1970). Ordered forms are also exhibited in solution by poly(glycly-L-prolylglycine) and several other sequential copolymers at room temperature (Oriel and Blout, 1966; Segal, 1969) or at low temperatures (Brown *et al.*, 1969). Furthermore, poly(L-prolylglycyl-L-proline) and poly(glycyl-L-prolyl-L-proline) are reported to undergo a heat-induced conformational transition in solution (Engel *et al.*, 1966; Doyle, 1970) while poly(L-prolyl-L-prolylglycine) shows a threefold reduction in molecular weight in the disordered form (Kobayashi *et al.*, 1970).

Sequential copolypeptides containing L-proline with glycine not located at the third residue have been investigated and reported to give ordered structures in solution, similar to poly-L-proline III (Mattice and Mandelkern, 1970, 1971a,b). Also, each chain of the double layered sheet in poly(L-prolylglycylglycine) exists in a poly-L-proline II type structure (Traub, 1969).

In the present paper we wish to report the synthesis and conformational properties in solution of the sequential copolypeptide poly(L-prolyl-L-α-phenylglycyl-L-proline), where the glycyl residue, responsible for very important features of collagen, is replaced by an L-α-phenylglycyl group, which has greater steric requirements and is therefore less likely to give the collagen-like triple helix.

Synthesis of Poly(L-prolyl-L-α-phenylglycyl-L-proline). The polymer was synthesized by condensation of the tripeptide active ester, L-prolyl-L-α-phenylglycyl-L-proline pentachlorophenyl ester hydrobromide (VII), according to Scheme I.

SCHEME I



The *N*-benzyloxycarbonyl-L-α-phenylglycyl-L-proline pentachlorophenyl ester (III) was prepared by coupling *N*-benzyloxycarbonyl-L-α-phenylglycine (I) with L-proline pentachlorophenyl ester hydrobromide (II) *via* the mixed anhydride method (Anderson *et al.*, 1967). The hydrobromide salt of the dipeptide active ester IV was obtained by reaction of compound III with hydrogen bromide in acetic acid. The protected tripeptide VI was synthesized by coupling *N*-benzyloxycarbonyl-L-proline (V) with L-α-phenyl-

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glycyl-L-proline pentachlorophenyl ester hydrobromide (IV) by the mixed anhydride procedure. Treatment of the N-protected tripeptide active ester VI with hydrogen bromide in acetic acid gave the hydrobromide salt of the tripeptide active ester VII. Poly(L-prolyl-L-α-phenylglycyl-L-proline) (VIII) was obtained by polymerizing a concentrated solution of VII in purified dimethylformamide in the presence of triethylamine. The molecular weight of the sequence polymer VIII was determined in the ultracentrifuge by the sedimentation equilibrium method (Schachman, 1959) and was found to be 12,500.

Experimental Section

All melting points are uncorrected. The microanalyses were carried out by Micro Analysis Co., Maryland. Infrared spectra were obtained using potassium bromide pellets on a Perkin-Elmer Model 221 spectrophotometer.

*N-Benzyloxycarbonyl-*L*-α-phenylglycine (I).* L-α-Phenylglycine (23.7 g; 157 mmol) was dissolved in 200 ml of 0.88 N sodium hydroxide and cooled to 5°. Benzyloxycarbonyl chloride (29.1 g; 170 mmol) and 0.88 N sodium hydroxide (200 ml) were simultaneously added dropwise over a 30-min period while maintaining vigorous stirring. After the addition, stirring was maintained for an additional 2 hr at 0°. The alkaline phase was thoroughly extracted with ether and acidified to pH 2 with hydrochloric acid. The white solid that formed on cooling was filtered, washed with ice–water and dried. Yield 40.1 g (89.8%); mp 128–129.5°; $[\alpha]^{23}D$ 112.7° (*c* 0.56, ethyl acetate). *Anal.* Calcd for $C_{16}H_{15}NO_4$: C, 67.4; H, 5.26; N, 4.91. Found: C, 67.3; H, 5.21; N, 5.09.

