Conformational Properties of Poly(L-proline) and Poly(γ-hydroxy-L-proline). 1. Cis Imide Bonds and Evidence for Two Trans Conformers

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ABSTRACT: The conformations of poly(L-proline) and poly(γ-hydroxy-L-proline) chains were analyzed from the results of proton and carbon-13 high-resolution NMR spectroscopy and the characteristic ratios in pure solvents. The absence of cis residues in the poly(L-proline) chain in trifluoroethanol, coupled with its low characteristic ratio in this solvent, requires the population of another trans conformer in the vicinity of $\Psi = -50°$. This result is consistent with one group of conformational energy calculations. The results for each of the polymers in D_2O, where the presence of cis residues is established, can be given a consistent interpretation in accord with the above. It is also shown that in concentrated aqueous salt solution poly(γ-hydroxy-L-proline) undergoes trans → cis isomerization about the imide bond as had been established for poly(L-proline). Previous reports to the contrary can be attributed to the fact that the signal-to-noise ratio in the spectrum was too low.

In contrast to the polypeptides of the α-amino acids, those formed from the imino acids, L-proline (Pro) and γ-hydroxy-L-proline (Hyp), are unique in their ability to exist in a cis or trans configuration about the imide bond either in the solid state or in solution. A major reason for the isomerization of poly(Pro) is that similar steric interactions occur in both the cis and the trans configurations.[1,2] For non-Pro residues, on the other hand, the steric contacts are quite different between the two forms.[2-6] The crystal structures for more than 20 globular proteins have shown that cis peptide units are almost completely absent, with the exception of the particular sequence X-Pro which occasionally has been observed in the cis configuration. An isolated cis non-Pro residue in a string of trans residues has much less conformational freedom than a similarly placed trans residue.[1] On the other hand, for a similar sequence which contains a Pro residue, the difference in the conformational freedom between the cis and trans forms is not as great because of the restricted rotation imposed by the pyrrolidine ring.[1] As a consequence, there is a greater probability for sequences which contain a Pro residue rather than a non-Pro residue to contain a cis peptide bond.[1,2] Thus, the Pro cis peptide unit is an important conformer to be considered in the structural analysis of polypeptides and proteins.

Recent theories concerned with the mechanism of protein renaturation[7-9] have argued strongly that the isomerization of Pro residues is responsible for the slow kinetic step that is observed. This concept has also been further extended to the renaturation of non-Pro containing proteins where cis–trans isomerization has been assigned to other residues.

Hyp residues would also be expected to exist in the cis configuration for the same reasons cited above. However, while poly(Pro) is known to exist in two solid state forms—form I with all-cis imide bonds[10] and form II with all such bonds trans[11,12]—poly(Hyp) has only been definitely observed in a form designated as "A", with all trans imide bonds.[13,96] Poly(Hyp) in contrast to poly(Pro) has not as yet been observed to undergo mutarotation in solution. Because of the important role played by these residues, an analysis which details the specificity of allowed and observed conformations is appropriate. The results that have been obtained to date have not been unique and have lead to confusion in interpretation.

Both of these homopolymers have very similar average dimensional properties in solution. The characteristic ratios, $\langle r^2\rangle_0/n_p l_p^2$, of poly(Pro) and poly(Hyp) in H_2O at 30 °C are 13.7 ± 0.9[14] and 15.9 ± 1.6,[15] respectively. These ratios are much lower, and well beyond the experimental error, than the values that are calculated from conformational energy maps[16-29] for the all-trans polymers which are based on crystallographic pyrrolidine ring geometries.[12,13,30] The ring geometries used for these calculations were either planar, with the dihedral angles taken from the available crystallographic data of Pro peptides, or excessively puckered, usually at the C^γ atom. These type calculations typically allow for only one low-energy minimum in the vicinity of $\Psi = 160°$. On the other hand, if the restrictions imposed by the crystallographic data are relaxed, either by choosing a planar ring with the appropriate ϕ angle[29,31,32] or by allowing moderate ring puckerings[29,31-35] at the C^δ and C^β atoms as well as at the C^γ atom, a new low-energy region near $\Psi = -50°$ is predicted.[36] More details of these calculations will be given below.

Schimmel and Flory,[17] as well as Tanaka and Scheraga,[26] have pointed out that the presence of only a small fraction of cis residues will markedly decrease the average chain dimensions that are deduced for these polymers from the former type conformational energy map. They can in fact be brought into close accord with the experimental results.[26] Recent high-resolution ^{13}C NMR spectroscopy, from our laboratory, has shown that in D_2O both polymers contain a small but clearly measurable cis imide bond content.[37,38] Poly(Pro) has about 2–3% of their residues in this configuration while for poly(Hyp) this value is 0.5–1.5%. Thus, these conjectures with regard to dimensional properties[17,26] need to be given serious consideration in view of these experimental results.

On the other hand, a small population of conformational states, in the low-energy region located about $\Psi = -50°$, also reduces the dimensional properties calculated for the all-trans homopolymers, and they can be brought into experimental concordance.[29,32] Thus, there are alternative explanations for the low characteristic ratios observed. In this paper, we present the first evidence that the rotational state near $\Psi = -50°$ exists and is populated for both poly(Pro) and poly(Hyp). Thus a major difficulty in the conformational analysis of this class of polymers is resolved.

In another aspect of the conformational properties of these molecules, it has been shown that certain classes of salts (see ref 39 and 40 for general reviews), such as LiBr and $CaCl_2$, for example, drastically affect the hydrodynamic and optical properties, such as intrinsic viscosity and

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circular dichroism, and cause a cooperative disordering of the polymer chains. This disordering process in both polymers had been interpreted as arising from either an increase in the accessible range of the C^{α}—C=O rotational angle Ψ[41-44] or the formation of random sequences of cis and trans peptide bonds.[14,42,45-49] Torchia and Bovey[48] and Dorman et al.[50] have demonstrated the presence of cis imide bonds in concentrated aqueous salt solutions of poly(Pro) by 220-MHz ^{1}H NMR and 15- and 25-MHz ^{13}C NMR. The appropriate resonances and identification for the cis and trans isomers were established by studying the mutarotation in aqueous solution.[48,51,52] On the other hand, in marked contrast, it has been reported that poly(Hyp) does not undergo trans-cis isomerization in 6 M LiBr.[53] If this observation is correct, then poly(Pro) and poly(Hyp) differ in this respect. A mechanism other than imide bond isomerization would be necessary to explain the decrease in the dimensions of poly(Hyp) in the presence of these salts as well as the changes that are observed in the optical properties.

