Peptide Flexibility and Calculations of an Ensemble of Molecules[§]

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Abstract: Conformational averaging fast on the NMR time scale has been examined by computer simulations of multiple copies of the molecule and application of NOE and coupling constant restraints as an ensemble average. The calculation is illustrated for a model cyclic peptide, cyclo[-D-Pro-Ala²-Ala³-Ala⁴-Ala⁵-], for which conformational averaging is taking place. There is a well-defined type II' β-turn about the D-Pro-Ala², while no single conformation can be ascribed to the other half of the molecule which fulfills the NMR observables. From the ensemble calculations, four different conformations can be described for Ala⁴; a γ- and γ'-turn and two conformations involving a rotation of one or the other amide bond so that both amide protons are oriented in the same direction, either above or below the plane of the β-turn. The NMR observables can only be described by averaging over the ensemble containing these four conformations.

Introduction

One problem in the determination of conformation from NMR data is the possibility of large, significant conformational changes fast on the NMR time scale. The NMR observables and restraints developed from them will be consistent with an average structure that may not exist in solution or that is not even physically possible. There have been a number of cases in the literature where the experimental data does not fit one conformation.[1-6] Recently attention has been given to the problem in the structure refinement from NMR data.[7-13] Although these methods differ in the computational details, most assume that the experimental observables arise from ensembles of conformations,[8-12] the idea of "the" structure is not suitable.[14] The difficult task is then to calculate the appropriate population of each member of the ensemble.[9,10]

To identify significant conformational changes (fast on the NMR time scale), we have proposed the application of conformational constraints which average differently;[15] the distances derived from NOEs average as a function of the distance to the inverse sixth (or third) power,[4,16] while coupling constants average as a cosine series of the dihedral angle subtended by the coupled atoms (i.e., $\langle \cos^2 \theta \rangle$ and $\langle \cos \theta \rangle$). The application of each of the restraints separately, and together, should allow for the unambiguous identification of conformational averaging and provide insight into the nature of the averaging.

Here we illustrate this method using the cyclic pentapeptide cyclo[-D-Pro-Ala²-Ala³-Ala⁴-Ala⁵-]. The 20 NOEs, coupling constants (both homo- and heteronuclear couplings have been measured for the ϕ dihedral angle of the alanines), and temperature coefficients cannot be fulfilled by one single conformation.[17] Using an ensemble approach,[11,18] where the experimental restraints are applied as averages over multiple copies of the molecule, different families of conformations are obtained, which taken together can reproduce the experimental data. The incorporation of coupling constants into this method has been shown to successfully reproduce the side-chain rotamers calculated by the Pachler equation,[19] even for cases where the couplings indicate flexibility.[20]

Methods

The DG calculations were carried out using a modified version of the DISGEO program[21-23] following standard procedures previously described.[11,23,24] Using random metrization,[23,25] 50 structures were cal-

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Table 1. Experimental Restraints and Distances Calculated from Ensemble Simulations of cyclo[-D-Pro-Ala²-Ala³-Ala⁴-Ala⁵-] Using Either NOEs, ^3J Coupling Constant Restraints, or Both Experimental Restraints[a]

atoms		expt		calculation		
		upper	lower	both	NOE	^3J
Pro Hα	Ala² HN	2.37	2.00	2.23	2.20	**2.63**
Pro Hα	Ala³ HN	3.97	3.28	3.89	3.73	**5.61**
Pro qδ₂	Ala⁵ HN	4.87	3.61	4.46	4.60	3.86
Pro qδ₂	Ala⁵ Hα	3.05	2.11	2.51	2.44	2.27
Pro qδ₂	Ala⁵ qβ₃	4.60	2.65	2.81	2.85	3.05
Ala² HN	Ala² Hα	2.98	2.71	2.87	2.88	**2.70**
Ala² HN	Ala² qβ₃	3.12	2.56	2.81	2.76	**3.22**
Ala² HN	Ala³ HN	2.78	2.30	2.36	2.32	**3.16**
Ala² Hα	Ala³ HN	3.12	2.58	**3.20**	3.00	2.68
Ala² qβ₃	Ala³ HN	3.83	2.76	3.53	**3.84**	**3.88**
Ala³ Hα	Ala³ HN	2.96	2.44	2.78	2.83	2.50
Ala³ Hα	Ala⁴ HN	3.12	2.58	3.01	3.00	2.79
Ala³ HN	Ala³ qβ₃	3.52	2.66	3.14	3.19	3.12
Ala³ HN	Ala⁵ HN	4.19	3.46	3.77	3.81	**6.03**
Ala⁴ HN	Ala⁴ Hα	2.98	2.46	2.51	2.59	2.50
Ala⁴ HN	Ala⁵ HN	2.72	2.24	2.52	2.68	**4.11**
Ala⁴ Hα	Ala⁵ HN	3.64	3.02	3.19	3.56	**2.34**
Ala⁴ qβ₃	Ala⁵ HN	3.66	2.61	2.79	**2.54**	3.45
Ala⁵ HN	Ala⁵ Hα	2.98	2.64	2.70	2.90	**2.25**
Ala⁵ HN	Ala⁵ qβ₃	3.54	2.65	2.99	2.75	3.37

[a] Distances in Å. Pseudoatoms are represented with q and the restraints are adjusted following standard procedures.[54] The distances outside the experimental range are shown in bold.

