^{15}N Nuclear Magnetic Resonance Spectroscopy. Changes in Nuclear Overhauser Effects and T_1 with Viscosity

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Abstract: A procedure is described for probing changes in ^{15}N T_1 relaxation rates and nuclear Overhauser effects (NOE) with viscosity as a function of temperature. The large freezing-point depression and high viscosities of 8.8 M dimethyl sulfoxide (DMSO)−water solutions allowed study of the molecular motions of several ammonium salts, amides, and heterocycles on the pico- to nanosecond time scale. Dipole−dipole interactions provide the dominant form of relaxation for the ammonium salts, but chemical-shift anisotropy (CSA) also plays a significant role in the relaxation of amides and heterocycles. For pyridine, CSA is a particularly important mechanism and the shielding anisotropy of pyridine in 8.8 M DMSO−water is estimated to be on the order of 325 ppm. The ^{15}N NOE of NH_4Cl is greater than the theoretical maximum for intramolecular dipolar relaxation at high viscosities, and can be accounted for either by significant *inter*molecular contributions operating on a different time scale or by rapid motional averaging.

Introduction

^{15}N NMR spectroscopy can be very useful for structural determinations.[1] For this purpose, the ^{15}N nucleus possesses several attractive features, including a wide range of chemical shifts (∼900 ppm), a high sensitivity to its chemical environment, and a magnetogyric ratio opposite in sign to that of the proton. The latter is responsible for the negative ^{15}N−^{1}H nuclear Overhauser effect (NOE) that, when efficient, results in emission rather than absorption signals. The proton-induced NOE is capable of amplifying the intensity of ^{15}N NMR signals by nearly 4-fold, but is variable with respect to molecular motion and incursion of non-dipolar modes of relaxation. The time scale where the magnitude of the NOE is most sensitive coincides with that of the segmental motions experienced by oligopeptides and oligonucleotides. Not surprisingly, the application of the ^{15}N NOE in conjunction with spin−lattice (T_1) relaxation times is particularly useful for probing issues relating to biomolecular conformations and dynamics.[2] Despite this, relatively few attempts have been made to correlate the ^{15}N NOEs and T_1s as functions of molecular motion. Furthermore, the low natural abundance, small magnetic moment, and often long T_1 relaxation times of the ^{15}N nuclei impose severe detection limits, and hamper the routine acquisition of ^{15}N NMR signals, except for isotopically enriched samples.

Molecular tumbling in solution is commonly described by the autocorrelation time τ_c, which in turn can be considered to be a function of viscosity and temperature as formulated by Debye in eq 1,

$$\tau_c = \frac{4\pi a^3 \eta_s}{3kT} \tag{1}$$

where a is the effective radius of the solute, η_s is the microviscosity of its environment, and τ_c is the time for a molecule to rotate roughly one radian. The viscosities of conventional fluids are known to have an exponential dependency on inverse temperature,[3] so τ_c can be expressed as a function of temperature, i.e. $\log(\tau_c) = A/2.303T + \log(1/T) + C$, the exponential coefficient A being an empirical factor relating η_s and T.

We present here the results of experiments in which ^{15}N NOEs and T_1 relaxation times have been measured over a considerable range of viscosities as a function of temperature. A mixed solvent system (8.8 M DMSO in water) was chosen that possesses a large freezing-point depression and also allows reasonably high solute concentrations. The viscosities available with 8.8 M DMSO from 298 to 193 K range from 4 to 80000 cP, and correspond to molecular motions of solutes from a pico- to a nanosecond time scale. Other temperature-dependent ^{15}N NMR studies have been reported for several compounds, but most of these measurements have been limited to lower viscosities.[4]

A number of biochemically interesting amines, amides, and heterocycles were examined. Most of these are assumed to tumble essentially isotropically in solution and to have ^{15}N relaxation primarily via dipole−dipole mechanisms. Where other modes of relaxation become significant, their impact on overall T_1 relaxation can be monitored by the efficiency of the NOE. Correlation of experimental spin−lattice relaxation rates with NOE enhancements in small molecules provides an interesting complement to systems subject to more complex molecular dynamics, such as those of the amide nitrogens in peptide backbones.[2]

For practical ^{15}N NMR spectroscopy, our experiments suggest how ^{15}N signals can be collected more efficiently by using

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viscosity to shorten T_1 with little sacrifice of the NOE,[5] provided that the ^{15}N nuclei relax predominantly by dipolar interactions. It is well-known that the steady-state signal-to-noise ratio is proportional to the square root of the number of accumulations, and the amplitude of the NOE-enhanced signal is relatively unaffected by changes in the molecular tumbling rates faster than ω_H^{-1}, where ω_H is the resonance frequency for ^1H nuclei.

