JOURNAL OF MAGNETIC RESONANCE 97, 359–375 (1992)

Pulse Sequences for Removal of the Effects of Cross Correlation between Dipolar and Chemical-Shift Anisotropy Relaxation Mechanisms on the Measurement of Heteronuclear T_1 and T_2 Values in Proteins

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Received July 18, 1991; revised September 10, 1991

The effects of cross correlation between dipolar and chemical-shift anisotropy relaxation interactions on the measurement of heteroatom T_1 and T_2 relaxation times in proteins is considered. It is shown that such effects can produce errors of approximately 25% in the measurement of ^{15}N transverse relaxation times at a field strength of 11.8 T. Cross correlation has a less significant effect on the measurement of ^{15}N spin–lattice relaxation rates and for proteins the errors in T_1 decrease as a function of increasing molecular weight. Nevertheless, for T_1 measurements at 11.8 T errors of approximately 15 and 5% are calculated for proteins with correlation times, τ_c, of 5 and 9 ns, respectively. Pulse sequences which eliminate dipolar and chemical-shift anisotropy cross-correlation effects are described. These sequences are used to make more accurate measurements of ^{15}N T_1 and T_2 values of staphylococcal nuclease and to determine errors in these parameters that result when cross correlations are present.

NMR relaxation experiments can, in principle, provide a detailed description of protein dynamics. This is most often accomplished using T_1, T_2, and NOE experiments which map out the spectral density functions describing such motions at a number of frequencies (*1–6*). Recently ^1H-detected sensitive one- and two-dimensional heteronuclear NMR experiments have been described for measuring the relaxation properties of insensitive nuclei such as ^{15}N and ^{13}C (*4, 7–10*). This advance, coupled with the availability of nearly complete heteroatom assignments of spectra of uniformly ^{15}N- and ^{13}C-labeled proteins as a result of advances in multidimensional NMR techniques (*11–14*), provides access to a large number of probes of dynamics throughout the entire molecule under investigation. It has thus been possible to study backbone dynamics on a per residue basis in the proteins staphylococcal nuclease (*4*) (SNase, MW 17.5 kDa) and interleukin-1β (*5*) (IL-1β, MW 17.5 kDa).

The simplest and most common approach for the analysis of heteroatom relaxation rates is to assume that the interactions governing relaxation are uncoupled and hence

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0022-2364/92 $3.00

can be treated in an independent manner. This was the approach taken in the analysis of the ^{15}N relaxation data for SNase (*4*) and IL1-β (*5*) where the effects of cross correlation between the dipolar and ^{15}N chemical-shift anisotropy (CSA) relaxation mechanisms were neglected. In this way, for example, the relaxation of a backbone ^{15}N spin in a protein is the sum of contributions from dipolar interactions involving the directly bonded NH proton as well as the ^{15}N CSA term while the relaxation of a carbon spin in an AX_3 spin system is the sum of each $^1H-^{13}C$ dipolar contribution as well as the ^{13}C CSA component. However, studies by the Volds (*15*) and Werbelow and Grant (*16*) have shown that such cross-correlation effects can be significant. Recently we showed that for ^{13}C transverse relaxation in a methyl group attached to a molecule tumbling in the limit $\omega\tau_c \gg 1$, where τ_c is the overall correlation time, the transverse magnetization associated with the outer components of the ^{13}C quartet could decay at a rate of up to nine times faster than the inner components due to the effects of cross correlation between the three $^1H-^{13}C$ dipole vectors (*17*). This translates into an error of as much as a factor of three in measured ^{13}C T_2 values if such effects are ignored. A recent paper by Boyd *et al.* (*18*) has shown that dipolar and CSA cross correlation can significantly affect the recovery of ^{15}N longitudinal magnetization for the protein hEGF (MW 6.5 kDa). This result has stimulated us to consider the effects of cross correlation between dipolar and CSA interactions on previously measured heteronuclear T_1 and T_2 relaxation rates in SNase and evaluate the errors that can be introduced through the neglect of such effects. We show that measurements of ^{15}N T_1 values in larger proteins such as SNase are not strongly affected by dipolar/CSA cross correlations because the latter are effectively attenuated by the rapid spin flips of the NH protons. We further show that T_2 measurements are significantly affected by dipolar/CSA cross correlation even in larger proteins. Pulse schemes that eliminate dipolar/CSA cross correlations in T_1 and T_2 measurements are presented and their utility is demonstrated on a sample of uniformly ^{15}N-labeled SNase complexed with pdTp and Ca^{2+}.

THEORY

Recently Goldman has presented rigorous expressions for the transverse relaxation of a heterospin, A, in an AX spin system assuming that the relaxation of spin A is governed by both dipolar and CSA relaxation mechanisms (*19*). In the limit that $\omega_A\tau_c \gg 1$ and assuming further that the unique axis of the CSA tensor and the AX bond vector are colinear, Goldman showed that the decay of the transverse multiplet components of spin A is given by

$$\frac{d}{dt}A_{tr}^{(1)} = -(\lambda + \eta)A_{tr}^{(1)}$$

$$\frac{d}{dt}A_{tr}^{(2)} = -(\lambda - \eta)A_{tr}^{(2)}, \qquad [1]$$

where $\lambda = 0.2\tau_c\{\gamma_A\gamma_X h/(2\pi r^3)\}^2 + \frac{4}{45}\tau_c\{\omega_A(\sigma_{\parallel} - \sigma_{\perp})\}^2$ and $\eta = -\frac{4}{15}\tau_c\gamma_A\gamma_X h/(2\pi r^3)\omega_A(\sigma_{\parallel} - \sigma_{\perp})$. In Eq. [1], $A_{tr}^{(j)}$ is the A transverse magnetization associated with multiplet component j, γ_i is the magnetogyric ratio of spin i, h is Planck's constant,

r is the distance between spins A and X, $\sigma_{\|}$ and $\sigma_{\perp}$ are the principal components of the axially symmetric chemical-shift tensor, τ_c is the overall correlation time describing the isotropic tumbling of the molecule, and ω_A is the angular frequency of spin A. For the case of NH–^{15}N spin systems in proteins with a value of $(\sigma_{\|} - \sigma_{\perp}) = -160$ ppm (*20*), $r = 1.02$ Å and $\omega_A = 50.68$ MHz (11.8 T field), and where the principal axis of the ^{15}N chemical-shift tensor and the NH–^{15}N bond is parallel (note that the angle between the ^{15}N chemical-shift tensor and the NH–^{15}N bond is typically between 0° and 30° in proteins (*20*)), Eq. [1] indicates that the decay rates of the individual multiplet components are very different with a T_2 ratio of 7.75! The decay of net transverse A magnetization,

$$A_{tr}(t) = A_{tr}^{(1)}(t) + A_{tr}^{(2)}(t) = 0.5A_{tr}(0)\{\exp[-(\lambda + \eta)\tau] + \exp[-(\lambda - \eta)\tau]\}, \quad [2]$$

is thus predicted to be biexponential in the limit of an isolated two-spin pair with $(\lambda + \eta)/(\lambda - \eta) \sim 8$, independent of the value of τ_c. Clearly this large of an effect can result in a serious overestimation in measured T_2 values if not properly accounted for in the interpretation of the relaxation data or adequately suppressed.