Table 2. Experimental and Theoretical Coupling Constants Calculated from Ensemble Simulations of Cyclo[-D-Pro-Ala²-Ala³-Ala⁴-Ala⁵-] Using Either NOEs, ^3J Coupling Constant Restraints or Both Experimental Restraints

residue	expt ^3J	calculation			$\Delta\delta/\Delta T$[b]
		both	noe	^3J	
Ala²					8.0
$^3J_{HN-H\alpha}$	8.5	8.50	9.81	8.50	
$^3J_{HN-C\beta}$	1.5	1.49	1.14	1.50	
Ala³					2.7
$^3J_{HN-H\alpha}$	8.5	8.49	7.53	8.50	
$^3J_{HN-C\beta}$	2.1	2.08	0.99	2.10	
Ala⁴					4.0
$^3J_{HN\cdot H\alpha}$	6.8	6.80	2.88	6.80	
$^3J_{HN-C\beta}$	0.4	0.42	3.46	0.41	
Ala⁵					0.3
$^3J_{HN-H\alpha}$	8.8	8.81	7.53	8.34	
$^3J_{HN-C\beta}$	0.2	0.23	0.99	0.63	

[a] The temperature coefficients for the amides protons of the alanines are given. Coupling constants are reported in Hz. The coupling constants have been calculated for each member of the ensemble and then averaged over the ensemble. [b] The temperature coefficients given in -ppb/K.

culated and optimized using distance[26,27] and angle driven dynamics (DADD) with the addition of a penalty function for coupling constants, V_J. The "force field" of DADD calculations is

$$V = V_{Hol} + V_{NOE} + V_J$$

The holonomic function, V_{Hol}, maintains the topology of the molecule and consists of two terms: (1) a chiral term using oriented volumes to maintain planarity of amide groups and aromatic rings and the configuration of tetrahedral carbon atoms and (2) a distance matrix containing the upper and lower bounds on the interatomic distances between the atoms of the molecule.[11,22,23] The metrization and refinement were carried out three times using different restraints: (1) only NOEs (i.e., standard DDD simulation),[26,27] (2) only coupling constants, and (3) both NOEs and couplings as restraints (DADD), producing 32, 31, and 38 low-energy structures, respectively.

Each of the structures from the above calculations were copied 10 times producing ensembles of 320, 310, and 380 structures. These were

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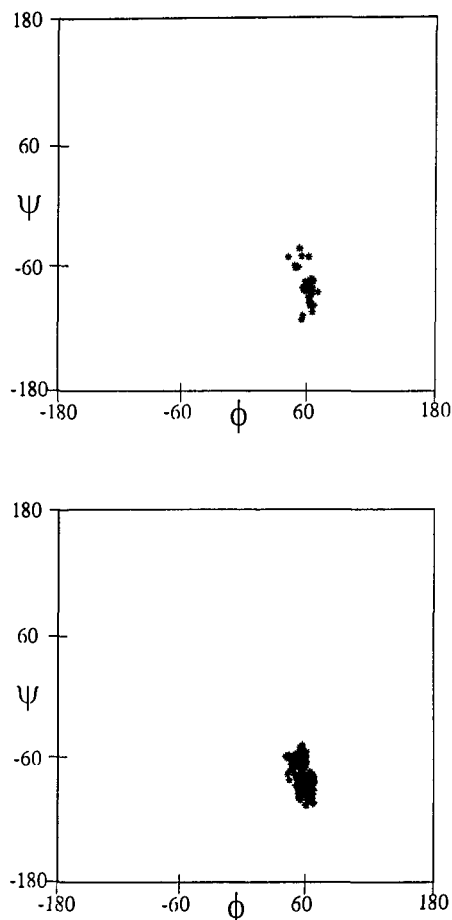
Figure 1. The results from the DG calculation and DDD optimization (above) and the ensemble simulations using both NOE and coupling constant (below) restraints. A Ramachandran plot is shown for each of the five amino acids of cyclo[-D-Pro-Ala²-Ala³-Ala⁴-Ala⁵-]: D-Pro.

then used as starting structures for three extended ensemble calculations with application of only NOEs, only coupling constants and both of the restraints, respectively. In the ensemble calculations,[11,18] the energy and forces for the experimental restraints are generated from an ensemble average. This is illustrated for the coupling constants below

$$V_J = K_J(J_{exp} - \langle J_{theo} \rangle)^2$$

where J_{theo} is the coupling constant calculation from a Karplus type equation,[28] J_{exp} is the experimental coupling, and K_J is a force constant which can be adjusted for each individual coupling constant according to the accuracy of the A, B, and C coefficients of the Karplus equation and the experimental coupling. The average coupling constants are calculated using

