Proline-Containing β-Turns in Peptides and Proteins. II. Physicochemical Studies on Tripeptides with the Pro-Gly Sequence*

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Synopsis

Amino acids are known to differ in their individual preferences for each of the four positions of the β-turn conformation formed by tetrapeptide segments. Proline and glycine show relatively high preferences for positions 2 and 3, respectively, of the β-turn. Using tripeptides of the type N-acetyl-Pro-Gly-X-OH, where X = Gly, Ala, Leu, Ile, and Phe, we have sought to study the influence of the 4th residue X on the stability of the β-turn conformation in these tripeptides. Our nmr and CD results show that the β-turn stability is quite significantly governed by the nature of the amino acid residue at this position in the following order: Leu > Ala > Ile, Gly > Phe.

INTRODUCTION

The β-turn is an important secondary structure in globular proteins and involves four consecutive amino acid residues causing the polypeptide chain to fold back on itself.1 It is also frequently found to be present in cyclic peptides of biological importance, where the occurrence of Pro in the β-turn is a fairly common feature.2 In globular proteins, Pro is seen to have the highest preference for the 2nd position of the β-turn formed by tripeptide segments of the type R₁-R₂-R₃-R₄. Gly is found to show the highest preference for the 3rd position.3 A schematic representation of the β-turn containing the Pro-Gly sequence is shown in Fig. 1.

Considerable work has been done in recent years on the conformational details and stability of the β-turn.3-5 The conformational studies have been concerned mainly with the stereochemical and energetic criteria for the 2nd and 3rd position residues. The analysis of Chou and Fasman3 has shown that the preferences of individual amino acid residues for the 1st and 4th positions are quite distinct. No systematic study has, however,

* For Paper I of this series, see Ref. 8.
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been available on the influence of these extreme residues on the formation and stability of the β-turn peptides segments. This paper reports the results of our own studies in this direction.

We have sought to study the effect of the C-terminal residue on the β-turn formed by synthetic peptides containing the Pro-Gly sequence in the middle. Using tripeptides with the general sequence N-acetyl-Pro-Gly-X-OH (where X = Gly, Ala, Leu, Ile, and Phe), we have analyzed by means of spectroscopic techniques the effect of the X residue on the β-turn stability. Besides their relevance to β-turns in globular proteins, the results obtained from this study are expected to be useful in understanding the conformation of collagen, which contains substantial amounts of Pro-Gly segments in its primary structure.

**EXPERIMENTAL**

Microanalysis was carried out by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley. Before the analysis, all the samples were dried over Drierite at room temperature under high vacuum. Melting points were determined on a Thomas Hoover melting point apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer model 141 polarimeter. Precoated silica gel G plates (E. Merck) were used for thin-layer chromatography (tlc) with the following solvent systems: I, butanol–acetic acid–water (4:1:1); II, butanol–acetic acid–pyridine–water (15:10:3:2); III, methanol–pyridine–water (20:1:5); IV, diisopropylether–methanol–acetic acid (71:10:5); V, chloroform–methanol (9:1); VI, chloroform–methanol–acetic acid (70:10:5). The compounds were detected by ninhydrin, chlorax, or uv light. Throughout the experiment, the water-soluble 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride is used. Electrophoretic mobilities were recorded relative to the mobility of histidine under similar conditions and designated as $E_{His}$. Electrophoresis was done at pH 1.85 on a Savant instrument using 5000 V for 20 min.
Synthesis of the Tripeptides, N-Ac-Pro-Gly-X-OH

The materials used for the synthesis have been described elsewhere.\(^6\)

**Benzyloxycarbonyl-L-Prolylglycylglycine Benzyl Ester (I)**

Benzyloxy carbonyl-L-prolylglycine\(^6\) (4.6 g, 15 mmol) and glycine benzyl ester p-toluene sulfonate (5.1 g, 15 mmol) were dissolved in a solvent containing 50 mL acetonitrile and 15 mL chloroform. The solution was cooled to 0°C and treated with the carbodiimide hydrochloride (2.9 g, 15 mmol). Triethylamine (2.1 mL) was then added. The reaction was allowed to take place overnight at 4°C, then at room temperature. The reaction mixture was evaporated to dryness under vacuum. The residue was dissolved in a mixture of ethyl acetate (300 mL) and water (50 mL). The ethyl acetate layer was serially washed with water (2 × 75 mL), 1N HCl (3 × 75 mL) and again with water (3 × 75 mL). The ethyl acetate layer was dried over anhydrous sodium sulfate and then evaporated to a white solid. This was recrystallized from ethyl acetate–petroleum ether to get 5 g (74%) of the final product of mp 108–109°C, \((\alpha)_D^{26} = -26.6^\circ\) (c 0.6, MeOH). The compound showed a single spot on tlc: \(R_f\) IV, 0.27; \(R_f\) V, 0.8; \(R_f\) VI, 0.84.