The NOE enhancement in a two-spin system of I and S relaxing solely by dipolar mechanisms can be described by the Solomon eq 2.[6]

$$\eta = \frac{\dfrac{\gamma_S}{\gamma}\left(\dfrac{6}{1+(\omega+\omega_S)^2\tau_c^2} - \dfrac{1}{1+(\omega-\omega_S)^2\tau_c^2}\right)}{\left(\dfrac{1}{1+(\omega-\omega_S)^2\tau_c^2} + \dfrac{3}{1+\omega^2\tau_c^2} + \dfrac{6}{1+(\omega+\omega_S)^2\tau_c^2}\right)} \quad (2)$$

Here η is the NOE amplification for nucleus I, γ and γ_S are the magnetogyric ratios of the two spins I and S, ω is the Larmor frequency of spin I, and $\omega_S = (\gamma_S/\gamma)\omega$ is the Larmor frequency of spin S. For the two-spin system in which I = ^{15}N and S = ^1H, $\gamma_H/\gamma_N = -9.87$; thus for sufficiently short τ_c, the maximum theoretical NOE for ^{15}N is -4.93, in the opposite sense of the unamplified signal. When correction for the normal-intensity signal is included, the actual maximum enhancement is $1 + \eta = -3.93$ (see Figure 1).

The denominator of eq 2 also describes the intramolecular dipole–dipole relaxation rate constant $T_1(dd)$, and can be written for the two-spin system as eq 3. Here r is the interatomic distance between the I and S nuclei.

$$\frac{1}{T_1(dd)} = \left(\frac{\mu_0}{4\pi}\right)^2 \frac{h^2\gamma^2\gamma_S^2\tau_c}{10r^6}\left(\frac{1}{1+(\omega-\omega_S)^2\tau_c^2} + \frac{3}{1+\omega^2\tau_c^2} + \frac{6}{1+(\omega+\omega_S)^2\tau_c^2}\right) \quad (3)$$

Under extreme narrowing conditions ($\tau_c \ll \omega$), eq 4 can be used when the interatomic distances of M neighboring ^1H nuclei are taken into account.

$$\frac{1}{T_1(dd)} = \left(\frac{\mu_0}{4\pi}\right)^2 h^2\gamma_H^2\gamma^2\tau_c \sum_{i=1}^{M}\frac{1}{r_i^6} \quad (4)$$

This linear dependency is potentially useful for determining τ_c from $1/T_1(dd)$, given precise $^{15}N-^1H$ interatomic distances (Figure 1). Experimental T_1 values, however, often involve contributions from several different relaxation mechanisms as expressed by eq 5.

$$\frac{1}{T_1} = \frac{1}{T_1(dd)} + \frac{1}{T_1(dd)^*} + \sum\frac{1}{T_1^*} \quad (5)$$

Here, $T_1(dd)^*$ represents intermolecular dipolar relaxation and T_1^* accounts for alternate relaxation processes such as chemical-shift anisotropy (CSA) or spin rotation, most of these being associated with single-quantum transitions that detract from the
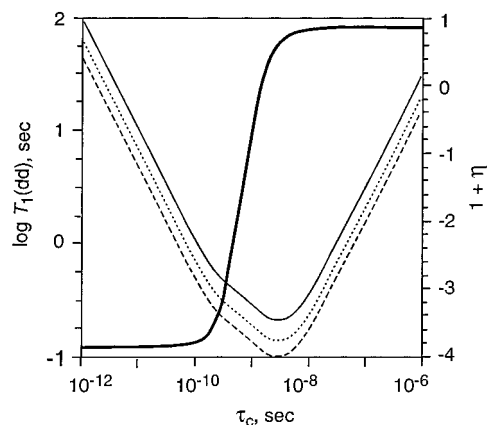

Figure 1. T_1 relaxation via intramolecular dipole−dipole interactions for an isotropically tumbling ^{15}N nucleus with two (—), three (---), or four (− − −) directly attached protons. NOE enhancement of a ^{15}N nucleus relaxing solely by intramolecular dipole−dipole interactions (▬).

efficiency of the NOE.[7] We consider intermolecular dipolar processes because of the high concentration of protons in the solvent medium (*vide infra*).

The relative contributions from the various T_1 processes are not easily quantified for any but the simplest of compounds, and this complicates the estimation of correlation times. Nonetheless, relative values for an effective correlation time τ_e can still be derived which will show the effects of size and structure on molecular motion. In some cases, correlation of the NOE to T_1 provides insight into relaxation mechanisms which compete with $T_1(dd)$.