$$\langle J_{theo} \rangle = \frac{1}{N}\sum_{i=1}^{N} J_i$$

The mean force calculated using these equations is applied to each individual structure within the ensemble.[18] The NOEs are handled in a similar fashion,[11]
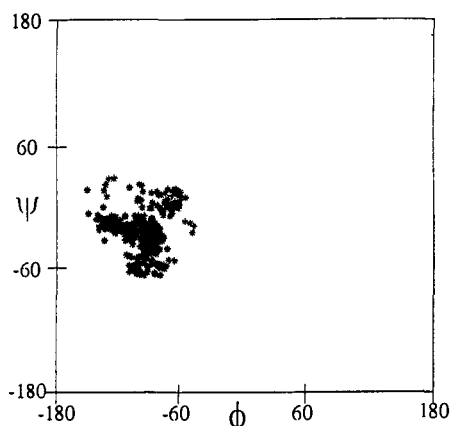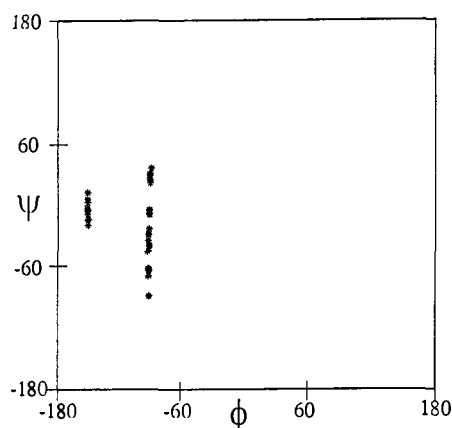
$$V_{NOE} = K_{NOE} \sum_{\langle d_{ij}\rangle > u_{ij}} \left(1 - \left[\frac{\langle d_{ij}\rangle}{u_{ij}}\right]^2\right)^2 + \sum_{\langle d_{ij}\rangle < l_{ij}} \left(1 - \left[\frac{\langle d_{ij}\rangle}{l_{ij}}\right]^2\right)^2$$

where the average distance is averaged over the ensemble as an inverse power of three

$$\langle d_{ij} \rangle = \frac{1}{N}\sum_{k=1}^{N}[(d_{ij})^{-3}]^{-1/3}$$

Of course, the distance can be averaged differently (i.e., d^{-6}), depending on the scale of the dynamics or motions which are of interest.[4,16]

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Figure 2. See Figure 1: Ala².

Figure 3. See Figure 1: Ala³.

The ensemble calculations were carried out for 20 000 steps with a step size of 5 fs at a temperature of 500 K with a tight coupling to a temperature bath.[29] After this the temperature was set to 1 K, and 5000 steps were carried out with a weak coupling to the temperature bath (every 75 steps). The theoretical coupling constants were calculated using A, B, and C coefficients of 9.5, -1.6, and 1.9, respectively.[30] Energy minimizations were carried out using a full energetic force field (GROMOS)[31] and a conjugate gradient algorithm for 200 steps. Short molecular dynamics simulations were carried out both with and without using the experimental restraints at 300 K for 20 ps following procedures previously described.[32,33] All minimizations and MD simulations were carried out with explicit DMSO solvent molecules.[32]

Results

The experimental data determined for cyclo[-D-Pro-Ala²-Ala³-Ala⁴-Ala⁵-] in DMSO[17] is listed in Tables 1 and 2. The distance restraints were obtained using the two-spin approximation and were adjusted by plus and minus 10% for the upper and lower distance restraints, respectively. The $^3J_{HN-H\alpha}$ and heteronuclear $^3J_{HN-C\beta}$, obtained from the HETLOC experiment,[34,35] measured for the alanines are given in Table 2.

The distances and coupling constants are consistent with a βII′-turn about the D-Pro-Ala². In the other "half" of the molecule, all of the restraints cannot be fulfilled by one single conformation.

The small temperature coefficient of Ala⁵ immediately suggests a γ-turn about Ala⁴. However, the short distance between the amide protons of Ala⁴ and Ala⁵ is inconsistent with a γ or γ′-turn. More likely is an equilibrium between both γ-turn structures and conformations with both amide protons pointed in the same direction. When the amide protons are pointed in a similar direction, the NOE is fulfilled, while with either of the γ-turns, the temperature coefficient is fulfilled. Such "flip-flop" of amide bonds in peptide structures has been previously observed.[4,6,36-41] This indication of mobility and averaging of conformational restraints seemed like an ideal situation to apply the ensemble calculation procedure and the application of NOE and coupling constant restraints. Therefore, three separate driven dynamics and ensemble calculations were carried out using NOEs, J couplings, and both of these restraints.

The distances between the atoms for which an NOE was observed calculated for each of the ensembles using d^{-3} averaging are given in Table 1. The coupling constants were calculated for each member of the ensemble and then averaged (listed in Table 2).