*N-Benzyloxycarbonyl-*L*-α-phenylglycyl-*L*-proline Pentachlorophenyl Ester (III).* To *N*-benzyloxycarbonyl-L-α-phenylglycine (I) (17.2 g; 60.35 mmol) in 250 ml of chloroform, cooled to −20° in a methanol–Dry Ice bath, were added consecutively *N*-methylmorpholine (6.58 ml; 60.35 mmol) and isobutyl chloroformate (8.33 ml; 60.35 mmol). The mixture was stirred at −20° for 15 min and L-proline pentachlorophenyl ester hydrobromide (II) (26.82 g; 60.35 mmol) was added, followed by triethylamine (8.44 ml; 60.35 mmol). After stirring for 3 hr at −20°, the reaction mixture was concentrated under reduced pressure and the residue distributed between 500 ml of chloroform and 300 ml of distilled water. The chloroform layer was washed with 300 ml of 0.25 N sodium bicarbonate, 300 ml of distilled water, 300 ml of 0.5 N hydrochloric acid, and three times with 300 ml of distilled water. The solvent was then evaporated under reduced pressure. The oily residue was taken up in isopropyl alcohol and stored for a few days at room temperature, filtered, and dried under vacuum. The crude hygroscopic product crystallized from isopropyl alcohol on storage at −10°. Yield 18.1 g (47.5%); mp 156–158°; $[\alpha]^{25}D$ 50.3° (*c* 0.51, chloroform). *Anal.* Calcd for $C_{27}H_{21}N_2O_5Cl_5$: C, 51.42; H, 3.35; N, 4.44; Cl, 28.10. Found: C, 51.21; H, 3.26; N, 4.50; Cl, 28.40.

*L-α-Phenylglycyl-*L*-proline pentachlorophenyl Ester Hydrobromide (IV).* *N*-Benzyloxycarbonyl-L-α-phenylglycyl-L-proline pentachlorophenyl ester (III) (3 g; 4.76 mmol) was dissolved in 15 ml of dioxane with a little warming. After the mixture was cooled to 23°, 10 ml of 44% hydrogen bromide in acetic acid was added. Stirring was continued for 50 min, after which the solution was concentrated under reduced pressure and taken up in 15 ml of dry methanol to which 320 ml of absolute ether was added to the cloud point. After storage for 4 hr at −10° the product crystallized. It was filtered, washed with anhydrous ether, and dried under vacuum. Yield 2.25 g (82%); mp 156–157°; $[\alpha]^{24}D$ 67.3° (*c* 1.02, chloroform). *Anal.* Calcd for $C_{19}H_{16}N_2O_3Cl_5Br$: C, 39.52; H, 2.79; N, 4.85; Cl, 30.69; Br, 13.83. Found: C, 39.34; H, 2.77; N, 4.70; Cl, 30.55; Br, 13.79.

*N-Benzyloxycarbonyl-*L*-prolyl-*L*-α-phenylglycyl-*L*-proline pentachlorophenyl Ester (VI).* To *N*-benzyloxycarbonyl-L-proline (V) (2.6 g; 10.7 mmol) in 200 ml of chloroform, cooled to −20° in a methanol–Dry Ice bath, were added consecutively *N*-methylmorpholine (1.13 ml; 10.7 mmol) and isobutyl chloroformate (1.4 ml; 10.7 mmol). The mixture was stirred at −20° for 15 min and L-α-phenylglycyl-L-proline pentachlorophenyl ester hydrobromide (IV) (6.2 g; 10.7 mmol) was added, followed by triethylamine (1.5 ml, 10.7 mmol). After stirring for 3 hr at −20°, the reaction mixture was concentrated under reduced pressure and the residue distributed between 300 ml of chloroform and 150 ml of distilled water. The chloroform layer was washed with 150 ml of 0.25 N sodium bicarbonate, 150 ml of distilled water, 150 ml of 0.5 N hydrochloric acid, and three times with 150 ml of distilled water. The solvent was then removed under reduced pressure. The oily residue was taken up in absolute ethanol and stored for 2 days at 0°. After crystallization the product was filtered and dried under vacuum. Yield 4.2 g(53.7%); mp 147–148°, $[\alpha]^{25}D$ 16.0° (*c* 0.54, chloroform). *Anal.* Calcd for $C_{32}H_{28}N_3O_6Cl_5$: C, 52.78; H, 3.84; N, 5.78; Cl, 24.39. Found: C, 52.66; H, 3.90; N, 5.73; Cl, 24.30.

*L-Prolyl-*L*-α-phenylglycyl-*L*-proline Pentachlorophenyl Ester Hydrobromide (VII).* *N*-Benzyloxycarbonyl-L-prolyl-L-α-phenylglycyl-L-proline pentachlorophenyl ester (VI) (10 g; 13.8 mmol) was dissolved in 30 ml of acetic acid and 30 ml of 42% hydrogen bromide in acetic acid added to this solution. The reaction mixture was stirred for 50 min, concentrated under reduced pressure, and taken up in 15 ml of dry methanol to which 600 ml of absolute ether was added to the cloud point. After the mixture stood overnight at −10°, the product crystallized; it was filtered, washed with anhydrous ether, and dried *in vacuo*. Yield 8.9 g (96%); mp 170–171°; $[\alpha]^{25}D$ 22.9° (*c* 0.89, methanol). *Anal.* Calcd for $C_{24}H_{23}N_3O_4Cl_5Br$: C, 42.73; H, 3.44; N, 6.23; Cl, 26.23; Br, 11.84. Found: C, 42.53; H, 3.56; N, 6.10; Cl, 26.10; Br, 11.94.

