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Incorporation of a Potentially Helix Breaking D-Phe-Pro Sequence into the Center of a Right Handed 16 Residue Peptide Helix

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Non protein amino acids with strong secondary structure preferences are potentially useful in peptide design. α-Aminoisobutyric acid (Aib) is a powerful 'stereochemical director' of polypeptide chain folding, stabilizing helical conformations in diverse oligopeptide sequences. In an approach to the *de novo* design of α,α motifs, the 16 residue peptides Boc-Val-Ala-Leu-Aib-Val-Ala-Leu-Xxx-Pro-Val-Ala-Leu-Aib-Val-Ala-Leu-OMe (Xxx = D-Phe **1**; Xxx = L-Phe **2**) have been spectroscopically studied in solution. Analysis of nuclear Overhauser effects, delineation of solvent shielded NH groups and circular dichroism spectra establish helical conformations in both the peptides. Despite the presence of a potentially helix breaking, central, (D)Phe-(L)Pro segment, peptide **1** is forced into a continuous helical fold presumably as a consequence of the overriding stereochemical dominance of the Aib residues.

One of the approaches to the *de novo* design (1,2) of synthetic protein mimics requires the use of non coded amino acid residues in the construction of stereochemically well defined secondary structural modules, followed by their covalent assembly using linking segments into more complex folds (3,4). The use of α-aminoisobutyric acid (Aib, one letter code used is U) residues in nucleating and stabilizing helices is well established (5,6). Helical modules developed for use in a '**Meccano set approach**' to synthetic protein design, ranging in length from seven to sixteen residues, have been crystallographically characterized (7,8). Attempts to link α-helical modules have used flexible ω amino acid linkers (9). An approach to interrupt helical segments centrally to generate two distinct elements of secondary structure involved insertion of a Gly-Pro fragment (10), in an attempt to mimic α,α corners observed in protein structures (11). We report in this communication an examination of the conformational effect of insertion of a dipeptide Xxx-Pro where Xxx = (D) Phe or (L) Phe into the middle of a potentially helical, sixteen residue sequence Boc-Val-Ala-Leu-Aib-Val-Ala-Leu-Xxx-Pro-Val-Ala-Leu-Aib-Val-Ala-Leu-OMe (Xxx = D-Phe **1**; Xxx = L-Phe **2**). The choice of the D residue was dictated by the fact that helices in proteins are often terminated by a residue adopting a left handed (α_L)conformation (12), a feature which may be stabilized by

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inversion of residue configuration. The central Xxx residue was chosen as Phe in order to permit straightforward, unambiguous assignment of NMR resonances. The Pro residue was positioned so as to act as a C-terminal breaker of the first helix and as an N-terminal nucleator of the second helix (13,14). Furthermore, the D-Phe residue in the tripeptide benzyloxycarbonyl-L-Ala-D-Phe-L-Pro adopts ϕ,ψ angles characteristic of a β strand conformation in crystals (15). Helical conformations of the heptapeptide modules have been established crystallographically (9,16). The results demonstrate that although the sequence contains only two Aib residues (positions 4 and 13), the helical propensities are strong enough to force both peptides into right handed helical conformations.

MATERIAL AND METHODS

The peptides **1** and **2** were synthesized by solution phase procedures with a final segment condensation of the 7+9 type. The protected heptapeptide ester Boc-Val-Ala-Leu-Aib-Val-Ala-Leu-OMe has been previously described (16). Addition of the dipeptide Boc-D-Phe-Pro-OH to the N- deprotected heptapeptide afforded the nonapeptide. Addition of the N-deprotected 9-residue C-terminal fragment to the heptapeptide acid gave the final peptides. All couplings were mediated by dicyclohexylcarbodiimide-1-hydroxybenzotriazole in DMF. Crude peptides were purified by MPLC on 30-60μm C_{18} columns using methanol-water elution, followed by HPLC on a 10μm C_{18} column (4x250mm). Homogeneity was demonstrated by analytical HPLC on a C_{18} 5μm column (6x150 mm) and by complete assignment of 400 MHz ^{1}H NMR spectra obtained on a Bruker AMX-400 spectrometer. 2D-COSY and NOESY spectra (τ_m 400msec) were used for complete sequence specific assignments. CD spectra were recorded on a JASCO J-500 spectropolarimeter.

