The solvent molecules which solvate p-$NH_2C_6H_4S\cdot$ are desolvated from the radical in the transition state. The solvent polarity is a main factor in governing the solvation ability of p-$NH_2C_6H_4S\cdot$. In addition specific interactions are present in the hydrogen-bonding solvents, π-electron-accepting solvents, and alkylamines.

Experimental Section

p-Aminobenzene disulfide was prepared by the air oxidation of the corresponding thiolate anion and recrystallized from aqueous ethanol.[32] Styrene and solvents were distilled before use. The flash photolysis apparatus was of standard design; the half-duration of the xenon flash lamps (Xenon Corp. N-851C) was ca. 10 μs. The transient absorption spectra were measured by a multichannel photodetector (Union Co. LTD.). The measurements were performed in a cylindrical cell (optical path = 10 cm) at 23 ± 1 °C. The oxygen concentration in solvent was controlled by dissolving oxygen under an appropriate pressure after degassing up to 10^{-4} torr; the oxygen concentrations were calculated from Henry's law.

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Registry No. Styrene, 100-42-5; p-$NH_2C_6H_4S\cdot$, 31053-95-9; $(p$-$NH_2C_6H_4)_2S_2$, 722-27-0.

Determination of Proline Ring Nonplanarity from Proton Spin–Spin Coupling Constants: Applications to Two Cyclic Pentapeptides

Alvin C. Bach, II,[1a,b] Aksel A. Bothner-By,*[1a] and Lila M. Gierasch*[1b]

Contribution from the Departments of Chemistry, Carnegie-Mellon University, Pittsburgh, Pennsylvania 15213, and University of Delaware, Newark, Delaware 19711.
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Abstract: A complete analysis of the 600-MHz proton nuclear magnetic resonance spectra of the proline residues in two cyclic pentapeptides, *cyclo*(D-Phe-Pro-Gly-D-Ala-Pro), I, and *cyclo*(Gly-Pro-Gly-D-Ala-Pro), II, has been performed. The proline spectral data have been treated by using a new correlation of vicinal coupling constants and ring nonplanarity. It is shown that if the vicinal coupling constants obey a population-weighted Karplus curve of the type $^3J = K_0 + K_1\langle\cos\theta\rangle + K_2\langle\cos^2\theta\rangle$, where the angular brackets indicate population-weighted averages, then the relation $^3J_c + {}^3J_{c'} + 2({}^3J_t + {}^3J_{t'}) = 6K_0 + 3K_2$ must be satisfied. The experimental data on the eight methylene pairs in I and II fit this relation within experimental error, yielding $6K_0 + 3K_2 = 40.0 \pm 0.3$ Hz (standard deviation). Two functions, $\hat{\chi}$ and $\tilde{\chi}$, of measured coupling constants are defined. The first, $\hat{\chi}$, is derived from $\langle\cos\chi\rangle$ and is a measure of the magnitude of twist in the particular C–C bond but does not give information about the sign of χ. The second, $\tilde{\chi}$, is derived from $\langle\sin\chi\rangle$ and indicates, by its sign, preference for positive or negative χ. Equality of χ from the two measures implies a single nonplanar form of the ring, while large $\hat{\chi}$ and small $\tilde{\chi}$ values indicate a ring interconverting between two conformers, the Ramachandran A and B forms. Each of the model compounds contains one proline ring of each kind. The results indicate that one of the rings is subject to motional constraints in the crystal which are removed in solution.

Very high field NMR provides a means of analyzing complex spin systems such as the seven-spin system found in the imino acid proline. Proline rings are of intrinsic interest for their impact on the conformations of peptides and proteins. The cyclic side chain of proline limits the possible dihedral angles of the peptide backbone and causes a bend to occur in the peptide chain. Prolines occur with a very high frequency in reverse turns in peptides and proteins.[2,3]

A further aspect of the proline ring of importance in peptide and protein structure is its puckering motion. Ramachandran et al.[4] noted in an examination of numerous X-ray diffraction structures of peptides and proteins that the proline ring is usually nonplanar in crystals. They classified the conformations encountered as A and B, respectively, according to whether the γ ring carbon was out of plane on the opposite side as the proline carbonyl group (exo) or on the same side (endo) (see Figure 1). Ramachandran A and B forms can also be characterized by $\chi^1 < 0$ for A and $\chi^1 > 0$ for B. Evidence for rapid endo–exo interconversion of proline rings in peptides in solution has been obtained from measurements of ^{13}C longitudinal relaxation times (T_1's);[5-8] longer T_1's have been interpreted in terms of increased mobility due to puckering. A number of small peptides containing prolines have been examined in solution by high-resolution proton NMR spectroscopy.[9-12] Partial or complete analysis of the spin systems was performed. A search was then made for a single conformer which would yield coupling constants matching the observed ones, based on Karplus curves.[13] This method should work well when a single conformer is present, but the presence of two or more rapidly interconverting conformations may either

