importance of strong internal hydrogen bonds (if they occur) in aqueous solution. These should in turn provide direct experimental information closely connected to the preferred conformations of these molecules in solution.

A Study of L-Proline, Sarcosine, and the Cis/Trans Isomers of *N*-Acetyl-L-proline and *N*-Acetylsarcosine in Aqueous and Organic Solution by ^{17}O NMR[1]

**Roger N. Hunston, Ioannis P. Gerothanassis, and Jürgen Lauterwein***

Contribution from the Institut de chimie organique de l'Universite, CH-1005 Lausanne, Switzerland. Received August 10, 1984

Abstract: ^{17}O NMR at 48.8 MHz has been used to study the cis/trans isomerism of *N*-acetyl-L-proline and *N*-acetylsarcosine in both aqueous and organic solution, and the results have been discussed in terms of possible hydrogen-bonded structures in the solvents examined. L-Proline, sarcosine, and their *N*-acetylated derivatives have been selectively enriched in ^{17}O either at the carboxyl or at the amide group and their spectra measured in aqueous solution throughout the whole pH range. Two resonances were observed for the carboxyl group of *N*-acetyl-L-proline, and these could be assigned to the cis and trans isomers due to differences in their signal intensity at low pH. The chemical shift difference of the two isomers was independent of the protonation state of the carboxyl group. This difference, as well as the difference in pK_a, is explained by an electric field effect of the amide group. The amide resonances of *cis*- and *trans*-*N*-acetyl-L-proline were poorly resolved at 8.4 T, and those of *N*-acetylsarcosine were inseparable. A low-frequency shift was observed for the amide resonances on deprotonation of the remote carboxyl group. The appearance of the carboxyl and amide resonances of the two isomers of *N*-acetyl-L-proline in methanol was similar to that of water. In contrast, using the solvents acetone and chloroform a large separation of the amide resonances was accompanied by a coalescence of the carboxyl resonances. The chemical shift of the cis amide resonance of *N*-acetyl-L-proline was found to be concentration dependent in acetone. This along with the chemical shift difference of the cis and trans amide resonances at low concentration (17.5 ppm) is discussed in terms of a γ-turn structure in the trans isomer. For *N*-acetylsarcosine the chemical shift difference was only 6.4 ppm, indicating a lower γ-turn probability. In chloroform, the chemical shifts and line widths of both the amide and carboxyl oxygens of *N*-acetyl-L-proline and the cis/trans isomer ratio are concentration dependent, indicating an increase in aggregation of the system with concentration. The chemical shift difference of the cis and trans amide resonances at dilute concentrations (22 ppm) proves the high tendency of formation of the γ-turn structure in this solvent.

Numerous studies have been reported on the hindered internal rotation of amide and peptide bonds, and a variety of spectroscopic techniques have been applied.[2] The presence of a cyclic side chain and/or alkyl substitution of the peptide bond as in proline and sarcosine is of particular interest because of the resulting increase in population of the cis isomer about the X-Pro bond and the restriction in conformational freedom.[3–5]

The compounds *N*-acetyl-L-proline (AcProOH) and *N*-acetyl-L-proline *N'*-methylamide (AcProNHMe) have been extensively studied as models for secondary structure in proline-containing peptides. The assignment of their cis and trans isomers was originally done by ^{1}H NMR,[6] and it was indicated that the trans isomer may exist as an intramolecularly hydrogen bonded

Scheme I

CIS   TRANS
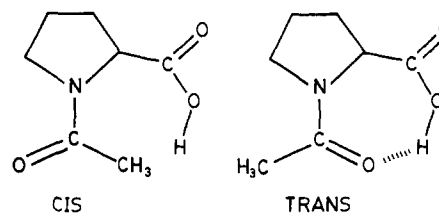
C_7 conformer (γ-turn structure, Scheme I) in nonpolar solvents, this model being subsequently extended to the situation in aqueous solution at low pH.[7]

The complexity of the ^{1}H NMR spectra of proline derivatives led to an early application of ^{13}C NMR;[8] however, the possible formation of an intramolecular hydrogen bond was not considered. The pH titration curves for the ^{13}C NMR resonances of AcProOH were interpreted[9] as supporting a γ-turn structure for the trans

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isomer in acidic aqueous solutions. From a combined ^1H and ^{13}C NMR study of the pH dependence of N-acetylsarcosine (AcSarOH) and various N-substituted dipeptides it was concluded[10] that the intramolecular hydrogen bond between the carbonyl oxygen of the C-terminal peptide bond and the carboxylic acid proton is a general phenomenon for the trans isomers. However, a subsequent ^{13}C NMR study of AcProOH and H-(L-Ala)$_n$-L-ProOH (n = 1, 2, 3)[11] concluded that a hydrogen bond between the terminal carboxyl group and the preceding amide carbonyl could be ruled out in polar media, this result being confirmed by a later application of ^{15}N NMR.[12]

With respect to AcProNHMe, both ^1H NMR and CD investigations support the formation of a γ-turn structure for the trans isomer in nonpolar solvents but indicate that in water the hydrogen bond is probably absent, or exists in a low and difficult to determine population.[13,14] London[15] proposed that a quantitative evaluation of the γ-turn structure in proline-containing peptides could be made on considering the chemical shift difference between the proline carbons C_β and C_γ.

It seems clear from the many problems encountered in characterizing the γ-turn structure in peptides by ^1H, ^{13}C, or ^{15}N NMR that a further nucleus could prove very useful for the NMR investigation of hydrogen-bonded structures. In view of the importance of oxygen atoms in the formation of intra- and intermolecular hydrogen bonds, ^{17}O NMR can be considered as an obvious candidate. Although large effects of hydrogen bonding on the ^{17}O chemical shifts have been recognized as early as 1963 by Christ and Diehl,[16] it is only in recent years that this technique received attention as a structural probe in amino acids,[17-21] amides,[22] and peptides.[23,24] The reasons for the difficultly in performing ^{17}O NMR experiments, i.e., low sensitivity, large line widths, and rolling base lines, are documented elsewhere.[25] However, high magnetic fields and modern NMR instrumentation have helped to alleviate some of the problems.[26] Furthermore, both Fiat and co-workers[24] as well as our group[20,27] have synthetized amino acids and peptides which are specifically enriched in ^{17}O.

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We have recently shown[28] that the ^{17}O resonances of the carboxyl group of *cis*- and *trans*-AcProOH in aqueous solution are well distinguished and that the ^{17}O chemical shifts upon deprotonation of the carboxyl function are large and specific so as to exclude an appreciable amount of intramolecular hydrogen bonding in the trans isomer. We present here a complete ^{17}O NMR study of the specifically enriched carboxyl and amide oxygens of AcProOH and AcSarOH. For comparative reasons the amino acids L-proline and sarcosine are included in the study. The conformational state of these molecules was investigated in aqueous and organic solution. It is shown that the effects of hydrogen bonding are extremely large on the amide oxygen chemical shifts. From this an attempt will be made to evaluate the γ-turn probabilities of AcProOH and AcSarOH in acetone.

