Long Range Hydrogen Bond Mediated Effects in Peptides: ^{15}N NMR Study of Gramicidin S in Water and Organic Solvents

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Abstract: The chemical shifts of the ^{15}N's of the cyclic peptide gramicidin S in dilute aqueous solution were measured and assigned with use of polarization transfer via scalar coupling (INEPT). Long-range perturbations were detected in the ^{15}N spectrum from comparison of these results and those in organic solvents. The effects are transmitted from one peptide linkage to another via an intramolecular hydrogen bond across a total of six bonds. The variation with temperature of the ^{15}N shifts in these solvents further corroborates the roles of the intramolecular hydrogen bond in the propagation of shift effects. From the long-range effects it is possible to examine the relationship between solvent–peptide interactions and the properties of internal hydrogen bonds.

The influence of the solvent on the conformations attained by peptides and proteins in solution is crucial. Although gross structural effects induced by solvent can sometimes be monitored by numerous spectroscopic techniques[2] and solvent exposure and isolation of individual N—H and C=O groups may be characterized by ^1H and ^{13}C NMR,[3] methods are lacking for elucidating the specific interactions with solvent and for investigating their perturbations on internal hydrogen bonding. The key position occupied by the nitrogen atom in the peptide linkage makes it an ideal NMR probe for addressing the questions of these inter- and intramolecular interactions. Its potential, however, to provide new understanding of these molecules has been limited by the inappropriateness of ^{14}N for high-resolution studies, due to its quadrupole moment, and by the intrinsic insensitivity of the ^{15}N nucleus in NMR.[4]

Recent improvements in the design of spectrometers have increased the sensitivity and allowed for larger samples facilitating some studies of peptides by ^{15}N NMR. Even more important has been the introduction of techniques for spin-polarization transfer from ^1H to ^{15}N in solution, using the scalar spin-coupling interaction,[5-7] e.g., INEPT and DEPT. These methods offer significant advantages, providing an improvement of an order of magnitude over the intensity of the original ^{15}N signal, and they are not subject to the possibility of signal nulling from partial NOE that has been found in peptides of moderate size.[8] The polarization transfer methods work well for ^{15}N of peptides since these molecules have a number of similarly coupled protons. Doubts about the effectiveness of these techniques in studying macromolecules[9] are not supported by our results in the study of several peptides. These new techniques have made it possible to break away from the constraints that exclude studies of many biologically interesting peptides on the basis of limited availability and solubility. Our results on the ^{15}N spectrum of the decapeptide antibiotic gramacidin S at natural abundance in water (Figure 1) reported here are an excellent example of what can readily be achieved in studies on peptides in dilute solutions, 10 mM in this instance.

With the improved ability to observe ^{15}N NMR of peptides, the development of its use as a probe depends on understanding the origins of chemical shift effects. It is important in this regard to study examples where variables of conformation, intramolecular interactions, and solvent–solute interactions can be controlled. Gramacidin S is one of the best such cases. Studies using ^1H NMR,[3,10,11] ^{13}C NMR,[12] ^{15}N NMR[8,13-15] and IR[16] in organic solvents are all consistent with the solvent-invariant backbone structure and hydrogen bonding scheme illustrated in Figure 1. Recent ^1H NMR results in water[11,17] provide support for the existence of this structure in aqueous solution. The extension of these studies to examining the ^{15}N NMR of this peptide in water would place on firmer ground the structural interpretation of the ^{15}N shifts of biochemically important molecules in a biologically relevant solvent and provide insight into the water–peptide interaction. The ^{15}N shift results for gramicidin S in water and the temperature dependence of the shifts in water, dimethyl sulfoxide (Me_2SO), methanol, and trifluoroethanol (TFE) which we report here and the shifts in methanol, Me_2SO, and TFE,[13] remeasured here against an internal standard for greater accuracy, have made it possible to suggest a novel mechanism by which ^{15}N chemical shift perturbations are transmitted through a six-bond chain including an internal hydrogen bond. The detection of this long-range effect allows the investigation of the specifics of inter- and intramolecular interactions.

Experimental Section

Gramacidin S was purchased from Sigma Chemical Co. Material for study in water was first lyopholyzed from a 5:2 water:dioxane solution so that it would dissolve more rapidly in water.[11] Other solutions were made up with the material as supplied. Samples were dissolved in 10.5-mL solutions with 95% protonated solvent and 5% deuterated solvent except for the TFE sample in which 5% CD_3OD was used. This latter addition had no effect on the chemical shifts. The deuterated solvents were used to supply a lock signal. As a reference, 100 μL of Me_4Si was added to the organic samples, and 100 μL of *tert*-butyl alcohol was added to the aqueous samples. The spectra were observed on a Nicolet NT-300 widebore system with use of a 20-mm ^{15}N probe and 20-mm sample tubes. The proton spectra of the samples, used for referencing, were