Experimental Section

^{15}N-Enriched Samples. The following ^{15}N-enriched substrates (95−99% ^{15}N content) were purchased from commercial sources: $^{15}NH_4$-Cl, $^{15}N_2$-urea, ^{15}N-pyridine (Prochem B. O. C. Ltd.); $^{15}N_2$-L-glutamine (Cambridge Isotope Laboratories); and ^{15}N-L-alanine, potassium ^{15}N-phthalimide (Merck, Sharp, and Dohme). $^{15}N_2-(\pi,\tau)$-L-histidine monohydrochloride was very kindly supplied by Professor W. W. Bachovchin of Tufts Medical School. ^{15}N-Benzylamine was prepared from ^{15}N-enriched potassium phthalimide by a modified Gabriel synthesis.[8] ^{15}N-L-Pyroglutamic acid was obtained by heating a sample of $^{15}N_2$-L-glutamine in the presence of trifluoroacetic acid in aqueous 8.8 M DMSO at 100 °C.[9] Nonlabeled substrates were mixed with the labeled materials in several cases to maintain constant molarities.

Preparation of Samples. All samples, except for alanine, were dissolved in aqueous 8.8 M DMSO (9:1 DMSO:DMSO-d_6) and degassed by thoroughly sparging with argon. Alanine was dissolved in 7.9 M DMSO and degassed as above. Either trifluoroacetic acid (CF_3CO_2H) or KOH was added to many of the samples to avoid complications from rapid proton exchange. These samples were prepared at the following concentrations and ^{15}N-enrichment levels: alanine plus 1 molar equiv of CF_3CO_2H, 0.52 M (50% ^{15}N); ammonium chloride plus 0.2 molar equivs of CF_3CO_2H, 0.58 M (30.8% ^{15}N); ammonium pyroglutamate plus 1 molar equiv of CF_3CO_2H, 0.125 M (95% ^{15}N); benzylamine plus 1 molar equiv of CF_3CO_2H, 0.58 M (15% ^{15}N); glutamine plus 1 molar equiv of CF_3CO_2H, 0.125 M (95% ^{15}N); histidine hydrochloride, 0.066 M (95% ^{15}N); pyridine plus 0.06 molar equiv of KOH, 1.22 M (46% ^{15}N); pyridine plus 1.15 molar equiv of CF_3CO_2H, 1.10 M (46% ^{15}N); and urea, 0.58 M (30.8% ^{15}N).

NMR Measurements. Unless otherwise stated, ^{15}N NMR spectra were recorded at 50.684 MHz using 10-mm-bore broad-band probes

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(7) Although eq 5 is of heuristic value, at best it can only approximate overall spin−lattice relaxation as the sum of separate components because of cross-correlation effects: Boyd, J.; Hommel, U.; Campbell, I. D. *Chem. Phys. Lett.* **1990**, *175*, 477 and references therein.

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(Bruker Magnetics). In general, measurements were made at room temperature (ca. 300 K), then in 5-deg decrements for temperatures below 273 K until T_1 reached a minimum. Temperature readings were calibrated using the chemical-shift difference between the hydroxyl and methyl peaks of pure methanol at 10-deg intervals; the uncertainties in the temperature measurements are estimated to be ± 1 K. All T_1 relaxation times were measured by the inversion-recovery method using a minimum of seven points; the standard deviations were typically less than 2%. All ^{15}N NMR spectra were acquired using a relaxation delay of at least four times the determined T_1 value. NOE-enhanced spectra were obtained using composite pulse proton decoupling (WALTZ-16 sequence), with a brief (1−4 s) pause following each acquisition for heat dissipation. Quantitative NOEs were calculated by area integration of the NOE-enhanced decoupled signal versus the unenhanced signal; the estimated uncertainties in measurement were typically less than 5%.

Viscosity Measurements. Kinematic viscosity of 8.8 M DMSO in water was measured using routine viscometers (Cannon-Fenske, Cannon Instrument Co.) of the appropriate gauge. All measurements were performed in a constant-temperature bath in duplicate.

Results and Discussion

Viscosity-Temperature Relationship. The kinematic viscosity of aqueous 8.8 M DMSO has a biexponential relationship with inverse temperature between $T = 183$ K and 298 K, as shown in Figure 2 and as expressed by eq 6.

$$\eta_s = ae^{A/T} + be^{B/T} \tag{6}$$

The coefficients A and B obtained by analysis of the linear regions of the curve are estimated to be 3075 and 6050, respectively; the term corresponding to A is dominant at higher temperatures ($T > 250$ K). The kinematic viscosity of 8.8 M DMSO containing 0.58 M NH$_4$Cl was also measured at selected temperatures, and was found to be within 5% that of the pure solvent. Thus, the essentially exponential viscosity−temperature relationship of this solvent system seems unaffected by the presence of solutes.

