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

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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 T, 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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Fig. 1. (a) Two-dimensional pulse sequence for measuring heteronuclear T2 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 \( 1/4J_{XH} \). 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 \( \mu \)s separated by delays. The spin lock consisted of contiguous 180° pulses of 174 \( \mu \)s without separating delays. Phase cycling is as follows: (A) 4(+y)4(−y), (B) +x, −x, (C) 2(+x)2(−x) + 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) + 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 T2 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 T1p values. The utility of T1p as another means of measuring T2 in liquids was first described by Redfield (8) and later by Freeman and Hill (9). For resonances at the spin-lock frequency, T1p is equal to T2, provided the protein behaves approximately as a rigid, spherically symmetric tumbler with a rotational correlation time, \( \tau_c \), much less than reciprocal of the spin-lock field strength. Thus, for spin-lock
strengths in the kilohertz range, we demand $\tau_c \ll 10 \mu s/\text{rad}$; this is satisfied by proteins that have $\tau_c$ in the nanosecond regime (2, 3, 5). More detailed aspects of $T_{1p}$ are given elsewhere (5). For resonances away from the spin-lock carrier frequency, the ratio of $T_{1p}$ to $T_2$ is approximately proportional to $1/\sin^2 \beta$, where $\beta$ is the tip angle of the effective field in the rotating frame. For example, a $^{15}$N sweep width of $\pm 700$ Hz (1.4 kHz) has tip angles of $77^\circ$ at the edges of the spectrum using a 3 kHz spin lock. $T_{1p}$ 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 $(\tau/2)-180°-(\tau/2)$ period, the net coherence oscillates between $N_x$ and antiphase $2I_zN_y$ coherence during either half of the $\tau$ 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 $^1H-^1H$ 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 $^1H-^1H$ 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)

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

where

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

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

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

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

\[
J(\omega) = \frac{2}{5} \frac{\tau_c}{1 + (\omega \tau_c)^2},
\]
if we model the protein as an isotropically tumbling rigid body with a rotational correlation time, \( \tau_c \). Note from Eq. [2] that \( 1/T_{2z(\text{isol})} \) is identical to \( 1/T_2 \) except that it lacks a \( 6J(\omega_i) \) 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 \( J(\omega_i) \) 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

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

where

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

and

\[
\rho_{ij} = \frac{\hbar^2 \gamma_i^4}{4r_{ij}^6} \left\{ J(\omega_{ii} - \omega_{ij}) + 3J(\omega_{ii}) + 6J(\omega_{ii} + \omega_{ij}) \right\} \tag{7}
\]

\[
\sigma_{ij} = \frac{\hbar^2 \gamma_i^4}{4r_{ij}^6} \left\{ 6J(\omega_{ii} - \omega_{ij}) - J(\omega_{ii} + \omega_{ij}) \right\}. \tag{8}
\]

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 \( \rho_{ij} \) 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 \( \sigma_{ij} \) terms indicate cross-relaxation pathways \((15, 16)\) to antiphase coherences involving spins that do not experience the one-bond heteronuclear coupling, \( J_{I_N} \). 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 \( ^1H-\text{H} \) 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 \( 180^\circ \) opposed in the case of \( 2I_{iz}N_{(y,x)} \). However, the \( I_i \) spin flips caused by \( I_i-I_i \) 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 \( I_i \pm N_x \) in the dipolar Hamiltonian; thus \( 1/T_{2z(\text{isol})} \) is independent of the \( I_i \) transition frequency, \( \omega_i \).

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-spin (proton) $T_1$ values to satisfy $T_1 \ll 1/2\pi J_{IN}$ (about 1.77 ms/rad for $^{15}$N–
$^1$H 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 re-
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 $\tau$ periods between 180° refocusing
pulses must satisfy $\tau \ll 1/2J_{IN}$. For $^{15}$N–$^1$H spin systems, $J_{IN}$ is about 90 Hz; thus, $\tau$
should be well below 5.5 ms. However, such short $\tau$ periods demand significant duty
cycles at high power on the X-nucleus channel and may be unfeasible for some spec-
trometers. If sufficiently short $\tau$ 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 $\tau$. In our studies, we were limited to 180° $^{15}$N pulses of 100 ms 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
$^{15}$N-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 $2I_{lz}N_x$
coherence. Under the spin-lock and resonance-offset provisions given above, these
experiments provide $T_2$ and $T_{2x}$ values, respectively. Note that the spin locking prevents
the mixing of in-phase and antiphase magnetizations in both sequences; thus, $T_2$ and
$T_{2x}$ are measured separately. The resulting relaxation times clearly show that $T_2$ is
significantly longer than $T_{2x}$ for all residues in eglin c. Examples of the comparative
$T_2$ and $T_{2x}$ fits are shown as upper and lower curves, respectively, in Fig. 2 for the
amide $^{15}$N 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 $T_{1p} = T_2$ 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 $T_2^*$ fits for residues Val 14 and Arg 53 of the proteinase inhibitor eglin c. $T_2$ and $T_2^*$ 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 $F_2$ ($^1$H) 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, $T_2^* = 146 \pm 3$ ms. (b) Arg 53: $T_2 = 278 \pm 3$ ms, $T_2^* = 112 \pm 3$ 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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