The Observation of Cis Residues in Poly(L-proline) in Aqueous Solution

C. C. Wu, R. A. Komoroski, and L. Mandelkern*

Department of Chemistry and Institute of Molecular Biophysics, Florida State University, Tallahassee, Florida 32306. *Received June 3, 1975*

ABSTRACT: Proton Fourier transform NMR spectra at 270 MHz were obtained for three different molecular weight samples of poly(L-proline) in D_2O. A weak, but clearly discernable, resonance at 4.3 ppm was observed in each case with an integrated intensity about 2–3% of the C^α trans proton resonance. Based on the Torchia–Bovey assignment this resonance is attributed to the C^α cis proton. The presence of cis residues, even in this small concentration, necessitates a reexamination of the conformational properties of this polymer. Definite conclusions cannot be reached from spectra obtained in organic solvents because of the much smaller separation between the cis and trans resonances.

The conformation of poly(L-proline) in dilute solution has been subject to extensive theoretical and experimental investigations.[1,2] However, no firm conclusion has as yet been drawn. Theoretical expectations are based on conformational energy calculations. Along with the usual assumptions that are made,[3] there is the additional complication in this case of having to properly take into account the twisting or puckering of the pyrrolidine ring.[2,4,5] Several efforts in this direction have lead to quite different conformational energy maps and thus to different predicted average properties as manifested, for example, in the characteristic ratio.[1,6] The characteristic ratio of this polymer has been deduced from experiment, using the same principles that have been applicable to other polypeptides and chain molecules.[6] This procedure assumes that the molecule is statistically conformed. The characteristic ratio determined in this way is very much smaller than that predicted for a planar ring with all trans imide bonds.[1,7] However, Schimmel and Flory[7] have pointed out that the presence of only a very small fraction of units in the cis configuration will markedly decrease the chain dimensions. The energy difference between the cis and trans configuration of the C^α carbon in poly(L-proline) is such as to warrant consideration of this possibility.[8] It is, therefore, important to determine whether a relatively small proportion of units in the cis configuration are present in solvents wherein the chain has been thought to be completely trans. This problem needs to be resolved if further progress is to be made in defining and understanding the conformation of poly(L-proline). In the present paper, we use high resolution proton Fourier transform NMR techniques to examine this problem.

Experimental Section

Three samples of poly(L-proline), M_w = 1,800, 16,300, and 97,000, which have previously been characterized,[6] were used in the main body of this work. The D_2O, "100.0%", was obtained from Diaprep; the acetic-d_4 acid and formic-d_2 acid were purchased from Merck and Co., Inc. To prepare the solution a polymer sample (20 mg) was first dissolved and the solution lyophylized. The polymer sample was then dissolved in either D_2O (1 ml) or the organic solvents (1 ml), and the solution was filtered in a dry atmosphere before obtaining the NMR spectrum.

Proton Fourier transform NMR spectra were obtained at 270 MHz using a Bruker HX-270 FT-NMR spectrometer. Field frequency stability was maintained by locking on the solvent deuterium. The probe temperature was kept at 14 ± 2° using a Bruker variable temperature control unit. At this temperature the residual HDO proton resonance does not overlap with the C^α trans proton resonance[9] (see Figure 1). The width of the 90° radiofrequency pulses used was 23 μsec. A pulse repetition rate of 1.5 sec was used in all cases reported here. This delay time is more than three times greater than the longest proton T_1 in poly(L-proline) in D_2O, which is approximately 450 msec for the α-trans proton.[10] High-sensitivity spectra were acquired using the block averaging method; 50–99 blocks (4096 or 8192 data points) of 200–400 transients were individually accumulated and later added as time domain spectra. The above procedure allowed for optimum analog-to-digital conversion with the 12-bit digitizer of the Nicolet 1089 computer. The resultant free induction decay was Fourier transformed after appropriate exponential filtering to enhance sensitivity.