Experimental T_1 and NOE values were determined for various ^{15}N-containing solutes in 8.8 M DMSO over a wide range of temperature-dependent viscosities, and plotted as functions of inverse temperature (*vide infra*). Linear relationships between $\log T_1$ (or $-\log(1/T_1)$) and $1/T$ are apparent at higher temperatures, suggesting activation energies for isotropic reorientation (E_A) in the range of 2.4 to 5.4 kcal/mol (see Table 1). An effective correlation time τ_e can be determined using eq 7, given an appropriate constant τ_o.

$$\tau_e = \tau_o e^{E_A/RT} \tag{7}$$

From eq 4, τ_c is directly proportional to $1/T_1$ in this temperature range, so if $\tau_c \approx \tau_e$ then the exponential coefficient $A \approx E_A/R$, where R is the ideal gas constant. Equation 4 yields values between 1220 and 2725 K^{-1}, most of these significantly lower than calculated from the viscosity data. This difference can be rationalized in three ways: (1) the overall rotational motion of many of the substrates is not wholly isotropic, thus compromising the $\tau_c \approx \tau_e$ approximation, (2) the microviscosity term η_s is not a linear function of kinematic viscosity, a bulk property, and (3) the Debye expression itself is not an exact relationship, but rather a descriptive model for molecular tumbling.

Our observations are at odds with earlier theoretical derivations by Bloembergen and co-workers, who used the Debye expression and eq 4 to assume a linear relationship for $1/T_1$-
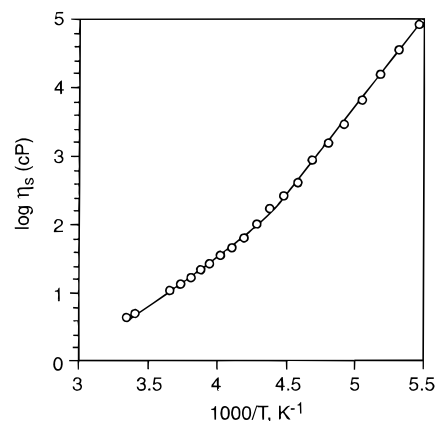
Figure 2. Kinematic viscosity of 8.8 M DMSO in water as a function of temperature.

Table 1. Estimated E_A and τ_o Values of ^{15}N-Containing Compounds at 50.684 MHz in 8.8 M DMSO

compd	E_A, kcal/mol	T at minimum T_1, K	τ_o, s
amines			
NH$_4^+$	4.4	194	2.9×10^{-14}
benzylamine-H$^+$	3.7	211	4.0×10^{-13}
alanine-H$^+$	3.5	222	1.1×10^{-12}
glutamine-H$^+$	3.1	232	3.6×10^{-12}
amides			
urea	4.4	216	1.0×10^{-13}
pyroglutamate	4.0	228.5	4.9×10^{-13}
glutamine-H$^+$ (amide)	3.3	230	2.0×10^{-12}
heterocycles			
pyridine	5.4	213.5	8.5×10^{-15}
histidine-H$^+$ (N1)	2.4	241	1.9×10^{-11}
histidine-H$^+$ (N3)	3.2	241	3.9×10^{-12}

(dd) and η_s/T for short correlation times.[10] Supporting experimental evidence came from T_1 studies of the protons in ethyl alcohol, in which the ratio of $\log(T_1)$ against $\log(\eta_s/T)$ was shown to have a slope of roughly -1. However, by nature, log−log plots can often be easily misinterpreted. Other spin-relaxation studies have encountered similar difficulties in using the Debye approximation to describe the reorientation of small molecules, and require the use of *ad hoc* correction factors based on glass-transition temperatures[11] or effective molecular volumes.[12]

The empirical correlation of ^{15}N NMR data with temperature is still very useful for determining the relative contributions by various processes to overall relaxation. The studies have been subdivided into three categories to illustrate the relationship between NOE and T_1 relaxation and molecular structure.

Ammonium and Alkylammonium Ions. The data for ^{15}N ammonium chloride and the trifluoroacetate salts of benzylamine, alanine, and glutamine are shown in Figure 3. These systems possess 3 or 4 protons directly attached to the ^{15}N nucleus, and intramolecular dipolar interactions are assumed to be the dominant mode of relaxation. Approximate values for τ_o can be calculated from E_A and eq 7 by assuming the theoretical value for τ_c at the minimum T_1 of a given system. At Larmor frequencies of 50.684 and 500.135 MHz for ω_N and

(10) Bloembergen, N.; Purcell, E. M.; Pound, R. V. *Phys. Rev.* **1948**, *73*, 679.

(11) McClung, R. E. D.; Kivelson, D. *J. Chem. Phys.* **1968**, *49*, 3380 and references therein.

(12) Deslauriers, R.; Smith, I. C. P. In *Biological Magnetic Resonance*; Berliner, L. J., Reuben, J., Eds.; Plenum Press: New York, 1980; Vol. 2, pp 243−344.

