# Why Cyclic Peptides? Complementary Approaches to Conformations

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*Received May 30, 1975*

Three-dimensional structures of peptide chains can be related to biological functions of peptides and proteins on the molecular level. In order to deduce the conformations of biological macromolecules, physical, chemical, and theoretical techniques have been developed and applied. Analysis by x-ray crystallography[3a] is a potent method for solid-state structure determination, but it does not provide information on dynamic processes, on compounds in solution (usually the physiological milieu), or on biologically important molecules which have not been crystallized. In addition, the conformation in crystals may be unique to the solid state—especially for small peptides whose crystals contain little solvent.[3b] Thus, the quest continues for techniques which yield detailed structural information to complement that obtained from crystallographic studies.

We describe herein approaches to the deduction of peptide conformation in solution by the use of three techniques: $^{13}C$ nuclear magnetic resonance, conformational energy calculations, and circular dichroism. Sections of this paper illustrate the types of conformational information that can be derived from each of the *individual* methods for synthetic and naturally occurring cyclic peptides, but for complete conformational analysis a *combination* of techniques is usually necessary, or, as has been aptly stated, ". . . if the restraints on validity sometimes seem demoralizing, they remain so only as long as one set of data, one type of method, is considered separately. Viewed in consort with other methods, . . . there can be strength in converging weakness."[4]

## Why Cyclic Peptides?

In choosing molecules which might be suitable models for conformational analysis, our goal has been to combine biological relevance with structural simplicity, i.e., the molecules should have a few discrete conformational states and be simple enough so that these states can be characterized by experimental and predictive techniques. The discussion below shows that these criteria are met by cyclic peptides which contain proline and glycine.

Proline is found frequently in naturally occurring biologically active peptides,[5] including *peptide hormones,* such as angiotensin, bradykinin, oxytocin, vasopressin, MSH-releasing-inhibiting hormone (MSH, melanocyte stimulating hormone), TRF hormone (TRF, thyroid-releasing factor); *peptide antibiotics,* such as gramicidin S and actinomycin; and the *peptide antitoxin,* antamanide. In addition, the importance of proline has been established as a conformational determinant in structural proteins such as collagen[6] and as a frequent participant in the reversal of direction of peptide chains in globular proteins.[7,8]

The simplest amino acid, glycine, also plays an important structural role in peptide and protein chains. Because it lacks a side chain, glycine fits into secondary structures where larger amino acids would be excluded. Since proline and glycine residues often comprise much of the amino acid sequence in turn regions of secondary structure in proteins, it is perhaps no coincidence that peptide chains which contain proline and glycine have a higher probability of cyclizing than chains which contain other amino acids[9] (as manifested by isolated yields in cyclic peptide synthesis reactions).

The large number of possible conformational states available to a linear peptide chain—of even a few amino acids—is substantially reduced by cycliza-

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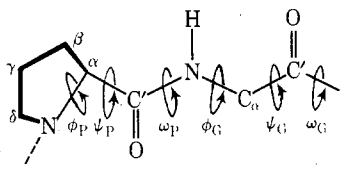
tion. The number of possible conformers is further reduced by building prolines into cyclic peptide sequences, since the proline side chain (a) severely restricts rotations about the N–$C_\alpha$ bond and (b) limits the rotations of neighboring groups. In addition, highly symmetric primary sequences (i.e., repeating units of two or three amino acids) amplify conformational preferences by repetition in a manner similar to cooperative stabilization of regular structures in polymers.

By synthesizing proline-containing cyclic peptides having high sequential symmetry and containing 2 to 12 amino acid residues, we have obtained molecules which have certain preferred conformers. These conformers have been characterized by the techniques which will be described herein. In contrast, such discrete conformational states are less likely for non-proline-containing cyclic peptides[10,11] (or for linear peptides).

An added bonus of studying proline-containing compounds is that they afford an opportunity to determine factors which affect the equilibrium between cis and trans peptide bonds. Although trans is the usual molecular arrangement of peptide bonds, cis peptide bonds (at the amino terminus of proline residues) have been found in proteins,[12] in the naturally occurring cyclic peptide, antamanide,[13] as well as in synthetic linear[14] and cyclic peptides.[15]

## Determination of Conformation

The approach to conformational analysis which will be presented depends largely on data from $^{13}C$ and $^{1}H$ nuclear magnetic resonance (NMR) spectroscopy and potential energy calculations, as well as on theoretical and experimental circular dichroism (CD) spectroscopy. As the extensive contributions made to conformational analysis in this field by proton NMR have been reviewed,[5] they will not be discussed herein. For nonstrained molecules, such as cyclic hexapeptides, specification of conformation reduces to determining dihedral angles for rotations about single bonds ($\phi$, $\psi$, $\omega$) for the peptide backbone.



**Carbon-13 Nuclear Magnetic Resonance.** Molecules of biological significance are being studied increasingly by $^{13}C$ NMR spectroscopy.[16] The simplicity of the spectra and narrowness of resonances in proton-decoupled spectra provide a clear set of lines which often can be assigned to individual carbon atoms.