For this reason, we have also quantitatively studied both poly(Pro) and poly(Hyp) at both intermediate and high concentrations of several salts. We limit ourselves here to the presentation of a typical ^{13}C NMR result which clearly demonstrates that poly(Hyp) also undergoes salt-induced cis-trans isomerization. In a subsequent paper, the properties of these polymers in the aqueous salt system will be discussed in detail.

Experimental Section

The poly(Pro) and poly(Hyp) samples were obtained from Sigma Chemical Co. in forms I and A, respectively. The form I of poly(Pro) was converted into form II by allowing a formic acid solution to stand overnight at room temperature. The reported molecular weight of both samples was 12000. These values are in good agreement with the viscosity average molecular weights of 10000 for poly(Pro) and 14000 for poly(Hyp) which we determined from intrinsic viscosity measurements using relationships previously established.[54] The polymers were exhaustively dialyzed against deionized distilled water and recovered by lyophilization before use. For experiments where the polymer concentration was critical, the solutions were prepared in volumetric flasks using polymer which had been dried under vacuum with a dry ice/2-propanol trap. In the NMR experiments, the solute concentration is not critical. Therefore, the polymer solutions were prepared directly in the NMR tubes. In this case an estimate of the solution volume was taken from the final height. For these experiments, the polymer concentration was in the range of 30 to 50 mg/mL.

The lithium bromide was of reagent grade and was used without further purification. The D_2O was obtained from Merck, Sharp and Dohme Isotopes and had a 99.7 atom % D content. The trifluoroethanol, TFE, was used as received from Marstan Chemical Laboratories.

Viscosity. Flow times were measured using Cannon-Ubbelohde semimicrodilution viscometers in baths where the temperature was controlled to ±0.03 °C. Flow times for the solvents were 112–140 s. Kinetic energy corrections are not required with these viscometers. Since the intrinsic viscosities of these samples were low, the effect of rate of shear should be negligible as was found to be the case.

NMR. Carbon-13 spectra were obtained using a Bruker HX-270 spectrometer with quadrature detection operating at 67.905 MHz. Samples were contained in 15-mm tubes. Field/frequency stabilization was provided by a deuterium lock. A 90° pulse width of approximately 54 μs was used.

In an effort to minimize temperature gradients along the sample tube, when a high concentration of salt was present, the following procedure was used to maintain samples at ca. 30 °C. Dry nitrogen was passed through a dry ice/2-propanol heat exchanger. Two-level proton decoupling was employed. Typical power levels were 5 W forward and $^1/_2$ W reflected for the high power part of the decoupler cycle. The low power portion was about 1 W, just sufficient to maintain the nuclear Overhauser effect but causing no significant heating of the sample. The heating produced by the decoupling radiofrequency was balanced by the cooling effect of the cold nitrogen flow through adjustments of the nitrogen flow rate and the delay time between pulses (in effect controlling the duty cycle of the decoupler). The pulse delay was always $>5T_1$.[55] Temperatures were measured after a 30-min equilibration period and again after the experiment was completed. Control at elevated temperatures was maintained using a Bruker B-ST 100/700 variable temperature unit.

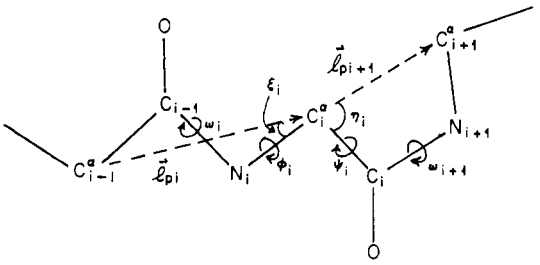

Figure 1. Schematic representation of the backbone of a polypeptide chain with residue i in the trans configuration and residue $i + 1$ in the cis configuration.

For the poly(Pro) sample in trifluoroethanol, it was necessary to null the solvent resonances in order to achieve sufficient dynamic range. Since the T_1's of the solvent nuclei were much longer than those of the peptide nuclei, an inversion–recovery pulse sequence was used with a delay between pulses sufficiently long to allow observation of the fully relaxed peptide resonances but a postacquisition delay time short enough to effectively null the solvent methylene resonances and nearly null the methyl resonances. The actual parameters were 180°–1.02 s–90°–2.41 s. This sample also contained a coaxial 4-mm capillary tube with D_2O for a lock signal.

Proton spectra were obtained at 80 °C with the HX-270 spectrometer operating in the Fourier transform mode. The analysis of chemical shifts and coupling constants was performed using the ITERCAL version of LAOCN3[56] on a Nicolet 1080 series computer.

Computations. The influence of cis residues on the mean-squared unperturbed end-to-end distance, $\langle r^2\rangle_0$, was obtained by applying Flory's rotational isomeric state theory.[60,61] Figure 1 illustrates the skeletal backbone of a polyimide chain with residue i in the trans configuration and residue $i + 1$ in the cis configuration. The virtual bond vector $\mathbf{l}_{p,i}$ and the angle ξ_i are dependent on the state of rotation about ω_i. The angle η_i is dependent on the state of rotation about ω_{i+1}. Therefore, the transformation matrix, $\mathbf{T}_i$, not only depends on ϕ_i and Ψ_i but also on ω_i and ω_{i+1}.[59] It is given by

$$\mathbf{T}_i = \mathbf{T}_i(\omega_i,\omega_{i+1},\phi_i,\Psi_i) = \mathbf{R}(\xi_i,-\omega_i - \pi)\,\mathbf{R}(\theta^{\alpha},-\phi_i)\,\mathbf{R}(-\eta_i,-\Psi_i - \pi) \quad (1)$$