(13) Bond length from neutron-diffraction studies: Keiter, E. A. Ph.D. Thesis, University of Illinois, 1986.
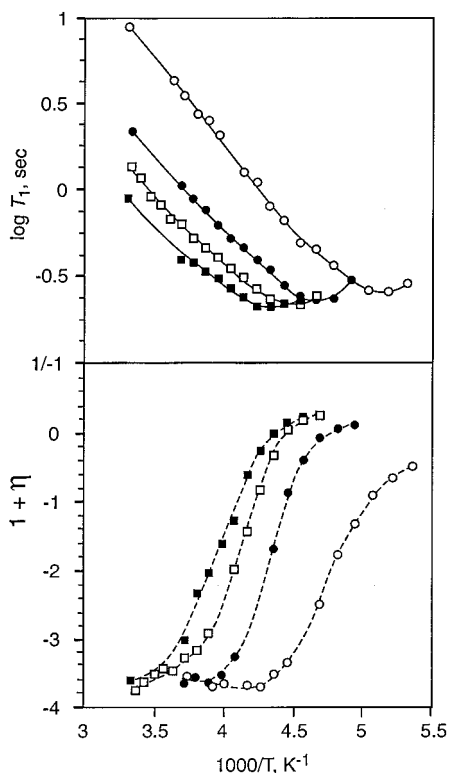
Figure 3. T_1 relaxation data for NH_4Cl (O), benzylamine-H^+ (●), alanine-H^+ (□), and glutamine-H^+ (■) as a function of temperature. The corresponding NOE data as a function of temperature use the same symbols, but with dashed lines and with the scale on the lower half of the graph.

ω_H, respectively, and an assumed r_{NH} length of 1.02 Å,[13] τ_c obtained from eq 3 is approximately 3×10^{-9} s. There is a general trend for higher molecular weight molecules to have longer τ_o times, consistent with their slower tumbling rates. More precise methods for determining τ_o have been developed,[14] but require the accurate measurement of multiple physical parameters, whereas the estimates presented here are sufficient for demonstrating correlations to a first approximation.

The curves generated by the experimental NOE data of the ammonium species are in accord with the theoretical NOE curve of ^{15}N nuclei experiencing intramolecular dipolar relaxation, and the NOE values are roughly consistent with the molecular correlation times defined by their respective τ_o values. The baseline NOE values of the spherically symmetrical NH_4^+ cation and the benzylammonium cation ($1 + \eta = -3.5$) are somewhat less than the theoretical maximum at higher temperatures, which suggests a small contribution from spin-rotation relaxation.[15] Unlike dipole-dipole dependent processes, relaxation rates arising from spin rotation are inversely proportional to $\omega\tau_c$,[16] and attenuate quickly with increasing viscosity and molecular size. We therefore expect this mechanism to be negligible at lower temperatures. Conversely, the NOE values of the alkylammonium salts, and of NH_4Cl in particular, are somewhat greater than theoretically expected at lower temperatures; one explanation for this is an efficient intermolecular dipolar mechanism (*vide infra*).

Amides. The ^{15}N T_1 and NOE values of urea, the terminal amide of glutamine, and pyroglutamic acid were also measured and are plotted as functions of temperature (Figure 4). Like
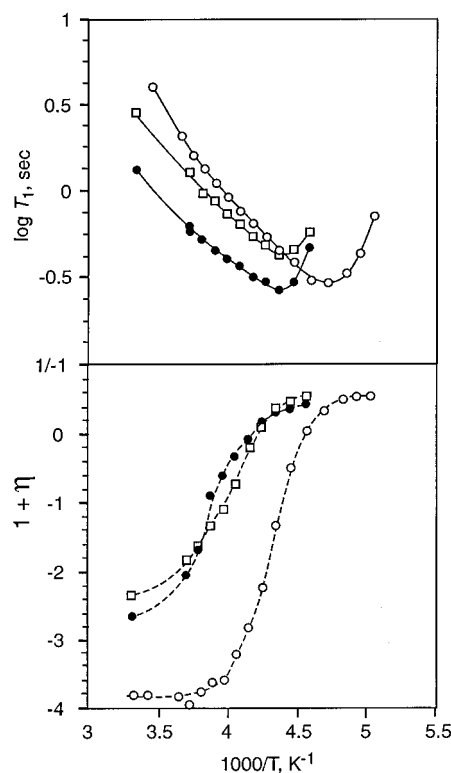
(14) Farrar, T. C.; Jablonsky, M. J. *J. Phys. Chem.* **1991**, *95*, 9159.

(15) Burke, T. E.; Chan, S. I. *J. Magn. Reson.* **1970**, *2*, 120.

(16) Spin rotation is more significant at lower frequencies (4−6 MHz) for $^{15}NH_4Cl$, as well as some other ^{15}N-containing molecules:[4] Lichter, R. L.; Roberts, J. D. *J. Am. Chem. Soc.* **1971**, *93*, 3200.
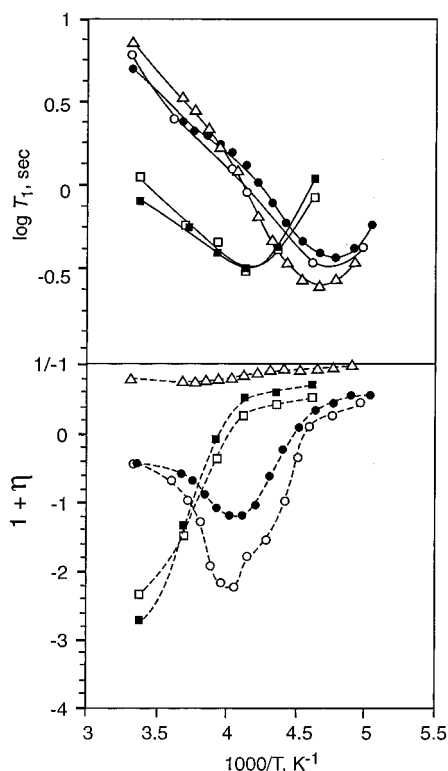