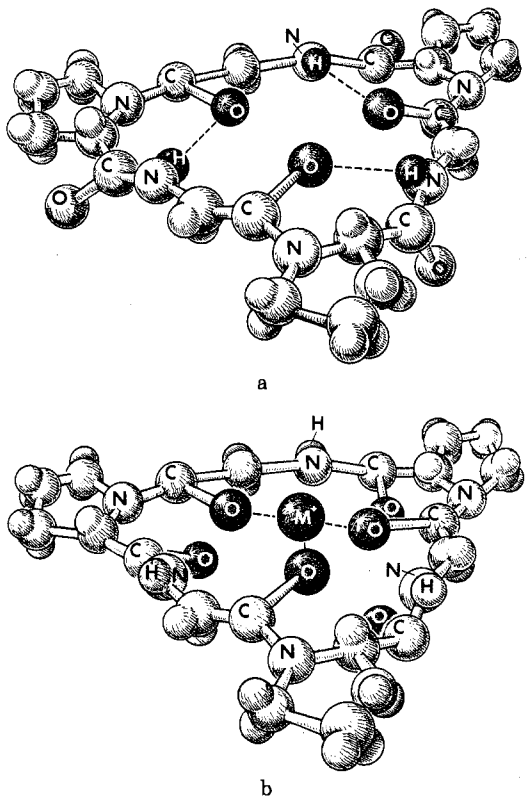


**Figure 1.** Schematic representations of conformations deduced for *cyclo*(Pro-Gly)$_3$ in solution: (a) free peptide in $\gamma$-turn conformation, containing all-trans peptide bonds and three intramolecular 1←3 hydrogen bonds; and (b) cation complex, formed from $\gamma$-turn conformation by rotation (about Pro $\psi$ and Gly $\phi$ angles) of the peptide bond units between Pro and Gly residues. The position of the cation, shown interacting primarily with the three Gly carbonyl groups, has not been definitely established.[17]

$^{13}C$ spectra of cyclic peptides can reveal the degree of conformational symmetry. Thus, spectra of a cyclic hexapeptide, such as *cyclo*(Pro-Gly)$_3$ (see Figure 1), which has $C_3$ symmetry in its primary amino acid sequence, may contain only one resonance for each carbon within the Pro-Gly repeat unit—indicating $C_3$-*conformational* symmetry. If the symmetry persists at low temperature (say, −60°), it may be concluded that the peptide has *inherent* conformational symmetry, as opposed to *apparent* symmetry due to averaging among asymmetric conformers. Such experiments have established that the *cyclo*(Pro-Gly)$_3$ conformer in nonpolar solvents, such as chloroform, is $C_3$ symmetric.[17]

If, on the other hand, the conformation of *cyclo*-(Pro-Gly)$_3$ is asymmetric under the experimental conditions, then, barring chemical shift degeneracy, *three* resonances should be observed for each type of carbon atom in the molecule. Generally, species whose intrinsic chemical shift difference is at least 10 Hz will give rise to discrete lines in NMR spectra at about 25° if the barrier to interconversion of the

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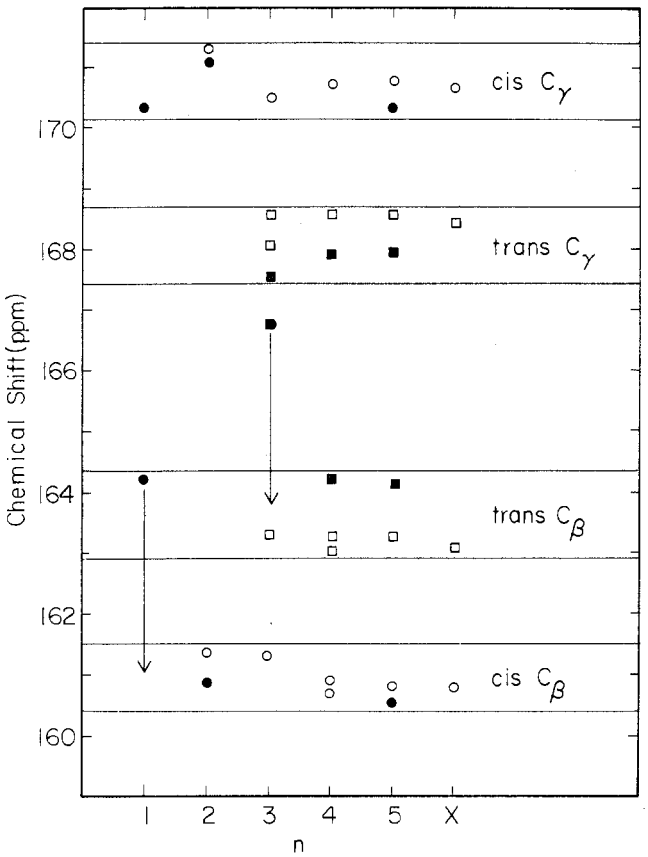


**Figure 2.** $^{13}C$ chemical shifts of proline $C_\beta$ and $C_\gamma$ in a series of *cyclo*(Pro-Gly)$_n$ peptides, $n$ = 1–5, and in linear poly(Pro-Gly), $n$ = X (average degree of polymerization for polymer sample not determined). The horizontal lines enclose the normal ranges of chemical shifts found for each type of carbon. Squares indicate that the Gly-Pro peptide bond is trans; circles that it is cis. For the open symbols the solvent was $D_2O$; filled symbols, $CDCl_3$. Arrows indicate that the chemical shifts of two unusual $C_\beta$ resonances, those of *cyclo*(Pro-Gly)$_1$ and *cyclo*(Pro-Gly)$_3$ in $CDCl_3$, deviate significantly from the "expected" region (see text).

species is greater than 15 kcal/mol. Sometimes observations of asymmetry can be associated with the occurrence of isomers about the X–Pro peptide bonds, as the activation energy for this process is about 20 kcal/mol. In polar solvents *cyclo*(Pro-Gly)$_3$ exhibits tripling of resonances for each type of carbon—indicating an asymmetric conformer. Further, it has been established that this conformer has one cis Gly-Pro peptide bond.[17,18]

$^{13}C$ chemical shifts depend primarily on the chemical nature of the carbon atom. However, there is wide variation (several parts per million) within the region for a given carbon type, and there have been efforts to correlate these variations with conformation. For example, it has been noted in a variety of linear, cyclic, and polypeptides[19,20] that the chemical shift of Pro $C_\gamma$ is ca. 168 ppm for a trans X–Pro peptide bond but about 170 ppm for a cis bond (vs. external $CS_2$). Thus, $^{13}C$ spectra may be used to establish the isomeric state of the X–Pro peptide bond. This correlation appears valid for the X–Pro bond independent of possible variations in proline ring conformation from one molecule to another.[21] Even though proline-containing cyclic dipeptides[22] and *cyclo*(Pro)$_3$[23] have quite different proline ring conformations, both have Pro $C_\gamma$ chemical shifts indicative of their cis peptide bonds (an average of 170.4 ppm in $CDCl_3$ for several *cyclo*(Pro-X) diketopiperazines which have similar ring conformations[22]; 170.5 ppm for *cyclo*(Pro)$_3$ in $CDCl_3$).