Experimental Section

N-Acetyl-L-[^{17}O]proline (~10 atom % ^{17}O). To a stirred solution of sodium methoxide in methanol (0.28 mL, 4 M) was added 1 equiv of H_2O (20 μL, 1.1 mmol) enriched to 20 atom % ^{17}O (Yeda), followed by a solution of *N*-acetyl-L-proline methyl ester[29] (171 mg, 1 mmol) in methanol (1 mL). The mixture was allowed to stir overnight at room temperature, the solution was then cooled to 0 °C and acidified to pH 3, and the solvent was evaporated under reduced pressure. The residue was dissolved in a minimum of cold 1 N HCl (~1 mL) and further acid was added if necessary to give pH 1. The cold solution was then extracted with 5 × 3 mL of ethyl acetate and evaporated at room temperature under reduced pressure. The resulting solid was dried under vacuum over P_2O_5 to give Ac^{17}ProOH in 81% yield (mp 114-115 °C).

L-[^{17}O]Proline and [^{17}O]Sarcosine (~10 atom % ^{17}O). The above compounds were prepared in a similar way to that just described by saponification of their methyl esters in the presence of $H_2^{17}O$. After acidification to pH 3 and evaporation of the solvent under reduced pressure the resulting product could be used directly for the ^{17}O titration curve measurements, the product and salt concentration being adjusted to our standard conditions (see below).

N-[^{17}O]Acetyl-L-proline Methyl Ester (~1 atom % ^{17}O). To a cold, stirred suspension of sodium [^{17}O]acetate[30] (1 atom % ^{17}O)(164 mg, 2 mmol) in 10 mL of acetonitrile containing 22 mg of 18-crown-6 was added ethyl chloroformate (200 μL, 2.1 mmol). After being stirred overnight at room temperature the mixture was cooled to -5 °C and a solution of proline methyl ester·HCl (Fluka) (350 mg, 2.1 mmol) in 10 mL of chloroform containing triethylamine (295 μL, 2.1 mmol) was added. The mixture was allowed to reach room temperature and stirred overnight. The solvent was then removed under reduced pressure and the product taken up in 20 mL of ether. The salt precipitate was filtered and washed with a further 5 mL of ether, and the combined ether extracts were evaporated under reduced pressure. The resulting oil was chromatographed on silica gel with ethyl acetate/hexane as eluant to give pure ^{17}AcProOMe (253 mg, 74% yield).

N-[^{17}O]Acetyl-L-proline (~1 atom % ^{17}O). To a solution of ^{17}AcProOMe (342 mg, 2 mmol) in methanol (0.5 mL) was added 2 mL of 2 M aqueous sodium hydroxide. The mixture was allowed to stir several hours at room temperature, acidified to pH 3, and evaporated under reduced pressure. The residue was redissolved in 5 mL of water and the solution brought to pH 1 and extracted with 5 × 5 mL of ethyl acetate. Evaporation of the solvent and drying over P_2O_5 gave ^{17}AcProOH in 85% yield.

The same procedure has also been used to obtain ^{17}AcProOH enriched to ~10 atom %. The ^{17}O NMR spectrum in Figure 6 was recorded with such a sample.

N-Acetyl[^{17}O]sarcosin (~10 atom % ^{17}O) and *N*-[^{17}O]Acetylsarcosine (~1 atom % ^{17}O). These compounds were prepared by procedures analogous to those just described.

The ^{17}O enrichments were estimated from the way in which the compounds were prepared and the original ^{17}O composition of the water and were found to be in good agreement with measurements made by mass spectrometry and by integration of the ^{17}O NMR resonances in methanol, using the solvent signal as reference.

^{17}O NMR Measurements. Organic solvents were spectral grade and have been dried over activated molecular sieves. All spectra were recorded at 40 ± 1 °C. If not stated otherwise, the solute concentrations were 0.1 M. The aqueous solutions also contained 1 M NaCl and 0.0005

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Table I. ^{17}O Titration Shifts and pK_a Values of Proline, Sarcosine, N-Acetyl-L-proline and N-Acetylsarcosine[a]

compd	resonance	isomer	δ_1[b]	δ_2[c]	Δ_{12}[d]	pK_a
Pro[e]	COOH		250.2	266.7	16.5	1.68 ± 0.02
Sar	COOH		255.0[f]	272.4[f]		
AcProOH	COOH	cis	251.1	269.3	18.2	2.79 ± 0.02
		trans	246.1	265.0	18.9	3.36 ± 0.02
	CONR	cis	297.2	288.9	−8.3	2.79[g]
		trans	298.4	290.3	−8.1	3.43 ± 0.14
AcSarOH	COOH	cis	252.0	271.5	19.4	2.98 ± 0.02
		trans	249.5	269.6	20.1	3.37 ± 0.02
	CONR	cis	300.2[h]	291.5[h]	−8.7[h]	h
		trans				

[a] Measured in 0.1 M solution in H_2O which contained 1 M NaCl and 0.005 M EDTA; $T = 40\ °C$. The chemical shifts (ppm) were obtained from nonlinear least-squares fits of one-proton titration curves[20] to the experimental data. They were measured relative to 1,4-dioxane used as external reference, +0.2 ppm relative to water. Their errors were estimated ±0.5 ppm for the carbonyl resonances and ±1 ppm for the amide resonances. [b] δ_1 is the chemical shift at acid pH (carboxyl oxygens protonated. [c] δ_2 is the chemical shift at neutral pH (carboxyl oxygens deprotonated). [d] Δ_{12} values are the chemical shift changes on deprotonation of the carboxyl group. Positive values indicate deshielding. [e] The chemical shift after deprotonation of the α-amino group was $\delta_3 = 265.8$ ppm; $\Delta_{23} = -0.9$ ppm; p$K_a = 9.99 \pm 0.6$. [f] The chemical shifts were taken at individual pH values: δ_1 at pH 0.5; δ_2 at pH 6.0. The value at pH 12.5 was $\delta_3 = 271.3$ ppm. [g] The pK_a was fixed according to that of the more precise titration curve of the carboxyl oxygen. [h] Only a composite resonance of the cis and trans isomers is observed at 48.8 MHz.

M EDTA. These are our standard conditions[19,20] for the comparison of ^{17}O NMR results of amino acids and peptides. EDTA was added because of the observation of line broadening effects from paramagnetic metal ion impurities.[21]

The ^{17}O NMR spectra were obtained at 48.82 MHz with a Bruker WH-360 instrument equipped with a high-resolution probe (10 mm sample tubes). No field/frequency lock was used. The chemical shifts were determined relative to the resonance position of 1,4-dioxane, measured in a separate experiment.[26] At 40 °C the chemical shift of dioxane relative to water is +0.2 ppm. Data manipulations were carried out on an Aspect-2000 computer. The following spectral parameters were used: spectral width = 40 kHz; 90° pulse length = 32 μs; quadrature phase detection; acquisition time $T_{acq} \gtrsim 5T_2$; preacquisition delay $\Delta t = 30\ \mu s$; no relaxation delay T_d; zero-filling up to 16 K before FT.

Overlapping resonances were usually resolution enhanced by a multiplication of the FID with a Gaussian-exponential function.[31] This function has the form $\exp(at - bt^2)$ where a and b are adjustable parameters and are related to the Aspect-2000 parameters (LB) [in Hz, (LB) < 0] and (GB) [0 < (GB) < 1] as follows: $a = -\pi(LB)$ and $b = a/[2(GB)T_{acq}]$. Some spectra were processed by using exponential line broadening [(LB) > 0].

