conditions either conformer can be crystallized. The ease with which these conformers can be crystallized suggests that their energy difference and/or barrier to internal rotation are relatively small, and this has been corroborated by the most recent MO energy calculations.[9] That conformation is relevant to function, for thyroid hormones, is accepted, but just *how* is not clearly defined. Structure–functional analysis of thyroid hormones must take into account three important conformational facts: (1) both the distal and proximal orientations of a 3′ substituent are possible in solution, (2) there is a definite pattern in the flexibility of the two phenyl rings observed in the diphenyl ether conformation, and (3) there is a predictable degree of variability in the amino acid conformation.

The conformation observed in this structure agrees with the *in vivo* results which show a need for a distally oriented substituent for activity. The *in vivo* requirements for the amino acid conformation are still uncertain.

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Cyclic Peptides. IX. Conformations of a Synthetic Ion-Binding Cyclic Peptide, *cyclo*-(Pro-Gly)$_3$, from Circular Dichroism and ^{1}H and ^{13}C Nuclear Magnetic Resonance[1]

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Abstract: The solution conformers of a synthetic ion-binding cyclic hexapeptide, *cyclo*-(L-prolyl-glycyl)$_3$ [*cyclo*-Pro-Gly)$_3$], are derived from the complementary information of circular dichroism (CD) spectra, ^{1}H and ^{13}C nuclear magnetic resonance (nmr) spectra, theoretical CD spectra, and computed intramolecular potential energies. In polar solvents, such as water and dimethyl sulfoxide, *cyclo*-(Pro-Gly)$_3$ adopts an asymmetric conformation which contains one cis Gly-Pro peptide bond. In the nonpolar solvents, dioxane and chloroform, the *cyclo*-(Pro-Gly)$_3$ conformer is C_3 symmetric, has all peptide bonds trans, and is stabilized by three 1 ← 3 hydrogen bonds (γ turns). *cyclo*-(Pro-Gly)$_3$–cation complexes form a second class of all-trans C_3-symmetric conformers which do not contain intramolecular hydrogen bonds. Among the alkali metal cations, *cyclo*-(Pro-Gly)$_3$ shows selectivity for Li^+ and Na^+ over K^+ and larger cations. Among the divalent alkaline earths of corresponding ionic radii, *cyclo*-(Pro-Gly)$_3$ selectively binds Mg^{2+} and Ca^{2+} in preference to Ba^{2+}. The measured *cyclo*-(Pro-Gly)$_3$–cation binding constants are comparable to those observed for related, naturally occurring cyclic peptides. For example, the binding constant of *cyclo*-(Pro-Gly)$_3$ for Ca^{2+} in acetonitrile is 1.1×10^5 M^{-1}. Magnesium forms three distinct complexes with *cyclo*-(Pro-Gly)$_3$ with stoichiometries of Mg^{2+}:*cyclo*-(Pro-Gly)$_3$ of 1:2, 1:1, and 2:1.

The effects of amino acid sequence, solvent, and temperature on the stability of peptide structures can be evaluated through the study of synthetic cyclic peptides whose entire conformational space can be explored. A family of cyclic peptides, *cyclo*-(L-prolyl-glycyl)$_n$ (n = 1, 2, 3, . . .), provides excellent models for such studies of conformational determinants. Since members of the *cyclo*-(Pro-Gly)$_n$ family bind cations, they are also valuable in assessing relative binding strengths and selectivities for a series of biologically relevant cations.

Previous ^{1}H and ^{13}C nuclear magnetic resonance (nmr) studies have identified three conformational classes for *cyclo*-(L-prolyl-glycyl)$_3$ [*cyclo*-(Pro-Gly)$_3$].[2,3] Two of these conformers are C_3 symmetric, as evidenced by the magnetic equivalence of the three Pro-Gly units. However, the third conformer is asymmetric and gives separate resonances for each Pro-Gly unit. The deduced symmetries, proton–proton coupling constants, and Corey–Pauling–Koltun (CPK) molecular models were used to make preliminary conformational assignments.[2,3] These earlier studies indicated likely conformational states, but the data were not sufficient to define each of the three conformers in detail.

In a subsequent theoretical study,[4] the intramolecular potential energy (the sum of van der Waals and dipolar interactions) was computed for all possible *cyclo*-(Pro-Gly)$_3$ conformers. Utilizing standard coordinates for the peptide units, this investigation considered only dihedral angle variations. In addition, only trans Pro-Gly peptide bonds were considered, since the trans isomer for secondary peptide groups is at least 2 kcal/mol more stable than the cis isomer.[5] In contrast, both

(1) For paper VIII in this series, see C. M. Deber, E. T. Fossel, and E. R. Blout, *J. Amer. Chem. Soc.*, **96**, 4015 (1974).
(2) C. M. Deber, D. A. Torchia, S. C. K. Wong, and E. R. Blout, *Proc. Nat. Acad. Sci. U. S.*, **69**, 1825 (1972).
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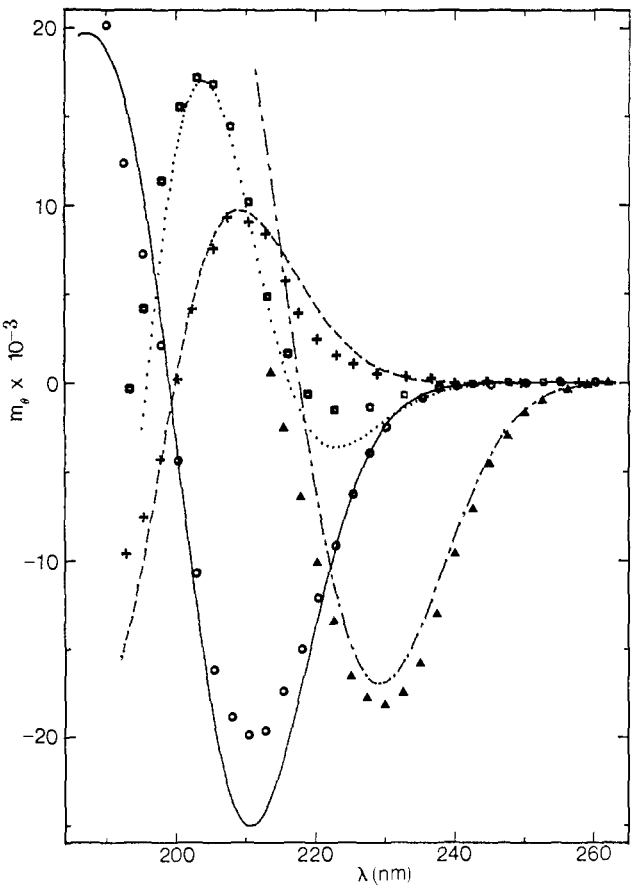

Figure 1. Comparison of theoretical (lines) and experimental (points) CD spectra for various *cyclo*-(Pro-Gly)$_3$ conformers. Peptide concentrations were 0.03–5.00 × 10^{-4} *M*. The dihedral angles of the conformers are given in Table I. For the theoretical spectra: all bandwidths were 14 nm, the $\pi \rightarrow \pi^*$ wavelengths were those calculated for the exciton system (for conformer S_2^* the bands were shifted, see text), and the $n \rightarrow \pi^*$ wavelengths were assumed to be 212, 228, 208, and 215 nm for conformers A, S, S_1^*, and S_2^*, respectively. Experiment: (○○○) in water, (▲▲▲) in dioxane, (□□□) 0.06 *M* $Mg(ClO_4)_2$ in acetonitrile, and (+ + +) 0.5 *M* $Ca(ClO_4)_2$ in water. Theory: (———) conformer A, (— — — —) conformer S, (· · · · · ·) conformer S_2^*, (------) conformer S_1^*.

cis and trans isomers were considered for Gly-Pro peptide bonds, since for tertiary peptide groups the two isomers have comparable stability.[5,6] The complete exploration of conformational space *via* computer unearthed a class of C_3-symmetric conformers which had been overlooked in examinations of molecular models, namely, a class having all peptide bonds trans and three intramolecular hydrogen bonds. While the computed conformational distribution is not quantitatively accurate due to neglect of solvent–peptide interactions and imperfections in the intramolecular potential functions, the computations are a qualitative guide to the relative stability of different conformers and allow the ensuing analysis to focus on the regions near local potential energy minima.

