A Conformational Study of Peptides with the General Structure Ac-L-Xaa-Pro-D-Xaa-L-Xaa-NH_2: Spectroscopic Evidence for a Peptide with Significant β-Turn Character in Water and in Dimethyl Sulfoxide

B. Imperiali,* S. L. Fisher, R. A. Moats, and T. J. Prins

Contribution No. 8458 from the Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, California 91125. *Received September 24, 1991*

Abstract: Several tetrapeptides, Ac-Val-Pro-D-Ser-His-NH_2, in particular, show significant type II β-turn character in water and in dimethyl sulfoxide. Evidence for this turn population is provided by 2D-rotating frame nuclear Overhauser effect (ROESY) spectroscopy, ^{1}H NMR amide temperature coefficients, and circular dichroism (CD) studies. To further investigate which residues specifically contribute to the integrity of the turn, studies on 10 tetrapeptides, having the general sequence Ac-L-Xaa-Pro-D-Xaa-L-Xaa-NH_2, are described. The results show the effects of sequence variations on the type II β-turn forming propensity of these peptides in solution. Conclusions from these studies indicate that a cooperative effect between a sterically hindered, β-branched amino acid at the (i) position and a small, non-β-branched D-amino acid at the (i+2) position promotes turn formation. Implications for use of these sequences as structural nucleation elements in de novo protein design are discussed.

Introduction

Reverse turns play an important structural role in the compact globular architecture of native folded proteins.[1] However, although the reverse turn has been the subject of numerous experimental[2] and theoretical[3] investigations, the explicit role of the turn motif in the protein folding process in nature remains an open issue.[4] It is, in fact, probable that this small motif is simply a versatile player in the hierarchy of protein structure, and the specific responsibility in folding, be it active or passive, depends very much on the local amino acid sequence.[5] A current predicament in the de novo design and synthesis of proteins concerns the ability to precisely predict and control the three-dimensional architecture of a protein-like biopolymer.[6] In this context, we reasoned that it would be advantageous to delegate a specific function to the reverse turn in the protein infrastructure. Therefore, our objective in this work has been to identify and construct a conformationally well-characterized turn motif, which could be used to actively nucleate[7] and stabilize folded protein structures. Progress in this area would complement the recent developments in α-helix and β-sheet stabilization, and the utilization of those structural motifs in de novo protein design.[6]

Herein we report that several tetrapeptides, Ac-Val-Pro-D-Ser-His-NH_2, in particular, adopt a significant amount of reverse turn (β-type II)[1c] character in both water and dimethyl sulfoxide (DMSO). We present spectroscopic evidence for this, along with an investigation of the effects of variations within the general peptide sequence, Ac-L-Xaa-Pro-D-Xaa-L-Xaa-NH_2, on turn formation. Invariant features of this sequence include a proline at the (i+1) position, a D amino residue at the (i+2) position, and blocking groups on the amino and carboxyl termini (see Figure 1).

Conformational analysis of the tetrapeptides in solution in dimethyl sulfoxide (DMSO) and water is based on several experimental criteria. These include an evaluation of nuclear Overhauser effects (NOE's), amide temperature coefficients, and circular dichroism (CD) spectra. NOE effects are observed using two-dimensional rotating-frame nuclear Overhauser effect spectroscopy[8] (ROESY), and results are compared with those predicted for protein secondary structure by Wuthrich.[9] NOE studies provide unequivocal evidence for through-space interactions between protons, which would be unique to a folded structure. Variable temperature (VT) studies allow for an assessment of whether or not exchangeable protons are solvent shielded and hence potentially involved in hydrogen-bonding interactions.[1c,2c] Temperature coefficients can be influenced by many factors beyond backbone hydrogen-bonding; however, comparisons made in this paper between closely related peptides are very useful for providing auxiliary information pertaining to substitution trends in solution conformations. Finally, CD measurements are carried out in water and the spectra obtained compared with those predicted[10] and observed[11] for reverse turn motifs. The CD spectra of certain reverse turn structures are distinct from those of other secondary structural motifs and random chain conformers; therefore, the CD studies in water provide a further, *independent*, indicator of solution state conformations.

In general, the main limitation of these data is that they cannot provide a quantitative measure of the absolute population of a specific conformation in linear peptide models; however, the evidence under evaluation can afford an indication of the *existence* of a population of structures with a defined conformation. Furthermore, a comparison of relative populations within the series of peptides can be used to indicate which features of the sequence

(1) (a) Chou, P. Y.; Fasman, G. D. *J. Mol. Biol.* **1977**, *115*, 135. (b) Smith, J. A.; Pease, L. G. *CRC Crit. Rev. Biochem.* **1980**, *8*, 315. (c) Rose, G. D.; Gierasch, L. M.; Smith, J. A. *Adv. Protein Chem.* **1985**, *37*, 1.

(2) (a) Gierasch, L. M.; Deber, C. M.; Madison, V.; Niu, C.-H.; Blout, E. R. *Biochemistry* **1981**, *20*, 4730. (b) Aubry, A.; Cung, M. T.; Marraud, M. *J. Am. Chem. Soc.* **1985**, *107*, 7640. (c) Dyson, H. J.; Rance, M.; Houghten, R. A.; Lerner, R. A.; Wright, P. E. *J. Mol. Biol.* **1988**, *201*, 161. (d) Stradley, S. J.; Rizo, J.; Bruch, M. D.; Stroup, A. N.; Gierasch, L. M. *Biopolymers* **1990**, *29*, 263. (e) Narasinga Rao, B. N.; Kumar, A.; Balaram, H.; Ravi, A.; Balaram, P. *J. Am. Chem. Soc.* **1983**, *105*, 7423. (f) Kopple, K. D.; Schamper, T. J.; Go, A. *J. Am. Chem. Soc.* **1974**, *96*, 2597.

(3) (a) Nishikawa, K.; Momany, F. A.; Scheraga, H. A. *Macromolecules* **1974**, *7*, 797. (b) Zimmerman, S. S.; Scheraga, H. A. *Biopolymers* **1978**, *17*, 1885. (c) Tobias, D. J.; Sneddon, S. F.; Brooks, C. L., III *J. Mol. Biol.* **1990**, *216*, 783.

(4) (a) Lewis, P. N.; Momany, F. A.; Scheraga, H. A. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 2293. (b) Hiltner, W. A.; Walton, A. G. *J. Mol. Biol.* **1975**, *92*, 567. (c) Montelione, G. T.; Arnold, E.; Meinwald, Y. C.; Stimson, E. R.; Denton, J. B.; Huang, J. G.; Clardy, J.; Scheraga, H. A. *J. Am. Chem. Soc.* **1984**, *106*, 7946.

(5) Schultz, G. E.; Schirmer, R. H. *Principles of Protein Structure*; Cantor, C. R., Ed.; Springer-Verlag: New York, 1978; pp 74-75.

(6) (a) DeGrado, W. F. *Adv. Protein Chem.* **1988**, *39*, 51. (b) Richardson, J. S.; Richardson, D. C. *Trends Biochem. Sci.* **1989**, *14*, 304.