The angle θ^{α} is the supplement of the N–C^{α}–C^{β} angle. To simplify matters for present purposes, we assume that only two rotational states are accessible to ω, the trans, t, and the cis, c, state. The configurational partition function, Z, for a polypeptide chain containing n_p peptide bonds can be calculated from eq 2. The

$$Z = \mathbf{J}^{*}U_1^{n_p}\mathbf{J} \quad (2)$$

$$U = \begin{bmatrix} \mathbf{U}_{tt} & \mathbf{U}_{tc} \\ \mathbf{U}_{ct} & \mathbf{U}_{cc} \end{bmatrix}_i \quad (3)$$

$$\mathbf{J}^{*} = [11\ldots1] \quad (4)$$

$$\mathbf{J} = \mathrm{col}(100\ldots0) \quad (5)$$

superstatistical weight matrix, U_i, for residue i is defined by eq 3, and the terminal matrices are defined by eq 4 and 5.

In this formulation, the rotations of neighboring residues are interdependent upon one another since the rotational states of residue i depend upon the state of residue $i + 1$, as compared to the usual notation.[64] Here, $\mathbf{U}_{\zeta\eta,i}$ represents the statistical weight matrix for the peptide bond of residue i, i.e., ω_i, in state ζ and the peptide bond of residue $i + 1$, ω_{i+1}, in state η. In this case,

the states are either trans, t, or cis, c. Symbolism of the type U_a^b denotes the product of b successive matrices, commencing with U_a.

The fraction of peptide bonds in the cis state, f_c, can be obtained from eq 6. The super matrix for internal peptide units is defined by eq 7, and the terminal matrices are given by eq 8 and 9.

$$f_c = Z^{-1} n_p^{-1} \hat{U}_1^{n_p} \tag{6}$$

$$\hat{U}_i = \begin{bmatrix} U & U \\ \mathbf{0} & U \end{bmatrix}_i \tag{7a}$$

and

$$U_i = \begin{bmatrix} \mathbf{0} & \mathbf{0} \\ \mathbf{U}_{ct} & \mathbf{U}_{cc} \end{bmatrix}_i \tag{7b}$$

$$\hat{U}_{[1} = \boldsymbol{J}^* [\mathbf{E} \quad \mathbf{0}] \hat{U}_1 \tag{8}$$

$$\hat{U}_{n_p]} = \hat{U}_{n_p} \, \mathrm{col}(\mathbf{0}, \mathbf{E}) \boldsymbol{J} \tag{9}$$

The identity matrix of the appropriate order is $\mathbf{E}$, while $\mathbf{0}$ represents the rectangular null matrix.

The mean-square unperturbed end-to-end distance, $\langle r^2 \rangle_0$, can then be calculated from

$$\langle r^2 \rangle_0 = Z^{-1} \boldsymbol{G}_1^{n_p} \tag{10}$$

where

$$G_i = \begin{bmatrix} \|\mathbf{G}\|_{tt}(\mathbf{U}_{tt} \otimes \mathbf{E}_s) & \|\mathbf{G}\|_{tc}(\mathbf{U}_{tc} \otimes \mathbf{E}_s) \\ \|\mathbf{G}\|_{ct}(\mathbf{U}_{ct} \otimes \mathbf{E}_s) & \|\mathbf{G}\|_{cc}(\mathbf{U}_{cc} \otimes \mathbf{E}_s) \end{bmatrix}_i \tag{11}$$

Here $\|\mathbf{G}\|_{\zeta\eta,i}$ is a diagonal array of generator matrices for ω_i in state ζ, while ω_{i+1} is in state η. The individual generator matrix, $\mathbf{G}_{\xi o,\zeta\eta,i}$, for residue i in the ϕ, Ψ rotational state ξ and residue $i + 1$ in the ϕ, Ψ state o, with ω_i in state ζ and ω_{i+1} in state η is given by

$$\mathbf{G}_{\xi o,\zeta\eta,i} = \begin{bmatrix} 1 & 2\mathbf{l}^{\mathrm{T}}{}_{p\zeta}\mathbf{T}_{\xi o,\zeta\eta} & \mathbf{l}^2{}_{p\zeta} \\ 0 & \mathbf{T}_{\xi o,\zeta\eta} & \mathbf{l}_{p\zeta} \\ 0 & 0 & 1 \end{bmatrix}_i \tag{12}$$

The virtual bond vector, $\mathbf{l}_{p,i}$, and its transpose, $\mathbf{l}^{\mathrm{T}}{}_{p,i}$, for residue i are dependent only on the state of the ith peptide bond, ζ. As indicated above, the transformation matrix for residue i, $\mathbf{T}_{\xi o,\zeta\eta,i}$, is dependent on the state of the ϕ, Ψ rotational angles for residue i, ξ, and for residue $i + 1$, o. $\mathbf{T}_i$ also depends on the state of ω_i, ζ, and ω_{i+1}, η. The terminal matrices in eq 10 are defined as

$$\boldsymbol{G}_{[1} = \boldsymbol{J}^* \otimes [10000] \boldsymbol{G}_1 \tag{13}$$

$$\boldsymbol{G}_{n_p]} = \boldsymbol{G}_{n_p} \boldsymbol{J} \otimes \mathrm{col}(00001) \tag{14}$$

Although in the above formulation ω has been allowed only two rotational states, the development is completely general and can be easily expanded to accommodate additional rotational states.

