Critical Chain Length for the Formation of α- and β-Polypeptide Conformations in the Solid State. Syntheses and Conformations of Oligopeptides with Alternate L-Alanyl and L-Leucyl Residues [1]

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A series of oligopeptides with alternate L-alanyl and L-leucyl residues Nps-(L-Leu-L-Ala)$_n$-OEt and Nps-L-Ala-(L-Leu-L-Ala)$_m$-OEt (n = 1—6 and 8, and m = 1—5) have been prepared by stepwise synthesis with *o*-nitrophenylsulphenyl *N*-carboxy-α-amino-acid anhydrides for lower oligopeptides and fragment condensation with *N*-hydroxysuccinimide and dicyclohexylcarbodi-imide for higher oligopeptides; assignment of conformation to the products in the solid state has been made on the basis of i.r. and for i.r. spectroscopic results and *X*-ray diffraction measurements. α-Helical conformations have been found in deca- and higher peptides. The β-structure, on the other hand, appears to begin at the heptapeptide level.

THERE is considerable interest in the conformation of well-defined synthetic oligopeptides in the solid state [2–5] with respect to the development of secondary structures in peptide systems. *X*-Ray diffraction measurements and i.r. and c.d. spectroscopy of comparable low-molecular weight homo-oligopeptides protected by t-butoxycarbonyl and methyl ester groups demonstrate that above a critical size (the pentapeptide) and up to the heptamer they invariably exist in the β-form; the α-helix conformation has not been found in these low molecular-weight oligopeptides. We have synthesized a number of higher oligopeptides, each containing L-alanine, L-leucine, and glycine, and have shown that the α-helical conformation is, initially, observable at the pentadeca- or hexadeca-peptide stage.[6–9] Not only do the glycyl residues in peptides destabilize their

H-Ala-OEt (1) —(Nps-Leu-NCA)→ Nps-Leu-Ala-OEt (2) —(HCl)→ HCl·H-Leu-Ala-OEt (2′) —(NEt$_3$, Nps-Ala-NCA)→

Nps-Ala-Leu-Ala-OEt (3) —(HCl)→ —(NEt$_3$, Nps-Leu-NCA)→ Nps-(Leu-Ala)$_2$-OEt (4) —(HCl)→ HCl·H-(Leu-Ala)$_2$-OEt (4′)

—(NEt$_3$, Nps-Ala-NCA)→ Nps-Ala-(Leu-Ala)$_2$OEt (5) —(HCl)→ —(NEt$_3$, Nps-Leu-NCA)→ Nps(Leu-Ala)$_3$-OEt (6)

—(HCl)→ HCl·H-(Leu-Ala)$_3$-OEt, (6′)

(2) —(OH$^-$)→ —(H$^+$)→ [Nps-Leu-Ala-OH], (2″) (3) —(OH$^-$)→ —(H$^+$)→ [Nps-Ala-Leu-Ala-OH] (3″)

(4) —(OH$^-$)→ —(H$^+$)→ Nps-(Leu-Ala)$_2$-OH, (4″)

(3″) + (4′) —(NEt$_3$, DCCI, HONSu)→ Nps-Ala-(Leu-Ala)$_3$-OEt, (7)

(2″) + (6′) → Nps-(Leu-Ala)$_4$-OEt (8) —(HCl)→ HCl·H-(Leu-Ala)$_4$-OEt, (8′)

(3″) + (6′) → Nps-Ala-(Leu-Ala)$_4$-OEt, (9)

(2″) + (8′) → Nps-(Leu-Ala)$_5$-OEt (10) —(HCl)→ HCl·H-(Leu-Ala)$_5$-OEt, (10′)

(3″) + (8′) → Nps-Ala-(Leu-Ala)$_5$-OEt, (11)

(2″) + (10′) → Nps-(Leu-Ala)$_6$-OEt (12) → HCl·H-(Leu-Ala)$_6$-OEt, (12′)

(4″) + (12′) → Nps-(Leu-Ala)$_8$-OEt. (16)

SCHEME

α-helical conformation [10-15] but the critical chain lengths for the formation of the α-helix in such oligopeptides do not parallel those for non-glycyl containing peptides. Since peptides without glycyl residues form helices at the hepta- or octa-peptide level in solution,[16-22] the critical peptide size in the solid state must be shortened in non-glycyl containing peptides.

