Conformational and Ion Binding Studies of a Cyclic Pentapeptide. Evidence for β and γ Turns in Solution[1]

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Abstract: The cyclic pentapeptide, *cyclo*-(glycyl-L-prolylglycyl-D-alanyl-L-prolyl) [*cyclo*-(Gly$_1$-Pro-Gly$_2$-D-Ala-Pro)], has been synthesized and studied by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) and circular dichroism (CD). We find that the predominant conformation in chloroform, acetonitrile, dimethyl sulfoxide, and water contains all trans peptide bonds and displays *two* N-H's (those of Gly-1 and D-Ala) which by the usual criteria (solvent, concentration, and temperature independence of ^{1}H chemical shifts) do not participate in any intermolecular interactions. Infrared spectra (IR) in chloroform support the interpretation that the conformation contains N-H's which are intramolecularly hydrogen bonded. ^{13}C NMR chemical shifts and CD spectra suggest strongly that one of the hydrogen bonds is in a γ turn involving one of the Pro residues. A model containing one β turn (1←4 hydrogen bond) and one γ turn (1←3 hydrogen bond) is proposed for the solution conformation based on all of the spectral data. Dr. I. L. Karle has completed a crystal-structure determination, reported in an accompanying paper, on *cyclo*-(Gly-Pro-Gly-D-Ala-Pro), which reveals that the same β,γ-turn conformer is present in the solid state. Ion binding studies with *cyclo*-(Gly-Pro-Gly-D-Ala-Pro) establish a new class of synthetic cyclic peptide complexing agents, cyclic pentapeptides. The peptide has been found to bind the divalent cations Mg^{2+}, Mn^{2+}, Ca^{2+}, and Ba^{2+}, and the alkali metal Li^+, strongly. Titration curves from CD data demonstrate at least two complexes of probable stoichiometry 1 peptide:1 cation (PC) and 1 peptide:2 cations (PC$_2$). NMR data suggest that the cyclic pentapeptide adopts a conformation with one cis X-Pro bond in a PC complex, and that this complex may be in equilibrium with an all-trans PC species. A model for the specific mechanism of binding is discussed.

Cyclic peptides are well-established models for conformational features which are important in protein structure.[2-9] Recent investigations of the details of protein tertiary structure[10-12] and of the preferred conformations of cyclic[2-9] and linear[13-15] peptides, both natural and synthetic, have revealed the prevalence and importance of hydrogen-bonding arrangements in which the peptide chain reverses direction. Two particular schemes of hydrogen bonding have been identified frequently: a 1←4 (C=O···H–N) type, termed a β turn;[16,17] and a 1←3 type, termed a γ turn.[2,3,11,18] Although both types of so-called "reverse turns" have been observed in model compounds (for example, β turns in *cyclo*-(X-L-Pro-Y)$_2$ hexapeptides[17] and γ turns in *cyclo*-(L-Pro-Gly)$_3$),[2] it was our intention to create a situation wherein the two turns could be compared within a single compound.[19] Of particular interest are the spectral parameters, particularly nuclear magnetic resonance (NMR), circular dichroism (CD), and infrared (IR) (e.g., sensitivity of N–*H* chemical shifts to temperature, solvent, and concentration perturbations; CD characteristics; ^{13}C chemical shifts of proline ring carbons; etc.) associated with each specific structural feature.

We report the synthesis and conformational analysis of a cyclic pentapeptide which was designed so that a likely conformation would contain both a β turn and a γ turn, and hence two different types of transannular hydrogen bonds. The formation of a β turn was made probable by the use of the sequence: Gly-L-Pro-Gly-D-Ala. Previous studies suggest strongly that the Pro-Gly unit can be readily accommodated in a type II β turn, which in the above sequence would then lead to a 1←4 hydrogen bond from the Gly preceding Pro to the D-Ala. The completion of the pentapeptide cycle with a proline residue introduces no other amide proton, and from inspection of molecular models seems to direct formation of a 1←3 hydrogen bond from D-Ala to Gly-1. Therefore, the compound chosen for study was *cyclo*-(Gly-Pro-Gly-D-Ala-Pro) (see diagram).

Data from ^{1}H and ^{13}C NMR, from solution IR, and from circular dichroism are used in this study in the development of a consistent model for the solution conformation of the cyclic pentapeptide. In an accompanying paper,[20] the crystal structure of *cyclo*-(Gly-Pro-Gly-D-Ala-Pro) is reported. Since the
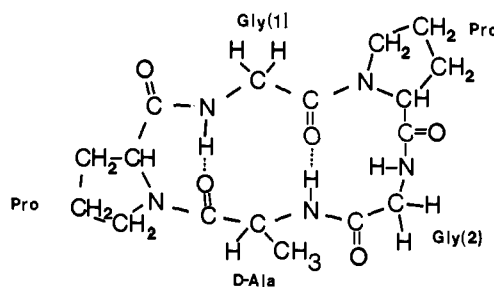
present peptide is expected to be stabilized by strong intramolecular interactions, we anticipated that a significant correlation would exist between the solution and crystal conformations. Such a correlation allows meaningful comparisons to be drawn between the details of the crystal structure and the observed spectral parameters in solution. Furthermore, any differences between the two states of the peptide can be discussed in terms of the influences of intermolecular interactions (with solvent or with other peptides) on the conformational energetics.

In addition, in light of recent work on other cyclic peptides of various sizes and sequences, we then focus attention on the capability of the present cyclic pentapeptide to complex cations. Previous studies have established the potential for binding alkali metal and alkaline earth cations of several types of cyclic peptide: those in the (Pro-Gly)$_n$ series,[2,3] those of (X-Pro-Y)$_2$ sequence,[21] and those that are analogues of valinomycin, such as *cyclo*-(D-Val-L-Pro-L-Val-D-Pro)$_3$.[22-24] The present study constitutes the first investigation of ion binding by a homodetic cyclic pentapeptide. The details of the molecular interactions important in ion binding in complex biomolecular systems can be probed in the relatively simple model compounds. Studies of synthetic peptide complexing agents can potentially provide insight into mechanisms which may occur in membrane transport and other biological events associated with ion binding.

