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Quantitative comparison of errors in ^{15}N transverse relaxation rates measured using various CPMG phasing schemes

Wazo Myint¹, Yufeng Cai², Celia A. Schiffer², and Rieko Ishima^{1,*}

¹Department of Structural Biology, University of Pittsburgh School of Medicine, 3501 Fifth Avenue, Pittsburgh, PA 15260

²Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, 364 Plantation Street, Worcester, MA 01605

Abstract

Nitrogen-15 Carr-Purcell-Meiboom-Gill (CPMG) transverse relaxation experiment are widely used to characterize protein backbone dynamics and chemical exchange parameters. Although an accurate value of the transverse relaxation rate, R_2 , is needed for accurate characterization of dynamics, the uncertainty in the R_2 value depends on the experimental settings and the details of the data analysis itself. Here, we present an analysis of the impact of CPMG pulse phase alternation on the accuracy of the ^{15}N CPMG R_2 . Our simulations show that R_2 can be obtained accurately for a relatively wide spectral width, either using the conventional phase cycle or using phase alternation when the r.f. pulse power is accurately calibrated. However, when the r.f. pulse is miscalibrated, the conventional CPMG experiment exhibits more significant uncertainties in R_2 caused by the off-resonance effect than does the phase alternation experiment. Our experiments show that this effect becomes manifest under the circumstance that the systematic error exceeds that arising from experimental noise. Furthermore, our results provide the means to estimate practical parameter settings that yield accurate values of ^{15}N transverse relaxation rates in the both CPMG experiments.

Keywords

relaxation; Monte Carlo; dynamics; protein; NMR; CPMG

Introduction

NMR relaxation is one of powerful methods to characterize internal motion of individual sites of proteins (Bruschweiler 2003; Dayie et al. 1996; Farrow et al. 1995; Fushman & Cowburn 2001; Igumenova et al. 2006; Ishima & Torchia 2000; Jarymowycz & Stone 2006; Kay 2005; Palmer 2001; Peng & Wagner 1995; Redfield 2004). Several relaxation rates, including the transverse relaxation rate (R_2), are typically used to characterize the degree of internal motion in biomolecules (Kay et al. 1989; Lipari & Szabo 1982a; Lipari & Szabo 1982b; Nirmala & Wagner 1988). R_2 is also used to detect slow conformational changes in

*Corresponding author: Rieko Ishima, Rm 1037, Biomedical Science Tower 3, 3501 Fifth Avenue, University of Pittsburgh School of Medicine, Pittsburgh, PA 15260, Phone: 412-648-9056, Fax: 412-648-9008, ; Email: ishima@pitt.edu.

uncertainties, magnitudes of the uncertainties other than those due to the experimental noise are estimated (Ishima & Torchia 2005). Our results show that in CPMG experiments performed by employing a radio-frequency of $B_1 > 5$ kHz, R_2 obtained using the conventional CPMG ($XXXX$) phase scheme is close to the one obtained using the alternative phase scheme ($XXY\bar{Y}$) at the off-resonance frequency, $\omega_{off}/2\pi$, up to 1400 Hz. However, the conventional CPMG is expected to introduce higher systematic error even with $\omega_{off}/2\pi < 1400$ Hz presumably due to non-exponential behavior of magnetization decay. We conducted ^{15}N CPMG relaxation experiments to verify the results of the calculations. Experimental results further showed that the differences in R_2 uncertainties in the two schemes are manifest only when the noise-to-signal ratio is significantly smaller than the fractional systematic error on R_2 .