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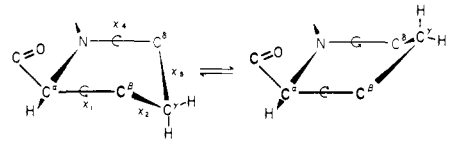

Figure 1. Ramachandran A (left) and B (right) forms of proline.[4] For Ramachandran A the χ ranges are as follows: χ^1, 0 to −30°; χ^2, +15 to +50°; χ^3, −15 to −30°; χ^4, +5 to +25°. For Ramachandran B the ranges are as follows: χ^1, +20 to +35°; χ^2, −30 to −40°; χ^3, +20 to +35°; χ^4, −5 to −20°. Figure is based on: London, R. *J. Am. Chem. Soc.* **1978**, *100*, 2678.

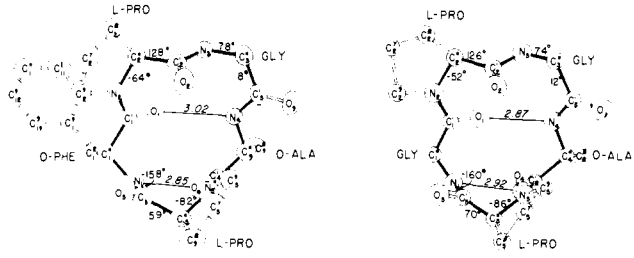

Figure 2. X-ray structures of the two cyclic peptapeptides: *cyclo*(D-Phe[1]-Pro[2]-Gly[3]-D-Ala[4]-Pro[5]) (I) and *cyclo*(Gly[1]-Pro[2]-Gly[3]-D-Ala[4]-Pro[5]) (II) (from ref 16 and 17).

escape detection or give ambiguous results.

In the present work we have examined the high-resolution 600-MHz proton NMR spectra of two proline-containing model peptides: *cyclo*(D-Phe[1]-Pro[2]-Gly[3]-D-Ala[4]-Pro[5]) (I) and *cyclo*(Gly[1]-Pro[2]-Gly[3]-D-Ala[4]-Pro[5]) (II). The backbone conformations of these two peptides in solution have been previously determined;[14,15] both are characterized by the presence of two transannular hydrogen bonds, one in a β turn (with hydrogen bonding between the C=O of residue i and the N—H of residue $i + 3$) and the other in a γ turn (with hydrogen bonding between the C=O of residue i and the N—H of residue $i + 2$) (see Figure 2). In addition, X-ray diffraction studies have been performed on single crystals of both peptides, and the structures in the solid elucidated.[16,17] A comparison of high-resolution ^{13}C NMR data for compound I in solution and in the solid state indicated essentially identical structures,[18] as do comparisons of the observed $J_{HN-C^{\alpha}H}$ coupling constants measured in solution with the dihedral angles observed in crystals.[15,19] It was our intention to perform complete spin–spin analysis of the spectra arising from the proline rings of these two peptides and to attempt to relate the NMR parameters with the conformations demonstrated by X-ray diffraction. It was anticipated that such a comparison would reveal puckering motions present in solution but not in the solid. In our analysis, we have found a correlation of ring nonplanarity with vicinal coupling constants that should be of general utility in treating proline rings whether they are rapidly interconverting between two forms or are present as a single conformer.

Experimental Section

NMR Spectroscopy. Samples were prepared with 5 mg of each compound (synthesized as described previously[14]) dissolved in 0.5 mL of $CDCl_3$ (Norell Chemical Co. Inc.) with 2% Me_4Si (Aldrich) added as an internal reference and lock source.

