Study of Internal Rotations in Gramicidin S by Means of Carbon-13 Spin–Lattice Relaxation Measurements

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Abstract: Spin–lattice relaxation times of individual resonances in the proton-decoupled natural-abundance ^{13}C Fourier transform nmr spectrum of gramicidin S in methanol at 43° yield information about rates of internal rotation of the side chains. The correlation times for internal rotation about C^{α}–C^{β} of the phenylalanine and valine residues are $(3.3 \pm 0.7) \times 10^{-10}$ and $(5.1 \pm 1.2) \times 10^{-10}$ sec, respectively. Because of an ambiguity in the spectral assignment for C^{β} of leucine, the correlation time for rotation about C^{α}–C^{β} of this residue is either $(6.9 \pm 0.9) \times 10^{-11}$ or $(5.2 \pm 0.5) \times 10^{-11}$ sec. Our measurements also yield qualitative information concerning internal rotations about C^{β}–C^{γ} and beyond.

Proton nuclear magnetic resonance has been used extensively to study the properties of gramicidin S in solution.[1] Recently, the ^{13}C nmr spectrum of gramicidin S has been reported.[2-4] As in the case of other complex molecules,[5-7] the proton-decoupled natural-abundance ^{13}C nmr spectrum of gramicidin S is more resolved than the corresponding proton nmr spectrum; nearly every nonequivalent carbon yields a resolved single-carbon resonance. One attractive feature of proton-decoupled natural-abundance ^{13}C nmr spectra is that one can take advantage of the great spectral resolution to measure *individual* ^{13}C spin–lattice relaxation times (T_1) of all carbons that yield resolved resonances, by means of partially relaxed Fourier transform (PRFT) nmr spectra.[8,9] Moreover, ^{13}C spin–lattice relaxation times of *protonated* carbons of large molecules are relatively easy to relate to dynamic properties of the molecules in solution, because the ^{13}C relaxation of such carbons is overwhelmingly dominated by a single relaxation mechanism, namely the ^{13}C–^{1}H dipolar interaction with the *directly bonded* hydrogen or hydrogens.[9] We present here T_1 measurements for all resolved ^{13}C resonances of gramicidin S in methanol, recorded at 15.18 MHz with the use of our 20-mm probe.[10,11] These measurements yield information about the rotational motion of the molecular backbone of gramicidin and about internal rotations in the side chains. The resulting values for the rates of internal rotation about carbon–carbon bonds of side chains in gramicidin S will be useful in the interpretation of similar data for proteins.

Experimental Section

Gramicidin S was purchased from Schwartz/Mann, Orangeburg, N. Y., and dissolved in methanol (150 mg/ml). The solution was filtered to remove a small amount of an insoluble impurity. No attempt was made to remove a small amount of soluble impurity that gave rise to a single resonance in the ^{13}C spectrum (see below). Proton-decoupled natural-abundance ^{13}C Fourier transform nmr spectra were recorded at 15.18 MHz and 43 ± 1° on our "home-built" Fourier transform nmr instrument[9,12] with the use of a 20-mm probe[10,11] and a Nicolet-1085 computer but before the incorporation of the special crystal filter described in ref 11. Chemical shifts were measured digitally with respect to internal tetramethylsilane (TMS), using a digital resolution of 0.37 Hz (16,384 time-domain points and 2994-Hz sweep width). The chemical shifts of well-resolved resonances are accurate to ±0.05 ppm. ^{13}C spin–lattice relaxation times of individual resonances were measured by means of PRFT spectra.[8,9] We recorded separate PRFT spectra of the nonprotonated carbon region (downfield) and protonated carbon region (upfield), with 1000- and 2000-Hz spectral widths, respectively. To accumulate each spectrum, 8192 memory addresses were used. For the nonprotonated and protonated carbon regions, 1024 and 4096 accumulations per spectrum, respectively, were used. The carrier frequency was set to a suitable high frequency for the downfield region and a suitable low frequency for the upfield region, so as to avoid interference from "folded" resonances.[13] For the PRFT spectra of the protonated carbon resonances, the recycle time (interval between successive 90° radiofrequency pulses) was about $3.5T_{1,\max}^{CH} + \tau$, where $T_{1,\max}^{CH}$ is the longest protonated carbon T_1 value, and τ is the interval between a 180° radiofrequency pulse and the following 90° radiofrequency pulse. For the less accurate measurements of the T_1 values of nonprotonated carbons, the PRFT spectra of the downfield region were obtained with a recycle time of about $2T_{1,\max}^{C} + \tau$, where $T_{1,\max}^{C}$ is the longest nonprotonated carbon T_1 value. Fourier transformation was done with 16,384 time-domain points, by placing 8192 addresses with a zero value at the end of each 8192 accumulated data points. In this way, there was one point every 0.122 and every 0.244 Hz in the frequency-domain spectra of the downfield and upfield resonances, respectively. The resulting signal-to-noise ratios are displayed in Figure 1, which shows all the resonances resulting from the normal spectra (without applying any 180° radiofrequency pulses) obtained using the conditions described above. The signal-to-noise ratio is greater than 60 for all protonated carbon resonances. The signal-to-noise ratio is lower for the nonprotonated carbons, because fewer accumulations per spectrum were used, and

