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Further Study on Peptides Having the L-Alanyl-L-leucylglycyl Repeating Unit. Syntheses and Conformations of the Peptides up to the Hexatriacontapeptide Level

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ABSTRACT: A series of peptides having the L-alanyl-L-leucylglycyl repeating unit, Nps-(L-Ala-L-Leu-Gly)$_n$-OEt (n = 7–10 and 12), has been synthesized by the fragment condensation method, using the active ester Nps-(L-Ala-L-Leu-Gly)$_2$-ONSu. Conformations of these peptides as well as those of the lower homologues with n = 1–6, which had been prepared in an earlier study, were studied by X-ray powder diffraction measurement and IR spectroscopy for the solid state and CD spectroscopy for solutions. The 15-peptide (n = 5) takes two different conformations, an α helix or a β structure, depending upon the casting procedure from a solution in trifluoroethanol. The β structure was found in the solid sample obtained by evaporation of the solvent, a slow phase transformation from the solution to the solid state, and the α helix was found in the sample obtained by reprecipitation, a rapid phase transformation. The conformations of the peptides obtained by the slow phase transformation were the β structure for the peptides with n = 1–5 and the α helix for the peptide with n = 7. The 18-peptide (n = 6) was in a transition area from the β structure to the α helix. The CD study demonstrated that the onset of helicity begins at the 12-peptide (n = 4) for these peptides in TFE. The intensity of the CD bands at 207 and 222 nm and the total molar optical rotation were found to be related to the conformations of these peptides in the solid state.

Oligopeptides consisting of L-alanyl-L-leucylglycyl repeating units begin forming α helices at the 15-peptide in the solid state when the peptide samples are reprecipitated from solution by addition of diethyl ether.[1] We have examined the solid-state conformations of these peptides when obtained by another casting procedure involving slow evaporation of the solvent from solutions. The observed conformations of the samples thus obtained are different from those of the samples obtained by reprecipitation. The 15-peptide takes the β structure and the 18-peptide has a conformation similar to the α helix. This experimental fact shows that the solid-state conformations of the oligopeptides can vary with the casting procedure. An analogous phenomenon has been observed for homo-oligo(L-methionine) bound with poly(ethylene glycol).[2] This variation of conformation should be prominent for oligopeptides having rather short peptide chain lengths which cannot form enough hydrogen bonds to stabilize a specific conformation, and there should be a critical peptide chain length for the formation of the α helix which is independent of the casting procedure. Thus we have prepared peptides having longer peptide chain lengths, Nps-(L-Ala-L-Leu-Gly)$_n$-OEt (n = 7–10 and 12), and studied the conformation of the solid samples obtained by slow evaporation of the solvent. The conformation of the peptides with n = 1–10 and 12 in solution was also studied since it may be related to the solid-state conformation and since we were seeking information concerning its variation with solid-state conformation.

Experimental Section

Syntheses of Peptides. Nps-L-Ala-L-Leu-Gly-ONSu and Nps-(L-Ala-L-Leu-Gly)$_2$-ONSu. These active esters were prepared by the procedure reported in our earlier study.[3]

Nps-(L-Ala-L-Leu-Gly)$_8$-OEt as an Example for the Fragment Condensation. The 18-peptide Nps-(L-Ala-L-Leu-Gly)$_6$-OEt[3] (1.65 g, 1 mmol) was dissolved in 5 mL of 1,1,1,3,3,3-hexafluoropropan-2-ol (HFIP), and to the solution was added 0.5 mL of 4 N hydrochloric acid in ethanol to precipitate the 18-peptide ethyl ester hydrochloride. The precipitate was collected on a glass filter and washed with diethyl ether until the yellow color of the solid disappeared. The white solid was reprecipitated from HFIP. The product was dissolved in 100 mL of dimethyl sulfoxide (Me_2SO), and triethylamine (0.154 mL, 1.1 mmol) and Nps-(L-Ala-L-Leu-Gly)$_2$-ONSu (0.75 g, 1 mmol) were added with stirring at room temperature. The reaction was monitored by thin-layer chromatography as will be mentioned in the text. After 5 h, the active ester (0.23 g, 0.3 mmol) was added to the solution, and stirring was continued for an additional 5 h. After the reaction, the reaction system was diluted with 500 mL of water to precipitate the product. The product was collected on a glass filter and washed with methanol until the filtrate did not show the yellow color. Then the product was washed with diethyl ether and dried. The crude product was dissolved in warm Me_2SO, and the solution was diluted with water to give a precipitate. The product was collected on a glass filter, washed with methanol, tetrahydrofuran (THF), and diethyl ether, and dried over P_2O_5 to give 1.96 g (92% yield) of pure Nps-(L-Ala-L-Leu-Glu)$_8$-OEt (**5**).

