SYNTHESIS AND CONFORMATIONAL INVESTIGATION OF B-TURN FORMING TETRA- AND HEXAPEPTIDES

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Tetra- and hexapeptides containing Pro-Gly or Gly-Pro or Aib-Pro in their sequences were synthesized using the liquid-phase method. The high solubility of the poly(ethylene glycol) bound peptides in water and in organic solvents enables the application of the singlet-singlet energy transfer method for conformational investigation of these peptides. The conformational study in solid state by IR and in solution by CD were carried out in parallel to the energy transfer method. The qualitative results generated by IR and CD were found to be in good agreement with the quantitative end-to-end distances given by the energy transfer method.

In the last few years a number of papers have appeared in the literature in which the efficiency of energy transfer between two luminophores attached to the same macromolecular substrate has been used to determine the interluminophore distance on the basis of the Förster equation[1]. The validity of this equation has been successfully tested with model compounds[2-4]. In the field of linear polypeptides this technique was first applied to conformational study of ACTH[5,6]. In these experiments both natural (Tyr,Trp) and synthetic fluorescent amino acids have been used. This approach has in comparison to other spectroscopic methods the advantage of needing low substance concentration (10^{-4} to 10^{-5} mol l^{-1}), where the peptide–peptide interaction exhibits no great influence on the studied conformation.

The possibility of investigating the conformation of solubilized oligopeptides through attachment to a suitable polymer, *e.g.* poly(ethylene glycol) (PEG), has recently opened the door to carry on such energy transfer investigations in solution. The aim of this work is (*i*) to provide a deeper insight into the relation between conformation and sequence composition and (*ii*) to make a comparison between the results given by CD and IR and that given by energy transfer method.

The earlier investigations carried out by Mutter and coworkers[7,8] have demonstrated that the attachment of poly(ethylene glycol) showed no influence on the peptide conformation neither in solid state nor in solution. Also, since poly(ethylene glycol) contains only C—C, C—O, C—H and O—H bonds exhibiting no electronic transitions above 180 nm the measurements in the region of the peptide absorption suffer no spectral interference from the polymer.

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The peptides chosen in this work have in their sequences Pro-Gly or Gly-Pro or Aib-Pro in the positions i + 1 and i + 2 because of the tendency of such arrangement to make a β-turn[9-11].

EXPERIMENTAL

The details of the synthesis of tetrapeptides *I—III* are reported in ref.[12].

Boc-Trp-Ile-Pro-Gly-Ile-Tyr-PEG M, Boc-Trp-Ile.Gly-Pro-Ile-Tyr-PEG M and Boc-Trp-Ile-Pro-Aib-Ile-Tyr-PEG M

The co-oligopeptides *IV—VI* are covalently bound to monofunctional poly(ethylene glycol)-monomethyl ether (PEG-M) of molecular weight 5 000. All the compounds were chromatographically and analytically pure. Analytical controls were carried out after each step of the synthesis (starting with 5 g PEG-M). Purity was indicated by amino acid analysis and thin layer chromatography. All the amino acids were Boc-protected at the α-amino group. The Boc group was removed by treatment of the PEG-peptide for 30 min with trifluoroacetic acid–dichloromethane (1 : 1) using 10 ml of the deprotecting agent per 1 g PEG-peptide. The volume of the solution was then reduced by flash evaporation to an oil and the poly(ethylene glycol)-peptide was precipitated by the addition of anhydrous ether under vigorous stirring. The mixture was stirred another 15—30 min at 0°C, the precipitate was filtered, washed with ether, and dried under vacuum. The coupling reactions were carried out *via* the *in situ* symmetrical anhydride method[13] applying excess anhydride component. To this end, the Boc-protected amino-acid derivative was dissolved in a minimum amount of dichloromethane and the solution was cooled to 0°C. 0·48 equiv. of dicyclohexylcarbodiimide in a 2M stock solution of dichloroethane was added, and the mixture was allowed to stand 30 min at 0°C. The precipitated dicyclohexylurea was removed by filtrating the anhydride solution directly into a flask containing the deprotected amino component in dichloromethane. The extent of coupling was monitored first by qualitative fluorescamin test on thin layer plates. Quantitative ninhydrin and fluorescamin tests[14] were carried out after isolation of the protected poly(ethylene glycol)-peptide by precipitation. The coupling yield was not lower than 99·5%.