The longitudinal relaxation of the A multiplet components in an AX spin system is given by

$$\frac{d}{dt}A_z^{(1)} \sim -(\lambda' + \eta')A_z^{(1)}$$

$$\frac{d}{dt}A_z^{(2)} \sim -(\lambda' - \eta')A_z^{(2)}, \quad [3]$$

where $A_z^{(1)}$ and $A_z^{(2)}$ refer to the z magnetization associated with the two multiplet components, $\lambda' = [\tau_c/\{1 + (\omega_A\tau_c)^2\}][0.3\{\gamma_A\gamma_X h/(2\pi r^3)\}^2 + \frac{2}{15}\{\omega_A(\sigma_{\|} - \sigma_{\perp})\}^2]$, $\eta' = [-\tau_c/\{1 + (\omega_A\tau_c)^2\}][0.4\gamma_A\gamma_X h/(2\pi r^3)]\omega_A(\sigma_{\|} - \sigma_{\perp})$. Equation [3] assumes that $J(\omega_A) \gg J(\omega_X), J(\omega_A \pm \omega_X)$, where $J(\omega) = \tau_c/\{1 + (\omega\tau_c)^2\}$. In this limit, the longitudinal relaxation of A magnetization is biexponential and the ratio of ^{15}N longitudinal relaxation rates for the multiplet components again is 7.75 (11.8 T).

An intuitive understanding of Eqs. [1] and [3] describing the effects of dipolar/CSA cross correlation on measured relaxation rates is easily obtained by considering simple local field arguments in the limit when $\omega\tau_c \gg 1$. Consider the two-spin system that Goldman examined and let us focus on transverse relaxation. Figure 1 shows an energy-level diagram for an AX two-spin system with direct product basis set eigenfunctions and with the eigenvalues of each state i indicated by E_i. Thus the state $\alpha\beta$ corresponds to spins A and X having spin states α and β, respectively. The two multiplet components associated with transverse A magnetization are given by transitions connecting states $\alpha\beta$, $\beta\beta$ and states $\alpha\alpha$, $\beta\alpha$. The Hamiltonian, $\mathcal{H}$, that will contribute to relaxation is given by

$$\mathcal{H} = \mathcal{H}_D + \mathcal{H}_{CSA},$$

where

$$\mathcal{H}_D = -[\gamma_A\gamma_X h/(2\pi r^3)](3\cos^2\theta - 1)\mathcal{A}_z\mathcal{X}_z \quad [4]$$

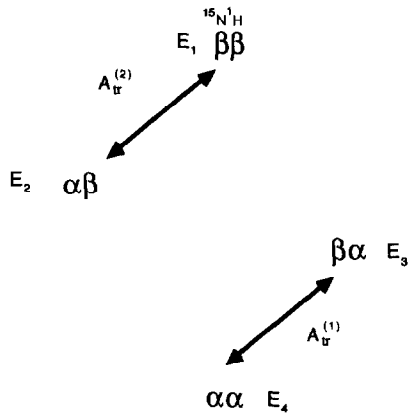

FIG. 1. Energy-level diagram for an AX (A = ^{15}N, X = ^{1}H) two-spin system with direct-product basis-set eigenfunctions and with eigenvalues of each state i indicated by E_i. The two multiplet components associated with transverse A magnetization connecting states $\alpha\beta$, $\beta\beta$ and states $\alpha\alpha$, $\beta\alpha$ are indicated by arrows. $A_{tr}^{(j)}$ is the transverse A magnetization associated with multiplet component j. Using the product-operator description (*30*), $A_{tr}^{(1)} = 0.5(A_{tr} + 2A_{tr}X_z)$, $A_{tr}^{(2)} = 0.5(A_{tr} - 2A_{tr}X_z)$.

and

$$\mathcal{H}_{CSA} = \tfrac{1}{3}\omega_A(\sigma_{||} - \sigma_\perp)(3\cos^2\theta - 1)\mathcal{A}_z,$$

where $\mathcal{A}_z$ and $\mathcal{X}_z$ are the spin operators corresponding to the z components of angular momentum of spins A and X, θ describes the orientation of the A–X dipole vector and the axially symmetric chemical-shift tensor (assumed colinear) with respect to the external magnetic field, and all other symbols are defined previously. This model is valid for describing the transverse relaxation of a ^{15}N or ^{13}C spin which is dipolar coupled to only one ^{1}H spin in the protein. The effect of $\mathcal{H}$ on the energies of the spin states indicated in Fig. 1 is to change E_i by the amounts

$$\begin{aligned} \Delta E_1&: \quad d/4 - c/2 \\ \Delta E_2&: \quad -d/4 + c/2 \\ \Delta E_3&: \quad -d/4 - c/2 \\ \Delta E_4&: \quad d/4 + c/2, \end{aligned} \qquad [5]$$

where $d = -[\gamma_A\gamma_X h/(2\pi r^3)](3\cos^2\theta - 1)$ and $c = \frac{1}{3}\omega_A(\sigma_{||} - \sigma_\perp)(3\cos^2\theta - 1)$. Since the contributions to the transverse relaxation rate of the multiplet components corresponding to the transitions connecting $\alpha\beta$ and $\beta\beta(1/T_{2(1,2)})$ and connecting $\alpha\alpha$ and $\beta\alpha(1/T_{2(3,4)})$ are proportional to $\overline{(\Delta E_1 - \Delta E_2)^2}$ and $\overline{(\Delta E_3 - \Delta E_4)^2}$, respectively, we can write

$$T_{2(3,4)}/T_{2(1,2)} = \overline{(d/2 - c)^2}/\overline{(d/2 + c)^2}, \qquad [6]$$

where the bar denotes the appropriate average over all space. Equation [6] simplifies to $T_{2(3,4)}/T_{2(1,2)} = (\lambda - \eta)/(\lambda + \eta)$ as indicated by Eq. [1] and described in detail by

Goldman (*19*). Thus, the interference between dipolar and CSA interactions results in a situation in which the individual multiplet components of a two-spin system relax with different transverse relaxation rates. In general, this is the case for more complex spin systems, and as indicated by Eq. [3] a similar situation occurs for longitudinal relaxation as well.