**ANAL.:** Calcd. for C\(_{24}\)H\(_{27}\)O\(_8\)N\(_5\): C, 63.56; H, 6.00; N, 9.23. Found: C, 63.85; H, 5.78; N, 9.42.

**L-Prolylglycylglycine (II)**

Compound I (2 g) was dissolved in a solvent containing acetic acid: water:95% ethanol (1:4:7) in about 150 mL. Palladium, 10% (0.6 g), on charcoal was added and kept magnetically stirred. The reaction mixture was hydrogenated at room temperature for 5 h, filtered, and evaporated under vacuum to get 1.9 g of a white solid. The compound was put on a Dowex-50 column in H\(^+\) form, washed with distilled water (10 times the bed volume), then eluted with 15 bed volumes of 5% ammonia solution. The ammonia solution was lyophilized to get a white solid. The compound showed a single spot on tlc: \(R_f\) I, 0.13; \(R_f\) II, 0.29; \(R_f\) III, 0.27. A single spot resulted on electrophoresis: \(E_{His}\) 0.67.

**N-Acetyl-L-Prolylglycylglycine (III)**

The crude product from II (0.61 g) was dissolved in 3 mL acetic acid, cooled, treated with acetic anhydride (0.51 g, 5 mmol), and kept stirred magnetically. After about 7 h, the reaction mixture was lyophilized to get a thick oily mass. This was dissolved in 10 mL water, 100–200 mg of Dowex-50 in the H\(^+\) form was added to remove the unacetylated peptide, and the mixture was filtered. The filtrate was evaporated to get 0.63 g (86%). The residue was recrystallized from ethanol–acetone–petroleum ether, mp 145–147°C, \((\alpha)_D^{25} = -70.5\) (c 0.9, H\(_2\)O). The compound showed a single spot on tlc: \(R_f\) I, 0.26; \(R_f\) II, 0.47; \(R_f\) III, 0.61.
**Benzoyloxycarbonyl-L-Prolylglycyl-L-Alanine Benzyl Ester (IV)**

This compound was synthesized as described for I utilizing benzoyloxycarbonyl-L-prolylglycine (6.1 g, 20 mmol), L-alanine benzyl ester p-toluene sulfonate (7 g, 20 mmol), 3.8 g of the carbodiimide, and 2.8 mL of triethylamine. The final product was a thick syrup, which was used as such in the following synthesis.

**L-Prolylglycyl-L-Alanine (V)**

Benzoyloxycarbonyl-L-prolylglycyl-L-alanine benzyl ester (2.35 g, 5 mmol) was hydrogenated and then purified on a Dowex column as described for II. The compound was recrystallized from ethanol–ether to get 0.94 g (78%) of an extremely hygroscopic powder, mp 131–135°C (not sharp), \(\left(\alpha\right)_{D}^{25} - 56.8^\circ\) (c 0.5, H2O). The compound showed a single spot on tlc: \(R_f\)I, 0.18; \(R_f\)II, 0.38; \(R_f\)III, 0.34. A single spot was found on electrophoresis, \(E_{His}\) 0.60.

**N-Acetyl-L-Prolylglycyl-L-Alanine (VI)**

Compound V (0.5 g, 2 mmol) was acetylated with 0.4 g (4 mmol) of acetic anhydride and treated as described for III to get a white solid. This was recrystallized from ethanol–acetone–petroleum ether to get 0.41 g (70%) of VI, mp 185–187°C, \(\left(\alpha\right)_{D}^{25} - 101.61^\circ\) (c 0.6, H2O). The compound showed a single spot on tlc: \(R_f\)I, 0.35; \(R_f\)II, 0.56; \(R_f\)III, 0.63.

**Benzoyloxycarbonyl-L-Prolylglycyl-L-Isoleucine Benzyl Ester (VII)**

This was synthesized as described for I, utilizing benzoyloxycarbonyl-L-prolylglycine (3 g, 10 mmol), L-isoleucine benzyl ester p-toluene sulfonate (3.9 g, 10 mmol), carbodiimide hydrochloride (1.0 g, 10 mmol) and triethylamine (1.4 mL). A thick syrup (4.34 g) was obtained, which was used as such for the the next step.

**L-Prolylglycyl-L-Isoleucine (VIII)**

Compound VII (2 g, 4 mmol) was hydrogenated and purified on the ion-exchange column as described for II. The product was recrystallized from ethanol–ether to get 0.63 g (75%) of an extremely hygroscopic powder,
and its melting point could not be determined. \((\alpha)_D^\circ = -43.8^\circ\) (c 0.5, H\(_2\)O). The compound showed a single spot on tlc: \(R_f\ I, 0.33; R_f\ II, 0.56; R_f\ III, 0.46.\) A single spot was also found on electrophoresis, \(E_{\text{His}} 0.056.\)

**ANAL:**  
Calcd. for C\(_{13}\)H\(_{23}\)N\(_3\)O\(_4\):  C, 54.72; H, 8.12; N, 14.73.  Found:  C, 54.5; H, 8.1; N, 14.6.

