tide/lipid ratio were necessary to observe sufficient TRNOE signals.[14] In the present UVRR experiments, on the other hand, enkephalin was mixed with neutral lipid at 1:10 mole ratio. Since TRNOE signal was very weak in the absence of charged lipids, contribution of weakly bound species to the UVRR spectrum may be neglected. Free peptides in the aqueous phase cannot be the major component either, because UVRR spectra showed large changes in the amide and aromatic side chain vibrations on going from aqueous solution to the peptide–liposome mixture. Accordingly, the binding mode of enkephalin revealed by the present UVRR experiments concerns the tightly bound peptides, which are the major component at low peptide/lipid ratios. The formation of tightly bound complex may account for the observation that NMR signals got broader and the signal-to-noise ratio of TRNOE signals decreased with increasing amount of the lipid added to the enkephalin solution.[14] A chromatographic study suggested the presence of tightly bound enkephalin, which remained bound even after gel filtration.[50] In living cells, enkephalin is likely to bind to the cell membrane in two steps. The initial step is weak binding to the cell surface through electrostatic interaction, and the next step is tight binding through hydrophobic interaction.

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Conclusions

Met-enkephalin and Leu-enkephalin exist as an ensemble of various extended conformers in aqueous solution. In the liposome-bound state, the peptide backbone takes a folded conformation predominantly. The Tyr side chain of Met-enkephalin is mostly inserted into the hydrophobic region of the lipid bilayer with its phenolic hydroxyl group hydrogen-bonded with a proton acceptor of the lipid, whereas the Phe side chain is located in the polar head group region or exposed to the aqueous phase. For Leu-enkephalin, the Tyr side chain is less buried in the membrane than for Met-enkephalin and the hydroxyl group is hydrogen-bonded with water. [Trp^4]Met-enkephalin also has a folded main-chain conformation in the membrane-bound state, and the Tyr and Trp side chains are located in the hydrophobic and hydrophilic regions, respectively, in a manner similar to the corresponding side chains of Met-enkephalin. The hydrophobicity of the Tyr environment in the membrane-bound state correlates with the receptor affinity and opiate activity. UVRR spectroscopy proves to be a useful tool for investigating the environments of aromatic side chains of membrane-bound peptides.

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Conformational Analysis of Cyclic Hexapeptides Containing the D-Pro-L-Pro Sequence To Fix β-Turn Positions

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Abstract: Two cyclic hexapeptides, cyclo(D-Pro-Pro-Gly-Arg-Gly-Asp) (**1**) and cyclo(D-Pro-Pro-Arg-Gly-Asp-Gly) (**2**), containing a D-Pro-Pro sequence introduced to define and fix the phase of a two-β-turn backbone were examined by proton NMR methods. The NMR data were used in constrained distance geometry conformation searches to identify the probable backbone conformations. The conformation searches were conducted using upper and lower bound constraints derived from observed nuclear Overhauser effects (NOEs) involving backbone protons and also using additional lower bound constraints from backbone and β-proton NOEs judged to be unobserved. The two procedures gave substantially the same results although the dihedral angle ranges were narrower with the additional constraints. In each case, the D-Pro-Pro sequence unequivocally adopted a type II′ β-turn conformation as anticipated and stabilized the second turn across the ring from it. In the case of the Arg-Gly sequence of **1**, a type II turn was predominant; for the Gly-Asp sequence of **2**, structures with type I, II, II′, and III turns were all returned by the constrained search, and there were no experimental grounds to suggest a preference.

Introduction

Synthetic cyclic peptides of stable backbone conformation can be tools for mapping the biologically active conformations of peptide sequences and, thus, an intermediate step on the path from polypeptide to peptidomimetic. We describe here the conformational component of one such study, in which two successive proline residues are used to limit the conformational freedom of cyclic hexapeptides.

The two-β-turn backbone conformation of cyclic hexapeptides and the ability of proline residues to define its phase by occupying the $i + 1$ position of one of the turns are both well documented,[1-15] as is the preference of proline for the $i + 1$ position of β-turns in proteins.[16] The principal conformation-determining feature of proline is the pyrrolidine ring-induced restriction of the backbone dihedral angle ϕ to the 50–90° ranges, the sign being negative

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Table I. Cyclic Hexapeptide Examples with Proline in the $i + 1$ Position of a Two-β-Turn Backbone

cyclic hexapeptide[a]			crystal structure	solution studies
Gly	Gly	**Pro**	1	12,13
Pro	Gly	Gly		
ala	Gly	**Pro**	2	14
Pro	Gly	Ala		
phe	Gly	**Pro**	3	15
Pro	Gly	phe		
phe	Ala	**Pro**	4	15
Pro	Ala	phe		
Trp	Phe	**pro**	5	5–7
Phe	Thr	Phe		
Ala	Gly	**Pro**	8,9	
Phe	Phe	Val		
Pro	Leu	**Pro**	10	11
Gly	Tyr	Gly		

[a] Residues in all lower cases are of the D configuration. Pro in the $i + 1$ position of a turn is indicated in boldface type.

for L-proline and positive for D-proline. These ranges are also those preferred for the ϕ_{i+1} angle of β-turns.[17,18] Table I lists the instances of cyclic hexapeptide backbones containing trans Xxx-Pro bonds in which X-ray crystallographic analyses demonstrate the expression of this conformational preference and cites references to relevant NMR studies.