The ensemble for which NOEs and ^3J both have been applied reproduce the distances and coupling constants extremely well. There is only one, very small distance violation (0.08 Å), while all coupling constants are in complete agreement. Therefore, by

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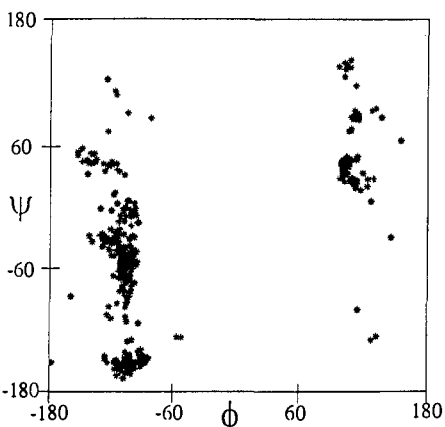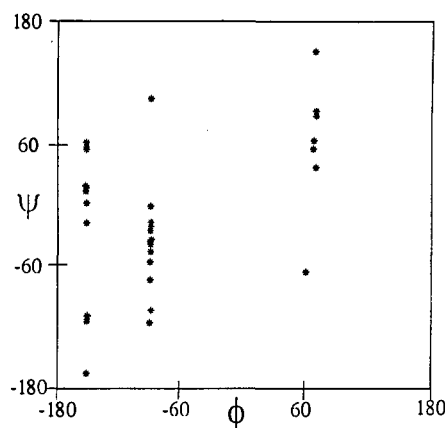(30) Bystrov, V. F. *Prog. NMR Spectrosc.* **1976**, *10*, 41–81.

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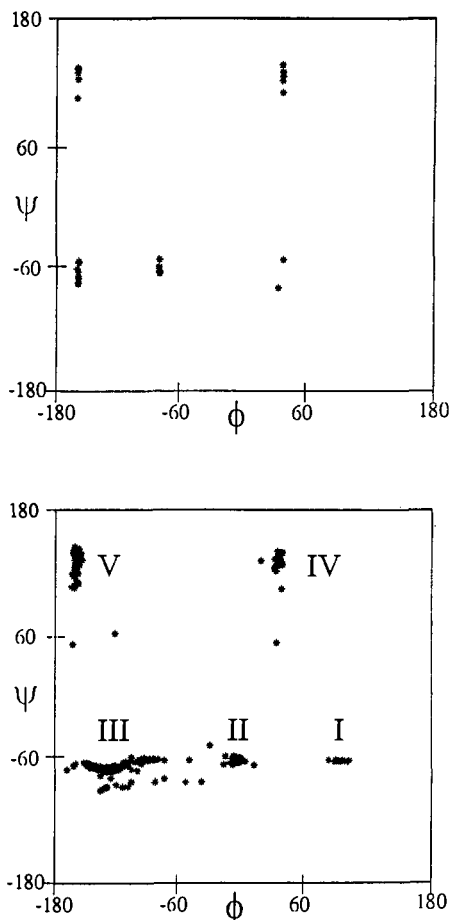
Figure 4. See Figure 1: Ala[4].

Figure 5. See Figure: Ala[5].

analysis of the restraints alone one may conclude that the structure of the peptide is well determined. However, the spread of the structures, especially for Ala[4], is extremely large. This can clearly be seen in the Ramachandran plots of the ensembles from the optimization and ensemble calculation using both restraints given in Figures 1–5.

The spread of conformations of the proline (Figure 1) is, of course, rather small. A similar result is observed for Ala[2]. Taken together this indicates the presence of the βII'-turn about D-Pro-Ala[2]. This is in accord with the low-temperature coefficient of Ala[3]. Very different results are observed for the other residues. Both the DADD and ensemble calculations of Ala[3] indicate a wide range of conformations. The range of the ϕ dihedral angle is smaller, consistent with the βII'-turn discussed above. Similar results are observed for Ala[5], where the ψ dihedral angle adopts a small range of values (consistent with the β-turn), while the ϕ torsion adopts a wide range of values, more or less, centered at −120° and 40°.

The greatest range of values are found for Ala[4]. These can be roughly divided using the ϕ, ψ values into five different families of conformations: (I) 90°,−60°, (II) 0°,−60°, (III) −120°,−60°, (IV) 30°, 120°, and (V) −170°, 120°. The violation ("energy" from the simplified force field of the ensemble program) of the first three families is approximately equal (∼13), while for families IV and V larger violations (∼23) are calculated. The difference in the error function comes from violations within the holonomic term, V_{Hol}, as bonds are too long and peptide bonds are forced out of planarity to meet the experimental restraints.

To obtain a more "realistic" view of these different families, a member from each was soaked with DMSO[32] and energy minimized using a full molecular mechanics force field. The results from these minimizations are shown in Figure 6A–E. The potential energies before minimization are in the same relative
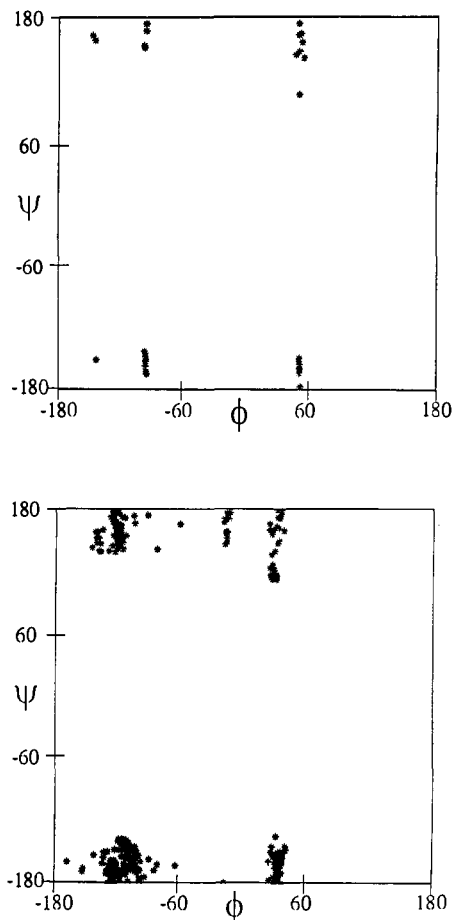
order as from the simplified force field although the magnitude of the difference is larger (i.e., the structures from I, II, and III are of similar energy, two orders of magnitude smaller than the energy of IV and V). After energy minimizations the energies for the five structures are practically the same.