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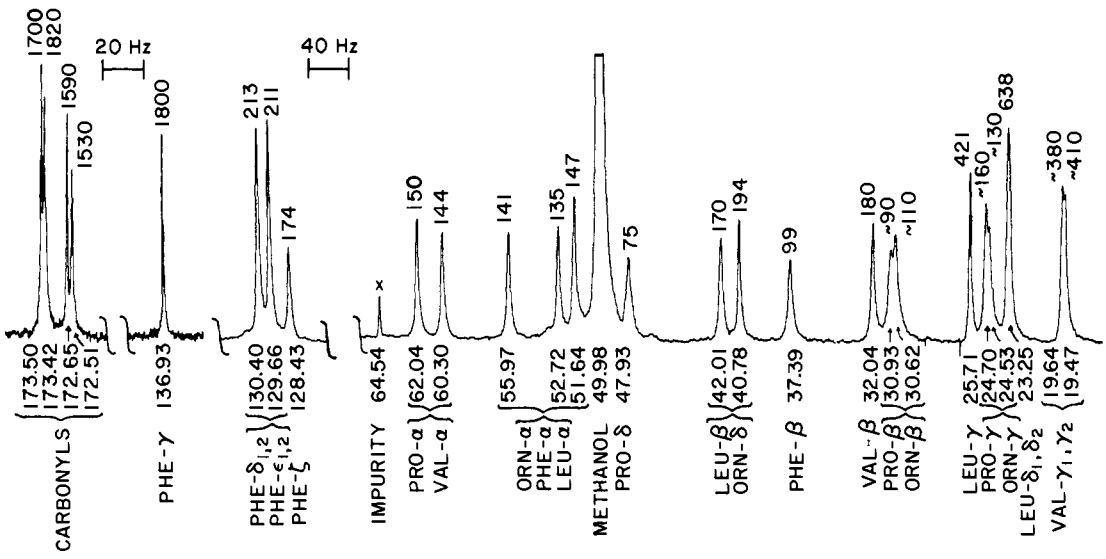

Figure 1. Proton-decoupled natural-abundance ^{13}C nmr spectrum of gramicidin S in methanol (150 mg/ml) at 43°, recorded at 15.18 MHz. Details are given in the Experimental Section. Numbers below each peak are chemical shifts in ppm downfield from internal TMS. Carbon designations are those of Figure 2. Numbers above each peak are T_1 values, in millisecond.

because no digital exponential broadening was applied to the accumulated time-domain signal. The time-domain data of each normal and PRFT spectrum of the protonated carbon region were multiplied by an exponential function with a negative time constant (corresponding to a digital broadening of 0.389 Hz) to improve the signal-to-noise ratio. Seven to twelve PRFT spectra with different τ values were used to determine each T_1. Computational details have been given elsewhere.[9] The estimated accuracy of the T_1 values is ±5 and ±10% for the protonated and the nonprotonated carbon resonances, respectively.