**N-Acetyl-L-Prolylglycyl-L-Isoleucine (IX)**

L-Prolylglycyl-L-isoleucine (0.4 g, 1.4 mmol) was acetylated as under II to get a white powder, which is very hygroscopic. The powder was dissolved in ethyl acetate, treated with heptane to precipitate a thick oil, and lyophilized to get a fluffy powder, 0.3 g (65%). The melting point could not be determined accurately due to its extreme hygroscopic nature. \((\alpha)_D^{25^\circ} = 44^\circ\) (c 0.5, MeOH). The compound showed a single spot on tlc: \(R_f\ I, 0.49; R_f\ II, 0.76; R_f\ III, 0.70.\)

**ANAL:**  
Calcd. for C\(_{15}\)H\(_{25}\)N\(_3\)O\(_5\):  C, 55.03; H, 7.70; N, 12.84.  Found:  C, 55.1; H, 7.8; N, 12.8.

**N-Acetyl-L-Prolylglycyl-L-Phenylalanine (X)**

L-Prolylglycyl-L-phenylalanine (0.6 g, 2 mmol) was acetylated as under III to get a thick oil, and the oil was crystallized from 95% ethanol–acetone–petroleum ether to get 0.58 g (80%) of white crystalline needles, mp 164–165°C, \((\alpha)_D^{25^\circ} = 22.1^\circ\) (c 0.43, MeOH). The compound showed a single spot on tlc: \(R_f\ I, 0.49; R_f\ II, 0.71; R_f\ III, 0.70.\)

**ANAL:**  
Calcd. for C\(_{18}\)H\(_{23}\)N\(_3\)O\(_5\):  C, 59.82; H, 6.42; N, 11.63.  Found:  C, 60.01; H, 6.37; N, 11.68.

**L-Prolylglycyl-L-Leucine (XI)**

Benzyloxy carbonyl-L-prolylglycyl-L-leucine\(^6\) (0.85 g, 2 mmol) was hydrogenated as described for II to get an oil. The oil was dissolved in 95% ethanol, and ethyl acetate was added to precipitate a solid material (0.54 g). The compound was recrystallized once again to get 0.45 g (78%), mp 208–210°C (decomp., showed color change at 198°C), \((\alpha)_D^{25^\circ} = 50.0^\circ\) (c 0.6, H\(_2\)O). The compound showed a single spot tlc: \(R_f\ I, 0.30; R_f\ II, 0.56; R_f\ III, 0.45; E_{\text{His}} 0.55.\)

**ANAL:**  
Calcd. for C\(_{13}\)H\(_{23}\)N\(_3\)O\(_4\):  C, 54.72; H, 8.12; H, 14.73.  Found:  C, 54.33; H, 8.11; N, 14.92.

**N-Acetyl-L-Prolylglycyl-L-Leucine (XII)**

XI (0.34 g, 1.2 mmol) was acetylated as described for III to get a white solid 0.30 g (77%), mp 182–183°C, \((\alpha)_D^{25^\circ} = 79.4^\circ\) (c 0.5, H\(_2\)O). The compound showed a single spot on tlc: \(R_f\ I, 0.50; R_f\ II, 0.74; R_f\ III, 0.69.\)

**ANAL:**  
Calcd. for C\(_{15}\)H\(_{25}\)N\(_3\)O\(_5\):  C, 55.03; H, 7.70; N, 12.84.  Found:  C, 54.86; H, 7.59; N, 12.69.
CD Spectra

The CD spectra of the tripeptides were recorded on a Jasco-J-20 automatic recording spectropolarimeter. The spectra were scanned using quartz cells of pathlengths 0.01–0.5 cm from 250 to 200 nm. Most of the measurements were done at a peptide concentration of 0.2 mg/mL for all peptides. Trifluoroethanol (TFE) was obtained from Sigma Chemical Co. and was distilled before use. The molar ellipticity $|\theta|_{\text{molar}}$ is expressed in deg cm$^2$ dmol$^{-1}$. Temperature variation of the sample was achieved by circulating water or ethyl alcohol from an external thermostat through a jacketed cell holder. The temperature of the sample was measured by a calibrated thermistor probe (Victor Engineering Co., USA).

IR Spectra

IR spectra were recorded in nujol mull on a Carl Zeiss UR-10 spectrometer using a NaCl cell. The spectra in CHCl$_3$ solutions were obtained using a Perkin-Elmer model 283 instrument with a 1-mm pathlength cell.