In order to reduce the amount of storage and computer time required to calculate $\langle r^2 \rangle_0$ for a poly(Pro) chain, certain simplifying assumptions were made. One assumption was that ω can only exist in one of two rotational states, 0 and 180°, the cis and trans states, respectively. It was also assumed that $\boldsymbol{U}_i$ is independent of rotations in neighboring residues. Because of the large barrier to rotation[41,62,63] about the imide bond, ω must be limited at best to small distortions (ca. ±15°)[5,64-67] about the two minima. These distortions will, however, tend to average out in the measured average dimensional properties. This assumption is thus a reasonable one. A second assumption that is made will not in general be correct, especially for the cis state. It is not mandatory, however, since the resolution of the interdependent rotational problem can be carried out exactly.[60] However, it is acceptable for present purposes, since we are interested in gross dimensional effects resulting from the incorporation of cis peptides into the chain. As will be seen below, extreme cases were treated which in turn compensate for the procedure adopted. With these assumptions, the internal supergenerator matrix, $\boldsymbol{G}_i$, can be replaced by

$$G_i = (\mathbf{A} \otimes \mathbf{E}_s) \begin{bmatrix} \langle G \rangle_{tt} & \langle G \rangle_{tc} \\ \langle G \rangle_{ct} & \langle G \rangle_{cc} \end{bmatrix}_i \tag{15}$$

The matrix $\mathbf{A}$ which is dependent only on the fraction cis, $\alpha = f_c$, is defined as

$$\mathbf{A} = \begin{bmatrix} 1 - \alpha & 0 \\ 0 & \alpha \end{bmatrix} \tag{16}$$

The average generator matrices were obtained in the usual manner,[57] except that $\mathbf{T}$ was evaluated using eq 1. The virtual bond lengths, l_p, and angles, ξ and η, used were 3.825 Å, 15.95°, and 18.95° for the trans state and 2.907 Å, 57.19°, and 58.81° for the cis state, respectively. These values were obtained from the structure of the internal dimer of poly(Pro) previously used by Nishikawa and Ooi.[31] The trans γ^1 conformational energy map of Mattice et al.[32] was used to obtain $\langle G \rangle$ at 30 °C.

Results and Discussion

Conformational Analyses in Pure Solvents. The characteristic ratio of poly(Pro) has been experimentally determined in several organic solvents including trifluoroethanol, TFE, where it was found to be 19.8 ± 1.3.[14] In water, the characteristic ratio is 13.7 ± 0.9.[14] Both values are substantially lower than those obtained from conformational energy maps for an all-trans chain, with one energy minimum restricted to the range 90° < Ψ < 220°. Only if one assumes a square energy well for this region is a characteristic ratio of 20 predicted. Thus to reconcile these results some other low-energy rotational state must be populated both in water and in organic solvents. A small fraction of *cis* peptide residues or a small population of the rotational state near Ψ = −50°, or a combination of the two, can lower the average chain dimensions to the experimentally observed values. Since the characteristic ratio is higher in the organic solvents, a decrease in the population of either one or both of these states is implied. Before pursuing the implication of this observation in more detail, it is helpful to review the major highlights of the myriad of conformational energy calculations that have been reported for poly(Pro) and poly(Hyp), which have been briefly alluded to in the introduction.

Many theoretical studies have been reported which attempt to predict the conformational properties of poly(Pro)[16-35] and poly(Hyp)[23,27-29] as well as Pro containing peptides.[2,68-76] Conformational energy maps have been obtained for the helical homopolymers,[16,18-20,24,28] for their internal dimers,[17,22,23,25-27,29,31-35] and for Pro preceding and succeeding other amino acids.[2,68-73,75] In one approximation, the geometry of the pyrrolidine ring has been fixed.[2,16-29,68-76,77] Without exception, where the planar trans configuration has the -X-Pro- sequence (where X is either a Pro or a non-Pro amino acid) this ring restriction has led to the prediction of only one broad low-energy minimum for Ψ_X near 160°.[81] On the other hand, the Pro-Y sequence (where Y is a non-Pro amino acid) yields two low-energy regions for Ψ_{Pro}. In addition to the region at Ψ near 160°, there is another low-energy region near Ψ = −50°. More recent calculations of Zimmerman and Scheraga[2,76] on -X-Pro- and -Pro-Y- sequences also reflect these trends for Ψ_X and Ψ_{Pro}.[82] The reason for these differences in the trans chain, under these assumptions, is that the δ carbon protons of the ring make severe steric contacts with the β-carbon protons of the preceding residue when its Ψ rotational angle is near Ψ = −50°. Thus this conformer is excluded in -X-Pro- peptides.

The above restrictions on the pyrrolidine ring structure have been shown to be quite severe by other energy cal-

culations of Pro peptides in which a range of angles was allowed for the pyrrolidine ring. For the examination of a single Pro residue, it has been found[33,35,80-82] that the dihedral angles of the pyrrolidine ring can accommodate a narrow range of angles (ca. ±20°). More refined calculations[33,35,82] of the pyrrolidine ring of Pro have shown the existence of two broad energy minima,[83] and it should, therefore, be quite flexible.[84] Ooi and co-workers[29,31,32] have demonstrated that inclusion of such pyrrolidine ring flexibility considerably alters the computed conformational distribution for Pro (or Hyp) peptides. Of particular importance was their finding that for trans peptides the Ψ_X rotational angle in -X-Pro(Hyp)- sequences had comparable energies for the regions near both Ψ = 160 and -50°. Venkatachalam et al.[33,34] have considered a wider variation of ring dihedral angles and also predict the existence of two low-energy minima for Ψ_X in the trans internal Pro dimer. Recently Madison[35] has calculated the intramolecular energy of five Pro peptides which were minimized with respect to all internal coordinates. In particular, the intramolecular energy of *N*-acetyl-L-proline-*N'*,*N'*-dimethylamide was calculated. This study predicted stable conformers for Ψ_{Pro} either near -50° or near 160° for both cis and trans peptides. Although this Pro peptide can serve as a good model for the *all-trans*-Pro-Pro sequence, it is not very satisfactory for the *all-cis*-Pro-Pro sequence. In this case the peptide group of the succeeding Pro residue would forbid any Ψ angles near -50° for the preceding residue because of very severe steric contacts experienced by the pyrrolidine ring. However, Madison's calculations suggest that this conformer for the preceding residue in the -Pro-Pro- sequence in which the preceding peptide bond is cis and the succeeding peptide bond is trans may exist.