A limitation in the ability of proline to define the $i + 1$ position arises from potential steric interference between the proline δ-methylene group and the side chain of the preceding residue when they are of the same stereochemical configuration.[19] Larger side chains in the preceding residue tend increasingly to force cis Xxx-Pro peptide bonds in cyclic hexapeptides, as has been observed both in solution[20,21] and in the crystal.[22,23] To avoid this interference and the resulting conformational ambiguity, D- rather than L-proline may be incorporated after an L-series residue, as in the cyclic hexapeptides incorporating the Phe-D-Pro-Phe sequence studied by Kessler et al.[5-7] However, this change introduces an uncertainty in the phase of a two-turn backbone, in that a turn with the L residue at $i + 1$ and D-Pro at $i + 2$ becomes a possibility. Proline in the $i + 2$ position of a turn, connected by a trans peptide bond to a preceding residue of the opposite configuration, occurs in gramicidin S and is demonstrated in cyclic hexapeptides.[24]

In some cyclic peptides, turns have been found with proline in the $i + 2$ position, preceded by a residue of the same configuration but joined to it by a cis peptide bond.[6,25-27] This introduces an additional degree of freedom when a single proline is used to establish the phase of the two-β-turn backbone. In this connection, the X-ray structure of cyclo(Pro-Pro-Gly-Pro-Leu-Gly), which exhibits cis Gly-Pro and Pro-Pro peptide bonds and a Pro-Leu β-turn, is also worth noting.[28]

Table II. Proton Chemical Shift and Coupling Constant Data for the Cyclic Hexapeptides in 5:3 DMSO-d_6/Sulfolane-d_8, 303 K

residue	HN, δ	J_{HNCH}, Hz	H^{α}, δ	$J_{\alpha\beta}$, Hz	H^{β}, δ	H^{δ}, δ
A. Cyclo(D-Pro-Pro-Gly-Arg-Gly-Asp) (**1**)						
D-Pro			4.55	8.3	2.10	3.65
				4.3	1.91	3.51
Pro			4.32	8.1	2.09	4.04
				2.6	1.95	3.62
Gly	7.34	8.2	4.21			
		2.7	3.45			
Arg	8.54	2.9	3.79	7.5	1.74	3.13
				7.5	1.63	
Gly	8.68	6.4	3.83			
		6.4	3.52			
Asp	7.56	8.1	4.85	9.7	2.59	
				3.5	2.36	
B. Cyclo(D-Pro-Pro-Arg-Gly-Asp-Gly) (**2**)[a]						
D-Pro			4.50	8.0	2.17	3.56
				7.5	1.80	3.52
Pro			4.43		2.02	3.86
						3.60
Arg	7.56	8.1	4.52	7.5	1.90	
				7.3	1.85	
Gly	8.58	5.2	3.79			
		4.9	3.53			
Asp	7.89	8.2	4.59	7.3	2.78	
				4.1	2.69	
Gly	7.27	5.8	4.18			
		2.6	3.77			

[a] Chemical shift temperature coefficients (ppm/deg) for the NH protons of **2**: Arg, 0.0027; Gly^4, 0.0080; Asp, 0.0040; Gly^6, 0.0017.

In most classes of β-turns, the ϕ_{i+1} and the ϕ_{i+2} angles are both in the ±(50–90°) ranges accessible to proline.[17,18] Therefore, two successive prolines of opposite stereochemical configuration ought to be a stronger and less ambiguous turn determinant for a cyclic peptide than an isolated proline. If the first proline is to be preceded by an L residue, the turn-determining sequence should be D-Pro-L-Pro.

In this paper, we report on the probable solution conformations of two cyclic hexapeptides containing the D-Pro-L-Pro sequence, cyclo(D-Pro-Pro-Gly-Arg-Gly-Asp) (**1**) and cyclo(D-Pro-Pro-Arg-Gly-Asp-Gly) (**2**), which are synthesized as conformationally defined examples of the Arg-Gly-Asp sequence important in certain cellular recognition processes. By means of a distance geometry search incorporating distance constraints derived from nuclear Overhauser effect (NOE) measurements, we have established that the D-Pro-L-Pro sequence does indeed adopt a type II′ β-turn conformation and that it thereby stabilizes a second, opposite, turn in the cyclic hexapeptide. Two distance geometry based conformation searches were carried out: one with upper and lower bound distance constraints derived from observed NOEs between backbone protons only, and one with these constraints plus the additional restrictions of lower bounds for backbone distances for which NOEs were judged to be unobserved (anti distance constraints).[29]

Methods

Synthesis. Cyclo(D-Pro-Pro-Gly-Arg-Gly-Asp) and cyclo(D-Pro-Pro-Arg-Gly-Asp-Gly) were synthesized by Dr. Fadia E. Ali. Their preparation and other characterization will be reported elsewhere.[30]

NMR Measurements. Proton NMR spectra of cyclo(D-Pro-Pro-Gly-Arg-Gly-Asp) (**1**) were obtained employing a JEOL GX500 spectrometer, and NMR data were processed using the program package FTNMR (Hare Research, Woodinville, WA). A 5 mM solution in 5:3 dimethyl sulfoxide-d_6/sulfolane-d_8 at 30 °C was used. Assignments were made from PECOSY,[31] TOCSY, and NOESY spectra. The TOCSY

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Table III. Observed Nuclear Overhauser Cross Peaks[a] and Derived Constraints for Cyclo(D-Pro[1]-Pro[2]-Gly[3]-Arg[4]-Gly[5]-Asp[6]) (**1**) in 5:3 DMSO-d_6/Sulfolane-d_8, 303 K

atom	atom	lower bound	upper bound
1HA	2HD2	1.92	2.45
1HA	2HD1	1.99	2.54
3HN	3HA1	2.28	2.91
3HN	3HA2	2.14	2.74
3HN	2HD1	2.46	3.14
4HN	3HA2	2.11	2.69
4HN	4HA	2.18	2.78
4HN	3HA1	2.05	2.62
5HN	5HA1	1.81	2.32
5HN	5HA2	1.99	2.54
5HN	4HA	2.01	2.56
5HN	6HN	2.41	3.08
6HA	1HD1	1.97	2.51
6HN	6HA	2.17	2.77
4HN	4HB1	2.20	2.81[b]
4HN	4HB2	2.12	2.70[b]
6HA	6HB1	1.80	2.29[b]
6HA	6HB2	1.81	2.32[b]

[a] NOEs from geminal pairs are not listed. [b] Constraints derived from these NOEs were not used. Because of assignment ambiguities, interactions between 5HA2 and 6HA and/or 1HD2 were also omitted. See text.

mixing time was 60 ms (MLEV-17). Coupling constants were measured from the PECOSY spectrum or from a 1-D spectrum. NOESY spectra were measured at mixing times of 75, 150, and 250 ms. The chemical shift and coupling constant data are reported in Table IIA.

