

Improved Accuracy of Heteronuclear Transverse Relaxation Time Measurements in Macromolecules. Elimination of Antiphase Contributions*

JEFFREY W. PENG,†‡ V. THANABAL,† AND GERHARD WAGNER†§

†Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Avenue, Boston, Massachusetts 02115; and ‡Biophysics Research Division, University of Michigan, 2200 Bonisteel Boulevard, Ann Arbor, Michigan 48109

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Heteronuclear T_2 relaxation measurements (^{13}C , ^{15}N) play an integral role in the characterization of internal motions in proteins by NMR (1–3). Recently there has been some controversy concerning the correct method of measuring ^{15}N or ^{13}C T_2 values because different experiments produced different (mainly too short) T_2 values (1–3). We propose and show experimental evidence that this is due to an oscillation between in-phase and antiphase coherence of the heteronucleus if chemical-shift refocusing and concomitant proton decoupling are achieved via a single (Carr–Purcell) or a train of (Meiboom–Gill) X-nucleus 180° pulses. This leads to a mixture of contributions from in-phase and antiphase relaxation, and the latter causes apparently faster T_2 relaxation. We propose to eliminate the antiphase contributions from the T_2 measurements by applying a spin lock on the heteronucleus. The result is a significant improvement in the accuracy of heteronuclear T_2 measurements.

A basic 2D pulse sequence for measuring heteronuclear T_2 values in a 2D NMR experiment (1) is shown in Fig. 1a. We are mainly concerned with ^{15}N relaxation and denote the heteronucleus as N and follow the product-operator formalism (4). In the pulse sequence of Fig. 1a, in-phase transverse coherence, N_x , relaxes during a refocusing delay τ , primarily due to dipole–dipole interactions between the N spins and their directly bonded protons (I spins). ^{15}N relaxation studies on the proteinase inhibitor eglin c reveal that the use of different refocusing pulse schemes (Fig. 1a: i, ii, iii) yields different transverse relaxation times (5). Specifically, the use of a single 180° pulse produces, in small proteins, relaxation times about 50% shorter than those obtained using a 3 kHz spin lock. Carr–Purcell–Meiboom–Gill (CPMG) (6, 7) relaxation times yield intermediate values, and the apparent T_2 values increase as the spacing between the 180° pulses is narrowed. However, with our spectrometer, we never came closer than to about 75% of the spin-lock values, being limited by a hard-pulse duty cycle of 2%. For *all* residues in the protein, the maximum transverse relaxation time is

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§ To whom correspondence should be addressed.