(7) Wetlaufer, D. B. *Trends Biochem. Sci.* **1990**, *15*, 414.

(8) (a) Bothner-By, A. A.; Stephens, R. L.; Lee, J.; Warren, C. D.; Jeanloz, R. W. *J. Am. Chem. Soc.* **1984**, *106*, 811. (b) Bax, A. *J. Magn. Reson.* **1988**, *77*, 134. (c) Bax, A.; Davis, D. G. *J. Magn. Reson.* **1985**, *63*, 207.

(9) Wuthrich, K.; Billeter, M.; Braun, W. *J. Mol. Biol.* **1984**, *180*, 715.

(10) (a) Woody, R. W. *Peptides, Polypeptides and Proteins*; Blout, E. R., Bovey, F. A., Goodman, M., Lotan, N., Eds.; Wiley: New York, 1974; p 338. (b) Manning, M. C.; Illangasekare, M.; Woody, R. W. *Biophys. Chem.* **1988**, *31*, 77.

(11) For examples, see: (a) Kawai, M.; Fasman, G. D. *J. Am. Chem. Soc.* **1978**, *100*, 3630. (b) Bush, C. A.; Sarkar, S. K.; Kopple, K. D. *Biochemistry* **1978**, *17*, 4951.

0002-7863/92/1514-3182$03.00/0 © 1992 American Chemical Society

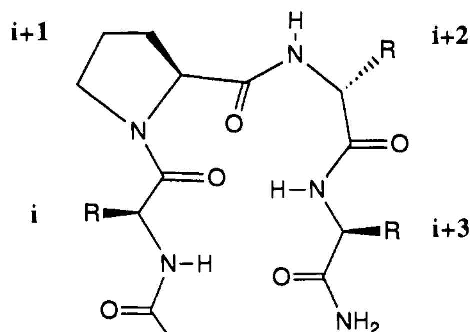

Figure 1.

are important for the structural integrity of the motif.

The results from these studies now provide information on the design of a viable, preprogrammed structural nucleation element for incorporation into larger polypeptides. The key attributes of the motif are 2-fold: firstly, the system that we have focused on emulates a short, internal sequence of a linear polypeptide; secondly, the motif appears to owe its stability only to a complementary combination of local steric and hydrogen-bonding effects and not to dominant interactions between oppositely charged groups[12] or specific effects due to strong side chain–main chain interactions.[13]

Experimental Section

Peptide Synthesis and Characterization. Commercially available starting materials and reagents for peptide synthesis were purchased from MilligenBiosearch or Sigma Chemical Co. Peptides were synthesized on a 0.1–0.2 mmol scale by solid phase methods using N^{α}-9-fluorenylmethoxycarbonyl (FMOC) protected amino acids[14] and benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate/1-hydroxybenzotriazole (BOP/HOBT)[15] mediated amide coupling chemistry on a Milligen 9050 automated peptide synthesizer. PAL resin[16] (Milligen GEN077483 substituted at ca. 0.30 mequiv/g) was used to afford carboxyl terminus primary amides. Activated esters were formed in situ using BOP, HOBT, and 0.451 M *N*-methylmorpholine in dimethylformamide (DMF). A typical protocol involved a 2-h coupling time with 4 equiv of amino acid. Deprotection of FMOC-protected amine groups was performed using a 7-min 20% piperidine/DMF wash. Peptides were N-acetylated on the resin using 20 equiv of acetic anhydride and 5 equiv of triethylamine in 3 mL of DMF. After shaking for 2 h, the resin was washed with dichloromethane and vacuum dried.

Peptides were deprotected and cleaved from the resin by treatment with trifluoroacetic acid (TFA)/thioanisole/ethanedithiol/anisole (90:5:3:2) for 2 h. After filtration of the resin, the combined filtrates were concentrated to ca. 2 mL volume and precipitated with ether/hexane 2:1. The supernatant was decanted and the peptides triturated with ether/hexane 2:1 (3 × 20 mL). The peptides were repeatedly lyophilized until no traces of the cleavage mixture remained. Peptides were purified, when necessary, using reverse phase HPLC (C_{18}) gradient elution with 0.08% TFA in acetonitrile added to 0.1% TFA in water.

Typical characterization of each peptide includes ^{1}H NMR in both DMSO-d_6 and 10% D_2O/90% H_2O, ^{13}C NMR (DMSO-d_6), R_f, and high resolution mass spectroscopy. A complete lising of these spectroscopic data for all peptides examined can be found in the supplementary material.

Spectroscopic Studies. (a) NMR. NMR experiments were run on either a Bruker Instruments AM500 or AMX500. Peak assignments were made by the use of two-dimensional correlation spectroscopy (2D COSY) experiment. 2D COSY experiments were run in either magnitude mode or phase sensitive mode, and the FID's were multiplied by a phase shifted sine bell apodization function prior to Fourier transformation.

(12) Such effects have been shown to assist in "locking" the conformation of a short peptide into a β-turn: (a) Mayer, R.; Lancelot, G. *J. Am. Chem. Soc.* **1981**, *103*, 4738. (b) Otter, A.; Scott, P. G.; Liu, X.; Kotovych, G. *J. Biomol. Struct. Dynam.* **1989**, *7*, 455.

(13) For a recent example of a study in which an amino acid side chain is involved in a strong, conformationally significant, interaction with peptide backbone, see: Abbadi, A.; Mcharfi, M.; Aubry, A.; Premilat, S.; Boussard, G.; Marraud, M. *J. Am. Chem. Soc.* **1991**, *113*, 2729.

(14) (a) Atherton, E.; Fox, H.; Harkiss, D.; Sheppard, R. C. *J. Chem. Soc., Chem. Commun.* **1978**, 539. (b) Carpino, L. A.; Han, G. Y. *J. Org. Chem.* **1972**, *37*, 3404.

(15) Hudson, D. *J. Org. Chem.* **1988**, *53*, 617.

(16) Biosearch Technical Bulletin No. 9000-02.

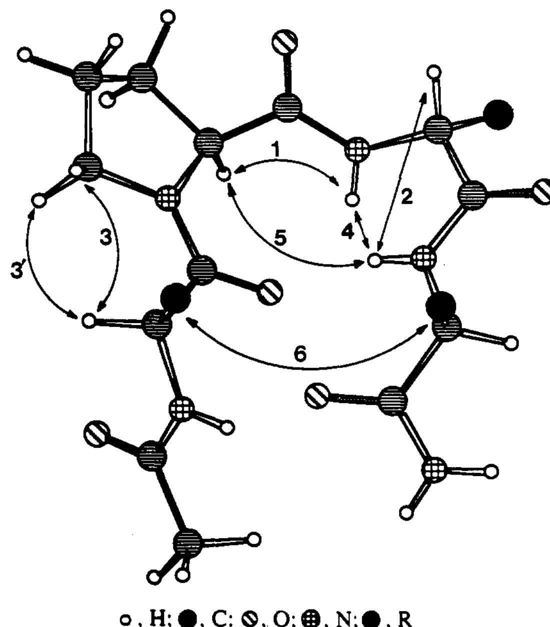

Figure 2. NOE interactions in β type II reverse turn motif: **1**, ProCα–(i+2)NH; **2**, (i+2)Cα–(i+2)NH; **3** and **3′**, (i)Cα–ProCδ; **4**, (i+2)NH–(i+3)NH; **5**, ProCα–(i+3)NH; **6**, (i) side chain–(i+3) side chain.