Methods

NMR Experiments

^{15}N transverse relaxation experiments were conducted using a 0.8 mM ^{15}N labeled ubiquitin at pH 4.5 on a Bruker Avance 900 NMR instrument. Ubiquitin sample was purchased and prepared as described previously (Myint et al. 2009). Three transverse relaxation experiments were performed with a conventional CPMG phase scheme ($XX-XX$, here noted 00-00), an alternative phase scheme ($XX-Y\bar{Y}$, here noted as 00-13), and a long alternative phase scheme ($XXY\bar{Y}-XXY\bar{Y}$, here noted as 0013-0013). Here, X and Y indicate pulse phases, and a hyphen indicates the timing applied to the ^1H 180° pulses to suppress cross-correlation by ^1H - ^{15}N dipolar interaction and ^{15}N chemical shift anisotropy (CSA) (Kay et al. 1992; Palmer et al. 1992). We used the same CPMG pulse sequence that was applied previously ((Freedberg et al. 2002), except for an additional semi-constant time for the t_1 evolution, not in the original sequence (Kay et al. 1992). The total phase cycle is 8 with a phase inversion for the CPMG period. All the ^{15}N pulses are applied even number of times at each pulse scheme. Delays for the 00-00 and 00-13 experiments were varied: 0, 8, 16, 24, 32, 40, and 48 ms for the 00-00 and 00-13. Delays for the 0013-0013 experiments were varied: 0, 16, 32, and 48 ms. The maximum delay is set shorter than the expected $1/R_2$ because of the high sensitivity of an instrument equipped with a cryogenic probe and to avoid significant sample heating (Myint et al. 2009). Radio-frequency power for the ^{15}N pulses was 5.56 kHz (90 μs as a 180° pulse), and a half duration between ^{15}N CPMG pulses, τ_{CP} , was 0.5 ms. Here, $2\tau_{CP}$ is the time between the centers of two adjacent CPMG pulses. ^{15}N pulses were applied at 130 ppm as a carrier frequency to investigate off-resonance effects. The number of scans was 8. Using the same pulse powers, ^{15}N longitudinal relaxation was recorded for the analysis of the 00-13 type relaxation data. The delays applied for the longitudinal relaxation measurements were: 0, 0.05, 0.1, 0.25, and 0.5 s.

^{15}N transverse relaxation experiments were also performed using a 250 μM Human Immunodeficiency virus-1 (HIV-1) protease at pH 5.8 on a Bruker Avance 600 MHz NMR instrument. The protease was over-expressed and purified mainly using the previous protocol (Nalam et al. 2007). Data was recorded using a conventional CPMG phase scheme ($XX-XX$) with delays of 0, 8, 16, 24, 32, 48, and 64 ms. Data were recorded by employing

the same r.f. field strength for the ^{15}N pulses, 5.56 kHz, as the same that of 900 MHz. However, the carrier frequency of the pulse was placed at 117 ppm that is approximately the center of the amide ^{15}N chemical shifts of the protease sample. 64 scans were accumulated for each free induction decay.

Data analysis

Free induction decay signals were zero-filled four times, apodized with a sine window function with 40% offset, and Fourier transformed in both dimensions using NMRpipe software (Delaglio et al. 1995). Signal intensities at individual positions were taken instead of peak volumes using the NMRdraw software (Delaglio et al. 1995). Line-shape fitting algorithm was not used to estimate peak heights. Experimental noise was obtained for each two-dimensional spectrum, but assumed to be the same in each CPMG experiments.

For each time-course of magnetization decay, a transverse relaxation rate, R_2 , was optimized assuming a mono-exponential decay function, $I(t) = I_0 \exp(-tR_2)$. In theory, if the initial intensity is correct, there is only one unknown parameter in this equation. However, since there is small loss of intensity at time zero, both I_0 and R_2 are typically optimized. Note that the same equation is used for R_1 determination as well as R_2 in protein ^{15}N NMR relaxation since the Freeman-Hill method is used (Freeman & Hill 1971).

Once R_2 values were optimized, Monte Carlo error estimation was performed to estimate uncertainty of R_2 . In this method, first unknown parameters, R_2 and the initial intensity, were optimized using the experimental data, and the set of the fit “ideal” intensities were back-calculated using the optimized parameters. Second, a hundred number of sets of synthetic intensity data were generated to allow a Gaussian distribution with the ideal intensities as the mean. Finally, optimization was repeated for these synthesized data sets to calculate the standard deviation of R_2 and the initial intensity. The Gaussian distribution to generate synthetic intensities was defined in two ways:

1. a root-mean-square deviation (rmsd) of the NMR spectral noise (Palmer et al. 1991; Skelton et al. 1993)
2. a standard deviation of the residual ($I_i - I^{\text{fit}}$) of the fit (Nicholson et al. 1992). In this article, R_2 errors that were calculated using the experimental noise are denoted $R_{2\text{noise_err}}$, and those were calculated using the residual of the fit are denoted $R_{2\text{fit_err}}$. The former reflects only experimental noise error and the later reflects overall fit-uncertainties that include both experimental statistic noise and systematic noise. By comparing the two types of R_2 uncertainties the uncertainties other than those due the experimental noise are estimated (Ishima & Torchia 2005).