Data for cyclo(D-Pro-Pro-Arg-Gly-Asp-Gly) (**2**), also from a 5 mM solution in 5:3 dimethyl sulfoxide-d_6/sulfolane-d_8 at 30 °C, were collected employing a Bruker AMX500 spectrometer and were processed using the program package FELIX (Hare Research, Woodinville, WA). Again, assignments were made from PECOSY, TOCSY (60 ms MLEV-17 mixing), and NOESY spectra, and coupling constants were measured from PECOSY or 1-D spectra. Chemical shifts and coupling constants are reported in Table IIB. NOESY spectra were measured at mixing times of 50, 100, 150, and 200 ms, and distance constraints were derived from buildup rates as detailed below.

The NOE cross peak volumes at 250 ms were used to determine the doubly bound distance constraints for distance geometry calculations on cyclo(D-Pro-Pro-Gly-Arg-Gly-Asp) (**1**). Data from two Gly H^{α} and two Pro H^{δ} geminal proton pairs were available for distance calibration. The maximum deviation of these volumes from their mean was 23% (average deviation, 12%), corresponding to a distance error of about 4% and indicating sufficient legitimacy of the assumption of a single effective correlation time for the backbone atoms. The mean buildup rate was taken to correspond to 1.75 Å. The Pro H^{α}–H^{β} couplings were sufficiently distinctive to allow prochiral assignment of the Pro β-protons.

For cyclo(D-Pro-Pro-Arg-Gly-Asp-Gly) (**2**), NOESY buildup rates were obtained by least-squares fitting of the four measured points. Only the buildup rates for which the linear fit gave correlation coefficients greater than 0.9 were used to determine the doubly bound constraints. Two NOEs for which the correlation coefficients were less than 0.9 were used to provide upper bound constraints. This molecule exhibited less chemical shift dispersion than **1**, and only two backbone geminal pairs were available for calibration. The mean of the buildup rates for the two cross peaks of the Pro[1] $H^{\beta 1}$–$H^{\beta 2}$ interaction was taken as corresponding to 1.75 Å. With that scaling, the Gly[6] $H^{\alpha 1}$–$H^{\alpha 2}$ distance was calculated from its cross peak buildup rates to be 1.72 Å. Prochiral assignments were not made.

For both peptides, only data for backbone protons, i.e., H^{α}, H^{N}, Pro H^{β}, and Pro H^{δ}, excluding intraresidue Pro H^{α}–H^{β} data, were used to determine upper and lower bound constraints for distance geometry calculations. The upper and lower bounds used were −10% and +15% of the distance calculated using the geminal proton reference. The observed nongeminal NOEs and the constraints derived from them are indicated in Tables III and IV.

Data from observed interactions between backbone protons and the Asp and Arg β-protons were not used for doubly bound constraints, since they almost certainly cannot correspond to single side chain conformations. In contrast to the situation in the interior of a protein molecule, there is no reason to expect that acyclic side chain motion will be constrained in molecules as small as these. For example, the observed H^{α}–H^{β}

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Table IV. Observed Nuclear Overhauser Cross Peaks[a] and Derived Constraints for Cyclo(D-Pro[1]-Pro[2]-Arg[3]-Gly[4]-Asp[5]-Gly[6]) (**2**) in 5:3 DMSO-d_6/Sulfolane-d_8, 303 K

atom	atom	lower bound	upper bound
1HA	2HD1	1.82	2.33
1HA	2HD2	1.84	2.35
1HD2	6HA1	2.18	2.78
2HD1	3HN	2.53	3.23
2HA	3HN	2.81	3.59
3HA	3HN	2.32	2.96
3HA	4HN	2.20[b]	2.51
4HN	4HA1	2.27[b]	2.55
4HN	4HA2	2.27[b]	2.57
4HA2	5HN	2.64	3.38
4HA1	5HN	2.20[b]	2.56
5HA	5HN	2.35	3.01
5HA	6HN	2.55	3.26
5HN	6HN	2.08	2.66
6HA2	6HN	2.21	2.82
6HA1	6HN	2.25	2.87
1HD1	6HA1	1.00[c]	3.00[c]
4HN	5HN	1.00[c]	4.00[c]
3HB2	3HN	2.25[d]	2.88[d]
3HB2	4HN	2.38[d]	3.05[d]
3HB1	4HN	2.15[d]	2.75[d]
5HA	5HB1	2.19[d]	2.79[d]
5HA	5HB2	2.05[d]	2.62[d]
5HN	5HB2	2.52[d]	3.22[d]
5HN	5HB1	2.47[d]	3.15[d]

[a] NOEs from geminal pairs are not listed. [b] Lower bound set at geometric limit instead of −10%. [c] Upper bound only set. See text. [d] Constraints derived from these NOEs were not used. Because of assignment ambiguities, interactions involving 1HG1, 1HG2, 1HB2 with 2HG1, and 2HG2 were omitted. See text.

coupling constants for Asp in **1**, 9.7 and 3.5 Hz, correspond to a dominant Asp χ^1 angle of −60° or 180°. However, the H^{α}–H^{β} NOEs (Table III) indicate two equal, short distances, which if taken at face value would correspond to $\chi^1 = +60°$. The contradiction is a consequence of the strong weighting of conformations with short interproton distances in the NOE average. In fact, when the data shown as unused in Tables III and IV were used as distance geometry input, seriously distorted peptide bond geometries were generated. On the other hand, the absence of NOEs to these β-protons could reasonably be included and were included among the anti distance constraints described below.

Potential interproton interactions that did not give rise to observed Overhauser cross peaks were used to provide additional lower bound constraints in a set of distance geometry calculations run in parallel with those using only observed data. These additional constraints are referred to as anti distance constraints (ADCs) by Brüschweiler et al.[29] Peaks were considered not observed when in the 250-ms experiment (**1**) or the 200-ms experiment (**2**) they were less than 3% of the intensity corresponding to the 1.75 Å distance of a geminal proton pair. That intensity would correspond to an interproton distance of >3.1 Å.