The chemical shift of the C^α cis proton resonance in D_2O was measured digitally with respect to the corresponding trans resonance that has been previously reported.[9] Chemical shifts of the resolved resonances in organic solvents were measured digitally with respect to tetramethylsilane as the internal reference.

Directly bonded ^{13}C–^1H coupling constants were measured from the undecoupled ^{13}C spectrum of poly(L-proline) at 67.9 MHz and 30°. The one-bond carbon–hydrogen coupling constants (in Hz) for poly(L-proline) are as follows: C^α, 147; C^β, 135; C^γ, 136; C^δ, 143 with an estimated accuracy of ±4 Hz.

Results and Discussion

The pertinent portions of the poly(L-proline) NMR spectra in D_2O are given in Figure 1 for three different molecular weight samples. The resonance at 4.7 ppm has been assigned to the C^α trans proton while the two resonances of almost equal area at 3.8 and 3.6 ppm, respectively, have been assigned to the C^δ protons.[9] From a study of the isomerization (cis → trans) of a low molecular weight sample of this polymer in D_2O, Torchia and Bovey[9] have assigned resonances at 4.3 and 4.4 ppm to the C^α cis protons. In the spectra shown in Figure 1, there is a weak, but clearly discernable, resonance at 4.3 ppm for the two highest molecular weight samples. For the lowest molecular weight this resonance is shifted 0.06 ppm upfield. In each case the integrated intensity of this resonance is about 2–3% of the C^α trans proton resonance. Hence, based on the Torchia–Bovey assignment we have attributed these resonances to the C^α cis proton.

The fact that the cis configuration is observed in such low concentrations using high-sensitivity NMR methods requires stringent precautions be taken to ensure that the assigned resonance does not arise from either impurities or experimental artifacts. Very minor impurities can contribute to the spectrum when it is necessary to detect a resonance which has a line width of approximately 20 Hz and is present to an effective concentration of roughly 0.04%. Hence a number of experimental checks were carried out to ensure the experimental reality of the resonance assigned to the C^α cis protons in D_2O.

Since the resonance at about 4.3 ppm is present in the spectra of three samples of widely different molecular weights obtained from different sources[6] at different times, it is unlikely that it can arise from an impurity directly associated with the polymer. While it is possible that the peak may arise from end groups of the lower molecular weight samples, this cannot be true for the 97,000 molecu-

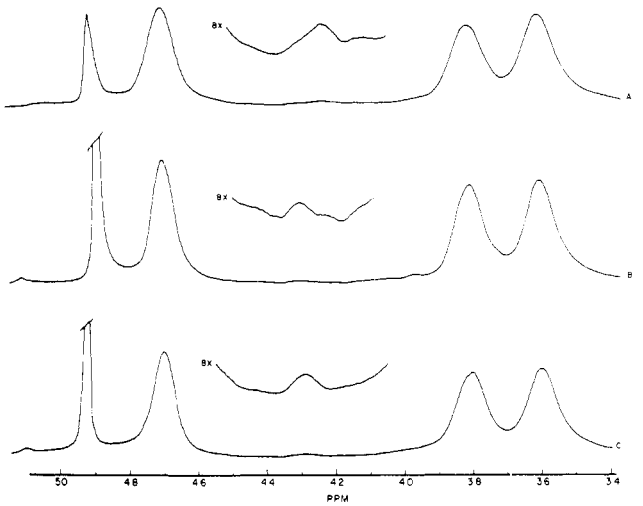

Figure 1. Partial proton Fourier transform NMR spectra at 270 MHz of poly(L-proline) in D_2O at a nominal temperature of 14°. (A) M_w = 1,800, 25,000 scans, 10.4 hr total time; (B) M_w = 16,300, 33,480 scans, 14.0 hr total time; (C) M_w = 97,000, 35,280 scans, 14.7 hr total time. All spectra were obtained using a 1501.5 Hz total spectral width (2048 frequency domain points), a pulse repetition rate of 1.5 sec, and an artificial broadening of 1.4 Hz due to exponential filtering to enhance sensitivity. Chemical shift scale is in ppm from TMS. The variation in the position of the HDO resonance is due to slight differences in temperature among the samples. The relative broadening of the HDO resonance in A compared to B and C is probably due to a slight temperature change during data accumulation.