Reversals in direction of peptide chains in globular proteins have been recognized as specific structural features.[7] One member of this class of structures, the β turn (a reversal in chain direction stabilized by a 1 ← 4 hydrogen bond), is also found in a group of synthetic cyclic peptides.[8] However, *cyclo*-(Pro-Gly)$_3$ is unlikely to form β turns due to the steric restrictions of the alternating L-proline residues but may form γ turns (a bend in the peptide chain stabilized by a 1 ← 3 hydrogen bond), another specific conformational feature found in proteins.[7]

In this paper circular dichroism (CD) and ^1H and ^{13}C nmr spectra are employed in conjunction with previous nmr data[2,3] to allow unambiguous identification of all *cyclo*-(Pro-Gly)$_3$ conformers. Once the conformational states are defined, the effects of conformational changes can be correlated with the observed spectral parameters. In addition to conformational and spectral data, the stoichiometries and binding constants of *cyclo*-(Pro-Gly)$_3$–cation complexes are reported and compared to results for related naturally occurring compounds.

Experimental Section

The synthesis and characterization of *cyclo*-(Pro-Gly)$_3$ have been reported.[9] Nmr spectra were obtained on Varian HA-100, XL-100-15, and CFT-20 spectrometers at ambient probe temperature, about 30°, using 0.01–0.04 *M* solutions. CD measurements were performed at 20° on a Cary 60 spectropolarimeter with Model 6001 CD attachment. The mean residue ellipticity was independent of concentration over the range 0.03–5.00 × 10^{-4} *M*. A Cary 15 spectrophotometer was used for absorption spectra. Spectral grade solvents and analytical reagent grade salts were employed.

The stoichiometry of cation–peptide complexes was determined by equilibrating a solution of peptide in an organic solvent (either methylene chloride or a mixture of methylene chloride and acetonitrile) with excess salt and then determining the amount of salt solubilized. The salt and the solvent system were chosen so that the salt was virtually insoluble in the pure solvent. The amount of salt solubilized by complexation with peptide was determined from the radioactivity of the cation or spectrophotometrically from the absorbance of the anion after subtraction of a solvent blank. The concentrations of peptide and anion were double-checked by extraction into water (which at the concentrations employed dissociates the complex); then the anion concentration was determined spectrophotometrically and the peptide concentration polarimetrically.

The extent of ion binding was determined from CD spectra assuming an equilibrium between two states (free and bound peptide). Ion-binding constants were then calculated from the extent of binding and the demonstrated stoichiometries. The extent of binding, $\alpha(c)$, for a given amount, c, of cation added is

$$\alpha(c) = (m_\theta^c - m_\theta^f)/(m_\theta^b - m_\theta^f)$$

where m_θ is the mean residue ellipticity of the peptide, and the superscripts denote experimental conditions: c, a given amount of cation added; b, sufficient cation added to bind all of the peptide; and f, free peptide (no cation).[10]

For the reaction, P + C ⇌ PC (with P denoting peptide and C denoting cation), the binding constant is

$$K = \frac{[PC]}{[P][C]} = \frac{\alpha(c)}{\{1 - \alpha(c)\}\{C_0 - \alpha(c)P_0\}}$$

with C_0 and P_0 denoting total concentrations of cation and peptide, respectively.

The numerical values reported for binding constants and stoichiometries are the means of three to ten measurements. The standard

(6) (a) C. M. Deber, F. A. Bovey, J. P. Carver, and E. R. Blout, *J. Amer. Chem. Soc.*, **92**, 6191 (1970); (b) V. Madison and J. Schellman, *Biopolymers*, **9**, 511 (1970).

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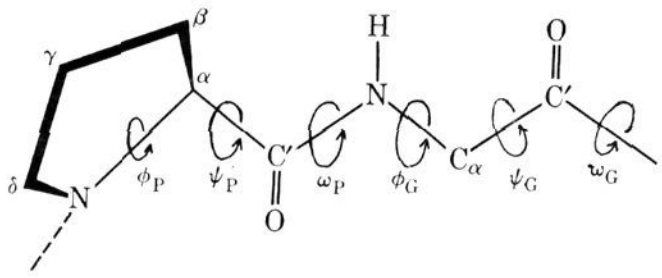
Table I. *cyclo*-(Pro-Gly)$_3$ Conformers

Conformer	Exptl conditions	Symmetry	$(\phi,\psi,\omega)^a$ Pro	$(\phi,\psi,\omega)^a$ Gly	ϕ_{Glv},[b] deg	$J_{N\alpha}$,[c] Hz	TPE,[d] kcal/mol
S	Nonpolar solvents	C_3	(100,250,0)	(350,18,0)	355	3.0, 4.5[e]	−6.3
S_1*	Sufficient concn of cations to form 1:1 complex	C_3	(138,310,0)	(264,352,0)	260	5.0, 5.5[e]	−4.8
S_2*	Sufficient $[Mg^{2+}]$ to bind $2Mg^{2+}$:*cyclo*-(Pro-Gly)$_3$	C_3	(112,330,0)	(249,30,0)	254	5.5, 7.5	−3.0
A	Polar solvents	C_1	(112,330,0) (112,339,0) (112,107,0)	(275,25,0) (225,64,0) (316,335,180)			0.4

[a] These dihedral angles are the best estimate of each conformer considering CD and nmr data in conjunction with theoretical results. As in previous studies on *cyclo*-(Pro-Gly)$_3$, the dihedral angle conventions of 1966 [J. T. Edsall, P. J. Flory, J. C. Kendrew, A. M. Liquori, G. N. Ramachandran, and H. A. Scheraga, *Biopolymers*, **4**, 121 (1966); *J. Biol. Chem.*, **241**, 1004 (1966); *J. Mol. Biol.*, **15**, 399 (1966)] were followed. [b] Calculated from observed $J_{N\alpha}$ using a Karplus-like relationship: M. Karplus, *J. Chem. Phys.*, **30**, 11 (1959). For parameters used in the Karplus equation, see V. F. Bystrov, S. L. Portnova, V. I. Tsetlin, V. T. Ivanov, and Yu. A. Ovchinnikov, *Tetrahedron*, **25**, 493 (1969). [c] Observed coupling constants between the glycine amide proton (N–H) and the two glycine H_α's. [d] Total intramolecular potential energy computed in ref 4. The global minimum in the calculated TPE is −7.5 kcal/mol for a conformer near S with dihedral angles (ϕ,ψ,ω) of (100,260,0) and (337,19,0) for Pro and Gly, respectively. [e] See ref 2.

deviation is approximated by the average of the absolute deviations from the reported mean.

A schematic diagram of the repeating Pro-Gly unit of *cyclo*-(Pro-Gly)$_3$, designating carbon atoms and dihedral angle nomenclature (with subscripts P and G denoting Pro and Gly angles), is given below.



Results

Circular Dichroism Spectra. For each of the *cyclo*-(Pro-Gly)$_3$ conformational classes, theoretical CD spectra computed within the low-energy portion of the conformational distribution[4] were utilized to derive molecular conformers from the experimental CD spectra. The dihedral angles of each derived conformer were adjusted to produce simultaneous agreement between theoretical and experimental CD spectra, between calculated and observed $J_{N\alpha}$ proton–proton coupling constants, and with the molecular symmetry deduced from nmr spectra. In Figure 1 the four types of experimental spectra are compared to theoretical spectra for each of the four conformers. The experimental conditions under which each conformer is observed and the dihedral angles deduced are given in Table I.

The good agreement between the experimental and theoretical CD spectra identifies the solution conformers with four regions centered upon the conformers given in Table I. This identification is unambiguous, since all of conformational space was explored, and each spectral type is predicted only for its cited region. (One minor exception is noted below.) In particular, the CD data indicate that *cyclo*-(Pro-Gly)$_3$ in nonpolar solvents has all peptide bonds trans and three intramolecular hydrogen bonds. The nonlinear 1 ← 3 hydrogen bonds (see Figure 2a) are responsible for the negative n → π^* Cotton effect observed at 230 nm (Figure 1). By contrast, a large positive CD band is predicted in this wavelength region for a class of conformers containing three cis Gly-Pro bonds. Previous nmr data[2,3] were unable to distinguish between the three-cis class and the all-trans conformer S (Table I, Figure 2a), since both are consistent with the $J_{N\alpha}$ proton coupling constants which have been observed.