The 600-MHz proton NMR spectra were obtained by using the spectrometer at the NMR Facility for Biomedical Studies, Carnegie-Mellon University, Pittsburgh, PA. Spectra were run in the correlation mode[20] and subjected to resolution enhancement. Individual multiplets

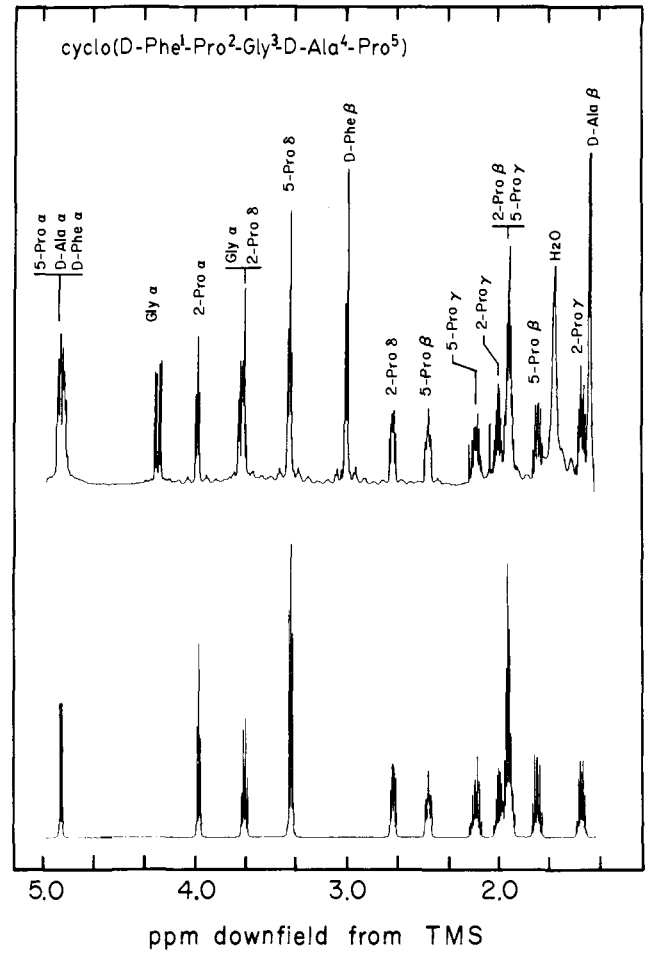

Figure 3. Top: 600-MHz ^1H correlation NMR spectrum of *cyclo*(D-Phe[1]-Pro[2]-Gly[3]-D-Ala[4]-Pro[5]) (number of scans, 300; concentration, 5 mg/0.5 mL $CDCl_3$; temperature, 22 °C; resolution enhanced). Bottom: simulated spectrum of the two prolines.

were plotted on a scale of approximately 1 cm/Hz, and these expanded plots were used to confirm the simulations. Line widths were normally 2–3 Hz. Assignments of proline ring protons to the two proline rings in each compound were made by decoupling each separately observable proton multiplet and observing the collapse of splitting produced.

Spectra were generally well resolved and separated at 600 MHz, except that in I, the signals from the $H^{\gamma exo}$[21] of Pro[5] and $H^{\beta exo}$ and $H^{\beta endo}$ protons of Pro[2] overlapped, while in II, the signals from the $H^{\gamma exo}$ of Pro[5] and the $H^{\beta exo}$ of Pro[2] overlapped, as did those of the $H^{\gamma endo}$ of Pro[5] and the $H^{\gamma exo}$ of Pro[2]. No ambiguity was encountered in making the assignments as to type of proton, in using decoupling techniques, or in finding a suitable set of approximate parameters on which to iterate. Stereochemical assignments were based on chemical shift arguments, for example, the proximity of the aromatic ring to Pro[2] in compound I.

Analysis of the Spectra. Simulations of the observed spectra were performed by using in part the LAOCN3 program[22] implemented for six spins on a Xerox Data Systems Sigma 5 computer and in part Bruker's ITRCAL and PANIC programs for seven spins on an Aspect 2000 computer.

The ITRCAL program was used to generate simulations of subsets of the seven spin systems. The LAOCN3 program was used to obtain error estimates for these subsets, while all final interactions and simulations were performed by using the PANIC program. Root-mean-square errors in line fitting ranged from 0.22 to 0.29 Hz, and derived coupling constants are assigned probable errors of ±0.1 Hz.