When quantitative coupling was achieved, the N-Boc-protected poly(ethylene glycol)peptide was precipitated by adding ether to the coupling mixture to obtain a partially crystalline product which could be readily filtered off and which resulted in a fine powder after drying. The phenolic OH group of Boc-Tyr was protected with benzyl group. At the end of the synthesis it was removed by catalytic hydrogenation, which was carried out in the least amount of methanol on a Pd catalyst (1 g per 3 g poly(ethylene glycol)peptide. The mixture was subjected to continuous stream of hydrogen while stirring for 12 h. The pure product was obtained by filtration of the solution and addition of ether while stirring at 0°C, the precipitate was filtered, washed with ether and dried under vacuum to yield pure peptide. Boc-Trp-Ile-Gly-Pro-Ile-Tyr-PEGM (*IV*): Yield 4·2 g; amino acid analysis: Tyr (0·8), Ile (1·7), Pro (0·9), Gly (1). Boc-Trp-Ile-Pro-Gly-Ile-Tyr--PEGM (*V*): Yield 4·4 g; amino acid analysis: Tyr (0·8), Ile (1·8), Pro (1·2), Gly (1). Boc-Trp-Ile--Pro-Aib-Ile-Tyr-PEGM (*VI*): Yield 4·6 g; amino acid analysis: Tyr (0·8), Ile (1·63), Aib (0·04), Pro (0·85) Aib shows resistance to hydrolysis.)

Spectroscopic Measurements

IR Absorption spectra were recorded using a Perkin Elmer Infracord. The samples were prepared in the form of KBr disc. The concentration used through this work was 1·5 mg peptide in 300 mg

KBr. Circular dichroism measurements were obtained with a Jouan Model CD 185 spectrometer. The used solutions were prepared as 1 mg peptide per 1 ml trifluoroethanol (TFE). The fluorescence spectra as in Fig. 1 were recorded using a home made instrument. Approximately $5 \cdot 10^{-5}$ mol l^{-1} peptide solutions in water at 25°C were used. The Tyr-Trp separations, r, were calculated using the relation: $r = (E^{-1} - 1)^{1/6} R_0$. Whereby, R_0, was computed using a published[15] value of the overlap integral, J_{AD}, and under the assumption of random donor-acceptor orientation ($x^2 = 2/3$). Transfer efficiency, E, was calculated from the measured donor quantum yields in the presence of Φ_D, and absence of Φ_D^0 of transfer as: $E = 1 - \Phi_D L \Phi_D^0$. The quantum yields of Tyr and Trp were determined by comparison with values in aqueous solution (0·14 and 0·13, respectively)[15,16]. Relative fluorescence intensities were determined through integration of the spectral area using OTT 32/75 planimeter.

RESULTS AND DISCUSSION

The IR study in the solid state confirmed that the two absorption bands of amide I and amide V are the most sensitive to conformational changes of the peptide chain length. An unordered conformation shifts the amide I and amide V bands near 1 655 cm^{-1} and 650 cm^{-1}, respectively, the various types of β-structure have strong bands near 1 635 cm^{-1} and 710 while absorptions characteristic for α-helix are at 1 650 cm^{-1} and 615 cm^{-1}[16].