The error in the R_2 value, measured using the 00-13 and 0013-0013 sequences, caused by R_1 relaxation during the r.f. pulses is recommended to be corrected using the equation introduced by Zuiderweg's group (Yip & Zuiderweg 2004):

$$R_2^{\text{obs}} = R_2 + d(R_1 - R_2)/4 = R_2(1 - d/4) + dR_1/4 \quad (1)$$

Here, d indicates a duty cycle of the r.f. pulse: total duration of the r.f. pulses divided by the entire CPMG delay. However, in the following analysis, no correction was made because the correction factor is calculated to be small. When pulse power $\gamma_N B_1/2\pi = 5.6$ kHz is applied with a reasonable delay ($\tau_{CP} = 0.45$ ms and 0.5 ms for simulation and experiments, respectively), the observed R_2 will be only 2.5% and 2.25% smaller than the correct R_2 , respectively when $R_1 = 0$ (equation (1)). When $0 < R_1 < R_2$, the difference becomes less than 2.5% and 2.25%, respectively. This is in contrast to the condition that was used by Yip and Zuiderweg in which ca. 8% correction of R_2 was needed (Yip & Zuiderweg 2004). Note that since this equation does not contain any off-resonance frequency, some of the error caused by the combination of the effects of pulse imperfection and off-resonance is not corrected by this equation. We did not use the 00130031 sequence (Yip & Zuiderweg 2004) because the minimum cycle, each CPMG loop ($n=1$) of $(00130031-00130031)_2$, needed to suppress cross correlation between ^{15}N CSA and ^1H - ^{15}N dipolar interactions is long, i.e., 32 ms when $\tau_{CP} = 0.5$ ms.

Simulation

The time evolution of bulk nuclear magnetization of a scalar-coupled ^{15}N - ^1H spin system was calculated to determine transverse relaxation rates. For this, we used a relaxation matrix that contains 16 Cartesian product operators as the base set, as described previously (Allard et al. 1997; Allard et al. 1998; Myint et al. 2009; Myint & Ishima 2009).

E/2	N_X	N_Y	N_Z	H_X	H_Y	H_Z	$2N_XH_X$
$2N_YH_X$	$2N_ZH_X$	$2N_XH_Y$	$2N_YH_Y$	$2N_ZH_Y$	$2N_XH_Z$	$2N_YH_Z$	$2N_ZH_Z$

Time dependence of the relaxation was calculated step by step for each time increment, t_1 , by solving the $M(t_0 + t_1) = \exp(R_2^* t_1) M(t_0)$. τ_{CP} is 0.45 ms, and each CPMG loop of $(\tau'_{CP} - 180_N - \tau'_{CP} - 180_N - \tau''_{CP} - 180_H - \tau''_{CP} - 180_N - \tau'_{CP} - \tau'_{CP} - 180_N - \tau'_{CP})_2$ was set to be a 7.2 ms, and incremented to a total 72 ms. Here, τ'_{CP} and τ''_{CP} satisfy $\tau_{CP} = \tau'_{CP} + 90_N$ and $\tau_{CP} = \tau''_{CP} + 90_N + 90_H$. Simulation was done twice with the starting ^{15}N transverse magnetization at X and -X and detection at X and -X, respectively, for the phase cycle. The output is the average time course of the two phase cycles. In the relaxation matrix, ^1H and ^{15}N chemical shift and the 90-Hz ^1H - ^{15}N J-coupling evolution, r.f. pulse effects, auto and cross relaxation terms, and cross-correlation terms were included (Allard et al. 1997; Allard et al. 1998; Myint et al. 2009; Myint & Ishima 2009). In the calculation, ^{15}N and ^1H r.f. pulses were applied at field strengths of 5.56 kHz and 25 kHz, respectively, and ^{15}N signal off-resonance frequency, $\omega_{off}/2\pi$, was varied from -3000 to 3000 Hz in 200 Hz steps. Off-resonance frequency of the ^1H signal was set at 2250 Hz, which corresponds to 7.5 ppm at 900 MHz NMR. Relaxation terms in the matrix were calculated assuming a simple Lorentzen spectral density function with a 5 ns rotational correlation time, 170 ppm ^{15}N CSA, and 1.02 Å N-H distance. Additional ^1H longitudinal and transverse relaxation rates of 10 s^{-1} and 20 s^{-1} were added, in addition to the ^1H - ^{15}N dipolar term, to the ^1H relaxation (Myint et al. 2009).