We also investigated the question of whether the $^3J_{cis}$ coupling constants between pairs of protons in adjacent methylene groups are demonstrably equal or unequal. Complete calculations using both LAOCN3 and PANIC were performed on both assumptions; either the cis coupling

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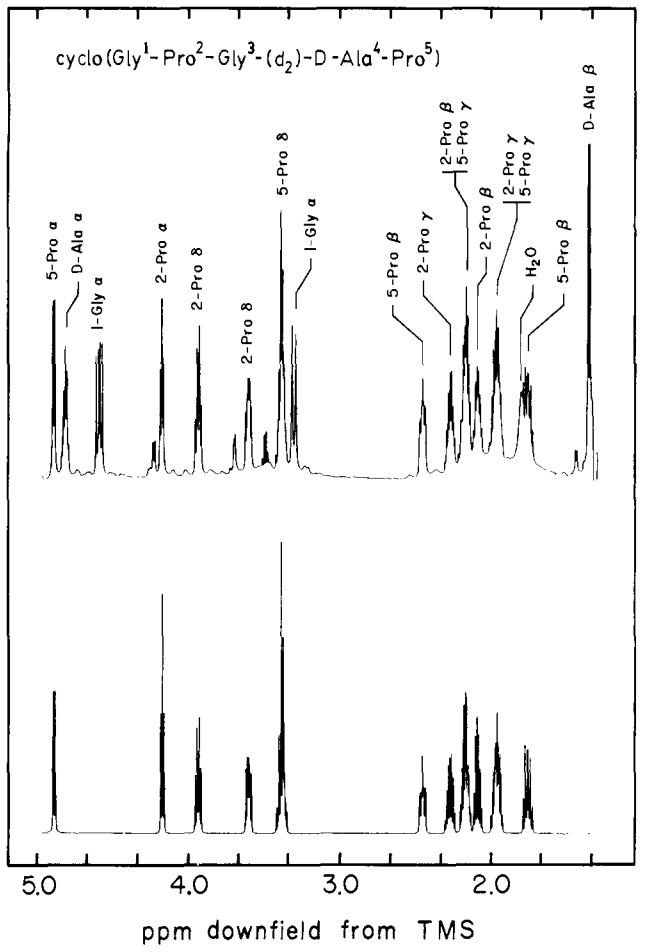

Figure 4. Top: 600-MHz ^1H correlation NMR spectrum of *cyclo*-(Gly^1-Pro^2-Gly^3-D-Ala^4-Pro^5) (number of scans, 300; concentration, 5 mg/0.5 mL $CDCl_3$; temperature, 22 °C; resolution enhanced). Bottom: simulated spectrum of the two prolines.

constants were allowed to vary independently or they were constrained to have equal values in pairs. The errors in fitting the spectrum were slightly larger when the constraint was applied, but the root-mean-square error in line fitting increased by only about 20% while the parameters were changed by less than the deduced probable error, which did not increase. Therefore, we could not demonstrate that the $^3J_{cis}$ coupling constants between pairs of protons in adjacent methylene groups were different. Accordingly, paired values are given in Table I.

Results and Discussion

Spectral Parameters of the Prolines in the Cyclic Pentapeptides. The spectrum of I is displayed in Figure 3, along with a simulation based on the ring protons of the two prolines only, so that signals from the D-Phe, Gly, and D-Ala residues are absent. In Figure 4 are shown analogous spectra for compound II. The NMR parameters derived from the spectral analysis are presented in Figure 5. No corrections for electronegativity were applied.

Note that in each cyclic pentapeptide the two proline rings have very different spectral parameters; this is particularly marked in the difference between the two $J_{\alpha\beta}$ values for the rings. The corresponding rings (Pro^2, Pro^5) in the two peptides have very similar values of NMR parameters, except for the chemical shifts of the proline ring in the β turn which differ significantly. This is probably a consequence of the orientation of the aromatic ring of compound I. Despite the chemical shift differences, the coupling constants remain close.

Determination of χ Values and Proline Ring Geometry. It seems reasonable to assume that the coupling constants between corresponding pairs of vicinal protons attached to proline rings in various environments will follow the same Karplus curve. The curve could have different constants if the vicinal pair were an α,β pair, a β,γ pair, or a γ,δ pair. A Newman projection of such a pair is given in Figure 6. There are four coupling constants—the