$^1$H-NMR Spectra

Proton nmr spectra were recorded on a Bruker WH-270 FT instrument at the Bangalore nmr facility. Chemical shifts were measured in $\delta$ (ppm) scale for all the samples with internal tetramethylsilane (TMS) as reference. TMS and DMSO-d$_6$ were obtained from Stohler Isotope Co. (USA). Absolute methanol was prepared by standard procedure. All the spectra were recorded in the Fourier transform mode using deuterium lock. For non-deuterated solvents, the homogeneity was adjusted by observing the FID of the solvent signal. In these cases, a gated decoupler was used to suppress the solvent signal, and a minimum of 100–400 scans were made to improve the signal-to-noise ratio. Temperature control was achieved to $\pm 1^\circ$C by passing dry N$_2$ gas through a heating coil for high temperatures and through a liquid nitrogen trap for low temperatures. At each temperature, the sample was equilibrated for 15–20 min before collecting the data. The sample concentration ranged from 3 to 10 mg/mL depending on the solubility of the material in the solvent used.

RESULTS AND DISCUSSION

The CD spectra of N-Ac-Pro-Gly-Leu-OH and N-Ac-Pro-Gly-Ile-OH in TFE at 25°C are shown in Fig. 2. This solvent is known to promote ordered structure in peptides.$^7$ The conformation of the Leu-tripeptide in TFE at a lower temperature ($-40^\circ$C) has been shown by us elsewhere$^8$ to be that of a nearly 100%, type II $\beta$-turn, using vacuum-uv–CD spectral data. The CD spectral characteristic of this conformation has been shown,$^9$ in accordance with the expectations from the theoretical calculation,$^9$ to be a relatively large value ($-12$) for the ratio $R = (\theta)_{201}/(\theta)_{225}$ and the presence
of a low-wavelength CD band at 180 nm. Figure 2 shows that although the Ile-tripeptide in TFE exhibits a negative CD band around 223 nm similar to the Leu-tripeptide, it does not show a well-defined maximum around 200 nm; this indicates a reduced content of the $\beta$-turn conformation. In water, both tripeptides lack the 225-nm band of the $\beta$-turn but show the CD spectral characteristics of a predominantly random-coil conformation with a negative band around 203 nm.

The CD spectra of N-Ac-Pro-Gly-Ala-OH in water and at different temperatures in TFE are shown in Fig. 3. Like the Leu- and Ile-tripeptides, the Ala-tripeptide also seems to exist in the disordered conformation in water giving rise to a strong negative CD band at 201 nm. In TFE, unlike the Ile-tripeptide, it shows the presence of greater amount of $\beta$-turn conformation as manifested by the increased magnitude of the positive CD band. On cooling the solution, there is a considerable increase in the extent of the $\beta$-turn conformation in this tripeptide, and at $-20^\circ$C its spectrum is similar to that observed for Leu-tripeptide in TFE at room temperature. However, the magnitude of the positive CD band of the Ala-tripeptide at the lowest temperature studied ($-20^\circ$C) is less than the expected theoretical value and the value obtained for the Leu-tripeptide.

N-Ac-Pro-Gly-Gly-OH show CD spectra similar to those of the Ile-tripeptide in TFE and water (Fig. 4). The CD spectrum of N-Ac-Pro-Gly-
Phe-OH in TFE at room temperature was obtained and was subjected to correction for the contribution of the phenylalanine chromophore as described by us elsewhere. The corrected spectrum is shown in Fig. 4. This spectrum is seen to resemble those of the Ile- and Gly-tripeptides in TFE.

It is interesting to note at this point that in spite of their close structural similarity, the various N-Ac-Pro-Gly-X-OH tripeptides, under identical conditions, show a considerable variation in their CD spectral characteristics; this reflects the influence of the X residue on the ordered conformation, which appears to be the β-turn.

IR Studies

The ir spectral data of the tripeptides in nujol mull indicated the presence of a hydrogen-bonded amide group in these compounds. Thus, in addition to the amide A band around 3400 cm⁻¹ expected for the free NH group, these tripeptides exhibited a relatively strong band around 3300 cm⁻¹, as
observed in the intramolecularly hydrogen-bonded conformation of \(N\text{-Ac-Pro-Gly-NHCH}_3\) by Stimson et al.\(^4\) The amide I and II bands were also found to occur in pairs. We were unable to carry out ir studies of these peptides in \(\text{CHCl}_3\) or \(\text{CCl}_4\) at different concentrations for the purpose of distinguishing the intra- from the intermolecular hydrogen-bond formation\(^1\) because of the poor solubility of the peptides in these solvents. However, such data were obtained on the close analogs of the Leu- and Ala-containing tripeptides, viz., \(\text{tBoc-Pro-Gly-Leu-OH}\) and \(\text{tBoc-Pro-Gly-Ala-OH}\). In dilute \(\text{CHCl}_3\) solutions, both these peptides exhibited ir bands that were very similar to those of the \(N\)-acetyl counterparts and were independent of the peptide concentration in the range of \(6-30 \times 10^{-3} \text{M}\). An intramolecular hydrogen-bonded conformation is thus indicated.

\(^1\)H-NMR Studies

The CD and ir studies presented in the previous sections indicated the occurrence of a hydrogen-bonded \(\beta\)-turn conformation in the tripeptides in nonpolar environment. The CD data also indicated that the extent to
which this ordered conformation occurs in these peptides is considerably influenced by the nature of the fourth residue. In order to get a more quantitative insight into the problem, we have resorted to $^1$H-nmr spectroscopy. In recent years, several workers$^{4,12,13}$ have demonstrated the presence of the folded or $\beta$-turn conformation in linear peptides containing proline residues by nmr spectroscopy.