In order to eliminate acoustic ringing problems several spectra, especially those of samples with low ^{17}O concentration, were recorded with the pulse sequence suggested by Ellis.[32]

$$90_x-\Delta t-FID(+)-T_d-180_x-90_{-x}-\Delta t-FID(+)-T_d-$$
$$-90_{-x}-\Delta t-FID(-)-T_d-180_x-90_x-\Delta t-FID(-)-T_d \qquad (1)$$

Applying this sequence $\Delta t = 20$–$30\ \mu s$ was chosen. Care was taken that the carrier frequency was within ±2 kHz from the spectral region of interest. Because of the short acquisition times used (~ 10 ms), the total experimental time was largely determined by the fixed software delay, $T_d = 20$ ms, which intervenes after each acquisition in the Bruker microprogram structure.

^1H and ^{13}C Measurements. The solutions were prepared in the same manner as for ^{17}O NMR except that deuterated solvents were used for locking purposes. The ^1H (360 MHz) and ^{13}C NMR (90.5 MHz) spectra were recorded with long pulse repetition times in order to allow complete recovery of the CH magnetization vectors. A composite pulse sequence (16 cycles) was applied for ^1H broad band decoupling.[33]

Viscosity Measurements. The viscosities were determined at 25 °C with a Contraves low shear 30 rotational rheometer based on the Couette principle.

Results and Discussion

(1) L-[^{17}O]Proline and [^{17}O]Sarcosine in Aqueous Solution. Figure 1 shows the pH dependence of the ^{17}O chemical shift of proline. Only one resonance is observed for the carboxyl group since the C=O and C—OH resonance positions are averaged by rapid intermolecular proton transfer.[34] Deprotonation of the
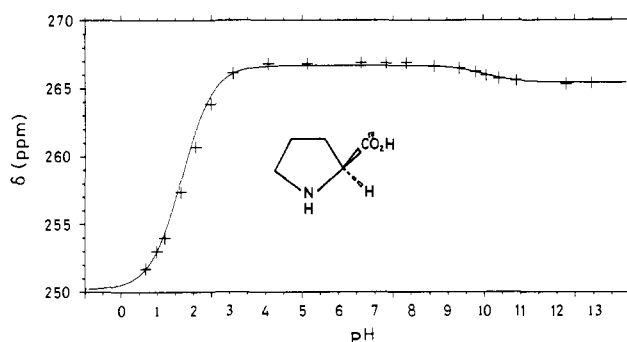

Figure 1. ^{17}O NMR chemical shift titration curve of L-proline, 0.1 M, in aqueous solution containing 1 M NaCl and 0.0005 M EDTA; $T = 40$ °C. The solid line was obtained from nonlinear least-squares fits of one-proton titration curves[20] to the experimental data (+).
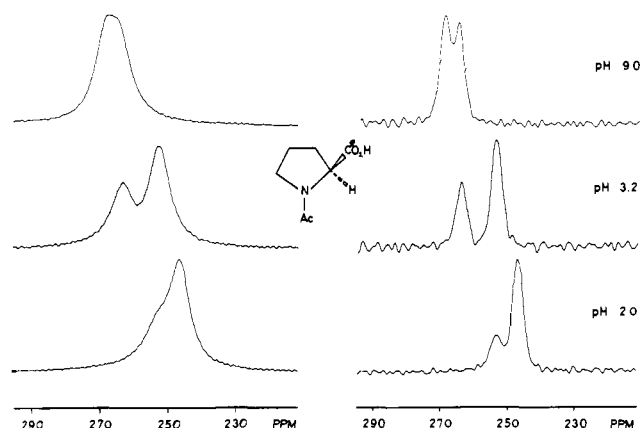

Figure 2. ^{17}O NMR spectra (48.8 MHz) of N-acetyl-L-[^{17}O]proline (10% enrichment) obtained under conditions as in Figure 1. The carboxyl resonances are shown at three different pH values. In all cases the resonance at high frequency corresponds to the cis isomer. $T_{acq} = 12$ ms; total experimental time is ca. 20 min. Left: normal spectra. Right: spectra after multiplication of the FID with a Gaussian-exponential function ((LB) = −300 Hz; (GB) = 0.6; see Experimental Section for definition of the parameters).

carboxyl group results in an ^{17}O chemical shift to high frequency with an inflection point at its pK_a. A second inflection was observed at the pK_a of the amino group (Figure 1), resulting in a decrease of the ^{17}O chemical shift by 0.9 ppm, smaller than in our previous observations on amino acids with primary amino

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¹⁷O NMR Study of Cis/Trans Isomerism

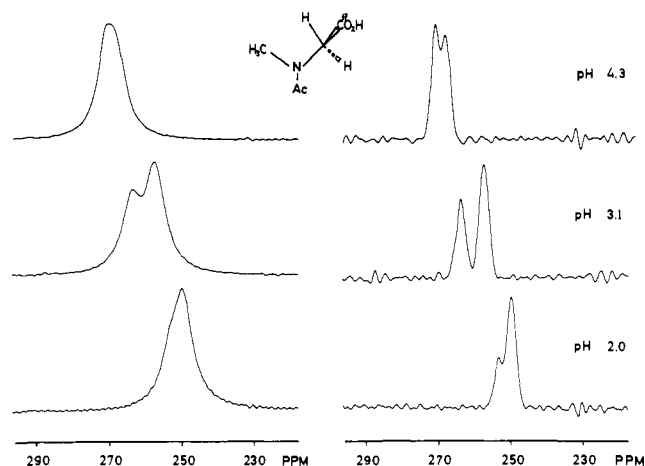*J. Am. Chem. Soc., Vol. 107, No. 9, 1985* 2657

Figure 3. ¹⁷O NMR spectra (48.8 MHz) of N-acetyl[¹⁷O]sarcosine (10% enrichment) obtained under the conditions of Figure 1 at three different pH values. In all cases the resonance at high frequency corresponds to the cis isomer. Data acquisition and processing are as described in Figure 2.

Table II. Cis/Trans Equilibrium of N-Acetyl-L-proline and N-Acetylsarcosine

			% cis[a]	
compd	solution conditions		this work[b]	lit.
AcProOH	H₂O, 1 M NaCl,	pH 2.0	25.0[c]	
	40 °C, 0.1 M	pH 3.2	36.5[d]/37.0[c]	
		pH 9.0	51.8[c]	
	H₂O, 30 °C	0.1 M H₂SO₄		25[e,f]
		pH 9		50[e,f]
	D₂O	pH 1.3		19[g,h]
		"high pH"		45[g,h]
	CH₃OH, 40 °C, 0.1 M		31.1[c]/29.0[h]	
	CH₃COCH₃, 40 °C	0.01 M	21.9[h]	
		0.02 M	21.0[h]	
		0.1 M	21.4[h]	
		0.6 M[j]	25.3[h]	
	CHCl₃, 40 °C	0.01 M	<2[f]	
		0.02 M	4.3[h]	
		0.1 M	9.5[h]	
		0.2 M	13.9[h]	
		0.5 M	20.8[h]	
AcSarOH	H₂O, 1 M NaCl,	pH 2.0	31.0[c]	
	40 °C, 0.1 M	pH 3.1	43.9[d]/46.4[c]	
		pH 4.3	52.2[c]	
	H₂O, 20 °C	neutral		30[g,h]
		anion		52[g,h]
	CH₃OH, 40 °C, 0.1 M		33.1[f]	
	CH₃COCH₃, 40 °C	0.005 M	32.5[f]	
		0.04 M	32.9[f]	
		0.14 M[j]	32.5[h]	