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Table I
Syntheses of Peptides Having L-Alanyl-L-leucylglycyl Residues

peptide[a]	yield,[b] %	$[\alpha]_D$,[c] deg	R_f[d]	calcd % C	calcd % H	calcd % N	found % C	found % H	found % N
4	90	−22.3	0.49	54.06	7.58	16.21	54.15	7.60	16.28
5	92	−17.5	0.44	54.14	7.62	16.45	54.16	7.65	16.32
6	88	−13.1	0.43	54.20	7.65	16.54	54.28	7.75	16.38
7	89	−9.62	0.41	54.26	7.68	16.63	54.37	7.86	16.51
8	88	−8.73	0.39	54.33	7.72	16.75	54.21	7.95	16.59

[a] Numbers for the peptides coincide with those in Scheme I. [b] Yields are the values after purification by reprecipitation. [c] *c* 1.0, 1:4 HFIP–TFE. [d] Eluent 2:1 HFIP–benzene. The peptides decompose over 270 °C.

The 21-peptide (n = 7) **4** was prepared by the same procedure as above from the 18-peptide (n = 6) **1** and the active ester Nps-L-Ala-L-Leu-Gly-ONSu (**2**). The 27-peptide (n = 9) **6** was obtained by the reaction of 1 equiv of **4** with 1.5 equiv of the active ester Nps-(L-Ala-L-Leu-Gly)$_2$-ONSu (**3**), and the reaction was sustained for 24 h at room temperature. The product was purified by reprecipitation from HFIP. The 30-peptide (n = 10) **7** and the 36-peptide (n = 12) **8** were prepared by the same procedure as for **6** from **5** and **7**, respectively.

Treatment of the Oligopeptides. Reprecipitation. The 15-peptide (0.5 g) was dissolved in 5 mL of trifluoroethanol (TFE). To the solution was added 100 mL of diethyl ether. The resulting precipitate was collected on a glass filter, washed with diethyl ether, and dried over P_2O_5 in a vacuum desiccator.

Casting from Solutions by Slow Evaporation of the Solvent. The peptide sample (0.2 g) was dissolved in 5 mL of TFE or a 1:1 mixture of TFE–HFIP in a test tube (diameter 1 cm, depth 5 cm). Then the tube was allowed to stand for 2 days at room temperature. The solid film or powder was obtained. The solid was dried under a high vacuum for 2 days.

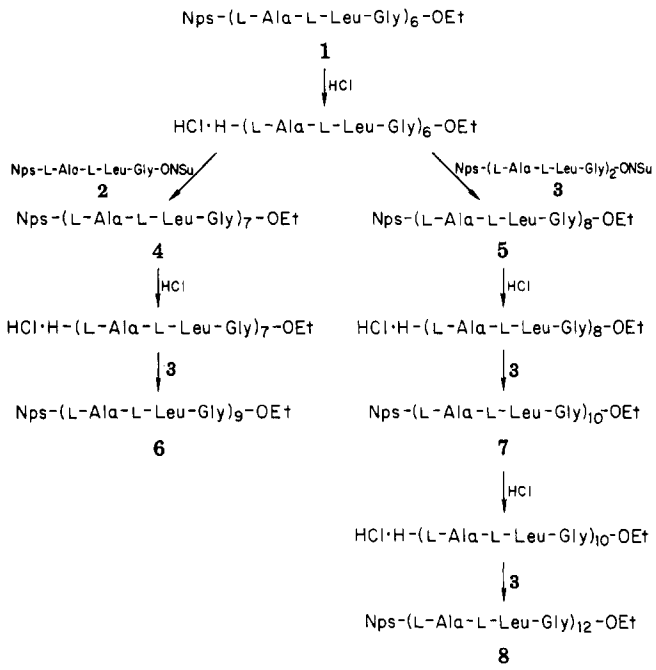
Measurements. The infrared (IR) spectra were obtained for KBr disks with a Jasco IR-A spectrophotometer. The far-IR spectra were obtained for Nujol mulls with a Jasco IR-F spectrophotometer. The X-ray powder diffraction patterns were recorded with a Rigaku GF-2012 X-ray diffractometer. The circular dichroism (CD) spectra were obtained with a Jasco J-20 CD spectrophotometer at a 1 mg/mL concentration of the sample in TFE at room temperature. The optical rotation was measured at the sodium D line with a Jasco DIP-SL automatic polarimeter. The molar rotation of the peptides, Φ, was calculated from the specific rotation $[\alpha]$ by the equation[4]