In our present studies, we have used solvent titration as well as the temperature dependence of the chemical shift as diagnostic tools$^{13,14}$ for identifying the extent to which these tripeptides adopt a $\beta$-turn or folded conformation in which the 4th residue NH is shielded from the solvent.

The $^1$H-nmr spectra of all the five tripeptides were obtained in DMSO-$d_6$, TFE, and MeOH. The assignments of the resonances were made by comparing the spectra with the published ones for the same amino acid residues in similar solvents and by use of area ratios, coupling patterns, and decoupling experiments.$^{15-18}$ Table I summarizes the data on the chemical shifts obtained at 20 ± 1°C of the NH protons in N-Ac-Pro$_2$-Gly$_3$-X$_4$-OH (where X = Leu, Ala, Gly, Phe, and Ile) tripeptides in DMSO-$d_6$, TFE, and MeOH.

In TFE and MeOH, the NH protons of all these tripeptides show single peaks. On the other hand, in DMSO-$d_6$, additional minor peaks are observed as shown in Fig. 5 (which gives the amide and acetyl proton resonances). The single resonances in TFE and MeOH were taken to represent the all-trans conformation for the peptides, while the additional minor resonances in DMSO-$d_6$ were assumed to arise from trans-cis isomerization about the acetyl-Pro bond that is commonly observed in other similar peptides.$^{4,19}$ As pointed out in a similar study,$^{20}$ the deblocked tripeptide H-Pro-Gly-Gly-OH was found by us to show no minor resonance for the terminal Gly NH proton in DMSO-$d_6$, in contrast to the corresponding N-acetyl derivative, indicating the involvement of the acetyl-Pro bond in the isomerization of the latter. As will be shown below, the relatively smaller temperature coefficients of the major amide resonance lines in TFE and MeOH support their assignment to a $4 \rightarrow 1$ hydrogen-bonded all-trans conformation in these solvents. (The details of the cis $\Rightarrow$ trans isomerization studies on the tripeptides will be presented elsewhere.)

**Solvent Dependence of Peptide NH Chemical Shifts**

In order to identify the NH protons that are solvent-shielded, we have chosen TFE and MeOH, since the conformation of the molecule in both these solvents is similar, viz., all-trans. Figure 6 shows the effect of altering the solvent on the chemical shifts of the Gly$_3$ NH and $X^4$ NH resonances of the three tripeptides containing Leu, Ala, and Ile. In all these cases, increasing the concentration of MeOH causes a large downfield shift of the Gly$_3$ NH proton of the tripeptides, whereas the shifts of the NH protons of the $X^4$ residues are much less, the actual extent of the shift varying from peptide to peptide. The other two tripeptides also exhibit (not shown in
<table>
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<th>Table I: &quot;H-NMR Chemical Shift Data for NH Protons of N-Ac-Pro-Gly-X-OH Tripeptidesa,b&quot;</th>
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<td><strong>Tripeptide</strong></td>
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<tr>
<td>N-Ac-Pro-Gly-Leu-OH</td>
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<td>N-Ac-Pro-Gly-Phe-OH</td>
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<td>N-Ac-Pro-Gly-Gly-OH</td>
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- Recorded at 20 ± 1°C to an accuracy of ±0.005 ppm with a 270-MHz nmr spectrometer.
- a: NH minor peak; M: major peak; d: doublet; t: triplet; q: quartet.
- b: ±0.05 accuracy, measured with a 100-MHz spectrometer.
- c: Both Gly NH signals overlap.
Fig. 5. The 270-MHz $^1$H-nmr spectrum of the amide region of (a) N-Ac-Pro$_2$-Gly$_3$-Leu$_4$-OH and (b) N-Ac-Pro$_2$-Gly$_3$-Ala$_4$-OH in DMSO-d$_6$ at 20°C. Major (M) and minor (m) resonances of the N-acetyl protons are also shown.

the figure) similar behavior, i.e., pronounced changes in the chemical shift of Gly$_3$ NH compared to $X^4$ NH proton on changing the solvent polarity. (It is also seen from Table I that a larger downfield shift of the Gly$_3$ NH proton when compared with the $X^4$ NH proton is observed on going from TFE to DMSO-d$_6$.) These results show that the 4th residue NH proton (i.e., $X^4$ NH) is solvent-shielded to a considerable extent, but the Gly$_3$ NH proton is quite solvent-exposed in all the five tripeptides. [The absence
Fig. 6. Solvent dependence of the chemical shift for NH protons of Ala (A), Leu (L), and Ile (I) tripeptides (represented as N-Ac³-Pro²-Gly³-X⁴-OH) in TFE-MeOH mixtures.

of any significant change in the CD spectra in going from TFE to MeOH (data not shown) may be taken to indicate that the chemical shift changes do not arise from conformational change in the peptides.