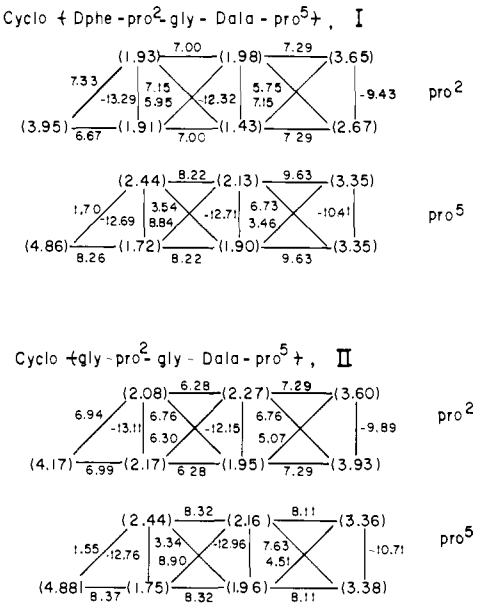

Figure 5. Shifts and coupling constants for the prolines of the cyclic pentapeptides. Chemical shifts in parts per million are given for each proton (in parentheses) in parts per million from Me_4Si. Coupling constants are indicated on the lines joining the protons. The α proton value is at the extreme left, the β protons are in the next vertical array, etc. J_1 are indicated by the lines slanting down to the right and $J_{1'}$ by lines slanting up to the right.

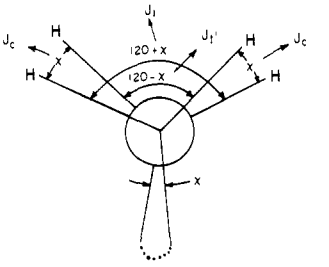

Figure 6. View along C–C bond of proline ring showing relationships between χ, J_c, J_1, and $J_{1'}$.

two cis coupling constants J_c and $J_{c'}$, with the dihedral angle χ^i, and two trans coupling constants J_1 and $J_{1'}$, with dihedral angles $120° + \chi^i$ and $120° - \chi^i$, respectively.

One form of the Karplus curve is

$$^3J = K_0 + K_1 \cos \phi^i + K_2 \cos^2 \phi^i \ldots \quad (1)$$

For both J_c and $J_{c'}$, this becomes

$$^3J_c = {}^3J_{c'} = K_0 + K_1 \cos \chi^i + K_2 \cos^2 \chi^i \ldots \quad (2)$$

If interconversion between conformers is rapid, with fractional populations, $f_1, f_2, f_3, \ldots$, then

$$\langle {}^3J_c \rangle = f_1 {}^3J_1 + f_2 {}^3J_2 + f_3 {}^3J_3 \ldots \quad (3)$$

$$\langle {}^3J_c \rangle = K_0 + K_1 \langle \cos \chi^i \rangle + K_2 \langle \cos^2 \chi^i \rangle \ldots \quad (4)$$

where $\langle \cos \chi^i \rangle$ and $\langle \cos^2 \chi^i \rangle$ are similarly weighted averages. For the trans coupling constants

$$\langle {}^3J_1 \rangle = K_0 + K_1 \langle \cos (120° + \chi^i) \rangle + K_2 \langle \cos^2 (120° + \chi^i) \rangle \quad (5)$$

$$\langle {}^3J_{1'} \rangle = K_0 + K_1 \langle \cos (120° - \chi^i) \rangle + K_2 \langle \cos^2 (120° - \chi^i) \rangle \quad (6)$$

Expanding the trigonometric functions and adding, one obtains

$$\sum \langle J_1 \rangle = 2K_0 - K_1 \langle \cos \chi^i \rangle - K_2 \langle \cos^2 \chi^i \rangle + 3K_2/2 \quad (7)$$

Combining eq 4 and eq 7, one gets

$$\sum \langle J_c \rangle + 2 \sum \langle J_1 \rangle = 3K_2 + 6K_0 \quad (8)$$

Table I. Comparison of Values of Torsional Angles[a] (Deg) in the Proline Rings of *cyclo*(D-Phe-Pro-Gly-D-Ala-Pro), I, and *cyclo*(Gly-Pro-Gly-D-Ala-Pro), II, Derived from Spin–Spin Coupling Constants and from X-ray Diffraction Data

compd	proline ring	methylene pair	$\hat{\chi}^{i}$ [c]	$\tilde{\chi}^{i}$ [c]	χ^{i}(X-ray)[d]
I	2	($\alpha\beta$)	35	−3	+25
		$\beta\gamma$	33	+3	−35
		$\gamma\delta$	31	−3	+31
	5	($\alpha\beta$)	25	+22	+28
		$\beta\gamma$	25	−13	−26
		$\gamma\delta$	11	+8	+14
II	2	($\alpha\beta$)	33	−2	−27
		$\beta\gamma$	37	+1	+39
		$\gamma\delta$	31	+4	−36
	5	($\alpha\beta$)	23	+23	+28
		$\beta\gamma$	24	−14	−24
		$\gamma\delta$	26	+8	+9

[a] Estimated error ±3°. [b] The $\alpha\beta$ pairs are calculated by using a "phantom" H^{α} (see text). [c] For definitions of $\hat{\chi}^{i}$ and $\tilde{\chi}^{i}$, see eq 12 and 14. [d] From ref 16 and 17.