$$\Phi = [\alpha](\text{mol wt})/10000 \qquad ((\text{deg}\cdot\text{cm}^{-2})/\text{mol})$$

Results and Discussion

Synthesis. The synthetic scheme to the longest peptide is set out in Scheme I. The 18-peptide (n = 6) **1** was used as the starting peptide, and the syntheses were carried out stepwise by the fragment condensation method, using purified active esters Nps-L-Ala-L-Leu-Gly-ONSu (**2**) and Nps-(L-Ala-L-Leu-Gly)$_2$-ONSu (**3**).[3] A typical example of the syntheses is given by the preparation of the 24-peptide (n = 8) **5** by the reaction of **1** with **3**. **1** was treated with hydrochloric acid in HFIP to remove the Nps protecting group. The resulting 18-peptide ethyl ester hydrochloride was dissolved in Me_2SO and treated with triethylamine and **3**. Since the Nps-peptides exhibit a strong yellow color, the reaction was successfully monitored by thin-layer chromatography, using a silica gel thin layer and THF eluent. The thin-layer chromatogram immediately after addition of **3** to the reaction system showed a single spot (R_f 0.77) due to **3**; after 1 h, two spots (R_f 0.77 and 0.00) appeared for the resulting product **5**; after 5 h, two spots (R_f 0.00 (major, **5**) and 0.31 (faint)) were evident for the byproduct Nps-(L-Ala-L-Leu-Gly)$_2$-OH (**3′**) resulting from hydrolysis of **3**. Thus an additional amount of **3** was added to the reaction system, and the reaction was allowed to continue for an additional 5 h. After the reaction, thin-layer chromatography showed three yellow spots (R_f 0.00 (major), 0.31 (faint), and 0.77 (minor)) and no ninhydrin-positive spot, which suggests the absence of unreacted starting 18-peptide ethyl ester. The solution was diluted with water to precipitate the product, which was collected on a glass filter, washed with methanol and THF to remove **3** and **3′**, and purified by reprecipitation from warm Me_2SO to give pure **5**.

Scheme I
Syntheses



The other peptides were prepared by the same procedure as above. In every reaction, the peptide active ester **3** was used (1.5 equiv; in two portions, 1.0 and 0.5 equiv) in the coupling reaction with the peptide ester hydrochloride. All reactions were performed in Me_2SO solvent and allowed to continue in a gelatinous solution state, and the product gave a single spot on thin-layer chromatography after purification. Results of the syntheses are summarized in Table I.

Conformation. Figure 1 shows the IR spectra of the 15-peptide for samples obtained by various casting procedures. The solid sample prepared by reprecipitation from a solution in TFE by addition of diethyl ether showed a band at 1655 cm^{-1} in the amide I region and bands at 528, 462, 395, and 363 cm^{-1} in the far-IR region. The amide I band at 1655 cm^{-1} can be assigned to the α-helix or random-coil structures.[5] Since the far-IR bands at 528 and 363 cm^{-1} and at 462 and 395 cm^{-1} are characteristic of L-alanine and L-leucine with the α-helical conformation, respectively,[6] the amide I band at 1655 cm^{-1} is assigned to the α helix. However, taking into account the rather short peptide length of the peptide, it still cannot be excluded that a considerable portion of the random coil may