Results and Discussion

Below each resonance in Figure 1 we show the corresponding chemical shift, in parts per million downfield from internal TMS, and the assignment of the resonances made by Gibbons, *et al.*,[2] and Johnson and Jankowski.[4] The standard IUPAC–IUB carbon designations used here are shown in the top part of Figure 2.

Above each peak in Figure 1 we show the corresponding ^{13}C T_1 value, in milliseconds. As expected,[9] the nonprotonated carbons have considerably longer T_1 values than the protonated ones. Because of the various possible important contributions to the dipolar relaxation of the nonprotonated carbons, and also because of the possibility of contributions from nondipolar relaxation mechanisms,[14] it was not possible to extract useful information from the T_1 values of C^γ of the phenylalanines nor from the T_1 values of the carbonyl carbons. We present below our interpretation of the T_1 values of the protonated carbons.

It has been shown that the relaxation of protonated carbons of large molecules is overwhelmingly dominated by ^{13}C–^1H dipolar interactions with the directly bonded hydrogens.[9] In the case of a molecule undergoing isotropic rotational reorientation, and in the absence of internal motions, T_1 is given by[15] eq 1, where N is the number of directly bonded hydrogens, γ_H and γ_C are the gyromagnetic ratios of ^1H and ^{13}C, respectively, r is the C–H bond length, τ_R is the correlation time for the

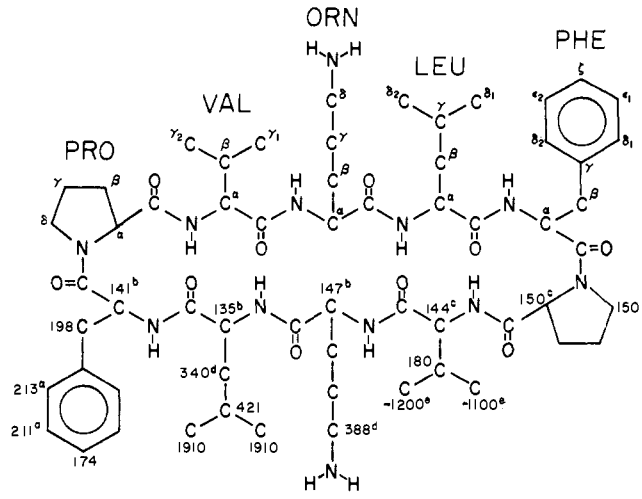

Figure 2. Structure of gramicidin S. Hdyrogens bound to carbon have been ommitted. Standard IUPAC-IUB carbon designations are shown in the top portion of the structure. Values of NT_1 (in millisecond) of protonated carbons are shown in the lower portion. NT_1 values with letter superscripts correspond to carbon resonances that have not been specifically assigned (see Figure 1). NT_1 values with the same superscript may have to be interchanged when the corresponding specific assignments become available.

$$\frac{1}{NT_1} = \frac{\hbar^2\gamma_H^2\gamma_C^2}{10r^6}\left[\frac{\tau_R}{1+(\omega_H-\omega_C)^2\tau_R^2}+\frac{3\tau_R}{1+\omega_C^2\tau_R^2}+\frac{6\tau_R}{1+(\omega_H+\omega_C)^2\tau_R^2}\right] \quad (1)$$

isotropic rotational motion of the molecule, and ω_H and ω_C are the resonance frequencies, in radians per second, of ^1H and ^{13}C, respectively. Equation 1 can be used to obtain τ_R from a measurement of T_1 of a protonated carbon, if the molecule as a whole rotates isotropically, *and* if the pertinent C–H groups are not participating in some internal rotation.[15] Thus, eq 1 is not applicable to the side-chain carbons of gramicidin S. Equation 1 is valid for the α carbons if any seg-