*Poly(*L*-prolyl-*L*-α-phenylglycyl-*L*-proline) (VIII).* A mixture of L-prolyl-L-α-phenylglycyl-L-proline pentachlorophenyl ester hydrobromide (VII) (5.4 g; 7.4 mmol), *N,N*-dimethylformamide (5.4 ml), and triethylamine (2.4 ml, 17.1 mmol) was shaken for 3 days at room temperature. The reaction mixture was diluted with 300 ml of ether, filtered, washed with ether, dry methanol, distilled water, and again with ether, and dried. Yield 1.94 g (80%). The ir spectrum showed peaks at 6.05 (amide I) and 6.55 μ (amide II); the pentachlorophenyl ester peak at 5.62 μ was absent. *Anal.* Calcd for $C_{18}H_{21}N_3O_3 \cdot H_2O$: C, 62.59; H, 6.72; N, 12.17. Found: C, 62.50; H, 6.41; N, 11.91.

Weight-Average Molecular Weight. The weight-average molecular weight ($\overline{MW}$) of the polymer VIII was determined in a Spinco Model E Analytical ultracentrifuge by the sedimentation equilibrium method in 0.1 M aqueous *N,N*-dimethylacetamide. The measurement was carried out at a rotor speed of 21,400 at 27° and in a concentration range of 0.2–0.5%. The $\overline{MW}$ of the polymer was found to be 12,500.

Spectroscopic Measurements. All circular dichroism

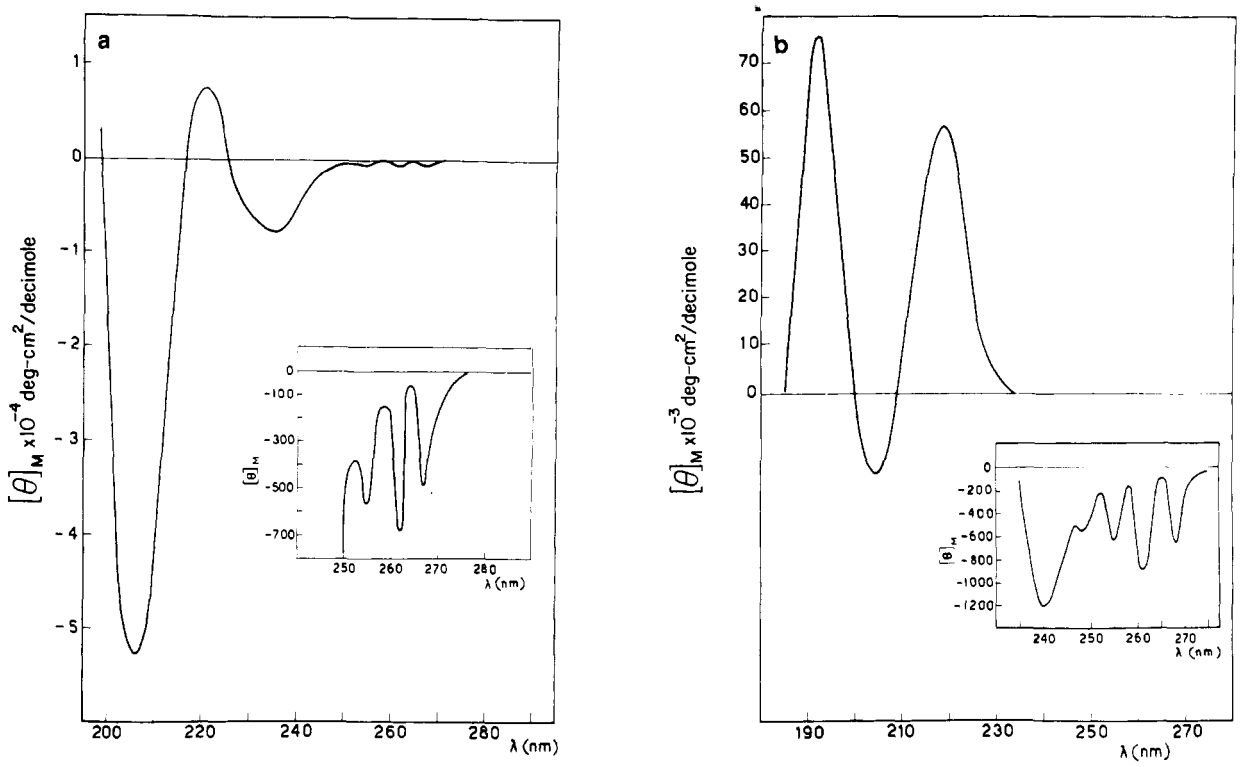

FIGURE 1: (a) CD spectrum of poly(Pro-Ph-Gly-Pro) in F_3EtOH (2.1 mg/ml). (b) CD spectrum of *N*-acetyl-Pro-Ph-Gly-Pro methyl ester in F_3EtOH (0.835 mg/ml).