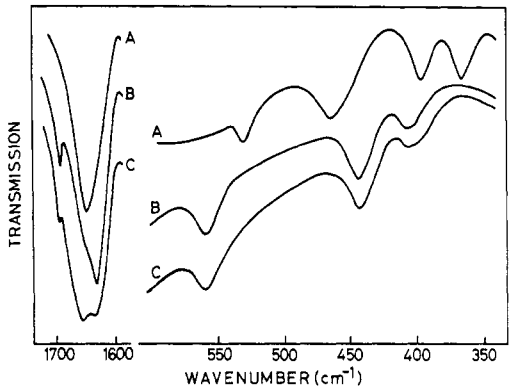


Figure 1. IR spectra of Nps-(L-Ala-L-Leu-Gly)$_5$-OEt in the solid state obtained by various casting procedures: (A) sample obtained by reprecipitation from TFE; (B) sample obtained by slow evaporation of the solvent from TFE; (C) sample obtained by slow evaporation of the solvent from 1:1 TFE–HFIP.

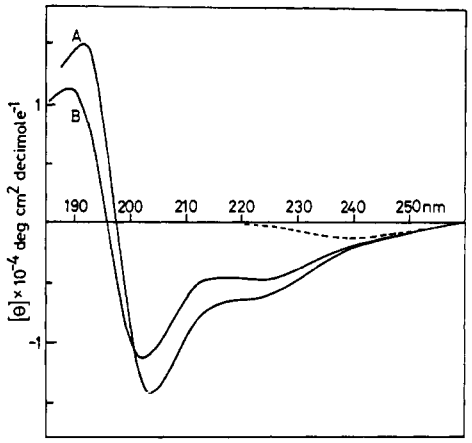


Figure 2. CD spectra of Nps-(L-Ala-L-Leu-Gly)$_5$-OEt: (A) in TFE; (B) in 1:1 TFE–HFIP.

contribute to the band in the amide I region. On the contrary, the samples prepared by slow evaporation of the solvent showed spectra different from that of the sample prepared by reprecipitation. The sample from TFE solution showed amide I bands at 1695 and 1629 cm^{-1}, a very weak shoulder near 1650 cm^{-1}, and a far-IR band at 444 cm^{-1}. The two amide I bands at 1695 and 1629 cm^{-1} are characteristic of the antiparallel β structure,[5] and the far-IR band at 444 cm^{-1} is characteristic of L-alanine with the β structure.[6] The shoulder near 1650 cm^{-1} suggests the presence of an α helix or random coil. Since the far-IR spectrum of the peptide has no bands characteristic of the α helix, the shoulder near 1650 cm^{-1} is assigned to the random coil. The sample from 1:1 TFE–HFIP solution showed a spectrum similar to that of the sample from TFE solution, except for a definite band at 1655 cm^{-1}, which suggests the presence of a considerable amount of random-coil structure. From this IR evidence, we can see that the conformation in the solid state of the 15-peptide varies with the casting procedure used to obtain the solid sample. The fact that the same peptide takes different conformations, α helix or β structure, in the solid state, depending upon the casting procedure used, suggests that a conformational change should be involved in the phase transformation of the peptide from solution to the solid state. In order to demonstrate this, we have to know the conformation of the peptide in solution. Figure 2 shows the CD spectra of the 15-peptide in TFE and 1:1 TFE–HFIP. In analyzing the CD spectra of the peptides protected by the Nps group, one should pay attention to the fact that, since the Nps protecting group has a Cotton effect at 240 nm, illustrated by the dashed line in Figure 2,[7] the CD curves resulting from the amide transitions may change in this wavelength range. Thus, the CD spectrum of the 15-peptide in TFE has a positive band with an ellipticity per residue [θ] = 15 000 at 192 nm (the amide $\pi \rightarrow \pi^*$ transition) and two negative bands with [θ] = −14 000 and −6500 at 204 nm (the amide $\pi \rightarrow \pi^*$ transition) and 222 nm (the amide n $\rightarrow \pi^*$ transition), respectively. The spectrum of the peptide in 1:1 TFE–HFIP has a positive band with [θ] = 11 200 at 189 nm and negative bands with [θ] = −11 400 and −4700 at 202 and 222 nm, respectively. Judging from the magnitude of [θ] and position of these bands,[8] this peptide takes an equilibrium conformation between the α helix and the random coil, with the proportion of the latter somewhat larger in 1:1 TFE-HFIP than in TFE.[9]