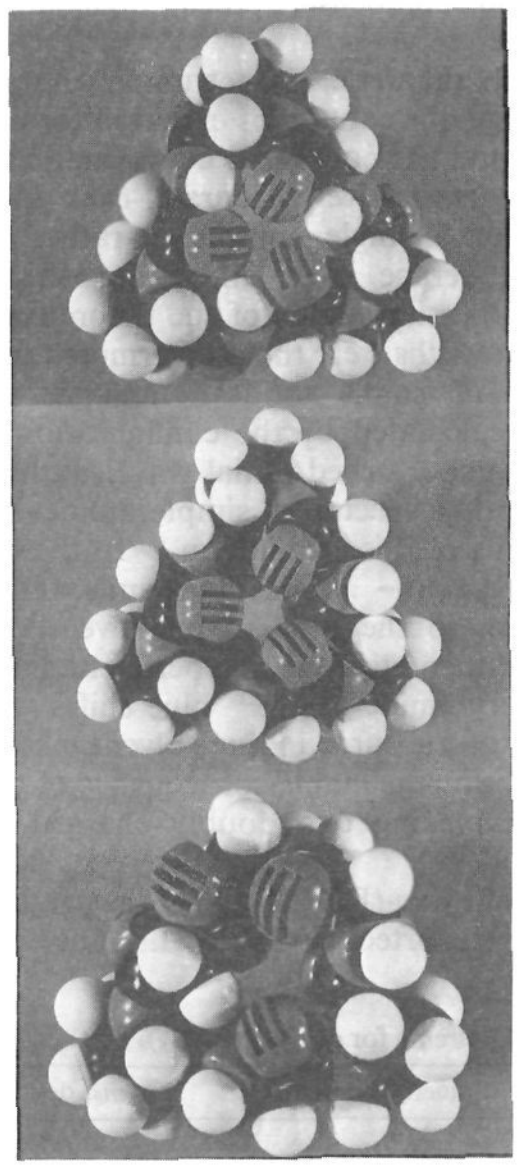


Figure 2. Photographs of CPK models of major conformational types (see Table I): (a, top) conformer S; (b, middle) conformer S*; (c, bottom) conformer A.

Nmr spectra have shown that *cyclo*-(Pro-Gly)$_3$–cation complexes have C_3 symmetry.[2,3] Therefore, regardless of the mode of complexation, the cation will be centered on an optical node (*i.e.*, on one of the planes defining the quadrant symmetry rule for the one-electron mechanism) of each carbonyl group. Thus, the cation does not contribute directly to the optical activity but produces CD changes upon formation of S_1* and S_2* *via* altered peptide conformation. Inspection of the di-

hedral angles in Table I reveals that the two types of cation complexes, conformers S_1* and S_2*, are minor variants within the same conformational class. Yet, the CD spectra (Figure 1) of these two conformers are distinct, which illustrates the sensitivity of the CD spectrum to minor conformational changes.

Theoretical CD spectra from two different conformational regions matched that observed for *cyclo*-(Pro-Gly)$_3$ in polar solvents. The spectral type observed for conformer A was predicted for conformers near the local minimum in intramolecular potential energy for structures with one cis Gly-Pro bond and, in addition, for a few high-energy conformers with two cis Gly-Pro bonds. The two-cis conformers are considered unlikely because of (1) their high intramolecular potential energy [4–8 kcal/mol above the cited one-cis conformer (Table I) and 3–7 kcal/mol above the local minimum for two-cis conformers] and (2) their lack of features which would compensate their high intramolecular potential energy.

Since each theoretical spectrum changes very little over the range of fluctuations expected for each class of conformer, and the computed conformational distribution is not quantitatively accurate, each spectrum was computed for a single conformer rather than for a Boltzmann average over all conformers. For the individual Gaussian bands of the theoretical CD spectra, the bandwidths were those determined from absorption spectra of simple amides,[11] and the wavelengths were those calculated by the configuration interaction theory with unperturbed initial wavelengths also taken from absorption spectra of simple amides. (See ref 4 for the details of this calculation.) In the case of conformer S_2*, the $\pi \rightarrow \pi^*$ excition bands were arbitrarily shifted 3 nm to the blue to improve the agreement between the theoretical and experimental CD spectra. As the tertiary amide $\pi \rightarrow \pi^*$ blue shifts with increasing polarity of the surrounding solvent medium, such a shift might be a reasonable consequence of the carbonyl oxygens complexing two doubly charged magnesium ions.

Extrema of *cyclo*-(Pro-Gly)$_3$ CD spectra in additional solvents are reported in Table II. The spectra from

Table II. CD Extrema for *cyclo*-(Pro-Gly)$_3$

Solvent	m_θ, deg	λ, nm
Water	−20,000	210
Methanol	−18,500	210
Dioxane	−18,300	230
Methylene chloride	−20,000	230
Acetonitrile	−12,000	212
	−8,600	225[a]
0.02 *M* calcium perchlorate in acetonitrile	11,000	208
0.06 *M* magnesium perchlorate in acetonitrile	18,000	202

[a] Shoulder.

which these extrema are abstracted show that (1) conformer A predominates in methanol as well as in water, (2) conformer S predominates in methylene chloride (or chloroform) as well as in dioxane, (3) the CD of conformer S_1* (complex with calcium) is the same in acetonitrile as in water but is distinct from the CD of S_2* (complex with two magnesium ions) in acetonitrile, and (4) *cyclo*-(Pro-Gly)$_3$ in acetonitrile is a 40:60 mixture of conformer S:conformer A.

Cation Binding Constants. For three group I cations and three group II cations of comparable radii, binding constants (Tables III and IV) were determined from CD spectra as described in the Experimental Section. For the cations and solvents given in Table III, the entire binding curve was determined by stepwise additions of salt solutions to a *cyclo*-(Pro-Gly)$_3$ solution. For Na^+ and Ca^{2+} the binding curves indicate a 1:1 stoichiometry. For Mg^{2+} in acetonitrile two separate steps are apparent in the binding curve (peptide concentration $\sim 3 \times 10^{-6}$ *M*). The experimental binding curve is well reproduced by assuming that the first step is

$$cyclo\text{-(Pro-Gly)}_3 + Mg^{2+} \rightleftharpoons cyclo\text{-(Pro-Gly)}_3\text{-}Mg^{2+}$$

and the second step is

$$cyclo\text{-(Pro-Gly)}_3\text{-}Mg^{2+} + Mg^{2+} \rightleftharpoons cyclo\text{-(Pro-Gly)}_3\text{-}Mg_2^{4+}$$

with equilibrium constants K_1 and K_2, respectively. In acetonitrile at higher peptide concentration ($\sim 1 \times 10^{-3}$ *M*), the Mg^{2+} binding curve has three distinct steps. The initial step is due to the formation of a complex of one Mg^{2+} ion and two *cyclo*-(Pro-Gly)$_3$ molecules. (This complex is also inferred from nmr spectra; see following section.) The succeeding two steps are due to the formation of Mg^{2+}:*cyclo*-(Pro-Gly)$_3$ complexes with stoichiometries of 1:1 and 2:1. Binding constants for the latter two steps are reported (Table III).

Table III. Binding Constants of Cations to *cyclo*-(Pro-Gly)$_3$

Cation[a]	Solvent	K, M^{-1}
Na^+	Water	2.2 ± 0.4
Ca^{2+}	Water	$1.3 \pm 0.1 \times 10^2$
Ca^{2+}	Acetonitrile	$1.1 \pm 0.4 \times 10^5$
Mg^{2+}	Acetonitrile	$1.0 \pm 0.6 \times 10^5$ (K_1)
		$6.4 \pm 1.8 \times 10^2$ (K_2)

[a] The anion was perchlorate.

Table IV. Cation Binding Constants for *cyclo*-(Pro-Gly)$_3$ in 80% MeOH/20% Water

Ion[a]	m_θ deg (4.00×10^{-3} *M* salt)	m_θ (sat.), deg	K, M^{-1} [b]
None	−20,000		
Li^+	−7,400	9,500	1.8×10^2
Na^+	−11,500	7,100	1.1×10^2
K^+	−17,000	*c*	2.9×10^1
Ca^{2+}	+7,100	12,000	1.4×10^3
Ba^{2+}	−1,850	8,700	4.2×10^2

[a] Anion was perchlorate. [*cyclo*-(Pro-Gly)$_3$] = 4.50×10^{-5} *M*. [b] Estimated error in the binding constant is ±20%. [c] K^+ salts were not sufficiently soluble to achieve saturation. The average of the ellipticities observed for Li^+ and Na^+ was used to calculate the binding constant.