[a] The concentration of the cis form is in percent of the total solute concentration. [b] The error is estimated <5% independent of the method. [c] Obtained from the relative peak heights in the resolution-enhanced ¹⁷O NMR spectra. [d] Obtained from simulation of the ¹⁷O NMR spectrum with Lorentzian lines. [e] Madison and Schellman.[6] [f] On the basis of the relative areas of the cis and trans resonances in the ¹H NMR spectrum. [g] Bedford and Sadler.[9] [h] On the basis of the relative peak heights of the cis and trans resonances in the ¹³C NMR spectrum (nuclear Overhauser enhancements are supposed to be independent of the isomer). [i] Evans and Rabenstein.[10] [j] Saturated solution.

groups.[20] The ¹⁷O chemical shifts corresponding to the three ionization states of proline are presented in Table I, together with the titration shifts and pK_a values. Table I also contains the ¹⁷O chemical shifts of sarcosine measured at three distinct pH values.

(2) N-Acetyl-L-[¹⁷O]Proline and N-Acetyl[¹⁷O]sarcosine in Aqueous Solution. The ¹⁷O NMR spectra of the carboxyl groups of AcProOH (Figure 2) and AcSarOH (Figure 3) revealed the existence of two resonances. Because of the different intensities
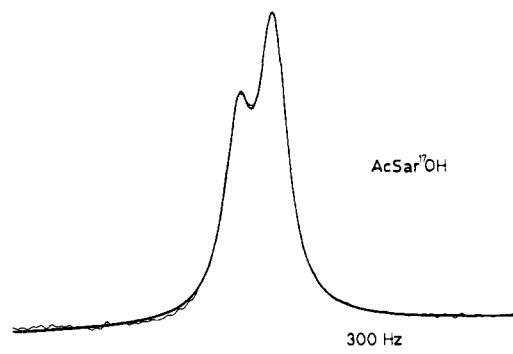

Figure 4. Simulation of the ¹⁷O NMR spectra of N-acetyl[¹⁷O]sarcosine recorded at pH 3.1 (solution conditions as in Figure 1). The spectrum is composed of two Lorentzian lines corresponding to the cis and trans isomer. The line widths were evaluated as $\Delta\nu_{1/2}$ = 324 and 289 Hz for the cis and trans isomer, respectively, with an estimated error of 5%.
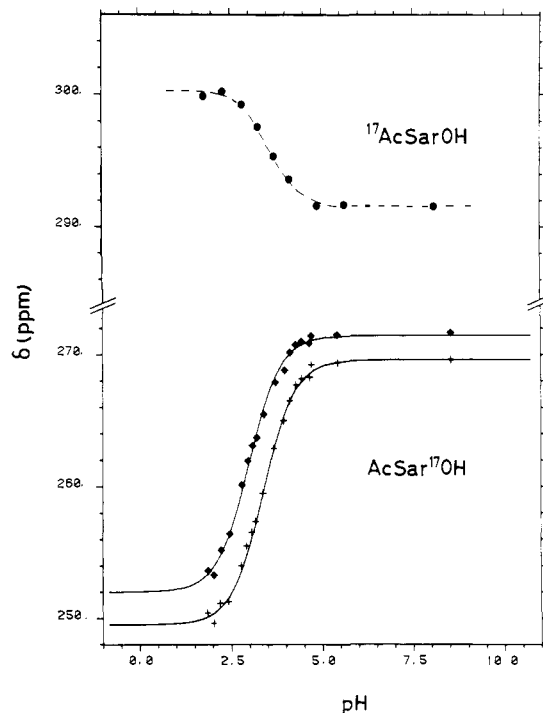

Figure 5. ¹⁷O NMR titration shifts of N-acetylsarcosine obtained under the conditions of Figure 1. The carboxyl resonance of the cis isomer is marked with a solid diamond and that of the trans isomer with a plus. The solid lines correspond to calculated one-proton titration curves.[20] Only a composite resonance (●) was observed for the amide oxygens of the cis and trans isomers.

at low pH, they could be attributed to the cis and trans isomers around the amide bond, the smaller one corresponding to the cis form (20–30% for both AcProOH and AcSarOH).[6,9,10] The resonances of AcSarOH were in general less well resolved because of the smaller difference in the chemical shift and pK_a values of the two isomers; however, in the pK_a region where the resonances were best separated, a simulation by Lorentzian lines could be performed (Figure 4). Assuming that the ¹⁷O line widths of the cis and trans isomers do not vary relative to each other over the whole pH range, the cis/trans ratios of AcProOH and AcSarOH were estimated from the relative peak heights of the resolution-enhanced spectra.[35] Table II gives the cis/trans ratios evaluated in this way at various pH values and compares them with the results obtained by ¹H and ¹³C NMR. The pH titration curves

(35) We are presently investigating the possibility of calculating the original line widths and integrals of resonances obtained after Gaussian filtering: Braoudakis, G.; Gerothanassis, I. P.; Lauterwein, J. *J. Magn. Reson.* **1983**, *83*, 288–292.

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of the carboxyl oxygens of AcProOH have already been communicated,[28] and those of AcSarOH are presented in Figure 5. Measurements were not made below pH 2 because of enrichment due to acid-catalyzed exchange. The ^{17}O chemical shifts corresponding to the two ionization states of the carboxyl group are collected in Table I.

Upon deprotonation of the carboxyl function of AcProOH, titration shifts of +18.2 and +18.9 ppm were observed for the carboxyl oxygens of the cis and trans isomers, respectively (Table I). They are larger than those observed for proline, sarcosine, and other free amino acids; however, they are in close agreement with the value found for N-glycylglycine.[23] Obviously, because of acylation at the nitrogen no titration shift would be expected at high pH.

The pK_a values of AcProOH observed on carboxyl titration (2.79 and 3.36 for the cis and trans isomers, respectively) are slightly smaller than those previously reported.[9] This may be attributed to the higher ionic strength of our solutions[20] and the use of deuterated solvents in the ^1H and ^{13}C NMR titration studies.[36] However, the difference of $\sim$0.6 unit between the pK_a values of the two isomers have been confirmed in the present work. The pK_a values for the cis and trans isomer of AcSarOH differed by $\sim$0.4 unit, in accordance with Evans and Rabenstein.[10]

The chemical shift difference between the carboxyl oxygen resonances in the cis and trans forms of AcProOH was 5.0 ppm at low pH and 4.3 ppm at high pH. That of the two isomers of AcSarOH was reduced ca. twofold relative to AcProOH (Table I). Thus ^{17}O NMR of the carboxyl groups of AcProOH and AcSarOH reflects clearly the sensitivity to the cis/trans isomerism.[37] However, in both cases the chemical shift difference has been found independent of the protonation state of the carboxyl group, i.e., two parallel titration curves were obtained (Figure 5) (Figure 2 of ref 28).