Nuclear Overhauser effects (NOE's) were detected using the 2D spin-locked ROESY experiment.[8] It should be noted that the ROESY cross peaks, while useful in obtaining qualitative proximity information (<3.5 Å), cannot be easily used to derive quantitative distance information in the same way as 2D NOE spectroscopy (NOESY) data.[17] 2D ROESY experiments were run in phase sensitive mode using time proportional phase increments. For the ROESY experiments the spin-lock power setting was calculated by determining the power of a 125-μs, 90° pulse. The experimental parameters, such as mixing time, water suppression parameters, spin-lock power, and offset frequency, were varied to optimize amide cross-peak intensity and to minimize HOHAHA artifacts[18] using the model peptide Tyr-Pro-Gly-Asp-Val which had previously been shown to exhibit the NOE's of interest in this study.[2c] In ROESY experiments on the AM500 spectrometer, the spin lock was applied through the decoupler channel using normal mode with O1/O2 coherence. The FID's were multiplied by a phase shifted squared sine bell apodization function prior to Fourier transformation. In a typical COSY or ROESY experiment, the relaxation delay was 1.3 s, and the transmitter offset was positioned in the center of the spectrum or on the water resonance; the data matrix consisted of 512 t_1 increments containing 2 K complex points. All COSY and ROESY experiments run in 90% H_2O/10% D_2O, solvent suppression, were obtained using presaturation at 278 K. Aqueous samples were run at pH 4.3.

The temperature dependence of the amide proton shifts was determined between 300 K and 325 K in DMSO and 278 K and 310 K in H_2O. A minimum of five temperature steps were recorded in each experiment. Calibration of the probe temperature was performed using either a methanol or ethylene glycol standard. In all cases, the chemical shifts were found to vary linearly with temperature.

(b) CD. CD spectra were recorded at 25 °C on a Jasco J-600 spectropolarimeter. Peptide solutions were prepared in double-distilled H_2O (4 mM concentration) and degassed using four freeze–pump–thaw cycles and stored under argon. Standard peptide spectral samples were prepared by serial dilution with double-distilled H_2O (degassed by argon sparging for 1 h) to 50 μM, pH adjusted to 4.5, and analyzed in a 1.0 cm path length quartz cell. Both the optics and sample chamber were flushed continuously with dry N_2 throughout each experiment. All spectra were obtained from 280 nm to 195 nm at a scan speed of 50 nm/min, a time constant of 0.5 s, and a band width of 1 nm. In all cases a minimum of eight scans were taken. CD spectra are reported in ellipticity/α-amino acid residue. Data were processed on a Macintosh IIci computer with KaleidaGraph software, version 2.1.1.

(17) Bauer, C. J.; Frenkiel, T. A.; Lane, A. N. *J. Magn. Reson.* **1990**, *87*, 144.

(18) Cavanagh, J.; Keeler, J. *J. Magn. Reson.* **1988**, *80*, 186.

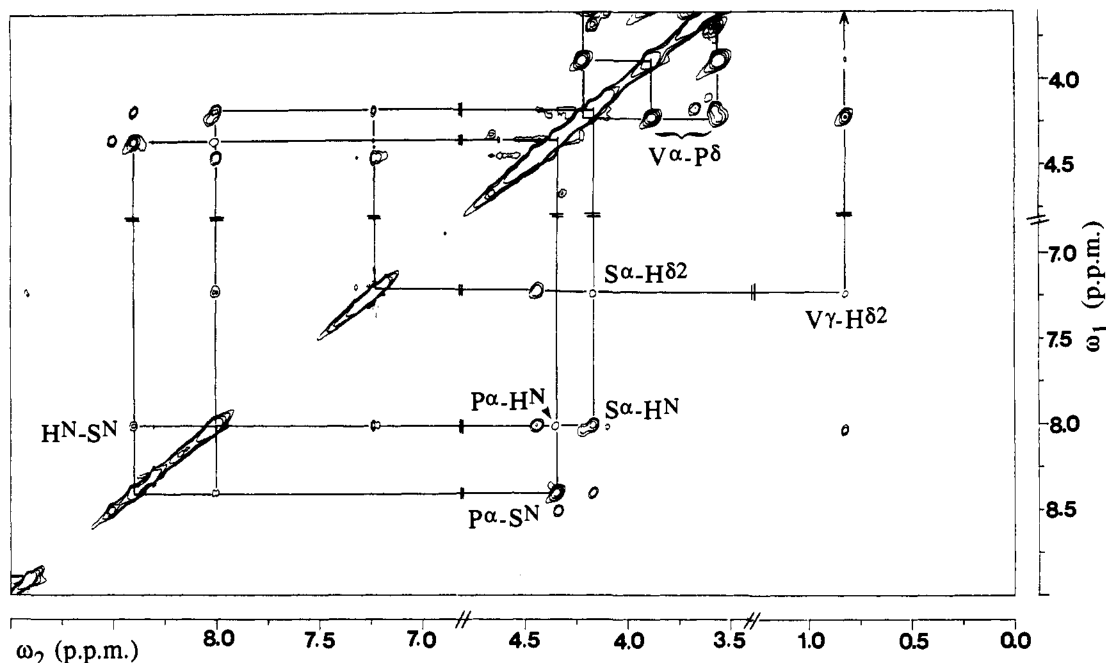

Figure 3. Sections of a 500-MHz ROESY spectrum of Ac-Val-Pro-D-Ser-His-NH_2 in DMSO at 298 K. The region encompassing cross peaks from the amide and α-carbon protons is shown. Labeled peaks represent those important for conformational analyses and can be assigned to the following interactions: d_{NN}(His, Ser), $d_{\alpha N}$(Pro, Ser), $d_{\alpha N}$(Pro, His), $d_{\alpha N}$(Ser, His), $d_{\alpha\delta}$(Val, Pro), $d_{\gamma\delta 2}$(Val, His), and $d_{\alpha\delta 2}$(Ser, His).

Results

Conformational Study of Ac-Val-Pro-D-Ser-His-NH_2 and Ac-Val-Pro-D-Ala-His-NH_2. NOE studies on Ac-Val-Pro-D-Ser-His-NH_2 and Ac-Val-Pro-D-Ala-His-NH_2 in water and DMSO provide the strongest evidence to indicate the presence of a turn population. Figure 2 illustrates the through-space connectivities which might be observed in a typical type II β-turn structure. Interactions **1**, **2**, **3**, and **4** are classified as "sequential" connectivities.[2c] Although interactions **1**, **2**, and **3** would be present, in varying intensity, in the spectra of many peptides, **4**, **5**, and **6** would be observed in a type II reverse turn folded structure.[9] The diagnostic regions of the ROESY spectra of Ac-Val-Pro-D-Ser-His-NH_2 in DMSO and water are shown in Figures 3 and 4, respectively. In DMSO, the sequential NOE's (**1**, **2**, and **3**; Figure 2) are strong. In addition, the long-range connectivities, d_{NN} (His, Ser), $d_{\alpha N}$ (Pro, His), and $d_{\gamma\delta 2}$ (Val, His) (**4**, **5**, and **6**, Figure 2), are observed. In water the sequential NOE's and the d_{NN} (His, Ser) are present; however, the remaining long-range interactions are not seen. The ROESY spectra of Ac-Val-Pro-D-Ala-His-NH_2 in both DMSO and water show the interactions **1**, **2**, **3**, and **4**, with the long-range interaction (**4**) being weak in water. The ROESY data for these and related peptides are summarized in Table I. A small amount of a second, ordered conformation (<10% in all peptides) is evident in the 1D NMR spectra. This minor component was not conformationally analyzed in detail.