For the eight sets of data obtained in this study, the sum $\sum\langle J_c\rangle + 2\sum\langle J_1\rangle$ is 40.0 ± 0.3 Hz (standard deviation). A plot of $\sum\langle J_c\rangle$ vs. $\sum\langle J_1\rangle$ is reasonably linear. The least-squares line through the points has a slope of −1.86 ± 0.45 (standard deviation), compared with the expected slope of −2.00. Thus the assumption of a generally applicable Karplus curve for vicinal proline methylenes is not contradicted.

In order to obtain a measure of the nonplanarity of the ring, it is necessary to have values for K_0, K_1, and K_2 and to use them with the observed coupling constants to estimate $\langle\cos\chi^{i}\rangle$. We suggest the values

$$K_0 = +1.0 \text{ Hz} \tag{9}$$

$$K_1 = -2.33 \text{ Hz} \tag{10}$$

$$K_2 = +11.33 \text{ Hz} \tag{11}$$

Various combinations of K_0, K_1, and K_2, in increments of a third of a hertz, were tried. The above values were chosen on the basis of their generation of a reasonable Karplus curve with a small coupling constant at 90°, cis planar coupling constant up to 10 Hz (as observed in some prolines), and other couplings in approximate agreement with values suggested in the literature.[23-25] The values are expected to be slightly different from those obtaining in open-chain compounds, because of orbital rehybridization in the strained twisted ring. The values may be adjusted over small ranges, and this changes the deduced χ angles by a few degrees but does not basically alter the pattern of the data or the conclusions.

Equation 4 cannot be solved exactly since it will not in general be true that $\langle\cos^2\chi^{i}\rangle = \langle\cos\chi^{i}\rangle^2$. However, the error in setting these two equal is not likely to be large, and proceeding with this assumption, one can calculate $\langle\cos\chi^{i}\rangle$ for each of the pairs. The results are given in Table I along with $\hat{\chi}^{i}$, which is defined by

$$\hat{\chi}^{i} = \cos^{-1}\langle\cos\chi^{i}\rangle \tag{12}$$

Note that $\hat{\chi}^{i}$ gives a measure of nonplanarity but gives no indication of the direction of twist, cos χ^{i} being an even function of χ^{i}.

In order to determine if A or B conformers are preferred, one may use the difference $\Delta\langle J_1\rangle = \langle J_1\rangle - \langle J_{1'}\rangle$, which from eq 5 and 6 can be shown to be

$$\Delta\langle J_1\rangle = (\sqrt{3}K_2/2)\langle\sin 2\chi^{i}\rangle - \sqrt{3}K_1\langle\sin\chi^{i}\rangle \tag{13}$$

Again, because of averaging, $\langle\sin 2\chi^{i}\rangle$ and $\langle\sin\chi^{i}\rangle$ will not satisfy exactly the usual trigonometric relation, but for small angles the error will be small, and an average angle, $\tilde{\chi}^{i}$, can be deduced. The results are given in Table I with $\tilde{\chi}^{i}$ defined by

$$\tilde{\chi}^{i} = \sin^{-1}\langle\sin\chi^{i}\rangle \tag{14}$$

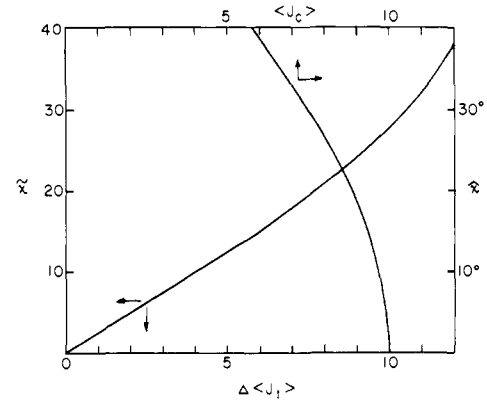

Figure 7. Plot of $\tilde{\chi}$ vs. $\Delta\langle J_1\rangle$ and $\hat{\chi}$ vs. $\langle J_c\rangle$, as derived from eq 4 and 13 in the text.