EXPERIMENTAL

For all experiments described in this paper a sample of 1.5 m*M* SNase uniformly ^{15}N-labeled and complexed with pdTp and Ca^{2+}, pH 6.4, was employed. Spectra were recorded on a Bruker AM500 spectrometer at 308 K. All data sets were recorded as 256×512 real matrices with 32 scans per t_1 point. The data sets were processed with software provided by New Methods Research (Syracuse, New York). Cross-peak volumes were obtained from surface-fitting routines provided in the software package. T_1 and T_2 values were extracted in a straightforward fashion by measuring the volumes of cross peaks in 2D maps as a function of relaxation delay, T, and fitting the volumes to an equation of the form $y = A \exp(-T/T_i) \{i = 1, 2\}$ using conjugate gradient minimization techniques. Precision limits of the extracted parameters, A and T_i, were obtained by a Monte Carlo approach described by Kamath and Shriver (*21*).

Figures 2a and 2b illustrate the pulse schemes that are employed to measure heterospin T_1 and T_2 values. The sequences are very similar to previously published methods (*4*) with the exception that during the T period ^{1}H 180° pulses are applied every 5–10 ms in order to interchange multiplet components and hence effectively suppress the effects of cross correlation between dipolar and CSA relaxation mechanisms. This approach is discussed in detail later in the text. Figure 2b (i) shows our previous CPMG (*22, 23*) scheme for measuring transverse relaxation times. Note that the single ^{1}H 180° pulse applied in the center of T helps suppress the intense water signal and also serves to considerably reduce the influence of dipolar and CSA cross-correlation effects. However, the sequence of Fig. 2b (ii) is preferred since cross-correlation effects are eliminated completely.

Multiplet component transverse relaxation rates were measured both with and without suppression of the dipolar/CSA cross-correlation contribution. In order to obtain ^{15}N transverse relaxation rates of individual multiplet components with the elimination of dipolar/CSA cross correlation the sequence of Fig. 2b (ii) was employed with the exception that the ^{1}H 180° pulse applied in the center of the t_1 period was removed. In addition, after the t_1 period magnetization was immediately transferred back to protons for detection by simultaneous application of ^{1}H and X 90° pulses (i.e., the 2τ period immediately following the t_1 interval was removed from the sequence). In this way the function describing the transfer of magnetization from X to ^{1}H is of the form $\sin(\pi J t_1)$ for a two-spin system and multiplet components are antiphase absorptive in F_1. ^{15}N transverse relaxation rates of multiplet components showing the effects of dipolar/CSA cross correlation were obtained using the scheme of Fig. 2b (ii) with the exception that ^{1}H 180° pulses were not applied during the CPMG portion of the sequence nor during the t_1 period. In order to achieve sufficient