VT studies on these two peptides provide data which are consistent with the NOE observations.[19] In an ideal β-turn [(i+3) to (i) hydrogen bond] in the absence of interactions between the side chain functionality and the peptide backbone, the temperature coefficients would be predicted to show maximum solvent shielding of the (i+3) residue NH and minimum shielding of the (i+2)

(19) Temperature coefficients are influenced strongly by solvent effects; in DMSO values range from 0 to (−)8 ppb/K [<(−)3 ppb/K suggests strong solvent shielding, (−)3 to (−)4.5 ppb/K denotes moderate shielding, and >4.5 ppb/K indicates that a proton is exposed to the bulk solvent]. In water the absolute values are a larger, and range from (−)2 to (−)11 ppb/K [<(−)4 ppb/K suggests strong solvent shielding, (−)4 to (−)6 ppb/K denotes moderate shielding, and >(−)6 ppb/K indicates that a proton is likely to be exposed to the bulk solvent]. As a comparison, the VT data for a constrained cyclic peptide which has been conformationally analyzed in detail are presented. The peptide cyclo-Pro-D-Tyr(Bzl)-Gly-Ile-Leu-Gln) has been shown to adopt two β-turns (a type I and a type II). Temperature coefficients for the (i+2) and (i+3) residues of the type II turn in $CDCl_3$–$(CD_3)_2SO$, 98:2, are (−)5.9 and (−)1.8 ppb/K, respectively (see ref 1c).

Table I. Observed ROESY Connectivities[a] in DMSO and Water

peptide	DMSO	water
Ac-Val-Pro-D-Ser-His-NH_2	**1**, **2**, **3**, **4**, **5**, and **6**	**1**, **2**, **3**, and **4**
Ac-Val-Pro-D-Ser-Phe-NH_2	**1**, **2**, **3**, **4**,[b] and **6**[c]	**1**, **2**, **3**, and **4**[d]
Ac-Val-Pro-D-Ser-Ile-NH_2	**1**, **2**, **3**, and **4**	**1**, **2**, **3**, and **4**
Ac-Val-Pro-D-Ala-His-NH_2	**1**, **2**, **3**, and **4**	**1**, **2**, **3**, and **4**[d]
Ac-Val-Pro-D-Ala-Phe-NH_2	**1**, **2**, and **3**	**1**, **2**, and **3**

[a] See Figure 2 for definition of interactions. [b] It is likely that interaction **5** is present in this spectrum due to the intensity of the relevant cross peak; however, the assignment cannot be made unequivocally due to the coincidence of the Pro-αCH and Phe-αCH. [c] $d_{ring\gamma}$ (Phe, Val). [d] Cross peak relatively weak.

Table II. Temperature Coefficients for Amide Protons in Dimethyl Sulfoxide ($\Delta\delta/\Delta T$)[a]

peptide	i	i+2	i+3
Ac-Val-Pro-D-Ser-His-NH_2	−7.3	−7.8	−2.9
Ac-Val-Pro-D-Ala-His-NH_2	−5.5	−5.0	−2.7
Ac-Val-Pro-D-Ser-Phe-NH_2	−5.0	−5.1	−2.9
Ac-Val-Pro-D-Ser-Ile-NH_2	−6.2	−4.6	−3.5
Ac-Val-Pro-D-Ala-Phe-NH_2	−5.8	−4.2	−4.5
Ac-Ala-Pro-D-Val-His-NH_2	−4.6	−3.8	−4.4
Ac-Ala-Pro-D-Ala-Phe-NH_2	−5.2	−3.8	−4.5
Ac-*t*-Leu-Pro-D-Ala-His-NH_2	−6.0	−4.8	−4.2
Ac-Ala-Pro-Gly-Phe-NH_2	−5.5	−3.9	−3.9
Ac-Ala-Pro-D-Leu-Phe-NH_2	−5.3	−3.2	−5.4
Ac-Ala-Pro-D-Val-Phe-NH_2	−5.3	−3.9	−5.4

[a] In parts per billion per degree Kelvin.

Table III. Temperature Coefficients for Amide Protons in Water ($\Delta\delta/\Delta T$)[a]

peptide	i	i+2	i+3
Ac-Val-Pro-D-Ser-His-NH_2	−8.7	−9.4	−5.0
Ac-Val-Pro-D-Ala-His-NH_2	−7.9	−8.1	−4.9
Ac-Val-Pro-D-Ser-Phe-NH_2	−8.7	−8.8	−5.8
Ac-Val-Pro-D-Ser-Ile-NH_2	−8.1	−9.0	−6.2
Ac-Val-Pro-D-Ala-Phe-NH_2	−8.4	−9.1	−6.2
Ac-Ala-Pro-D-Val-His-NH_2	−7.6	−8.8	−7.8
Ac-Ala-Pro-D-Ala-Phe-NH_2	−9.3	−8.5	−6.8
Ac-*t*-Leu-Pro-D-Ala-His-NH_2	−7.6	−9.0	−6.5
Ac-Ala-Pro-Gly-Phe-NH_2	−6.1	−7.3	−7.5
Ac-Ala-Pro-D-Leu-Phe-NH_2[b]	−8.2	−9.1	−8.9
Ac-Ala-Pro-D-Val-Phe-NH_2	−8.3	−9.9	−8.7

[a] In parts per billion per degree Kelvin. [b] In 10% DMSO/H_2O.