(CD) measurements were performed on a Cary 61 recording spectropolarimeter. The molar ellipticities are calculated per tripeptide unit. The concentration range was 1.5–3 mg/ml and the cell path length was 0.1–1.0 mm. The nuclear magnetic resonance (nmr) spectra were recorded on a Varian HR 220 spectrometer. The concentration ranged between 5 and 12 mg/ml. All chemical shifts are given in ppm from the internal standard (Me_4Si). Both CD and nmr spectra were measured at room temperature.

Results and Discussion

Circular Dichroism Studies. The CD sectrum of poly(L-prolyl-L-α-phenylglycyl-L-proline) [poly(Pro-Ph-Gly-Pro)] in F_3EtOH solution in the range 280–200 nm is shown in Figure 1a. It is characterized by an intense negative band at 206 nm (θ_M −53,000 per tripeptide unit), a positive band at 221 nm (θ_M +7500), a negative band at 235 nm (θ_M −7700), and small negative aromatic bands in the range 250–270 nm (θ_M −600 to −900).

The model tripeptide, *N*-acetyl-L-prolyl-L-α-phenylglycyl-L-proline methyl ester in the same solvent (Figure 1b), exhibits a negative dichroic band near 205 nm (θ_M −16,000) and a rather intense positive band at 218 nm (θ_M +57,500), a negative band around 239 nm (θ_M −1200), and the negative aromatic bands in the range 250–270 nm (θ_M −600 to −900). In summary, both compounds show two negative bands (206, 235 and 205, 239 nm, respectively), a positive one (at 218 and 221 nm, respectively) and negative aromatic bands, the position of the maxima being somewhat shifted to the red in the model tripeptide. However, the relative intensities of the various peaks are completely reversed in poly(Pro-Ph-Gly-Pro) compared with the model compound; the intensities of the negative 206- and 235-nm bands are much higher in the polymer, while the intensity of the 218-nm positive peak is much higher in the model compound. The aromatic region seems to be little affected by significant changes in going from the trimer to the polymer, the intensities of the 250–270-nm negative peaks being about the same.

The CD spectra of poly(Pro-Ph-Gly-Pro) and of the model compound in chloroform in the range 260–220 nm are presented in Figure 2a and b. The polymer is characterized by a large negative band around 231 nm (θ_M −35,000), whereas the trimer exhibits a negative peak at 236 nm (θ_M −8,500) and a positive peak at 222 nm (θ_M +16,300). In chloroform the small positive band observed for the polymer in F_3EtOH is absent, probably due to the large increase of the 230-nm negative band in this solvent, which obscures the positive contribution of the 221-nm Cotton effect. The model compound in chloroform also shows an increase in the 236-nm negative band and a decrease in the 222-nm positive band, compared with the corresponding bands in F_3EtOH.

Addition of small amounts of F_3CCOOH to the chloroform solution of poly(Pro-Ph-Gly-Pro) causes a dramatic change in the CD pattern (Figure 3): the 231-nm negative peak is greatly reduced in intensity (θ_M −5000) and red-shifted to 239 nm, and a positive peak appears at 220 nm (θ_M +42,400). The spectrum in acid is quite similar to that of the model compound in pure chloroform; it is noteworthy that only a small change is observed for the model compound itself upon addition of F_3CCOOH.

A plot of the molar ellipticity at 225 nm *vs.* the percentage of F_3CCOOH added to pure chloroform is presented in Figure 4. Clearly a sharp transition takes place between 0.4 and 1% F_3CCOOH. Comparing with the spectra shown in Figures 2b and 3, we can conclude that a conformational transition occurs from an ordered to an unordered form for poly(Pro-Ph-Gly-Pro) in chloroform upon addition of F_3CCOOH. This ordered form is also the same in F_3EtOH