Chemical shifts of the other proline ring carbons, including Pro $C_\beta$, are often sensitive to other conformational influences in addition to X–Pro peptide bond stereochemistry. Consider the $^{13}C$ chemical shift data from a series of *cyclo*(L-Pro-Gly)$_n$ peptides ($n$ = 1 to 5) and a linear poly(L-Pro-Gly) sample presented in Figure 2. In this series Pro-Gly is the repeating unit, and there are no end group effects. However, there are progressive variations in molecular size and shape. Nevertheless, the cis and trans Pro $C_\gamma$ resonance positions (in two solvents, water and chloroform) are relatively invariant, as are both cis and trans Pro $C_\beta$ resonances, except in a few instances where unusual chemical shifts are observed, as in Pro $C_\beta$ of *cyclo*(Pro-Gly)$_1$ and *cyclo*(Pro-Gly)$_3$ in chloroform. In these latter cases $^{13}C$ spectra can be employed to diagnose specific features of peptide structure (*vide infra*).

Among the more important effects of neighboring substituents on $^{13}C$ chemical shifts is the shift to higher field observed for a carbon atom when it is cisoid ("eclipsed") to a vicinal substituent.[24] In the rigid conformations of many proline-containing cyclic dipeptides, such as *cyclo*(Pro-Gly)$_1$, Pro $C_\beta$ is eclipsed by the Pro carbonyl oxygen atom, and relatively high-field positions are observed for the Pro $C_\beta$ resonance.[20b] Similar eclipsing occurs in the $\gamma$-turn conformer of *cyclo*(Pro-Gly)$_3$ (see Figure 1a), and again, Pro $C_\beta$ is at "abnormally" high field (see Figure 2). Upon formation of complexes of *cyclo*(Pro-Gly)$_3$ with various metal and alkylammonium cations (discussed below, see Figure 1b), the eclipsing of Pro $C_\beta$ is relieved, and its resonance shifts to the lower field position more usually found for trans Pro $C_\beta$ resonances. Thus, it appears that the downfield movement of Pro $C_\beta$ upon complex formation in *cyclo*(Pro-Gly)$_3$ can be correlated with movement from one preferred rotamer to another *within the Pro $\psi$ trans′ region.*[17] (The trans′ region describes the rotational states of the Pro $C_\alpha$–C′ bond in which the Pro $C_\alpha$–H is trans to the Pro carbonyl oxygen atom.) Experiments of this nature currently lead only to an inference of the rotational state about Pro $C_\alpha$–C′ bonds. However, they do provide evidence that $^{13}C$ chemical shifts may indeed be sensitive enough to be useful for the determination of such subtle conformational changes.

When cations form complexes with ionophores

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(such as valinomycin), $^{13}C$ chemical shift changes are often maximal at the carbonyl carbons[25] and may be due directly to redistribution of electron densities from *binding* to the carbonyl oxygen atoms. Significant chemical shift changes which also occur at carbons more distant from the binding site (such as the Pro $C_\beta$ shift discussed above) are probably caused by the *conformational* change of the ionophore in going from the free to the bound state. In certain cases, changes in chemical shifts, even in carbonyl carbons, due to conformational effects may be in the opposite direction from those due to binding; therefore, little or no net change may result. Such a fortuitous cancelation of effects may explain the fact that the chemical shift of the glycine carbonyl carbon in *cyclo*(Pro-Gly)$_3$ is invariant even though three different types of complexes with divalent magnesium are formed (wherein $Mg^{2+}$ is surely bound in some or all of the complexes to the Gly carbonyl oxygens).[17]

When amino acid salts (such as amino acid ester hydrochlorides which are alkylammonium salts of the type $RNH_3^+Cl^-$) and *cyclo*(Pro-Gly)$_3$ or its octapeptide homologue, *cyclo*(Pro-Gly)$_4$, are mixed in chloroform solutions, significant chemical shift changes in both components are observed in $^{13}C$ spectra.[26] It was found also that diastereomeric pairs of complexes between L- or D-amino acid salts with the cyclic peptides were distinguishable by the splitting of carbon resonances belonging to the amino acid salts; as seen in Figure 3, *cyclo*(Pro-Gly)$_4$ displays the capacity for enantiomeric recognition between D- and L-Pro-OBz·HCl. Further confirmation that the amino acid and the cyclic peptide have formed a complex and are now tumbling as an entity in solution[27] was obtained by measurements of $^{13}C$ spin–lattice relaxation times. These ($T_1$'s) were found to differ for individual $\alpha$ carbons in the free salt and the free *cyclo*(Pro-Gly)$_3$. However, when the salt and the peptide are mixed, the $NT_1$'s (where $N$ is equal to the number of protons attached to each carbon) converge to a common value. Such complexes may be considered to be formally analogous to the chiral macrocyclic ether–amino ester complexes described by Cram and coworkers.[28]

Recently, lanthanide shift reagents have been used to give geometric information on entire peptide molecules.[22] Various strategies to pinpoint cation binding sites via the effect of shift reagents on $^{13}C$ NMR spectra are currently being explored in our laboratories.