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Table I. ^{15}N Chemical Shifts and Temperature Dependencies of Gramacidin S in Four Solvents[a]

solvent	phenylalanine	leucine	ornithine	valine	proline	ornithine NH_3^+
Me_2SO[b,d]	127.04 (−21.8)	123.07 (−3.1)	125.21 (−5.3)	112.96 (−10.3)	136.6[c]	39.3[c]
MeOH[b]	127.11 (−15.8)	127.34 (−2.1)	125.99 (−12.5)	118.18 (−21.9)	139.4[c]	35.1[c]
H_2O	129.75 (−40.6)	128.23 (+12.5)	125.31 (−6.3)	120.05 (−31.3)	139.1	35.4
TFE + 5% CD_3OD[b]	126.15 (−16.5)	131.12 (−12.3)	125.19 (−12.3)	120.88 (−32.9)	141.6[c]	34.8[c]

[a] Shifts in ppm at 25 °C relative to NH_3.[4] Temperature dependence in parentheses in ppb/°C. Concentrations in H_2O 10 mM, in Me_2SO 42 mM, in MeOH 83 mM, and in TFE 42 mM. [b] Assignments reported by others used.[13] [c] Shift values are reported by Hawkes et al.[13] and corrected to NH_3 reference.[4] [d] Temperature coefficients in Me_2SO are different from those reported previously,[13] see text for explanation.

obtained with the samples in the ^{15}N probe by exchanging the prefilter and transmitter coupler modules and using a conventional ^1H one-pulse experiment. From the proton spectra the exact frequency of the signal of Me_4Si could be measured either directly or, as in the case of water samples, with a secondary reference. In order to be able to determine the zero position in the ^{15}N shift scale, the absolute frequencies of the ^1H of Me_4Si and the ^{15}N of CH_3NO_2 were determined in a sample of Me_4Si in CH_3NO_2. These were, with the external D_2O lock used, 300 066 985.6 Hz and 30 417 092.0 Hz, respectively. The position of the ^{15}N resonance in CH_3NO_2, as a secondary reference, is given as 380.23 ppm[4] relative to the primary reference ^{15}N signal of liquid NH_3. The frequency at 0 ppm for ^{15}N is then calculated to be 30 405 530.9 Hz in this sample. For other samples the zero position (f_0) of the ^{15}N scale is then given by

$$f_0^{^{15}N} = 30\,405\,530.9 + r\left(f_{Me_4Si}{}^{^1H} - 300\,066\,985.6\right) \quad (1)$$

where r is the ratio of the frequencies of f_0 for ^{15}N and ^1H, or 30 405 530.9/300 066 985.6 = 0.101 329 144 4, and should be consistent from spectrometer to spectrometer while the offset frequencies in (1) will vary with field strength. This method of measuring ^{15}N chemical shifts relative to Me_4Si provides a solution to the problem of a good internal standard for ^{15}N spectroscopy, giving more accurate and consistent results than those from the variety of external ^{15}N references, and it does not require any correction for bulk susceptibility.[18]

The temperatures of the samples were determined from the readout of the temperature controller on the spectrometer which is accurate to within 1 °C. Variable-temperature measurements were made over a range of 10 to 40 °C.

Connectivities between ^{15}N and ^1H transitions were established by using polarization-transfer selective suppression,[19] using the sequence $90°_X$ (^{15}N); 180° (^1H, selective); $90°_X$ (^{15}N); $90°_X$ (^1H)–τ–180° (^1H); 180° (^{15}N)–τ–$90°_Y$ (^1H); $90°_X$ (^{15}N)–2τ–acquire (^1H decoupler on). The inter pulse delay $\tau = (4J)^{-1} = 2.68$ ms and the selective ^1H pulse was 30 ms long.

The selective 180° pulse was applied at the respective frequencies of the (^1H–^{15}N) satellites, 45 Hz from the center of the normally observed ^1H signal. The lengths of 90° pulses for protons and ^{15}N were 110 and 95 μs, respectively.

Spectra were normally obtained by using INEPT,[5,6] with sweep widths of 1.5 KHz into 1024 locations. After filtering, zero filling, and Fourier transformation, the spectra were usually plotted in absolute magnitude.