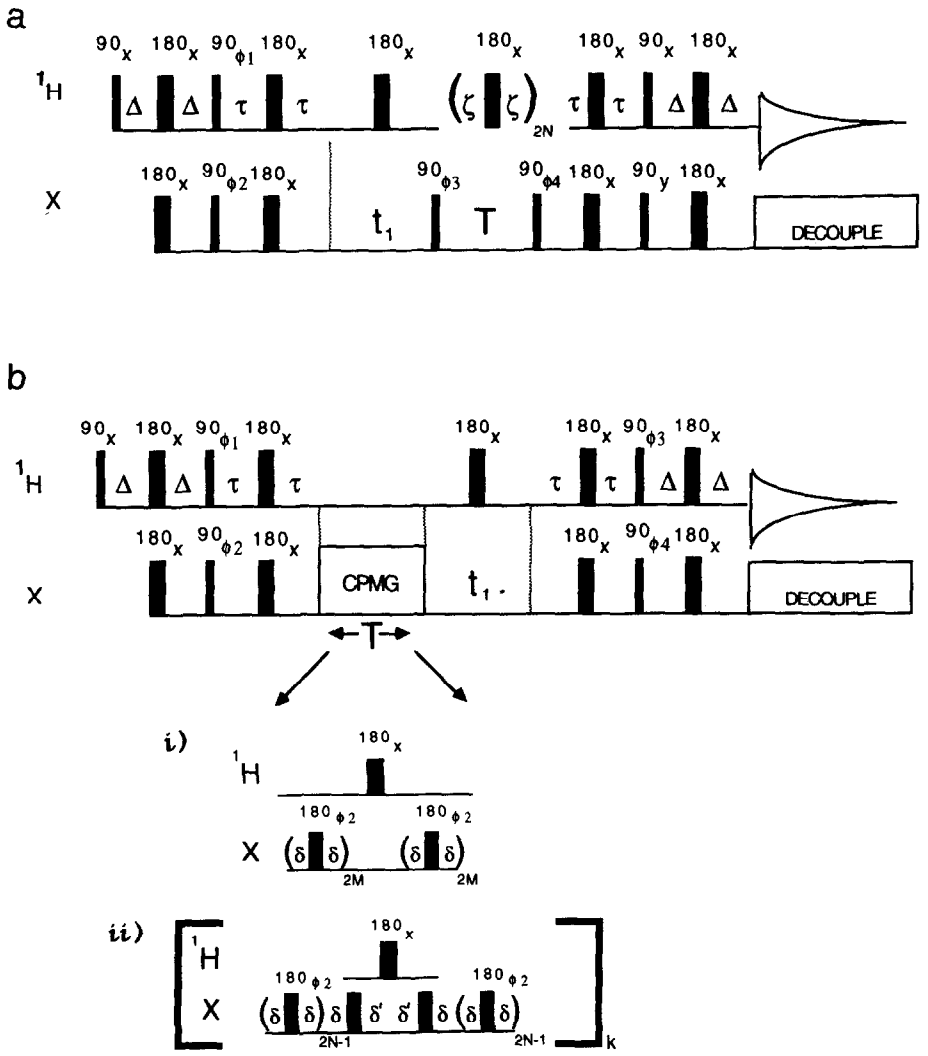

FIG. 2. Pulse sequences for measuring heteroatom T_1 (a) and T_2 relaxation times (b). Effective water suppression (if X = ^{15}N) is achieved with the use of an off-resonance DANTE sequence (*31, 32*). The value of Δ is set to slightly less than $1/(4J)$, where J is the ^{1}H–X coupling constant (2.3 ms for X = ^{15}N) to minimize relaxation losses. Because the transverse relaxation times for backbone ^{15}N spins are reasonably long, the value of τ is set to $1/(4J)$. In a, ^{1}H 180° pulses are applied every 5–10 ms (at a rate much greater than the decay rate of the fastest relaxing multiplet component) in order to suppress the effects of ^{1}H–^{15}N dipolar/CSA cross correlation. The phase cycling employed is $\phi_1 = 8(y), 8(-y)$; $\phi_2 = 4(x), 4(-x)$; $\phi_3 = -y, y$; $\phi_4 = 2(x), 2(-x)$; Acq = $x, 2(-x), x, -x, 2(x), -x$. The receiver phase is inverted every eight scans. A composite 180° pulse ($90^\circ_x 180^\circ_y 90^\circ_x$) is applied in the center of the t_1 evolution period. Quadrature in F_1 is achieved by TPPI (*33*) of ϕ_3. In b, the pulse scheme for measuring heteroatom T_2 values published previously (i) and the new sequence which completely suppresses the effects of ^{1}H–^{15}N dipolar/^{15}N CSA cross correlation (ii) are indicated. The modified CPMG sequence (ii) consists of applying ^{1}H 180° pulses every 5–10 ms at the peak of the spin echo in order to invert the ^{1}H spin state and hence average the relaxation rates of the multiplet components. Note that ^{1}H 180° pulses are positioned such that ^{15}N magnetization spends an equal amount of time associated with each of the multiplet components. The delay δ' is chosen such that $2\delta' + \delta_{H180°} = 2\delta$ ($\delta_{H180°}$ = ^{1}H 180° pulse width) with the value of δ set $<1/(2J)$ so that

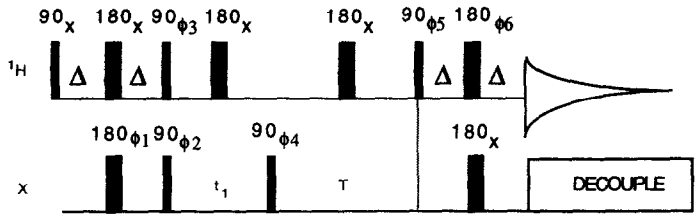

FIG. 3. Scheme for the measurement of T_{1zz}. For the case in which spectra are recorded in water (i.e., where X = ^{15}N), effective water suppression is achieved with the use of an off-resonance DANTE sequence described previously (*32*). In addition, a 180° ^{1}H pulse is applied in the center of the relaxation period, T, so that any water magnetization that relaxes along z is inverted. In this way at the end of T very little net water magnetization is along the z axis. The value of Δ is set to 2.3 ms. The phase cycling employed is $\phi_1 = 4(x), 4(-x)$; $\phi_2 = x, -x$; $\phi_3 = 2(y), 2(-y)$; $\phi_4 = 16(x), 16(-x)$; $\phi_5 = 8(x), 8(-x)$; $\phi_6 = 4(x), 4(y)$; Acq = $x, 2(-x), x, -x, 2(x), 2(-x), 2(x), -x, x, 2(-x), x$. The phase of the receiver is inverted every 16 scans. A composite 180° pulse is applied at the center of the t_1 evolution period. Quadrature in F_1 is achieved by TPPI of ϕ_2.

water suppression in these experiments it was necessary to saturate the water resonance for ~10 ms (12.5 kHz ^{1}H RF field) after the final X 90° pulse ($90^\circ_{\phi 4}$) and before the final ^{1}H 90° ($90^\circ_{\phi 3}$) pulse. Five 2D data sets with T values of 8, 42, 67, 92, and 125 ms were obtained in order to measure multiplet component relaxation rates.

In order to measure ^{15}N T_2 values of as many residues as possible, spectra were recorded with the application of a ^{1}H 180° pulse in the middle of the t_1 evolution period. ^{15}N T_2 values were measured both with and without suppression of the dipolar/CSA cross-correlation contribution using the sequences of Fig. 2b and recording six 2D data sets with T = 8, 25, 42, 92, 109, and 143 ms. Cross-correlation effects between the dipolar and CSA relaxation mechanisms were eliminated by application of ^{1}H 180° pulses every 8 ms (Fig. 2b (ii)). The delay between successive ^{15}N 180° pulses applied during the CPMG sequence was set to 900 μs (δ = 450 μs). ^{15}N T_1 values were measured with (Fig. 2a with ζ = 5 ms) and without suppression of the effects of dipolar/CSA cross correlation. For the case in which cross correlation was not suppressed the sequence of Fig. 2a was employed with the exception that ^{1}H 180° pulses were not applied during the relaxation delay T. T_1 relaxation times were obtained from six 2D data sets with T values of 10, 90, 190, 330, 490, 730, and 1150 ms. T_{1zz} relaxation rates were obtained with the pulse sequence indicated in Fig. 3. A 180° ^{1}H pulse is applied in the center of T to minimize the intense signal from water magnetization. T_{1zz} relaxation times were obtained from nine 2D spectra with T delays of 2, 25, 50, 75, 100, 150, 200, 250, and 300 ms.

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antiphase X magnetization does not build up. *If the condition $\delta \ll 1/(2J)$ is not satisfied, a serious underestimate in measured T_2 values can result.* The phase cycling employed is $\phi_1 = y, -y$; $\phi_2 = 2(x), 2(-x)$; $\phi_3 = 4(x), 4(-x)$; $\phi_4 = 8(x), 8(-x)$; Acq = $x, 2(-x), x, 2(-x, x, x, -x), x, 2(-x), x$. After 16 scans, the phase of the first 180° pulse on X after the evolution period, t_1, is inverted without changing the receiver phase. This pulse and the pulse at the center of the evolution period are composite 180° pulses. Quadrature in F is achieved via a TPPI of ϕ_4.