Insight into the dynamics taking place about Ala[4] can be obtained by application of the coupling constant and NOE restraints separately. The distances and coupling constants obtained from these ensembles are listed in Tables 1 and 2. The conformations within each of these ensembles are shown in Ramachandran plots in Figures 7–11.

For the proline the ensemble calculation using only NOEs is almost identical to that using both experimental restraints (compare Figures 1 and 7). The calculation employing only coupling constants indicates a wide range of ψ torsions, but this is to be expected considering that there are no experimental restraints on this dihedral angle.

Very similar results are obtained for Ala[2]. A very small range of conformations, centered about that expected for the i + 2 residue of a βII'-turn, is found for the NOE restrained ensemble. Only eight of the 320 conformations are observed in other conformations. The J restrained ensemble again displays a wide range of ψ torsions. The ϕ dihedral angle shows some dispersion, but the values are centered about −140° and 80°, both consistent with the coupling constants.

The NOE restrained ensemble indicates that Ala[3] adopts a small range of ϕ values, centered about 180°, and two distinct conformations of ψ torsions, −160° and 60°. The ensemble using only J restraints is interesting in that the ψ dihedral angle adopts two distinct values and not the smeared ranges found for the previous two residues. However, it is important to note that a specific ψ torsion is strongly coupled to the ϕ angle of the next residue. This arises from the necessity of maintaining the planarity

A

B

C

D

E
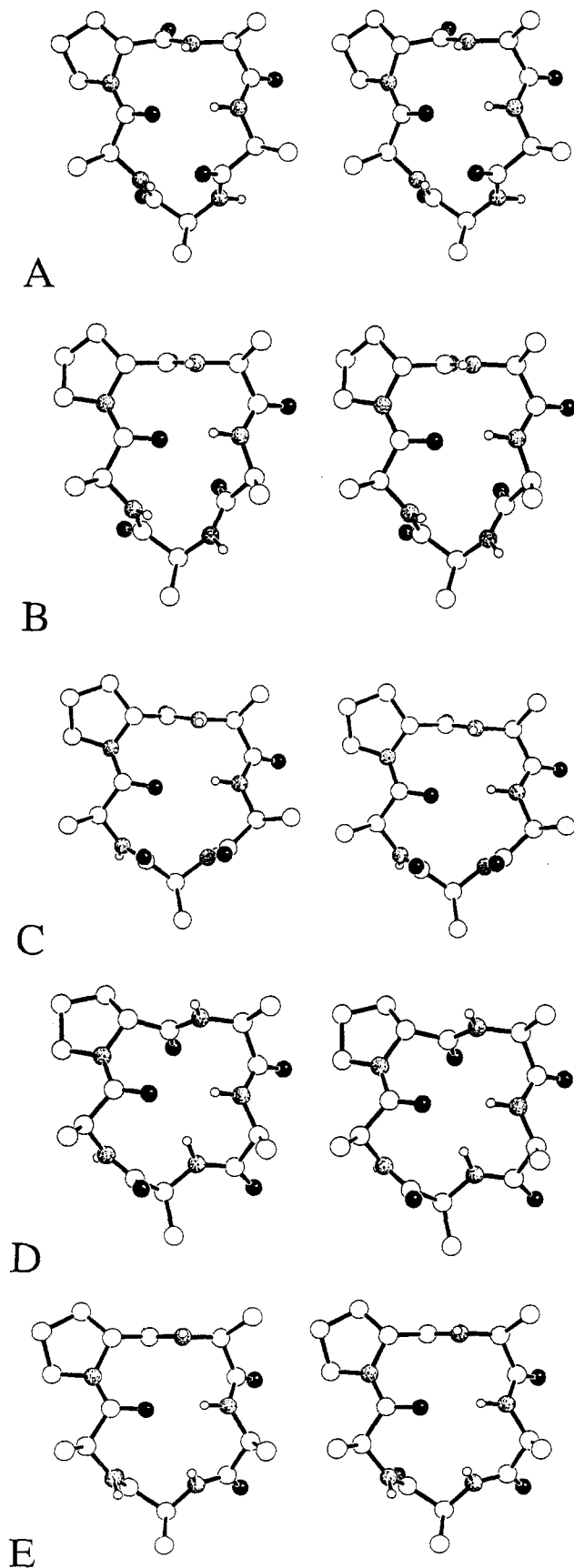
Figure 6. Stereoplots of representative structures from each of the five families obtained from the DDD calculation (see text), energy minimized for 200 steps of steepest descents using explicit DMSO solvent: (A) family I, (B) family II, (C) family III, (D) family IV, and (E) family V.