The IR spectra of peptides *I*–*III* are shown in Fig. 2 at the regions of amide I, V and A. The spectra of *I* and *II* exhibited no significant bands in the range of 680 to

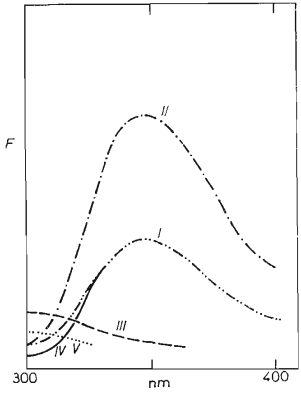

FIG. 1

Fluorescence spectra of a peptide bound by Tyr and Trp with excitation at 270 nm (curve *I*), 293 nm (curve *II*) and of the peptide bound only by Tyr with excitation at 270 nm (curve *III*). Curve *IV* was obtained by the normalization of curve *I* and *II* at 360 nm, where, curve *V* was obtained by subtraction of curve *IV* from curve *I* and represents the amount of energy transferred

750 cm^{-1} but in amide-I region there is a strong band at 1 635 cm^{-1}, a very strong shoulder at about 1 650 cm^{-1} and also a weak band near 1 690 cm^{-1} which turned to be very weak as going from *I* to *II*. A band in this region is usually assigned to the antiparallel β-structure. However, in the present case a conformational assignment on these basis would be ambiguous since the Boc group also presents an absorption at about 1 690 cm^{-1}. In the case of peptide *III* the spectrum contains two absorption bands one at 720 cm^{-1} and the other at 1 635 cm^{-1}. It has also a very strong shoulder at about 1 650 cm^{-1}.

The resulting spectra of *I*–*III* compared to the characteristic spectra of unordered conformation, β-structure and α-helix, show a significant difference which indicates the probability of the presence of a new form characterized by a band at 1 635 cm^{-1} and a strong broad shoulder at about 1 650 cm^{-1} (α-helix region). These spectra are in agreement with those obtained by Deber[17] who referred them to the presence of a β-turn. The β-turn contribution in the spectrum is demonstrated more clearly in case of the poly(ethylene glycol) bound peptides that for the free peptides.

Fig. 3 shows the IR spectra of peptides *IV*–*VI* in the region of amide V, I and A bands. The spectrum of the peptide *IV* has two bands in the region of amide V one of medium intensity at 695 cm^{-1} and a second one more intense at 745 cm^{-1}; in the amide-I region there is a shlouder at 1 635 cm^{-1} and a strong band at about 1 660 cm^{-1}. The overall shape of the spectrum is similar to that observed for the tetrapeptides *I*–*III*. In the spectrum of the peptide *V* two bands were investigated

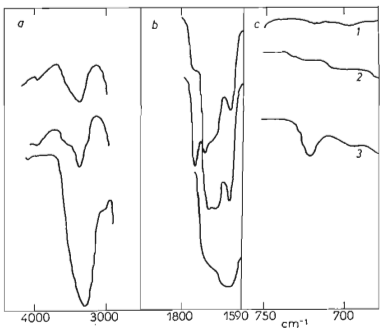

FIG. 2

IR Spectrum of the Peptides Boc-Val-Pro-Gly-Gly-PEG_{6000} (*1*), Boc-Val-Pro-Gly-Gly-PEG_{3000} (*2*) and Boc-Val-Pro-Gly-Gly-OH (*3*). *a* Amide A, *b* Amide I, *c* Amide V

at 745 cm^{-1} (amide V) and at 1 642 cm^{-1} (amide I). These bands point out an unordered conformation as the most probable. The spectrum of *VI* was found to be similar to that of *IV*. It exhibits two bands for amide *V* (at 695 cm^{-1} and at 745 cm^{-1}), and also a shoulder at 1 635 cm^{-1} and a strong band at 1 655 cm^{-1} (amide-I region). The splitting of the amide-I band in the case of peptides *IV* and *VI* may indicate a mixture of unordered and β-turn conformation.