TABLE 1

^{15}N Relaxation Times Measured for SNase

Amino acid	T_2^a (ms)	T_2^b (ms)	T_2^c (ms)	T_2^d (ms)	T_{1zz} (ms)
L7			164.3	149.9	92.0
H8			139.6	127.2	55.1
K9			113.8	106.0	58.2
E10	164.7/88.3	99.9/92.6	101.6	99.1	99.9
T13			134.5	117.5	113.8
L14	181.0/82.3	104.0/102.8	114.9	108.5	89.5
K16			114.2	107.5	86.2
A17			119.9	118.1	67.4
I18			140.2	129.5	84.8
D19	161.8/75.6	89.2/90.7	100.3	92.3	110.9
G20	147.1/98.3	104.5/90.2	123.1	111.6	63.2
T22			89.6	83.5	113.9
V23			92.9	89.5	104.4
K24			105.6	103.2	94.2
L25			117.4	105.5	83.0
M26	177.2/86.7	102.9/97.1	106.8	102.0	82.0
Y27	194.8/89.0	111.5/113.6	124.6	111.2	100.7
K28			124.5	113.1	73.3
G29	161.1/83.9	114.1/103.8	134.3	117.1	97.5
M32	194.9/90.7	115.2/111.1	102.8	97.4	83.4
T33					64.8
F34	173.2/80.5	106.3/101.2	113.8	105.4	88.6
R35	175.6/69.6	90.6/98.8	101.4	103.2	82.9
L36					62.8
L37	138.4/73.2	85.7/87.7	95.2	90.1	67.8
L38	149.5/90.3	117.4/116.4	119.6	112.8	65.3
V39	203.4/67.6	101.6/81.0	115.7	104.6	123.1
D40			101.2	100.1	83.6
T41	153.9/80.9		100.4	93.4	100.1
K45			121.1	113.8	61.1
K48			72.7	65.4	73.8
K49			134.7	122.0	84.7
G50			118.7	123.4	91.2
E52			41.6	43.8	97.1
K53			51.9	43.1	85.3
Y54	126.8/88.5	99.3/106.5	95.4	97.5	91.5
G55					88.8
A58					101.2
S59	165.0/81.3	96.3/92.6	105.7	98.1	100.8
A60			89.4	84.3	116.7
F61			96.7	90.7	101.7
K64			92.3	94.8	104.2
M65			99.3	98.4	107.9
V66			113.7	100.2	97.5
E67			92.1	87.0	97.2
N68	186.8/78.3	101.0/109.3	114.2	109.0	112.7
A69	252.2/72.5	106.2/113.0	118.0	113.2	144.2
K70	192.2/82.9	108.7/108.7	116.7	111.9	96.1
K71			108.8	104.8	116.9

TABLE 1—*Continued*

Amino acid	T_2^a (ms)	T_2^b (ms)	T_2^c (ms)	T_2^d (ms)	T_{1ZZ} (ms)
I72	200.4/84.9	113.5/103.3	118.3	108.9	109.6
E73			96.2	93.9	91.0
V74	156.4/79.0	88.2/98.4	100.2	94.5	95.0
D77	165.3/91.4	113.2/100.3	114.8	105.5	81.2
K78	140.5/80.2	98.7/98.6	107.0	101.5	98.0
G79	203.4/80.7	104.0/107.1	113.8	108.4	123.6
Q80			112.2	110.2	91.0
T82	244.5/81.2	115.1/103.4	125.4	111.2	134.7
D83			104.6	104.2	97.5
K84			101.4	99.6	43.4
Y85			108.8	99.3	101.4
G86			117.0	110.8	97.9
R87			101.0	100.2	91.4
G88					87.5
L89			97.1	98.9	82.7
A90	185.0/64.4	91.2/87.2	90.7	92.2	98.4
Y91			102.0	93.7	82.7
I92			97.4	97.6	95.7
Y93	159.9/78.1	100.0/91.4	123.0	114.9	108.4
A94			110.5	102.2	96.9
D95	166.9/59.3	105.0/109.8	109.7	103.8	90.7
G96	147.7/93.5		146.3	117.7	93.9
K97					113.2
M98			118.3	110.4	49.8
V99	159.8/73.6		116.9	107.3	73.2
N100	167.0/71.8	93.1/99.6	104.1	97.7	87.0
E101	161.8/74.1	98.6/108.9	113.7	101.7	79.4
A102					97.0
L103			98.1	94.6	89.9
V104	156.5/70.4	88.5/90.9	95.3	92.7	83.2
R105			89.3	85.2	92.0
Q106	187.3/71.3	100.3/95.6	110.0	107.6	102.4
G107	175.0/75.7	102.7/87.2	110.7	95.3	91.2
L108			104.8	97.3	88.2
K110			129.9	105.2	96.9
V111			104.3	97.9	93.0
A112	167.5/86.2	101.4/108.3	115.9	102.0	104.4
Y113	143.6/99.3	98.4/104.9	122.9	109.3	107.9
Y115	165.6/85.1	122.6/106.2	135.5	117.0	89.3
K116			123.6	113.4	51.2
N118	197.3/89.4	115.3/106.6	117.5	110.2	114.1
N119			106.4	100.2	123.0
T120	167.6/79.4	97.4/95.5	103.9	100.0	83.2
H121			120.8	118.0	93.4
E122			91.2	90.4	102.8
Q123			94.3	86.3	45.6
H124			90.9	87.9	73.3
L125			100.2	91.8	91.0
R126			100.9	91.5	93.0
S128			101.5	96.4	108.9
E129					80.2

TABLE 1—*Continued*

Amino acid	T_2^a (ms)	T_2^b (ms)	T_2^c (ms)	T_2^d (ms)	T_{1zz} (ms)
A130			92.3	85.8	113.4
Q131	174.3/66.3	88.5/90.4	96.9	95.2	108.7
A132					105.5
K133			97.1	91.9	102.3
K134			95.5	89.4	106.4
E135			111.2	98.8	98.1
K136			108.9	102.9	103.4
L137			92.0	96.5	98.1
N138	184.7/82.5	102.6/101.3	108.7	103.8	105.8
I139			86.0	81.0	67.9
S141			106.9	100.0	109.7
E142					120.8
D143			211.2	193.0	76.5
N144			319.5	321.6	54.4
A145			454.7	400.2	50.3
D146			452.1	407.0	82.1
S147			870.0	722.0	57.8
G148					50.0
Q149			1393.1	1033.8	481.9
Average error[e] (%)	7.9	4.0	3.2	2.5	1.6

[a] ^{15}N T_2 values for multiplet components. No attempt has been made to remove the effects of ^1H–^{15}N dipolar/^{15}N CSA cross correlation.

[b] ^{15}N T_2 values for multiplet components measured using a modified CPMG sequence where ^1H 180° pulses are applied at the height of the spin echo every 8 ms.

[c] ^{15}N T_2 values measured with the sequence of Fig. 2b (i). ^1H–^{15}N dipolar/^{15}N CSA cross-correlation effects are not completely eliminated.

[d] ^{15}N T_2 values measured with the sequence of Fig. 2b (ii). ^1H–^{15}N dipolar/^{15}N CSA cross-correlation effects are eliminated.

[e] The average error was calculated according to the formula error $= 1/N \cdot \sum_{i=1}^{N} |\text{error}(i)|$, where the summation is over all residues i. Residues D143–Q149 were not included in the calculation of the average error associated with the T_2 values. The errors in T_2 values for these residues are ~15%.

RESULTS AND DISCUSSION

In what follows, we will first consider the effects of correlation between the dipolar and chemical-shift relaxation mechanisms on transverse relaxation. These effects are muted considerably by proton spin flips in proteins and we examine how the spin-flip rate affects both T_1 and T_2 measurements. Finally, pulse schemes are presented which effectively suppress dipolar/CSA cross correlation.