of the peptide bond between the two dihedral angles. Here, ψ of Ala^3 is influenced by the ϕ of Ala^4 (which is found to adopt
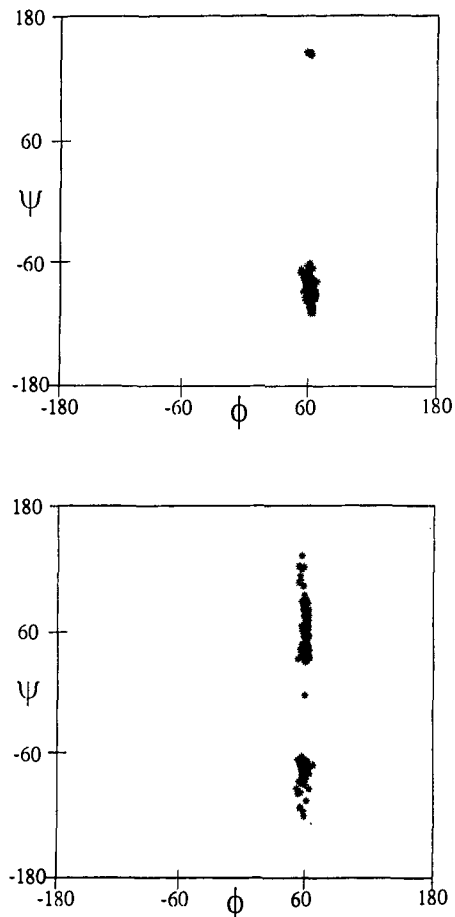
Figure 7. The results from the ensemble calculations using only NOE (above) or coupling constant (below) restraints. A Ramachandran plot is shown for each of the five amino acids of cyclo[-D-Pro-Ala^2-Ala^3-Ala^4-Ala^5-]: D-Pro.

two distinct conformations in the J restraint ensemble, as discussed below).

The NOE ensemble illustrates three distinct conformations for the ϕ torsion of Ala^4: $-120°$, $-55°$, and $120°$, all with ψ values of $-70°$. These are similar to the first three families found in the ensemble using restraints from NOEs and coupling constants simultaneously. However, the populations are different: the family with the positive ϕ value, the least populated with the application of both restraints, is highly populated here. The reverse is true for the conformations centered about a ϕ value of $-120°$ (i.e., it is sparsely populated here, highly populated with the use of both restraints). Using only the J restraints, Ala^4 adopts two conformations corresponding to families IV and V of the ensemble using both restraints discussed above. However, now these structures are of similar violation "energies", illustrating that the distortion arises from the NOEs, mainly two HN–HN NOEs (see Ala^4-Ala^5 and Ala^3-Ala^5 in Table 2). It is important to note that of the five families found for Ala^4 from the ensemble using both restraints, three are found from the NOEs, two using the couplings, with almost no overlap (10 structures from the J ensemble are found in the families developed using only the NOEs).

The ensembles using NOEs or J restraints indicate a small range of values for the ψ of Ala^5, in agreement with the stability of the βII'-turn. However, there is disagreement on the ϕ dihedral angle. The NOE ensemble contains a value of $-80°$, while the predominant conformation of the J ensemble has a value of $50°$. It is important to note that the two coupling constants measured for the ϕ torsion of Ala^5 do not produce the same dihedral angles, errors of 0.4 and 0.5 Hz are observed for the ensemble calculations using only the J restraints. Although, these errors are rather
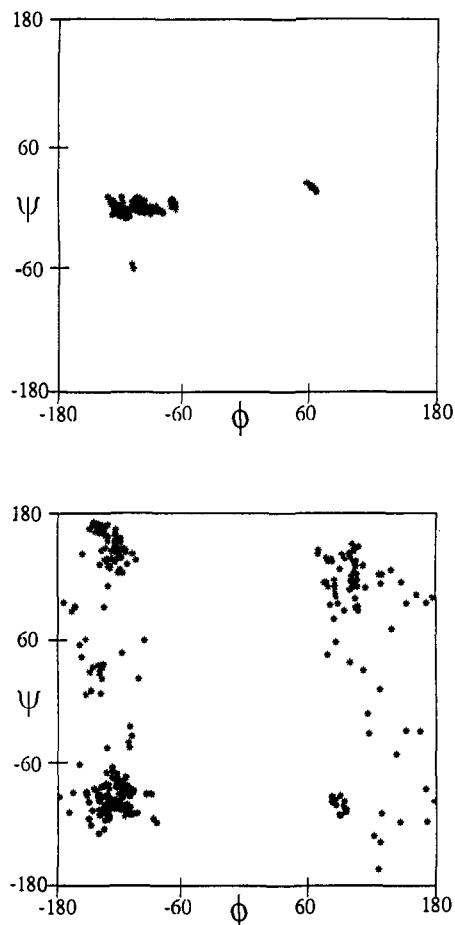
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Figure 8. See Figure 7: Ala².