Table III and IV) were determined from CD spectra as described in the Experimental Section. For the cations and solvents given in Table III, the entire binding curve was determined by stepwise additions of salt solutions to a *cyclo*-(Pro-Gly)$_3$ solution. For Na^+ and Ca^{2+} the binding curves indicate a 1:1 stoichiometry. For Mg^{2+} in acetonitrile two separate steps are apparent in the binding curve (peptide concentration $\sim 3 \times 10^{-6}$ *M*). The experimental binding curve is well reproduced by assuming that the first step is

$$cyclo\text{-(Pro-Gly)}_3 + Mg^{2+} \rightleftharpoons cyclo\text{-(Pro-Gly)}_3\text{-}Mg^{2+}$$

and the second step is

$$cyclo\text{-(Pro-Gly)}_3\text{-}Mg^{2+} + Mg^{2+} \rightleftharpoons cyclo\text{-(Pro-Gly)}_3\text{-}Mg_2^{4+}$$

with equilibrium constants K_1 and K_2, respectively. In acetonitrile at higher peptide concentration ($\sim 1 \times 10^{-3}$ *M*), the Mg^{2+} binding curve has three distinct steps. The initial step is due to the formation of a complex of one Mg^{2+} ion and two *cyclo*-(Pro-Gly)$_3$ molecules. (This complex is also inferred from nmr spectra; see following section.) The succeeding two steps are due to the formation of Mg^{2+}:*cyclo*-(Pro-Gly)$_3$ complexes with stoichiometries of 1:1 and 2:1. Binding constants for the latter two steps are reported (Table III).

For other cations, binding constants were determined at a single, fixed salt concentration (Table IV). Due to the qualitative similarity of the CD spectra upon complete complexation, all complexes in Table IV were assumed to be 1:1.

cyclo-(Pro-Gly)$_3$ selects among both group I and group II cations. For group II, Ca^{2+} and Mg^{2+} are

(11) E. B. Nielsen and J. A. Schellman, *J. Phys. Chem.*, 71, 2297 (1967).

about three times more strongly bound than Ba^{2+}. In group I, though, Na^+ is three times more strongly bound than K^+; Li^+ binds most strongly in the methanol–water mixture. Selectivity within each group of cations is probably related to ionic size. However, a definitive statement cannot be made due to the undetermined degree of solvation (hence, undetermined size) of the ions in the *cyclo*-(Pro-Gly)$_3$ complexes.

Stoichiometries of Cation Complexes. The extraction procedure described in the Experimental Section was adopted since, due to the solubility of *cyclo*-(Pro-Gly)$_3$ complex in water, the method of Pedersen[12] (extraction of ion–complex into an organic phase from aqueous solution) could not be applied. Within experimental error, the molar ratios of cation to peptide are 1:1 for Na^+ and Ca^{2+} but 2:1 for Mg^{2+} (Table V). The fact

Table V. Stoichiometries of *cyclo*-(Pro-Gly)$_3$–Cation Complexes[a]

Cation	Anion	Cation:peptide ratio
Na^+	Cl^-	0.93 ± 0.05[b]
Na^+	Picrate	0.91 ± 0.03
Na^+	2,6-Dichloroindophenolate	0.94 ± 0.04
Ca^{2+}	5-Hydroxy-1-naphthalenesulfonate	0.91 ± 0.08[c]
Mg^{2+}	8-Anilino-1-naphthalenesulfonate	2.0 ± 0.2

[a] Except for the noted exceptions, salt concentrations were determined from the ultraviolet absorbance of the anion; the peptide concentration range was 0.1–2.0 × 10^{-4} *M*, and the solvent was methylene chloride. [b] Concentration of Na^+ was determined *via* ^{22}Na γ-ray emission. [c] Peptide concentrations were 0.3–1.0 × 10^{-4} *M*, and the solvent was CH_2Cl_2–CH_3CN (7:3, v/v).

that the same stoichiometry was observed for a threefold (or greater) concentration range of *cyclo*-(Pro-Gly)$_3$ ensures that limiting solubility is not determining the results. For the sodium salts, the measured stoichiometries were independent of the anion employed. The form of the binding curves (discussed in the previous section) confirms that the reported stoichiometries (Table V) are retained for calcium and magnesium perchlorates.

Nmr Spectra. Further nmr data were especially important for *cyclo*-(Pro-Gly)$_3$ conformer S which occurs in nonpolar solvents. The original nmr data[2,3] were used to infer that conformer S contained three cis Gly-Pro peptide bonds. Subsequently, conformational energy calculations[4] revealed an alternative C_3-symmetric conformer which had all peptide bonds trans, was also consistent with the $J_{N\alpha}$ coupling constants observed for conformer S, and had a theoretical CD spectrum which agreed with the experimental one. The nmr experiments reported below verify that conformer S (reported in Table I) indeed has all peptide bonds trans.

One established method[6b,13] for determining peptide bond geometry is a benzene titration. This technique is based upon the preferential upfield shift of the *N*-alkyl group anti to the carbonyl oxygen of its peptide group and is presumably due to repulsion between the aromatic ring and the carbonyl oxygen, both of which are electron rich.[13] Although the conformation of *cyclo*-(Pro-Gly)$_3$ is solvent dependent, the following observations establish that *no* conformational change occurs upon adding benzene to a chloroform solution of *cyclo*-(Pro-Gly)$_3$: (a) the proton–proton coupling constants are unchanged, (b) the spectrum remains that expected from a C_3-symmetric conformer, and (c) the chemical shifts change monotonically with benzene mole fraction. For *cyclo*-(Pro-Gly)$_3$ in chloroform, benzene shifts the proline δ protons about three times further upfield than the proline α proton (Table VI),

Table VI. Benzene Titration of *cyclo*-(Pro-Gly)$_3$ in Chloroform[a]

Proton	$\frac{\Delta \text{ Hz}}{x_{\text{benzene}}}$
Gly N–H	−21
Gly H_α	39
Pro H_α	35
Pro H_δ (2 protons)	110
Pro H_β and/or H_γ (2 protons)	32
Pro H_β and/or H_γ (2 protons)	82

[a] Benzene was added in increments to give solutions 0.05–0.62 mol fraction of benzene at increments of about 0.1. The chemical shifts were linear in this range, and the slope of the chemical shift change (at 100 MHz) *vs.* mole fraction of benzene (Δ Hz/x_{benzene}) is reported. Positive values indicate shifts to higher field.

indicating that the δ protons are anti to the carbonyl oxygen, which is the molecular geometry with trans Gly-Pro peptide bonds.

In order to observe a direct transition between conformers S and S_1*, a further series of nmr experiments was performed in a mixed solvent (acetonitrile–chloroform, 3:1, v/v) due to the limited solubilities of salts and of *cyclo*-(Pro-Gly)$_3$–cation complexes in chloroform. In this solvent without salt, *cyclo*-(Pro-Gly)$_3$ is an approximately equimolar mixture of conformers S and A (Figure 3a). Upon addition of sodium thiocyanate (Figure 3b,c), the population of conformer A diminishes leaving a single resonance for the three N–H's of the C_3-symmetric conformers (S and S_1*). With increasing salt, this latter resonance shifts upfield, and the Gly $J_{N\alpha}$ coupling constants increase from values of 3.0 and 4.5 Hz, characteristic of conformer S, to values of 5.0 and 5.5 Hz, characteristic of conformer S_1*. The coupling constants were measured directly from the upfield portions of the spectra (not shown).

A ^{13}C nmr experiment, performed in acetonitrile–chloroform (1:1, v/v) to attain the higher peptide concentration needed, showed parallel results. With no added salt, *cyclo*-(Pro-Gly)$_3$ is a mixture of conformers S and A (Figure 4a). When sodium thiocyanate is added [molar ratio of NaSCN to *cyclo*-(Pro-Gly)$_3$ was 1:4.4], mainly single resonances are observed (Figure 4b) for each type of carbon. Minor resonances from a small population of conformer A are also apparent, especially at the ^{13}C-enriched glycine carbonyl carbon. Further additions of this salt were not possible due to limited solubility of the Na^+–*cyclo*-(Pro-Gly)$_3$ complex.

The observation of resonances typical of a single C_3-symmetric conformer in solutions which must contain both conformers S and S_1* (Figures 3 and 4) establishes that the two conformers are rapidly interconverting. The coalescence temperature for interconversion between the two conformers is less than 30° (the temperature at which the experiments were conducted). From this fact and the difference in chemical shift for the N–H's (in the ^1H nmr spectra) of the two pure con-

(12) C. J. Pedersen, *Fed. Proc. Fed. Amer. Soc. Exp. Biol.*, 27, 1305 (1968).
(13) J. V. Hatton and R. E. Richards, *Mol. Phys.*, 5, 139 (1962).