Experimental Section

Synthesis. *cyclo*-(Gly-Pro-Gly-D-Ala-Pro) was synthesized via cyclization of the linear pentapeptide glycylprolylglycyl-D-alanyl-
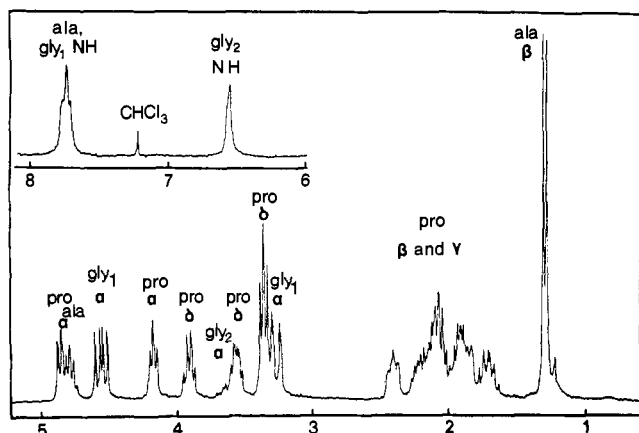
Figure 1. The ^{1}H NMR spectrum (270 MHz) of *cyclo*-(Gly-Pro-Gly-d_2-D-Ala-Pro) in CDCl$_3$. Chemical shifts are given downfield from internal tetramethylsilane (Me$_4$Si): peptide concentration, 27 mg/mL; temperature, 47 °C.

prolyl *p*-nitrophenyl ester hydrochloride (HCl·Gly-Pro-Gly-D-Ala-Pro-ONp) in a dilute solution of pyridine at elevated temperature. The cyclic pentapeptide was synthesized with the Gly-2 residue deuterated at the C$_\alpha$H$_2$ (78%) as well as in the undeuterated form. The deuterated analogue allowed the two glycine residues to be distinguished in NMR spectra. The couplings of precursor amino acids were done by the mixed anhydride method; amino protection was accomplished with the *tert*-butyloxycarbonyl (*t*-Boc) group, and carboxyl protection was accomplished by the use of benzyl esters (OBz). The purity of di- and tripeptide precursors was checked by thin-layer chromatography (TLC), nuclear magnetic resonance, and infrared spectroscopy. Detailed procedures commencing with the synthesis of the linear pentapeptide are described below.

t-Boc-Gly-Pro-Gly-D-Ala-Pro-OBz. A solution of 4.3 g (13 mmol) of *t*-Boc-Gly-Pro-Gly-OH in a minimum amount of chloroform was cooled to −20 °C in a dry ice/CCl$_4$ bath and treated with 1.45 mL (1 equiv) of *N*-methylmorpholine and 1.88 mL (1.1 equiv) of isobutyl chloroformate to form the mixed anhydride. The solution became cloudy, and after 30 min 4.05 g (13 mmol) of HCl·D-Ala-Pro-OBz and another equivalent of *N*-methylmorpholine were added. One hour of stirring at −20 °C was followed by a gradual increase to room temperature and stirring overnight. Successive extractions with 100 mL of 0.2 N HCl (2×), 3% NaHCO$_3$ (2×), and H$_2$O (2×), drying over anhydrous Na$_2$SO$_4$, and removal of solvent yielded the linear pentapeptide as a white foam (7.0 g, ca. 90% yield). Crystallization attempts were unsuccessful; TLC in 10% methanol/90% chloroform indicated a major product (>90%) with an R_f of 0.56. ^{13}C NMR gave positive identification of the *t*-Boc pentapeptide benzyl ester, and the material was used in the next step without further purification.

t-Boc-Gly-Pro-Gly-D-Ala-Pro-OH. The *t*-Boc pentapeptide benzyl ester (7.0 g) was dissolved in *tert*-butyl alcohol (100 mL) and treated with a catalytic amount of palladium on carbon. The mixture was hydrogenated overnight at 40 psi, filtered through a pad of Celite to remove catalyst, and dried to give 6.7 g of a white foam (96%), which showed no evidence of residual benzyl ester by TLC.

t-Boc-Gly-Pro-Gly-D-Ala-Pro-ONp. A solution of 5.5 g of the *t*-Boc-pentapeptide acid (11 mmol) and 1.5 g of *p*-nitrophenol (1 equiv) in 100 mL of chloroform was cooled to −20 °C in dry ice/CCl$_4$ and treated with a solution of 2.2 g (11 mmol) of dicyclohexylcarbodiimide in 20 mL of CHCl$_3$. Stirring was continued at −20 °C for 30 min, overnight at 4 °C, and 1 h at room temperature. The solution was filtered to remove dicyclohexylurea (DCU), treated with 3 drops of acetic acid, and stirred at room temperature for 30 min, at which point an additional amount of DCU was filtered. To ensure complete removal of DCU, the chloroform was evaporated, and the reaction mixture was dissolved in acetone. Two crops of DCU were collected from the acetone solution. The product was a pale yellow foam >90% pure by TLC. Attempts at crystallization were unsuccessful; yield, 6.2 g, 67%.

HCl·Gly-Pro-Gly-D-Ala-Pro-ONp. The *t*-Boc-pentapeptide active ester (6.0 g, 9.9 mmol) was dissolved in 20 mL of chloroform and 150 mL of ether (dried over alumina). The solution was cooled in an ice bath, and dry HCl gas was bubbled through for 30 min. The solution

was then stirred for an additional 30 min at 0 °C, after which the solvents were removed, and the oily product was triturated with ether to yield a white powdery solid. TLC of the triturant and the solid product showed the trituration removed some unreacted starting material, and that the solid was pure hydrochloride salt; yield, 5.5 g, 95%.

cyclo-(Gly-Pro-Gly-D-Ala-Pro). Cyclization of the pentapeptide *p*-nitrophenylester hydrochloride was accomplished by adding a solution of 5.3 g (9.5 mmol) of the peptide in 100 mL of dimethylformamide (dried over Na$_2$SO$_4$ and neutralized with a few drops of acetic acid) dropwise over a period of 3 h to stirred spectrophotometric grade pyridine (2 L) which was preheated to 50 °C. The reaction was allowed to proceed for 3 days (at 50 °C). Solvents were removed, and two 100-mL aliquots of methanol were added and evaporated to hasten removal of dimethylformamide. The remaining brownish yellow oil was triturated twice with ether and then treated with Rexyn I-300 ion exchange resin in 50% ethanol in water, with stirring, until the color was gone (three treatments of ca. 8 h were required). Removal of the solvents yielded an oil which solidified upon trituration with ether, giving 2.4 g of an off-white solid, which gave one spot on TLC in 10% methanol in chloroform (R_f 0.54). The crude yield was 66%. The cyclic pentapeptide was crystallized from methanol/ether; mp 260–261 °C. High-resolution mass spectrometry gave *m/e* 381.19939; predicted for C$_{17}$H$_{23}$O$_5$N$_5$D$_2$, 381.19812. Anal. (C$_{17}$H$_{25}$N$_5$O$_5$) C, H, N.

Spectroscopy. ^{1}H and ^{13}C NMR. Proton NMR spectra were recorded on either a Bruker HX-270 or a JEOL FX100 instrument, with 5-mm sample tubes, using solutions of concentration 0.03–0.1 M, in deuterated solvents obtained from Norell Chemical Co. All spectra were recorded in the Fourier transform mode. ^{13}C NMR data were obtained on either a JEOL FX60 or FX100, with 10-mm sample tubes. Concentrations for carbon spectra ranged from 0.12 to 0.25 M.

Infrared. Infrared spectra of solutions of the cyclic pentapeptide in chloroform (dried over molecular sieves) were measured through the range of concentrations 0.1–0.0025 M in a NaCl cell (path length 0.1 mm) on a Perkin-Elmer Model 327 double-beam spectrophotometer, with solvent alone in the reference beam. Absorbances at 3440 and 3300 cm^{-1} were determined, and the ratio (A(3440)/ A(3300)) plotted vs. concentration.