Table 1 (first column of numbers) shows the ^{15}N transverse relaxation decay rates of individual multiplet components for SNase. Clearly the difference in multiplet component T_2 values predicted by theory is borne out by the experimental results although the ratio of decay rates is only ~2:1 and not 8:1 as predicted. Differences between theory and experiment are the result of the fact that the theory (Eq. [3]) is

based on the assumption of an isolated spin pair, which is an invalid assumption for NH–^{15}N spin systems in proteins. For proteins the size of SNase very efficient proton spin-flip processes involving zero-quantum or near-zero-quantum transitions occur which maintain a uniform proton spin temperature across the protein. A spin flip involving the NH proton will interconvert the two ^{15}N multiplet components and this process will tend to average the intrinsic differences in multiplet T_2 values caused by interference of dipolar and CSA relaxation mechanisms.

In order to quantitate the rate of NH–^{1}H spin flips in the protein SNase and hence obtain an accurate measure of the extent of averaging of the relaxation rates of the transverse multiplet components, we have determined the rate of decay of NH–^{15}N heteronuclear J order, $2A_zX_z$. Once heteronuclear J order is established, mutual spin flips result in a very rapid equilibration of the magnetization of the NH protons and their proton neighbors. The process of equilibration of magnetization destroys the J order which was established. The decay of J order, when compared with the previously measured T_1 data (*4*), provides a direct measure of the rate of spin flips in the protein. The pulse scheme that we have employed to measure the decay of J order (T_{1zz}) is indicated in Fig. 3. This sequence is essentially identical to the one used by Boyd *et al.* in their study of hEGF (*18*) with the exception that a ^{1}H 180° pulse is applied in the middle of the relaxation period T to allow for efficient suppression of the water signal. Note that for the decay of NH–^{15}N J order in proteins the size of SNase, the influence of dipolar and CSA cross correlation is very small since the relaxation rate is governed by very rapid spin flips while the cross-correlation effects occur on a considerably slower time scale. Therefore we have made no attempt to suppress such effects in the sequence of Fig. 3. Table 1 shows the measured values of the decay of J order for SNase. Values of T_{1zz} of approximately 100 ms are obtained. The T_{1zz} decay curves for several residues in SNase are indicated in Fig. 4.

The effects of spin flips between NH protons and neighboring proton spins on the relaxation of the individual transverse ^{15}N multiplet components during a CPMG sequence with $\delta \ll 1/(2J)$, where 2δ is the delay between successive ^{15}N 180° pulses and J is the one-bond NH–^{15}N coupling constant, can be described in terms of a two-site exchange of magnetization between sites corresponding to each of the multiplet components. This can be expressed by

$$\frac{d}{dt}\begin{bmatrix} A_{tr}^{(1)} \\ A_{tr}^{(2)} \end{bmatrix} = \begin{bmatrix} a & k \\ k & b \end{bmatrix}\begin{bmatrix} A_{tr}^{(1)} \\ A_{tr}^{(2)} \end{bmatrix}, \qquad [7]$$

where $A_{tr}^{(1)}$ and $A_{tr}^{(2)}$ are the two ^{15}N multiplet components, $a = -(\lambda + \eta) - k$, and $b = -(\lambda - \eta) - k$, where λ and η are defined as in Eq. [1] and k is the exchange rate of magnetization between the multiplet components which is equal to the NH spin-flip rate given by the decay rate of NH–^{15}N heteronuclear J order (*24*). Several cases are of interest. The first case is that in which $k \ll \lambda \pm \eta$. In this limit Eq. [7] is readily solved to give the expression indicated in Eq. [2]. Alternatively if $k \gg \lambda \pm \eta$ then a decoupled line is obtained with a transverse relaxation time of $1/\lambda$. Thus, under the condition of NH spin flips occurring rapidly compared to the decay of the transverse components of magnetization, the net decay of transverse ^{15}N magnetization occurs as a single exponential with a rate given by the average relaxation rates of the two

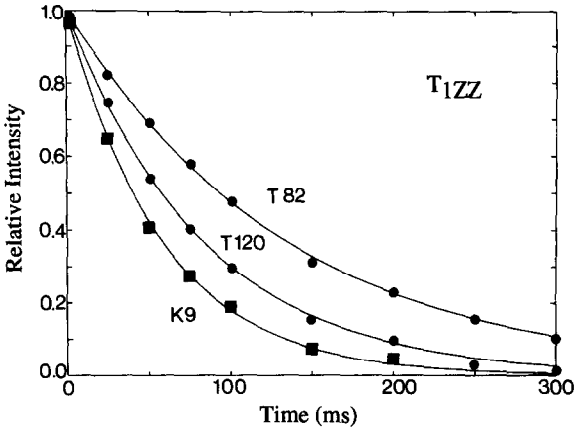

FIG. 4. Experimental NH–^{15}N T_{1zz} data of selected residues of SNase. The solid curves are the best fits of the data while the experimental data points are indicated by (●) for residues T82 and T120 and by (■) for residue K9.

multiplet components. In this limit, the effects of cross correlation between dipolar and CSA mechanisms vanish. Finally, for the case in which $k \sim \lambda \pm \eta$, it is easily shown that the decay of each of the transverse multiplet components occurs biexponentially with one time constant larger and one smaller than the average relaxation time. As an example of the latter situation, consider the case of ^{15}N transverse relaxation in the protein SNase. Previous studies have shown that this protein tumbles in solution isotropically with an overall correlation time $\tau_c = 9$ ns and an average NH–^{15}N order parameter $S^2 = 0.9$ (*4*). In this case, $\lambda + \eta = 18.2\ s^{-1}$ and $\lambda - \eta = 2.3\ s^{-1}$, and assuming an exchange rate of $8.5\ s^{-1}$ due to NH spin flips (see Table 1) each multiplet component decays biexponentially as

$$A_{tr}^{(1)} = 0.26 \exp(-7.1t) + 0.24 \exp(-30.4t)$$

$$A_{tr}^{(2)} = 0.60 \exp(-7.1t) - 0.10 \exp(-30.4t) \qquad [8]$$

and the detected magnetization is given by $A_{tr}^{(1)} + A_{tr}^{(2)}$. In this case, if one neglects the effects of cross correlation, samples the decay of transverse magnetization at regular intervals for $t \leq 200$ ms ($2T_2$ in the absence of cross correlation), and fits the data with a single exponential, an overestimate of T_2 by ~25% would be obtained.