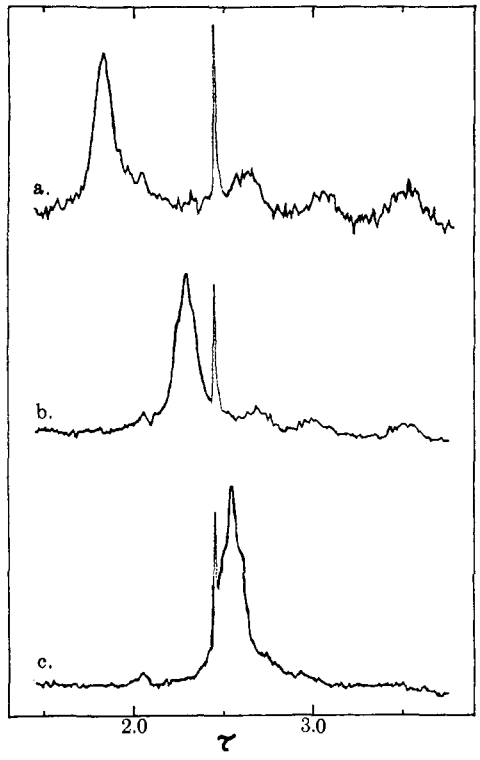


Figure 3. Downfield portions of proton magnetic resonance spectra (100 MHz) of *cyclo*-(Pro-Gly)$_3$ plus sodium thiocyanate in acetonitrile-d_3–chloroform-d_1 (3:1, v/v). Chemical shifts (τ scale) are relative to internal tetramethylsilane. The three N–H resonances of the asymmetric conformer occur at τ 2.6, 3.1, and 3.5. The larger resonance is due to the three N–H's of C_3-symmetric conformer(s). The sharp line near τ 2.5 is due to residual $CHCl_3$. The peptide concentration was 0.025 *M*. The concentrations of added sodium thiocyanate and, in parentheses, molar ratios of salt:peptide were (a) no salt (0); (b) 0.005 *M* (1:5); (c) 0.012 *M* (1:2). Addition of more salt caused precipitation of a NaSCN–*cyclo*-(Pro-Gyl)$_3$ complex.

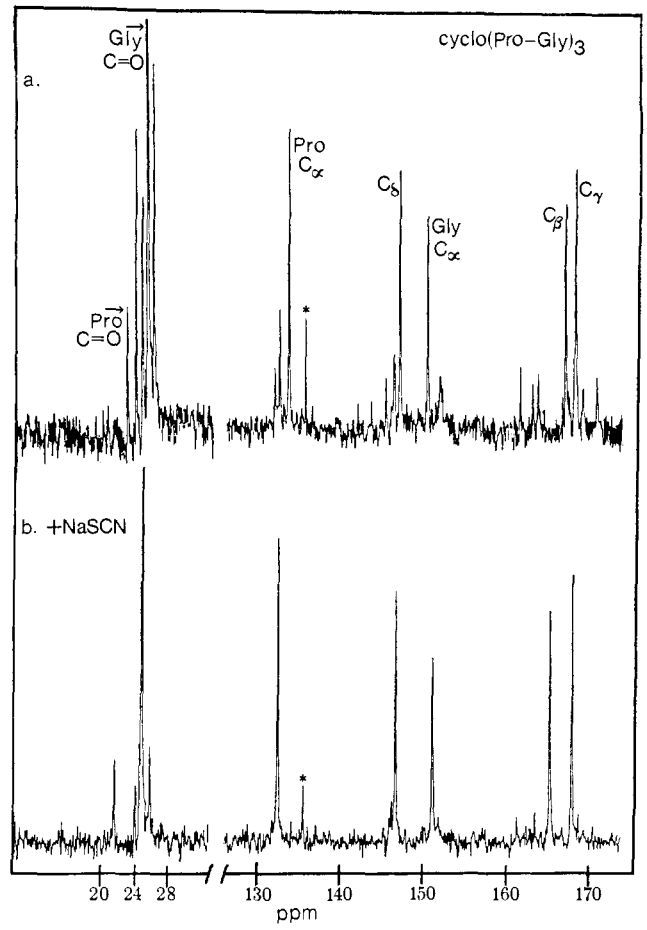


Figure 4. Fourier transform ^{13}C nmr (25.16 MHz) of *cyclo*-(Pro-Gly)$_3$ in acetonitrile-d_3–chloroform (1:1, v/v). Chemical shifts are given in parts per million upfield from external CS_2. The starred (*) resonances are due to a trace of ethanol in the chloroform. The glycine C′ was enriched 20% with ^{13}C, and the major Gly C′ resonance is off-scale in each spectrum. The peptide concentration was 0.04 *M*. The spectra are (a) free peptide; (b) sodium thiocyanate added to a concentration of 0.009 *M* (molar ratio of salt:peptide, 1:4.4).

formers (at least 75 Hz, see Figure 3), the energy barrier to interconversion must be *less than* 15 kcal/mol.[14,15] Since the barrier for cis–trans isomerization of peptide bonds is about 20 kcal/mol,[16] conformers S and S_1* must have the same distribution of cis and/or trans bonds.[17]

The change in $J_{N\alpha}$ coupling constants from conformer S (3.0 and 4.5 Hz) to conformer S_1* (5.0 and 5.5 Hz) implies *via* a Karplus-type relationship (see Table I) a change in glycine ϕ from 355° for conformer S to 260° in conformer S_1*. These glycine ϕ angles are in good agreement with the values proposed in Table I for the all-trans conformers S and S_1*, respectively. While the ϕ_{Gly} angles determined for conformer S can be accommodated by a three-cis conformer of high intramolecular potential energy (9 kcal/mol above the all-trans conformer), steric constraints prevent the conformational change to the ϕ_{Gly} angles indicated by $J_{N\alpha}$ for conformer S_1* while maintaining three cis bonds. Thus, these experiments also lead to the conclusion that both conformers S and S_1* have all peptide bonds trans.

The chemical shift changes seen in Figures 3 and 4 are consistent with the present conformational assignments. First, upon adding salts, the upfield shift of the Gly N–H resonances from the interconverting C_3-symmetric conformers (Figure 3) is consistent with the exposure of these protons to solvent in conformer S_1* after breaking the 1 ← 3 hydrogen bonds of conformer S (see Figure 2). This conformational change is also reflected in the $^{13}C_\beta$ chemical shifts (Figure 4, Table VII). In conformer S, C_β is eclipsed (ψ_{Pro} ~250°) and apparently shielded by the carbonyl oxygen. The C_β chemical shift of *cyclo*-(Pro-Gly)$_3$ conformer S is similar to that observed in proline diketopiperazines[18a]

(14) See H. Shahan-Alidi and K. H. Bar-Eli, *J. Phys. Chem.*, **74**, 961 (1970), for the method of estimating energy barriers from coalescence temperature.

(15) For analysis of systems with exchange among three chemical sites, see H. S. Gutowsky and A. Saika, *J. Chem. Phys.*, **21**, 1688 (1953).

(16) W. E. Stewart and T. H. Siddall, III, *Chem. Rev.*, **70**, 517 (1970).

(17) Binding of sodium thiocyanate to *cyclo*-(Pro-Gly)$_3$ in dimethyl sulfoxide was investigated by Deber, *et al.*[2] Figure 5b,c in that reference shows a resonance labeled "S" which does not average with the resonance due to the peptide–cation complex (S*) nor with those due to the asymmetric conformer (A). The area of peak S is only *ca.* 5% of the total area of peaks due to the amide protons. The origin of peak S is not known, but it could be due to a small population of one of the following: (a) a C_3-symmetric conformer with three cis Gly-Pro bonds; (b) a second type of asymmetric conformer (with additional resonances either accidentally degenerate or obscured by the major resonances); or (c) an all-trans conformer S (provided that the solvent-dependent interconversion rate between S and S* is slow in dimethyl sulfoxide).

(18) (a) D. E. Dorman and F. A. Bovey, *J. Org. Chem.*, **38**, 2379 (1973); (b) D. E. Dorman, D. A. Torchia, and F. A. Bovey, *Macromolecules*, **6**, 80 (1973); (c) K. Wüthrich, A. Tun-Kyi, and R. Schwyzer, *FEBS Lett.*, **25**, 104 (1972); (d) *cf.* ref 1 and 8.