Circular Dichroism. CD spectra were obtained for solutions of the peptide in water (distilled, deionized), trifluoroethanol (Eastman Kodak Reagent Grade), and acetonitrile (Spectro-grade, Matheson Coleman and Bell), on a Jasco J-40A automatic recording spectropolarimeter with a Princeton Model 128A lock-in amplifier and Morvue photoelastic modulator power supply. Solutions were 0.005–1.0 mM, and were run in quartz cells of path length 0.1 cm from 250 to 185 nm. Data are reported as mean residue ellipticities (m_θ).

Binding Studies. Cation binding by the cyclic pentapeptide was studied by circular dichroism and by ^{13}C NMR, in both cases using perchlorate salts and acetonitrile as solvent. Quantitative binding constant analysis was performed based on the observed changes in circular dichroism during titrations of a peptide solution with a solution of the same peptide concentration which also contained salt. The actual calculation of binding constants follows the methods described elsewhere.[2,23,25] All salts were dried overnight in vacuo at 110 °C prior to use, and acetonitrile was dried over molecular sieves (Davison, 4 Å), and filtered immediately before solution preparation.

Results

Free Peptide. ^{1}H NMR. Proton NMR spectra of *cyclo*-(Gly-Pro-Gly-D-Ala-Pro) in CD$_3$CN, Me$_2$SO-d_6, and CDCl$_3$ show single resonances for each type of proton present in the peptide, demonstrating that in these solvents *one* conformation exists (on the NMR time scale). In Figure 1 the ^{1}H NMR spectrum (270 MHz) of *cyclo*-(Gly-Pro-Gly-d_2-D-Ala-Pro) in CDCl$_3$ is shown. Assignments were determined by spin decoupling and by comparisons with similar peptides. Resonances of Gly-1 and Gly-2 were distinguished by comparing spectra of the peptide and its deuterated analogue. The vicinal coupling constants between the N-H's and C$_\alpha$-H's ($J_{N\alpha}$) of the cyclic pentapeptide in the three solvents are very similar (within 0.5 Hz). In CDCl$_3$, the values are: D-Ala, $J_{N\alpha} = 8.5$

Hz; Gly-1, $J_{N\alpha}$ = 9.5 and 2.5 Hz; and Gly-2, $J_{N\alpha}$ = 6.0 and 7.0 Hz.

Several experiments were performed in order to determine the accessibility of the peptide N–H's. First, temperature dependences of the N–H resonances in both Me$_2$SO-d_6 (0.07 M) and CDCl$_3$ (0.07 M) solutions were measured. The CDCl$_3$ solution was of a sufficiently high concentration (as judged by a concentration dependence study (see below)) so that some aggregation of peptide was occurring. The temperature dependences reflect the extent of peptide–peptide interaction in CDCl$_3$, rather than the relatively weak peptide–solvent interactions. In both experiments, *two* of the three N–H's (those of the D-Ala and Gly-1) had *small* temperature coefficients (see below), indicating relatively little interaction of these N–H's with solvent in Me$_2$SO-d_6, or, in the case of CDCl$_3$, with other peptide molecules.

	$\Delta\delta/\Delta T$ (ppm/deg) × 10³		
	D-Ala	Gly-1	Gly-2
CDCl$_3$	3.2	1.6	13.9
Me$_2$SO-d_6	0.5	0	4.5

Second, in solutions of varying concentrations in CDCl$_3$ only the resonance of the Gly-2 N–H showed a marked change in its chemical shift. A concentration increase from 0.07 to 0.26 M caused this peak to shift downfield by 1.3 ppm, while the D-Ala and Gly-1 N–H resonances moved downfield by only 0.2 and 0.1 ppm, respectively. These chemical-shift changes also reflect the potential for intermolecular interactions, i.e., accessibility, of the N–H's, as it is expected that higher concentrations in CDCl$_3$ will favor peptide–peptide interactions.

Third, a titration was performed in which the ^{1}H NMR spectrum of a chloroform solution of the cyclic pentapeptide (0.31 M) was examined as acetone was added up to 50% (v/v), while keeping the peptide concentration constant. In this experiment, acetone serves as a hydrogen bond acceptor, and downfield shifts of N–H resonances are expected for those N–H's which are exposed to solvent. Indeed, the Gly-2 N–H resonance shifted downfield by 0.30 ppm, while the peaks for the D-Ala and Gly-1 N–H's moved 0.06 ppm downfield and 0.07 ppm upfield, respectively, between 100% CDCl$_3$ and 50% acetone solutions.

^{13}**C NMR.** In Figure 2 are presented upfield regions of ^{13}C NMR spectra (25.05 MHz) of *cyclo*-(Gly-Pro-Gly-D-Ala-Pro) in CDCl$_3$, CD$_3$CN, Me$_2$SO-d_6, and D$_2$O. Resonances were assigned by examining ^{1}H coupled spectra and by comparison with spectra of related compounds. It is again evident that *one* conformation predominates in all solvents studied. The resonances of the C$_\beta$ and C$_\gamma$ of both prolines occur within the range normally associated with trans X-Pro bonds,[25-26] indicating that the major conformer in all solvents examined contains all trans peptide bonds.

Even though the resonances of both prolines can be associated with a trans X-Pro bond, there are large differences in the peak positions (in particular of the C$_\alpha$ and C$_\beta$) of the two prolines in the sequence. Note especially the *upfield* position of one of the Pro C$_\beta$'s; in CDCl$_3$, 168.04 ppm, as compared to 163.94 for the other C$_\beta$. The usual range for a C$_\beta$ in a trans X-Pro bond is 162.5–164.5 ppm.[26] The signals for the two Pro C$_\alpha$'s are also markedly different: 131.30 and 134.22 ppm. This distinction between the two prolines seems to persist in all solvents examined, with minor variations. For example, in D$_2$O the Pro C$_\beta$ resonances occur at 164.03 and 167.01 ppm, while the C$_\alpha$ signals are observed at 130.76 and 133.05 ppm in this solvent.