Once the time dependence was calculated, R_2 and its uncertainty, $R_2^{\text{fit_err}}$, were determined by Monte-Carlo error estimation using the standard deviation of the residual ($I_i - I_i^{\text{fit}}$) of the fit as the uncertainty in I_i (Nicholson et al. 1992). However, $R_2^{\text{noise_err}}$ was not calculated

because experimental noise itself was not assumed in the simulation. The effect of the inhomogeneity of the B_1 field was simulated by assuming that relative magnetization intensities of 0.375, 0.5, and 0.125 experienced respective r.f. field strengths of $1.0B_1$, $1.05B_1$ and $1.1B_1$, respectively, where B_1 is the correctly calibrated field strength. Then, R_2 and $R_2^{\text{fit_err}}$ were determined from simulations of the weighted time course of the average magnetization. The simulation analysis for B_1 inhomogeneity was performed for the [00-00] and the [00-13] schemes individually. Calculations were performed using MATLAB software (The Mathworks Inc., Natick, MA).

Results and Discussions

The purposes of this study are to further identify systematic errors, that persist even when experiments are repeated (Bevington & Robinson 1992; Heinrich & Lyons 2007), in ^{15}N transverse relaxation rates, which are used to characterize protein backbone dynamics. In particular, uncertainties in CPMG R_2 values caused by systematic errors present when using phase schemes, [00-00], [00-13], and [0013-0013] are compared. For this purpose, we will firstly compare values of R_2 and $R_2^{\text{fit_err}}$ derived from simulations of measurements obtained using phase-schemes, [00-00] and [00-13]. Next, we will compare experimental results obtained using the [00-00], [00-13], and [0013-0013] schemes. In the evaluation of the experimental results, R_2 errors estimated from two sets of Monte-Carlo methods, $R_2^{\text{fit_err}}$ and $R_2^{\text{noise_err}}$, are compared as well as the R_2 values to identify systematic errors.

Simulated off-resonance frequency dependence of [00-00] and [00-13] at a practical r.f. power level

The time course of magnetization in a scalar-coupled two-spin system was calculated for the conventional phase [00-00] and the alternative phase [00-13], using r.f. pulse power of $\gamma_{\text{N}}B_1/2\pi = 5.6 \text{ kHz}$ (i.e., $90 \mu\text{s}$, 180° pulse) at varying off-resonance frequency, $\omega_{\text{off}}/2\pi$. Time courses of magnetization obtained by employing the [00-00] scheme at $\omega_{\text{off}}/2\pi = \pm 2400 \text{ Hz}$ exhibit non single-exponential decay profiles (Figure 1A). The profiles are basically consistent to the previous results (For example, Fig. 4 of the reference, (Yip & Zuiderweg 2004)). In contrast, the time course of magnetization obtained by employing the [00-13] scheme under the same conditions exhibited profiles that are closer to a single-exponential decay (Figure 1B). This result is consistent with previous observations (Bain et al. 2011; Yip & Zuiderweg 2004). Note that our simulation shows slightly different profiles at positive and negative $\omega_{\text{off}}/2\pi$. Such a difference is not observed in the simulation for a single spin system, or when ^1H signal is located at the r.f. carrier frequency in the two-spin system (data not shown). Thus, although an even number of ^1H pulses is applied in each CPMG cycle, the difference in profiles is most likely due to an asymmetry caused by an ^1H off-resonance effect. The effect of the coupled spin was not taken into account in the previous CPMG simulations for the alternative phase schemes (Bain et al. 2011; Yip & Zuiderweg 2004).

Once the time course of magnetization was simulated, R_2 was determined by assuming that each time course decayed as a single-exponential function. The resultant R_2 values are shown as a function of the absolute value of $\omega_{\text{off}}/2\pi$ in Figure 2A. R_2 values were

determined using both [00-00] and [00-13] schemes and are denoted as R_2^{00-00} and R_2^{00-13} , respectively. Both R_2 values were quite similar up to an off-resonance frequency, $\omega_{\text{off}}/2\pi = 1400$ Hz (i.e., $\omega_{\text{off}}/(\gamma_N B_1) = 0.25$), with average difference in R_2 1.2% and with the maximum difference in R_2 of 2.2% (Figure 2A). Within each individual scheme, the fractional root-mean square deviations of R_2 values in the range from $\omega_{\text{off}}/2\pi$ 0 to 1400 Hz were 0.20% and 0.17% for R_2^{00-00} and R_2^{00-13} , respectively. These results were obtained including all data except for the R_2^{00-00} point at 1200 Hz. As shown by an arrow in Figure 2A, the R_2^{00-00} at 1200 Hz was significantly higher than others. When the magnetization along X-axis starts CPMG duration and the pulses are applied from the X-axis, the Y-component of the transverse magnetization vector rotates through the Y-Z plane during the pulsing whereas the X-component stays mostly in the transverse plane. Thus, pulse imperfection effect caused by chemical shift precession during the pulsing becomes largest when a magnetization vector undergoes almost 360° precession between the adjacent CPMG pulses, as has been shown in other simulation and experimental results (Bain et al. 2011; Yip & Zuiderweg 2004).