The presence of the solvent-shielded amide protons in these tripeptides argues for the occurrence of a well-defined ordered structure in solution. The 4 → 1 hydrogen-bonded β-turn conformation¹ (Fig. 1) involving the acetyl carbonyl in a hydrogen bond with the 4th residue (X) NH is consistent with the above nmr data, as well as with the conclusions reached from the CD and ir studies.

Temperature-Dependence of Peptide NH Chemical Shifts

We have studied the temperature dependence of peptide NH proton chemical shift for all the five tripeptides in DMSO-d₆, since in this solvent the temperature coefficients for solvent-shielded and solvent-exposed NH protons have been characterized and extensively documented.⁴,¹³,²¹ Typical chemical shift versus temperature data for the amide protons [both major (M) and minor (m) associated with the trans and cis conformers, respectively] in the Leu tripeptide in DMSO-d₆ are shown in Fig. 7. The temperature dependence of the NH chemical shifts was found to be linear in DMSO-d₆ in the range of 20–70°C, indicating the absence of any significant change in the conformation of the tripeptides. (The details of the nmr data for the Phe-tripeptide have been presented elsewhere,²⁰ along with single-crystal x-ray diffraction data for this tripeptide.) As mentioned
earlier, in DMSO-d₆ there are some minor peaks that are attributable to a relatively small percentage of cis conformer in solution. A similar observation has also been made by Scheraga and coworkers⁴ for N-Ac-Pro-Gly-NHCH₃ in DMSO-d₆.

The temperature coefficient $d\delta/dT$ values, as well as the 0°C intercept values, for these tripeptide NH protons in DMSO-d₆ are listed in Table II. For comparison, the temperature coefficient values of the NH protons of N-Ac-Pro-Gly-NHCH₃ are also given in Table II. In general, the $X^4(M)$ NH protons show lower values, indicating considerable hydrogen-bonded character. On the other hand, in the cis conformer, the $X^4(m)$ NH proton for all the tripeptides is almost completely solvent-exposed, indicating the absence of any hydrogen bond involving the 4th residue NH in the cis conformer. The Gly³ (M) NH resonances in the Leu-, Ala-, and Ile-tripeptides, as well as in N-Ac-Pro-Gly-NHCH₃,⁴ show relatively large temperature coefficients, indicating that these protons are considerably solvent-exposed in DMSO-d₆. However, in the Gly- and Phe-tripeptides, there appears to be a significant shielding of the Gly³ (M) NH proton from the solvent; this may possibly arise from a hydrogen bond involving this proton and the carbonyl oxygen of the acetyl group in a C₇-conformation of the tripeptide with trans acetyl-Pro-bond.²⁰ The data on the temperature dependence of the Gly³ (m) NH resonances (which were found to be relatively small and broad, Fig. 5) were rather difficult to obtain; and in the cases where these are reported (Table II), the experimental errors associated with them are expected to be rather large. Therefore, no attempt has been made to interpret these data.

A 4 → 1 hydrogen-bonded β-turn conformation observed with other peptides containing the -Pro²-Gly³-sequence⁴,¹³,¹⁴,²²-²⁴ is compatible with the temperature-coefficient data obtained for all the tripeptides. Very
### Temperature Dependence of Peptide NH Proton Chemical Shifts of N-Acetyl-Pro-Gly-

**Proline-Containing β-Turns**

**TABLE II**

Temperature Dependence of Peptide NH Proton Chemical Shifts of N-Acetyl-Pro-Gly-

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<th>Tripeptide</th>
<th>Proton</th>
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| N-Ac-Pro$_2$-Gly$_3$-Leu$_4$-OH | Gly$^3$ (M) NH | -5.8 | -8.50 |
| N-Ac$_4$-Pro$_2$-Gly$_3$-Ala$_4$-OH | Gly$^3$ (M) NH | -5.5 | -8.45 |
| N-Ac$_4$-Pro$_2$-Gly$_3$-Ile$_4$-OH | Gly$^3$ (M) NH | -4.8 | -8.35 |
| N-Ac$_4$-Pro$_2$-Gly$_3$-Gly$_4$-OH | Gly$^3$ (M) NH | -6.1 | -8.31 |
| N-Ac$_4$-Pro$_2$-Gly$_3$-Phe$_4$-OH | Gly$^3$ (M) NH | -3.9 | -7.96 |
| N-Ac$_4$-Pro$_2$-Gly$_3$-NH$_4$CH$_3$ | Gly$^3$ (M) NH | -5.4 | -8.37 |
|                      | Gly$^3$ (m) NH | -6.0 | -8.13 |
|                      | Gly$^3$ (M) NH | -4.4 | -7.88 |
|                      | Gly$^3$ (M) NH | -4.7 | -8.40 |
|                      | Gly$^3$ (m) NH | -6.4 | -8.31 |
|                      | Gly$^3$ (M) NH | -4.2 | -8.12 |
|                      | Gly$^3$ (M) NH | -4.7 | -8.33 |
|                      | Gly$^3$ (m) NH | -6.3 | -8.33 |
|                      | Gly$^3$ (M) NH | -4.6 | -8.08 |
|                      | Gly$^3$ (M) NH | -5.6 | -8.25 |
|                      | Gly$^3$ (m) NH | -3.0 | -7.87 |
|                      | Gly$^3$ (M) NH | -3.3 | -7.89 |

*To be multiplied by $10^{-3}$ to get coefficients in ppm/deg and by 2.7 for Hz/10°C at 270 MHz.