**Conformational Energy Calculations.** The principal result of early conformational energy calculations in which atoms were treated as hard spheres is that an amino acid in a peptide chain is restricted to a few distinct conformations.[29] Subsequent introduction of intramolecular potential functions has allowed estimates of the relative stability of various conformational regions. For glycine only about 75%

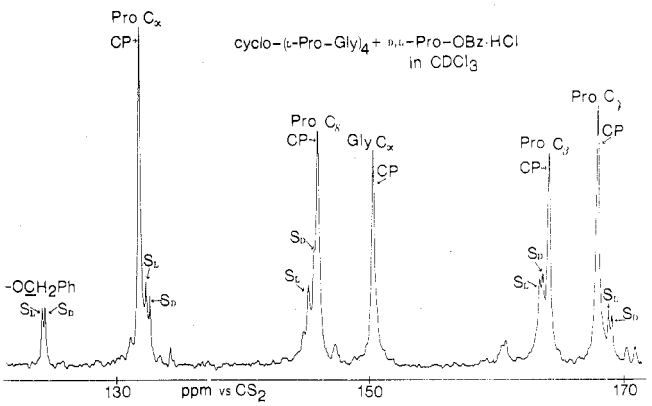


**Figure 3.** $^{13}C$ NMR spectrum (20 MHz) in chloroform-*d* of the upfield region (122–172 ppm vs. external $CS_2$) of *cyclo*(L-Pro-Gly)$_4$ complexed with D,L-Pro-OBz·HCl. Concentration of cyclic peptide is 50 mg/ml. The solution contains 0.5-equiv each of L-Pro-OBz·HCl and D-Pro-OBz·HCl per equivalent of *cyclo*(Pro-Gly)$_4$. Assignments of resonances (CP = cyclic peptide, S = salt) were made by comparison with related peptides. Other small resonances correspond to minor populations of cis peptide bond-containing conformations of *cyclo*(Pro-Gly)$_4$.[26a]

of the conceivable conformers are sterically reasonable, while for all other amino acids the set of available conformers is substantially reduced by the steric bulk of the side-chain atoms to less than 25% of the total.[30]

At present it is believed that computed intramolecular energy differences of <1 kcal/mol are insignificant. Furthermore, the majority of conformational energy calculations (largely as a matter of expediency) consider only intramolecular interactions, and the computed potential energies correspond to enthalpies rather than free energies of the structures. The latter approximation is usually acceptable, as the entropies of the various intramolecular states are often comparable. However, the neglect of peptide–solvent interactions is less justified, since such interactions often give significant contributions both to the enthalpy and entropy. The validity of initial attempts to include general aspects of peptide solvation in energy computations[31] remains to be demonstrated. In our opinion, the lack of an adequate representation of peptide–solvent free energies is the major shortcoming of present conformational energy calculations. The importance of solvent as a conformational determinant is emphasized by our studies of *cyclo*-(Pro-Gly)$_3$, where the conformer observed in polar solvents (water, methanol, dimethyl sulfoxide) has computed *intra*molecular energy 7 kcal/mol *above* the global minimum.[17]

Shown in Table I are typical potential functions and parameters[32] which are utilized to calculate (1) the change in a conformational variable ($\Delta x_1$) which will increase the energy 1 kcal/mol above minimum and (2) the additional increment ($\Delta x_2$) which will raise the energy another 1 kcal/mol (2 kcal/mol above the minimum). Three so-called "hard variables"—chemical bond lengths, bond angles, and one category of dihedral angles, namely, those which involve rotation about double bonds or partial double bonds

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Table I
Deformation of Various Conformational Variables to Give Indicated Increase in Energy[a]

| Type of deformation | Expression for potential energy | Typical value of parameter ($K$) | Deformation to give 1-kcal/mol increment | | Linear displacement, Å | |
|---|---|---|---|---|---|---|
| | | | $\Delta x_1$ | $\Delta x_2$ | $\Delta l_1$ | $\Delta l_2$ |
| Bond length | $K_b(\Delta b)^2$ | 200 | 0.07 Å | 0.03 Å | 0.07 | 0.03 |
| Bond angle | $K_\theta(\Delta\theta)^2$ | 0.0091 | 10° | 5° | 0.28 | 0.11 |
| Dihedral angles | | | | | | |
| $\omega$ | $\frac{1}{2}K_\omega \times (1 - \cos 2\omega)$ | 20 | 13° | 5° | 0.34 | 0.14 |
| $\chi$ | $\frac{1}{2}K_\chi \times (1 + \cos 3\chi)$ | 3 | 24° | 12° | 0.63 | 0.33 |
| $\phi$ and $\psi$ | $\frac{1}{2}K \times (1 + \cos 3X)$[b] | 0.2 to 0.5 | 180° | | 3.0 | |
| Nonbonded van der Waals | $K^*[(r^*/r)^{12} - 2(r^*/r)^6]$ | 0.2 | 0.40 Å | 0.14 Å | 0.40 | 0.14 |
| Electrostatic energy of a hydrogen bond | $CQ_1Q_2/(\epsilon r)$ | | 0.40 Å | 0.70 Å | 0.40 | 0.70 |

[a] Typical potential functions and parameters are employed. See Karolus and Lifson[33] for a fuller explanation of the potential functions and the variation of the parameters with the atoms involved. Appropriate units are used for $K$, so that the potential energies are in kcal/mol. $\Delta x_1$, change in parameter to give 1-kcal/mol increase above the minimum in potential energy. $\Delta x_2$, additional change in parameter to increase the energy a second kcal/mol. $\Delta l_1$, linear displacement corresponding to $\Delta x_1$. For angular variables $\Delta l = r\Delta\theta$ with $r$ = 1.5 Å. $\Delta l_2$, linear displacement corresponding to $\Delta x_2$. $r^*$ is the van der Waals radius (3.0 Å in this case). $r$ is the interatomic distance. Energy increments were calculated for decreases of $r$ from the van der Waals radius. $C$ is a constant (332) to convert the energy to kcal/mol. $Q_1$ and $Q_2$ are electronic charges as a fraction of the charge of an electron. In this case $Q_H$ = 0.27, $Q_O$ = −0.42. $\epsilon$ is the effective dielectric constant assumed to be 4. Energies were calculated for increase of the interatomic distance, $r$, from a typical hydrogen bond distance, 1.8 Å. [b] $X$ stands for either $\phi$ or $\psi$.