RESULTS AND DISCUSSION

The CD spectra of **1** and **2** in methanol (Figure 1) are characteristic of right handed helices (17). While the L-Phe peptide shows band intensities corresponding to a completely helical conformation, significantly lower intensities are observed for the D-Phe peptide. The CD spectra in trimethylphosphate are similar and remain largely unchanged upto 85°C, suggesting that the conformations are thermally stable. Figure 2 is a partial 400 MHz NOESY spectrum for peptide **1** in $CDCl_3$ while Figure 3 summarizes the NOE results for the two peptides. In both peptides a succession of d_{NN} connectivities characteristic of a helix are observed, while $d_{\alpha N}$ connectivities are either weak or absent (18). Interestingly, the intensities of the observed NOEs are stronger in peptide **1**. Solvent perturbation of NH chemical shifts were measured in $CDCl_3$-DMSO-d_6 mixtures. The values are summarized in Figure 3. In **1** and **2** the three N-terminal NH resonances show dramatic downfield shifts with increasing DMSO concentration, indicative of their exposure to solvent (19). All other NH groups appear solvent shielded supporting an alpha helical conformation in both cases. $^3J_{HN\alpha H}$ values for the 13 NH doublets could be estimated for **1**. For nine residues J lies between 4.0 and 6.0 Hz, while for Val(1) J<3.0 Hz. Values of 7-7.7 Hz are observed for Leu(12), Ala(15) and Leu(16). The coupling constants are compatible with a helical conformation at all non-Aib/Pro residues. The solvent shielded nature of Val(10) suggests that the 4⟶1 hydrogen bond involving the A(6)CO-V(10)NH groups is maintained in both peptides indicating a helical conformation at D/L Phe and Pro residues. Further support for this conformation is obtained by an analysis of the NOEs involving Pro(9) $C^\delta H_2$ protons. In the L-Phe peptide **2** the strong NOEs Phe(8)NH ⟷ Pro(9) $C^\delta H_2$ and Pro(9) $C^\delta H_2$ ⟷ Val(10)NH are observed. In the D-Phe pep-

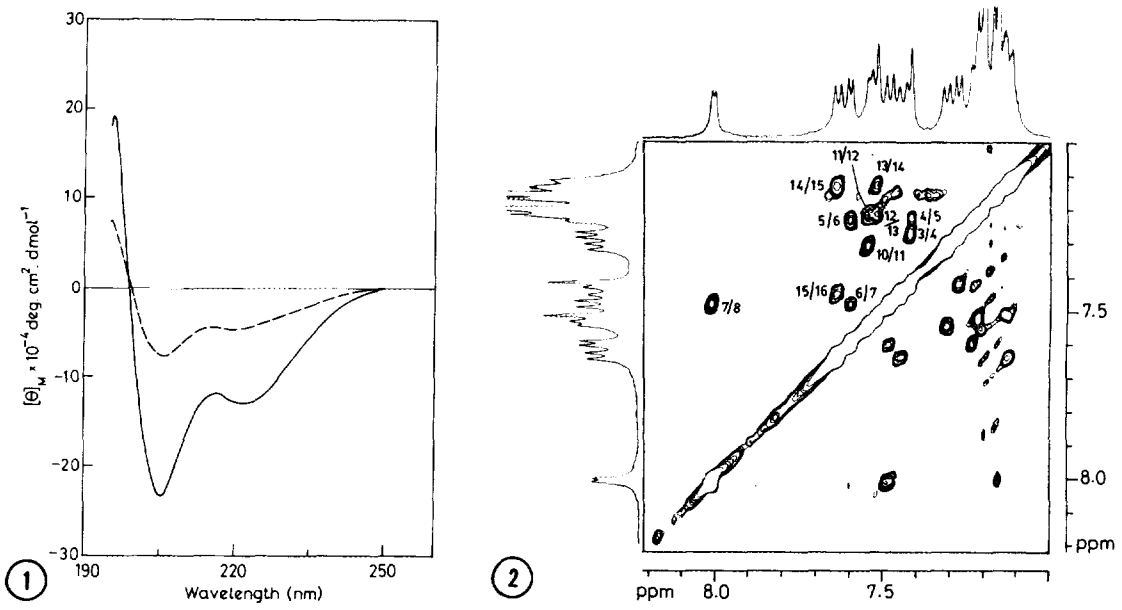

Fig. 1. CD spectra of peptides **1** (broken lines) and **2** (continuous line) in methanol.

Fig. 2. Partial 400 MHz NOESY spectrum of peptide **1** in $CDCl_3$ showing the NH_i/NH_{i+1} connectivities.

tide **1** the following intense NOEs, characteristic of a helical conformation at the linking segment are detected: Phe(8) NH $\longleftrightarrow$ Pro(9)$C^{\delta}H_2$, Phe(8) $C^{\alpha}H \longleftrightarrow$ Pro(9) $C^{\delta}H_2$ and Pro(9) $C^{\delta}H_2 \longleftrightarrow$ Val(10)NH. The simultaneous observation of both d_{NN} and $d_{\alpha N}$ NOEs at L residues in peptides is a feature of a left handed helical (α_L) conformation (20,21). In the present cases the $C^{\delta}H_2$ group of Pro(9) occupies a spatial position that is similar to the NH group in a non Pro residue. The observation of both $d_{N\delta}$ and $d_{\alpha\delta}$ NOEs at D-Phe (8) in peptide **1** confirms an α_R conformation at this residue (20,21). Using standard dimensions for amino acid residues the following distances (Å) were obtained on a computer generated model for the segment Ac-Xxx-(L)Pro-NHMe in a right handed alpha helical conformation ($\phi = -47°, \psi = -57°$): C^{α}(D)Ala-C^{δ}(L)Pro = 3.4 / 2.2 Å,C^{α}(L)Ala-C^{δ}(L)Pro = 4.1 / 3.6 Å. Analysis of intensities of these NOEs in the two peptides is in accordance with the distances obtained above. The observed interresidue NOEs confirm the *trans* stereochemistry of the D-Phe-Pro peptide bond in $CDCl_3$.