Here, we report the syntheses and conformations in the solid state of oligopeptides without glycyl residues but containing alternate L-alanyl and L-leucyl residues. We believe that this oligopeptide system may have the shortest known critical chain-length for development of the α-helix, because L-alanine and L-leucine form the most stable α-homo-polypeptide known [23] and are found most frequently in the α-helical moieties of proteins.[24]

The synthesis of oligopeptides containing alternate L-alanyl and L-leucyl residues is set out in the Scheme. L-Alanine ethyl ester was chosen as a *C*-terminal amino-acid residue. The lower oligopeptides from di- to hexa-peptides were prepared by stepwise elongation of the peptide chain by using *o*-nitrophenylsulphenyl *N*-carboxy-anhydrides (Nps-NCAs) of L-leucine and L-alanine.[25] L-Alanine ethyl ester was treated with Nps-L-leucine-NCA in tetrahydrofuran for 2 h at room temperature to give Nps-L-leucyl-L-alanine ethyl ester. After removal of the Nps-protecting group from the dipeptide derivative by treatment with hydrochloric acid in dioxan, the resulting dipeptide ester hydrochloride was allowed to react with Nps-L-alanine-NCA to yield Nps-L-alanyl-L-leucyl-L-alanine ethyl ester. The tetra-, penta-, and hexa-peptides were analogously prepared. The peptides from hepta- to dodeca-peptides were prepared by the fragment condensation method with least danger of racemization in the condensation step using dicyclohexylcarbodi-imide in the presence of *N*-hydroxysuccinimide:[26-28] Nps-(L-Leu-L-Ala)$_n$-OEt and Nps-L-Ala-(L-Leu-L-Ala)$_n$-OEt were prepared by the condensation of H-(L-Leu-L-Ala)$_{n-1}$-OEt with Nps-L-Leu-L-Ala-OH and Nps-L-Ala-L-Leu-L-Ala-OH, respectively. The hexadecapeptide Nps-(L-Leu-L-Ala)$_8$-OEt was also prepared by the condensation of the dodecapeptide ester H-(L-Leu-L-Ala)$_6$-OEt with the Nps-tetrapeptide Nps-(L-Leu-L-Ala)$_2$-OH. All peptide derivatives were purified by repeated recrystallization. After purification, they showed a single spot on t.l.c. (silica gel). Results of the syntheses are summarized in the Table.

Syntheses of oligopeptides having alternate L-alanyl and L-leucyl residues

Peptide [a]	Yield * (%)	M.p. (°C)	$[\alpha]_D$ [b] (°)	R_F	Found (%)			Required (%)		
(2)	89	110—111	−66.5	0.78 [c]	58.5	7.6	11.6	58.1	7.2	12.0
(3)	86	134—135	−70.4	0.37 [c]	57.3	7.5	13.0	56.9	7.2	13.3
(4)	82	203—205	−96.9	0.34 [c]	58.5	7.9	12.8	58.3	7.7	13.1
(5)	85	229—231	−73.6	0.89 [d]	58.0	7.5	14.0	57.4	7.7	13.85
(6)	76	257—260	−101.3	0.32 [e]	57.8	8.3	13.2	58.4	8.0	13.6
(7)	66	260—265 †	−79.2	0.13 [e]	57.2	8.1	14.6	57.7	7.9	14.2
(8)	60	>270 †	−84.6	0.49 [f]	58.9	8.6	13.3	58.4	8.1	13.9
(9)	62	>270 †	−56.2	0.42 [f]	58.5	8.0	13.8	57.9	8.1	14.4
(10)	54	>270 †	−52.1	0.41 [f]	59.1	8.0	13.8	58.5	8.2	14.2
(11)	60	>270 †	−42.5	0.41 [f]	57.2	8.4	14.0	58.0	8.2	14.5
(12)	62	>270 †	−50.0	0.40 [f]	59.6	8.0	14.8	58.5	8.3	14.3
(16)	65	>270 †	−52.6	0.18 [f]	57.8	8.8	13.9	58.5	8.4	14.5

[a] Numbers for the oligopeptides coincide with those in the Scheme. [b] *c* 2.0 in hexafluoropropan-2-ol–trifluoroethanol (1 : 2). [c] Developing solvents, ethyl acetate–benzene (1 : 1). [d] Tetrahydrofuran. [e] Trifluoroethanol–benzene (1 : 2). [f] Trifluoroethanol.
* After purification. † Decomp.