Certain backbone proton–proton interactions were omitted from the constraint list. These omitted constraints included all geminal interactions, all intra side chain interactions, including H^{α}–H^{β}, and all observed H^{N}–H^{β}. In addition, specific constraints were omitted because of assignment ambiguities arising from chemical shift degeneracies. These are listed in the footnotes to Tables III and IV. Otherwise, unless a backbone H–H interaction (H^{α}, H^{N}, Pro H^{β}, or Pro H^{δ}) is explicitly listed as a distance constraint in Tables III and IV, it was assigned as an anti distance constraint with a lower bound of 3.0 Å.

Conformation Searches. A total of 500 conformations were generated for **1** and **2**, with and without ADCs, using the distance geometry program DGEOM (available from QCPE, Department of Chemistry, Indiana University, Bloomington, IN 47405). Torsion angle sampling was applied during the runs, and distance correlation was turned off.[32] All bonds were considered fully rotatable, with the exception of amide bonds which could be either cis or trans. For **1**, a total of 14 distance constraints derived from NMR NOE data (Table III) were applied with and without 307 anti distance constraints which were assigned a lower bound of 3.0 Å. For **2**, 18 distance constraints (Table IV), including two for which only upper bounds were assigned, and 277 anti distance constraints were used. Each conformer was subjected to a full minimization using the

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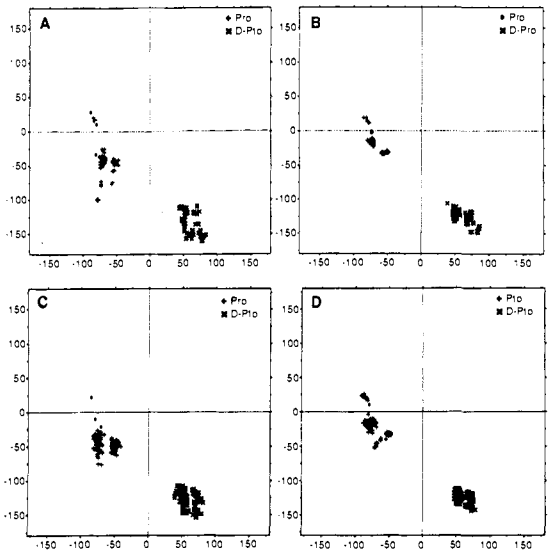

Figure 1. Ramachandran plots of D-Pro and Pro residues in NOE-constrained distance geometry search derived conformations meeting the energy and constraint violation criteria described in the text: A, cyclo(D-Pro-Pro-Gly-Arg-Gly-Asp) (1), distance constraints only (84 conformations); B, 1, distance constraints and anti distance constraints (142 conformations); C, cyclo(D-Pro-Pro-Arg-Gly-Asp-Gly) (2), distance constraints only (122 structures); D, 2, distance constraints and anti distance constraints (169 structures).

program AMBER 3.0[33] modified to accept distance bounds, with all partial charges set to zero to eliminate electrostatic energy terms. A full minimization is defined as either the norm of the gradient of the energy reaching 0.01 kcal/mol·Å or 10 000 iterations. In practice, no conformer required the full 10 000 iterations. The NMR-derived distance constraints were applied using a flat-bottomed well potential with harmonic sides such that no penalty was assessed for distances falling within the bounds. For the constraints assigned only upper bounds, a lower bound of 1 Å, a distance below van der Waals contact and thus unattainable, was used. For the anti distance constraints, an upper bound value of 999 Å, also unattainable, was used. A force constant of 50 kcal/mol·Å^2 was used for each constraint. Those conformers below 1.5 kcal/mol in constraint energy, a measure of the violation of the NOE bounds, were ordered on total energy, and the set of conformers within 10 kcal/mol of the lowest energy conformer was selected for analysis. For both molecules, the lowest energy conformer of the full 500 showed a constraint violation energy well below 1.5 kcal. As tests of conformational stability,[32] these selected conformers were subjected to an additional 100 iterations of minimization, this time including electrostatic terms (constant dielectric, $\epsilon = 1$; nonbond cutoff = 99 Å), and then to an additional 100 iterations with electrostatics, but without the NMR-derived constraints. For these calculations, AMBER 3.0, Revision A, was used.[34] As expected, some changes in dihedral angles occurred to favor hydrogen bonding and minimize electrostatic repulsion, but this procedure did not seriously distort the overall backbone conformations or move a conformation from one classification (see below) to another.

Results

Conformations. As described above in the Methods section, the NMR data constrained conformation searches for the two peptides were carried out under two protocols, one using only doubly bound distance constraints (DCs) derived from observed NOE data, the other including also a lower limit for additional interproton distances for which NOEs were not observed (anti distance constraints, ADCs[29]). The two protocols yielded substantially similar probable conformations, although the DC + ADC protocol yielded narrower dihedral angle distributions. The peptide conformations obtained are discussed here. Comment on the methodology appears in the next section.

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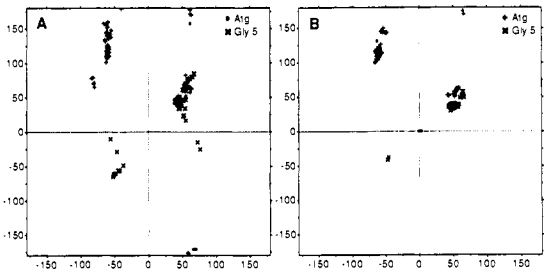

Figure 2. Ramachandran plots of Arg and Gly^5 (turn) residues in NOE-constrained distance geometry search derived conformations of cyclo(D-Pro-Pro-Gly-Arg-Gly-Asp) (1) meeting the energy and constraint violation criteria described in the text: A, distance constraints only; B, distance constraints and anti distance constraints.