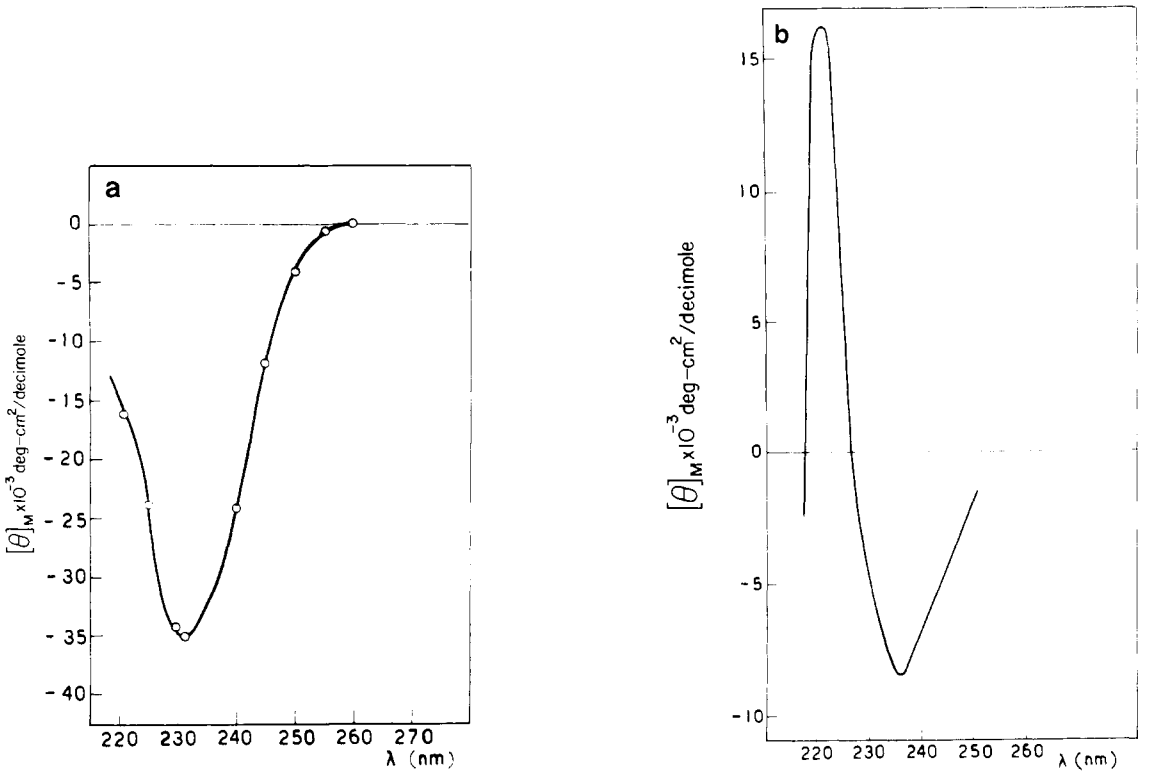

FIGURE 2: (a) CD spectrum of poly(Pro-Ph-Gly-Pro) in chloroform (2.34 mg/ml). (b) CD spectrum of *N*-acetyl-Pro-Ph-Gly-Pro methyl ester in chloroform (2.85 mg/ml).

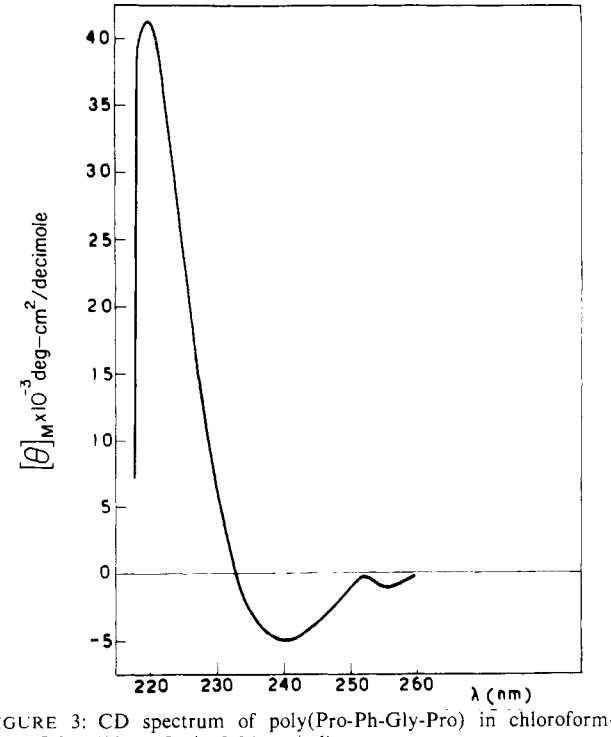

FIGURE 3: CD spectrum of poly(Pro-Ph-Gly-Pro) in chloroform-F_3CCOOH (98.5:1.5 v/v, 2.34 mg/ml).

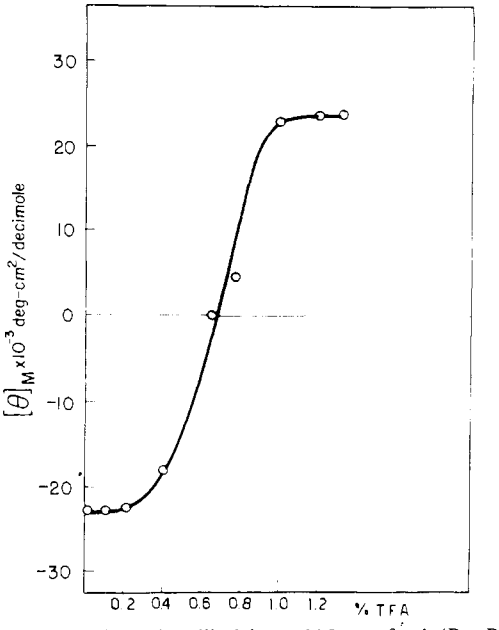

FIGURE 4: Plot of the molar ellipticity at 225 nm of poly(Pro-Ph-Gly-Pro) *vs.* per cent F_3CCOOH in chloroform-F_3CCOOH mixtures.

since the optical properties vary almost linearly in F_3EtOH-chloroform mixtures from pure F_3EtOH to pure chloroform.