The effects of interference between dipolar and CSA interactions on spin–lattice relaxation rates are predicted to be less severe than those for transverse relaxation rates. For the case of transverse relaxation, the exchange rate, k, is, in general, smaller than the rate of decay of the fastest decaying multiplet component and hence exchange is not fast enough to efficiently average the different T_2 values of the multiplet components. In contrast, for the case of proteins the size of SNase, the proton spin-flip rate is much greater than the longitudinal decay rates of the individual multiplet components. Consequently, efficient averaging of the longitudinal relaxation rates of the multiplet components occurs. For example, assuming $\tau_c = 9$ ns and $S^2 = 0.9$ as

before, the individual multiplet components have longitudinal decay times of 340 ms and 2.6 s, and a single exponential fit of the recovery of longitudinal magnetization sampled for $T \leq 1.2$ s (1.2 s = $2T_1$ in the absence of cross correlation) gives a value of T_1 that is ~5% too long. It is important to recognize that the effects of cross correlation on longitudinal recovery rates become more important for proteins smaller than SNase. For the case of SNase, $\omega_N\tau_c \sim 3$ at 500 MHz (*4*), where ω_N is the ^{15}N resonance frequency. However, for smaller proteins where $\omega_N\tau_c$ is such that the ^{15}N T_1 is approaching the T_1 minimum, the longitudinal decay rates increase while the ^1H spin-flip rate decreases. This results in an error in T_1 values substantially larger than 5% if cross correlation is not included. For example, if $\tau_c = 5$ ns, $S^2 = 0.9$, $k = 4.7$ s^{-1}, and the recovery of longitudinal magnetization is sampled for time values $T \leq 0.825$ s (0.825 s = $2T_1$ in the absence of cross correlation), an error of ~15% is introduced if cross correlation is neglected. Note that the effects of dipolar/CSA cross correlation on longitudinal relaxation rates are much more sensitive to molecular size than is the case for transverse relaxation rates for proteins tumbling in the limit $\omega\tau_c \gg 1$. This is due to the fact that in this limit the dipolar and CSA contributions to the transverse relaxation rate and the ^1H spin-flip rate all increase linearly with correlation time. In contrast, for macromolecules with $\omega_N\tau_c > 1$ the contribution to the longitudinal relaxation rate from the dipolar and CSA interactions decreases with increasing correlation time.

Another potential source of nonexponentiality in the recovery of longitudinal magnetization is due to ^1H–X cross relaxation (*25*). However, the measured values of T_{1zz} that we have reported in this paper indicate conclusively that the effects of ^1H–X cross relaxation on measured longitudinal relaxation rates of amide nitrogens in proteins are negligible. For the case of SNase in which an average ^{15}N T_1 value of ~600 ms was recorded at 500 MHz as well as an average ^1H–^{15}N NOE of 0.8 (*4*), the value of the ^{15}N longitudinal relaxation rate ρ_N is ~50 times greater than the ^1H–^{15}N cross-relaxation rate, $\sigma_{N,NH}$. Even in the case of L7 which, except for the next to last residue G148, shows the highest degree of internal motion of all residues examined, the value of $\sigma_{N,NH}$ is only 6% of the ρ_N value. Thus, even for mobile backbone amides, the ^1H–^{15}N cross-relaxation rate is inefficient and no deviation from exponential recovery due to cross relaxation is observed. Furthermore, even if the NH spins are perturbed by ^1H–^{15}N cross relaxation, the highly effective cross-relaxation pathways between NH and aliphatic protons in proteins would very rapidly reestablish the equilibrium NH magnetization level. That is, since $1/T_{1zz} \gg \rho_N \gg \sigma_{N,NH}$, NH–$^{15}N$ cross relaxation is negligible.

Based upon the predicted large errors that occur in the measurement of relaxation rates due to the effects of cross correlation between dipolar and CSA relaxation mechanisms, it is important to use pulse schemes that remove such effects. This is accomplished in a straightforward manner for sequences which measure either T_1 or T_2 relaxation times. The previously published sequence for the measurement of heteronuclear NOEs (*4*) is not affected by dipolar/CSA cross correlation since the ^1H spins are saturated during the buildup of the NOE. For the measurement of longitudinal heteronuclear relaxation rates a pulse sequence is presented in which ^1H 180° pulses are applied during T at a rate at least five times faster than the decay rate of the fastest

decaying multiplet component. Typically ^{1}H 180° pulses are applied every 5–10 ms. Using this sequence, we find that measured T_1 values for SNase are, on average, 8–9% shorter than T_1 values recorded using the previously proposed sequence (*4*) that did not eliminate dipolar/CSA cross correlation. The average error caused by the cross-correlation effects is in reasonable agreement with the theoretical result discussed above. Alternative schemes for measuring T_1 values which eliminate cross correlation involve ^{1}H saturation or ^{1}H decoupling during the time allowed for longitudinal relaxation (T in Fig. 2a). This latter approach has been demonstrated by Boyd *et al.* (*18*) in order to obtain T_1 values free from cross correlation. For the measurement of transverse decay rates we employ a modified CPMG sequence (*22, 23*),

$$\begin{array}{ll} ^1\text{H:} & \left[\qquad\qquad\qquad\qquad 180^\circ \qquad\qquad\qquad\qquad\right] \\ \text{X:} & \left[(\delta\text{—}180^\circ\text{—}\delta)_{2n-1}\text{—}\delta\text{—}180^\circ\text{—}\delta' \qquad \delta'\text{—}180^\circ\text{—}\delta\,(\delta\text{—}180^\circ\text{—}\delta)_{2n-1}\right]_k \end{array}.$$

The value of δ' is set so that $2\delta' + \delta_{H180^\circ} = 2\delta$, where δ_{H180° is the duration of the ^{1}H 180° pulse. Proton pulses are applied after an even number of spin echoes (every 5–10 ms) with the center of the pulse coinciding with peak of the echo. The value of δ is set to $\ll 1/(2J)$, where J is the one-bond ^{1}H–X heteronuclear coupling constant. For ^{15}N experiments a δ value of 450 μs is typically employed while δ is set to 250 μs for ^{13}C T_2 measurements. The frequencies at which ^{1}H and X 180° pulses are applied are very different so that heteronuclear Hartmann–Hahn effects do not occur.

Alternate schemes for measuring heteronuclear T_2 values that eliminate dipolar/CSA cross-correlation effects are also possible. For example, consider a sequence with ^{1}H decoupling during the transverse relaxation delay, T, and a 180° X pulse applied at $T/2$ (*26*). In this case ^{1}H decoupling eliminates cross correlation and ensures that antiphase X magnetization is not generated. This is important since the in-phase and antiphase components of X magnetization decay with different relaxation rates (*4*). Unfortunately, the quality of heteronuclear decoupling sequences such as WALTZ (*27*), GARP (*28*), or DIPSI (*29*) is degraded when there is scalar coupling between the proton(s) attached to the heteroatom and other protons in the molecule (*29*). This results in an increase in the measured X nucleus transverse decay rate and hence a decrease in the measured T_2 value.