(3) pK_a Values and Carboxyl Oxygen Chemical Shifts. The differences in pK_a values and carboxyl ^{17}O chemical shifts of the cis and trans isomers of AcProOH arise most probably from the orientation of the amide dipole with respect to the carboxyl group.[38] This amide dipole can exert its influence in three possible ways: first by formation of an intramolecular hydrogen-bonded structure in the trans isomer, second by a magnetic anisotropic effect, and third by interaction of its associated electric field with the carboxyl group.[39]

Hydrogen bonding in the trans isomer has already been postulated[10] as a possible explanation for the difference in pK_a, and indeed, modification of acid strength is a rather widely used criterion for the existence of an intramolecular hydrogen bond.[40]

The magnetic anisotropy of the amide bond should have no effect on the pK_a; however, the electric field is expected to influence considerably the deprotonation of the carboxyl group in the trans isomer where unfavorable charge interactions would occur.[11] It is therefore clear that both the hydrogen bonding and the electric field effect give plausible explanations for the difference in pK_a observed for the two isomers.

Let us now consider the difference in the carboxyl ^{17}O chemical shifts at high pH. The carboxyl group, now in its anionic form, can no longer participate in an intramolecular hydrogen bond, and this argument cannot be invoked to explain the ^{17}O chemical shifts. The magnetic anisotropy effect, because of its inverse third power dependence on the distance between the dipole and the

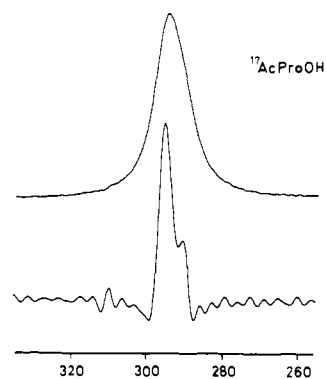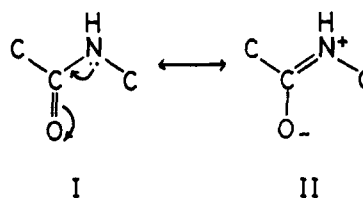**Figure 6.** ^{17}O NMR spectrum (48.8 MHz) of N-[^{17}O]acetyl-L-proline (10% enrichment) obtained under the conditions of Figure 1 at pH 3.7. T_{acq} = 12 ms; NS = 300000; total experimental time = 1 h. Upper trace: normal spectrum. Lower trace: after resolution enhancement ((LB) = -550 Hz; (GB) = 0.4).

Scheme II

I   II

nucleus under question, would be expected to have very little influence on the carboxylate chemical shift in the trans isomer (<1 ppm). This follows from the distance of $\sim$3.8 Å between the amide oxygen and the carboxylic acid oxygens in the trans form,[11] together with the consideration of the shielding contour maps of a carbonyl group[41] or from recent calculations of the magnetic anisotropy shifts due to the peptide bond.[42] Therefore the only explanation of the chemical shift difference at high pH can be an electric field-dependent contribution originating at the amide dipole.[43,44] The electric field distorts the electron distribution at the carboxyl group in the trans isomer, thereby altering the paramagnetic term of the ^{17}O screening constant.[45] This effect would exist throughout the whole pH range, the magnitude of which depends upon the polarizability of the carboxylic group relative to its anion. The observed parallel titration curves for the cis and trans isomers suggest that the electric field effect can be invoked to explain the chemical shift difference throughout the whole pH range and, in a similar fashion, explains the difference in pK_a of the two isomers and the decrease in population of the trans isomer in its carboxylate form.

Analogous parallel titration curves to those of AcProOH were also observed for AcSarOH (Figure 5). As before both hydrogen bonding and electrical field effect can be used to explain the difference in pK_a; however, the chemical shift difference at high pH can only be understood by the latter. The simultaneous decrease in the difference in pK_a and chemical shifts of the two

(36) Bundi, A.; Wüthrich, K. *Biopolymers* **1979**, *18*, 285–298.

(37) The ^{13}C chemical shift difference between the carboxyl groups of the cis and trans isomers of AcProOH is considerably smaller ($\sim$0.1 ppm).[9]

(38) The inductive effect, i.e., redistribution of the bonding electrons along the chain of the intervening atoms, can be considered equal in both isomers. However, it is certainly responsable for the decrease of acid strength of AcProOH relative to proline since the amide group is less electron attracting than the ammonium group of the amino acid (cf. the pK_a values in Table I).

(39) Robert, J. D.; Caserio, M. C. "Basic Principles of Organic Chemistry"; W. A. Benjamin: London, 1977; Chapter 18, pp 799–801.

(40) Ferguson, L. N. "The Modern Structural Theory of Organic Chemistry"; Prentice-Hall, Inc.: Englewood Cliffs, NJ, 1963, Chapter 1, pp 125–157. Bystrov, V. F.; Arseniev, A. S.; Gavrilov, Y. D. *J. Magn. Reson.* **1978**, *30*, 151–184.

(41) Jackman, L. M.; Sternhell, S. "Applications of NMR Spectroscopy in Organic Chemistry"; Pergamon Press: Oxford, 1969; pp 88–92.

(42) Clayden, N. J.; Williams, R. J. P. *J. Magn. Reson.* **1982**, *49*, 383–396.

(43) Batchelor, J. G. *J. Am. Chem. Soc.* **1975**, *97*, 3410–3415.

(44) Linear electric field effects have been proposed as a possible explanation for the chemical shift dependence of the C_β carbon in the trans isomer of AcProNHMe.[15] However, the chemical shift of C_β can also be explained by a γ-effect mechanism: Seidman, K.; Maciel, G. E. *J. Am. Chem. Soc.* **1977**, *99*, 659–671. A further complication for the explication of the pyrrolidine carbon shifts may come from ring puckering: London, R. E. *J. Am. Chem. Soc.* **1978**, *100*, 2678–2685.

(45) Chemical shifts or screening constants are usually discussed with respect to contributions from a diamagnetic and a paramagnetic term. It can be assumed for good reasons that the diamagnetic term is independent of change in the chemical environment of the oxygen atom: Ebraheem, K. A. K.; Webb, G. A.; Witanowski, M. *Org. Magn. Reson.* **1976**, *8*, 317–320. Thus the shift differences are essentially induced by the paramagnetic term. The latter is usually evaluated following the average excitation energy approximation: Karplus, M.; Pople, J. A. *J. Chem. Phys.* **1963**, *38*, 2803–2809.