In D$_2$O, an additional set of resonances whose intensities are approximately 20% of the major peaks can be seen. Both the fact that these signals are observed separately from those of the major conformer (and hence are due to a conformer which
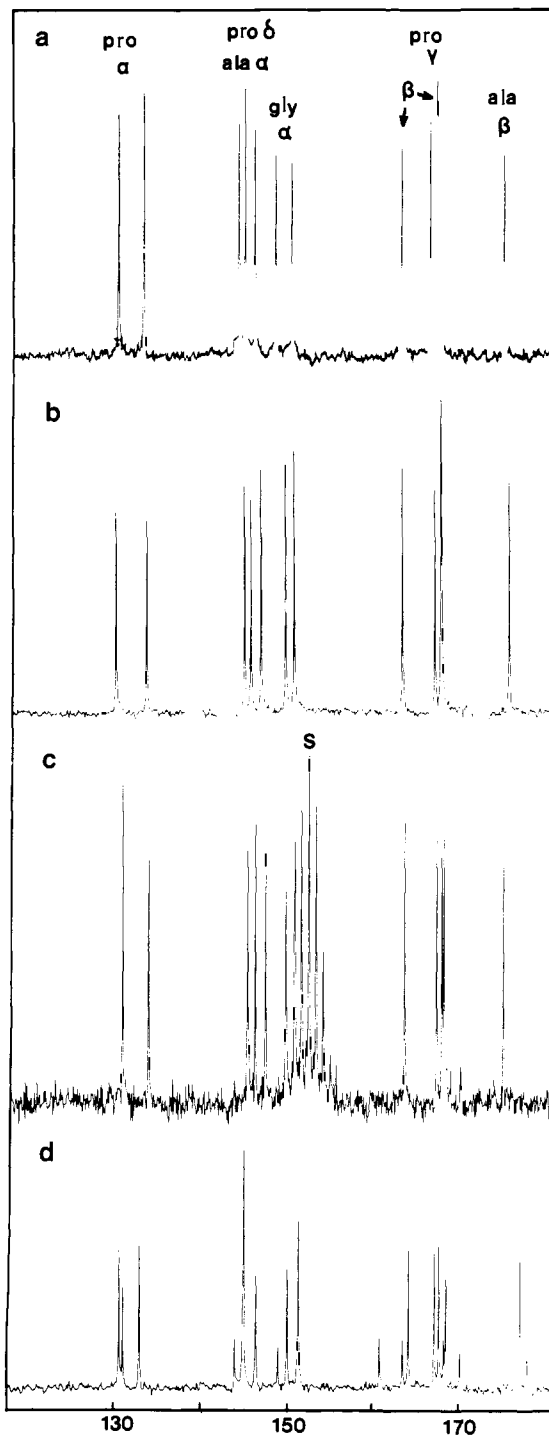

Figure 2. Upfield region of 25.05-MHz ^{13}C NMR spectra of *cyclo*-(Gly-Pro-Gly-D-Ala-Pro) in: (a) CD$_3$Cl, 200 mg/mL; (b) CD$_3$CN, 50 mg/mL; (c) Me$_2$SO-d_6, 27 mg/mL; and (d) D$_2$O, 50 mg/mL: ambient temperature (30 °C); chemical shifts reported are upfield from external ^{13}CS$_2$. On this scale, ^{13}C of Me$_4$Si resonates at 193.7 ppm.

interconverts slowly with it) and the occurrence of one Pro C$_\beta$ and one Pro C$_\gamma$ resonance in the region associated with cis X-Pro bonds lead to the identification of the minor conformer as a cis peptide bond containing species. It is clear from the Pro C$_\beta$ region that one of the X-Pro bonds in the minor conformer is cis, while the other is trans.

Infrared Studies. A plot of the intensity of absorption at 3440 cm^{-1} divided by that at 3300 cm^{-1} (from spectra in dry chloroform) vs. the concentration of peptide was extrapolated to infinite dilution. The value obtained was 0.5, and, although the measured intensity of the bands attributable to hydrogen-
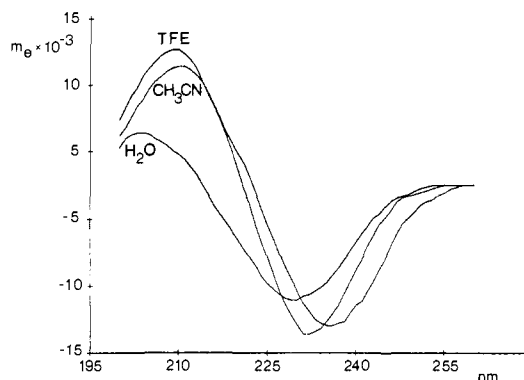
Figure 3. Circular dichroism spectra of *cyclo*-(Gly-Pro-Gly-D-Ala-Pro); peptide concentration, 1.06 mM.
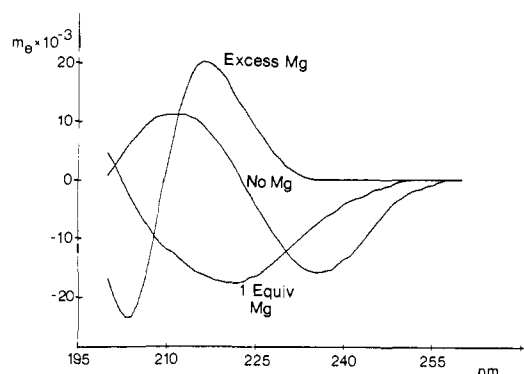

Figure 4. Circular dichroism spectra of *cyclo*-(Gly-Pro-Gly-D-Ala-Pro) in CH_3CN as $Mg(ClO_4)_2$ is added; peptide concentration, 1.06 mM.

Figure 5. Titration curve of *cyclo*-(Gly-Pro-Gly-D-Ala-Pro) in CH_3CN. Δm_θ vs. log [Mg^{2+}]; peptide concentration, 1.06 mM. Wavelengths of measurement are indicated (in nanometers).

Table I. Cation Binding Constants[a]

	Ionic radius, Å	K_1,[b] M^{-1}	K_2,[b] M^{-1}
Li^+ [c]	0.60	3.4×10^2	4.4×10
Mg^{2+} [d]	0.65	1.6×10^4	1.7×10^2
Mn^{2+} [c]	0.80	5.9×10^3	7.5×10

[a]Measured in acetonitrile solution by circular dichroism. Calculated according to ref 24 and 26. Estimated error ±20%. [b] Defined in the text. [c] Peptide concentration = 0.352 mM. [d]Peptide concentration = 1.06 mM.

bonded or non-hydrogen-bonded N–H's cannot be treated quantitatively without considering the absorption probability and line width, the observed ratio qualitatively suggests a high degree of intramolecular hydrogen bonding, particularly in comparison to other model peptides.[14]

Circular Dichroism. CD spectra of the cyclic peptide in acetonitrile, trifluoroethanol, and water all show the same general features (see Figure 3); a large negative band at about 235 nm, and a positive band at ca. 210 nm.

Ion Binding. Circular Dichroism. A qualitative assessment of binding of various cations by the cyclic pentapeptide revealed that several divalent cations were bound (as indicated by perturbations of the free peptide spectrum): Ba^{2+}, Ca^{2+}, Mg^{2+}, and Mn^{2+}, while among the monovalents tested (Na^+, K^+, NH_4^+, Li^+) only one was bound: Li^+. Quantitative binding studies were carried out on Mg^{2+}, Mn^{2+}, and Li^+. As can be seen in Figure 4, marked changes in the CD spectrum take place upon the addition of salt. In the case illustrated (Mg^{2+}), initially the maximum observed for free peptide at 210 nm decreases, until the concentration of cation is approximately the same as that of peptide, at which point the spectrum is characterized by a broad minumum at 220 nm. At cation concentrations significantly above this, the spectrum changes to that associated with saturated salt solutions: a maximum at 218 nm (with no long-wavelength minumum), and a minimum at 200 nm. These spectral changes are similar for each of the cations examined in detail.