The conformations of the oligopeptides in the solid state were examined by i.r. and far-i.r. spectroscopy and *X*-ray powder diffraction measurements. We have found in earlier studies on the conformations of oligopeptides that the samples as synthesized take the β-structure and are transformed into the α-form above the critical lengths after treatment with solvents.[6-8] We have also found that this β→α conformational transformation occurs not only in solution but is also brought about in the solid state by shear stress.[9] The oligopeptides having alternate L-alanyl and L-leucyl residues synthesised in the present study occurred in the β-form and remained unchanged even when treated with hexafluoropropan-2-ol. Application of shear stress to the samples so treated began to form the α-helix at the decapeptide stage. Figure 1 shows i.r. spectra in the Amide I region of the oligopeptides after addition of shear stress. The nonapeptide showed absorption at 1 630 cm^{-1} and a shoulder at 1 696 cm^{-1} characteristic of the β-structure. In contrast, the decapeptide showed absorption at 1 652 cm^{-1} and a weak shoulder at 1 630 cm^{-1}; the former absorption is characteristic of the α-helix or random coil conformation as were the far-i.r. spectrum and *X*-ray powder diffraction pattern. Thus the decapeptide takes predominantly the α-helical conformation. The undeca- and dodeca-peptides showed only absorption at 1 652 cm^{-1}. On this basis we may conclude that oligopeptides larger than the decapeptide exist in the α-helical conformation. This conclusion was supported by the far-i.r. spectra which are shown in Figure 2. Here the nonapeptide showed bands at 488 and 451 cm^{-1} which are characteristic of L-leucine [7,8] and L-alanine,[29-31] respectively, with a β-structure. In contrast, in the spectra of the deca- and higher oligopeptides, the band at 451 cm^{-1} disappeared and new bands appeared at 526, 393, and 369 cm^{-1}. The band at 393 cm^{-1} is characteristic of L-leucine associated with the α-helical conformation and those at 426 and 369 cm^{-1} are of L-alanine in a similar situation.[31] Thus the far-i.r.

spectra too suggest that the α-helical conformation develops above the decapeptide.

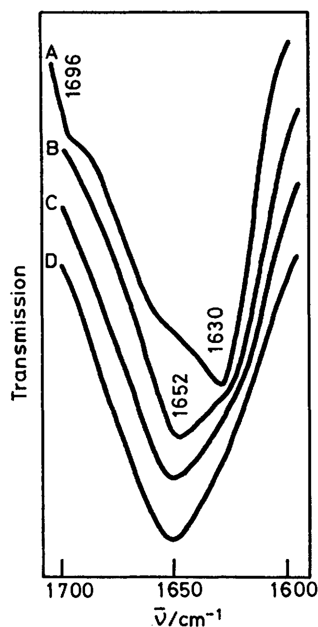

FIGURE 1 I.r. spectra of the peptides with alternate L-alanyl and L-leucyl residues after application of shear stress: A = nona-, B = deca-, C = undeca-, D = dodeca-peptides

X-Ray diffraction patterns of the oligopeptides supported this conclusion.[2] The nonapeptide showed the prominent peaks at $2\theta = 5.2, 10.3, 15.7, 18.9$, and $23.6°$. The peaks at $2\theta = 10.3$ and $18.9°$ can be assigned as the (020) and (110) planes of the orthorhombic unit cell of the peptide with the β-structure. The deca- and higher