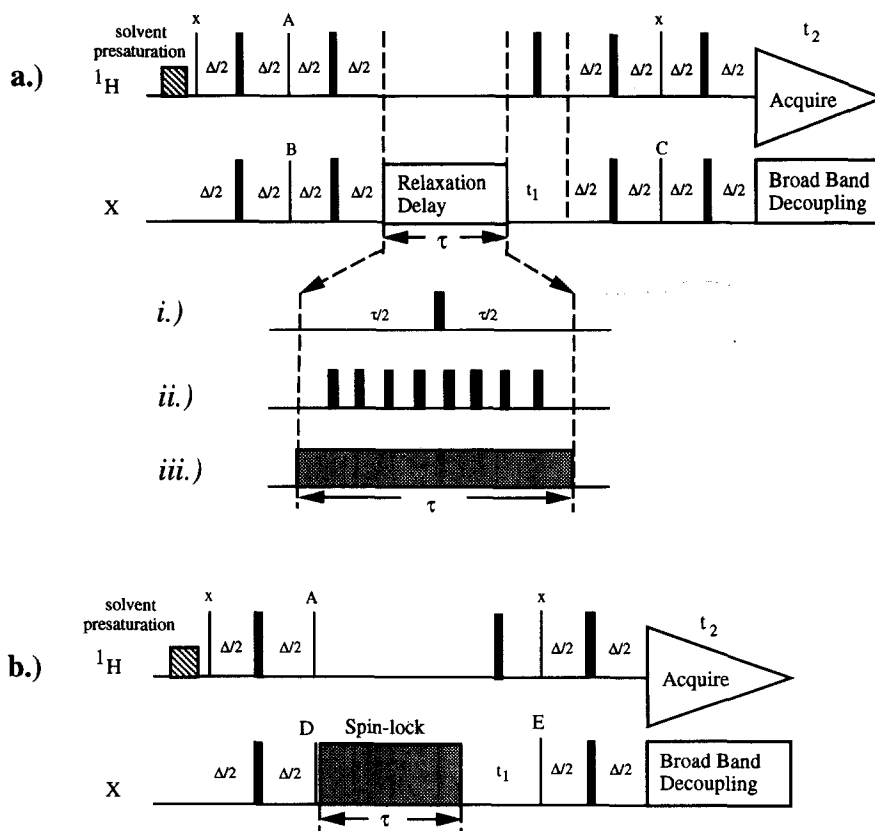


FIG. 1. (a) Two-dimensional pulse sequence for measuring heteronuclear T_2 values. The results are ^1H X correlation spectra with cross-peak intensities determined by the extent of transverse relaxation during the period τ ; 90° and 180° pulses are indicated by thin and thick vertical bars, respectively. The delays $\Delta/2$ are set to $1/4J_{\text{XH}}$. Pulse schemes for refocusing transverse X-nucleus magnetization during the relaxation delay include (i) a single 180° refocusing pulse, (ii) a CPMG pulse train, and (iii) a weak on-resonance spin lock. CPMG trains consisted of 180° pulses of $100 \mu\text{s}$ separated by delays. The spin lock consisted of contiguous 180° pulses of $174 \mu\text{s}$ without separating delays. Phase cycling is as follows: (A) $4(+y)4(-y)$, (B) $+x, -x$, (C) $2(+x)2(-x) + \text{TPPI}$. The receiver phases are $(-x, x, x, -x, x, -x, -x, x)$. (b) Two-dimensional pulse sequence for measuring spin-locked antiphase relaxation. The spin lock is of the same type as that in a. Additional phase cycling includes (D) $+y, -y$, (E) $2(+y)2(-y) + \text{TPPI}$. Spectra were recorded on both Bruker AMX500 and GE Omega 500 spectrometers.

observed using the spin lock. Therefore, it is unlikely that chemical-exchange phenomena as proposed previously (2) are responsible for the short T_2 values in the single 180° pulse scheme, since exchange processes are expected to be localized in certain regions of the protein structure. Note that the spin-lock relaxation times are rigorously heteronuclear $T_{1\rho}$ values. The utility of $T_{1\rho}$ as another means of measuring T_2 in liquids was first described by Redfield (8) and later by Freeman and Hill (9). For resonances at the spin-lock frequency, $T_{1\rho}$ is equal to T_2 , provided the protein behaves approximately as a rigid, spherically symmetric tumbler with a rotational correlation time, τ_c , much less than reciprocal of the spin-lock field strength. Thus, for spin-lock

strengths in the kilohertz range, we demand $\tau_c \ll 10 \mu\text{s}/\text{rad}$; this is satisfied by proteins that have τ_c in the nanosecond regime (2, 3, 5). More detailed aspects of $T_{1\rho}$ are given elsewhere (5). For resonances away from the spin-lock carrier frequency, the ratio of $T_{1\rho}$ to T_2 is approximately proportional to $1/\sin^2\beta$, where β is the tip angle of the effective field in the rotating frame. For example, a ^{15}N sweep width of ± 700 Hz (1.4 kHz) has tip angles of 77° at the edges of the spectrum using a 3 kHz spin lock. $T_{1\rho}$ is then expected to be about 5% greater than T_2 . The typical precision of the T_2 measurements is such that these overestimations are not deleterious to subsequent motional interpretation of the data. In our studies at 11.7 T all of the amide ^{15}N signals were contained within a sweep width of 1200 Hz. Thus, the spin lock can be reasonably approximated as on-resonance for all of these cross peaks. For much wider heteronuclear spectral widths, severe off-resonance effects can be eliminated simply by varying the spin-lock carrier position to complete the coverage of the desired spectral range.

We propose that the evolution of in-phase N_x coherence into antiphase $2I_zN_y$ coherence is the primary cause for the shorter T_2 values in the single 180° pulse experiment. Although N_x is refocused at the end of the $(\tau/2) - 180^\circ - (\tau/2)$ period, the net coherence oscillates between N_x and antiphase $2I_zN_y$ coherence during either half of the τ period. Accordingly, the relaxation times of both in-phase and antiphase coherence must be considered. The relaxation time of antiphase coherence, T_{2z} , is significantly shorter than T_2 for proteins, due to $^1\text{H}-^1\text{H}$ dipolar interactions experienced by the protons directly bonded to the X nuclei. Since the apparent transverse relaxation time reflects a mixture of both T_2 and T_{2z} , an erroneously short " T_2 " will be recorded.