Table III. ¹⁷O Chemical Shifts of N-Acetyl-L-proline and N-Acetylsarcosine in Various Solvents at 40 °C

compd	solvent	concn, M	δ(COOH)		δ(CONR)	
			cis	trans	cis	trans
AcProOH	H₂O[a]	0.1[b]	251.1[c]	246.1[c]	297.2[c]	298.4[c]
	CH₃OH	0.1[b]	255.6	248.6	316.4[d]	
	CH₃CH₂OH	0.1[b]	257.1	250.2	e	
	CH₃COCH₃	0.01	258.5[d]		354.7	337.2
		0.02	258.8[d]		354.7	337.2
		0.1	258.7[d]		352.0	336.9
		0.6[f,g]	258.0[d]		341.2	332.8
	CHCl₃	0.01	264.6[d]		h	313.7
		0.02	262.9[d]		335.5	313.9
		0.04	261.4[d]		335.2	313.8
		0.1	259.1[d]		332.7	316.0
		0.35[f]	257.2[d]		315.0[d]	
		0.6[f]	255.6[d]		315.6[d]	
AcSarOH	H₂O[a]	0.1[b]	252.0[c]	249.5[c]	300.2[c,d]	
	CH₃OH	0.1[b]	255.2	252.1	317.7[d]	
	CH₃COCH₃	0.005		258.6	354.9	348.6
		0.04	261.6	258.8	354.4	348.1
		0.14[f,g]	258.0[d]		350.8	344.9

[a] Solutions contained 1 M NaCl and 0.0005 M EDTA. [b] Chemical shifts were independent of concentration. [c] Extrapolated values for the fully protonated carboxyl group. [d] The cis and trans isomers appear as a composite resonance. [e] Not measured. [f] Measured at natural abundance in ¹⁷O. [g] Saturated solution. [h] Percent cis isomer <2%.

isomers of AcSarOH with respect to AcProOH can be explained by an increase in the average distance between the interacting oxygen bearing groups in the trans isomer as rotation around the N–C bond is no longer restricted.

(4) N-[¹⁷O]Acetyl-L-proline and N-[¹⁷O]Acetylsarcosine in Aqueous Solution. In aqueous solution the cis and trans amide resonances of N-acetyl-L-proline are poorly resolved, even at their pK_a where the chemical shift difference should be largest (Figure 6). However, using resolution enhancement techniques their chemical shifts could be measured over a large, if limited, pH range and their titration curves evaluated. The amide oxygen resonances of the cis and trans isomers of AcSarOH were inseparable at 8.4 T, and the titration curves could not be calculated (Figure 5). The ¹⁷O chemical shifts of AcProOH corresponding to the two ionization states of the carboxyl groups are presented in Table I, together with the approximate titration shifts of AcSarOH.

The chemical shifts of the amide oxygens of AcProOH and AcSarOH in aqueous solution fall in the same region as those previously reported for the amides and peptides.[22,23] They can be understood in terms of the contribution from the two amide resonance structures (Scheme II). The observation of a low-frequency shift of the amide oxygen upon deprotonation of the carboxyl function is in contrast to the behavior of the carboxyl oxygen itself. Replacement of the carboxyl groups by the less-electron-attracting carboxylate ion will favor structure II (Scheme II) and consequently lead to a shift to low frequency. The titration shifts of −8.3 and −8.1 ppm observed for the cis and trans isomers of AcProOH, respectively, agree well with that of N-glycylglycine (ca. −6 ppm).[23] On the other hand, the chemical shifts of AcProOH at both acid and neutral pH (298.4 and 290.3 ppm for the trans isomer) are quite different from those in N-glycylglycine (ca. 281 and 275 ppm).[23] This difference probably reflects the proximity of the N–terminal ammonium group in N-glycylglycine.

We have recently found that the chemical shift of the carboxylate oxygen in amino acids is closely related to the number of bonds from the ionization site, and this is of order of 1 to 3 ppm.[20] In contrast, the titration shifts of ca. 8 ppm for both the cis and trans isomers of AcProOH show the sensitivity of the amide oxygen to ionization several bonds away.

(5) N-Acetyl-L-proline and N-Acetylsarcosine in Organic Solution. The ¹⁷O NMR spectra of the carboxyl and amide groups of AcProOH and AcSarOH have been measured at various concentrations in methanol, ethanol, acetone, and chloroform. The chemical shifts of the cis and trans resonances are given in Table III. Assignments were based on the unequal population of the two isomers and on data from ¹³C and ¹⁵N NMR.[11,12] The spectra in methanol and ethanol were quite similar to those in water.
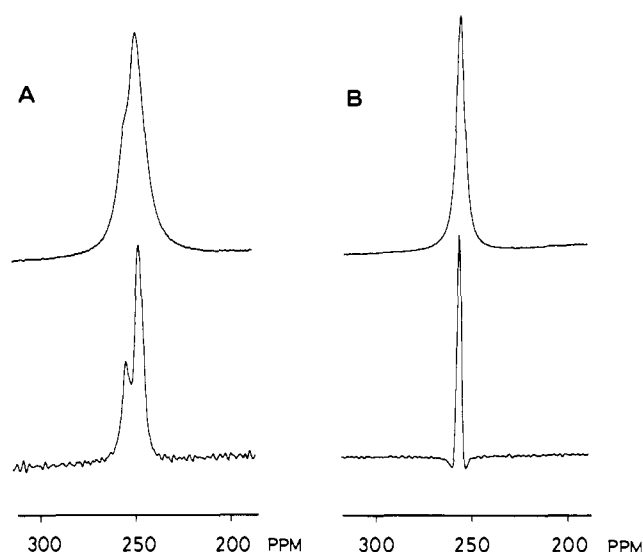
Figure 7. ¹⁷O NMR spectra (48.8 MHz) of N-acetyl-L-[¹⁷O]proline (0.1 M, 10% enrichment) recorded at 40 °C in (A) methanol and (B) acetone. T_{acq} = 12 ms; NS = 150 000; total experimental time = 30 min. Upper traces: normal spectra. Lower traces: after resolution enhancement ((LB) = −300 Hz; (GB) = 0.5).

Well-separated resonances were observed for the carboxyl oxygens of both isomers (Figure 7A) but only a single unresolved line for the amide oxygens (Figure 8A). No concentration dependence of the ¹⁷O chemical shifts and line widths of AcProOH was observed in methanol. The difference in the chemical shifts between the carboxyl oxygens was slightly larger in methanol and ethanol (6.9 ppm) than in water (5.0 ppm). The carboxyl resonances were shifted to high frequency in methanol relative to water (+4.5 and +2.5 ppm, respectively, for the cis and the trans isomer of Ac-ProOH). These shifts are in good agreement with the value +3.6 ppm found for a solution of 0.1 M acetic acid in the two solvents.[20,46] The dependence of the chemical shifts appears in agreement with the hydrogen-bonding strength of these solvents.

The amide oxygen resonances of the cis and trans isomers of AcProOH could not be resolved in methanol despite a population of ca. 30% of the cis isomer (Table II). They were shifted by ca. +19 ppm in methanol relative to water, a similar chemical shift