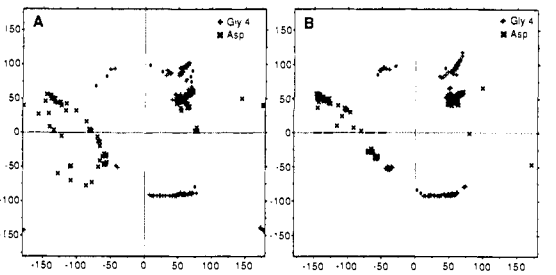

Figure 3. Ramachandran plots of Gly^4 and Asp (turn) residues in NOE-constrained distance geometry search derived conformations of cyclo(D-Pro-Pro-Arg-Gly-Asp-Gly) (2) meeting the energy and constraint violation criteria described in the text; A, distance constraints only; B, distance constraints and anti distance constraints.

One-dimensional spectra of the two cyclic hexapeptides did not reveal detectable minor components in slow exchange. Therefore it may be concluded that in each case a single cis–trans configuration of peptide bonds dominates, as the 15–18 kcal barrier to cis–trans peptide bond isomerization normally enforces slow exchange at room temperature. The absence of Xxx-D-Pro or D-Pro-Pro H^α–H^α NOEs indicates that cis peptide bonds are probably absent in the predominant conformations, and the full conformation searches do not yield energetically reasonable conformations with cis peptide bonds.

A total of 500 structures under each protocol were generated for each peptide. The full range of calculated total energies in a set of 500 structures was 120–150 kcal above the minimum, and the full range in constraint violation energy was 0 to 25–30 kcal. In each set of calculations, distorted or cis peptide bonds began to appear in those minimized structures that were about 15 kcal in total energy above the lowest energy structure. The conformations considered further, limited to a maximum violation energy of 1.5 kcal and 10 kcal in total energy above the lowest energy conformation meeting the 1.5 kcal condition, contained only undistorted trans peptide bonds.

Figure 1A–D, which shows ϕ–ψ plots of the proline residue conformations generated for the two peptides, illustrates two of the key results of this study. First, the D-Pro-L-Pro backbone dihedral angles correspond to type II′ β-turns only; other conformations for this residue pair are inconsistent with the NOE data and are not returned by the searches. Second, it can be seen that the use of the anti distance constraints narrows the dihedral angle ranges of the conformations returned by the searches and, in particular, brings the values for ψ(Pro) nearer the canonical 0° of the type II′ turn. β-turns in cyclic peptide crystal structures do in fact tend to have ψ_{i+2} near 0°. The effects of the anti distance constraints in narrowing the range of conformations are also seen in Figures 2 and 3, which show the conformation ranges of the residues that form the turn opposite the prolines. Results of the searches using both DCs and ADCs are examined below.

Probable Conformations of 1. Of the 500 structures of **1** generated by the distance geometry search and energy minimization protocol embodying both DCs and ADCs, 142 conformations meet the energy criteria set above. In each of the 142,

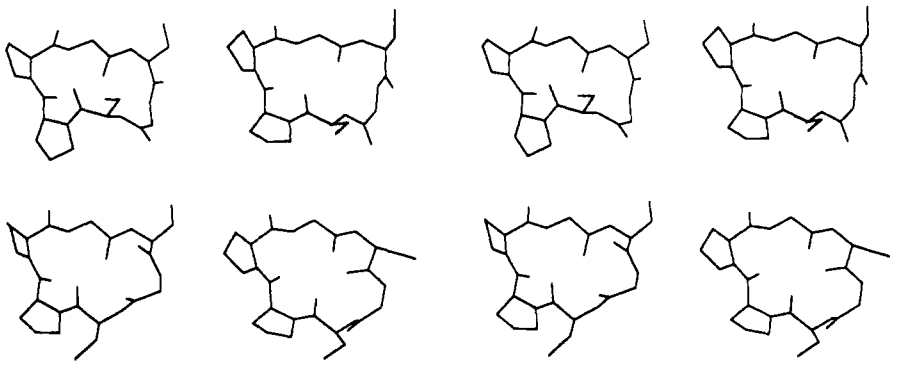

Figure 4. Stereoplot of the lowest energy exemplars of all four backbone types generated by the constrained distance geometry search for cyclo(D-Pro-Pro-Gly-Arg-Gly-Asp) (**1**). To avoid confusion of β-carbons with carbonyl oxygen atoms, γ-carbons, although not determined by NMR data, are shown for Arg and Asp residues. Top row, classes 1a and 1b; bottom, 1c and 1d.

Table V. Probable Conformations of Cyclo(D-Pro-Pro-Gly-Arg-Gly-Asp)

		D-Pro		Pro		Gly^3		Arg		Gly^5		Asp	
type[a]	E^b	φ	ψ	φ	ψ	φ	ψ	φ	ψ	φ	ψ	φ	ψ
1a (100)	2.6 (0.0)	54 ± 15	−121 ± 15	−70 ± 15	−7 ± 27	−108 ± 36	175 ± 5	−61 ± 5	116 ± 16	50 ± 8	35 ± 5	−162 ± 12	98 ± 28
1b (29)	4.2 (2.2)	62 ± 15	−123 ± 13	−63 ± 14	−23 ± 10	−150 ± 21	−178 ± 10	50 ± 9	57 ± 17	51 ± 5	37 ± 3	−160 ± 8	106 ± 22
1c (11)	8.9 (8.1)	85 ± 2	−143 ± 5	−66 ± 16	−6 ± 24	−149 ± 21	−168 ± 4	−53 ± 4	146 ± 4	63 ± 3	53 ± 5	98 ± 10	131 ± 5
1d (2)	9.2 (8.7)	62 ± 15	−143 ± 6	−67 ± 12	−23 ± 10	−157 ± 6	155 ± 2	65 ± 1	173 ± 2	−46 ± 1	−39 ± 1	−92 ± 5	124 ± 9

type	Gly		Pro		Leu		Pro		Gly		Tyr	
CLR[c]	65	−124	−83	−7	−92	163	−57	122	68	10	−159	92

[a] The number of occurrences of each conformation type is given in parentheses. [b] Average energy (no electrostatic terms) of class relative to the lowest energy conformation found in search. Lowest energy member of class is in parentheses. [c] Crystal structure conformation of cleromyrine II (Declercq, 1990).

the D-Pro^1-Pro^2 sequence forms a type II′ β-turn, as shown in Figure 1A. The 142 conformations may be grouped into only four classes. Dihedral angle ranges for these classes are given in Table V. In all but two of the 142 conformations, the Arg^4-Gly^5 sequence adopts a conformation that is in the range of a type II (or the closely related types III′ and I′) β-turn. The Arg-Gly distributions using both types of constraints are shown in Figure 2A.