At $\omega_{\text{off}}/2\pi > 1400$ Hz, deviations of R_2 by the off-resonance effects are observable in both R_2^{00-00} and R_2^{00-13} (Figure 2). In previous results, R_2^{00-13} profiles were reported to exhibit a smaller and smoother change than those of R_2^{00-00} (Bain et al. 2011; Yip & Zuiderweg 2004). This discrepancy between these results and ours is mostly caused by differences in experimental and simulation parameter: their applied B_1 field strength ($\gamma_N B_1/2\pi = 2.5$ kHz) was smaller than that of ours ($\gamma_N B_1/2\pi = 5.6$ kHz), and their τ_{CP} (0.35 ms) was shorter than ours (0.45 ms and 0.5 ms). The latter set of the parameters make the [00-00] profile unfavorable. In addition, even in the previous experimental results, reduced but clear deviations of R_2 caused by off-resonance effects were shown even in R_2^{00-13} (Bain et al. 2011; Yip & Zuiderweg 2004). Errors in R_2^{00-13} found at a large $\omega_{\text{off}}/2\pi$ values are not corrected by equation (1) because equation (1) does not contain off-resonance frequency as one of calibration parameters. Overall, using our practical conditions for ^{15}N relaxation measurements, R_2^{00-00} and R_2^{00-13} exhibit similar profiles almost within the entire experimentally required $\omega_{\text{off}}/2\pi$ range.

Uncertainty of each R_2 value, $R_2^{\text{fit-err}}$, was estimated from the Monte-Carlo method by assuming an average residual of the simulated intensities from the fit-intensities as a standard deviation for the normal distribution (Figure 2B). This $R_2^{\text{fit-err}}$ solely reflects a discrepancy from a single-exponential decay model. $R_2^{\text{fit-err}}$ for R_2^{00-13} exhibits very small uncertainties ($<0.1 \text{ s}^{-1}$ that correspond to $<1\%$ of R_2^{00-13}) in the entire $\omega_{\text{off}}/2\pi$ range. In contrast, $R_2^{\text{fit-err}}$ obtained for R_2^{00-00} exhibited large uncertainties at $\omega_{\text{off}}/2\pi > 1800$ Hz, reflecting non-exponential behavior of magnetization simulated using the [00-00] scheme (Figure 2B). Nevertheless, $R_2^{\text{fit-err}}$ values for R_2^{00-00} ($<2\%$) remained small for $\omega_{\text{off}}/2\pi < 1800$ Hz. Again this frequency range covers nearly all ^{15}N signals of backbone amides in diamagnetic proteins up to 20 ppm at a 91 MHz ^{15}N resonance frequency (Figure 2B). Overall, application of the practical B_1 field strength ($\gamma_N B_1/2\pi = 5.6$ kHz) provides similar R_2 values in both R_2^{00-00} and R_2^{00-13} schemes, within the spectral range of ± 1400 Hz.

Effects of pulse strength miscalibration on R_2 error, determined by simulation

Similar simulations of magnetization and the determinations of R_2 were performed varying the B_1 field strength keeping the pulse width constant (i.e., power miscalibration). At a 5% or 10% increase (miscalibration) of the B_1 field strength, R_2^{00-00} exhibits more variation of R_2 values at $\omega_{\text{off}}/2\pi < 1400$ Hz (Figure 3A), which is larger than the variation of R_2^{00-13} (Figure 3B). Similar to these observations, but more significantly, $R_2^{\text{fit_err}}$ for R_2^{00-00} (Figure 3D) became larger than that of R_2^{00-13} at the miscalibrated B_1 field strength (Figure 3E). In particular, the $R_2^{\text{fit_err}}$ for R_2^{00-00} increased in proportion to the power miscalibration (Figure 3D). Such differences between R_2^{00-00} and R_2^{00-13} , and between $R_2^{\text{fit_err}}$ for R_2^{00-00} and $R_2^{\text{fit_err}}$ for R_2^{00-13} are reduced when B_1 inhomogeneity is taken into account in the simulations (Figures 3C and F). Overall, the simulations demonstrate that the difference between R_2 obtained using R_2^{00-00} and R_2^{00-13} is insignificant for a relatively wide spectral width, provided that r.f. pulses are properly calibrated (Figure 2). However, the difference becomes significant when r.f. pulses are miscalibrated (Figure 3). In particular, the [00-13] scheme is more tolerant to miscalibration errors than the [00-00] scheme.