Table VII. ^{13}C Chemical Shifts of *cyclo*-(Pro-Gly)$_3$ and Its Complexes[a]

Resonance	Free peptide, ppm	NaSCN: *cyclo*-(Pro-Gly)$_3$ (1:4.4), ppm	$Mg(ClO_4)_2$: *cyclo*-(Pro-Gly)$_3$ (2:1), ppm
Pro C′	22.4	21.1	19.0
Gly C′	24.8	24.4	25.1
	(23.4, 24.2, 25.5)[b]		
Pro C_α	133.3	131.9	131.0
Pro C_δ	146.6	146.4	145.6
Gly C_α	149.9	150.8	151.1
Pro C_β	166.5	165.0	163.7
	(161.1, 162.5, 163.2)[b]		
Pro C_γ	167.8	167.7	167.8
	(168.7, 170.4)[b]		

[a] Peptide concentration was 0.04 *M*. The molar ratios of salt to peptide are indicated. The solvent was CD_3CN–$CHCl_3$ (1:1, v/v), except for the experiment with $Mg(ClO_4)_2$ in which CD_3CN–$CHCl_3$ (3:1, v/v) was used. Chemical shifts are upfield from external CS_2 calibrated by independent measurements of $CHCl_3$ at 114.61 ppm in 1:1 and 114.66 ppm in 3:1 CD_3CN–$CHCl_3$. [b] Resonances of Gly C′ and Pro C_β and C_γ in parentheses are those observed for the small population of asymmetric conformer A (see Figure 4).

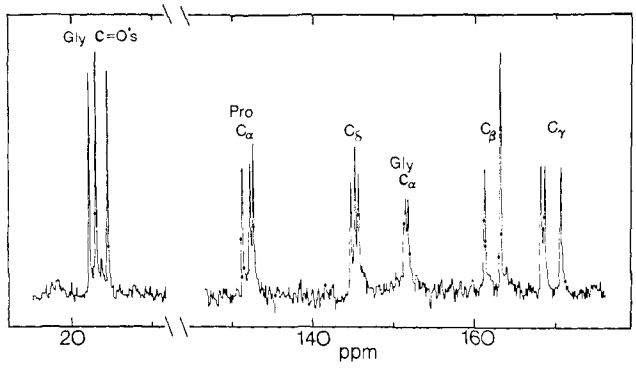


Figure 5. Fourier transform ^{13}C nmr spectrum (20 MHz) of *cyclo*-(Pro-Gly)$_3$ in D_2O. Peptide concentration was 0.065 *M*. Glycine carbonyl carbons were enriched *ca.* 10% with ^{13}C. Due to the short recycle time used in recording the spectrum (0.7 sec), the natural abundance proline carbonyl carbons were only barely observable near 19 ppm. Chemical shifts in parts per million upfield from external CS_2 are: Gly C′, 22.1, 23.0, 24.4; Pro C_α, 131.2, 132.2, 132.5; Pro C_δ, 144.6, 145.2, 145.6; Gly C_α, 151.5, 151.8; Pro C_β, 161.3, 163.2; and Pro C_γ, 168.0, 168.5, 170.5.

in which the carbonyl oxygen also eclipses C_β. By contrast, in conformer S_1*, C_β is not eclipsed, and the C_β resonance shifts downfield to the position observed in a variety of proline compounds in which eclipsing is also absent.[18] Throughout the addition of salt, the proline C_γ resonance remains at 167.8 ± 0.1 ppm (Figure 4, Table VII), the value typical for many compounds with trans X-Pro bonds.[18]

For the asymmetric conformer A, ^{13}C chemical shifts are consistent with the conformer which has one cis Gly-Pro peptide bond (Table I). In water solution conformer A predominates, as evidenced by the three resonances observed for each of the following carbon atoms: glycine C′, as well as proline C_α, C_δ, and C_γ (Figure 5). The C_γ resonance at 170.5 ppm (Figure 5) is typical for proline preceded by a cis peptide bond,[18] while the C_γ resonances at 168.0 and 168.5 ppm (Figure 5) are typical for proline with trans peptide bonds.[18] Although C_β chemical shifts are less reliable indicators of cis–trans isomerism than C_γ shifts, the C_β resonance at 161.3 ppm and the one at 163.2 ppm which has twice the intensity (Figure 5) are typical of values observed for cis and trans peptides, respectively[18] (in the absence of specific conformational effects). In acetonitrile–chloroform only a small fraction of the molecules assumes conformation A. The chemical shifts of the minor C_β and C_γ resonances (due to conformer A) are nearly the same as those observed in water. The C_γ resonances at 170.4 and 168.7 ppm (Figure 4, Table VII) are typical of cis and trans bonds, respectively.[18] (A third minor C_γ resonance may be coincident with the major resonance of conformer S at 167.8 ppm.) The C_β resonances at 161.1 and 163.2 ppm (Figure 4, Table VII) are typical of cis and trans peptide bonds, respectively. The third C_β resonance at 162.5 ppm is nearer values observed for trans bonds, but its intermediate position makes interpretation ambiguous.

The proposed one-cis conformer A (Table I, Figure 2c, has two N–H's exposed to solvent and one N–H shielded by the pyrrolidine ring. The N–H chemical shifts in dimethyl sulfoxide (DMSO) are consistent with this model. Two N–H's have chemical shifts attributed to those hydrogen bonded to DMSO (1.2 and 1.6 ppm on the τ scale),[2] while the third N–H (at 3.5 ppm)[2] seems to be shielded from DMSO.

Further characterization of conformational states A and S is possible from the effects of temperature on their nmr spectra. In dimethyl sulfoxide the three separate N–H resonances observed for conformer A coalesce at 110° to one resonance. The energy barrier estimated[14,15] from this coalescence temperature and the low-temperature chemical shift separation is comparable to that observed for cis–trans peptide bond isomerism.[16] This result indicates that the asymmetry of conformer A is due to the presence of one or two cis Gly-Pro peptide bonds. Evidence in favor of the one-cis alternative has already been presented. In contrast, the nmr spectrum of conformer S in methylene chloride is unchanged upon cooling to −75°, confirming that conformer S has *inherent* C_3 symmetry rather than *apparent* C_3 symmetry arising from interconversions between conformers separated by a significant energy barrier. Any conformational averaging in conformer S at −75° would involve an energy barrier less than 10 kcal/mol.

Proton nmr spectra of *cyclo*-(Pro-Gly)$_3$–magnesium complexes are reported in Figure 6. Resonances from at least two distinct complexes are observed. Nmr spectra of the magnesium complexes indicate C_3-symmetric conformers with chemical shifts and coupling constants similar to those observed for the *cyclo*-(Pro-Gly)$_3$–Na^+ complex (Figures 3 and 4). The glycine ϕ angle derived from the experimental $J_{N\alpha}$ (Table I) for the final state agrees well with the value proposed in Table I for conformer S_2*.

No resonances from free peptide are present for molar ratios of Mg^{2+}:*cyclo*-(Pro-Gly)$_3$ above 1:2. This implies that the first complex is a "peptide sandwich" with a stoichiometry of one Mg^{2+} cation per two *cyclo*-(Pro-Gly)$_3$ molecules. Extraction experiments in the presence of excess salt in nonpolar solvents (reported above) indicate formation of a "magnesium sandwich" with a stoichiometry of $2Mg^{2+}$:*cyclo*-(Pro-Gly)$_3$. Monitoring of ion binding *via* CD spectra indicates yet