In the most frequently generated conformation class, 1a in Table V, the Arg-Gly^5 sequence is best described as a type II β-turn. Neither of the residues, Gly^3 or Asp, connecting the D-Pro-Pro and Arg-Gly turns is fully extended, which is not an uncommon feature in reported two-β-turn cyclic hexapeptide crystal structures.[10,35-37] Conformation 1a has a close analogy in the crystal structure reported for cleromyrine II,[10] which is given for comparison as the last entry in Table V. The Gly-Pro and Pro-Gly turn sequences of cleromyrine II correspond to the type II′ D-Pro^1-Pro^2 and type II Arg^4-Gly^5 turns, respectively, of the class 1a conformation of cyclo(D-Pro-Pro-Gly-Arg-Gly-Asp). The 1a subset includes both the conformation with the lowest total energy of the 500 structures generated and the conformation with the lowest constraint violation energy of the full set. It also has the lowest average total energy (excluding electrostatic terms): 2.6 kcal above the minimum.

Another set of conformations of relatively low energy, the lowest 2.2 kcal above the minimum and the average 4.2 kcal above the minimum, is given as 1b in Table V. It has the Arg-Gly^5 sequence as a type III′ β-turn, and the Gly^3 residue is more extended than in 1a. These two backbone conformation classes, which account for 129 of the 142 structures within the energy limits chosen, are quite similar in shape.

Two other conformation classes of significantly higher energy, their lowest energy exemplars 8 kcal above the minimum, were also returned by the constrained search. Conformation 1c is similar to 1a in that the Arg-Gly sequence is near the type II β-turn conformation, but it is distinguished by an unlikely value for ϕ_{Asp} of ca. +100°. Conformation 1d, which occurred only twice within the energy limits used, has a distorted, more open Arg-Gly turn which does not fit any of the usual classifications.

Figure 4 shows the lowest energy conformations of all four backbone types. The relative importance of the four conformations may be estimated not only from their calculated energies, which tend to rule out significant populations of 1c and 1d, but also from coupling constant observations. The backbone H–N–C–H coupling constants (Table IIA) of Gly^3 and Arg are sufficiently different from average values to suggest dihedral angle information about the predominantly populated conformations. Although the Gly^3 observations, on examination, prove to be of no help in distinguishing among the conformation classes, the Arg coupling is suggestive. The measured value of $^3J_{HNCH}$ for Arg is 2.9 Hz, which is much closer to the expected value (ca. 4 Hz) for ϕ_{Arg} near −60°, as in conformation 1a, than to that expected for the cisoid H–N–C–H arrangement corresponding to the ϕ_{Arg} = 60° of 1b or 1d (ca. 7 Hz).[38] Also, any important contribution from conformation 1c, which predicts a small $^3J_{HNCH}$ for Asp, is inconsistent with the 8.1 Hz coupling constant observed.

On the basis of calculated energies and the Arg H–N–C–H coupling constant, therefore, it seems most probable that conformation 1a, with the type II Arg-Gly^5 turn, is the dominant conformation of cyclo(D-Pro-Pro-Gly-Arg-Gly-Asp-Gly) in solution. Figure 5 shows overlays of the 10 lowest energy structures of class 1a. The 46 lowest energy conformations generated by the constrained search are, perhaps coincidentally, of this type.

Probable Conformations of 2. As can be seen in Figure 1c, the D-Pro-Pro sequence in this peptide is also limited to the type II′ β-turn conformation. The N–H chemical shift pattern and the temperature coefficients of the N–H resonances are what would be expected for a typical two-β-turn cyclic hexapeptide backbone in a hydrogen bond accepting solvent,[39,40] in that there are low-temperature coefficients and high-field chemical shifts for the internally directed N–H protons of the residues (Arg, 7.56 ppm, 0.0027 ppm/deg; Gly^6, 7.27 ppm, 0.0017 ppm/deg) connecting the turns, relative to the externally directed N–H of the putative

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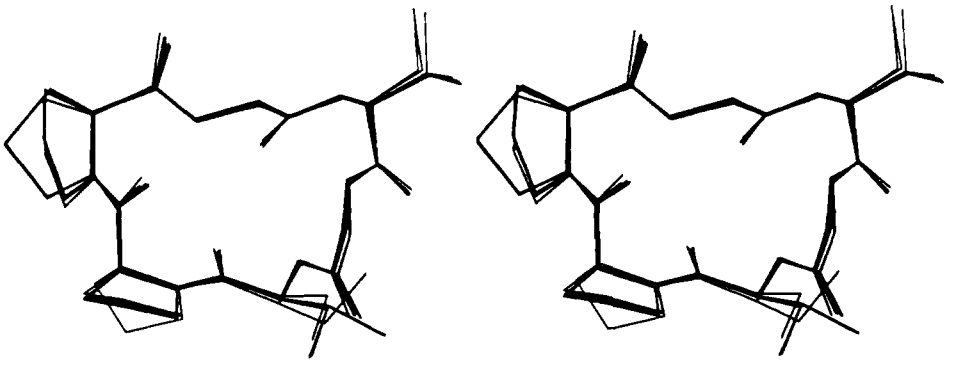

Figure 5. Stereoplot overlay of the 10 lowest energy backbone conformations of class 1a obtained in the constrained distance geometry search for cyclo(D-Pro-Pro-Gly-Arg-Gly-Asp) (1). The rms deviation of the C^{α} atoms is minimized in making the overlay. To avoid confusion of β-carbons with carbonyl oxygen atoms, γ-carbons, although not determined by NMR data, are shown for Arg and Asp residues.