Titration data for the three salts in acetonitrile were plotted as Δm_θ (m_θ at a certain cation concentration minus m_θ of the free peptide) at two or more wavelengths against log [cation]. In the curves obtained (see the example in Figure 5) two inflections can be observed, indicating that at least two types of complex are formed as the cation concentration is increased.
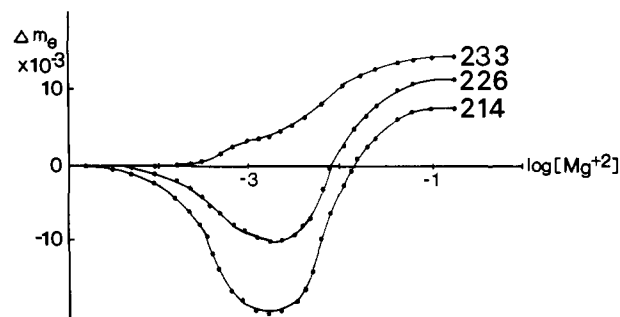
Definition of the particular complexation equilibria which are occurring as salt is added is not possible based on these CD data alone. An indication of the stoichiometry of the first binding step was obtained by plotting Δm_θ as a function of [salt]. The resulting curve was linear up to a [salt]/[peptide] ratio of 1:2, and then leveled off gradually at 1:1. The second inflection occurred at a much higher [salt]/[peptide] ratio. This finding suggests that the first species formed is probably a 1:1, PC complex. At higher cation concentration, the likely subsequent binding reaction would involve addition of one more cation, yielding a PC_2 species. Although the possibility of a P_nC ($n \geq 2$) type of species at low cation concentration cannot be excluded, apparent binding constants were calculated from the titration curves using the equilibria:

$$P + C \rightleftharpoons PC \qquad K = \frac{[PC]}{[P][C]}$$

$$PC + C \rightleftharpoons PC_2 \qquad K = \frac{[PC_2]}{[PC][C]}$$
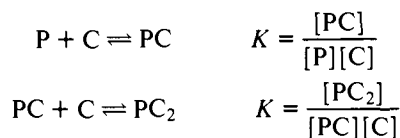
It was assumed that the observed CD curve at any cation concentration was a superposition of the curves for P, PC, and PC_2 species. The Δm_θ value for the PC species was obtained by extrapolation of the early linear portion of the plot of Δm_θ as a function of [salt] to a 1:1 [salt]/[peptide] ratio. The calculated binding constants are given in Table I.

¹³C NMR. A 0.05 M solution of *cyclo*-(Gly-Pro-Gly-D-Ala-Pro) in CD_3CN was titrated with $Mg(ClO_4)_2$ while the upfield region of the ¹³C spectrum was examined. The results are presented in Figure 6, where two main observations can be made. First, as the cation concentration increases, a new set of resonances, separate from the original peaks of the free peptide, appears and grows as cation is added. At concentrations greater than 1.0 equiv, these resonances decrease in size. Two C_β signals are seen for the new conformer, one in the trans region and one in the region associated with cis X-Pro bonds. Since this conformation appears when small amounts of cation are added, and decreases at cation concentrations greater than 1.0 equiv, it is most probably a complex of a 1:1 peptide/cation stoichiometry (PC, vide supra).[27] Furthermore, from the fact
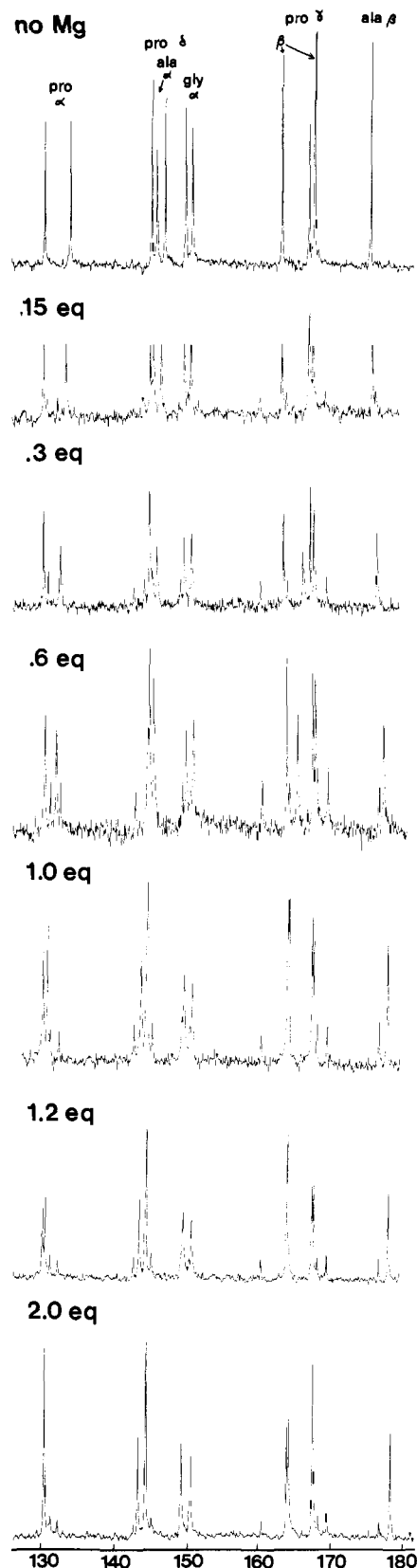
Figure 6. The 25.05-MHz ^{13}C NMR spectra of *cyclo*-(Gly-Pro-Gly-D-Ala-Pro) (0.05 M in CD$_3$CN) at various concentrations of Mg^{2+}. No new resonances are seen at higher salt concentrations; small shifts continue to occur in the major peaks up to ca. 3 equiv.
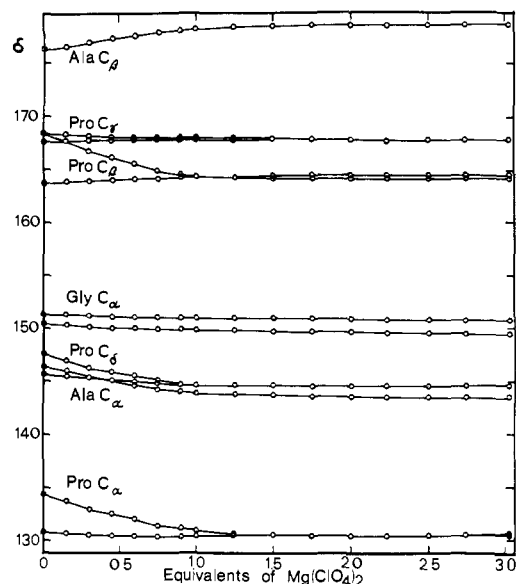

Figure 7. Chemical shifts (upfield from external ^{13}CS$_2$) of major (all-trans) ^{13}C resonances during Mg^{2+} titration (same conditions as Figure 6), plotted as a function of equivalents of added salt. Initial chemical shifts and assignments (confirmed by examining a ^{1}H-coupled spectrum) are: Pro C$_\alpha$, 130.8, 134.4; Pro C$_\delta$, 145.7; Ala C$_\alpha$, 146.4; Pro C$_\delta$, 147.6; Gly-2 C$_\alpha$, 150.4; Gly-1 C$_\alpha$, 151.3; Pro C$_\beta$, 163.8; Pro C$_\gamma$, 167.6, 168.3; Pro C$_\beta$, 168.3; Ala C$_\beta$, 176.2.

as cation is added. Note in particular that the separation of Pro C$_\alpha$ resonances decreases as the concentration of cation increases until they eventually cross, and separate further at higher added [Mg^{2+}]. Other marked changes occur in the Pro C$_\beta$ and C$_\gamma$ region. Most notable is the movement of the upfield C$_\beta$ which parallels that of the higher field C$_\alpha$; viz., as cation is added, its resonance can be seen to shift from under the most upfield peak in this region, to cross the next most upfield peak, and to continue downfield with added cation until it crosses the signal of the other C$_\beta$. Throughout these changes, both the larger C$_\beta$ and C$_\gamma$ signals remain in the region associated with trans X-Pro bonds. In addition, it can be inferred from the fact that shifts, rather than new peaks, are observed, that the complexation at higher cation concentration yields a species which is not separated by a high-energy barrier from the free peptide.