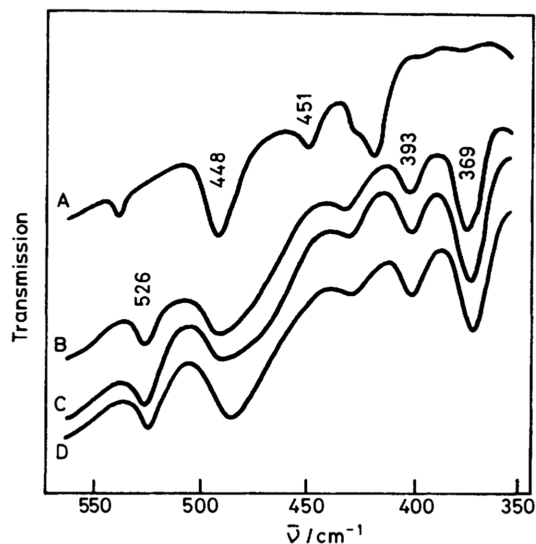

FIGURE 2 Far-i.r. spectra of the peptides after application of shear stress: A = nona-, B = deca-, C = undeca-, D = dodeca-peptides

peptides showed prominent peaks at $2\theta = 9.0$ and $19.0°$. The peak at $9.0°$ can be assigned as the (100) plane of the hexagonal unit cell of the peptides with the α-helix. From the above results, we conclude that the critical chain length for development of the α-helix of the oligopeptide having alternate L-alanyl and L-leucyl residues is the decapeptide. This critical length is shorter than those of the oligopeptides having glycyl residues.[6-9] Thus the shortening of the critical length results from the amino-acid component of the oligopeptide without glycyl residues which destabilize the α-helical conformation.

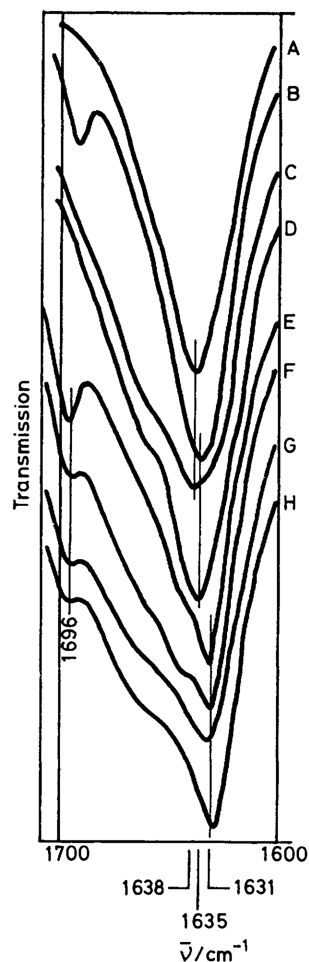

FIGURE 3 I.r. spectra of the peptides with alternate L-alanyl and L-leucyl residues as synthesized: A = tri-, B = tetra-, C = penta-, D = hexa-, E = hepta-, F = octa-, G = nona-, and H = deca-peptides

A further interesting problem concerned with the conformations of oligopeptides in the solid state is the formation of the β-structure. It has been demonstrated that there is a critical chain length for development of the β-structure [3-5] as well as the α-helix in the solid state. We studied the conformations of the synthesis of oligopeptides having alternate L-alanyl and L-leucyl residues by i.r. and far-i.r. spectroscopy. Figure 3 shows the i.r. spectra in the Amide I region. The tri- and penta-peptides showed absorption at 1 638 cm^{-1} and the tetra- and hexapeptides showed absorption at 1 635 cm^{-1}. The hepta- and higher peptides showed bands at 1 696 and 1 631