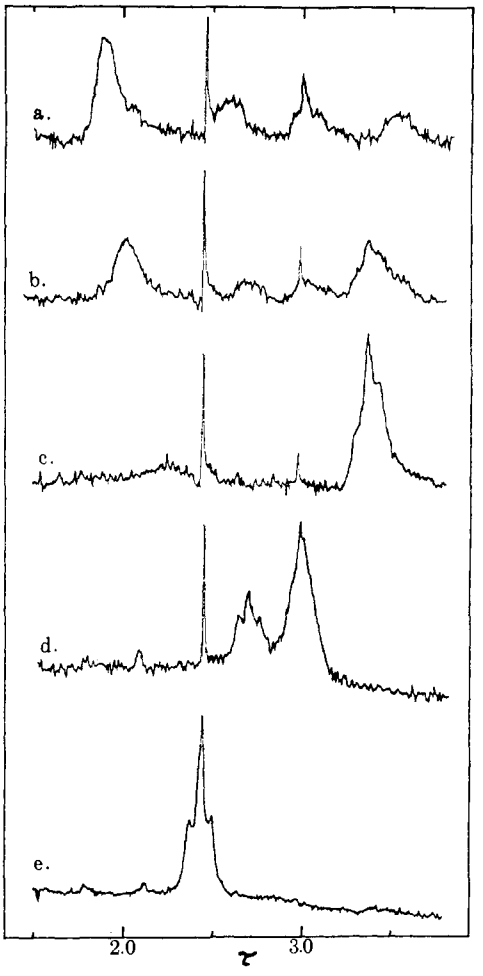


Figure 6. Downfield portions of proton magnetic resonance spectra (100 MHz) of *cyclo*-(Pro-Gly)$_3$ plus magnesium perchlorate in acetonitrile-d_3–chloroform-d_1 (3:1, v/v). Chemical shifts (τ scale) are relative to internal tetramethylsilane (see caption to Figure 3 for explanation of N–H resonances). The sharp line near τ 2.5 is due to residual $CHCl_3$. The peptide concentration was 0.012 *M*. The concentrations of added magnesium perchlorate and, in parentheses, molar ratios of salt:peptide were (a) no salt (0); (b) 0.003 *M* (1:4); (c) 0.006 *M* (1:2); (d) 0.012 *M* (1:1); (e) 0.024 *M* (2:1). The nmr spectrum was identical with spectrum e when the Mg^{2+} concentration was doubled to 0.048 *M*.

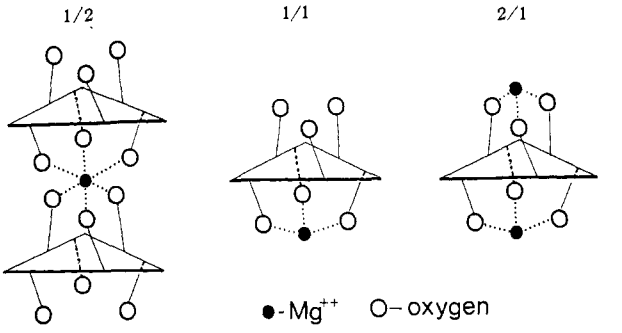


Figure 7. Schematic representation of probable *cyclo*-(Pro-Gly)$_3$ complexes with Mg^{2+}. The molar ratios of Mg^{2+}:peptide are indicated. In the text, these structures are referred to as 1:2 (peptide sandwich) and 2:1 (magnesium sandwich). Although the stoichiometries of these complexes were deduced from experimental data, the cation positions are hypothetical (see Discussion). The perchlorate counterions were omitted from this figure, but must play a vital role in reducing repulsion between the two magnesium ions in the 2:1 complex.

another magnesium complex with 1:1 stoichiometry. Possible structures of these complexes are presented schematically in Figure 7. As the two faces of *cyclo*-(Pro-Gly)$_3$ are different (the three proline carbonyls project in one direction and the three glycine carbonyls in the opposite direction), there are three possible types of "peptide sandwich" and two ways to form the depicted 1:1 complex. The resonance at 3.4 ppm (τ) in Figure 6b,c can be identified with the 1:2 complex and that at 2.4 ppm in Figure 6e with the 2:1 complex. The fractions of complexes of each stoichiometry at intermediate magnesium concentrations are uncertain.

Separate resonances (rather than averages) are observed for mixtures of free *cyclo*-(Pro-Gly)$_3$ and its magnesium complexes. Analyses of CD and nmr spectra indicate that all magnesium complexes of *cyclo*-(Pro-Gly)$_3$ lie within the same conformational region as the 1:1 complexes of other cations (conformer S_1* in Table I). As there are no large barriers to peptide conformational change within this all-trans class of conformers, slow exchange of magnesium must be the step which produces the separate resonances. For macrocyclic compounds several examples of slow cation exchange, with free energy of activation for the dissociation step up to 24 kcal/mol, have been observed.[19a] Valinomycin has an intermediate rate of K^+ exchange which produces broadened nmr bands but does not produce separate resonances for the complexed and uncomplexed states at room temperature.[19]

Discussion

Data presented here allow detailed conformational assignments in accord with nmr and CD data, as well as calculated intramolecular potential energies. Conformer S, which occurs in relatively inert solvents (such as chloroform and dioxane), is C_3 symmetric, has all peptide bonds trans, and is stabilized by three intramolecular 1 ← 3 hydrogen bonds (see Table I and Figure 2a). Formation of these 1 ← 3 hydrogen bonds (γ turns) requires that proline ψ be about 250°, which is near the lower limit of the trans′ region, and that the proline C_β and carbonyl oxygen atoms be eclipsed. As expected in nonpolar solvents, the observed conformer S is near the global minimum in the computed intramolecular potential energy. The computations indicated that the coulombic interaction energy of the polar atoms involved in the 1 ← 3 hydrogen bonds is a major stabilization factor for conformer S. The computed conformational distribution is fairly precise for *cyclo*-(Pro-Gly)$_3$ in nonpolar solvents, even though an explicit potential function for hydrogen bonding was not included.

Upon addition of cations to solutions of *cyclo*-(Pro-Gly)$_3$ to form conformer S* (Table I and Figure 2b) from conformer S, the Pro-Gly peptide unit must be rotated ~80° about the proline C_α–C′ bonds and concomitantly about the glycine N–C_α bonds. (The two bonds are nearly parallel.) This rotation breaks the intramolecular hydrogen bonds, exposes the N–H's to solvent, and brings the three proline carbonyl oxygens together to form an open cup on one face of the molecule. The glycine carbonyl oxygens form a similar cup on the opposite face. The conformers S* lie within a

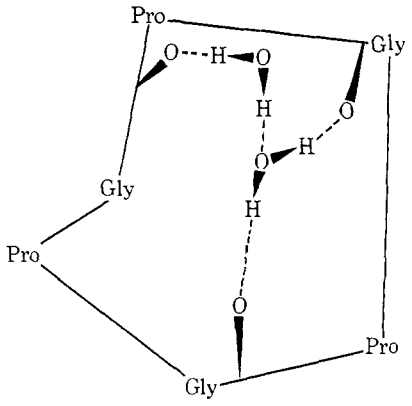
(19) (a) J.-M. Lehn, *Struct. Bonding (Berlin)*, **16**, 1 (1973); (b) D. J. Patel, *Biochemistry*, 12, 496 (1973).

second local minimum in the computed intramolecular potential energy and are 1.5–3.5 kcal/mol above the energy of conformer S. This increase in intramolecular potential energy must be compensated by favorable energy of solvation and/or by the energy of bonding cations to the peptide. The free energy of cation binding is considerable. For example, from the equilibrium constant of 1.1×10^5 M^{-1} (Table III), a free energy change ($-RT$ ln K) of 7 kcal/mol is calculated for binding Ca^{2+} to *cyclo*-(Pro-Gly)$_3$ in acetonitrile solution.

Two classes of cation–*cyclo*-(Pro-Gly)$_3$ complexes have been observed. The first class, conformer S_1*, is a 1:1 cation–peptide complex which includes complexes of Na^+, Ca^{2+} and, by inference, Li^+, K^+, and Ba^{2+}. The second type of complex, conformer S_2*, is a 2:1 cation–peptide complex formed by two Mg^{2+} ions and one *cyclo*-(Pro-Gly)$_3$ molecule. Perhaps a second binding site is favorable for magnesium (but not for the other cations), since Mg^{2+} combines a small ionic radius with a 2+ charge. Nevertheless, some bonding property peculiar to magnesium could also contribute; for example, magnesium compounds are known to have considerably more covalent character than compounds of other group II metals.[20]

While both conformers S (nonpolar solvents) and S* (cation complexes) are C_3 symmetric, have all six peptide bonds trans, and have the three proline ψ angles within the trans′ region, conformer A, which occurs in polar solvents, such as water and dimethyl sulfoxide, is asymmetric and appears to have one cis Gly-Pro peptide bond and one cis′ proline ψ angle (Table I and Figure 2c). The dihedral angles assigned to conformer A in Table I were selected by matching the theoretical with the experimental CD spectrum. Both one-cis and two-cis conformers have theoretical CD spectra which match the experimental spectrum. The one-cis conformers were selected mainly due to their lower computed intramolecular potential energy. For the asymmetric conformer A, overlap in the upfield portion of the proton nmr spectra prevents measurement of the $J_{N\alpha}$ coupling constants.