The positions of the major resonances were plotted as a function of the [Mg^{2+}]. The resulting curves (Figure 7) reveal that the largest shifts occur up to 1 equiv of added salt, at which point the resonance positions continue changing only gradually. Beyond 2.0 equiv of salt, very little shifting is seen. Note also that the most marked shifts occur in one set of proline signals and in the alanine signals.

Discussion

Free Peptide Conformation. Results from ^{1}H and ^{13}C NMR, IR, and CD studies of *cyclo*-(Gly-Pro-Gly-D-Ala-Pro) are all consistent with an all-trans conformation of the peptide which contains two intramolecular hydrogen bonds, one in a β turn and one in a γ turn. The N–H's of the Gly preceding Pro and of the D-Ala are *both* sequestered by the usual criteria. In addition, the observations both of an upfield Pro C$_\beta$ resonance and of a long-wavelength minimum in the circular dichroism spectrum are consistent with the presence of a 1←3 hydrogen bond (γ turn) involving one of the prolines.[2,3,25] Model building reveals that such a conformation is sterically allowed for the pentapeptide with the dihedral angles (which are also consis-
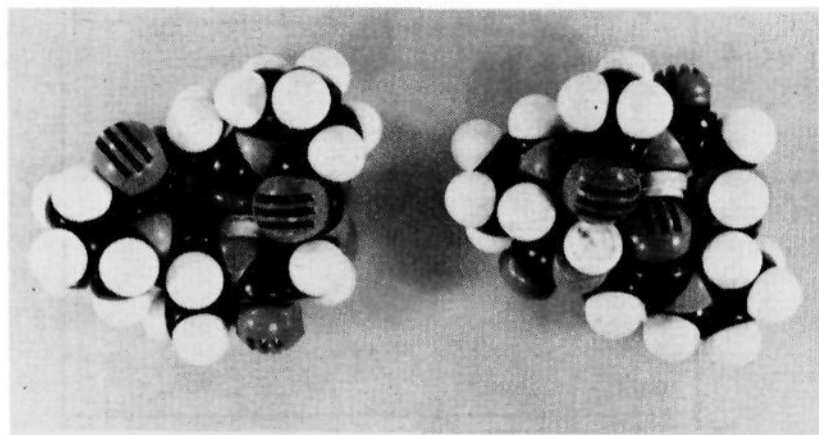
that it is observed separately from free peptide, and has the characteristic Pro C$_\beta$ and C$_\gamma$ signals, it contains one cis peptide bond.

Second, several notable shifts occur in the larger resonances

Figure 8. Photograph of Corey–Pauling–Koltun (CPK) model of proposed solution conformation of free cyclo-(Gly-Pro-Gly-D-Ala-Pro). Note in right-hand view that the γ-turn hydrogen bond is nonlinear.

Table II. Dihedral Angles of cyclo-(Gly$_1$-Pro-Gly$_2$-D-Ala-Pro)

	Gly-1	Pro	Gly-2	D-Ala	Pro
	From NMR, in Solution				
ϕ	120	−60	90	160	−80
ψ	180	120	0	−120	70
	From X-ray, in Crystal[20]				
ϕ	83	−52	74	134	−86
ψ	−134	126	12	−69	70
ω	174	−179	177	178	−160

tent with $J_{N\alpha}$ data) given in Table II.[28,29] The model satisfies the hydrogen-bonding requirement evidenced by the solvent shielding of the Gly-1 and D-Ala N–H's, and provides a consistent interpretation of the proline ^{13}C shifts (with the Pro whose C_β is upfield occurring in the γ turn, with an abnormally low ψ angle of 70°). The model was constructed with all trans, planar peptide bonds (ω = 180°). A Corey–Pauling–Koltun (CPK) model of the above conformation is shown in Figure 8. Note that the 1←3 hydrogen bond is nonlinear, but appears to have a short C=O⋯H—N distance.

The overall conformation of the cyclic pentapeptide resembles strongly a composite of the hydrogen-bonded turns present in cyclic hexapeptides studied previously. For example, the β turn is analogous to those seen in the (X-Pro-Y)$_2$ cyclic hexapeptides.[17] The 1←3 hydrogen bond is very similar to those postulated to occur in cyclo-(Pro-Gly)$_3$[2] and cyclo-(Pro-Gly)$_4$.[3] In the latter compounds, the ^{13}C NMR evidence for the occurrence of a γ turn (high upfield proline C_β) and the circular dichroism minimum at ca. 235 nm were present in nonpolar solvents, and paralleled the results presented here. Actually, the present peptide exhibits a greater upfield shift of the C_β resonance (168.04 ppm in CDCl$_3$ as compared to 166.5 ppm for that of cyclo-(Pro-Gly)$_3$ in the same solvent). Furthermore, the cyclic pentapeptide maintains the same conformation, including the transannular hydrogen bonds, in all solvents examined (chloroform, acetonitrile, dimethyl sulfoxide, trifluoroethanol, and water). This result is in marked contrast to the (Pro-Gly)$_n$ cyclic hexapeptide and octapeptide which both adopted an asymmetric conformation containing cis peptide bonds in polar solvents.

One of the goals of the present study was to examine the spectral parameters of the two different types of intramolecular hydrogen bond which occur in β and γ turns. The characteristic upfield-shifted C_β resonance of a proline in the 2 position of a 1←3 hydrogen bond has been alluded to. Another type of spectral parameter used in the present study to indicate the presence of two intramolecular hydrogen bonds was the dependence of N–H chemical shifts in ^{1}H NMR spectra on perturbations in the environment of the peptide, for example, temperature and peptide concentration. It was found throughout that *both* of the N–H's which were involved in intramolecular hydrogen bonds were relatively insensitive to external perturbations. In addition, the results show a small but significant difference in all experiments between the 1←4 and 1←3 hydrogen-bonded N–H's—in all cases, the Gly-1 N–H, which is involved in the β turn (1←4 hydrogen bond), was *less* sensitive to solvent, temperature, or concentration variations. This observation is consistent with a qualitative assessment from models of the accessibility of the two N–H's to solvent: namely, that the N–H in the γ turn is more exposed (see Figure 8). IR data in CHCl$_3$ were consistent with intramolecular hydrogen bonding, suggesting the possibility that

both the 1←3 and 1←4 hydrogen bonds affect the N–H stretch similarly.