The consequences of having even a small population of the Ψ conformer near -50° on the predicted average dimensions of the *all-trans* poly(Pro) or poly(Hyp) chain have been shown to be quite dramatic.[29,32] The fixed ring geometry conformational maps which show only one conformer near Ψ = 160° predict very large characteristic ratios, $\langle r^2\rangle_0/n_p l_p^2$, which differ significantly among one another.[85,86] The all-trans internal Pro dimer map of Schimmel and Flory[17] predicts a characteristic ratio at n_p = 1000 of 107, while those of Go and Scheraga[73] predict ratios of 29, 39, 68, and 70, depending on the different ring puckerings at C^γ. However, by allowing the Ψ conformer near -50° to be populated, the predicted characteristic ratio of the *all-trans*-poly(Pro) chain is reduced substantially. Mattice et al.[32] predict ratios of 17 and 12 from conformational maps of the flexible *trans*-Pro internal dimer which included 2 and 6% of the conformer near Ψ = -50°, respectively. Exclusion of this conformer from these maps predicts ratios of 29-30. The reason for this reduction in $\langle r^2\rangle_0/n_p l_p^2$ is that this conformer introduces a sharp bend in an otherwise extended but nonordered chain,[88] and thus a few sharp bends randomly distributed drastically reduce the overall chain dimensions.

Returning to the reason for the observed low characteristic ratios, it is clear that the question of whether cis imide groups are present in TFE solution, as they are in D_2O, is of prime importance. To study this problem we have obtained the ^{13}C NMR spectrum of poly(Pro) in this solvent.[97] Since the solution was relatively dilute for experiments, being only 4% in poly(Pro), it was necessary to null the solvent resonances to achieve sufficient dynamic range to permit observation of cis residues at a 0.5% level. This was accomplished by using an inversion-recovery pulse sequence previously described. The aliphatic region

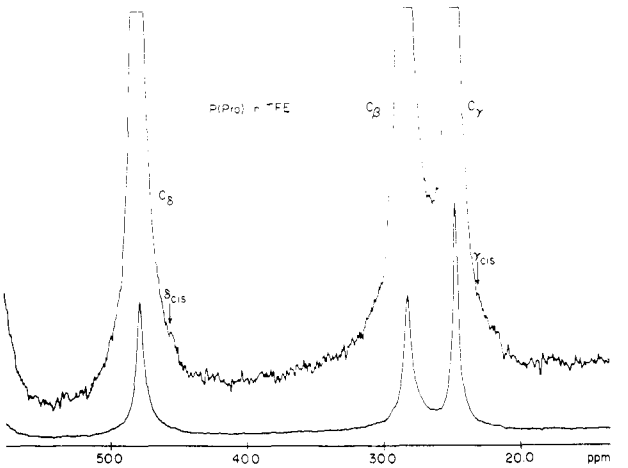

Figure 2. Partial carbon-13 spectrum at 67.9 MHz of poly(L-proline) in trifluoroethanol. The solvent resonances were suppressed as described in the text. The spectrum was obtained at room temperature and consists of 32 500 scans. The cis resonance positions for C^δ and C^γ are indicated. The chemical shift scale is in ppm from Me_4Si.

Table I
Characteristic Ratios and Fraction Cis for Poly(Pro) and Poly(Hyp) in H_2O and TFE

system	characteristic ratio	f_c
Poly(Pro)-H_2O	13.7 ± 0.9[a]	0.025 ± 0.005[c]
Poly(Hyp)-H_2O	15.9 ± 1.3[b]	0.010 ± 0.005[d]
Poly(Pro)-TFE	19.4 ± 1.6[a]	<0.005

[a] From ref 14. [b] From ref 15. [c] From ref 37. [d] From ref 38.

of the resulting spectrum is shown in Figure 2. The cis resonances were identified by dissolving poly(Pro) form I in TFE and recording the spectra during the mutarotation. The cis resonances for the δ, β, and γ carbons are all upfield from the corresponding trans resonances by 1.7, 0.5, and 2.2 ppm, respectively. The α carbon is obscured by the methylene carbon of TFE. Because of the larger chemical shift separations for the cis and trans isomers, the C^δ and C^γ resonances are best for detecting cis residues. The arrows in Figure 2 indicate the position where these cis resonances occur. As can be seen, cis resonances are not observed within the signal-to-noise limit of the spectrum. This limit is sufficient to detect cis isomers at a level of about 0.5%.

A summary of the characteristic ratios and fraction cis, f_c, for the two polymers is given in Table I. The data in the table indicate quite conclusively that there is a major difference in cis content for poly(Pro) in water and in TFE. In the latter solvent, the cis content is essentially absent, so that this configuration cannot be involved in determining the characteristic ratio in this case. An explanation for the difference in the cis population of poly(Pro) in the two solvents can be explained by the infrared observations of Strassmair, Engel, and Knof.[89] They found that organic alcohols, such as TFE and benzyl alcohol, bind to the carbonyl groups of poly(*O*-acetyl-γ-hydroxy-L-proline). The alcohol binding constants for the peptide carbonyl were two to five times greater when the peptide bonds were in the trans as opposed to the cis configuration. The side chain carbonyl did not show this preferential binding. Assuming that water has equal access to both the random cis and trans residues, then from their binding constants the trans residue at room temperature would be stabilized to 0.5-1.0 kcal/mol per residue with respect to the random

Table II
Comparison of Experimental Characteristic Ratios with Theoretical Fraction Cis Taken from the Curves in Figure 3

curve no.	conformational map	$90° < \Psi < 220°$	$-10° < \Psi < -70°$	exptl CR[a]	fraction cis[b]	polymer
1	Mattice et al.[c]	yes	no	19.4 ± 1.6[e]	0.025 ± 0.008	Pro
				15.9 ± 1.6[f]	0.035 ± 0.010	Hyp
				13.7 ± 0.9[g]	0.045 ± 0.010	Pro
2	Mattice et al.[c]	yes	yes	19.4 ± 1.6[e]	0.005 ± 0.005	Pro
				15.9 ± 1.6[f]	0.016 ± 0.009	Hyp
				13.7 ± 0.9[g]	0.026 ± 0.008	Pro
				calcd CR		
	Tanaka and Scheraga[d]	yes	no	29-30	0.0	Pro
				17-20	0.05	Pro

[a] At 30 °C. [b] The error limits reflect the breadth between the (a) and (b) curves of Figure 3 and the experiment error in the observed CR's. [c] γ^1 conformational energy map, ref 32. [d] Reference 25. [e] In trifluoroethanol, ref 14. [f] In H_2O, ref 15. [g] In H_2O, ref 14.

cis in an organic alcohol solvent as compared with water. This additional trans stabilization would cause a lowering of the fraction cis to between 0.01 and 0.005 in the organic alcohols. Thus the major reduction in the cis content can be attributed to preferential alcohol binding to the trans peptide carbonyl group.