The ^{15}N T_2 values for uniformly ^{15}N-labeled SNase measured using the sequence of Fig. 2b (ii) are presented in Table 1. For comparison we also report T_2 values measured using a CPMG scheme where only a single ^{1}H 180° pulse is applied during the T period (Fig. 2b (i)). This is the pulse sequence that we reported earlier (*4*). Note that when only a single ^{1}H 180° pulse is applied at $T/2$ the relaxation times are, on average, 7% too large due to the effects of dipolar and CSA cross correlation. (Residues D143–Q149 were not included in this average since T_2 values for these residues could not be measured accurately.) This number is considerably smaller than the error predicted by Eq. [8] since the effects of the single ^{1}H 180° pulse applied in the center of the CPMG interval were not included in the calculation. Inclusion of the effects of this pulse shows that the error is reduced to 8%, in good agreement with experiment. We should like to indicate that errors of 6–9% in the measured ^{15}N T_1 and T_2 values for SNase that we had reported previously (*4*) do not change any of

the conclusions of that work, although based on the present results the overall correlation time for SNase was overestimated by about 1–2%. In Table 1 we also report T_2 values of multiplet components measured using the modified CPMG sequence with a spacing between application of ^{1}H 180° pulses of 8 ms. It is clear that in this case the multiplet components decay with the same T_2 values and hence cross correlation between dipolar and CSA relaxation mechanisms has been effectively suppressed.

Recently Wagner and co-workers have proposed the use of spin-lock pulse sequences for the measurement of heterospin transverse relaxation rates (*26*). These sequences actually measure $T_{1\rho}$ values which are only identical to T_2 values in the limit of *on-resonance* spin-lock fields. For the off-resonance case $T_{1\rho}$ can become longer than T_2. For example, for a 2.9 kHz RF field and an offset of 1 kHz the value of $T_{1\rho}$ and T_2 will differ by $\sim$10% for ^{15}N T_2 and T_1 values of 100 and 600 ms, respectively. Of course for smaller offset values the differences between $T_{1\rho}$ and T_2 will decrease. The CPMG pulse sequence can also produce erroneous T_2 values if not used carefully. Figure 5 illustrates the ratio of the apparent T_2 value measured from the CPMG sequence vs the correct T_2 value as a function of the spacing between successive 180° pulses for an AX spin system in the absence of cross correlation. Figure 5 indicates that for a choice of $\delta \ll 1/(2J)$ accurate values of T_2 can be readily obtained. *However, we wish to emphasize that for* $\delta \sim 1/(2J)$ *significant underestimates of true* T_2 *values are obtained with this method.* The value of δ = 450 μs used in the present study results in an error of less than 1% in measured ^{15}N T_2 values. Given that the average

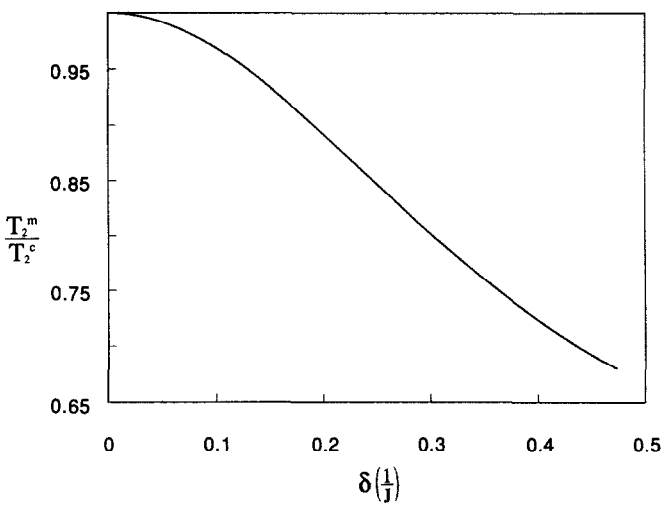

FIG. 5. Theoretical ratio of measured T_2 (T_2^m) and correct T_2 (T_2^c) as a function of δ (2δ is the delay between successive 180° pulses) in a CPMG pulse sequence. The delay value δ is measured in units of $1/J$, where J is the heteronuclear ^{1}H–X coupling constant. Values of J = 90 Hz, T_2^c = 100 ms, and T_{1f} = 100 ms are used, where T_{1f} is the time constant for ^{1}H spin flips. The ratio T_2^m/T_2^c is measured from an exponential sampling of the decay of transverse magnetization consisting of 10 time points for time values $T \leq 200$ ms ($2T_2^c$). Dipolar/CSA cross correlation is not included in this calculation.

power levels are considerably reduced for the CPMG sequence relative to its spin-lock counterpart and that if δ is chosen judiciously essentially error free T_2 values can be obtained, we strongly prefer this sequence for the measurement of T_2 values.

In summary, we have described the effects of dipolar and CSA cross correlation on the measurement of ^{15}N T_1 and T_2 relaxation times and presented pulse schemes for the effective removal of such effects. For the case of ^{15}N T_1 measurements the scheme that is presented is very similar to the sequence described by Boyd *et al.* (*18*). For SNase, a comparison of ^{15}N T_2 values obtained with the modified CPMG sequence vs values obtained with the CPMG sequence containing only a single ^1H 180° pulse applied in the middle of the CPMG relaxation interval, T, suggests that the effects of cross correlation result in measured T_2 values that are in error by approximately 7% when recorded at a field strength of 11.8 T. In the absence of any ^1H 180° pulses during the relaxation time, T, cross-correlation effects can be much more substantial, often resulting in errors as large as 25–30% in measured T_2 values. The effects of cross correlation on measured T_1 times are expected to be much smaller than those for T_2 values and errors of ~5 and 8.5% (11.8 T) are predicted and measured for SNase if cross correlation is ignored. Nevertheless the errors in measured T_1 values are not negligible and increase rapidly with decreasing molecular weight, necessitating the use of improved schemes for the measurement of T_1 values as well (*18*). While the emphasis in this paper has been on the measurement of ^{15}N relaxation times the pulse schemes that we have developed are, of course, equally valid for the removal of dipolar and CSA cross-correlation interference from ^{13}C relaxation time measurements.

ACKNOWLEDGMENTS

We thank New Methods Research (Syracuse, New York) for providing a copy of their NMR processing software. This work was supported by the Intramural AIDS Antiviral Program of the Office of the Director of the National Institutes of Health. L.E.K. acknowledges financial support from the Medical Research Council of Canada.

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