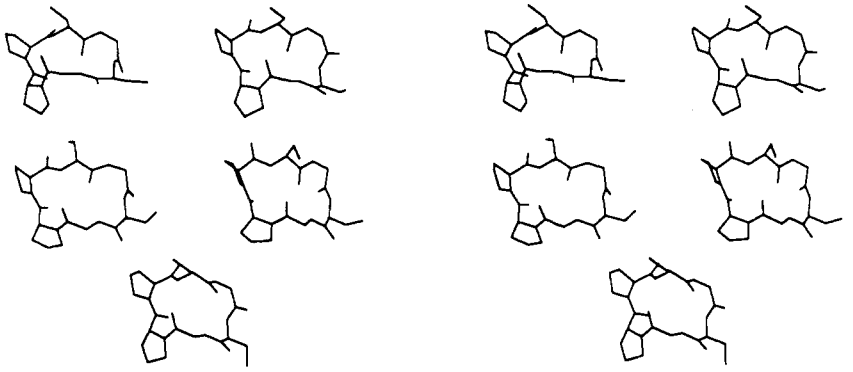

Figure 6. Stereoplot of the lowest energy exemplars of all five backbone types generated by the constrained distance geometry search for cyclo(D-Pro-Pro-Arg-Gly-Asp-Gly) (2). To avoid confusion of β-carbons with carbonyl oxygen atoms, γ-carbons, although not determined by NMR data, are shown for Arg and Asp residues. Top row, classes 2a and 2b, middle, 2c and 2d, and bottom, 2e.

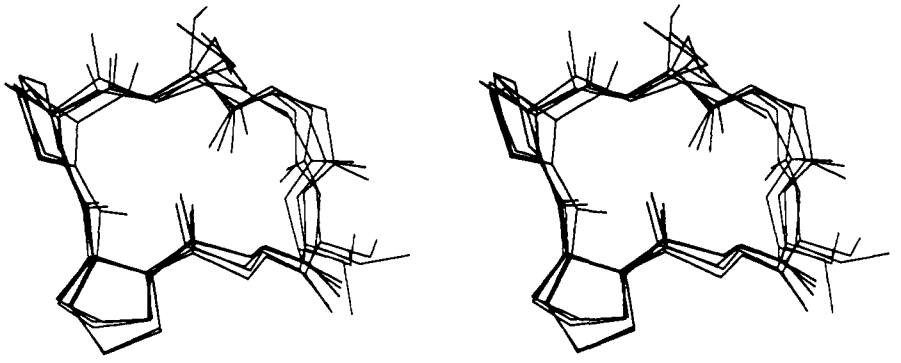

Figure 7. Stereo overlay of the five lowest energy exemplars of conformations found for cyclo(D-Pro-Pro-Arg-Gly-Asp-Gly) (2). The rms deviation of the C^{α} atoms is minimized to make the overlay. To avoid confusion of β-carbons with carbonyl oxygen atoms, γ-carbons, although not determined by NMR data, are shown for Arg and Asp residues.

Table VI. Probable Conformations of Cyclo(D-Pro-Pro-Arg-Gly-Asp-Gly)

		D-Pro		Pro		Arg		Gly^4		Asp		Gly^6	
type[a]	E^b	φ	ψ	φ	ψ	φ	ψ	φ	ψ	φ	ψ	φ	ψ
2a (20)	2.4 (0.0)	63 ± 12	−120 ± 7	−64 ± 14	−23 ± 10	−83 ± 8	161 ± 14	−39 ± 5	−53 ± 2	−119 ± 28	32 ± 24	−173 ± 6	86 ± 6
2b (9)[b]	5.3 (3.6)	61 ± 14	−126 ± 14	−69 ± 17	−2 ± 29	−116 ± 27	168 ± 7	−42 ± 12	94 ± 3	74 ± 24	55 ± 11	142 ± 60	115 ± 38
2c (26)	6.2 (4.1)	62 ± 12	−123 ± 11	−66 ± 18	−19 ± 15	−157 ± 13	152 ± 16	54 ± 10	58 ± 7	56 ± 8	45 ± 5	−173 ± 18	106 ± 32
2d (54)	6.7 (3.0)	60 ± 15	−126 ± 12	−69 ± 22	−8 ± 32	−131 ± 48	118 ± 60	38 ± 36	−86 ± 7	−101 ± 46	9 ± 48	151 ± 75	138 ± 67
2e (58)[c]	8.0 (3.5)	63 ± 14	−133 ± 11	−74 ± 13	−15 ± 37	−164 ± 21	121 ± 48	52 ± 17	99 ± 18	60 ± 10	51 ± 9	125 ± 60	135 ± 59

[a] The number of occurrences of each conformation type is given in parentheses. [b] Average energy (no electrostatic terms) of class relative to the lowest energy conformation found in search. Lowest energy member of class is in parentheses. [c] Excludes one high-energy outlier with Asp at −1, 153. [d] Excludes one high-energy outlier with Asp at −46, 179.

$i + 1$ residue Gly^4 (8.58 ppm, 0.0080 ppm/deg). However, reference to Figure 3, which presents the dihedral angle ranges for the Gly-Asp sequence opposite D-Pro-L-Pro, shows that the NOE observations are not at all so restrictive as in 1. The 169 conformations within the 1.5 kcal constraint violation energy and 10 kcal total energy limits could be divided into the five conformation classes listed in Table VI. The five classes all contain Gly-Asp turns. The conformations numbered 2a and 2d have turns in which the central peptide bond carbonyl points up relative to the average ring plane (peptide chain viewed as running clockwise), as in type I (or III) and II′ β-turns. Conformation 2a, which represents 20 of the 30 lowest energy conformers returned by the constrained search, contains a type I Gly-Asp turn; it is otherwise similar at each position around the ring to conformation 1a of 1. Conformations 2b, 2c, and 2e have the Gly-Asp amide plane orientation opposite to that in 2a and 2d. All five classes are

consistent with the N–H chemical shift and temperature coefficient data in predicting more or less internally directed N–H vectors for Arg and Gly.[6] The lowest energy versions of each are all within 4 kcal of each other; they are shown individually in Figure 6. A preference for one or another of these conformations cannot be established from coupling constant observations. However, except for the orientation of the Gly-Asp peptide bond, they have an overall similarity, illustrated in the overlay of Figure 7.