Figure 4. T_1 relaxation data for urea (O), the terminal amide group of glutamine-H^+ (●), and pyroglutamic acid (□) as a function of temperature. The corresponding NOE data as a function of temperature use the same symbols but with dashed lines.

the alkylammonium ions, intramolecular dipolar interactions are expected to be the major relaxation mechanism through isotropic tumbling. However, the shielding anisotropy about the nitrogen nucleus may potentially influence T_1 relaxation via CSA as described by eq 8,

$$\frac{1}{T_1(CSA)} = \frac{2}{15}\omega_N^2(\sigma_\| - \sigma_\perp)^2\left(\frac{\tau_c}{1 + \omega_N^2\tau_c^2}\right) \qquad (8)$$

where $\sigma_\|$ and $\sigma_\perp$ are the parallel and perpendicular components of the axially symmetric ^{15}N chemical shielding tensor.[6] It has been shown by solid-state NMR spectroscopy that a significant axially symmetric CSA exists for the secondary amide nitrogens in peptide bonds.[17] This has been used to approximate the ^{15}N CSA for proteins in solution; at 50.7 MHz, its relative contribution toward ^{15}N T_1 relaxation rates was estimated to be as high as 18%, in which slow isotropic tumbling ($\tau_c \gg \omega_N^{-1}$) was assumed.[2a]

The effect of CSA on the T_1 curve is expected to be similar to that of dipolar interactions, and essentially linear relationships are again observed between $\log(1/T_1)$ of the amides and $1/T$ at higher temperatures.[18] Values for E_A and τ_o were calculated and found to be within the same order of magnitude as those of the ammonium species, with a similar trend with respect to size (Table 1). The NOE curve is more sensitive to differences in relaxation mechanisms, as can be seen by comparison of the behavior of the three amide samples. The efficiency of the ^{15}N NOE in urea at higher temperatures is nearly at the theoretical

(17) Hiyama, Y.; Niu, C.-H.; Silverton, J. V.; Bavoso, A.; Torchia, D. A. *J. Am. Chem. Soc.* **1988**, *110*, 2378.

(18) Others have suggested that the T_1 relaxation of systems which experience significant CSA may not be well defined by a unique time constant, because of cross-correlation effects.[7] However, the small uncertainties accompanying our T_1 measurements seem consistent with a single rate constant.

Figure 5. T_1 relaxation data for pyridine ($\triangle$), pyridine-H$^+$ at 50 MHz ($\bullet$) and 30 MHz ($\bigcirc$), and the N1 ($\blacksquare$) and N3 ($\square$) nitrogens of histidine-H$^+$ as a function of temperature. The corresponding NOE data as a function of temperature use the same symbols, but with dashed lines.

maximum, indicating that its T_1 relaxation is dominated by dipole–dipole mechanisms. The magnitudes of the NOEs of pyroglutamate and glutamine in this temperature range are less, and suggest significant contributions of CSA to T_1 relaxation. Although the higher molecular weights of these compounds lead to longer τ_c values, this cannot be the major factor in NOE reduction because the NOE of the ammonium moiety of glutamine (Figure 3) is considerably larger than those of the corresponding amides at higher temperatures. Also, the number of attached protons is not directly responsible for NOE loss; the amides of glutamine and pyroglutamic acid possess nearly identical ^{15}N NOE profiles, despite the fact that one is primary and the other is secondary.

Heterocycles. CSA is expected to play a greater role in the T_1 relaxation of heterocycles. The effects of temperature and viscosity on the T_1 and NOE values for pyridine, pyridinium trifluoroacetate, and the heterocyclic nitrogens of histidine hydrochloride are shown in Figure 5. Pyridinium trifluoroacetate was investigated at two different frequencies (50.684 and 30.424 MHz) for dependency of its CSA on magnetic field strength.