(46) Gerothanassis, I. P.; Hunston, R. N.; Lauterwein, J., manuscript in preparation.
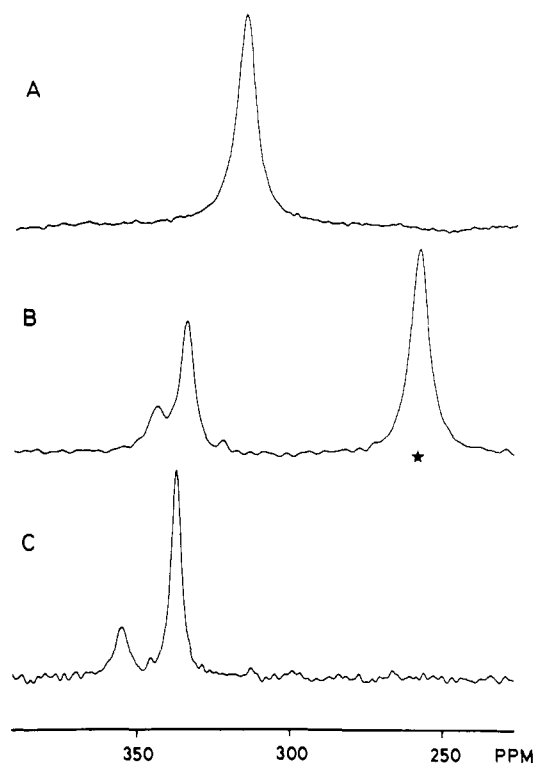
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Figure 8. ^{17}O NMR spectra (48.8 MHz) of *N*-[^{17}O]acetyl-L-proline (1% enrichment): (A) 0.1 M in methanol, T_{acq} = 10 ms, NS = 200 000, total experimental time = 32 min, (LB) = 50 Hz; (B) 0.4 M (natural abundance) in acetone, an asterisk marks the carboxyl oxygen resonance, T_{acq} = 5 ms, NS = 1 900 000, total experimental time = ca. 10 h (as a consequence of the application of the pulse sequence 1, see Experimental Section), (LB) = 50 Hz; (C) 0.1 M in acetone, T_{acq} = 10 ms, NS = 1 350 000, total experimental time = ca. 8 h (pulse sequence 1), (LB) = 50 Hz. All spectra were recorded at 40 °C.

difference to that observed for acetone in these solvents (ca. +25 ppm).[47]

The situation was reversed in the aprotic solvent acetone where the carboxyl oxygen resonances from the cis and trans isomers of AcProOH were strongly overlapped (Figure 7B); however, a large separation was observed for the amide oxygen resonances (Figure 8, B and C). AcSarOH in acetone showed a similar behavior. A study of the concentration dependence of the resonances was performed in acetone between 0.01 and 0.6 M of AcProOH and between 0.05 and 0.14 M of AcSarOH (natural abundance spectra were recorded at concentrations >0.1 M). Integration of the amide resonances showed that the concentration has only little effect on the cis/trans ratio, in agreement with our results from ^{1}H and ^{13}C NMR spectroscopy (Table II). However, the chemical shifts of the amide oxygens showed a characteristic concentration dependence (Figure 8, B and C; Table III). The resonances of both isomers shifted to low frequency on increasing the concentration, being more remarked in the case of the cis isomer, indicating an increase in hydrogen bonding of the amide oxygens. In addition, the increase in concentration was accompanied by a line broading of the two resonances (compare Figure 8 parts B and C), indicating an increase in intermolecular interactions in acetone.

The connection between line width and aggregation is based on the fact that the relaxation rate, $1/T_2$, is related to molecular motion and consequently to the size of the complex containing the resonating nucleus. In diamagnetic solutions the ^{17}O nucleus relaxes predominantly by the quadrupolar mechanism.[25] In the extreme narrowing limit the expression for the ^{17}O line width is

$$\Delta\nu_{1/2} = \frac{1}{\pi T_2} = \frac{12\pi}{125}\left(1 + \frac{\eta^2}{3}\right)\left(\frac{e^2qQ}{h}\right)^2\tau_c \qquad (2)$$
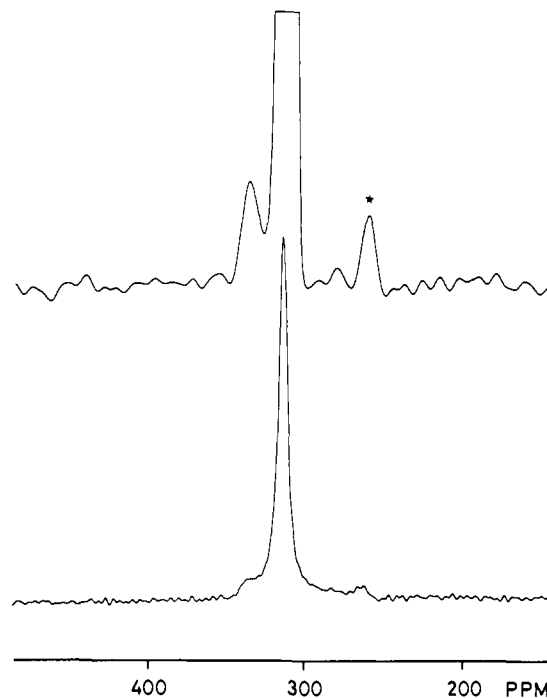
(47) Reuben, J. *J. Am. Chem. Soc.* **1969**, *91*, 5725–5729.

Figure 9. ^{17}O NMR spectrum (48.8 MHz) of *N*-[^{17}O]acetyl-L-proline (0.04 M, 1% enrichment) in chloroform at 40 °C. T_{acq} = 25 ms; NS = 1 500 000; 12 h (pulse sequence 1). Lower trace: normal spectrum. Upper trace: after resolution enhancement ((LB) = –300 Hz; (GB) = 0.15). An asterisk marks the carboxyl oxygen resonance at natural abundance.

where e^2qQ/h is the quadrupole coupling constant, η is the asymmetry parameter, and τ_c is the rotational correlation time. For roughly spherical molecules the Stokes–Einstein model predicts that τ_c should be proportional to the molecular weight.[48] Therefore, ^{17}O line widths can give information on the effective size of the aggregates under study, provided that they are stable with lifetimes larger than τ_c.

The carboxyl oxygens of AcProOH were shifted to high frequency in acetone relative to water (+7.4 and +12.4 ppm respectively for the cis and the trans isomer). A similar shift (+10.5 ppm) was obtained for dilute acetic acid in the two solvents.[20,46] This can be explained due to the absence of a proton-donating ability of the acetone molecules. The chemical shifts of the carboxyl oxygens of AcProOH and AcSarOH in acetone are independent of the concentration (Table III). This can be explained by the fast that, in contrast to the amide oxygen, the carboxyl OH undergoes hydrogen bonding throughout the whole concentration range, either with the amide oxygen through intra- or intermolecular interactions or with the solvent, acetone.

In chloroform, the chemical shifts and line widths of both the amide and carboxyl oxygens of AcProOH and the cis/trans isomer ratio are concentration dependent. Figure 9 shows the ^{17}O NMR spectrum of a 0.04 M solution of ^{17}AcProOH (1 atom % ^{17}O) in chloroform. The cis amide resonance appears at high frequency with an intensity corresponding to a population of the cis isomer by only 7% and is thus comparable to the intensity of the natural abundance signal from the carboxyl oxygens. The trans amide resonance was shifted to low frequency relative to the cis by –21.4 ppm (Figure 9). At concentrations <0.02 M the cis resonance was no longer observable (Table III). Its disappearance with decrease of the concentration is in accordance with the observation from ^{13}C NMR (<2% cis at 0.01 M AcProOH; cf. Table II). However, since the ^{17}O chemical shifts of the cis and trans amide resonances of AcProOH changed only little in the concentration range between 0.01 and 0.04 M, the chemical shift difference of ca. –22 ppm, in a very good approximation, should correspond

(48) Deslauriers, R.; Grzonka, Z.; Schaumburg, K.; Shiba, T.; Walter, R. *J. Am. Chem. Soc.* **1975**, *97*, 5093–5100.