Since the fraction of cis residues in poly(Pro) is so low, if not negligible, in TFE the rotational state near $\Psi = -50°$ *must* be populated in order to explain the low characteristic ratio observed in this solvent. This conclusion is more quantitatively demonstrated by the plot of Figure 3 and the data summarized in Table II. Figure 3 is a plot of the calculated characteristic ratio against the fraction of cis residues using the methods previously described. The γ^1 conformational energy map of Mattice et al.[32] for the trans peptide chain was used for these calculations. Curve 1 is obtained by restricting Ψ to the region between 90 and 220° for the trans residues. Curve 2 is obtained by including 3% of the energy minimum near $\Psi = -50°$ for the trans residues. Curve (a) is obtained by assuming the ϕ, Ψ angles of the cis residues to be those of the form I helix (i.e., −83°, 158°, respectively).[10] Curve (b) is obtained by assuming the ϕ, Ψ angles of the cis residues equal to the broad low-energy region of the trans residues, thus restricting Ψ between 90 and 220°. Curves (a) and (b) represent upper and lower limits for the influence of random cis residues on the characteristic ratio and thus justify the simplifications used in the calculations previously described. Although the average virtual bond length, $\bar{l}_p$, varies with the fraction of cis residues, it was held constant at the all-trans length of 3.83 Å. This procedure allows for a more direct comparison between the calculated and the experimental characteristic ratios. The backbone geometry was the same as that used by Nishikawa and Ooi.[31]

Figure 3 demonstrates clearly that a small fraction of cis residues has a dramatic effect on the calculated characteristic ratios, in agreement with previous predictions.[17,26] However, if Ψ for the trans residues is restricted to the region between 90 and 220°, i.e., curve 1, the fraction of cis residues required to lower the characteristic ratio to the experimental values for poly(Pro) and poly(Hyp) in water, and in particular for poly(Pro) in TFE, is greater than is observed experimentally. However, if only a small fraction of trans residues with rotational states near $\Psi = -50°$ are included, i.e., curve 2, then the theoretically estimated fraction of cis residues and the experimental fraction agree quite well.

This point is further illustrated in Table II. The fraction cis listed in this table was taken from the curves in Figure 3 and corresponds to the observed characteristic ratios. The error limits reflect those for the experimental characteristic ratios and the breadth between curves (a) and (b).

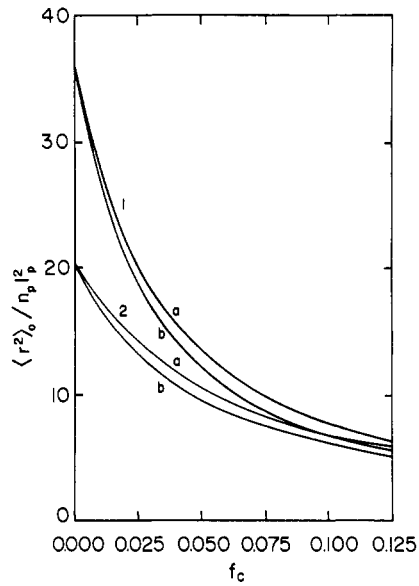

Figure 3. Plot of the characteristic ratio, $\langle r^2\rangle_0/n_p l_p^2$, as a function of the fraction cis for poly(L-proline) or poly(γ-hydroxy-L-proline) with $n_p = 1024$ and $l_p = 3.83$ Å at 30 °C. Curve 1, trans residues restricted to the region −80°, $90° < \phi, \Psi < -40°$, 220° only; and curve 2, 97% of the trans residues restricted to the region −80°, $90° < \phi, \Psi < -40°$, 220° with the remaining 3% in the region −80°, $-10° < \phi, \Psi < -60°$, −70°. See text for further details.

A comparison of the observed fraction cis with the values required to satisfy the results for both poly(Pro) and poly(Hyp) in H_2O indicates that either of the two general type conformational energy maps would be acceptable. An unequivocal decision cannot be made in this case. However, for poly(Pro) in TFE the experimental characteristic ratio of 19.8 requires a fraction cis of 0.025 using curve 1. This value is much higher than the experimentally determined quantity and is incompatible with the results. However, by including a small fraction of the trans states near $\Psi = -50°$, i.e., using curve 2, excellent agreement is found between the estimated and experimental fraction cis for poly(Pro) in TFE and also for poly(Pro) and poly(Hyp) in H_2O. Future conformational energy calculations for the trans or cis residues may alter the general appearance of Figure 2. However, it is very unlikely that they would cause a decrease in the characteristic ratio much below 30 without the inclusion of either of the two rotational states we have described.