CD measurements on F_3EtOH-F_3CCOOH mixtures have also been performed up to 12% F_3CCOOH. Plots of the molar ellipticities in these solvent systems at 220, 234, and 262 nm show that no transition takes place in the range 0–12% F_3CCOOH since the circular dichroism shows no sudden variation with solvent composition.

If we now compare the spectrum of poly(Pro-Ph-Gly-Pro) in F_3EtOH (Figure 1a) with the reported spectrum of poly-L-proline II in the same solvent (Bovey and Hood, 1967) in the range 200–230 nm, we find that they resemble each other very closely, both showing a very intense negative band at 206 nm and a small positive peak around 221–225 nm (this peak is about 4 nm blue-shifted in our polymer compared with poly-L-proline II probably due to overlap

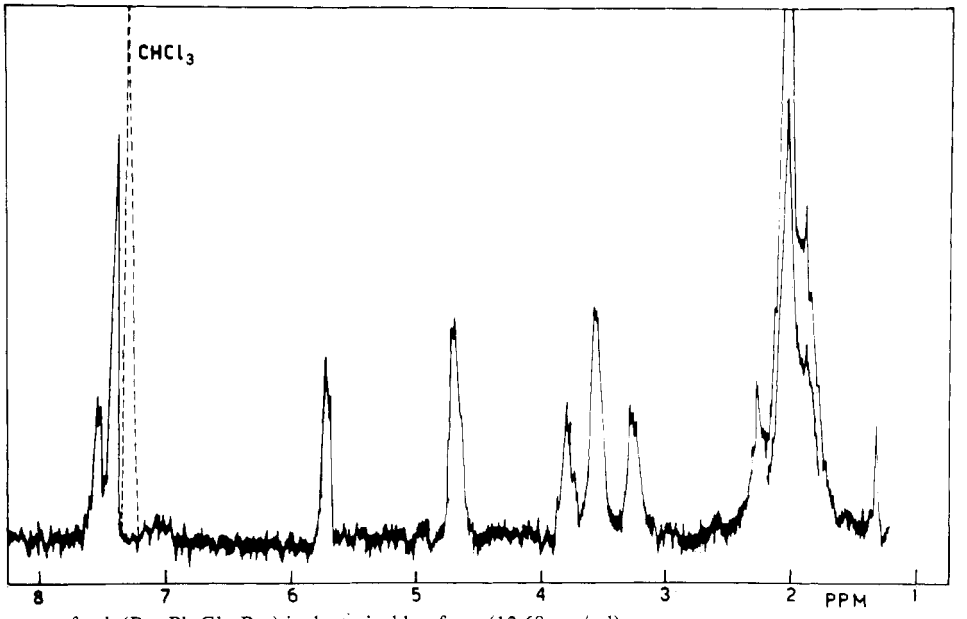

FIGURE 5: Nmr spectrum of poly(Pro-Ph-Gly-Pro) in deuteriochloroform (12.68 mg/ml).