The faster relaxation of antiphase coherence as compared to in-phase coherence has been established in the literature (10-12). For example, Vold and Vold (10) discuss the effects of random fields at the I spin upon the relaxation rates of transverse N-spin coherences. For our case, these random fields are due to $^1\text{H}-^1\text{H}$ dipolar interactions which induce transitions in the I spins. We briefly highlight the causes for the faster relaxation here. If we first consider an isolated IN two-spin system, then antiphase (i.e., $2I_zN_y$, $2I_zN_x$) coherence decays as (13-15)

$$\frac{d\langle 2I_zN_{(y,x)} \rangle}{dt} = -\frac{1}{T_{2z(\text{isol})}} \langle 2I_zN_{(y,x)} \rangle, \quad [1]$$

where

$$\frac{1}{T_{2z(\text{isol})}} = \frac{(\hbar\gamma_I\gamma_N)^2}{8r_{\text{IN}}^6} \{4J(0) + J(\omega_I - \omega_N) + 3J(\omega_N) + 6J(\omega_I + \omega_N)\}. \quad [2]$$

This can be compared to familiar expression for transverse relaxation rate of in-phase coherence

$$\frac{1}{T_2} = \frac{(\hbar\gamma_I\gamma_N)^2}{8r_{\text{IN}}^6} \{4J(0) + J(\omega_I - \omega_N) + 3J(\omega_N) + 6J(\omega_I) + 6J(\omega_I + \omega_N)\}. \quad [3]$$

The spectral density function, $J(\omega)$, becomes a Lorentzian distribution,

$$J(\omega) = \frac{2}{5} \frac{\tau_c}{1 + (\omega\tau_c)^2}, \quad [4]$$

if we model the protein as an isotropically tumbling rigid body with a rotational correlation time, τ_c . Note from Eq. [2] that $1/T_{2z(\text{isol})}$ is identical to $1/T_2$ except that it lacks a $6J(\omega_1)$ term. Therefore, if one considers only dipole-dipole interactions between the I and N spins, the antiphase relaxation times would actually be longer than T_2 . However, in proteins $J(\omega_1)$ may be very small. More importantly, the proton directly bonded to the X nucleus (I_i spins) can enjoy significant spin-lattice relaxation due to other nonbonded protons (I_j spins) within about 5 Å distance. When we include the effects of I_i - I_j dipole-dipole interactions, the antiphase coherence for the i th IN bond decays as

$$\frac{d\langle 2I_{iz}N_{(y,x)} \rangle}{dt} = -\frac{1}{T_{2z}} \langle 2I_{iz}N_{(y,x)} \rangle - \sum_{j \neq i} \sigma_{ij} \langle 2I_{jz}N_{(y,x)} \rangle, \quad [5]$$

where

$$\frac{1}{T_{2z}} = \frac{1}{T_{2z(\text{isol})}} + \sum_{j \neq i} \rho_{ij} \quad [6]$$

and

$$\rho_{ij} = \frac{\hbar^2 \gamma_1^4}{4r_{ij}^6} \{ J(\omega_{I_i} - \omega_{I_j}) + 3J(\omega_{I_i}) + 6J(\omega_{I_i} + \omega_{I_j}) \} \quad [7]$$

$$\sigma_{ij} = \frac{\hbar^2 \gamma_1^4}{4r_{ij}^6} \{ 6J(\omega_{I_i} - \omega_{I_j}) - J(\omega_{I_i} - \omega_{I_j}) \}. \quad [8]$$