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mental motion of the decapeptide ring is much slower than the overall rotational reorientation of the molecule. Although we cannot completely rule out the possibility of fast segmental motion of the gramicidin backbone, our α-carbon T_1 values suggest that segmental motion, if present, is slower than the overall rotational motion. The effective rotational correlation time for segmental motion of random-coil poly(γ-benzyl L-glutamate) in a $CDCl_3$–CF_3COOH mixture at 40° is 8×10^{-10} sec.[16] From our measured α-carbon T_1 values, eq 1 yields an effective τ_R value of $(3.3 \pm 0.2) \times 10^{-10}$ sec for gramicidin S in methanol at 43° (see below). It is unlikely that segmental motion of the backbone of a *small cyclic* peptide would be considerably faster than that of a random-coil linear polypeptide. Moreover, a τ_R value of 3.3×10^{-10} sec is comparable to previously measured correlation times for *overall rotation* of species with a molecular weight of about 1000. For example, methine carbons of the rigid corrin ring of aqueous dicyanocobalamin at 56° have T_1 values of about 100 msec,[7] which corresponds to a τ_R value of 4.8×10^{-10} sec. Some workers object to making comparisons of T_1 values in solvents of different viscosities. The viscosity of methanol at 40° is 4.49×10^{-3} P, while that of water at 56° is 4.99×10^{-3} P,[17] a difference of only 10%. A rigid gramicidin S structure was also inferred from circular dichroism studies.[18]

On the basis of the above considerations, we believe that it is safe to assume that the T_1 values of the α carbons of gramicidin S in methanol are determined mainly by the overall rotational motion of the molecule. Further analysis of our data relies on this assumption.

If the five different α carbons had identical ^{13}C T_1 values, this would be strong evidence for isotropic rotation of the gramicidin molecules in methanol.[9] The measured values (Figure 1) range from 135 ± 7 to 150 ± 8 msec. Therefore, the five α-carbon T_1 values are indeed identical or nearly identical, and the gramicidin molecules rotate isotropically or nearly isotropically. Further analysis of our data relies on the assumption of isotropic overall rotation.

Equation 1 can now be used to determine τ_R from the α-carbon T_1 values. Previously derived equations[15] can then be used to obtain correlation times for internal rotation about C^α–C^β from T_1 values of the β carbons. The arithmetic average of the five α-carbon T_1 values is 143.4 msec. Introduction of this value into eq 1 yields two solutions for τ_R: 3.3×10^{-10} and 1.1×10^{-7} sec. The second solution can be rejected not only on the basis of known correlation times for molecules of comparable molecular weight[7,9] but also because the computed line widths corresponding to the two solutions[15] are 2.2 and 152 Hz, respectively. The experimental α-carbon line widths are about 4 Hz, which includes an instrumental broadening of about 1 Hz from various sources. Therefore, we take 3.3×10^{-10} sec as the τ_R value for gramicidin S in methanol at 43°. The estimated accuracy is $\pm 0.2 \times 10^{-10}$ sec.

At our magnetic field of 14.2 kG, a τ_R value of 3.3×10^{-10} sec satisfies the extreme narrowing limit

$$(\omega_H + \omega_C)^2 \tau_R^2 \ll 1 \qquad (2)$$

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In the extreme narrowing limit, eq 1 becomes

$$1/NT_1 = \hbar^2 \gamma_H^2 \gamma_C^2 r^{-6} \tau_R \qquad (3)$$

When τ_R satisfies eq 2, the spin–lattice relaxation time (T_1^β) of a protonated β carbon with N^β directly attached hydrogens is given by[15]

$$\frac{1}{N^\beta T_1^\beta} = \frac{\hbar^2 \gamma_H^2 \gamma_C^2}{r^6} \tau_R \left[A + B \frac{6\tau_G}{6\tau_G + \tau_R} + C \frac{3\tau_G}{3\tau_G + \tau_R} \right] \qquad (4)$$

where τ_G is the correlation time for internal motion, as defined previously,[9] and