Values for some of the Gly$^3$ (m) NH were not possible to obtain due to their overlapping resonances with the major Gly$^3$ (M) NH signal. In all the five cases, the $X^4$ (M) NH resonance is significantly upfield-shifted compared to the $X^4$ (m) NH signal. This has been suggested as arising from the magnetic anisotropy of the carbonyl moiety of the 2nd residue when the peptide segment is in the β-turn conformation (Ref. 4).

Data taken from Stimson et al. (Ref. 4).

Recently, we demonstrated the presence of the 4 → 1 hydrogen-bonded type II β-turn conformation in one of the tripeptides, namely, NAc-Pro-Gly-Phe-OH, by x-ray crystallography.

**Extent of β-Turn Formation**

The CD, ir, and nmr studies described above, when taken together, point out the presence of a 4 → 1 hydrogen-bonded β-turn conformation in solution of the tripeptides. The proportion of this conformation (in equilibrium with non-hydrogen-bonded, disordered structures) is seen to depend on the nature of the side chain of the C-terminal residue in the sequence. Parallel observations can be made in globular proteins, where, as shown by us elsewhere, the β-turn is a highly favorable conformation for Pro-Gly-containing tetrapeptide segments and the extent of β-turn is
governed by the C-terminal residue. In order to obtain quantitative information on the latter observation, we have attempted to estimate the mole fraction of $\beta$-turn conformation for the individual tripeptides used in this study from the CD and nmr data by making certain simplifying assumptions.

In the case of the CD spectral data, this is done by making use of the ratio $R = |\theta_{201}/\theta_{225}|$, a parameter proportional to the extent of $\beta$-turn conformation in these peptides. The values of $R$ have earlier been shown by us to be approximately -12 for 100% $\beta$-turn conformation. A similar value is also obtained for the CD spectra recorded for Boc-Val-Pro-Gly-Gly-OMe, which was also shown by the nmr temperature-coefficient method to have 97% $\beta$-turn conformation. These values are in excellent agreement with the theoretically calculated $R$ value for the type I $\beta$-turn. As mentioned earlier, the Gly-Ala-, and Ile-tripeptides do not exhibit any ordered structure in water and have an average value for $R = +4$. This value can thus be considered to be a good approximation for characterizing the disordered (i.e., 0% $\beta$-turn) conformation ($R_c$) while the value of $R = -12$ represents the 100% $\beta$-turn conformation ($R$) in these peptides. With these approximations, we have evaluated the mole fraction of the $\beta$-turn conformation in the individual tripeptides in TFE at 25°C as follows:

$$X_{CD} = (R_{obs} - R_c)/(R - R_c)$$

The results are presented in Table III. The assumptions involved in the calculation of the $X_{CD}$ values, as well as the fact that the CD spectrum of the $\beta$-turn is concentration-dependent (see Figs. 2 and 3 of paper I), would tend to make the $X_{CD}$ data quantitatively less useful. However, the data in Table III do indicate that the side chains of the 4th residue follow the order Leu > Ala > Ile, Gly > Phe in stabilizing the $\beta$-turn conformation.

A similar estimate can also be obtained from the $^1$H-nmr data on the variation of the chemical shift (from 0.17 to 0.48) at the $X$-residue NH protons with the solvent (Table I). The order in which the $\beta$-turn conformation is stabilized is found to be Leu > Ala > Ile, Gly > Phe. We have not attempted to calculate quantitatively the mole fraction of the peptides in $\beta$-turn in solution from the data in Table I, since, as pointed out by other workers, a significant effect on NH solvent shift can arise from the interaction of the solvent with the carbonyl group of the same peptide moiety.