Results

The ^{15}N spectrum in water of the four protonated amide nitrogens in gramicidin S is shown in Figure 1. These shifts, temperature dependencies, and assignments are listed in Table I. The assignments were made with use of a pulse sequence based on the INEPT experiment,[19] and examples of the spectra used in assignment are available in the supplementary material. In addition to reporting the ^{15}N spectrum and assignments in water, we have measured the shifts and temperature dependencies in Me_2SO, methanol, and TFE with use of internal referencing to Me_4Si. This has provided improved values for the solvent- and temperature-induced shifts. The assignments in TFE were also reinvestigated in light of the two differing reports in the literature,[13,14] and our results were consistent with those of Hawkes et al.[13] Additional confirmation of the shift of the Phe N in water comes from the observation that this resonance loses intensity first as the pH of the solution is raised from 3. This is a result of the fact that the amide proton on the Phe nitrogen has, for gramicidin S amides, the fastest exchange rate with water, and as the pH is increased

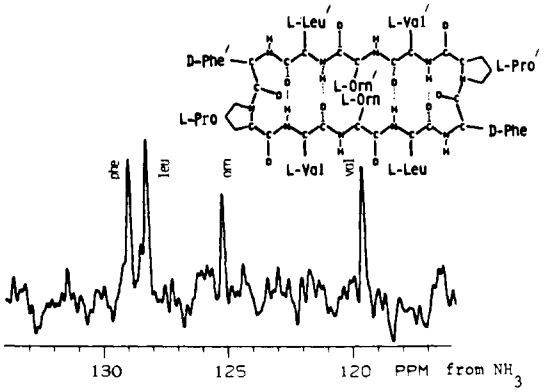

Figure 1. Structure and ^{15}N NMR spectrum of 10 mM gramacidin S in 95% H_2O–5% D_2O. The spectrum was acquired in about 90 min on a Nicolet NT-300 with use of a 10-mL sample in a 20-mm sample tube at 40 °C with INEPT[5,6] pulse sequence. Shifts relative to NH_3.[4]

the rate increases.[11] When the exchange rate is comparable to the interpulse delays of the INEPT sequence, then polarization transfer is lost. Proton exchange rendered the observation of the Orn side chain amine impossible with INEPT methods. The signal was measured after substantial time averaging with use of conventional single pulse methods with NOE. The resonance of the Pro nitrogen was not observed under the experimental conditions with use of INEPT that were applicable to the other amides since it lacks a directly bonded proton. Efforts to achieve polarization transfer through the smaller couplings to remote protons in Pro were unsuccessful. In the absence of an attached proton little or no NOE is expected and the T_1 relaxation is long, precluding the possibility of observing this resonance in a 10-mM sample by conventional methods. It was possible, however, to observe the Pro N resonance as a 7 mol % dioxane/water solution where the solubility was 80 mM. Since the shifts of the other amides were not significantly affected by the addition of dioxane, the value observed for the Pro N in this solvent mixture is probably a good representation of the shift in pure water.

At pH 3.5 the $^1J(^{15}N$–$^1H)$ couplings for the protonated amides were between 93.2 and 94.8 Hz, which are consistent with trans peptide bonds.[4]

Discussion

The change in the peptide ^{15}N chemical shifts between solvents reflects the variation in the hydrogen bonding to the particular peptide linkage.[20] Both hydrogen bond donation by the amide proton and acceptance by the amide carbonyl result in a downfield shift of the amide nitrogen resonance with the effects from each end being additive.[20] For the Val, Leu, and Pro nitrogens in gramicidin S (Table I) the solvent-induced shifts are consistent with the expected local changes in the ability of the solvents to hydrogen bond to the Pro, Orn, and Phe carbonyls, respectively (see Figure 1), with Me_2SO not bonding and methanol, water, and TFE bonding, thus inducing increasingly lower field shifts for the nitrogens. In contrast the Phe and Orn nitrogens should show roughly equal upfield shifts over the same sequence of solvents arising from a reduction in solvent hydrogen bond acceptance. They show little effect, however. Proton results[3] rule

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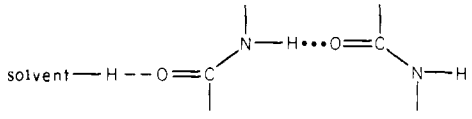
out solvent isolation of these amide protons as an explanation for the latter results. Our explanation of the lack of solvent-induced shifts on the Phe and Orn nitrogens is that the direct effect from the loss of hydrogen bonding of the amide protons to Me_2SO is compensated for by a chain of perturbations generated by the hydrogen bonding of the methanol, water, and TFE to the carbonyls of the amides across the intramolecular hydrogen bonds, six bonds away. For example, the ornithyl carbonyl is solvent hydrogen bonded in a protic solvent resulting in increased acidity of the internally hydrogen bonded leucyl amide proton of this linkage. The proton is then able to hydrogen bond more effectively to the valyl carbonyl oxygen across the gramicidin S ring, and the increase in hydrogen bonding causes a downfield shift in the adjacent ornithyl′ nitrogen—the ornithyl across the ring from the initially affected ornithyl carbonyl.



This compensates for the loss of direct hydrogen bonding of the Orn′ amide proton that is present in Me_2SO. The same sequence can serve to connect the Phe nitrogen shift to interactions at the Pro carbonyl.