(such as the peptide bond)—allow only relatively small conformational changes (Table I). Of these, bond lengths allow the least variation. The angular variables allow somewhat larger displacements, and these variations can be amplified for atoms which are distant from the rotation axis. In addition, there are two minima in the torsional potential energy for partial double bonds, corresponding to cis and trans isomers. Rotation about single bonds—corresponding to the peptide dihedral angles $\phi$ and $\psi$ (as well as the side-chain dihedral angles)—is the most effective means of producing conformational change (Table I). Dihedral angles in this second category are called "soft variables". Torsional barriers for rotations about the N–$C_\alpha$ bond ($\phi$) and the $C_\alpha$–C′ bond ($\psi$) have generally been considered to be small (0.2 to 0.5 kcal/mol), so that these dihedral angles would be free to assume any value if it were not for steric restrictions.

At room temperature $RT$ is 0.6 kcal/mol, and a structure 1 kcal/mol above the local potential energy minimum would have a Boltzmann weighting factor of 0.19 (compared to 1.0 at the minimum), so that thermal fluctuations of the order $\Delta x_1$ are likely (see Table I). Furthermore, more than one local minimum may be significantly populated—a second conformer 1 kcal/mol above the minimum would contain about 15% of the population. These caveats should be kept in mind to counter the tendency to regard the minimum energy conformer as *the* exact state.

For strained systems, such as cyclic dipeptides, the hard variables (bond lengths and bond angles) and soft variables (dihedral angles) are highly interdependent, so that both sets are adjusted to produce an energy minimum.[22,33] However, for larger molecules such an energy minimization would require astronomical amounts of computer time; yet there could be no assurance that a true minimum (rather than a local minimum) would be reached. Fortunately, most large molecules (such as cyclic hexapeptides) are unstrained, so that the hard variables can be fixed at standard values—obtained primarily from x-ray diffraction studies of crystals. Thus, only rotations about single bonds remain to be considered. It should be noted that the assumptions of standard bond lengths and angles are precisely those made in constructing molecular models. Nevertheless, with a computer and a set of potential functions, one can systematically explore many more conformers than with a set of molecular models, and the computations result in *semiquantitative* estimates of relative conformational stability rather than esthetic judgments of what is *apparently* most reasonable.

When only rotations about single bonds are considered (e.g., $\phi$ and $\psi$ rotations), nonbonded van der Waals and electrostatic potentials dominate the intramolecular potential energy. The repulsive term of the van der Waals energy is only a slight modification of a "hard-sphere potential" and defines sterically accessible conformational regions (i.e., those with no extensive overlap of nonbonded atoms). The attractive term of the van der Waals energy for an individual contact is small (only a few tenths of a kcal/mol at the van der Waals radius, the distance of minimum energy). For neutral molecules the electrostatic potential is significant only for polar atoms within a few angstroms of each other. These properties of the van der Waals and electrostatic terms bias intramolecular energy calculations toward structures with many atoms in contact (at or near the sum of their van der Waals radii), while inclusion of solute—solvent interactions would shift the conformational distribution toward more extended structures (intramolecular contacts being replaced by solute—solvent contacts).

The restricted conformational freedom of cyclic peptides compared to their linear counterparts is a result of steric contacts and the fact that the cyclization conditions can be used to specify six dihedral angles in terms of the remaining, independently variable, dihedral angles.[34] A systematic exploration of conceivable *cyclo*(Pro-Gly)$_3$ conformers[35] uncovered

(33) S. Karplus and S. Lifson, *Biopolymers*, 10, 1973 (1970).

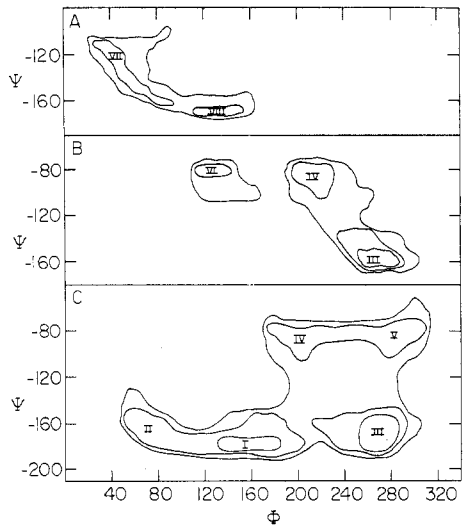


**Figure 4.** Potential energy contours for *cyclo*($Gly_1$-Pro-$Gly_2$)$_2$ $C_2$-symmetric conformers. $\phi_{Pro}$ was fixed at −68°. The $(\phi,\psi)$ angles of $Gly_2$ are determined from the cyclization conditions as a function of the three independently variable dihedral angles, $(\phi,\psi)_{Gly_1}$ and $\psi_{Pro}$. Energy contours are plotted at 1-kcal/mol intervals vs. $(\phi,\psi)_{Gly_1}$. The Roman numerals indicate local energy minima (see Table II). $\psi_{Pro}$ angles were selected near these minima: (a) $\psi_{Pro}$ = −60°; (b) $\psi_{Pro}$ = 170°; (c) $\psi_{Pro}$ = 100°.

an important low-energy class which is stabilized by 1←3 hydrogen-bonded turns (see Figure 2). Precise models for other *cyclo*(Pro-Gly)$_3$ conformational types were derived from experimental CD and NMR spectra utilizing the framework provided by the energy calculations.[17]

NMR experiments on a second type of cyclic hexapeptide, *cyclo*(X-Pro-Y)$_2$, where X and Y are various amino acids, have shown that the majority of the conformers for these cyclic hexapeptides are $C_2$ symmetric.[36] Since glycine has more conformational freedom than any other amino acid, the stable conformational regions for all *cyclo*(X-Pro-Y)$_2$ peptides will be contained within the regions found for *cyclo*(Gly-Pro-Gly)$_2$. Local intramolecular potential energy minima for $C_2$-symmetric conformations of *cyclo*(Gly-Pro-Gly)$_2$ are indicated in Figure 4 and Table II. NMR experiments have demonstrated that many of the *cyclo*(X-Pro-Y)$_2$ hexapeptides form structures containing two $\beta$ turns, which may or may not be stabilized by 1←4 intramolecular hydrogen bonds.[37a] The regions numbered I, VII, and VIII in Figure 4 contain such $\beta$-turn structures (types II, II′, and I, respectively, according to the nomenclature of Venkatachalam).[37b] Examination of molecular models revealed that structures within other regions of Figure 4 (II, V, and VII) have four carbonyl oxygens clustered in an array which appeared suitable for cation binding. This observation suggested experiments which have now demonstrated that *cyclo*(X-Pro-Y)$_2$ peptides bind cations with equilibrium constants and selectivities comparable to those of naturally occurring peptides.[38]