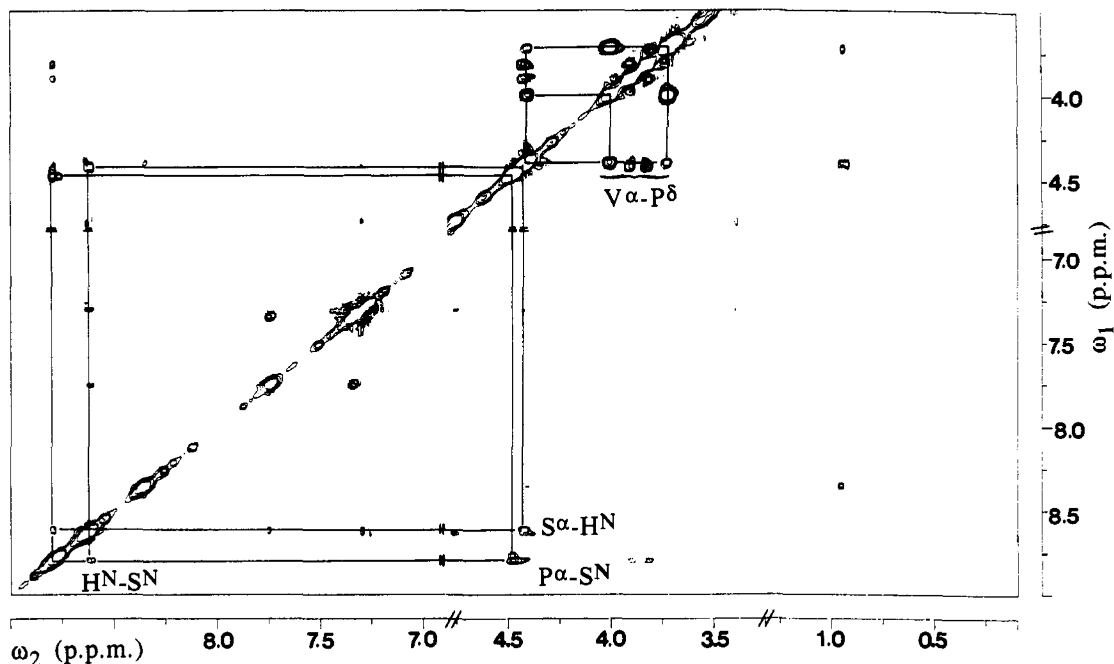

Figure 4. Sections of a 500-MHz ROESY spectrum of Ac-Val-Pro-D-Ser-His-NH_2 in 90% H_2O/10% D_2O at 278 K. The region encompassing cross peaks from the amide and α-carbon protons is shown. Labeled peaks represent those important for conformational analyses and can be assigned to the following interactions: d_{NN}(His, Ser), $d_{\alpha N}$(Pro, Ser), $d_{\alpha N}$(Ser, His), $d_{\alpha\delta}$(Val, Pro).

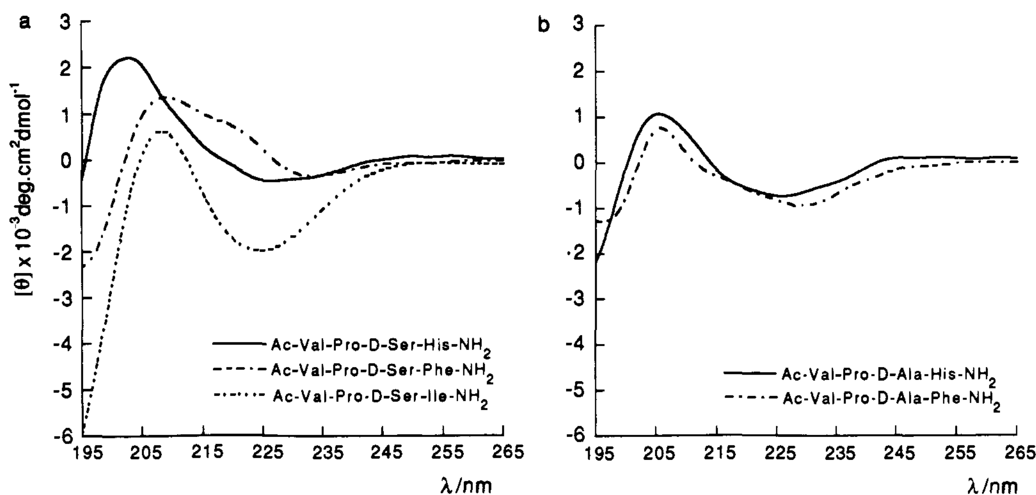

Figure 5. CD spectra in H_2O at 25 °C: (a) Ac-Val-Pro-D-Ser-Xaa-NH_2 (Xaa = His, Phe, and Ile); (b) Ac-Val-Pro-D-Ala-Xaa-NH_2 (Xaa = His and Phe).

residue NH.[1] In a structure containing a γ-turn, [(i+2) to (i) hydrogen bond] involving the proline and D-amino acid residue, the converse would apply.[1] The amide proton temperature coefficients of the tetrapeptides examined are summarized in Tables II and III. In both DMSO and water, the VT data for Ac-Val-Pro-D-Ser-His-NH_2 and the D-alanine-containing analogue indicate some β-turn population; temperature coefficients for the (i+3) NH show strong solvent shielding in DMSO and moderate shielding in water.

The CD spectra of Ac-Val-Pro-D-Ser-His-NH_2 and Ac-Val-Pro-D-Ala-His-NH_2 in water are shown in Figure 5, parts a and b. CD spectra of reverse turns are distinct from those of other secondary structural motifs.[10,11] The most common, and distinct, spectral type associated with a type II β-turn, classified as a class B spectrum, is characterized by negative ellipticities >220 nm and <190 nm and a positive ellipticity in the 200–210 nm range. All spectra in parts a and b of Figure 5 show maxima and minima in the appropriate wavelength range for a type II β-turn; however, the absolute value for θ is weaker for Ac-Val-Pro-D-Ala-His-NH_2 than Ac-Val-Pro-D-Ser-His-NH_2. The relative spectral intensities lend support to the NOE observations which suggested that the latter peptide was a stronger turn. The intensity of the positive ellipticity for the D-serine-containing peptide approaches a value for θ of 2300 deg cm^2 $dmol^{-1}$. (It should be noted that it is not possible to directly compare the CD spectra of peptides in this study with those calculated or in model systems, since the latter are based on only a core triamide unit.)

The CD spectrum of Ac-Val-Pro-D-Ser-His-NH_2 is invariant over the concentration range from 5 μM to 5 mM; therefore, it is assumed that intermolecular interactions are not affecting the measurements. Also, the upper concentration limit for the CD studies approaches that used in the NMR experiments, so the observations made in the two experiments can be correlated.

Collectively, these spectroscopic observations indicate the presence of population of an ordered conformation in Ac-Val-Pro-D-Ser-His-NH_2. Ac-Val-Pro-D-Ala-His-NH_2 also shows evidence of a similar structure, but to a lesser degree. We chose to examine a series of related peptides in order to explore which aspects of the sequence were important for the turn induction in Ac-Val-Pro-D-Ser-His-NH_2. Since turn formation is a complex process involving many interdependent effects, it is not feasible to carry out an exhaustive survey. However, we have demonstrated that in this closely related series of peptides, the opportunity exists for a significant amount of conformational variability. All peptides