The quantity $1/T_{2z(\text{isol})}$ is the antiphase relaxation time for an isolated IN spin pair given in Eq. [2]. The expression given in Eq. [5] shows that the direct relaxation rate is increased from $1/T_{2z(\text{isol})}$ in the simpler IN case to $1/T_{2z} = 1/T_{2z(\text{isol})} + \sum_{j \neq i} \rho_{ij}$. The ρ_{ij} terms are the constituent spin-lattice relaxation rates of the directly bonded proton I_i , due to dipole-dipole interactions with nonbonded protons I_j (15, 16). The σ_{ij} terms indicate cross-relaxation pathways (15, 16) to antiphase coherences involving spins that do not experience the one-bond heteronuclear coupling, J_{IN} . As such, these coherences cannot refocus and are not detected in the 2D experiments described here (Fig. 1). In-phase N_x coherence is not relaxed by the ^1H - ^1H dipolar interactions and therefore T_{2z} is significantly shorter than T_2 . This fact can be understood by recalling that the N-spin transverse coherence can be resolved into two component coherences corresponding to the I_i spin up and spin down, respectively. These components have the same phase in the transverse plane in the case of N_x and are 180° opposed in the case of $2I_{iz}N_{(y,x)}$. However, the I_i spin flips caused by I_i - I_j dipolar interactions connect only N-spin coherences with the same phase. Thus, the net amount of in-phase N_x coherence is unaffected, while the net amount of antiphase coherence is decreased. In contrast, I_i spin flips caused by I_i -N dipolar interactions invert the phase of the N-spin coherences as a result of the spin operators $I_i \pm N_z$ in the dipolar Hamiltonian; thus $1/T_{2z(\text{isol})}$ is independent of the I_i transition frequency, ω_{I_i} .

Note that the present explanation for the artificially short T_2 values emphasizes two steps: coherent antiphase evolution followed by antiphase relaxation. This contrasts to alternative explanations which blame the short T_2 values on scalar relaxation of

the second kind (2). Scalar relaxation of the second kind, as described by Abragam (13), results from the random modulation of the IN scalar interaction, due to rapid relaxation of the I spin. The rapid relaxation forces the collapse of the N-spin doublet. Thus, in order for scalar relaxation of the second kind to be applicable, we require the I_i-spin (proton) T_1 values to satisfy $T_1 \ll 1/2\pi J_{IN}$ (about 1.77 ms/rad for ¹⁵N–¹H bonds). Since the proton T_1 values are at least on the 100 ms time scale, an explanation of the apparently short T_2 values cannot be given in terms of scalar relaxation of the second kind.

Clearly, to obtain correct T_2 measurements, it is necessary to suppress the antiphase evolution during the transverse relaxation delay. This is precisely the effect of the low-power spin lock shown in the pulse sequence of Fig. 1a(iii). The CPMG method also suppresses antiphase evolution, although the extent of suppression depends on the spacing between the 180° pulses. Specifically, the τ periods between 180° refocusing pulses must satisfy $\tau \ll 1/2J_{IN}$. For ¹⁵N–¹H spin systems, J_{IN} is about 90 Hz; thus, τ should be well below 5.5 ms. However, such short τ periods demand significant duty cycles at high power on the X-nucleus channel and may be unfeasible for some spectrometers. If sufficiently short τ values cannot be implemented, then artificially short T_2 values will be recorded. Moreover, the recorded values will depend on the choice of pulse spacing τ . In our studies, we were limited to 180° ¹⁵N pulses of 100 μ s and $\tau = 5$ ms (2% duty cycle), resulting in the uniformly shorter T_2 values reported above. Thus, in these situations, the use of a low-power spin lock is a superior alternative to the CPMG method for obtaining accurate T_2 measurements.

To confirm that antiphase evolution and relaxation are responsible for the short T_2 measurements, we have performed both spin-locked in-phase and spin-locked antiphase ¹⁵N-relaxation experiments on the protein eglin c. The antiphase experiment shown in Fig. 1b simply applies the spin lock after the initial creation of antiphase $2I_{iz}N_x$ coherence. Under the spin-lock and resonance-offset provisions given above, these experiments provide T_2 and T_{2z} values, respectively. Note that the spin locking prevents the mixing of in-phase and antiphase magnetizations in both sequences; thus, T_2 and T_{2z} are measured separately. The resulting relaxation times clearly show that T_2 is significantly longer than T_{2z} for all residues in eglin c. Examples of the comparative T_2 and T_{2z} fits are shown as upper and lower curves, respectively, in Fig. 2 for the amide ¹⁵N nuclei of Val 14 and Arg 53.