By comparing the IR spectral evidence in the solid state and the CD spectral evidence in solution, we deduce the conformational change during the phase transformation. In TFE, the peptide takes the α helix and random coil. By reprecipitation of this peptide from TFE solution by addition of diethyl ether, the peptide takes mainly the α helix. Then the development of α-helical conformation of this peptide in the solid state can be explained by maintenance of the α-helical conformation during the rapid phase transformation. On the contrary, this peptide takes the β structure and random coil for the solid samples prepared by slow evaporation of the solvent from the TFE and 1:1 TFE–HFIP solutions. In this case, the α-helical conformation in these solutions is apparently transformed to the β structure in the solid state during the slow phase transformation. We suppose that this conformational change happens, as the α-helical conformation of this peptide formed in the solutions is not so stabilized. The α-helical conformation consisting of a short peptide length in a dilute solution is in dynamic equilibrium with the random coil. In the slow evaporation of the solvent, the peptide system changes its phase from solution to the solid state through a very highly concentrated solution state, where the associated β structure stabilized by intermolecular hydrogen bonding would be thermodynamically more stable than the α helix, which can be stabilized only by limiting intramolecular hydrogen bonding. Then the peptide changes conformation from the α helix to the β structure probably via a random-coil structure. This conformational change is expected to occur for peptides having rather short peptide lengths and may not occur for peptides having longer peptide chain lengths which can form stable α-helices in solution. Then we should find a peptide length taking the α-helical conformation which is independent of the casting procedure. In other words, it should be expected that there is a peptide length which is capable of forming an α helix stable enough to be maintained even in a concentrated solution during the slow phase transformation.

We studied the conformation of the peptides with n = 5–10 and 12 for the solid sample obtained by slow evaporation of the solvent from solution in TFE, 1:1 TFE–HFIP, and HFIP. The results obtained are independent of the kind of solvent. Therefore, the results are illustrated for the case of 1:1 TFE–HFIP. Figure 3 shows the X-ray powder diffraction patterns of the peptides with n = 5–7 and 10. The 15-peptide (n = 5) showed three prominent peaks at 2θ = 11.3, 19.1, and 23.1°. The first and second reflections can be assigned to the (020) and (110) planes of the orthorhombic unit cell of the peptide taking the β structure.[10] On the contrary, the 18-peptide (n = 6) and higher peptides (n = 7 and 10) showed two prominent

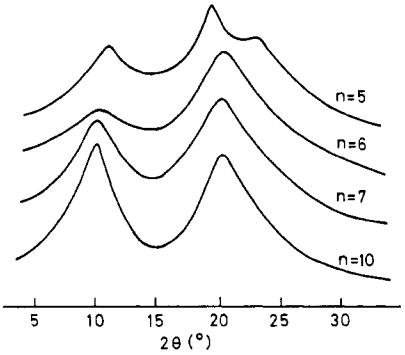


Figure 3. X-ray powder diffraction patterns of Nps-(L-Ala-L-Leu-Gly)$_n$-OEt obtained by slow evaporation of the solvent from 1:1 TFE–HFIP.

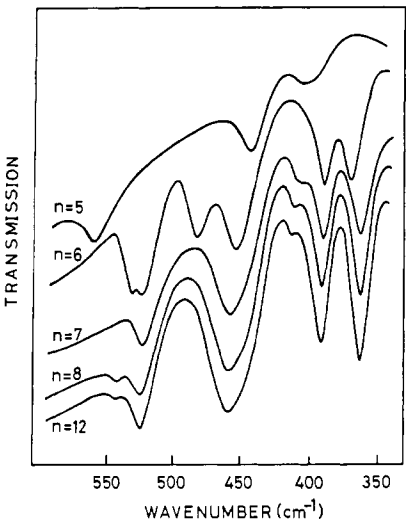


Figure 4. Far-IR spectra of Nps-(L-Ala-L-Leu-Gly)$_n$-OEt obtained by slow evaporation of the solvent from 1:1 TFE–HFIP.