**Table II**
**Minimum Energy Conformers Computed for *cyclo*($Gly_1$-Pro-$Gly_2$)$_2$[a]**

| Region | $(\phi,\psi)_{Gly_1}$ | $\psi_{Pro}$ | $(\phi,\psi)_{Gly_2}$ | PE |
|---|---|---|---|---|
| I | 160, −180 | 100 | 65, 74 | −6.3 |
| II | 80, −160 | 80 | 80, 154 | −6.1 |
| III | −80, −180 | 100 | 133, −80 | −6.6 |
| IV | −160, −80 | 140 | 85, −73 | −5.1 |
| V | −60, −80 | 100 | 101, −166 | −4.9 |
| VI | 120, −80 | 170 | −56, 97 | −6.8 |
| VII | 60, −110 | −50 | −70, 77 | −4.2 |
| VIII | 150, −170 | −60 | −100, 76 | −3.7 |

[a] All conformers are $C_2$ symmetric. Proline $\phi$ was fixed at −68°. All angles in degrees according to 1970 convention (IUPAC–IUB Commission on Biochemical Nomenclature, *Biochemistry*, **9**, 3471 (1970)). PE in kcal/mol computed as the sum of van der Waals and electrostatic terms assuming an effective dielectric constant of 4. Since bond lengths and angles were fixed at standard values, no contribution was included for bond stretching or bending potentials.

**Circular Dichroism.** Many types of spectroscopy (NMR, infrared, and absorption) primarily measure properties of individual groups with conformational effects providing minor perturbations of the basic spectrum. However, optical activity (circular dichroism and optical rotatory dispersion) depends uniquely upon molecular geometry. For chromophores which have a plane of symmetry (such as the peptide group), optical activity is a consequence only of the asymmetric array of interacting chromophores and perturbing polar groups. In view of this, optical activity measurements have been widely utilized to monitor conformational states of biopolymers.[39]

Because relatively little material is required (0.01 to 1 mg of peptide) and peptide concentrations can routinely be varied over a 1000-fold range ($10^{-6}$ to $10^{-3}$ *M*), CD spectra provide an attractive means for the measurement of peptide–cation complexes. CD spectra characteristic of *cyclo*(Pro-Gly)$_3$ and three of its cation complexes are shown in Figure 5. The variations in CD reflect changes in the relative orientations of the peptide groups upon binding. At the higher peptide concentrations ($10^{-3}$ *M*) the amount of certain peptide complexes becomes nearly linearly dependent on the amount of added cation (see Figure 6) due to binding of almost all (>95%) of the cation added. Thus, both stoichiometries and limiting ellipticities can be determined directly. For complexes with smaller formation constants, stoichiometries can be inferred from the shape of the binding curve.

Determination of equilibrium constants often requires analysis of binding curves which contain free peptide and two or more types of complex. The concentrations of all species can be calculated from the ellipticities at selected wavelengths and the total concentrations of peptide and cation. Expressions for various equilibrium constants, relationships between them, and numerical values for *cyclo*(Pro-Gly)$_3$ binding of $Mg^{2+}$ are given in Table III.

In determining peptide conformation, symmetry

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(35) V. Madison, *Biopolymers*, **12**, 1837 (1973).
(36) (a) L. G. Pease, C. M. Deber, and E. R. Blout, *J. Am. Chem. Soc.*, **95**, 258 (1973), and references therein; (b) L. G. Pease and C.-H. Niu, unpublished data.
(37) (a) V. Madison in "Peptides, Polypeptides and Proteins", E. R. Blout, F. A. Bovey, M. Goodman, and N. Lotan, Ed., Wiley, New York, N.Y., 1974, pp 89–98; (b) C. M. Venkatachalam, *Biopolymers*, **6**, 1425 (1968).
(38) C.-H. Niu, V. Madison, L. G. Pease, and E. R. Blout, manuscript in preparation.
(39) For reviews, see: (a) I. Tinoco, Jr., and C. R. Cantor, *Methods Biochem. Analy.*, **18**, 81 (1970); (b) E. R. Blout in "Fundamental Aspects and Recent Developments in Optical Rotatory Dispersion and Circular Dichroism", F. Ciardelli and P. Salvadori, Ed., Heyden & Son, Ltd., London, 1973, pp 352–372.