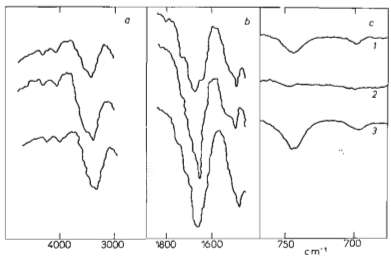

FIG. 3

IR Spectra of the Peptides *1* Boc-Trp-Ile-Pro-Gly-Ile-Tyr-PEG (*IV*), *2* Boc-Trp-Ile-Gly-Pro-Ile--Tyr-PEG (*V*) and *3* Boc-Trp-Ile-Pro-Aib-Ile-Tyr-PEG (*VI*). *a* Amide A, *b* Amide I, *c* Amide V

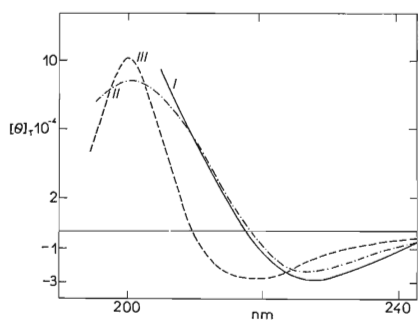

FIG. 4

CD Spectra of Boc-Val-Pro-Gly-Gly-PEG_{6000} (*I*), Boc-Val-Pro-Gly-Gly--PEG_{3000} (*II*) and Boc-Val-Pro-Gly-Gly-OH (*III*) in TFE

Fig. 4 shows the CD spectra of peptides *I*–*III*. The peptide *III* resulted from cleavage of the poly(ethylene glycol) from peptides *I* and *II*, either by catalytic hydrogenation or by alkaline hydrolysis.

The spectra exhibited a negative Cotton effect in the range of 217·5–225 nm which was assigned to $n-\pi^*$ transition. The $\pi-\pi^*$ transition appears between 199–200 nm as a positive Cotton effect and the zero crossing point shines between 209 and 216 nm. The overall shape of the curves looks like that of a β-structure. The only observed difference is that the $n-\pi^*$ transition band is shifted to a higher wavelength thus moving the zero crossing point in the same direction. This observed red shift ranged between 4 and 10 nm and is similar to that reported by Woody[18] as an indication of a β-turn formation.

The previously mentioned $n-\pi^*$ red shift was found to be greater in the case of poly(ethylene glycol) bound peptides compared to that of free peptides. The presence of poly(ethylene glycol) may be responsible of changing the formed β-turn from type 3-β-turn into type 4-β-turn as theoretically calculated[18].

CD Spectra of hexapeptides *IV*–*VI* are given in Fig. 5. The CD spectrum of peptide *IV* shows three negative peaks of different magnitude. The first is a weak shoulder at about 225–235 nm, the second is of stronger intensity at 210–215 nm while the third is the strongest one at about 200 nm. The bands at 200 and 215 nm point out that the peptide may be a mixture of β-structure and unordered conformation.

The spectrum of peptide *V* exhibited a middle intense negative Cotton effect at 215 nm and a strong one at about 200 nm. If we compare the overall shape of this

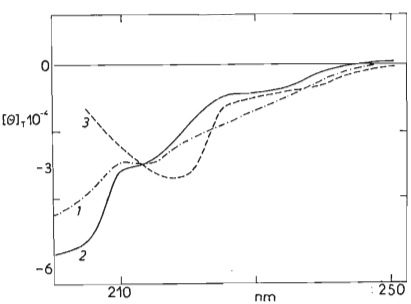

FIG. 5

CD Spectra of *1* Boc-Trp-Ile-Pro-Gly-Ile-Tyr-PEG (*IV*), *2* Boc-Trp-Ile-Gly-Pro-Ile-Tyr-PEG (*V*) and *3* Boc-Trp-Ile Pro-Aib-Ile-Tyr-PEG (*VI*) in TFE

spectrum with those investigated by Mutter and coworkers[19], for Boc-$(Val)_5$--Gly-PEG in water (conc. $5{\cdot}5 \cdot 10^{-4}$ mol l^{-1}) at 20°C and 70°C, we may state that the spectrum of peptide *V* looks similar to that recorded at 20°C. From the above similarity it can be suggested that peptide *V* might be in an associated form.