The intramolecular potential energy computed for conformer A is 3.4–6.7 kcal/mol above that of conformers S and S*. Computations have shown that interactions of *cyclo*-(Pro-Gly)$_3$ with *bulk* solvent will not shift the equilibrium to populate conformer A.[4] Thus, the large intramolecular potential energy difference between conformer A and the C_3-symmetric conformers must be compensated by strong, *specific* peptide–solvent interactions. Water would be particularly efficacious in stabilizing conformer A, since four water molecules can form eight, nearly linear, hydrogen bonds to bridge the six carbonyl oxygens in two groups of three. Two water molecules bridging three carbonyls are illustrated schematically in the structure below. Similarly, the remaining three carbonyls could be bridged by two more water molecules. However, conformer A is also stable in methanol (which could hydrogen bond to the carbonyls but form no bridges) and in solvents which are only hydrogen bond acceptors, such as dimethyl sulfoxide and tributyl phosphate. Since water was not rigorously excluded from these solutions, it is possible that water bridges also contribute



to the stability of conformer A in the latter solvents. Hydrogen bond acceptor solvents provide stabilization by bonding two out of the three glycine N–H's as is apparent from the N–H chemical shifts in dimethyl sulfoxide (see Results and ref 2). Nevertheless, conformer A in hydrogen bond acceptor solvents cannot be stabilized solely by the two N–H to solvent hydrogen bonds, since conformers similar to S* without bound salt have lower computed intramolecular potential energy than conformer A, and the S*-type conformers could be further stabilized by *three* N–H to solvent hydrogen bonds. However, experimental data show that the conformers S* do not exist without the additional stabilization provided by cation–peptide bonds.

Even in aqueous solution, *cyclo*-(Pro-Gly)$_3$ binds cations much more strongly than would be expected from its constituent amide groups acting singly. From the data of von Hippel, *et al.*,[21] sodium and calcium perchlorates in aqueous solution bind to an individual amide group of polyacrylamide with binding constants of 0.10 and 0.21 M^{-1}, respectively. The binding constants for these two salts with *cyclo*-(Pro-Gly)$_3$ (Table III) are, respectively, 3.7 and 110 times the polyacrylamide values for six peptide groups. As in the case of polyacrylamide,[21] the doubly charged cations have considerably larger binding constants to *cyclo*-(Pro-Gly)$_3$ than the singly charged cations (Tables III and IV). The selectivity by *cyclo*-(Pro-Gly)$_3$ among ions of a given charge is also greater than that of polyacrylamide. The concerted action of *cyclo*-(Pro-Gly)$_3$ peptide groups in binding cations is also evident from a comparison with the cyclic tetrapeptide, *cyclo*-(Pro-Gly)$_2$ (see ref 9 for the synthesis and characterization of this compound). Severe steric constraints in the tetrapeptide[1] prevent adjustment of the peptide carbonyls to form an ideal binding site. For calcium perchlorate in acetonitrile solution, the binding constant to *cyclo*-(Pro-Gly)$_2$ is only 2.0×10^2 M^{-1}, while that to *cyclo*-(Pro-Gly)$_3$ is 1.1×10^5 M^{-1}.

The binding behavior of *cyclo*-(Pro-Gly)$_3$ parallels that of the naturally occurring cyclic peptide, antamanide,[22a] and the enniatin cyclodepsipeptides.[22b] In acetonitrile the binding constants of calcium to *cyclo*-(Pro-Gly)$_3$ and antamanide are equal, and for other ions and solvents, binding constants for the two peptides are

(20) F. A. Cotton and G. Wilkinson, "Advanced Inorganic Chemistry," 3rd ed, Interscience, New York, N. Y., 1972, p 206 ff.

(21) P. H. von Hippel, V. Petrolas, L. Schack, and L. Karlson, *Biochemistry*, **12**, 1256 (1973).

(22) (a) T. Wieland in "Chemistry and Biology of Peptides," J. Meienhofer, Ed., Ann Arbor Science Publishers, Ann Arbor, Mich., 1972, p 377; (b) V. T. Ivanov, A. V. Evstratov, L. V. Sumskaya, E. I. Melnik, T. S. Chumburidze, S. L. Portnova, T. A. Balashova, and Yu. A. Ovchinnikov, *FEBS Lett.*, **36**, 65 (1973).

comparable. Antamanide shows higher selectivity among cations of a given charge, though both antamanide and *cyclo*-(Pro-Gly)$_3$ show preference for Na^+ and Ca^{2+} (which have the same ionic radius) over larger cations. Though the enniatins show a preference for K^+, *cyclo*-(Pro-Gly)$_3$ mimics their ability to form sandwich complexes (see Figure 7). Ivanov, *et al.*,[22b] have shown that the sandwich complex of one potassium ion with two enniatin B molecules is the species which transports potassium across lipid bilayers. These authors suggested that the sandwich complex might screen the cation from solvent more effectively than a 1 : 1 complex. Thus, for both enniatin B and *cyclo*-(Pro-Gly)$_3$, "club sandwich complexes" (formed by stacking the elements in Figure 7) could provide a conduit whose length could be adjusted precisely to span a membrane. Ions could then be conducted by passing them along the chain of peptide molecules (*via* "peristalsis") from one surface to the other. A further, somewhat unique, property of *cyclo*-(Pro-Gly)$_3$ is the fact that its cation complexes are completely extracted from organic phases by water, suggesting that this cyclic peptide may have unusual effects in ion transport.

X-Ray diffraction results on a number of cation complexes of cyclic peptides, cyclic depsipeptides, and macrocyclic compounds show that the cation binding site is distal to carbonyl bonds.[23] (The term, distal, is used to refer to positions on the opposite side from C′ of a plane perpendicular to the C′=O bond and passing through the oxygen nucleus. The term, proximal, refers to positions on the same side of this plane as C′.) This binding preference implies that the cations bind the nonbonded electrons of the oxygen atom, rather than forming an adduct with the peptide π system. Since a cation in the *central cavity* of *cyclo*-(Pro-Gly)$_3$ conformer S* would be proximal to the C=O bond (hence, away from the nonbonded electrons), it seems unlikely that the central cavity is the binding site. Rather, the cation is probably bound by one (or both) of the open cups formed by the three carbonyls on either face of *cyclo*-(Pro-Gly)$_3$ (see Figures 2b and 7). Presumably, additional cation coordination sites would be filled by anion and/or solvent molecules, as has been observed in crystals of lithium–antamanide complex.[23a]

A detailed description of the cation binding site in *cyclo*-(Pro-Gly)$_3$ is not possible from present spectroscopic data. It has been proposed that the carbonyl groups involved in complexation of diamagnetic cations can be identified by downfield shifts of these carbonyl carbons in ^{13}C nmr spectra of the ionophores.[24] Upon formation of *cyclo*-(Pro-Gly)$_3$ complexes with Na^+, both the proline and the glycine carbonyl carbons shift downfield (Table VII, ref 3). By contrast, for conformer S_2* [$2Mg^{2+}$:*cyclo*-(Pro-Gly)$_3$] in which it seems that all six carbonyls would be involved in complexation, the proline carbonyls shift downfield 3.4 ppm, while the glycine carbonyls shift upfield 0.3 ppm (Table VII). Therefore, the relative involvement of the two sets of carbonyls in ion binding remains uncertain.

Preliminary data[25] on *cyclo*-(Pro-Gly)$_4$, taken in conjunction with results herein, establish a correlation between the size of the cavity formed by the carbonyl oxygens of *cyclo*-(Pro-Gly)$_n$ peptides and the ionic radii of selectively bound cations.

Conclusions

This study illustrates the fusion of complementary information from nmr spectra, CD spectra, and theoretical computations in deriving the conformational states of an ion-binding cyclic peptide in solution. For a cyclic hexapeptide of relatively restricted conformational freedom, *cyclo*-(Pro-Gly)$_3$ exhibits an unusual number of conformational states. Both conformers S and S* fall within the regions of low intramolecular potential energy. The observation of the asymmetric conformer, which has much higher computed intramolecular potential energy, emphasizes the importance of *specific* solvent–solute interactions in stabilizing peptide conformers. Further investigations of *cyclo*-(Pro-Gly)$_n$ peptides and naturally occurring peptides will be necessary for the assessment of various determinants of peptide conformation, as well as strength and selectivity of cation binding.

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