Experimental R_2 determined using [00-00], [00-13], and [0013-0013] sequences

To verify the predictions of these simulations, we conducted ^{15}N R_2 experiments for ubiquitin using a 900 MHz NMR instrument, and evaluated experimental results only for signals that were fit by a single-exponential decay function with $R_2^{\text{err}} < 1 \text{ s}^{-1}$ (Figure 4). Most of the R_2^{00-00} values were very similar to R_2^{00-13} values (Figure 4A). As shown in Figure 4C, R_2^{00-00} and R_2^{00-13} did not exhibit significant difference for signals at $\omega_{\text{off}}/2\pi < 500$ Hz, but the R_2 values increasingly differ as $\omega_{\text{off}}/2\pi$ (off-resonance) increases. In contrast, although the effect of resonance off-set for $R_2^{0013-0013}$ is basically similar to that of R_2^{00-13} (Figure 4B), $R_2^{0013-0013}$ exhibits differences from R_2^{00-13} even when, $\omega_{\text{off}}/2\pi < 500$ Hz (Figure 4D). Since the [0013-0013] scheme is more symmetric with respect to the ^1H 180° pulses than the [00-13] scheme, a possible explanation of the poor performance of the [0013-0013] scheme when $\omega_{\text{off}}/2\pi$ is small may be incomplete suppression of CSA-dipolar cross correlation. In the [0013-0013] scheme the period between ^1H 180° pulses is twice that of [00-13] scheme. As shown previously (Kay et al. 1992; Palmer et al. 1992), the cross correlation is suppressed in a two-spin system. However, artifacts in R_2 measurements may become significant because of the interactions with external protons that have been neglected in our simulations.

Monte-Carlo error estimation was calculated for ubiquitin R_2 values in two ways: using the spectral noise ($R_2^{\text{noise_err}}$) and using the residual of the fits ($R_2^{\text{fit_err}}$), to generate the synthetic data. Since ubiquitin is a rigid, small protein, amide signal heights were quite uniform (except for E24 that undergoes chemical exchange and exhibits severe line broadening at 900 MHz) as is evident in the R_2 correlations plotted in Figure 4. Therefore, $R_2^{\text{noise_err}}$ was approximately the same for all three datasets (Figure 5, circles). In contrast, $R_2^{\text{fit_err}}$ exhibits quite different profiles for R_2^{00-00} (Figure 5A, $\times$ -symbols) compared with R_2^{00-13} and $R_2^{0013-0013}$ (Figures 5B and 5C, $\times$ -symbols). $R_2^{\text{fit_err}}$ for R_2^{00-00} begins to increase at $\omega_{\text{off}}/2\pi$ ca. 1000 Hz, and becomes over 2 s^{-1} at $\omega_{\text{off}}/2\pi > 1800$ Hz (Figure 5A, $\times$ -symbols). This profile is consistent with the simulations that incorporate the effect of pulse miscalibration, Figure 3F, demonstrating the excellent performance of the calculations.

$R_2^{\text{fit_err}}$ for R_2^{00-13} and $R_2^{0013-0013}$ exhibits a more gradual increases as a function of $\omega_{\text{off}}/2\pi$, and stays smaller than $R_2^{\text{fit_err}}$ for R_2^{00-00} even at $\omega_{\text{off}}/2\pi \sim 1500$ Hz. This small gradual increase of R_2^{err} for the phase alternation experiments is also consistent with the simulation results, Figure 3F. Overall, in both R_2^{00-00} and R_2^{00-13} , $R_2^{\text{fit_err}}$ almost equals $R_2^{\text{noise_err}}$ when $\omega_{\text{off}}/2\pi$ is small, and increases as $\omega_{\text{off}}/2\pi$ increases. This increasing discrepancy between the $R_2^{\text{fit_err}}$ and $R_2^{\text{noise_err}}$ as $\omega_{\text{off}}/2\pi$ increases is clear evidence of the off-resonance related systematic error.