$$A = {}^1/_4(3 \cos^2 \theta - 1)^2 \qquad (5)$$

$$B = 3 \sin^2 \theta \cos^2 \theta \qquad (6)$$

$$C = {}^3/_4 \sin^4 \theta \qquad (7)$$

Here θ is the angle between the C^β–H vector and the axis of internal rotation. We will assume that bonding at the β carbons is tetrahedral ($\cos^2 \theta = 1/9$). Then

$$A = 1/9 \qquad (8)$$

$$B = 8/27 \qquad (9)$$

$$C = 16/27 \qquad (10)$$

If τ_R is taken as 3.3×10^{-10} sec (see above), then eq 4 can be used to obtain τ_G for rotation about C^α–C^β from a measurement of the β-carbon T_1 value. It is instructive to compute first how fast the internal motion has to be before it measurably affects T_1^β. In the absence of internal motion ($\tau_G = \infty$), T_1^β satisfies eq 3. Taking into account that $N^\alpha = 1$, we conclude that

$$N^\beta T_1^\beta \ (\tau_G = \infty) = T_1^\alpha$$

It is possible to determine τ_G accurately only when the measured $N^\beta T_1^\beta$ differs from T_1^α by much more than the experimental error in the T_1 measurements. Since T_1^α satisfies eq 3, it follows from eq 4 that

$$\frac{T_1^\alpha}{N^\beta T_1^\beta} = A + B \frac{6\tau_G}{6\tau_G + \tau_R} + C \frac{3\tau_G}{3\tau_G + \tau_R} \qquad (11)$$

It is easily shown that $A + B + C = 1$. Introduction of this result into eq 11 shows that $T_1^\alpha \leq N^\beta T_1^\beta$. However, T_1^α *may* be greater than $N^\beta T_1^\beta$ if the extreme narrowing limit for τ_R does not apply,[15] as may be the case when dealing with biopolymers. For a tetrahedral θ, eq 11 becomes

$$\frac{T_1^\alpha}{N^\beta T_1^\beta} = \frac{1}{9}\left[1 + \frac{16}{6 + \rho} + \frac{16}{3 + 2\rho} \right] \qquad (12)$$

where

$$\rho = \tau_R/\tau_G \qquad (13)$$

In the limit $\rho \to 0$ (slow internal rotation), eq 12 yields the expected result that $T_1^\alpha = N^\beta T_1^\beta$. In the limit $\rho \to \infty$ (fast internal motion), eq 12 predicts that $N^\beta T_1^\beta = 9T_1^\alpha$, a result derived previously.[9]

Let us assume now that the smallest difference between T_1^α and $N^\beta T_1^\beta$ that we will accept as evidence for internal rotation about C^α–C^β is 20% (four times the experimental error of ±5%). Then we can use eq 12

to establish what is the largest value of τ_G (slowest internal rotation) that we can measure. We must have

$$T_1^{\alpha}/N^{\beta}T_1^{\beta} \lesssim 0.8$$

Then eq 12 yields

$$\rho \gtrsim 0.6$$

Therefore, measurable τ_G values are restricted to

$$\tau_G \lesssim 2\tau_R \qquad (14)$$

It should also be noted that when the internal motion becomes very fast ($\tau_G \ll \tau_R$), then $N^{\beta}T_1^{\beta}$ approaches the limiting value $9T_1^{\alpha}$ and again becomes very insensitive to changes in τ_G.

In the lower half of Figure 2 we show NT_1 values of all resolved *protonated carbon* resonances of gramicidin S. With the exception of proline, all other residues show a measurable increase in NT_1 when going from the α carbons to the side chains. In the case of proline, no significant internal motion is expected, and thus C^{β}, C^{γ}, and C^{δ} should have the same NT_1 value as the α carbon. This is indeed the case for C^{δ}. The T_1 values of C^{β} and C^{γ} could not be measured, because of overlap of their resonances with those of C^{β} and C^{γ}, respectively, of ornithine (Figure 1).