Figure 9. See Figure 7: Ala³.

small, certainly within the associated experimental error, they are much larger than those observed for the other three alanines.

Discussion

The direct application of coupling constants as a penalty function, first proposed by Kim and Prestegard,[42] has been shown to be useful in the examination of peptides where assumptions about allowed ranges of dihedral angles (e.g., for proteins it is often assumed that ϕ is negative) may not be appropriate.[43–45] In addition to the conformational constaints introduced by the couplings, evidence of conformational averaging is obtained. In the presence of such averaging, both NOE and coupling constant restraints cannot be fulfilled by the same conformation. Of course, this requires that during the structure refinement an attempt is made to drive the restraints to zero.

The distances and coupling constants calculated from the ensembles, Tables 1 and 2, clearly indicate that the NMR observables cannot be accounted for by the application of only one of the restraints. Of course, the NOE ensemble fulfills the NOE restraints (there are two small restraint violations, 0.01 and 0.07 Å), and the ensemble using only J restraints fulfills the coupling constants, although the agreement with the coupling constants of Ala⁵ is not as good as observed for the other residues. It is important to note that the coupling constants are better fulfilled by the ensemble using both of the experimental restraints. The same is true for the NOEs, although the difference is smaller.

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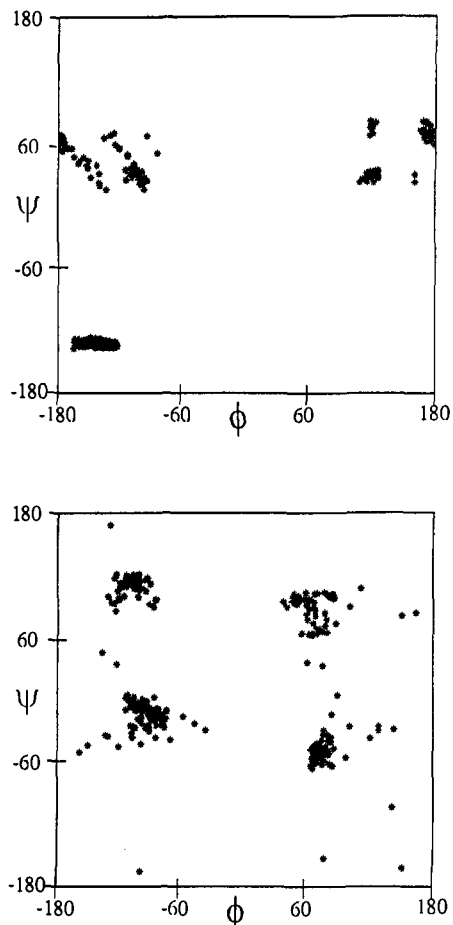(45) Mierke, D. F.; Golic-Grdadolnik, S.; Kessler, H. *J. Am. Chem. Soc.* **1992**, *114*, 8283–8284.

Each of the three populations used for the ensemble simulations were created using the DADD approach (constant restraints), which in the case of conformational averaging, would produce unrealistic structures. Three additional ensemble simulations were carried out (one each with only NOEs, only J restraints and with both), starting with 400 structures created from a DADD calculation using *no restraints*. There is no bias in this starting ensemble; however, the results from these three simulations are statistically identical with the results reported in Figures 1–5 and 7–11.

The "energy" calculated for the five different families developed from the examination of the ϕ, ψ of Ala⁴, in principle, can be used to obtain an estimate of the populations. However, the ensemble "force field" is rather crude (i.e., nonbonded interactions are treated as hard spheres) and does not provide an accurate estimate of the energy. Using a full force field produced large differences in energies, which would suggest that families IV and V are sparsely populated. However, it is important to note that after energy minimization using explicit DMSO (the same solvent as the NMR investigations) the potential energies for the five families are roughly the same. These minima are produced with adjustments of the bond length and out-of-plane terms; very small changes of the conformation are necessary to produce structures of equal energy (RMS difference less than 0.2 Å). No transitions between the families are observed. Even during MD simulations, no transitions are observed either with or without application of the experimental restraints. Of course, with much longer MD simulations it may be possible to observed such transitions.