The inexorable conclusion that is drawn from this analysis is that the conformational state near $\Psi = -50°$ for the trans residue must be accessible for poly(Pro) in

Table III
Comparison of Vicinal Couplings for Various Pyrrolidine Ring Conformations with the Values for Poly(Hyp)[a]

vicinal couplings	C^{γ}_{exo}[b]	C^{β}_{exo}[c]	$(0.9C^{\gamma}_{exo} + 0.1C^{\beta}_{exo})$[d]	poly-(Hyp)[e]	vicinal couplings	C^{γ}_{exo}[b]	C^{β}_{exo}[c]	$(0.9C^{\gamma}_{exo} + 0.1C^{\beta}_{exo})$[d]	poly-(Hyp)[e]
$J_{\alpha\beta_1}$	8.38	9.25	8.47	8.0	$J_{\gamma_2\delta_1}$	1.57	3.35	1.75	2.0
$J_{\alpha\beta_2}$	8.45	2.01	7.81	8.0	$J_{\gamma_2\delta_2}$	4.60	8.1	4.95	5.0
$J_{\beta_1\gamma_2}$	1.57	4.82	1.90	2.0	σ[f]	0.51		0.29	
$J_{\beta_2\gamma_2}$	4.60	7.89	4.93	5.0					

[a] In Hertz. [b] C^{γ}_{exo} ($X_1 = -X_4 = -25°$, $X_2 = -X_3 = 45°$, $\Phi = -60°$). [c] C^{β}_{exo} ($X_1 = -X_2 = 16°$, $X_3 = 10°$, $X_4 = 0°$, $\Phi = -70°$). [d] 10% of the couplings for the C^{β}_{exo} conformation added to the couplings for the C^{γ}_{exo} conformation. [e] Reference 94 and this work, uncertainty ±1.0 Hz. [f] $\sigma = [\Sigma_{i=1}^{N}(J_i(\text{calcd}) - J_i(\text{obsd}))^2/(N-2)]^{1/2}$.

TFE, and it is highly probable that it is also available to poly(Pro) and poly(Hyp) in water as well. We, therefore, have presented the first experimental evidence for the existence of this rotational state in poly(Pro).

The question as to whether the energy barrier for rotation about Ψ from about 160 to −50° is sufficiently high to yield two distinct NMR resonances should be considered. Several estimates of this barrier have been made for isolated Pro peptides.[33-35,93] The calculated values are 8,[93] 10,[35] and 15[33,34] kcal/mol. Although the calculated barrier of 15 kcal/mol[33,34] suggests that two distinct NMR resonances could be observed, they apparently are not observed in the homopolymer spectra. Roques et al.[94] did not observe any splitting in the trans ^{13}C resonances of the dipeptide Pro-Hyp in CD_3OD in the temperature range between −20 and −70 °C. These results suggest that the rotational barrier must be lower than 9.7 kcal/mol assuming minimum frequency separation.[95] This value is in good agreement with that calculated by Madison[35] for the proline peptide, $AcProMe_2A$, using a molecular mechanics approach; it is also close to the value calculated by Tonelli.[93] In the homopolymer, this barrier does not appear to be significantly enhanced. Here the rotational state near $\Psi = 160°$ produces a relatively extended structure so that medium- and long-range chain contacts are minimized. The state near $\Psi = -50°$ produces a bend in this relatively extended structure. However, unless there are consecutive residues in this more compact state, the medium range contacts are no more severe than for the all extended chain, and long-range contacts are minimized. Since only a small percentage of residues with rotational states near $\Psi = -50°$ are required to reduce the dimensional properties, medium-range contacts will be minimized. Therefore, the energy barrier for the isolated Pro peptide should be representative of the barrier in the homopolymers.

Since the accessibility of the region near $\Psi = -50°$ is crucially dependent on the pyrrolidine ring geometry, we have reexamined the conclusions reached from studies of proton vicinal coupling constants. Torchia[90] has analyzed the 220-MHz proton spectrum of poly(Pro) in D_2O. From a Karplus analysis of the vicinal coupling constants, it was concluded that in solution the proline ring rapidly interconverts between two conformations puckered at C^{γ}, C^{α}, C^{β}, or C^{δ}. Twisted conformations also were compatible with the data. This NMR study offers further evidence of ring flexibility. A similar analysis by Torchia[91] of the 220-MHz proton spectrum of poly(Hyp) appeared to indicate the existence of only one predominant ring conformation. This conformation is the one required for the formation of an intrachain hydrogen bond. Torchia and Lyerla[55] examined the ^{13}C T_1's for poly(Pro), poly(Hyp), and the glycyl copolymers. From an analysis of the effective correlation times of the side chain, they concluded that there was less ring mobility for poly(Hyp) than for poly(Pro). A recent neutron diffraction study of γ-hydroxy-L-proline indicates, however, that in the solid state the ring may be quite flexible.[92]

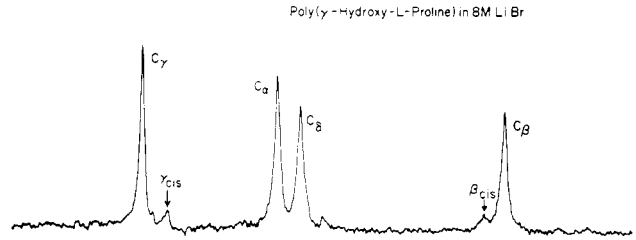

Figure 4. Partial carbon-13 spectrum at 67.9 MHz of poly(γ-hydroxy-L-proline) in 8 M LiBr D_2O solution. The spectrum was obtained at 54 °C and consists of 20 000 scans with a delay of 1.7 s between 90° pulses. The cis resonance assignments are indicated.

Because of the slightly higher magnetic field available to us, it was decided to investigate the 270-MHz proton spectrum of poly(Hyp) in D_2O. Iterative analysis of the proton spectrum yielded, within experimental error, the same chemical shifts and coupling constants as reported by Torchia. These original values were also used as starting parameters for our analysis. However, the errors in the individual coupling constants are relatively large in both cases (ca. ±1 Hz) because of the line widths. Thus a contribution from a second conformation of relatively low population could escape notice.

The conformation which Torchia[91] proposed for poly(Hyp) was a C^{γ}_{exo} ring puckering. One of the ring conformations which can produce a low-energy minimum near $\Psi = -50°$ is a shallow C^{β}_{exo} conformation.[32] This shallow puckering conformation allows the C^{β}_{i} protons to move away from the C^{δ}_{i+1} protons, thereby avoiding severe contacts between these protons. Using the Karplus equation and the Karplus constants from Torchia's analysis[91] of poly(Hyp), it is possible to calculate the expected vicinal coupling constants for these two conformations. The estimated conformational dihedral angles and the calculated vicinal coupling constants are shown in Table III along with the experimental coupling constants taken from Torchia's analysis.