An alternative interpretation for the obervation of both types of interresidue NOEs at D-Phe(8) might invoke populations of distinct conformational states, with helical and semi-extended structures in dynamic equilibrium. However this possibility may be ruled out because of the low solvent accessibility of the Val(10)NH group in the NMR solvent perturbation experiment ($\Delta\delta$ 0.04 in **1** and 0.03 in peptide **2** (Figure 3)), which strongly suggests the involvement of this NH group in intramolecular hydrogen bonding. Population of structures which correspond to semi- extended conformations at D-Phe(8) ($\phi \simeq 120°$) would be expected to interrupt the helical hydrogen bonding scheme resulting in exposure of Val(10)NH. Furthermore, the stability of the CD spectrum over a wide temperature range in a strongly solvating medium like trimethylphosphate(TMP), argues for the population of a major stable conformation.

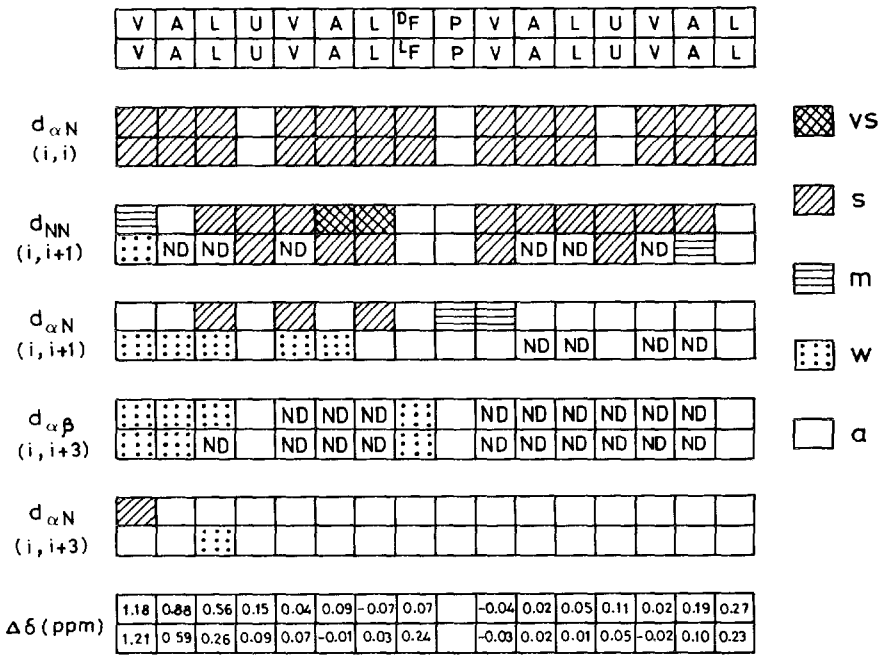

Fig. 3. Summary of NOE and solvent perturbation data. For each parameter the upper row of boxes correspond to peptide **1** and the lower row of boxes to peptide **2**; vs(strong), s(strong), m(medium), w(weak) and a(absent) refer to NOE Intensities. ND is an NOE that is obscured due to overlap of resonances and hence not determinable. The $\Delta\delta$ values in the lowermost boxes indicate chemical shift differences between solutions in $CDCl_3$ and 50% $CDCl_3$-DMSO. A value greater than 0.2 ppm can be taken as indicative of solvent exposure.

The above results conclusively demonstrate that the central D-Phe-Pro segment is accommodated without appreciable disturbance of the helical conformation in a sixteen residue peptide. Indeed earlier work (22) has shown that a single D-residue is accommodated into a right handed helix with only a slight destabilizing effect with respect to urea denaturation. The apparent difference in the CD spectra of peptides **1** and **2** may arise from aromatic sidechain contributions in the far UV region. Indeed the importance of considering cotributions of aromatic residues to CD spectra in the region 190 - 230nm has been emphasized in studies of model peptides (23), proteins with a low helical content like the bovine pancreatic trypsin inhibitor(BPTI) (24) and in an extensive analysis of barnase and several other engineered proteins (25). Thus in **1** and **2** the conflicting chiral sense of the aromatic residues may result in sidechain CD bands which are opposite in sign to the bands arising from the right handed helical peptide backbone. The overwhelming tendency of Aib residues to promote helix formation in peptides results in forcing the D-Phe-Pro segment into a right handed helix. Investigations on longer spacer segments containing several potential helix breaking residues are presently underway.

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