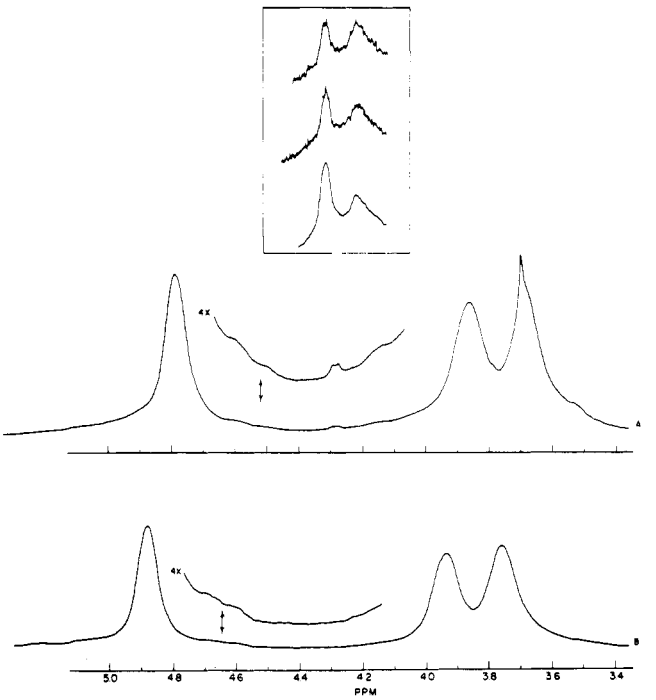

Figure 2. Partial proton Fourier transform NMR spectra at 270 MHz of poly-L-proline (M_w = 97,000) in organic solvents at 14°. (A) In acetic-d_4 acid, 2,500 Hz total spectral width (2048 frequency domain points), 19,800 scans, 8.3 hr total time; (B) in formic-d_2 acid, 4,000 Hz total spectral width (4096 frequency domain points), approximately 24,000 scans, 10 hr total time. An artificial broadening of 1.6 Hz is present due to exponential filtering to enhance sensitivity. Chemical shift scale is in ppm from TMS. The narrow resonances at 4.26 and 3.7 ppm (superimposed on a C^δ proton resonance) in A arise from solvent impurity and were present in a solvent blank. The insert shows the C^α trans and C^α cis proton resonances of poly(L-proline) form I (M_w = 13,500) in acetic-d_4 acid at 14° as a function of time after dissolution. Top, about 15 min after dissolution; middle, about 1 hr after dissolution; bottom, about 2 hr after dissolution. Sample was brought to room temperature for about 0.5 hr between accumulation of the middle and bottom spectra. The spectra given in the insert were taken only to establish the positions of the C^α trans and C^α cis proton resonances, and not to accurately measure the kinetics of the isomerization process.

lar weight polymer. A high-sensitivity proton spectrum of a solvent blank showed no broad resonance at 4.3 ppm. Thus, this peak cannot be due to a D_2O impurity. Low molecular weight impurities would be expected to yield considerably narrower resonances than that observed here at 4.3 ppm. This fact can be taken as further evidence that this peak arises from the presence of cis residues. Other weak resonances appear in the complete spectra of poly(L-proline) in D_2O. Most of these are found in the lower moleclar weight samples. Some can be identified as spinning side bands of the relatively narrow HDO resonance. Others may arise from nonpolymeric impurities or from end effects in the low molecular weight samples.