The negligible influence of temperature on the ^{15}N NOE of pyridine indicates the virtual absence of ^{15}N–^{1}H dipole–dipole interactions so that its T_1 relaxation is dominated by non-dipolar mechanisms. The E_A for molecular reorientation, 5.4 kcal/mol, is relatively high compared to the other substrates. Assuming exclusive CSA relaxation and a Larmor frequency of 50.7 MHz for ω_N, the effective correlation time constant τ_e at minimal T_1(CSA) from eq 8 is also approximately 3×10^{-9} s, resulting in a rather short τ_o (Table 1). Although contributions from spin rotation have been suggested for ^{15}N in neat pyridine,[4c] its role in relaxation is likely to be strongly attenuated in the considerable viscosity of the DMSO–water solvent, as was mentioned above for ^{15}NH$_4$Cl. Furthermore, the parabola-shaped ^{15}N T_1
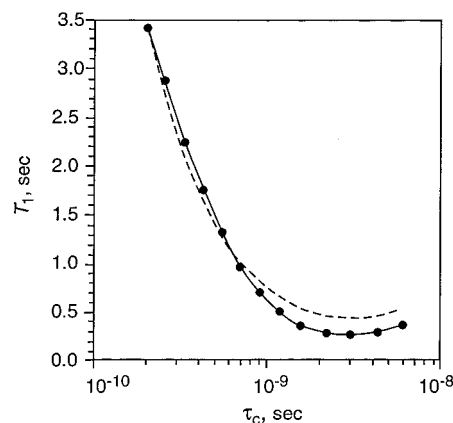

Figure 6. Determination of the shielding anisotropy of ^{15}N-pyridine in 8.8 M DMSO at 50.684 MHz: ($\bullet$) experimental T_1 data as a function of τ_c using eq 7; (---) best fit to Lorentzian line shapes in accord with eq 8.

curve of pyridine in 8.8 M DMSO is substantially different than that found for neat pyridine.

The assumption of CSA as the dominant ^{15}N relaxation mechanism for pyridine in DMSO–water allows calculation of the shielding anisotropy as a single term $\Delta\sigma = \sigma_{\parallel} - \sigma_{\perp}$ by lineshape analysis of the T_1 relaxation data. With the aid of a molecular correlation time scale derived from E_A and τ_o and a regressive line-fitting algorithm (KaleidaGraph), we estimate a $\Delta\sigma$ of 325 ppm (Figure 6). This is smaller than the earlier estimates of 672 and 987 ppm for neat pyridine,[4c,19] but it is fairly close to the theoretical $\Delta\sigma$ of 276 ppm.[20] It is unclear whether discrepancies arise from the theoretical premises and/or differences in substrate environment, in which hydrogen bonding could be an important factor for DMSO–water.[21] Supportive evidence for the latter is provided by a ^{15}N chemical-shift difference of 18.5 ppm between neat and aqueous pyridine.[23a] Other reasons for suggesting the influence of hydrogen bonding are discussed below.

Protonation of pyridine introduces a competing dipolar relaxation pathway for the ^{15}N nucleus. Dipole–dipole interactions surpass CSA in relaxation efficiency as temperature decreases, resulting in an unusual dip in the lineshape of the NOE vs temperature plot of pyridinium trifluoroacetate (Figure 5). At 50.7 MHz, the NOE enhancement factor $1 + \eta$ reaches a maximum of -1.23, or 45% efficiency, at 241 K. In comparison, $1 + \eta$ for urea at the same temperature (and viscosity, presumably) is -2.80, or 77% efficiency. Relaxation of the pyridinium ^{15}N nucleus by CSA becomes even less effective at 30.4 MHz, the maximum signal enhancement being -2.28 at 245 K.

The non-sigmoidal character of the NOE lineshapes of the pyridinium ion is a strong indication that the CSA and intramolecular dipolar mechanisms are operating on different

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time scales. The rotational motion of many axially symmetric aromatic structures are known to be somewhat anisotropic;[22] this would belie the necessary assumption of isotropic tumbling in solution for approximating a single correlation time τ_c.

Surprisingly, the overall ^{15}N T_1 relaxation rate for the pyridinium ion at 50.7 MHz is the same or slightly slower than that for pyridine itself, in spite of the assistance of the dipolar relaxation pathways. This is consistent with a loss in chemical-shift anisotropy for the pyridinium ion. Indeed, the ^{15}N chemical shift of pyridine in 8.8 M DMSO changes from 78.1 ppm at 300 K (upfield from neat $CH_3^{15}NO_2$) to 176 ppm upon protonation.[23] This behavior further supports the influential role of hydrogen bonding on the CSA of pyridine in DMSO−water.

The NOE and T_1 relaxation data for histidine monohydrochloride in 8.8 M DMSO at 50.7 MHz indicate that relaxation associated with CSA is attenuated to such a degree that dipole−dipole interactions provide the dominant relaxation mechanism. The NOE and T_1 plots of both nitrogen nuclei in histidine closely resemble those of the amide nitrogen in glutamine. The small contribution of CSA relaxation in histidine most likely arises from the presence of the directly attached protons on the ring nitrogens, but also reflects the reduction of anisotropy within the imidazole ring system relative to that of pyridine.