peaks at 2θ = 10.4 and 20.1°. The peak at 2θ = 10.4° can be assigned to the (100) plane of the hexagonal unit cell of the peptides taking the α helix.[11] These X-ray diffraction patterns suggest that the 15-peptide takes the β structure and that the 18-peptide and higher peptides form the α helix. However, the reflection of the (100) plane of the hexagonal unit cell for the 18-peptide (n = 6) is too weak for us to determine that this peptide forms a definite unit cell packed with the peptide chains taking the α-helical conformation. Far-IR spectroscopy gave evidence which is consistent with the results of the X-ray diffraction measurement. It could especially account for the weak X-ray reflection at 2θ = 10.4° of the 18-peptide. Figure 4 shows the far-IR spectra of these peptides. As was shown in Figure 1, the 15-peptide showed the band at 444 cm^{-1} characteristic of the β structure. The 21-peptide (n = 7) and higher peptides (n = 8 and 12) showed strong bands at 529 and 362 cm^{-1} and at 462 and 395 cm^{-1} characteristic of L-alanine and L-leucine with the α helix, respectively. The 18-peptide (n = 6) showed a spectrum similar to those of the higher peptides taking the α-helical conformation. It has bands at 536, 528, 487, 459, 393, and 372 cm^{-1}, four of which are located close to those of the α helix: 528–529 cm^{-1} and 372–362 cm^{-1} for L-alanine and 459–462 cm^{-1} and 393–395 cm^{-1} for L-leucine. These bands suggest that this peptide takes a secondary structure similar to the α helix. The presence of two other strong bands at 536 and 487 cm^{-1}, however, also suggests that there may be other conformers which disturb the hexagonal packing of the peptide chains taking the α-helix-like structure. From these X-ray and IR studies, it can be concluded that in the

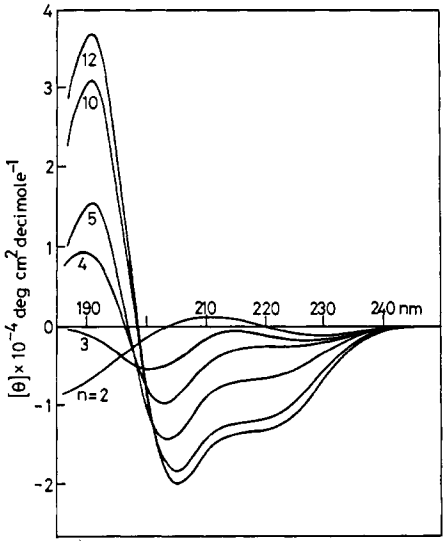


Figure 5. CD spectra of Nps-(L-Ala-L-Leu-Gly)$_n$-OEt in TFE.

solid state developed by the slow evaporation of the solvent, a series of peptides having the L-alanyl-L-leucylglycyl repeating unit take the β structure at the 15-peptide level and the α-helix at the 21-peptide. The 18-peptide is considered to be in a transition area from the β structure to the α helix. These results demonstrate that this series of peptides needs 21 amino acid residues to form a stable α helix to be maintained in a very highly concentrated solution of fluoro alcohols. This length is considerably longer than the critical length for the formation of the α helix of the peptides, 15 amino acid residues, in the solid state obtained by rapid phase transformation, reprecipitation from the same solution.[1]

With regard to these solid-state conformations, it is interesting to study the conformation of these peptides in solution. Figure 5 shows the CD spectra of these peptides in TFE. In these CD spectra, the CD band resulting from the Nps protecting group has been subtracted off. The shape of the CD spectra changes gradually as the chain length increases.[12] For the 6-peptide (n = 2), negative CD below 204 nm is followed by a positive weak band at 212 nm and a negative weak band at 230 nm. The 9-peptide (n = 3) showed two negative bands at 201 and 228 nm. The spectra of higher peptides have a positive band at about 189–192 nm and negative bands at 203–207 and 222 nm. The intensity of these bands increased with the chain length of the peptides. This change of the CD spectra resembles those for homooligopeptides consisting of γ-ethyl L-glutamate,[13] γ-methyl L-glutamate,[14] and L-methionine.[15] The appearance of the three bands near 195, 205, and 220 nm for the 12-peptide (n = 4) and higher peptides suggests that onset of helicity begins at the 12-peptide level. In order to show more clearly the onset of the helicity, plots of the ellipticity of the bands at 207 and 222 nm vs. repeating unit of the peptides are shown in Figure 6, which also contains a plot of total molar optical rotation[4] vs. repeating unit. The total molar rotation should change linearly in the absence of secondary structure and deviation from a straight line suggests the presence of secondary structure.[16,17] For the peptides in this study, a marked deviation from the straight line was observed at the 12-peptide. On the other hand, the ellipticity of the band at 222 nm should be constant in the absence of the α helix, and decreasing of the ellipticity suggests the onset of helicity.[13] For these peptides, the ellipticity of the band at 222 nm is almost zero from n = 1 to n = 3 and definitely decreases at the 12-peptide. From these results, we con-