Figure 7 shows a plot of $\tilde{\chi}^{i}$ vs. $\Delta\langle J_1\rangle$ and of $\hat{\chi}^{i}$ vs. $\langle J_c\rangle$, using the values K_1 and K_2 assumed above. The values are also entered in Table I for comparison with $\hat{\chi}^{i}$. *The values of $\langle\sin 2\chi^{i}\rangle$ and $\langle\sin\chi^{i}\rangle$ are odd functions of χ^{i}, and accordingly $\tilde{\chi}^{i}$ is positive or negative as χ^{i} is positive or negative. It measures the tendency, therefore, for preference for positive or negative values of χ^{i}.*

Values of $\hat{\chi}^{1}$ and $\tilde{\chi}^{1}$ may be evaluated from the ^3J_c and $^3J_1$ magnitudes, even though one of the CH_2 protons on the α-carbon is "missing", having been replaced by the carbonyl group. To evaluate these angles, we assume a "phantom" proton in place of the carbonyl and obtain the coupling constants to it from

$$J_{c'} = J_c \tag{A}$$

$$J_{1'} = 20 - J_c - J_1 \tag{B}$$

derived from eq 8.

Looking at Table I, it is observed that Pro^2 of both compounds I and II give $\hat{\chi}^{i}$ values of 31–37° for all three dihedral angles, while the $\tilde{\chi}^{i}$ values range from −3 to +4°. This implies that the ring is markedly nonplanar and that it rapidly interconverts between A and B forms with about equal populations. $Proline^5$ in both compounds, on the other hand, shows $\hat{\chi}^{i}$ values of 11–26° while $\tilde{\chi}^{i}$ values range from −14 to +23°. The magnitude of $\tilde{\chi}^{i}$ is in each case a substantial fraction of $\hat{\chi}^{i}$, indicating a pronounced preference for one Ramachandran conformation. The signs of the $\tilde{\chi}^{i}$ values indicate that this is the B form.

It is interesting to relate these findings to the structures observed in the X-ray diffraction studies. In both compounds in the crystalline state, Pro^2 is markedly nonplanar, with χ^2 and χ^3 angles of −35° and +31° in I and +39° and −36° in II, agreeing well with the $\hat{\chi}^{i}$ values. The twist is opposite in the two compounds, however. This appears to be due to constraints produced by the crystal lattice forces, since the A and B forms are nearly equally populated in solution.

Pro^5 has values of −26° and +14° for χ^2 and χ^3 in I and −23° and +9° for χ^2 and χ^3 in II. The values of $\hat{\chi}^{i}$ agree remarkably well. The values of $\tilde{\chi}^{i}$ imply that the indicated conformer is the major one.

The preference for one conformer may stem substantially from the inclusion of the proline ring in a γ turn, which would introduce a torsion of the N–C^{α} bond through the exocyclic C^{α}–CO and CO–N bonds.

Comparison with Other Proline Ring Analyses. There have been several approaches taken to determination of dihedral angles in 5-membered heterocycles, including proline. Lambert et al.[26,27]

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have applied the R value method to a variety of 5-membered carbocycles and heterocycles; the results, while indicating trends, were not interpretable quantitatively. Lambert et al. have pointed out that the evaluation of an average dihedral angle from the $^3J_{cis}$ coupling constants was more successful and suggest that this may be due to rehybridization of the carbons giving angles between trans protons in the planar state other than 120°.

Kuo and Gibbons[11] have used a double Karplus curve–ring closure method to ascertain values for χ^1, χ^2, and χ^3 in the Pro^5 in tyrocidine A in $(CD_3)_2SO$. It is interesting to compare their results with those obtained by applying the analysis developed in this paper. For χ^1, Kuo and Gibbons reported +30°, while our method gives $\bar{\chi}^1 = 28°$, $\tilde{\chi}^1 = +26°$. The near equality of $\bar{\chi}^1$ and $\tilde{\chi}^1$ implies little torsion about the $\alpha\beta$ C–C bond, and the agreement between the two methods is excellent. For χ^2, Kuo and Gibbons obtain −34°, while we obtain $\bar{\chi}^2 = 31°$, $\tilde{\chi}^2 = +21°$. For χ^3, Kuo and Gibbons obtain +30°, while we get $\bar{\chi}^3 = 26°$, $\tilde{\chi}^3 = +21°$. In each case, therefore, we agree well with the estimate of average nonplanarity but differ in that we deduced a significant population of conformer with lesser or opposite twist.