In water solution, the all-trans conformer of cyclo-(Gly-Pro-Gly-D-Ala-Pro) described above exists in equilibrium with a conformation which contains one cis peptide bond. The all-trans conformation is preferred (ratio of trans:cis = 4:1). No further studies have been done on the minor conformer, but model building suggests that for steric reasons the Gly-Pro bond is more likely to isomerize than the D-Ala-Pro bond. The resulting conformation can therefore maintain the 1←3 hydrogen bond but *not* the 1←4 interaction. Further evidence is necessary to substantiate this suggestion.

Comparison of Solution and Crystal Conformations. In an accompanying paper,[20] the results of a crystal-structure determination on cyclo-(Gly-Pro-Gly-D-Ala-Pro) are presented. The conformation observed in the crystal is very similar overall to that which occurs in solution. It is also all-trans, and is stabilized by the same two intramolecular hydrogen bonds. The dihedral angles are given in Table II for purposes of comparison. Those angles which differ from the ones in the proposed solution conformation by ≥20° are in italics. Note that two main differences between the two conformations can be described. First, the crystal structure is less planar than the proposed solution conformation; the Gly-1 and D-Ala ϕ, ψ changes between the two conformations constitute a "puckering" or folding of the cyclic peptide ring in the crystal structure relative to the solution conformation. Second, there is a nonplanar peptide bond between residues 2 and 3 in the γ turn (i.e., the Pro-D-Ala bond) in the crystal. There is no way of establishing such a nonplanarity in the solution structure from available data, but the magnitude of the energy required for such a distortion from planarity (2–3 kcal/mol)[30] led to the proposal in solution of all planar (within 5°) bonds. It would be valuable to seek out a means of demonstrating whether nonplanarity indeed also occurs in the solution conformer.

Other than the distinctions discussed above, the solution and crystal structures of cyclo-(Gly-Pro-Gly-D-Ala-Pro) are closely analogous. The fact that they are so similar suggests strongly that the intramolecular interactions present in this cyclic peptide are energetically very important in stabilizing the observed conformation. This conclusion is also supported by the result that the all-trans β,γ turn conformer is predominant in all solvents examined, regardless of their polarity or capability for hydrogen bonding.

Ion Binding. The results of a qualitative survey of cation binding by cyclo-(Gly-Pro-Gly-D-Ala-Pro) revealed that divalent ions bound better than monovalents, and that among the monovalents the only strong binding was to the smallest cation, Li$^+$. Clearly, a high charge to size ratio favors binding to the pentapeptide.

Detailed CD binding studies were performed with the three cations Mg^{2+}, Mn^{2+}, and Li$^+$. In all three cases, the existence of at least two types of complex was inferred from CD analysis. For the purpose of calculating apparent binding constants, these were tentatively identified as PC and PC$_2$ species, as suggested by the formation of the first maximally at 1:1 [salt]/[peptide], and the second at higher salt concentration.
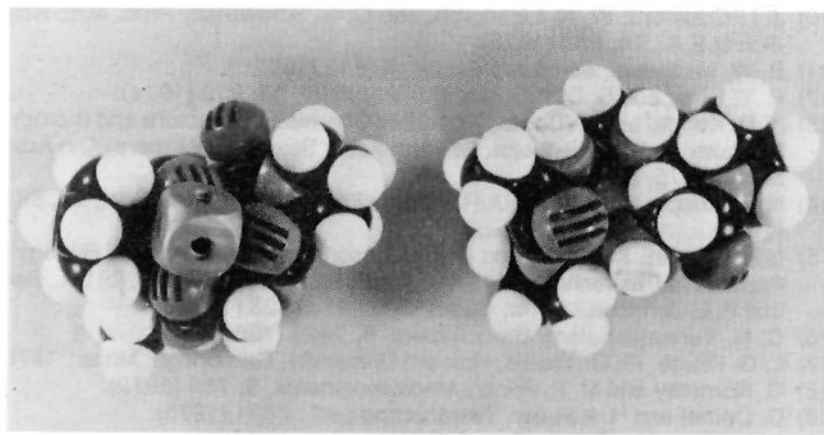
Figure 9. Photograph of CPK model of proposed one-cis 1:1 complex. Note binding site formed from the Pro (two) and Gly-2 carbonyls.
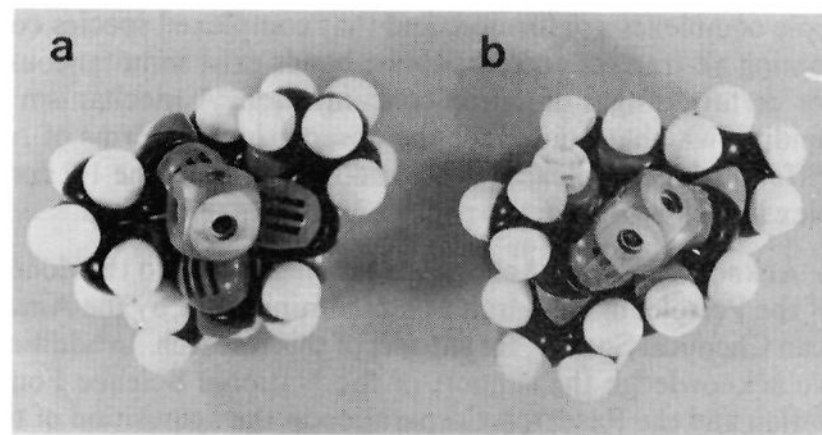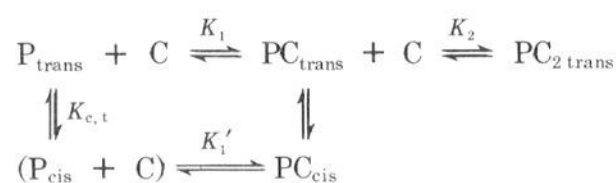

Figure 10. Photograph of CPK model of proposed all-trans conformer involved in complexation. Note two potential binding sites: (a) that which is proposed to bind in both PC and PC_2 species, formed from the two Pro and Gly-2 C=O's, and (b) that which binds the second cation in PC_2, formed from the Gly-1 and D-Ala C=O's.

^{13}C NMR spectra obtained during a titration of the peptide in acetonitrile with Mg^{2+} indicated that *two* processes were occurring at low cation concentrations (up to 1:1): (1) a conformation containing one cis X-Pro bond was becoming populated; and (2) an all-trans species distinct from the free peptide but rapidly interconverting with it (on the NMR time scale) was being formed, as shown by the *shifts* in the major (all-trans) NMR signals. Furthermore, the all-trans complex was deduced to be a 1:1 PC complex from the monotonic shifts of the major resonances up to 1 equiv of added salt. The proportion of peptide, as estimated from NMR signal intensities, which occurs in the one-cis species increases up to about 0.75 equiv of added Mg^{2+}, stays fairly constant up to 1.0 equiv, and then gradually decreases at higher [salt]. These changes are interpreted most readily in terms of a one-cis 1:1 complex, (PC_{cis}), which interconverts slowly with both the free peptide (P) and the all-trans 1:1 complex (PC_{trans}).[27] (Note: the existence of additional species of stoichiometry P_nC where $n = 2$ or greater cannot be definitively ruled out.)