The $N^{\beta}T_1^{\beta}$ values of the phenylalanine, leucine, and valine residues are all sufficiently longer than the α-carbon T_1 values to permit a determination of τ_G for the rotation about C^{α}–C^{β}. The resulting τ_G values for the phenylalanine and valine residues are $(3.3 \pm 0.7) \times 10^{-10}$ and $(5.1 \pm 1.2) \times 10^{-10}$ sec, respectively. The $N^{\beta}T_1^{\beta}$ value of the leucine residues is either 340 or 388 msec (Figure 2), because of the ambiguity in the assignments for C^{β} of leucine and C^{δ} of ornithine (Figure 1). If $N^{\beta}T_1^{\beta}$ is 340 msec, then τ_G for the rotation about C^{α}–C^{β} of leucine is $(6.9 \pm 0.9) \times 10^{-11}$ sec. If $N^{\beta}T_1^{\beta}$ is 388 msec, then τ_G is $(5.2 \pm 0.5) \times 10^{-11}$ sec.

The valine and leucine residues show further increases in NT_1 when going beyond C^{β}, with the largest increase occurring at the methyl groups. A quantitative interpretation of these NT_1 values must await further theoretical developments. The trend along the ornithine side chains cannot be observed in detail, because only C^{δ} yields a well resolved resonance whose T_1 can be measured accurately (see Figure 1).

In the case of phenylalanine, the axis of rotation about C^{β}–C^{γ} coincides with the C^{ζ}–H bond. Therefore, rotation about C^{β}–C^{γ} cannot affect the T_1 value of C^{ζ}. As a result, C^{ζ} should have the same NT_1 value as C^{β}. However, the difference in the observed values (Figure 2) is outside experimental error. This difference may be caused by a difference of C–H bond lengths of about 3%. Throughout our discussion we have assumed that all C–H bond lengths are 1.09 Å. A change of 3% in bond lengths would change the T_1 value of a protonated carbon by 19%. C–H bond lengths are not known with sufficient accuracy to establish if indeed a change in C–H bond length when going from an aliphatic to an aromatic carbon can account for the observed difference between NT_1^{β} and NT_1^{ζ} of the phenylalanine residues.

Internal rotation about C^{β}–C^{γ} of the phenylalanine residues should lengthen NT_1^{γ} and NT_1^{δ}. The experimental NT_1 values of C^{γ} and C^{δ} are indeed longer than NT_1^{ζ}.

Acknowledgment. This research was supported by the National Science Foundation (Grant GP-17966), by the donors of the Petroleum Research Fund, administered by the American Chemical Society, by the National Institutes of Health (Grant NS-10977), and by Eli Lilly and Co.

Thermodynamics of the Two Dissociation Steps of *N*-Tris(hydroxymethyl)methylglycine ("Tricine") in Water from 5 to 50° [1]

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Abstract: *N*-Tris(hydroxymethyl)methylglycine ("tricine"), a zwitterion, is a derivative of glycine containing a strongly hydrophilic substituent. Both of its acidic groups are stronger than those of the parent glycine, and consequently tricine is a useful buffer substance for the physiological region of pH 7.2 to 9. The equilibrium constants for the two dissociation steps of tricine have been determined at ten temperatures by measurement of the emf of cells without liquid junction containing hydrogen gas electrodes and silver–silver bromide electrodes. For the dissociation of protonated tricine, pK_1 is 2.023 at 25°, while pK_2 for the dissociation of tricine itself is 8.135 at 25°. The standard changes of Gibbs energy, enthalpy, entropy, and heat capacity have been derived from the change of the equilibrium constants for these two processes with temperature.

Considerable interest attaches to the acid–base behavior of glycine, the simplest amino acid. Inasmuch as glycine exists in a zwitterionic form, the thermodynamic constants for the dissociation equilibria are uncommonly informative. Furthermore, glycine buffer solutions have long found use for the control of pH in the range 8.5 to 10.5. Careful thermodynamic studies

(1) This work was supported in part by the National Science Foundation under Grant GP 14538 and by the donors of the Petroleum Research Fund, administered by the American Chemical Society.

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