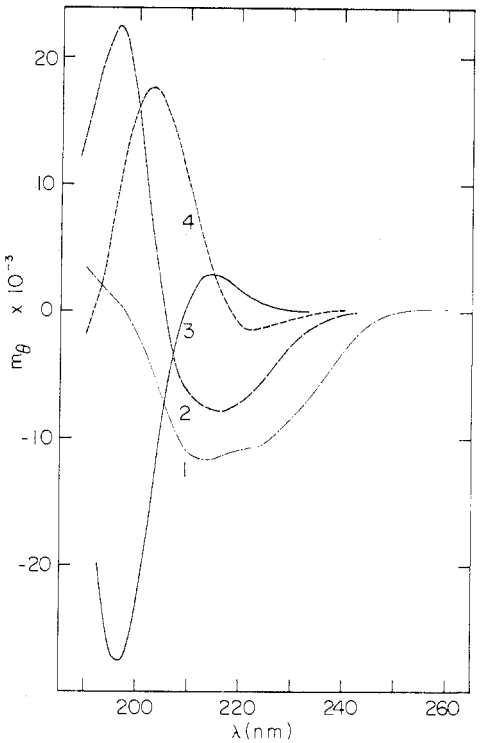


**Figure 5.** CD spectra (mean residue ellipiticity) of *cyclo*(Pro-Gly)$_3$ and its $Mg^{2+}$ complexes in acetonitrile. Below [P] will denote peptide concentration. The various species and experimental conditions are: (1) free peptide, [P] = $8.10 \times 10^{-4}$ *M*, [$Mg^{2+}$] = 0.0 (— - — - — - —); (2) peptide sandwich, [*cyclo*(Pro-Gly)$_3$]$_2$—$Mg^{2+}$, [P] = $8.10 \times 10^{-4}$ *M*, [$Mg^{2+}$] = $4.05 \times 10^{-4}$ *M* (— — — —); (3) 1:1 complex, [P] = $2.80 \times 10^{-5}$ *M*, [$Mg^{2+}$] = $1.40 \times 10^{-4}$ *M* (———); (4) ion sandwich, *cyclo*(Pro-Gly)$_3$–$Mg_2^{4+}$, [P] = $8.10 \times 10^{-4}$ *M*, [$Mg^{2+}$] = $2.00 \times 10^{-2}$ *M* (- - -).

rules can be very useful, especially when a particular local interaction dominates the spectrum and provided that the mechanism responsible for the optical activity can be identified.[40] For *cyclo*(Pro-Gly)$_3$ the peptide quadrant rule[40] for the one-electron mechanism allows the conclusions that (a) nonplanar hydrogen bonds between two planar peptide groups are primarily responsible for the large negative CD band observed in nonpolar solvents (Figure 7) and (b) direct contributions to the circular dichroism by bound cations are precluded by the $C_3$ symmetry of its metal complexes. In general, derivation of conformational details from CD spectra—even for small peptides—requires prediction of the complete spectrum via digital computer.

For the prediction of theoretical CD of discrete peptide structures, the configuration interaction method developed by Schellman and coworkers[41] has been adopted. This method simultaneously considers all interactions between excited states and yields slightly more realistic molecular states than the additive treatments of first-order perturbation theory (which was featured in the pioneering work on oligopeptides of Woody and Tinoco[42]). In our work only the two lowest energy transitions of each amide group are considered, since these two transitions have been well characterized experimentally. The restricted theory (two transitions/amide) is an approxi-

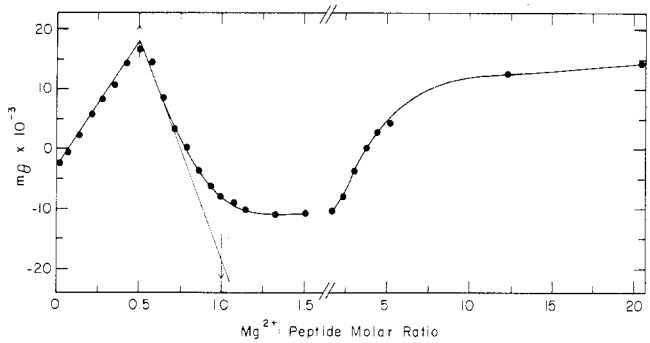


**Figure 6.** Variation of *cyclo*(Pro-Gly)$_3$ mean residue ellipticity at 200 nm with $Mg^{2+}$ concentration. Peptide concentration was $8.10 \times 10^{-4}$ *M* in acetonitrile solution. Extrapolations of linear portions of the plot to $Mg^{2+}$:peptide molar ratios of 0.5 and 1.0 are indicated.

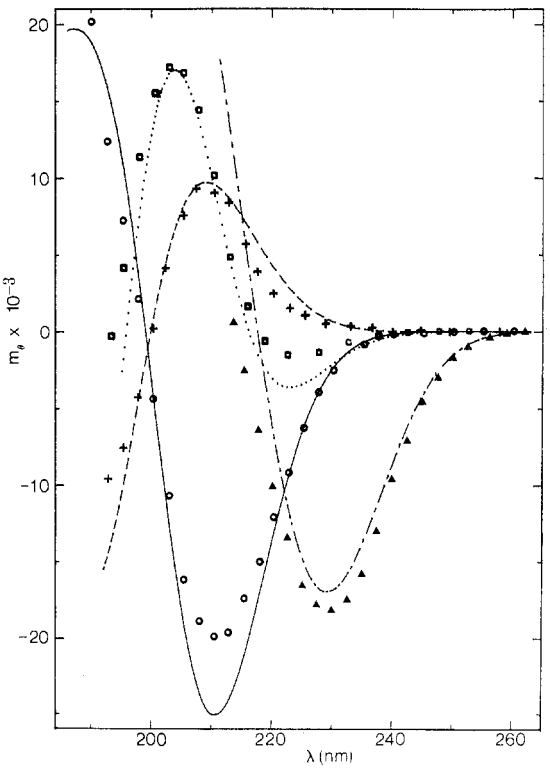


**Figure 7.** Comparison of theoretical (lines) and experimental (points) CD spectra for various *cyclo*(Pro-Gly)$_3$ conformers. See ref 17 for details. Experiment: (○) in water, (▲) in dioxane, (□) 0.06 *M* $Mg(ClO_4)_2$ in acetonitrile, and (+) 0.5 *M* $Ca(ClO_4)_2$ in water. Theory: (———) asymmetric conformer with one cis bond, (— - — - — -) γ-turn $C_3$-symmetric conformer, (· · · ·) 2:1 $Mg^{2+}$ peptide complex, (- - - -) 1:1 $Mg^{2+}$ peptide complex.

mation, as further transitions of the amide group and those of other chromophores (including alkyl groups) in the molecule are not included. Nevertheless, the restricted theory has proven successful in predicting the optical properties of small peptides,[43] polypeptides,[42,44] and segments of globular proteins.[45]

One method of comparing theoretically predicted CD with that experimentally observed is to resolve the experimental spectrum into component bands

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(42) R. W. Woody and I. Tinoco, Jr., *J. Chem. Phys.*, **46**, 4927 (1967).