At higher cation concentrations (>1 equiv) the NMR data reveal that small changes continue occurring in the positions of the all-trans signals, while the cis resonances decrease in size. An interpretation consistent with these findings is that an additional cation is bound by PC_{trans} resulting in an all-trans PC_2 complex, but that there is no cis form of this complex formed. High cation concentration would eventually favor the all-trans PC_2 at the expense of PC_{trans} and PC_{cis}, leading to a decreased intensity of cis signals, as observed.

A summary of this scheme for the major equilibria taking place as *cyclo*-(Gly-Pro-Gly-D-Ala-Pro) binds Mg^{2+} is given:

$$P_{trans} + C \overset{K_1}{\rightleftharpoons} PC_{trans} + C \overset{K_2}{\rightleftharpoons} PC_{2\,trans}$$
$$\Big\Vert K_{c,t} \qquad\qquad \Big\Vert$$
$$(P_{cis} + C) \overset{K_1'}{\rightleftharpoons} PC_{cis}$$

where $K_{c,t} = [P_{cis}]/[P_{trans}]$, $K_1 = [PC_{trans}]/[P_{trans}][C]$, $K_1' = [PC_{cis}]/[P_{cis}][C]$, and $K_2 = [PC_2]/[PC_{trans}][C]$. Clearly, $K_{c,t} \ll 1$, as no cis free peptide is observed in acetonitrile. Correlating the above with the observed CD changes as Mg^{2+} is added to peptide leads to the conclusion that the first step seen in the CD titration curve encompasses formation of both PC_{cis} and PC_{trans}. The second step then corresponds to the addition of a cation to PC_{trans} to form PC_2. Hence, the apparent binding constants calculated from CD data (Table I) are composites of K_1, K_1', and K_2 above. They are useful in roughly comparing binding capabilities, since the above scheme cannot be solved rigorously with the available data.

In conformational terms, the proposed description of binding is reasonable. Model building reveals that conformations with

three carbonyls oriented favorably for binding can be found either with all-trans peptide bonds, or with one cis X-Pro bond, namely the Gly-Pro bond. The likely conformers are shown in Figures 9 and 10. In both cases, there are no intramolecular hydrogen bonds. The binding sites appear to be quite similar in these two conformers, and both are formed from the two Pro C=O's and the C=O of Gly-2. However, the change in the orientation of the Gly-1 carbonyl between the cis and trans species alters the configuration of atoms on the *opposite* face markedly. In the trans form (proposed PC_{trans}), there is a possible second binding site composed of the Gly-1 and D-Ala carbonyls (see Figure 10b). Since the Gly-1 C=O is oriented outward (toward solution) in the cis form, no such secondary site exists. These proposals are consistent with the binding of an additional cation to PC_{trans}, but not to PC_{cis}. The second binding would involve interaction of the cation with only two carbonyls. This, in combination with cation–cation repulsion, would lead to a weaker binding, as is observed.

Further studies are in progress to establish the detailed conformations of the bound species. It is of particular interest to define precisely the mode of binding to only *two* carbonyls, as other synthetic and naturally occurring ion-binding peptides have employed at least three carbonyls.[2,3,19,24,31,32] In addition, analogues of this peptide which may bind preferentially in cis or trans complexes are being synthesized.

Conclusions

cyclo-(Gly-Pro-Gly-D-Ala-Pro) was synthesized and its conformation examined in a variety of solvents. In all cases, *one* conformation was predominant, and only in water was a competing conformation (one-cis) observed. The preferred conformation was shown to contain both 1←3 and 1←4 intramolecular hydrogen bonds (in so-called β and γ turns). The peptide provides the first definitive example of a γ turn stable in polar solvents, particularly in water. In addition, it was found that neither the β- nor the γ-turn hydrogen-bonded N–H's interacted with solvent by the usual criteria. This finding, together with the characteristic high-field C_β and long-wavelength CD minimum previously associated with the presence of a 1←3 hydrogen bond, demonstrates that several spectral parameters can be used to signal a γ turn. In addition, solution IR spectra showed that the N–H's in both the 1←3 and 1←4 hydrogen bonds present in the cyclic pentapeptide had frequencies typical of hydrogen-bonded model amides. A comparison of the solution and crystal structures of *cyclo*-(Gly-Pro-Gly-D-Ala-Pro) revealed striking similarities, especially in the presence of the two transannular hydrogen bonds.

cyclo-(Gly-Pro-Gly-D-Ala-Pro) was also found to bind strongly to cations. NMR and CD studies revealed that mul-

tiple complexes are formed, and that complexed species containing all-trans or one-cis peptide bonds exist simultaneously for certain ranges of cation concentration. A mechanism of binding which accounts for the observations in terms of *two* different 1:1 complexes (PC_{cis} and PC_{trans}) and one 1:2 complex (PC_2) with all-trans peptide bonds was proposed.

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Crystal Structure and Conformation of *cyclo*-(Glycylprolylglycyl-D-alanylprolyl) Containing 4→1 and 3→1 Intramolecular Hydrogen Bonds

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Abstract: *cyclo*-(Gly_1-Pro_2-Gly_3-D-Ala_4-Pro_5), a synthetic pentapeptide, contains two transannular C=O···HN bonds; one is the familiar 4→1 (type II) bond encompassing Pro_2-Gly_3 and the other is the recently encountered 3→1 bond encompassing Pro_5. All the peptide units are in the trans conformation with essentially planar amide linkages except for Pro_5 where $\omega_5 = -160°$. Conformational angles for the 4→1 bond are: $\phi_2 = -52°$, $\psi_2 = 126°$, $\phi_3 = 74°$, and $\psi_3 = 12°$. For the 3→1 bond they are $\phi_5 = -86°$ and $\psi_5 = 70°$. The space group is $P2_12_12_1$ with $a = 10.254$ (2) Å, $b = 21.320$ (5) Å, $c = 8.565$ (1) Å, and $Z = 4$. The structure was solved by direct phase determination.

The folds in peptide chains are often stabilized by the formation of intramolecular hydrogen bonds. Cyclic peptides are constrained to contain bends in the backbone and offer good models for studying the various possible types of bends containing intramolecular hydrogen bonds and for establishing the molecular dimensions and conformational angles for such bends. Thus far, 3→1, 4→1 (three types), and 5→1 (two

types) bonds have been observed in crystalline cyclic peptides[1,2] (Figure 1). The 3→1 and 4→1 bonds are also known as γ bends and β bends, respectively.

The present paper concerns the crystal structure and conformation of the cyclopentapeptide *cyclo*-(Gly-Pro-Gly-D-Ala-Pro) synthesized by Pease and Watson.[3] It is the first example of a cyclic peptide containing both a 3→1 and a 4→1