In conclusion, the correct measurement of heteronuclear T_2 values demands that the in-phase transverse coherence be prevented from evolving into antiphase coherence. Failure to do this will produce erroneously short transverse relaxation times, since antiphase magnetization experiences significant proton–proton relaxation. If spectral density $J(\omega)$ is Lorentzian and the spin lock is sufficiently on-resonance, then $T_{1\rho} = T_2$ and a spin-lock series can be used to measure the heteronuclear T_2 values. Alternatively, the CPMG method can be used if the pulse spacing is sufficiently narrow. However, if the latter method is used, additional experiments should be run to ensure that the observed T_2 values do not increase significantly as the pulse spacing is narrowed. The advantage of the spin-lock experiment shown in Fig. 1a(iii) is that the relaxation times obtained do not suffer from the ambiguity associated with this spacing between refocusing pulses.

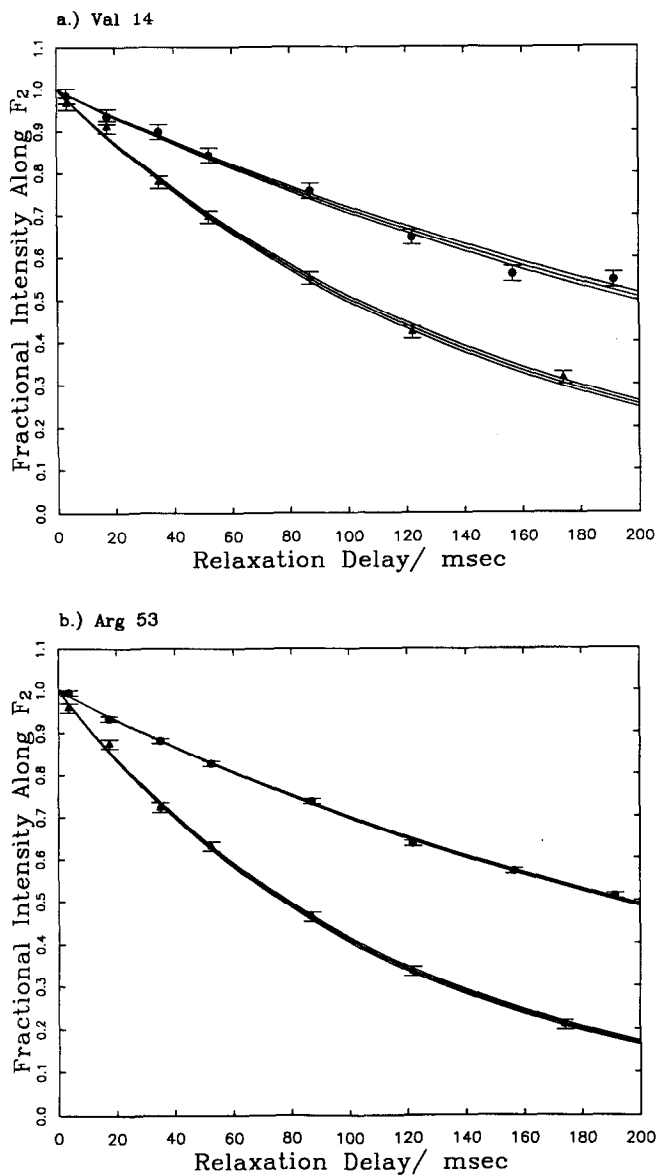


FIG. 2. Examples of comparative T_2 and T_{2z} fits for residues Val 14 and Arg 53 of the proteinase inhibitor eglin c. T_2 and T_{2z} values were measured using the spin-lock pulse sequences in Figs. 1a and 1b. Relaxation times are extracted by fitting integrals of cross peaks along the F_2 (^1H) dimension to an exponential decay using a nonlinear least-squares fitting procedure from the software package PLOT (New Unit Inc., Ithaca, New York). The T_2 fits are always the upper set of curves. (a) Val 14: $T_2 = 294 \pm 5$ ms, $T_{2z} = 146 \pm 3$ ms. (b) Arg 53: $T_2 = 278 \pm 3$ ms, $T_{2z} = 112 \pm 3$ ms. For each fit, three curves are plotted. The central curve corresponds to the fitted relaxation time while the bounding curves correspond to the quoted uncertainties. Error bars indicate the root-mean-square deviation from the fitted function to the peak intensities.

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