The spectrum of peptide *VI* shows a negative Cotton effect at about 218 nm and a zero cross point at about 203 nm. The shape of this curve is similar to that observed for the type 4-β-turn as previously mentioned. It can be concluded that the replacement of Gly by Aib in the peptide sequence developed the secondary structure.

Fig. 6 shows the CD spectrum of peptide *IV* in water which pointed out the development of its conformation into β-turn structure at 25°C. The increase of temperature consequently increased the intensity of the negative band at 217·5 nm which means further ordering. On the other hand, the spectrum of peptide *V* at 25°C indicates no change in water. As the temperature is raised to 70°C one band at 205 nm is formed. This band which is red shifted from the original position of the unordered form at 200 nm, can be considered as an indication of the conformational change from the unordered conformation towards the β-structure. The spectrum of peptide *VI* in water at 25°C seems to be unchanged from that in trifluoroethanol. This

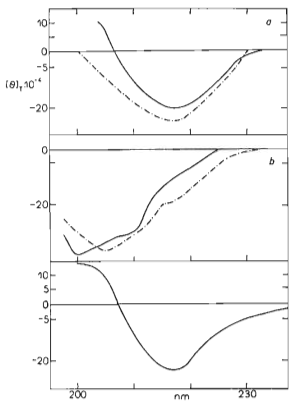

FIG. 6

CD Spectra of (*a*) Boc-Trp-Ile-Pro-Gly-Ile-Tyr-PEG (*IV*), (*b*) Boc-Trp-Ile-Gly-Pro-Ile-Tyr-PEG (*V*) and (*c*) Boc-Trp-Ile-Pro-Aib-Ile-Tyr-PEG (*VI*) in water at 25°C (———) and 70°C (— — —)

indicates that the Aib-containing peptide is unaffected by the change of solvent.

In conformational studies by energy transfer two sets of oligopeptides were used, one of them containing Tyr and Trp at the two terminals of the peptide chain having the form Boc-Trp-(Pep.)-Tyr-Peg M, the other having only Tyr in the form Boc--(Pep)-Tyr-PEG M. The Tyr-Trp separations r are as follows: *IV* 1·5794, *V* 1·54, *VI* 1·533.

The results given above were found to be in good agreement with those calculated or measured according to this theory for peptides of the same number of amino acids. For example, Englert and coworkers[20] calculated the end-to-end distance of Tyr-$(Ala)_4$-Trp = 1·45 nm and Hasa and coworkers[21], determined the distance for glutamine bound by naphthalene and Dansyl as D-Glu-N = 1·52 nm.

The results also pointed out two facts, first, the two peptides *IV* and *V* which are composed of the same amino acids with only a difference in their arrangement in positions 3 and 4 show a difference in their intramolecular distance of about 0·4Å. This fact can explain the observed difference in their CD spectra in water, where the peptide *IV* shows a greater tendency to form a β-turn than the peptide *V*. It is known that Pro-X bends occur with high frequency in proteins, while X-Pro bends are less frequent[11]. Secondly, comparing the measured distances of peptides *IV* and *VI* where Gly was replaced by Aib, an increase of about 0·07 nm was noticed, although both have a tendency to form a β-turn in water.

These observations indicate that the arrangement Pro-Aib is less flexible than Pro-Gly. This conclusion agrees with the results of Burgess and Leach[22] showing that the driving force for formation of the Aib-Pro bend results from the restricted range of conformational space available to Abi residues ($\phi \approx -50°$, $\varphi \sim -50°$ or $\varphi \sim +50°$, $\psi \sim +50°$).

The results given above point out that the energy transfer method is suitable for quantitative investigation of peptides conformations synthesized by liquid-phase method. There is series of papers in the way including out investigations on applying this method to the other known forms of conformations that take place by polypeptides, namely unordered conformation, β-structure and α-helix.

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