Off-resonance systematic error is not observed in large proteins

To test if off-resonance effects are observable in the case of a larger protein with less spectra sensitivity than ubiquitin, the CPMG experiments were also performed for HIV-1 protease using a 600 MHz instrument. In this experiment, the ^{15}N pulse carrier was placed at 117 ppm which is almost the center of the ^{15}N chemical shifts of the protease backbone amides. R_2^{00-00} for the 92 residues are on average 15.22 s^{-1} ($\pm 2.1 \text{ s}^{-1}$), which is approximately 1.5 times larger than that of ubiquitin and consistent with previous protease measurements (Freedberg et al. 2002). R_2^{00-00} values are relatively uniform in this $\omega_{\text{off}}/2\pi$ range (Figure 6A). $R_2^{\text{noise_err}}$ is 0.17 s^{-1} whereas $R_2^{\text{fit_err}}$ is 0.22 s^{-1} (Figure 6B). The $R_2^{\text{noise_err}}$ values are approximately 2% of R_2^{00-00} . Although some $R_2^{\text{fit_err}}$ are higher at $\omega_{\text{off}}/2\pi > 500$ Hz, the differences between $R_2^{\text{noise_err}}$ and $R_2^{\text{fit_err}}$ are not significant. Thus, differences in R_2^{err} estimated by two methods are not observed because the noise error is larger than the off-resonance error. This result is in contrast to that obtained for ubiquitin.

Summary

In this study, ^{15}N CPMG experiments with and without the phase alternation were investigated at the practical high pulse power ($\gamma_{\text{N}}B_1/2\pi = 5.6 \text{ kHz}$) and also with ^1H 180° pulses that are applied to suppress dipolar/CSA cross correlation. Since CPMG performance is determined by the field strength of the r.f. pulse ($\gamma_{\text{N}}B_1/2\pi$), the delay between the CPMG pulses (τ_{CP}), the off-resonance frequency ($\omega_{\text{off}}/2\pi$), and relaxation rates and scalar couplings, it is important to investigate the performance at optimal parameter settings. First, our results showed that, in the high power condition, both pulse phase schemes yield similar results unless resonance off-sets are very large. Our simulations reproduced the reduction of the measurement accuracy as $\omega_{\text{off}}/2\pi$ increases even with the phase alternation method, which is consistent with the experimental results. Second, when the pulse power is miscalibrated, the fit error becomes larger in the conventional CPMG than the phase alternation approach, even when the resonance off-set is small. Third, although the full (longer) phase alternation cycle has a better performance in theory, our experimental results show that the actual performance of the longer cycle was not improved in the ^{15}N experiments with ^1H 180° pulses.

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Abbreviations

CPMG	Carr-Purcell-Meiboom-Gill
R_2	transverse relaxation rate
R_2^{err}	uncertainty of transverse relaxation rate
$\omega_{\text{off}}/2\pi$	off-resonance frequency
r.f	radio frequency

References

- Akke M, Palmer AG III. Monitoring Macromolecular Motions on Microsecond to Millisecond Time Scales by R1 ρ -R1 Constant Relaxation Time NMR Spectroscopy. *J Am Chem Soc.* 1996; 118:911–912.
- Allard P, Helgstrand M, Hard T. A Method for Simulation of NOESY, ROESY, and Off-Resonance ROESY Spectra. *J Magn Reson.* 1997; 129:19–29. [PubMed: 9405212]
- Allard P, Helgstrand M, Hard T. The complete homogeneous master equation for a heteronuclear two-spin system in the basis of Cartesian product operators. *J Magn Reson.* 1998; 134:7–16. [PubMed: 9740725]
- Bain AD, Anand CK, Nie Z. Exact solution to the Bloch equations and application to the Hahn echo. *J Magn Reson.* 2010; 206:227–40. [PubMed: 20692859]
- Bain AD, Anand CK, Nie Z. Exact solution of the CPMG pulse sequence with phase variation down the echo train: application to R2 measurements. *J Magn Reson.* 2011; 209:183–194. [PubMed: 21316277]
- Bevington, PR.; Robinson, DK. Data Reduction and Error Analysis for the Physical Sciences. McGraw-Hill; New York: 1992.
- Bretthorst GL, Hutton WC, Garbow JR, Ackerman JJH. Exponential parameter estimation (in NMR) using Bayesian probability theory. *Concepts Magn Reson.* 2005; 27A:55–63.
- Bruschweiler R. New approaches to the dynamic interpretation and prediction of NMR relaxation data from proteins. *Curr Opin Structl Biol.* 2003; 13:175–183.
- Carver JP, Richards RE. General 2-Site Solution for Chemical Exchange Produced Dependence of T2 Upon Carr-Purcell Pulse Separation. *J Magn Reson.* 1972; 6:89–105.
- Chandrasekhar I, Clore GM, Szabo A, Gronenborn AM, Brooks BR. A 500 ps molecular dynamics simulation study of interleukin-1 beta in water. Correlation with nuclear magnetic resonance spectroscopy and crystallography. *J Mol Biol.* 1992; 226:239–250. [PubMed: 1619653]
- Czisch M, King GC, Ross A. Removal of systematic errors associated with off-resonance oscillations in T2 measurements. *J Magn Reson.* 1997; 126:154–157. [PubMed: 9252288]
- Davis DG, Perlman ME, London RE. Direct Measurements of the Dissociation-Rate Constant for Inhibitor-Enzyme Complexes Via the T-1-Rho and T-2 (Cpmg) Methods. *J Magn Reson, Ser B.* 1994; 104:266–275. [PubMed: 8069484]
- Dayie KT, Wagner G, Lefevre JF. Theory and practice of nuclear spin relaxation in proteins. *Annual Review of Physical Chemistry.* 1996; 47:243–282.
- Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A. Nmrpipe - a Multidimensional Spectral Processing System Based on Unix Pipes. *J Biomol NMR.* 1995; 6:277–293. [PubMed: 8520220]
- Eriksson MA, Berglund H, Härd T, Nilsson L. A comparison of 15N NMR relaxation measurements with a molecular dynamics simulation: backbone dynamics of the glucocorticoid receptor DNA-binding domain. *Proteins.* 1993; 17:375–90. [PubMed: 8108380]
- Farrow NA, Zhang O, Forman-Kay JD, Kay LE. Comparison of the backbone dynamics of a folded and an unfolded SH3 domain existing in equilibrium in aqueous buffer. *Biochemistry.* 1995; 34:868–878. [PubMed: 7827045]