(43) (a) T. M. Hooker, Jr., P. M. Bayley, W. Radding, and J. A. Schellman, *Biopolymers*, **13**, 549 (1974); (b) E. B. Nielsen and J. A. Schellman, *ibid.*, **10**, 1559 (1971).

(44) (a) R. W. Woody, *Biopolymers*, **8**, 669 (1969); (b) R. W. Woody, *J. Chem. Phys.*, **49**, 4797 (1968); (c) E. S. Pysh, *J. Chem. Phys.*, **52**, 4723 (1970).

(45) V. Madison and J. Schellman, *Biopolymers*, **11**, 1041 (1972).

Table III
Equilibrium Expressions and Binding Constants for Various Peptide–Cation Complexes

| Symbol[a] | Equilibrium expressions: Concentrations[b] | Equilibrium expressions: Mole fractions[c] | Interrelationships[d] | Numerical values[e] for *cyclo*-(Pro-Gly)$_3$–$Mg^{2+}$ |
|---|---|---|---|---|
| $K_{0,½}$ | $[P_2C]/([P]^2[C])$ | $X_2/(2P_0X_1^2[C])$ | | $5.0 \times 10^9\ M^{-2}$ |
| $K_{0,1}$ | $[PC]/([P][C])$ | $X_3/(X_1[C])$ | $(K_{0,½}K_{½,1})^{½}$ | $1.0 \times 10^5\ M^{-1}$ |
| $K_{0,2}$ | $[PC_2]/([P][C]^2)$ | $X_4/(X_1[C]^2)$ | $K_{0,1}K_{1,2}$ | $6.4 \times 10^7\ M^{-2}$ |
| $K_{½,1}$ | $[PC]^2/([P_2C][C])$ | $2P_0X_3^2/(X_2[C])$ | $K_{0,1}^2/K_{0,½}$ | 2.5 |
| $K_{½,2}$ | $[PC_2]^2/([P_2C][C]^3)$ | $2P_0X_4^2/(X_2[C]^3)$ | $K_{0,2}^2/K_{0,½}$ | $8.2 \times 10^5\ M^{-2}$ |
| $K_{1,2}$ | $[PC_2]/([PC][C])$ | $X_4/(X_3[C])$ | $K_{0,2}/K_{0,1}$ | $6.4 \times 10^2\ M^{-1}$ |

[a] Subscripts on $K$ indicate cation:peptide stoichiometry, the first subscript for reactant, the second for product. [b] P is used to denote peptide; C to denote cation; and $P_0$ to denote total peptide concentration. [c] $X$'s are mole fractions of peptide: $X_1$ for P, $X_2$ for $P_2C$, $X_3$ for PC, and $X_4$ for $PC_2$. [d] Only three of these constants are independent. [e] $K_{0,½}$, $K_{0,1}$, $K_{½,1}$, and $K_{1,2}$ were experimentally determined within estimated errors of 10–20% from CD spectra in acetonitrile solutions.

whose rotatory strengths (magnitudes) and transition energies (wavelengths) can be directly compared to the corresponding predicted values. This method is preferable when a unique correspondence can be established between each experimental band and a molecular transition. Due to the breadth of CD bands, only rarely can more than four or five bands be resolved in the region of peptide transitions. Thus, for molecules with more than four (or five) nondegenerate transitions (i.e., more than three (or four) peptide groups), it is usually preferable to compute theoretical spectra rather than attempting to resolve the experimental spectrum into component bands. In computing theoretical spectra, bandwidths must be determined independently (e.g., from absorption spectra of model compounds), since prediction of bandwidths is beyond the realm of simple theories.

If a unique correspondence can be established between CD predicted for a given conformational type and that experimentally observed, it can be inferred that this is the type of conformer present in solution. The procedure we follow, when feasible, is to predict CD for all conformational regions of low intramolecular energy. In cases where exploration of all conformers is impractical, the molecule may be restricted to a certain symmetry or arrangement of cis and trans peptide bonds as indicated by NMR experiments. For *cyclo*(Pro-Gly)$_3$ all conformational regions were explored and each region has a unique type of theoretical CD spectrum.[17] A very good match is obtained between each of four theoretical spectral types and experimental CD observed under four sets of conditions (Figure 7). In this case, the four conformational states can be assigned on the basis of the CD data alone. These conformational assignments are supported by a variety of NMR data.[17] In contrast, for *cyclo*(X-Pro-Y)$_2$ peptides conformational assignments are difficult on the basis of CD data only, since for these molecules the predicted CD spectra are qualitatively similar for several conformational regions. Thus, additional data are necessary to distinguish among these regions.

## Conclusions

Since it is not always possible to duplicate native conditions and since naturally occurring peptides are often quite complex, we have designed and synthesized some peptides which are more amenable to conformational analysis. We have illustrated the types of conformational information which can be extracted from NMR and CD spectra of such molecules with the aid of theoretical computations of CD spectra and conformational energies. $^{13}C$ NMR spectra (1) identify cis and trans isomers of X–Pro bonds, (2) indicate (along with $^1H$ spectra) the degree of conformational symmetry (or asymmetry), and (3) can be used to study conformational changes—for example, those accompanying interactions between cations and peptides. Potential energy calculations yield a qualitative guide to relative potential energies so that further conformational details can be extracted from experimental data. Conversely, experimental spectra may indicate that the conformational distribution need be computed only for certain types of structures (e.g., $C_2$ symmetric). CD spectra are a convenient means of monitoring conformational change and, when coupled with theoretical treatments, can yield details of three-dimensional molecular structure. This interplay of techniques augments the predictive power of the collective methods and should aid in delineating the relative influences of sequence and surroundings on conformation of biological macromolecules.

*We are pleased to acknowledge the continuing support of our work in this field by U.S. Public Health Service Grants AM07300 and AM10794. In addition, during part of the period the work reported here was in progress, C.M.D. and V.M. were holders of National Institutes of Health postdoctoral fellowships.*