Jones, Kuo, and Gibbons[12] have performed a partial analysis of the proton spectrum of gramicidin S in CD_3OD and have deduced $\langle\chi^i\rangle = 25°$. From the reported values of J_c and J_l we obtain $\bar{\chi}^1 = 25°$, $\tilde{\chi}^1 = +23°$, in complete agreement.

Analyses of L-proline itself have been reported by Ellenberger et al.[28] and by Pogliani et al.[29] Anteunis et al.[30] have synthesized *trans*-2,3-dideuterio-L-proline, and their analysis is consistent with those reported by the other two groups. For L-Pro, using Ellenberger's values for the coupling constants, we obtain $\bar{\chi}^1 = 22°$, $\tilde{\chi}^1 = +4°$, $\bar{\chi}^2 = 24°$, $\tilde{\chi}^2 = +1°$, and $\bar{\chi}^3 = 28°$, $\tilde{\chi}^3 = 0°$, confirming that in free proline all puckering modes are active and that there is little A–B preference.

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Direct Observation of the ^{43}Ca NMR Signals from Ca^{2+} Ions Bound to Proteins

Thomas Andersson,* Torbjörn Drakenberg, Sture Forsén, Eva Thulin, and Marianne Swärd

Contribution from the Departments of Physical Chemistry 1 and 2, Chemical Center, University of Lund, S-220 07 Lund 7, Sweden. *Received June 5, 1981*

Abstract: The ^{43}Ca NMR signals from Ca^{2+} ions bound to the Ca^{2+} binding proteins parvalbumin, troponin C, and calmodulin have been observed. The observation was made possible through the combined use of isotopically enriched $^{43}Ca^{2+}$, FT techniques, high magnetic fields, and a solenoid type of probe design. Measurements of the apparent longitudinal relaxation rate, R_1, and the transverse relaxation rate, R_2, provide values of both the quadrupole coupling constant and the correlation time. The magnitude of the calculated correlation times is in good agreement with the rotational correlation time for the entire protein molecules, indicating the Ca^{2+}-binding sites to have a comparatively rigid structure.

Much of the pioneering works on the NMR of the ^{43}Ca nucleus created an undue pessimism about the usefulness of this method for the study of Ca^{2+}–macromolecule interactions.[1] Many of these investigations were performed at the time when Fourier transform NMR spectrometers and cryomagnets were not available. During the last few years several studies have shown that ^{43}Ca NMR can give interesting information about Ca^{2+} binding to proteins even at millimolar concentrations.[2,3] These studies were performed on systems with relatively fast chemical exchange of the Ca^{2+} ion between the free and protein-bound states; i.e., the observed ^{43}Ca signal is an average signal sensitive to the exchange rate. The application of this method is limited to proteins with Ca^{2+} affinities less than ca. 10^5–10^6 M^{-1}.

In the work presented here we show that the direct observation of the ^{43}Ca NMR signal of the protein-bound Ca^{2+} ions is in some cases possible and that relevant physical and biological information can be obtained from such studies. As examples of this approach we have used three Ca^{2+}-binding proteins: carp muscle parvalbumin, pI = 4.25, rabbit skeletal muscle troponin C, and bovine testes calmodulin.

Experimental Section

Bovine testes calmodulin (CaM) and rabbit skeletal muscle troponin C (TnC) were prepared as described in ref 4 and ref 3, respectively. Carp muscle parvalbumin component, pI = 4.25 (PA), was generously supplied by Professor J. Parello, Montpellier, France.

Ca^{2+}–free TnC and CaM were prepared by passing an aqueous solution of the proteins through a column of Chelex-100. The solutions were then lyophilized and at the time of use dissolved in doubly distilled water. The Ca^{2+} content of TnC and CaM after this treatment, as measured by atomic absorption spectrophotometry, was ≤0.2 mol of Ca^{2+}/mol of protein. For PA, this procedure did not give a sufficient degree of deionization due to the higher affinity of PA for Ca^{2+}. Consequently, Ca^{2+} was replaced by Cd^{2+} ions by simply adding an excess of Cd^{2+} ions to the protein (PA binds Cd^{2+} more strongly than Ca^{2+}), and the excess

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