The long-range through-hydrogen-bond shift effect is independently corroborated by studies of the temperature coefficients of the ^{15}N resonance (Table I). The greater and lesser ^{15}N temperature coefficients in the four diverse solvents do not correlate with the simple local solvent exposure, as do the proton temperature dependence results.[3] Rather, the ^{15}N coefficients pair on the basis of which amide groups share a common intramolecular hydrogen bond, i.e., Phe and Val nitrogens and Leu and Orn nitrogens. These results confirm the importance of the internal hydrogen bond in mediating the shifts of the nitrogens in the peptide groups involved. These data are probably reflecting contributions from the effect of temperature on the internal hydrogen bond itself and those derived from the modulation of the solvent–peptide interaction. The magnitude and range of the ^{15}N temperature coefficients suggest that they will be quite useful in themselves in studying peptide interactions. The ultimate understanding of these temperature effects depends on the examination of peptides with structured and random conformations, and such investigations are currently under way.

A set of ^{15}N temperature coefficients for gramicidin S in Me_2SO has been previously reported,[13] but they do not agree with our data. We attribute this discrepancy to a failure of those authors to correct for the temperature dependence of their D_2O lock signal which would induce a temperature dependent field drift. We have found that the position of the D_2O resonance moves with temperature at about 10 ppb/°C. The earlier results are then a superposition of the ^{15}N and D_2O temperature coefficients. When the contribution of the temperature shift in the D_2O lock resonance is substracted out, their data agree with ours. This further substantiates the importance of using internal referencing for such ^{15}N studies.

Results reported for the temperature coefficients for the ^{15}N shifts of alumichrome in Me_2SO[20] are also consistent with the results reported here. The effect of temperature on the shifts of the Gly^2 and Orn^3 nitrogens in the two amide linkages that participate in the single amide to amide internal hydrogen bond in that molecule are similar.[20] The Gly^2 nitrogen, which is the solvent-exposed N–H of the pair, does not show the complete shift compensation effect between Me_2SO and TFE solutions[20] that we have found in analogous arrangements in gramicidin S, however. The Orn^3 nitrogen, attached to the solvent-exposed carbonyl of the pair in alumichrome, exhibits a shift between these two solvents of only half what we find in gramicidin S in such cases. This suggests that the solvent interaction at the Orn^3 is weaker, an effect that would reduce the degree of change in the internal hydrogen bond and hence the shift compensation to the Gly^2 nitrogen.

If internal hydrogen bonds in β sheet type structures are affected by solvent interactions to the exposed C=O's, as indicated by the ^{15}N results, then this effect should also be observable in the ^{13}C shifts of the C=O's involved. Although not analyzed this way when originally reported,[3,21] the ^{13}C shifts for gramicidin S are completely consistent with this change in the internal hydrogen bonds. The ^{13}C of the Orn and Pro C=O's, which are solvent exposed, show the expected downfield solvent shifts of 3.2 and 3.5 ppm relative to Me_4Si respectively between Me_2SO and TFE, while the internally bonded Leu and Val C=O's have downfield shifts of 2.2 and 2.7 ppm. Since it has been shown[21] with several model peptides in several solvents that the ^{13}C=O shift is only sensitive to interaction at the C=O oxygen, then the shifts for the Leu and Val C=O's must result from an increase in the internal hydrogen bond strength.

The high sensitivity of ^{15}N NMR to changes in the hydrogen bonding in peptides makes it an ideal probe to characterize better the nature and degree of solvent interactions and the changes in internal hydrogen bonds. Trends of the ^{15}N shifts of the Leu, Val, and Pro nitrogens in gramicidin S show the increasing strength of the solvent hydrogen bonding to the C=O as the solvents become more acidic. The magnitude of the changes for the range of solvents studied is about 8 ppm. This is slightly bigger than the range reported by Hawkes et al.,[13] and we attribute the difference to our use of more precise internal referencing. From the data for Phe and Orn one can conclude the increased solvent hydrogen bonding to the Orn and Val C=O's, and the consequential increase in the hydrogen bonding ability of the proton attached to the nitrogen bonded to those carbons compensates in a proportional way for the reduction in the solvent hydrogen bonding to the exposed amide protons. The changes imply that although solvent hydrogen bonding to C=O can enhance the internal hydrogen bonding of the covalently linked amide proton, the solvent interaction with the exposed amide proton does not have a substantial effect on the internal hydrogen bond, though it may increase the polarization of the C=O bond.

These results illustrate the great potential of using ^{15}N NMR to study inter- and intramolecular interactions in peptides and their relationship to secondary structure. The increases in signal strength obtainable with spin-polarization transfer techniques and the related techniques for assignment make this a practical approach.

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Supplementary Material Available: NMR spectra of gramicidin S (1 page). Ordering information is given on any current masthead page.

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