| Tripeptide | $R = |\theta_{201}/\theta_{225}|$ | $X_{CD}$ |
|------------|---------------------------------|---------|
| Leu        | -2.9                            | 0.43    |
| Ala        | -0.7                            | 0.29    |
| Ile        | +0.1                            | 0.24    |
| Gly        | +0.1                            | 0.24    |
| Phe        | +0.6                            | 0.21    |
A quantitative analysis can, on the other hand, be made of the data on the temperature dependence of the chemical shift presented in Table II. Following the arguments advanced by Urry and Long\textsuperscript{13} and Stimson et al.\textsuperscript{4}, we may assume average temperature coefficients of $-2 \times 10^{-3}$ and $-6 \times 10^{-3}$ ppm/deg, respectively, for the hydrogen-bonded and non-hydrogen-bonded NH protons. The amount of hydrogen-bonded character can be determined from the observed temperature coefficient by a linear interpolation between these values. Using the $d\delta/dT$ values for the $X^4 (M) NH$ proton listed in Table II we have calculated $X_{\text{nmr}}$, the mole fraction of $\beta$-turn conformation assuming that the conformation is unaffected in each of the tripeptides in DMSO-\textit{d$_6$} between 20 and 70°C. The $X_{\text{nmr}}$ values were obtained by first calculating the mole fraction of solvent-shielded protons, $X_S$, for each tripeptide and multiplying this by the mole fraction of the \textit{trans} isomer present $X_T$ (obtained from the area ratios of the major and minor peaks). Table IV lists the values of $X_S$, $X_T$, and $X_{\text{nmr}}$ for the tripeptides, along with corresponding data on \textit{N}-acetyl-Pro-Gly-NHCH$_3$\textsuperscript{4}.

It should be pointed out that the \textit{cis}/\textit{trans} ratio in the peptides is likely to be affected by temperature and thus cause some uncertainty in the $X_{\text{nmr}}$ values [although, as mentioned earlier, the $X^4 (m) NH$ proton resonances show high-temperature coefficients, indicating little solvent shielding of these protons in the \textit{cis} conformation]. This and the inherent experimental error in the determination of the temperature coefficients themselves (estimated to be about $\pm 0.1 \times 10^{-3}$ ppm/deg) will make the calculated $X_{\text{nmr}}$ values less useful for a quantitative analysis. We may, however, utilize them for obtaining a semiquantitative indication of the extent of $\beta$-turn conformation in the tripeptides and compare this with the information obtained from the CD data. [The difference in the peptide concentration used for the CD (0.2 mg/mL) and nmr (3–10 mg/mL) studies is assumed not to affect the relative order $\beta$-turn stability obtained from either of these studies.]

It is interesting to observe that the trends obtained from the $X_{\text{nmr}}$ and

\begin{table}[h]
\centering
\caption{Calculated Mole Fractions of $\beta$-Turn for \textit{N}-Ac-Pro-Gly-\textit{X}-OH Based on Temperature-Dependence Chemical Shift Studies in DMSO-\textit{d$_6$}}
\begin{tabular}{lllccc}
\hline
Peptide & $d\delta/dT \times 10^3$ & Mole Fraction* of & $\textit{trans}$ Isomer & Shielding & $\beta$-Turn \\
Residue (\textit{X}) & (ppm/deg) & & $(X_T)$ & $(X_S)$ & $(X_{\text{nmr}})$ \\
\hline
Leu\textsuperscript{4} (M) NH & $-3.3$ & & 0.76 & 0.68 & 0.50 \\
Ala\textsuperscript{4} (M) NH & $-3.9$ & & 0.68 & 0.53 & 0.36 \\
Ile\textsuperscript{4} (M) NH & $-4.4$ & & 0.70 & 0.45 & 0.32 \\
Gly\textsuperscript{4} (M) NH & $-4.2$ & & 0.69 & 0.40 & 0.28 \\
Phe\textsuperscript{4} (M) NH & $-4.6$ & & 0.68 & 0.35 & 0.24 \\
NHCH\textsubscript{3} (M)\textsuperscript{b} & $-3.3$ & & 0.78 & 0.68 & 0.53 \\
\hline
\end{tabular}
\footnotesize{* Calculated at 20°C. \\
\textsuperscript{b} Data taken from Stimson et al. (Ref. 4).}
\end{table}
$X_{CD}$ data are quite comparable and show the same order of $\beta$-turn stability in the tripeptides, viz., Leu $>$ Ala $>$ Ile, Gly $>$ Phe. The data on the tripeptides thus imply the involvement of the nonbonded interaction on the stability of the $\beta$-turn conformation. A clearer understanding of these observations must await theoretical studies taking into account such long-range interactions. What is significant, however, is the fact that in addition to the conformational preferences of the residues in the 2nd and 3rd positions of the $\beta$-turn (Pro and Gly, respectively, in our studies), our data bring out the importance of the 4th residue in influencing the stability of the $\beta$-turn conformation. This receives support from the analysis of the available data on $\beta$-turns in globular proteins. We have postulated elsewhere that the $\beta$-turn conformation at the Pro-Gly segments in nascent procollagen is the site of enzymatic hydroxylation of the proline residues and that the extent of the hydroxylation is dependent on the contribution of the residues adjoining the Pro-Gly segment to the stability of the $\beta$-turn conformation. The results presented here demonstrate the possibility of the $\beta$-turn conformation in simple tripeptides containing the sequences found in procollagen and may lead to a possible correlation between the conformation of the individual segments in procollagen and the extent of proline hydroxylation.

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PROLINE-CONTAINING $\beta$-TURNS


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