Interestingly, the ^{15}N NOE enhancements of both heterocyclic nitrogens are greatly attenuated when measured at 30.4 MHz $(1 + \eta(N1), 1 + \eta(N3) = -0.35, -1.18$ at 295 K), and are complemented by shorter T_1 times as well $(T_1(N1), T_1(N3) = 0.64, 0.78$ s). Competing exchange-modulated relaxation is one possible explanation for this change, because the imidazolium protons are known to exchange rapidly with the solvent faster than the millisecond time scale.[24] In this regard, Irving and Lapidot have convincingly demonstrated the effect of (and difficulty in removing) trace paramagnetic impurities on the ^{15}N NOE,[25] but their data suggest that the magnitudes of these effects correspond with the rate of proton exchange. Although it has been argued that $^{15}N-^1H$ scalar relaxation is an inefficient T_1 mechanism,[25,26] the notable dependency of the ^{15}N NOEs of histidine on frequency implicates chemical exchange as an important factor.[27] Further studies are necessary to determine the exact nature of this relationship.

Inter- vs Intramolecular Dipole−Dipole Relaxation. Intermolecular dipolar relaxation is dependent on diffusion-mediated (translational) motions and can be mathematically expressed by an equation similar to eq 3, with the intermolecular $^{15}N-^1H$ distances being time dependent and the variable τ_c replaced by τ_t, the correlation time for molecular diffusion in solution. Whereas τ_c is described by the Debye expression (eq 1), τ_t is described by the closely related Stokes-Einstein formula (eq 9), in which τ_t is the time required for a molecule to move a distance of $2a$.[12]

$$\tau_t = \frac{2\pi a^3 \eta_s}{kT} \qquad (9)$$

Despite their similar dependencies on viscosity and temperature, τ_c and τ_t are independent variables and may have disparate values. It is often difficult (if not impossible) to distinguish τ_c- and τ_t-dependent processes from one another. However, we observe in several cases evidence for a significant intermolecular dipolar relaxation component operating on a different time scale.

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(27) The ^{15}N NOEs of histidine have also been measured in an acidic aqueous solution at 9.12 MHz, and were observed to be at maximum intensity.[24]
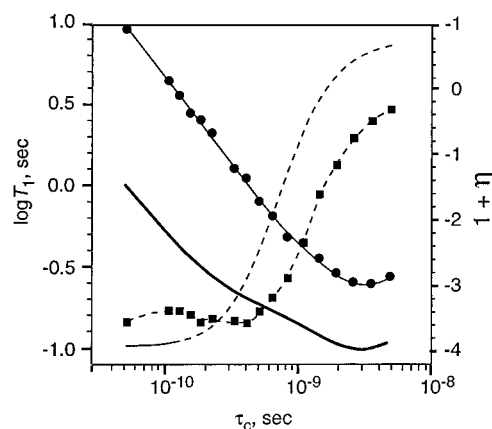

Figure 7. Experimental NOE (■) and T_1 (●) data for $^{15}NH_4Cl$ in 8.8 M DMSO at 50.684 MHz plotted as a function of τ_c using eq 6. The theoretical NOE (---) and the theoretical T_1 (▬) are shown on the assumption of solely intramolecular dipolar relaxation.

Our observation is that the NOE efficiency is sometimes significantly *greater* than predicted for purely intramolecular dipolar relaxation. The most pronounced case for which the NOE is sustained is NH_4Cl; the NOE enhancement is clearly still negative even as T_1 begins to increase (Figure 7). The NOEs of benzylamine-H^+, glutamine-H^+, and urea show similar behavior, but to a lesser extent. An intermolecular contribution to dipolar relaxation could explain the larger-than-theoretical T_1 relaxation times if τ_t is substantially faster than τ_c.

Other forms of relaxation are unlikely to be responsible for the additional NOE enhancement, as most of these should increase single-quantum transition probability, thus diminishing the NOE. Scalar relaxation arising from rapid chemical exchange is known to affect the NOE of systems with nuclei of comparable Larmor frequencies, but it would also reduce the NOE by increasing zero-quantum transitions.[28]

Alternatively, the sustained ^{15}N NOE of NH_4Cl may be explained by rapid librational averaging, not adequately described by either rotational or translational motion. This mechanism increases the probability of double-quantum transitions which give rise to the NOE. The ammonium nucleus is completely rigid and spherical, but its small size relative to solvent could give rise to fluctional behavior very different from that expected for a classical sphere tumbling isotropically in solution. It has been shown by molecular-dynamics calculations that local motional averaging of amino acid residues in proteins on the picosecond time scale can substantially increase their ^{13}C T_1 values.[29]

Our ^{15}N NMR studies suggest that in highly viscous solutions, even simple molecules are capable of complex motional behavior not readily explained by the present levels of theory for estimating the intermolecular contribution toward their ^{15}N dipole−dipole relaxations.

(28) In addition, effects due to rapid proton exchange could be ruled out by the acidification of the sample with CF_3COOH; the ^{15}N NMR spectrum then showed a clear quintet.

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