Table IV. [17]O Line Widths of *N*-Acetyl-L-proline in Chloroform at 40 °C

concn, M	$\Delta v_{1/2}(COOH)^a$		$\Delta v_{1/2}(CONR)^a$		η'^d
	cis	trans	cis	trans	
0.02	394[b]		c	315	0.55
0.04	385[b]		c	325	0.56
0.1	540[b]		c	480	0.58
0.35	750[b]			670[b]	
0.4					0.73
0.6	1090[b]			1184[b]	0.79

[a] Line widths (Hz) of the resonances at half-height corrected for the line broadening factors. Estimated error <10%. [b] The cis and trans isomers appear as a composite resonance. The effective line width can be considered as originating from the trans since the cis is populated <20% (cf. Table II). [c] The cis resonance can be well resolved only after Gaussian-exponential filtering (cf. Figure 9). However, by this procedure, the Lorentzian line shapes are modified.[35] [d] Viscosities (cP) measured at 25 °C.

to the case where intermolecular interactions are no longer significant. The monomeric state of the trans isomer is also indicated by the resonance line width of $\sim$300 Hz observed under these dilute conditions (Table IV). The narrow, low-frequency resonance is explained by the presence of the γ-turn structure (see below).

On increasing the concentration of AcProOH we observed a slow convergence and strong line broadening of the cis and trans amide resonances, the cis moving to low frequency and increasing in intensity indicating a stabilization through intermolecular hydrogen bonds. At high concentrations (>0.3 M) the resonance of the majority trans isomer obscured the smaller cis resonance ($\Delta v_{1/2}$ = 1.2 kHz at 0.6 M; cf. Table IV). The modes of intermolecular interactions are thought to be between the amide group of one with the carboxyl of another molecule,[49] either by self-association (cis/cis and trans/trans) or by hybrid association (cis/trans).[50] The resonance of the trans isomer, however, experienced a small high-frequency shift suggesting a reduction in the hydrogen bonding of the amide oxygen. This can be explained by the formation of oligomers where the chemical shift of the N-terminal free amide group carries great weight relative to those contributions of the hydrogen-bonded groups.

The aggregation of AcProOH in chloroform was also observed from the combined cis and trans carboxyl oxygen resonances which gave a steady increase in line width from that observed at low concentration ($\sim$390 Hz at 0.02 M) to the value of $\sim$1100 Hz at 0.6 M concentration (Table IV).[51]

(6) Evaluation of the γ-Turn Structure of *N*-Acetyl-L-proline and *N*-Acetylsarcosine. The observation that the concentration dependence of the amide oxygen chemical shift of the trans isomer of AcProOH is considerably smaller than that of the cis isomer suggests that the trans form is largely involved in intramolecular hydrogen bonding. In the case of ArSarOH the concentration dependence of the two isomers is quite similar, and a lower amount of trans isomers is expected to exist in the γ-turn structure.

A quantitative evaluation of the γ-turn conformation of AcProOH and AcSarOH in the aprotic solvents should be available from the analysis of the amide oxygen chemical shifts at low concentrations where self-association of molecules can be excluded. The amide resonances of the cis isomers of dilute AcProOH and AcSarOH in acetone are shifted by +57.5 and +54.7 ppm, re-

spectively, relative to those in water (Table III). These chemical shifts are in close proximity to the values of +52 and +52–55 ppm found for acetone[47] and a series of amides[22] in these solvents. However, the resonances of the trans isomers in acetone show a much smaller chemical shift relative to those in water (+38.8 and +48.4 ppm for AcProOH and AcSarOH, respectively). A similar differential chemical shift was observed for the cis and trans isomer of AcProOH in chloroform (Table III), and the cause of this must be intramolecular hydrogen bonding.

Once intermolecular self-association has been eliminated, the only possible hydrogen-bonding mechanism in acetone and chloroform is the formation of a γ turn for the trans isomers. If *f* is the fraction of trans molecules with a γ-turn conformation, we can write

$$\Delta\delta_{obsd} = f\Delta\delta_{\gamma turn} + (1 - f)\Delta\delta_{open} \qquad (3)$$

where $\Delta\delta_{obsd}$ is the observed chemical shift of the amide oxygen in the trans isomer, $\Delta\delta_{\gamma turn}$ is the chemical shift when the trans isomer is exclusively in the γ turn, and $\Delta\delta_{open}$ is the weighted average of the chemical shifts corresponding to the open conformations of the trans isomer. In eq 2, the chemical shifts of the trans isomer are expressed relative to the observed chemical shift of the cis isomer which is the weighted average of its (exclusively open) conformations. The use of the shift differences has the advantage of providing an internal standard, although for a given solvent most of the change seems to reflect the low-frequency shift of the trans resonance. The quantitative evaluation of *f* requires the knowledge of $\Delta\delta_{\gamma turn}$ and $\Delta\delta_{open}$. In a good approximation $\Delta\delta_{open}$ of AcProOH and AcSarOH can be taken as the chemical shift difference of the cis and trans isomers in aqueous solutions ($\sim$1 ppm; cf. Table I), since the γ-turn probability was found to be negligibly small in water. The estimation of $\Delta\delta_{\gamma turn}$ is, however, not straightforward since a chemical shift value for 100% γ-turn structure of the trans isomer is needed. Delaney and Madison[52] have shown by IR spectroscopy that for dilute AcProNHMe in chloroform ca. 93% of the trans isomer exists in its intramolecular hydrogen-bonded form. If we assume a similar large γ-turn probability for AcProOH, the difference in the chemical shifts between the unassociated cis isomer and the intramolecularly bound trans isomer at low concentration in chloroform (−22 ppm) should be a good estimate of $\Delta\delta_{\gamma turn}$. Indeed, this value is in excellent agreement with that value evaluated for the saturation of one oxygen lone pair from dilution studies of amides.[53] Then, from eq 2 and from $\Delta\delta_{obsd}$ = −17.5 ppm we calculate a γ-turn probability of 78% for the trans isomer of AcProOH in acetone.

With respect to AcSarOH we can assume that the hydrogen bond strength is comparable to that in AcProOH, and therefore from $\Delta\delta_{obsd}$ = −6.3 ppm we calculate a γ-turn probability of 25%. Thus the trans isomer of AcProOH favors intramolecular association to a ca. threefold larger extent than that of AcSarOH. It is evident from these results that ^{17}O NMR is a highly effective tool in the study of hydrogen bonding interactions.

(49) A similar structure was observed from X-ray of AcProNHMe: Matsuzaki, T.; Iitaka, Y. *Acta Crystallogr., Sect. B* **1971**, *B27*, 507–516.

(50) As discussed by London,[15] there is no evidence for excluding one of these modes of intermolecular association. The viscosity change observed on increasing the concentration (Table IV) can only partially explain the line broadening of the [17]O resonances.

(51) Upon dilution beyond 0.02 M again an increase of the line width of the carboxyl signal was observed. The amount of the broadening was field dependent. We assign it to a decrease of the rate of the intermolecular proton transfer between the nonequivalent carboxyl oxygens. An ^{17}O NMR analysis is in progress.

(52) Delaney, N. G.; Madison, V. *J. Am. Chem. Soc.* **1982**, *104*, 6635–6641.

(53) St. Amour, T. E.; Burgar, M. I.; Valentine, B.; Fiat, D. *J. Am. Chem. Soc.* **1981**, *103*, 1128–1136.