From Table III it is clear that while the C^{β}_{exo} conformation cannot be a major contributor to the observed coupling constants, if as little as 10% of this conformation is included, the agreement between the calculated and the experimental couplings is comparable to that obtained for the pure C^{γ}_{exo}. Thus the ^1H NMR data do not exclude the possibility of more than one ring conformation and hence do not preclude ring flexibility in poly(Hyp). It, therefore, is consistent with the conformational energy analysis and characteristic ratio of this type chain.

Isomerization of Poly(Hyp) in Salt Solution. It has been reported that, in contrast to poly(Pro),[50] poly(Hyp) does not undergo salt isomerization.[53] This result would appear to be in contradiction to the similarity in other properties of the two polymers. Hence we have reexamined this particular problem. In Figure 4 we present the ^{13}C spectrum of poly(Hyp) in 8 M LiBr that we have obtained. It is clear from this spectrum that poly(Hyp)

does indeed undergo isomerization in this salt. The previous conclusion for the same system is thus not correct and can probably be attributed to the fact that the signal-to-noise ratio in the previous report[53] was so low that the cis resonances were obscured. Thus both types of pyrrolidine polymers isomerize under these conditions. The fraction of cis residues for this system, as determined from the spectrum of Figure 4, is 0.07 ± 0.01. With the removal of this anomaly, a quantitative study of isomerization of both poly(Pro) and poly(Hyp) by a variety of salts can be undertaken.

In a subsequent paper we shall discuss the results of the isomerization on properties and propose a model which allows for a quantitative explanation of the dimensional changes observed.

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(78) Hopfinger and Walton (ref 20 and 28) have calculated in addition to the minimum near $\Psi = 160°$ another broad minimum near $\Psi = -50°$ for their dimer and trimer. However, the particle charges they have assigned to the proline residues result in a net positive charge for the oligomers. Because of this and because their total energy relies heavily on their particle charges, one cannot give serious consideration to these calculations.
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(97) ^{1}H NMR proved unsuccessful for this purpose because the cis α-proton resonance overlaps the trans resonance in organic solvents.

Conformational Energy Calculations of Enzyme–Substrate and Enzyme–Inhibitor Complexes of Lysozyme. 2. Calculation of the Structures of Complexes with a Flexible Enzyme[1]

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ABSTRACT: Conformational energy calculations were carried out to investigate the most favored binding modes of oligomers of β-D-*N*-acetylglucosamine (GlcNAc) to the active site of lysozyme. Both the substrate and the side chains of the enzyme were allowed to undergo conformational changes and relative motions during energy minimization. It was found that, regardless of whether the side chains of the enzyme were held rigidly or were allowed to move, $(GlcNAc)_6$ with standard geometry had a clear preference for binding to the active site cleft with its last two residues on the "left" side of the cleft region. This region contains such residues as Arg 45, Asn 46, and Thr 47, compared with the "right" side of the cleft which contains such residues as Phe 34 and Arg 114. This result was obtained irrespective of the location of the fourth residue, i.e., irrespective of how deeply it was buried in the active site cleft. Our earlier calculation (with the enzyme held rigidly), starting with the model-built structure (from the literature), with residue 4 buried deeply in the D site on the right side of the cleft and having a half-chair conformation, led to a high-energy structure. However, when the side chains of the enzyme were allowed to undergo changes of conformation, the energy of this structure was lowered significantly because of favorable contacts on the right side of the cleft. Nevertheless, the conformational energy of this structure was still higher (by about 5 kcal/mol) than that of the most stable hexamer having standard geometry (i.e., without distortion of residue 4) and situated on the left side of the cleft. In addition, a hexamer with standard geometry could bind with the same low conformational energy as that of the energy-minimized model-built structure on the right side without distortion of residue 4. However, the conformational energy of this structure could be lowered by ~14 kcal/mol, if distortion of the fourth residue, binding in the D site, were allowed. The conformational energy of this latter structure was the lowest of all those found in the energy search, if the strain energy for the fourth residue were ignored. If it were taken into account, however, the conformational energy of this species would probably increase so that it would become equal to or higher than that for the lowest energy hexamers binding to the left side of the active site. The finding of three low-energy structures, an undistorted mode on the left side of the active site, an undistorted mode of higher conformational energy on the right side, and a distorted mode on the right side, correlates well with recent experimental kinetic data on the binding of $(GlcNAc)_6$ to lysozyme. Finally, the presence of the *N*-acetyl group on GlcNAc provides sufficient interactions to account for the fact that GlcNAc oligomers bind to lysozyme, whereas glucose oligomers bind with much lower affinity.

In a series of publications,[3-5] we have used conformational energy calculations to compute the structures of complexes of oligomers of β-D-*N*-acetylglucosamine (GlcNAc) with the active site of hen egg white lysozyme. The ultimate purpose of this work is to determine the interactions involved in the specific binding of substrates and inhibitors to this enzyme.

Until now, under the assumption that the enzyme has features "built into" its *native* structure that allow for recognition of the substrate, we allowed the substrates to undergo rigid body and internal motion, but we kept the enzyme rigidly fixed. The calculations were carried out in four distinct steps:

1. The low-energy conformations of *isolated* oligomers and polymers of GlcNAc and *N*-acetylmuramic acid (MurNAc) were determined.[3]
2. Using a fragment of a disaccharide molecule to map the conformational space, the low-energy regions for binding $(GlcNAc)_2$ to the *rigid* active site of lysozyme were identified.[4]
3. The energies of a representative set of conformations of $(GlcNAc)_2$ (determined in step 1) in *all* low-energy binding regions (identified in step 2) were minimized,[4] with the conformation of the enzyme held rigidly fixed.
4. The lowest-energy structure of the disaccharide in the complex, obtained in step 3, was lengthened by adding

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