The ^{13}C–^1H coupling constant for the C^α trans carbon and its directly bonded proton was found to be 147 ± 4 Hz. This value eliminates the possibility that the resonance at 4.3 ppm is actually the ^{13}C satellite of the C^α trans proton resonance, since the peaks in question are separated by 108 Hz in the spectrum for this high molecular weight sample. This 108-Hz separation also ruled out the possibility that the peak at 4.3 ppm was an artifact due to 120 cycle modulation of the C^α trans proton resonance. Moreover, the position of the line assigned to the C^α cis proton does not depend on the position of the radiofrequency carrier frequency.

The resonance at 4.3 ppm is also present in a spectrum accumulated without sample spinning. Thus the possibility that this peak is a spinning side band of a major polymer resonance can be eliminated. This resonance was always present in spectra that were acquired after several major reshimmings of the magnet, and thus the possibility that this peak was due to an artifact associated with field shimming can be dismissed.

Based on these experimental checks we can conclude that the resonance observed at 4.3 ppm is real. Following the work of Torchia and Bovey[9] it is assigned to the C^α cis proton. These investigators assigned the resonances they observed at 4.4 and 4.3 ppm to a unit within a sequence of cis units and to a cis–trans junction, respectively.[9]

Hydrodynamic and thermodynamic properties, and consequently the characteristic ratio, are influenced by the choice of solvent. Trifluoroethanol and organic acids appreciably increase the characteristic ratio.[6] Consequently it is of interest to determine the cis concentration in such solvents. In the present case the choice of solvent is limited by isotopic purity. We have found that either acetic-d_4 acid or formic-d_2 acid are satisfactory for present purposes. It is necessary, however, to establish the position of the C^α cis proton in these solvents. Previous work with Poly(Pro)[11] and Poly(ProGly)[12] has shown that the separation between the cis and trans protons decreases when organic solvents are used. To properly locate the two resonances a low molecular weight sample (M_w = 13,500), predominantly in the cis configuration, was dissolved in either acetic-d_4 acid or formic-d_2 acid. The solution was immediately placed in the spectrometer at 14°. Fourier transform spectra of about 5 min total accumulation time were acquired at several time intervals after dissolution. The results in acetic acid, pertinent to the isomerization, are shown in the insert of Figure 2. The intensity of one of the resonances increases with time at the expense of the other. Thus, we can assign the

resonance at 4.52 ppm to the C^{α} cis proton and that at 4.77 ppm to the C^{α} trans proton. A similar procedure was used to establish the corresponding resonance positions in formic acid. The isomerization rate is much greater in formic than in acetic acid.

The separation between the cis and trans protons is found to be about 0.2 ppm in organic solvents, as compared to 0.4 ppm in aqueous solution. The chemical shifts of the ring protons in formic acid are as follows: C^{α} trans, 4.87; C^{α} cis, 4.65; $C^{\delta 1,2}$, 3.91, 3.74; $C^{\beta 1,2}$, 2.37, 2.12; $C^{\gamma 1,2}$, both 2.12. In acetic acid, the C^{δ}, C^{β}, and C^{γ} protons are partially overlapped by the solvent resonances. Hence, we do not report these chemical shifts.

Once the chemical shifts for the C^{α} trans and cis protons in acetic and formic acid have been established we are in a position to examine the spectrum of the predominantly trans polymer in these solvents. The pertinent features of these spectra, for the highest molecular weight sample, are given in the main body of Figure 2. An examination of Figures 1 and 2 shows that the resonances assigned to the C^{α} trans protons exhibit broad wings in both types of solvents. Although this poses no problem in D_2O, it does present a formidible one in the organic acids. The vertical arrows in Figure 2 indicate the expected position for the resonance assigned to the C^{α} cis proton from the isomerization studies in the organic acids. It is clear that the detection of a weak, broad resonance in the vicinity of the expected location is very difficult so that a definitive assignment is impossible. The reason for this difficulty is the much smaller chemical shift difference between the C^{α} cis and C^{α} trans proton resonances in organic solvents as compared to D_2O. In the organic solvents the "wings" can very easily arise from the ^{13}C satellites which, given the line width involved, can readily merge with the main resonance. Therefore, in contrast to the conclusion that can be drawn from the spectra in Figure 1, the interpretation of the spectra in Figure 2 must by necessity remain equivocal at present.