cm^{-1}. The absence of absorption near 1 650 cm^{-1}, for the oligopeptides shows that they are not in the α-helix or random coil form. The bands at 1 696 and 1 631 cm^{-1} of the hepta- and higher peptides are assigned as those of the antiparallel β-structure. The lower peptides have the Amide I band at higher wavenumbers, 1 638 and 1 635 cm^{-1}, than those characteristic of a typical β-structure and these vary with the chain length and the amino-acid sequence: Nps-L-Ala-L-Leu-L-Ala-OEt shows a band at 1 638 cm^{-1}, Nps-(L-Leu-L-Ala)$_2$-OEt at 1 635 cm^{-1}, Nps-L-Ala-(L-Leu-L-Ala)$_2$-OEt at 1 638 cm^{-1}, and Nps-(L-Leu-L-Ala)$_3$-OEt at 1 635 cm^{-1}. These facts suggest that the hexa- and lower peptides are not in a typical β-structure but, rather, a β-like structure in which the peptide chains loosely tie intermolecularly through hydrogen bonds. These results are supported by the far-i.r. spectra shown in Figure 4. The band at 488 cm^{-1} characteristic of L-leucine with the β-structure is found for the octa- and higher peptides and the heptapeptide showed a band at a slightly higher wavenumber, 490 cm^{-1}, which too can be assigned as that of the β-structure. The hexa- and lower peptides had no such band. In addition, the band at 452 cm^{-1} characteristic of L-alanine with this structure is found in the samples of the hepta- and higher peptides. Therefore, we conclude that in the peptides having alternate L-alanyl and L-leucyl residues, the critical chain length for the formation of the β-structure in the solid state is the heptapeptide. This contrasts with the critical length for the homo-oligopeptides of L-alanine and L-leucine [4,5] protected by t-butoxycarbonyl and methyl ester groups which is the pentapeptide. We believe that this difference results not from a difference in the protecting groups but, rather, from the sequence of amino-acids. The heterogeneity of the side chain of the peptides in this study disturbs intermolecular association and arrangement of the peptide main chains leading to the longer critical length.

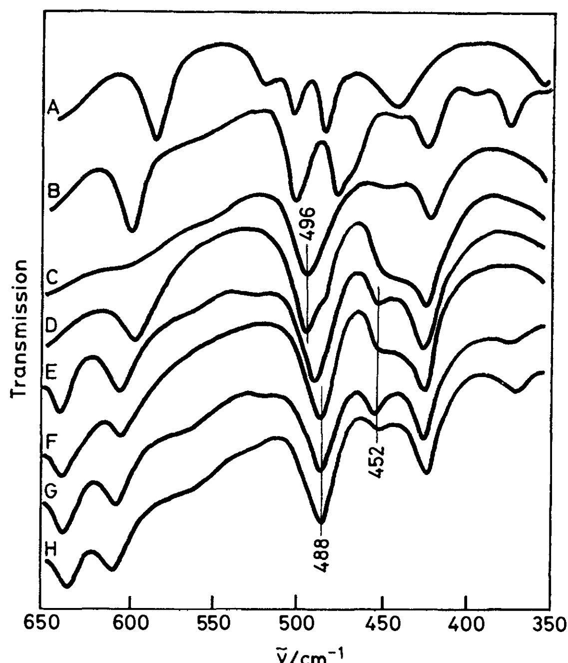

FIGURE 4 Far-i.r. spectra of the peptides as synthesized: A = tri-, B = tetra-, C = penta-, D = hexa-, E = hepta-, F = octa-, G = nona-, and H = deca-peptides

EXPERIMENTAL

*Stepwise Synthesis using Nps-NCAs.—Nps-*L*-Leu-*L*-Ala-*OEt. L-Alanine ethyl ester hydrochloride (15.4 g, 0.1 mol) was dissolved in tetrahydrofuran (200 ml) and triethylamine (14 ml) was added. The resulting salt was filtered off. To the solution was added with stirring Nps-L-leucine-NCA (34.2 g, 0.11 mmol) and the stirring was continued for 2 h at room temperature. The solution was diluted with ethyl acetate (200 ml) and washed with 5% sodium hydrogen carbonate, water, 5% citric acid, and water. The solution was dried (Na_2SO_4) and concentrated under reduced pressure to give an oily residue which upon addition of hexane crystallized to give the dipeptide derivative. The crude product was recrystallized from ethyl acetate.

*Nps-*L*-Ala-*L*-Leu-*L*-Ala-*OEt.— Nps-L-leucyl-L-alanine ethyl ester (38.3 g, 0.1 mol) was dissolved in 4M-hydrochloric acid (50 ml) in anhydrous dioxan and the solution was concentrated under reduced pressure. Diethyl ether was added to the residue to extract the yellow Nps-chloride; this was repeated until the yellow colour of the oil disappeared. The oil was then dissolved in tetrahydrofuran (300 ml) and triethylamine (14 ml) was added. The resulting crystals were filtered off. To the filtrate was added Nps-L-alanine-NCA (29.3 g, 0.11 mol). The solution was treated in a similar way to that described for the dipeptide synthesis to give the crude crystalline product; this was recrystallized from tetrahydrofuran.