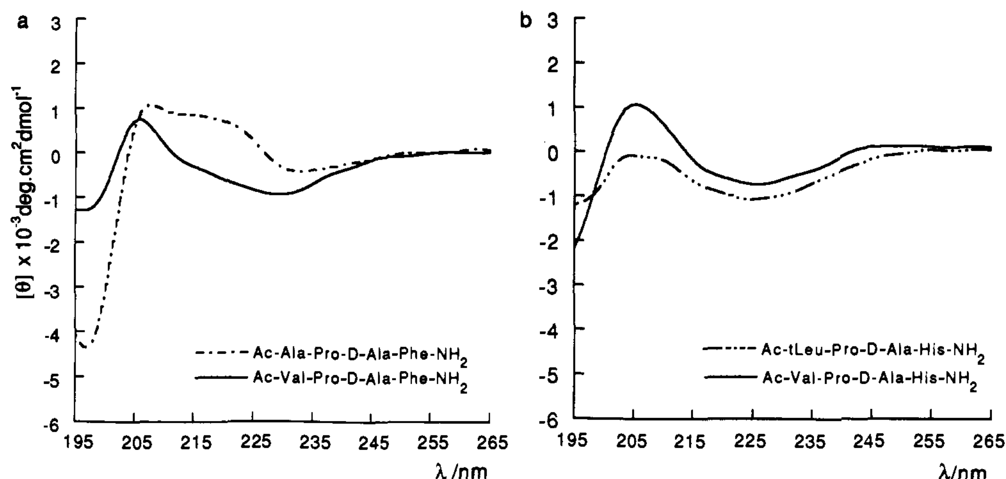

Figure 6. CD spectra in H_2O at 25 °C: (a) Ac-Xaa-Pro-D-Ala-Phe-NH_2 (Xaa = Ala and Val); (b) Ac-Xaa-Pro-D-Ala-His-NH_2 (Xaa = *t*-Leu and Val).

examined are based on the general structure Ac-L-Xaa-Pro-D-Xaa-L-Xaa-NH_2.

Conformational Consequences of Residue Substitution

(i+3) Histidine. In order to address the importance of histidine as the (i+3) residue, related peptides with single residue replacements were examined. All spectroscopic data in water are collected at pH < 4.5; thus the histidine side chain would be ionized. Ac-Val-Pro-D-Ser-**Phe**-NH_2 and Ac-Val-Pro-D-Ser-**Ile**-NH_2 can be compared with Ac-Val-Pro-D-Ser-**His**-NH_2, and Ac-Val-Pro-D-Ala-**Phe**-NH_2 compared with Ac-Val-Pro-D-Ala-**His**-NH_2.

NOE studies of these peptides in DMSO and water essentially indicate that a diminution of the turn population accompanies replacement of either D-serine at (i+2) or histidine at (i+3). For example, ROESY spectra of Ac-Val-Pro-D-Ser-Ile-NH_2 in DMSO lack the $d_{\alpha N}$ [(i+1), (i+3)] and long-range connectivities observed in the corresponding histidine-containing peptide.[20] Also, the d_{NN} [(i+2), (i+3)] interaction in Ac-Val-Pro-D-Ala-His-NH_2 is absent in Ac-Val-Pro-D-Ala-Phe-NH_2 in water. The VT data, presented in Tables II and III, show similar trends, indicating that histidine at (i+3) has a positive, but not unique, effect on the conformation. In the unordered peptide, Ac-Ala-Pro-D-Val-His-NH_2 (also documented in Tables II and III) the histidine NH temperature coefficient is typical of a solvent exposed proton. This latter observation rules out the possibility that the low coefficients are due only to a side chain specific effect.

CD studies for the D-serine- and D-alanine-containing peptides illustrated in Figure 5, a and b, respectively, support the conclusions reached in NMR experiments. Essentially, the peptides which show NMR evidence of being better turns are characterized by a CD spectra with more class B character.

(i) Valine. The side chain substituent of the amino acid residue at the (i) position experiences steric crowding from the δ-methylene of the proline ring in a β-turn.[21] Therefore, we chose to probe the influence of the steric effects of residues in this position. Specifically, Ac-**Val**-Pro-D-Ala-Phe-NH_2 was compared with Ac-**Ala**-Pro-D-Ala-Phe-NH_2, and Ac-*tert*-**Leu**-Pro-D-Ala-His-NH_2 with Ac-**Val**-Pro-D-Ala-His-NH_2. The ROESY spectrum of Ac-Val-Pro-D-Ala-Phe-NH_2 does not show any long-range interactions indicative of a folded structure; therefore, it is likely that the population of the folded structure(s) is low. However, the VT data for these two pairs of peptides in water and DMSO (Tables II and III) and the CD spectra in water *collectively* provide an idea of the influence of the (i) residue substitutions. The CD spectra for each pair of peptides are illustrated in parts a and b of Figure 6. In contrast to the spectra observed with

(20) The $d_{\alpha N}$ (Pro, His) interproton distance in a perfect type II turn is 3.3 Å,[9] which is close to the limits of detection in the ROESY experiment; thus this interaction might not always be apparent, even in a viable turn.

(21) Schimmel, P. R.; Flory, P. J. *J. Mol. Biol.* **1968**, *34*, 105.

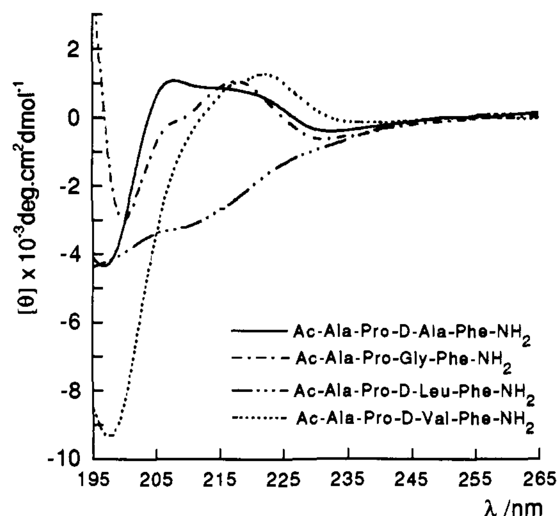

Figure 7. CD spectra in H_2O at 25 °C of Ac-Ala-Pro-Xaa-Phe-NH_2 (Xaa = Gly, D-Ala, D-Val, and D-Leu).

the previous five peptides (in which the CD spectra showed a distinct positive ellipticity maximum between 200 and 210 nm), Ac-Ala-Pro-D-Ala-Phe-NH_2 shows a very broad positive ellipticity ranging from 205 to 225 nm, and the *tert*-leucine-containing peptide exhibits no positive ellipticity. Thus it appears that either increasing or decreasing the steric bulk beyond valine perturbs the turn conformation in a negative way.

Influence of (i+2) Residue. The effect of varying the steric bulk of the (i+2) residue was also investigated. For this purpose, a homologous series of four peptides, of general structure Ac-Ala-Pro-**Xaa**-Phe-NH_2 (**Xaa** = Gly, D-Ala, D-Val, and D-Leu) were studied to see if a change in the (i+2) residue might alter the turn population. The temperature coefficients for the amide protons of these peptides in both DMSO and water are documented in Tables II and III, respectively. In DMSO there appears to be no type II β-turn population, and, in fact, in some cases the (i+2) NH coefficients show some solvent shielding (e.g., for Ac-Ala-Pro-D-**Leu**-Phe-NH_2, (−)3.2 ppb/K). This observation suggests the presence of an alternate folded structure such as a γ-turn or a reverse turn in which the amide NH of the D-leucine is hydrogen-bonded to the carbonyl group of the acetyl cap. In water, changes in the steric demand of the (i+2) residues to the small, flexible residue glycine, or more sterically demanding residues D-valine or D-leucine, weaken the turn; the D-alanine-containing peptide has the lowest coefficient for the (i+3) NH. In water there is no indication of an alternate folded structure.