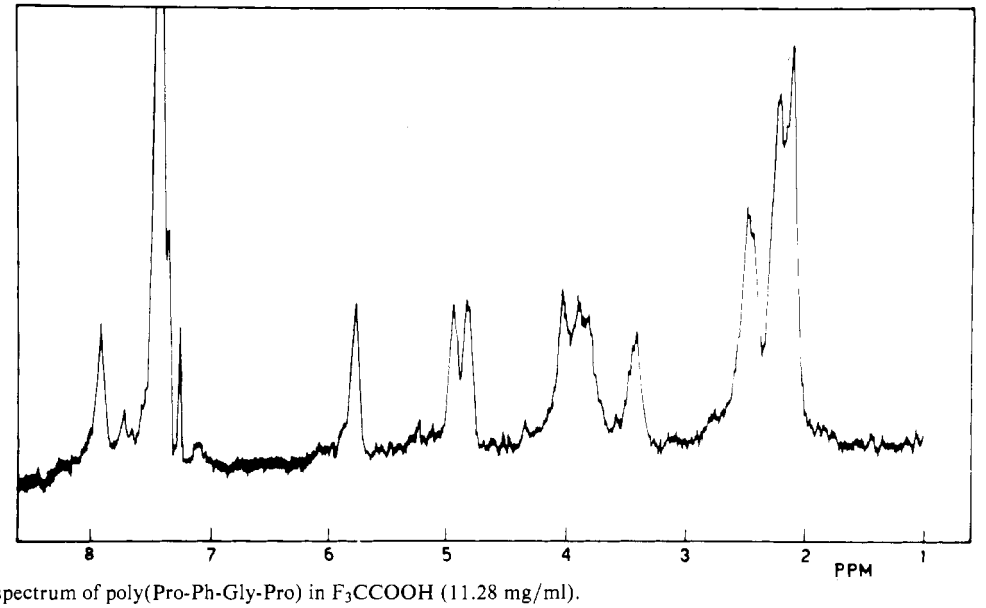

FIGURE 6: Nmr spectrum of poly(Pro-Ph-Gly-Pro) in F_3CCOOH (11.28 mg/ml).

with an adjacent negative band). We therefore conclude that the ordered structure of poly(Pro-Ph-Gly-Pro) in pure chloroform and F_3EtOH is a helical form related to poly-L-proline II. However, it should be clear that this kind of comparison cannot establish the extent of conformational ordering that takes place in the polymer. A collagen-like triple helix as in poly(L-prolylglycyl-L-proline) (Traub and Yonath, 1966; Engel *et al.*, 1966) is not to be expected, since L-α-phenylglycine replaces glycine at every third residue, thus preventing association of the polymer for steric reasons.

It is interesting to notice that the perturbing effect of the phenyl group in the region of the amide $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions is not great enough to influence the CD pattern of poly(Pro-Ph-Gly-Pro) in the range 200–230 nm in F_3EtOH. Furthermore, as we pointed out, the forbidden aromatic $\pi \rightarrow \pi^*$ transition at 250–270 nm is rather insensitive to conformational changes of the polymer, probably because the interactions of the phenyl groups are not very great even in the ordered structure, as they are separated by at least two proline residues from each other. Considering the different behavior of poly(Pro-Ph-Gly-Pro) in chloroform and F_3EtOH, we can also conclude that the ordered structure is probably much more stabilized in the latter, not being disrupted by up to 12% F_3CCOOH. However, the possibility of hydrogen bonding between F_3CCOOH and F_3EtOH, reducing the disrupting power of the former, cannot be excluded (Ferrara and Temussi, 1973).

Nuclear Magnetic Resonance Studies. To support our CD results, nmr studies on poly(Pro-Ph-Gly-Pro) were undertaken in deuteriochloroform, F_3CCOOH, and deuteriochloroform–F_3CCOOH mixtures.

The nmr spectra of the polypeptide in deuteriochloroform and in F_3CCOOH are presented in Figures 5 and 6. In deuteriochloroform it shows a multiplet at δ 4.70 ppm, corresponding to the prolyl α-CH's, a doublet at δ 5.71 ppm, attributed to the phenylglycyl α-CH, a resonance at δ 7.36 ppm for the phenyl group, and a doublet at δ 7.53 ppm for

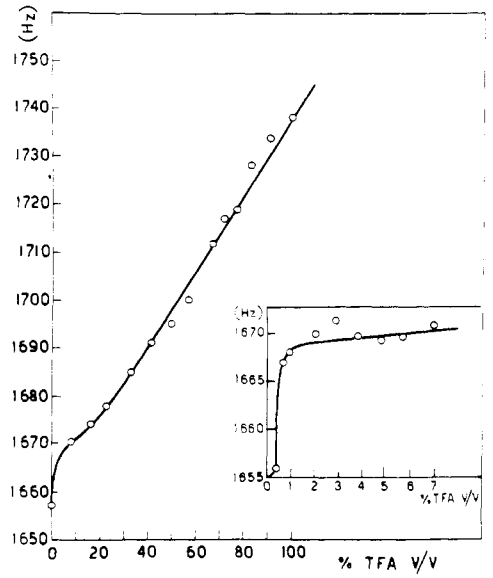

FIGURE 7: Plot of poly(Pro-Ph-Gly-Pro) NH chemical shift (Hz from Me_4Si) *vs.* solvent composition in deuteriochloroform-F_3CCOOH mixtures.

the phenylglycyl NH. In F_3CCOOH two different prolyl α-CH's are observed at δ 4.85 and 4.96 ppm, respectively, the phenylglycyl α-CH resonance appears at δ 5.80 ppm, the phenyl peak at 7.39 ppm, and the phenylglycyl NH at δ 7.90 ppm. Changes are also visible in the proline δ-proton region (δ 3.30–3.80 ppm) and in the more shielded β- and γ-proton regions (δ 2.0–2.5 ppm). It is noteworthy that the prolyl α-CH chemical shift for our polymer in deuteriochloroform is almost identical with that reported in the literature for poly-L-proline II (Deber *et al.*, 1970). Furthermore, while only one α-CH resonance is visible for both L-proline residues in the repeating sequence of the polymer when the solvent is chloroform, two separate resonances appear in F_3CCOOH.