The cyclization of a pentapeptide produces enough constraint to drastically reduce the number of conformations possible for the linear molecule. Cyclization also introduces strain, best illustrated by the findings of significant populations of conformations which are not observed in proteins. It is therefore possible
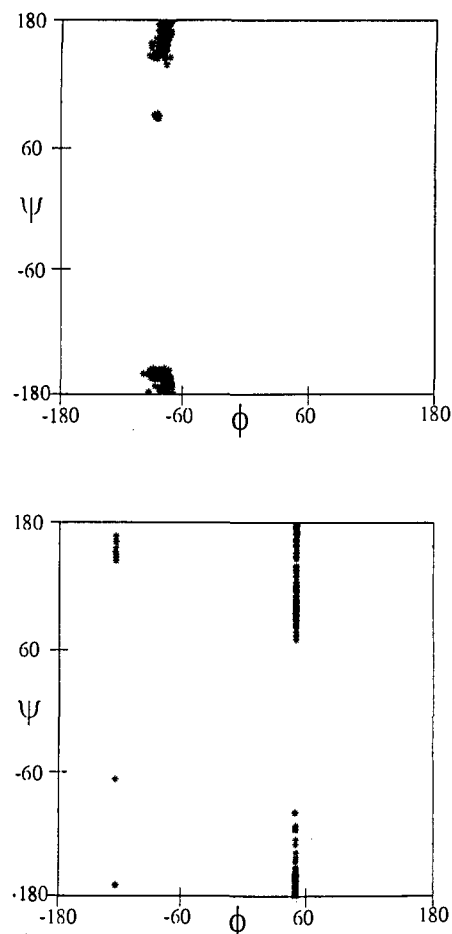
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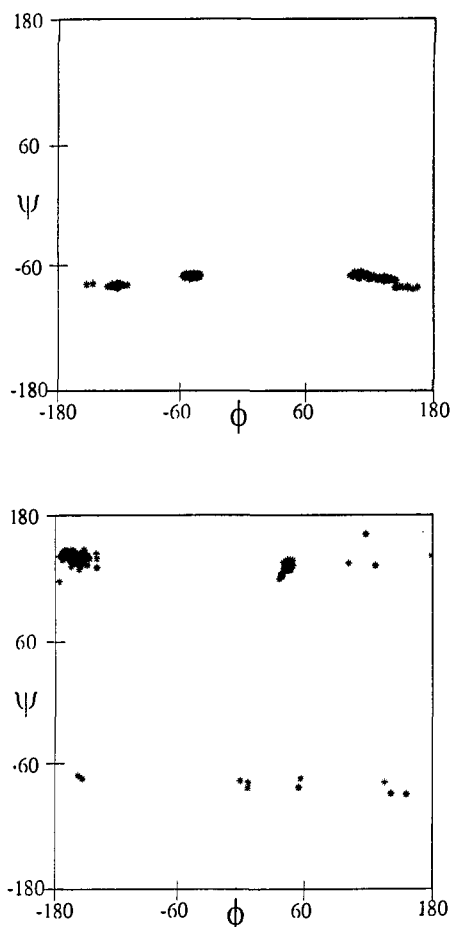
Figure 10. See Figure 7: Ala[4].

Figure 11. See Figure 7: Ala[5].

that an equilibrium of a few conformations account for the observed NMR data. The strain in the cyclic structure may prevent interconversion on the MD time scale, while on the NMR time scale the rate of interconversion is fast. Often it is not possible to freeze out these conformations either because the equilibration is still too fast or one of the conformations dominates at lower temperatures. Therefore, only the incompatibility between structurally relevant NMR parameters can indicate the presence of fast, conformational interconversion. Note that fulfillment of NMR parameters can easily be obtained with insufficient experimental data. Here the large data set based on different structure-dependent parameters is critical for the detection of conformational equilibration.

Several cyclic pentapeptides have been previously investigated by NMR and X-ray analysis.[17,46–53] Two common structural motives have been found: β- and γ-turns of different types.[47] If the cyclic pentapeptide contains one D-amino acid and four L-amino acids a βII'γ turn conformation is observed in almost all cases with the D-residue preferring the $i + 1$ position of the βII' turn.[53] Most of these structures were originally based on temperature gradients and chemical shifts (the most relevant NMR parameters up to the early 1980s) or maybe a few NOE-

derived distances. In the best of cases, coupling constants were only interpreted qualitatively by comparison of the experimental value with the coupling constant properly calculated from the MD trajectory[4,14] (i.e., it is important to stress that the coupling constant calculated from the average structure is meaningless). A rough agreement between the calculated and experimental values was almost always found. However, one problem often observed was that the experimentally derived distance between the amide protons in the $i + 1$ and $i + 2$ position of the γ-turn was too short.[17,52,53] It was not possible to explain the significance of this discrepancy on the basis of NOE distances alone. In addition, MD simulations performed in our group, in which different weighting function of the NOEs were applied, were unsuccessful in representing the experimental data with one or two conformations. This shows the importance of applying as many reliable restraints as possible in an ensemble method.

For the discussion of biologically relevant conformations one has to take all accessible conformations into account. The previously obtained βII'γ or βII'γ' conformations for cyclic pentapeptides are still important. The point illustrated here, is that in the γ-turn region other significant conformations must be considered to obtain an agreement with all of the available NMR data.

Conclusions

A model cyclic peptide, cyclo[-D-Pro-Ala^2-Ala^3-Ala^4-Ala^5-], has been examined with ensemble calculations using NOEs and coupling constants as restraints. From these calculations insight into the dynamic averaging of the NMR observables, which can only be described by averaging over the ensemble of molecules calculated using both of the restraints, is obtained. Ensembles generated using only the NOEs or coupling constants do not

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account for all of the NMR restraints. It is important to stress that the portion of the molecule well determined by the experimental parameters, the type II' β-turn, is reproduced very well with the simulations shown here; only the portion which is undergoing averaging shows a wide range of conformations. The better fit of the experimental parameters by the ensemble method is not solely from increasing the allowed range of the experimental restraints, but instead because it is a better representation of what is actually taking place within the NMR.