Discussion

Cyclic Peptide Conformations. In both cyclic hexapeptides, the D-Pro-L-Pro sequence unequivocally adopted the type II′ β-turn conformation as had been anticipated. This stable β-turn appears to stabilize another turn, formed by residues across the cyclic hexapeptide ring, to result in the frequently observed two-β-turn cyclic hexapeptide backbone. However, although the D-Pro-Pro region of both peptides is well defined, the other portions of these molecules are less so, perhaps because of the presence of two glycine residues. It would appear that **1**, with an Arg-Gly turn, is somewhat more narrowly defined than **2**, which has a Gly-Asp turn. The most probable Arg-Gly turn in **1** appears to be type II-like, i.e., the central amide carbonyl is directed up with the peptide chain viewed as running clockwise. The presence of a low equilibrium fraction of type I-like turn cannot be excluded for **1** on the basis of the NMR evidence. X-ray crystallographic studies of cyclo(Xxx-Gly-Gly)$_2$ analogs indicate that both type I and II forms are stable for Xxx-Gly β-turns,[1,41] and NMR studies of such C_2-symmetric sequences indicate averaging between type I and II turns. For **2**, both type I- and type II-like Gly-Asp turns are returned by the search, with calculated energy considerations favoring the former. However, in the few instances in which Gly^{i+1}-L-Xxx^{i+2} turns are reported in crystal structures of cyclic hexapeptides they are of type II′, i.e., type I-like in regard to the sense of the central C=O bond.[35,37,42] It is probable that both Gly-Asp amide plane orientations are present in the distribution of solution conformations of **2**.

The dihedral angle ranges consistent with the NOE data for the non-proline residues of **2**, particularly the between-turn residues, are in general broader than those found for **1**, even though there are 18 NOE-derived distance constraints for **2** and only 14 for **1**. As reference to Tables III and IV will indicate, the NOE-derived distances for **1** are in general nearer the lower geometric limits for hydrogen–hydrogen distances dictated by a single backbone torsion angle than are those for **2**. When the −10%,+15% lower and upper bounds are applied, the longer distances found for **2** are not very restrictive of torsion angles. Experimentally, the longer distances derive from weaker NOEs, but in flexible molecules weaker NOEs may very well result from a mixed population of conformers. Considering that the D-Pro-Pro sequence is equally narrowly determined in **1** and **2** and noting that the somewhat broader H$^\alpha$ shift and H–N–C–H coupling constant dispersion in **1**, it seems likely that the non-proline regions in **2** are in fact more conformationally mobile than those in **1**. We are not certain why this should be.

Although some of the conformations shown for **2** in Figures 6 and 7 may appear unattractive on electrostatic grounds because of the relative orientation of carbonyl dipoles, they cannot be excluded as potential contributors in a polar environment. It may also be noted that a number of two-β-turn cyclic hexapeptide crystal structures also lack good transannular hydrogen bonds and feature opposing carbonyl dipoles.[2,3,43]

Conformation Search Methodology. The incorporation of anti distance constraints in the NMR-constrained distance geometry searches in general reduces the range of dihedral angles returned by the searches, and in particular, it drives the values found for ψ_{i+2} of the D-Pro-Pro turn in both molecules from a mean near −45° to a mean near −20°. The $\psi_{i+2} = 0 \pm 30°$ range is often observed in crystal structures.[1,3-5,8,10] Therefore, the fact that the ADCs bring ψ_{i+2} into this range may be taken as an indication that they represent meaningful experimental input. The choice of the lower bound for these constraints must be made with care, however. In these studies, experimentally significant cross peak volumes corresponding to distances longer than 3.1 Å are not observed, so that use of a 3.0-Å lower bound for the anti distance constraints, while not highly restrictive, is at least safe.

For a molecule undergoing conformational exchange that is rapid on the chemical shift time scale, it is possible for the sum of the NOE data to be inconsistent with any single backbone conformation, particularly because of the weighting of closest approaches between interacting protons.[44] To describe such a system, it is then necessary to devise, if possible, a model combining multiple conformations in a manner reproducing the observations.[29,45-48] As mentioned earlier, for example, had we attempted to include observed NOEs involving the Asp and Arg β-methylene protons, multiple models incorporating different α,β rotamers for each backbone conformation would have been necessary. We did not attempt to deal with side chain conformation and, therefore, were able to exclude observed NOEs involving Arg and Asp β-protons.

At the other extreme, all of the observations instead may be consistent with more than one structure. This is the case in the present work for the backbone NOE data from **1** and **2** in the regions away from the D-Pro-Pro sequence. The observed NOEs involving the backbone and directly attached proline groups, plus the indications from the unobservability of other NOEs involving these groups, are all consistent with each of the conformational classes listed in Tables V and VI. In the absence of evidence to the contrary, it must be presumed that all of the lower energy conformations returned by the searches are possible contributors to the distribution in solution. It is important therefore that constrained searches with good sampling be used.

In summary, we conclude from this study that the D-Pro-Pro sequence favors a well-defined type II′ β-turn conformation and is a sufficient conformational determinant to ensure that a β-turn appears across from it in a cyclic hexapeptide ring. We also conclude that the use of lower bound distance constraints derived from absent NOEs, with the lower bound chosen on the basis of what is experimentally observed in the system, may be used to provide additional meaningful structural information. In earlier studies,[32] we used such information to exclude conformations generated by the distance geometry search procedure. In the present work we have incorporated them directly and more systematically into the search.

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