The presence of about 2–3% of cis residues in D_2O necessitates a reexamination of the conformational energy calculations, the characteristic ratio deduced therefrom, and the basis for the experimental determination of this quantity. Energy calculations for the all-trans chain, which yield a minimum restricted to a narrow region of ψ (rotational angle about the C^{α}–C' bond), invariably yield very high characteristic ratios.[1,2,7] However, Tanaka and Scheraga,[13] utilizing a conformational energy of this kind,[2] have shown by Monte-Carlo calculation that the random introduction of about 5% cis residues leads to a characteristic ratio which agrees with experiment. This concentration of cis units is not incompatible with the results presented here. However, conformational energy calculations, of the type produced by Mattice, Nishikawa, and Ooi,[5] which yield two widely separated minima in ψ, of not too differing energies, can also explain the characteristic ratio for a chain with all-trans imide units. The assignment of the small resonance at 4.3 ppm to the cis peptide bond is based on the original work of Torchia and Bovey.[9] If this assignment is incorrect the isomer could possibly be assigned to the other low-energy state of the all-trans chain.[5,14]

The present work does not definitively establish the distribution of cis units. If it is assumed that they are not in blocks, or ordered sequences, then the possibility must be considered that the molecule exists in solution as a highly ordered structure which is occasionally disrupted, i.e., an interrupted form II helix. If this were the case, then the basis for deducing the characteristic ratio from the intrinsic viscosity and virial coefficient may no longer be valid since a Gaussian distribution of chain elements is required.

The dilemma in the conformational analysis of poly(L-proline), caused by the presence of a few percent of cis residues, does not appear resolvable by indirect methods. It requires that the direct determination be made of the radius of gyration as a function of molecular weight over an extended molecular weight range and including very high molecular weight species.

Acknowledgment. This work was supported by a contract with the Energy Research and Development Administration, Division of Biophysics.

References and Notes

(1) L. Mandelkern and W. L. Mattice, *Jerusalem Symp. Quantum Chem. Biochem.*, **5**, (1973).
(2) S. Tanaka and H. A. Scheraga, *Macromolecules*, **7**, 698 (1974).
(3) P. J. Flory, "Statistical Mechanics of Chain Molecules", Interscience, New York, N. Y., 1969.
(4) D. A. Torchia, *Macromolecules*, **4**, 440 (1971).
(5) W. L. Mattice, K. Nishikawa, and T. Ooi, *Macromolecules*, **6**, 443 (1973).
(6) W. L. Mattice and L. Mandelkern, *J. Am. Chem. Soc.*, **93**, 1769 (1971).
(7) P. R. Schimmel and P. J. Flory, *Proc. Natl. Acad. Sci. U.S.*, **58**, 52 (1967).
(8) G. N. Ramachandran and V. Sasisekharan, *Adv. Protein Chem.*, **23**, 284 (1968).
(9) D. A. Torchia and F. A. Bovey, *Macromolecules*, **4**, 246 (1971).
(10) C. C. Wu, R. A. Komoroski, and L. Mandelkern, unpublished observation.
(11) C. M. Deber, F. A. Bovey, J. P. Carver, and E. R. Blout, *J. Am. Chem. Soc.*, **92**, 6191 (1970).
(12) D. A. Torchia, *Biochemistry*, **11**, 1462 (1972).
(13) S. Tanaka and H. A. Scheraga, in press.
(14) D. A. Rabenold, W. L. Mattice, and L. Mandelkern, *Macromolecules*, **7**, 43 (1974).