The tetra-, penta-, and hexa-peptides were prepared stepwise by the same procedure as above.

*Fragment Condensation.—Nps-*L*-Ala-(*L*-Leu-*L*-Ala)$_3$-*OEt. Nps-L-leucyl-L-alanyl-L-leucyl-L-alanine ethyl ester (5.67 g, 10 mmol) was treated with 4M-hydrochloric acid (5 ml) in ethanol. Diethyl ether was added to the solution to give a crystalline product which was filtered off, washed with diethyl ether, and recrystallized by dissolution in ethanol followed by addition of diethyl ether to give the pure tetrapeptide ester hydrochloride.

In one experiment, Nps-L-alanyl-L-leucyl-L-alanine ethyl ester (5.45 g, 12 mmol) was dissolved in acetone–methanol (1 : 1) (20 ml). To the solution was added 1M-sodium hydroxide (12 ml) and the solution was set aside for 1 h. The solution was then diluted with water (50 ml), washed with diethyl ether (30 ml), and acidified with 5% citric acid; this was then extracted with ethyl acetate (3 × 50 ml). The combined extracts were washed with water, dried (Na_2SO_4), and concentrated under reduced pressure to give Nps-L-alanyl-L-leucyl-L-alanine free acid. The above-prepared tetrapeptide ester hydrochloride was then dissolved in *NN*-dimethylformamide (100 ml), and triethylamine (1.4 ml) and *N*-hydroxysuccinimide (2.3 g, 20 mmol) were added to it. The solution was then cooled to −10 °C and the Nps-tripeptide free acid and dicyclohexylcarbodi-imide (2.1 g, 10 mmol) were added; the mixture was then stirred for 5 h at −5 °C and for an additional day at room temperature. The resulting urea was filtered off and the solution diluted with water (500 ml) to give a precipitate. The product was filtered off and washed with water, methanol,

and diethyl ether. The crude product was recrystallized from *NN*-dimethylformamide.

All other octa- to dodeca-peptides were prepared by the same fragment condensation method as above.

Nps-(L-*Leu*-L-*Ala*)$_8$-OEt.—Nps-(L-Leu-L-Ala)$_2$-OEt (3.85 g, 6.8 mmol) was saponified in a mixture of methanol–tetrahydrofuran (1 : 1) by 1M-sodium hydroxide to give the free acid, Nps-(L-Leu-L-Ala)$_2$-OH, in 90% yield; m.p. 215—220 °C (decomp.); R_F 0.60 [ethyl acetate–methanol (1 : 1)]; $[\alpha]_D$ $-79.6°$ (*c* 0.5 in tetrahydrofuran). The acid (1.62 g, 3 mmol) and *N*-hydroxysuccinimide (0.52 g, 4.5 mmol) were dissolved in *NN*-dimethylformamide (30 ml) and the solution was cooled to -10 °C. Dicyclohexylcarbodi-imide (0.72 g, 3.4 mmol) was added to the reaction mixture which was then stirred for 5 h at -10 °C. The resulting urea was filtered off and the filtrate added to a mixture of the dodecapeptide ester hydrochloride HCl·H-(L-Leu-L-Ala)$_6$-OEt (1.78 g, 1.5 mmol) and triethylamine (0.21 ml) in dimethyl sulphoxide (200 ml). The system was stirred for 2 d at room temperature and then diluted with water (500 ml). The product was filtered off, washed with water, methanol, tetrahydrofuran, and diethyl ether, and dried. The crude product was recrystallized from hexafluoropropan 2-ol.

Treatment of the Oligopeptides.—The sample as synthesized (0.1 g) was dissolved in hexafluoropropan-2-ol (5 ml). To the solution was added methanol or diethyl ether to precipitate the product, which was filtered off, washed with diethyl ether and dried (P_2O_5). The treated sample was ground in an agate mortar.

Measurements.—I.r. spectra were recorded with a JASCO IR-A spectrophotometer for KBr discs. Far-i.r. spectra were recorded with a JASCO IR-F spectrophotometer for Nujol mulls. *X*-Ray powder diffraction was measured with a JEOL Rotex JRX-12 *X*-ray diffractometer.

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