Figure 7 shows the CD spectra for this series of peptides in water. Following a similar trend to the water VT data in which Ac-Ala-Pro-D-Ala-Phe-NH_2 demonstrates a slight turn population,

the CD spectrum shows a negative ellipticity at 197 nm and a broad positive ellipticity from 208 nm to 220 nm. The spectra of the glycine, D-valine, and D-leucine analogues are quite varied, and lack any positive ellipticity in the region characteristic of the type II turns.

Discussion

The short linear peptides examined in this study could potentially adopt many conformations. However, the prolyl-D-amino acid dipeptide core of the sequence prevents adoption of either ordered α-helical or β-sheet structures; thus, we have chosen to limit discussion to the three predominant types of interconverting conformers.[22] These would be represented by (i) "open" (unfolded) structures with no hydrogen-bonding interactions, (ii) a γ-turn structure [(i+2) to (i) hydrogen bond], and (iii) β-turn structures [(i+3) to (i) hydrogen bond]. The β-turn could be either a type I or II structure; however, the latter is expected in the heterochiral sequence studied, since this conformation results in reduced steric interactions between the carbonyl oxygen of the central amide and the side chain of the (i+2) residue.[23]

The combined spectroscopic data for the peptide Ac-Val-Pro-D-Ser-His-NH_2 show evidence of a significant population of a folded conformation, and also allow identification of the likely conformation. The NOE experiments indicate the presence of a type II β-turn structure. In both solvents, the observed NOE $d_{\alpha N}$(Pro, Ser) (1, Figure 2) is very strong, indicating that these two protons are on the same face of the molecule as is characteristic of a type II turn (typical proton–proton distances in turns: type II, 2.2 Å; type I, 3.4 Å). In addition, the interaction between the δ-protons on the proline ring with the α-CH of the (i) residue is characteristic of a trans amide rotamer to proline (in the trans isomer the $d_{\alpha\delta}$(Val, Pro) distance is 2.0–3.9 Å, whereas in the cis isomer it is 4.3–5.0 Å[9]). A small amount of a second conformer, assigned as the cis amide rotamer to proline (based on chemical shift data), was noted in the 1D NMR. It is surprising that the contribution of the cis rotamer is as low as it is, particularly with a bulky residue such as valine in the (i) position. For comparison, the distribution of conformers for the peptide cyclo-(L-Val-L-Pro-Gly)$_2$ is biased very strongly in favor (>80% in D_2O or DMSO) of the isomer containing two cis amides.[2a] Also, studies on linear, uncapped pentapeptides of general structure Tyr-Pro-L-Xaa-L-Yaa-L-Zaa (uncapped terminii) also show high proportions of the cis amide rotamer.[2c] It appears that the balance of steric bulk at the (i), (i+2), and (i+3) positions around the turn, coupled with the turn type, may be responsible for the shift in equilibrium between the two rotamers. In those situations where congestion becomes an issue *and* the backbone cannot unfold to relieve this, the cis amide appears. In the peptides examined, the side chain of the D (i+2) residue would be removed from the crowded face of the molecule in the type II conformation. Both VT and CD studies are in agreement with the turn identification. In the VT experiments, the (i+3) amide NH is solvent shielded in both solvents. Furthermore, with regard to the CD studies, while either an open conformation or a type I turn would be characterized by negative ellipticities in the 200–210 nm range, and the γ-turn would show a maximum at longer wavelengths[1b] (approximately 230 nm), the type II turn would be expected to show a CD pattern similar to that illustrated in Figure 5a. The intensity of the positive ellipticity for the D-serine-containing peptide approaches a value for θ of 2300 deg cm^2 dmol^{-1} which is not strong. However, the absolute intensity of the CD spectra for reverse turns is always much lower than that for the regular α-helix and β-sheet, since it is composed of a "nonrecurring" arrangement of chromophores. In addition, low band intensities can be attributed to equilibrium between the turn conformation and unordered structures which show an intense negative band around 200 nm[24] and can therefore cancel out the intensity of the positive maximum.

(22) Boussard, G.; Marraud, M. *J. Am. Chem. Soc.* **1985**, *107*, 1825 and references therein.
(23) Venkatachalam, C. M. *Biopolymers* **1968**, *6*, 1425.
(24) Greenfield, N.; Fasman, G. D. *Biochemistry* **1969**, *8*, 4108.

The role of D-serine in stabilizing the type II β-turn motif is unclear. Although many reports have discussed type I turn stabilization by L-serine,[25] there is only one discussion of the Pro-D-Ser dipeptide influence on conformation. Studies of Boc-Pro-D-Ser-NHMe in solution in carbon tetrachloride and in the solid state have suggested that the serine hydroxyl group may provide additional stabilization to the turn conformation through a hydrogen-bonding interaction from the γ-O (Ser) to the amide NH;[22] however, the VT data for the blocked tetrapeptides in these studies do not indicate that this is the case in water or DMSO. In homochiral sequences, residues such as serine, and cysteine exhibit unique conformational properties which have been correlated with advantageous side chain interactions.[26] While the prolyl-D-serine dipeptide core of the sequence does seem to be a strong turn determinant, it is noteworthy that, even in the absence of the extra hydroxyl functionality, the turn is present, albeit to a lesser degree.

The studies with single residue replacements demonstrate the fine balance between the open and closed conformations. Probabilistic studies that examine the occurrence of residues localized within reverse turn structures in proteins[1a,27,28] illustrate that strong positional preferences exist for each amino acid residue involved in a reverse turn. The recent study by Wilmot and Thornton[28] analyzes the residue preferences within *specific* turn types and includes a data base of 101 type II turns; however, in contrast to the type I turn, there were very few statistically significant positional preferences in the type II turn beyond proline at (i+1) and glycine at (i+2).[29] Furthermore, this survey is of limited value in this discussion, because the majority of the peptides we have studied incorporate an unnatural D-amino acid at the (i+2) position. Thus, it is evident that as soon as an unnatural element is incorporated into the motif, in our case a D residue, studies must perforce rely on evaluation of model peptides.

The observed steric effects at the (i+2) position are consistent with the predicted dihedral angles for each residue, as derived from Ramachadran plots.[30] The ideal dihedral angles for the (i+2) residue in the type II turn are $\phi = 80°$ and $\psi = 0°$;[1c] β-branching, or incorporation of sterically demanding groups at the (i+2) amino acid residue, would be expected to restrict the conformational space open to that residue, and hence prohibit assumption of the ideal torsional angles. The spectroscopic data (both CD and VT studies) indicate that the tetrapeptides with either D-leucine or D-valine at the (i+2) position exist largely in an open conformation in water. In contrast, VT studies in DMSO suggest some population of an alternate folded structure (Ac-Ala-Pro-D-Leu-Phe-NH_2 [$\Delta\delta/\Delta T = -3.2$ ppb/K for (i+3) NH]). In the latter solvent, however, the ROESY studies did not provide further insight on the conformation of these peptides as no diagnostic NOE's were observed. Conformational analysis of simple dipeptides, of general structure *t*-BuCO-Pro-D-Xaa-NHMe,[2b] have indicated that the heterochiral dipeptides showed a high β-turn ratio in aprotic solvents, regardless of the steric bulk of the (i+2) residue. Our results imply that the full effect of steric bulk at the (i+2) position in the type II turn may not be felt in the absence of complete amino acids at the (i) and (i+3) location around the motif.