In order to ascertain whether the differences on changing from one solvent to the other are due only to solvent effects or possibly to a conformational transition, the spectra of the polymer were recorded in different deuteriochloroform–F_3CCOOH mixtures varying from pure chloroform to pure F_3CCOOH. A plot of the phenylglycyl NH chemical shifts *vs.* the solvent composition up to 100% F_3CCOOH is presented in Figure 7. A similar behavior is also observed for both the phenylglycyl and prolyl α-CH's whose nmr spectra in the range 0.0–1.1% F_3CCOOH are shown in Figure 8. A dramatic change occurs in this narrow range of composition, which cannot be explained simply in terms of solvent effects, which are, however, clearly the cause of the spectral changes in the solvent range 2–100% F_3CCOOH. These data are completely consistent with the above CD results and conclusions.

We also considered the possibility of cis–trans isomerism in the polypeptide chain, which has been demonstrated by recent studies in sequential copolypeptides of L-proline and glycine (Torchia, 1972). However, we find no conclusive evidence consistent with the presence of cis peptide bonds in poly(Pro-Ph-Gly-Pro) either in the ordered or in the unordered forms.

Conclusions

In this paper we have described the synthesis of a sequential polypeptide with the repeating sequence, L-prolyl-L-α-phenylglycyl-L-proline. It has been possible to demonstrate the formation of ordered structures in helix-supporting solvents, such as F_3EtOH and chloroform. We have demonstrated that no association takes place. The ordered form is more stable in F_3EtOH than in chloroform since it very readily undergoes a conformational transition on addition of a few per cent F_3CCOOH in the latter, while no such effect is observed in the former up to 12% F_3CCOOH. It is not surprising that a very small amount of F_3CCOOH is sufficient to disrupt the poly-L-proline II-like conformation in chloroform, since this solvent is a poor helix supporter. In

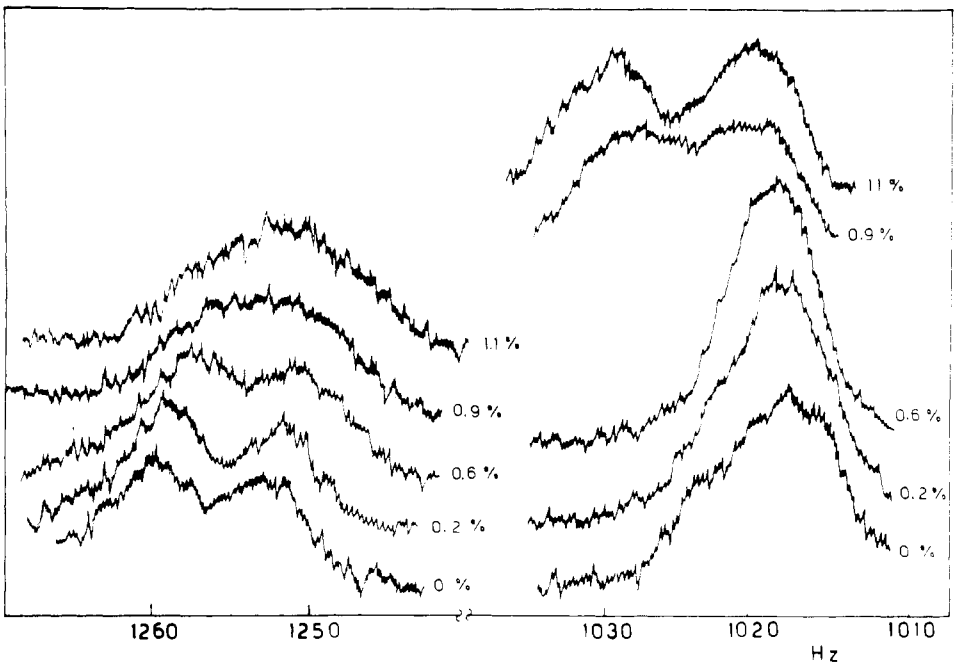

FIGURE 8: Nmr spectrum of the phenylglycyl and prolyl α-CH region in deuteriochloroform–F_3CCOOH mixtures up to 1.1% F_3CCOOH (11.34 mg/ml).

addition, the ordered structure is destabilized by distortions at the phenylglycyl residue, which lower the helix stability and allow the polymer to easily convert to a disordered form.

We have recently found that poly(Pro-Ph-Gly-Pro) exhibits X-ray diffraction patterns in the solid state typical of ordered structures. Our results will be published elsewhere.

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