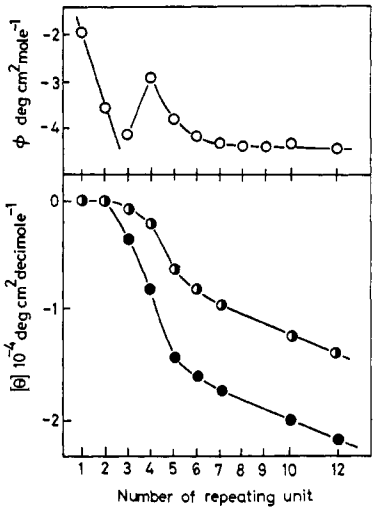


Figure 6. Change of the total molar rotation at the sodium D line and the ellipticities of the CD bands at 207 and 222 nm with the repeating unit in TFE: (○) total molar rotation; (◑) ellipticity of the band at 222 nm; (●) ellipticity of the band at 207 nm.

clude that the onset of helicity begins at the 12-peptide for this series of peptides. Another interesting fact is that the total molar rotation increases once at the 12-peptide (n = 4) and decreases again gradually to reach a constant value at the 21-peptide (n = 7). In accordance with this change of the total molar rotation, the ellipticities of the bands at 207 and 222 nm decrease once greatly to the 15-peptide (n = 5) and then decrease gradually from n = 5 to n = 7 followed by a constant decreasing at the 21-peptide. We think that the constant value of the total molar rotation and the constant decreasing of the ellipticities of the bands at 207 and 222 nm at the 21-peptide suggest the formation of a stable α-helical conformation at this peptide length. It should be noted that the 21-peptide is the critical peptide length for the formation of the α helix in the solid state obtained by the slow phase transformation.

In conclusion, we deduce a relationship between the conformations of these peptides in solution and those in the solid state. Though the α helix is first formed at the 12-peptide in TFE, it is not stable enough to be maintained during the phase transformation to the solid state. The α helix is more stabilized in a longer peptide, the 15-peptide (n = 5), as is shown by increasing ellipticity of the band at 222 nm. At this peptide length the α helix is maintained during the rapid phase transformation to the solid state during reprecipitation. However, the α helix at this peptide length is still not stable enough to be maintained and transformation to the β structure occurs during the slow phase transformation via a concentrated solution state. Finally, an α helix which is stable enough to be maintained even in the slow phase transformation process is formed at the 21-peptide.

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Comparative Conformational Studies of Polypeptides Containing a High Percentage of Proline

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ABSTRACT: In the search for models of a proline-rich protein isolated from human parotid saliva we were led to synthesize and study H-[Gly-(Pro)$_x$]$_n$-OH (x = 3, 4). Preliminary results concerning these polypeptides have already been reported[1] and suggest that in aqueous solution these peptides adopt a polyproline II (PPII) conformation in addition to some other structures (probably unordered). In this paper we present a new synthesis of these products and a more complete conformational study comparing the relative stabilities of the PPII helix adopted by these polymers.

Synthesis

The thermal polycondensation method previously set forth by Goodman et al.[2] is reported to be rapid when compared to solution techniques and to give higher yields and molecular weights.[3] The "monomers" used in the work reported here, H-Gly-(Pro)$_x$-OC$_6$H$_5$Cl$_5$·HCl (x = 3, 4), have already been described.[1] To carry out the thermal polycondensation they are mixed with a known quantity of Celite and desiccated by repeated evaporation in vacuo of a mixture of DMF and dioxane (1:3 v/v). Then they are heated in vacuo for 5 h at 40 °C and 3 days at 125 °C, extracted with acetic acid (5% in aqueous solution), and

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