The optimum residue at the (i) position, in the folded motif, should be one which favors an extended β-sheet conformation. In these investigations, we have focused principally on the influence of steric effects. It appears, from the limited study carried out thus far, that valine at the (i) position results in the highest turn population based on both CD and VT data. Increasing the steric demand of this residue to *tert*-leucine, or decreasing it to alanine,

(25) (a) Hollosi, M.; Kover, K. E.; Holly, S.; Fasman, G. D. *Biopolymers* **1987**, *26*, 1527. (b) Perczel, A.; Hollosi, M.; Fulop, V.; Kalman, A.; Sandor, P.; Fasman, G. D. *Biopolymers* **1990**, *30*, 763.
(26) Gorbitz, C. H. *Acta Chem. Scand.* **1990**, *44*, 584.
(27) Levitt, M. *Biochemistry* **1978**, *17*, 4277.
(28) Wilmot, C. M.; Thornton, J. M. *J. Mol. Biol.* **1988**, *203*, 221.
(29) This preference results from the severe steric conflict between the (i+1) carbonyl oxygen and the (i+2) side chain in any of the substituted L-amino acids.[23]
(30) Ramachandran, G. N.; Sasisekharan, V. *Adv. Protein Chem.* **1968**, *23*, 283.

weakens the integrity of the turn. These steric effects are rather pronounced as would be anticipated with residues preceding proline.[31] The influence of functionalized residues has not yet been evaluated, although it is generally agreed that, although amino acids with short polar side chains (such as asparagine, aspartic acid, serine, and cysteine) show a high positional preference in the type I turn, this is not the case with the type II turn.[28] The origin of this effect is related to the planarity of the backbone amide conformation. In the type I turn, the overall conformation of the polyamide backbone is twisted, allowing for stabilizing interactions between a functionalized side chain and the amide backbone. However, in the flatter type II conformation the angle of the side chain substituent, relative to the polyamide backbone, is such as to maximize the influence of steric effects, while at the same time not allowing for interaction between a short side chain and the peptide backbone.

With regards to the influence of amino acid replacements at the (i+3) position, three amino acids have been considered. In this case, in contrast to the (i) residue effect, β-branching, (e.g., in the peptide, Ac-Val-Pro-D-Ser-Ile-NH_2) does not stabilize turn formation relative to the two unbranched (i+3) amino acid-containing peptides. While the spectroscopic data shows that histidine at the (i+3) position stabilizes the turn motif, further investigation will be necessary to clarify the mechanism by which this occurs.

Conclusions

In general, short linear peptides, in which both amino and carboxyl terminii are derivatized, are considered to be conformationally unstructured in solution in polar aprotic and protic media. This instability is attributed to the fact that competition for intermolecular hydrogen-bonding interactions by solvent tends to diminish any advantageous stabilization which might be gained from intramolecular hydrogen bond formation which accompanies a folded structure. This study has presented spectroscopic evidence to indicate that the short linear peptide Ac-Val-Pro-D-Ser-His-NH_2 shows an observable population of a folded conformation in both DMSO and water. From the NMR and CD studies, this conformation has been identified as a type II β-turn. In this peptide, we suggest that an adventitious combination of steric effects influences the conformational space accessible to the peptide backbone in such a way as to promote intramolecular hydrogen-bonding and folding of the turn motif. The specific contribution of the hydroxyl and imidazole side chains in stabilizing this reverse turn structure have yet to be elucidated.

The heterochiral sequence, which forms the basis of the general structure of the peptides examined, is likely to be the key characteristic which contributes to the turn stability, since it promotes formation of a type II rather than a type I turn. The type II turn, specifically, is accompanied by a more planar conformation of the polyamide backbone; hence, the alignment of the hydrogen bond donor and acceptor groups (from the (i+3) amide hydrogen to the (i) carbonyl oxygen) is optimal. Thus, in an ideal situation the type II β-turn would be expected to be more intrinsically stable than the type I.[2a]

The demonstration that a short peptide, which is terminal capped to emulate an internal sequence within a long polypeptide, can adopt a sufficiently stable folded structure to be observed in aqueous solution now sets the stage for utilizing this short peptide as an engineered structural nucleation site for de novo protein design and synthesis. The heterochiral sequence provides an additional advantage in this regard, since it renders the structural motif "position-specific" within the long polypeptide chain, since the prolyl-D-amino acid dipeptide pair cannot adopt any other ordered secondary structure such as the α-helix or β-sheet. Finally the demonstration that the turn stability is observed with polar residues such as D-serine and histidine and to some degree with less polar residues such D-alanine and phenylalanine also means that the turn motif could be used as an exposed turn on the surface of a globular, folded structure or as an internal turn element.

Acknowledgment. This work was supported by the Caltech Consortium in Chemistry and Chemical Engineering (founding members: E.I. Du Pont de Nemours & Co., Inc., Eastman Kodak Co., and the Minnesota Mining and Manufacturing Co.) and in part by the National Science Foundation (CHE-9104445). Award of a Department of Education Predoctoral Fellowship to S.L.F., an NSF Predoctoral Fellowship to T.J.P., and a Du Pont Young Faculty Award (1990) to B.I. are also gratefully acknowledged.

Registry No. Ac-Val-Pro-D-Ser-His-NH_2, 139583-72-5; Ac-Val-Pro-D-Ala-His-NH_2, 139583-73-6; Ac-Val-Pro-D-Ser-Phe-NH_2, 139583-74-7; Ac-Val-Pro-D-Ser-Ile-NH_2, 139583-75-8; Ac-Val-Pro-D-Ala-Phe-NH_2, 139583-76-9; Ac-Ala-Pro-D-Val-His-NH_2, 139583-77-0; Ac-Ala-Pro-D-Ala-Phe-NH_2, 139583-78-1; Ac-*t*-Leu-Pro-D-Ala-His-NH_2, 139583-79-2; Ac-Ala-Pro-Gly-Phe-NH_2, 139583-80-5; Ac-Ala-Pro-D-Leu-Phe-NH_2, 139583-81-6; Ac-Ala-Pro-D-Val-Phe-NH_2, 139583-82-7.

Supplementary Material Available: A complete listing of the spectroscopic data for all peptides examined and additional ROESY spectra (8 pages). Ordering information is given on any current masthead page.

(31) Schimmel and Flory have noted that although, in general, the (ϕ, ψ) dihedral angles of residue (i) are independent of the neighbors (i−1) and (i+1), this is not true for residues succeeded by proline. In this case, interactions with the δCH_2 of proline affects the (ϕ, ψ) conformational space accessible to the preceding residue significantly.[21]