- Freedberg DI, Ishima R, Jacob J, Wang YX, Kustanovich I, Louis JM, Torchia DA. Rapid structural fluctuations of the free HIV protease flaps in solution. *Protein Sci.* 2002; 11:221–32. [PubMed: 11790832]
- Freeman R, Hill HDW. Fourier Transform Study of NMR Spin-Lattice Relaxation by “Progressive Saturation”. *J Chem Phys.* 1971; 54:3367–3377.
- Fushman D, Cowburn D. Nuclear magnetic resonance relaxation in determination of residue-specific N-15 chemical shift tensors in proteins in solution: Protein dynamics, structure, and applications of transverse relaxation optimized spectroscopy. In: *Methods in Enzymology. Nuclear Magnetic Resonance of Biological Macromolecules.* 2001; 339(Pt B):109–126.
- Gullion T, Baker DB, Conradi MS. New, Compensated Carr-Purcell Sequences. *J Magn Reson.* 1990; 89:479–484.
- Heinrich J, Lyons L. Systematic errors. *Ann Rev Nucl Part Sci.* 2007; 57:145–169.
- Horita DA, Zhang W, Smithgall TE, Gmeiner WH, Byrd RA. Dynamics of the Hck-SH3 domain: comparison of experiment with multiple molecular dynamics simulations. *Protein Sci.* 2000; 9:95–103. [PubMed: 10739251]
- Huang GS, Oas TG. Submillisecond folding of monomeric lambda repressor. *Proc Natl Acad Sci U S A.* 1995; 92:6878–82. [PubMed: 7624336]
- Igumenova TI, Frederick KK, Wand AJ. Characterization of the fast dynamics of protein amino acid side chains using NMR relaxation in solution. *Chem Rev.* 2006; 106:1672–1699. [PubMed: 16683749]
- Ishima R, Torchia DA. Protein dynamics from NMR. *Nat Struct Biol.* 2000; 7:740–3. [PubMed: 10966641]
- Ishima R, Torchia DA. Error estimation and global fitting in transverse-relaxation dispersion. *J Biomol NMR.* 2005; 32:41–54. [PubMed: 16041482]
- Ishima R, Wingfield PT, Stahl SJ, Kaufman JD, Torchia DA. Using amide H-1 and N-15 transverse relaxation to detect millisecond time-scale motions in perdeuterated proteins: Application to HIV-1 protease. *J Am Chem Soc.* 1998; 120:10534–10542.
- Istratov AA, Vyvenko OF. Exponential analysis in physical phenomena. *Rev Sci Instrum.* 1999; 70:1233–1257.
- Jarymowycz VA, Stone MJ. Fast time scale dynamics of protein backbones: NMR relaxation methods, applications, and functional consequences. *Chem Rev.* 2006; 106:1624–1671. [PubMed: 16683748]
- Kay LE. NMR studies of protein structure and dynamics. *J Magn Reson.* 2005; 173:193–207. [PubMed: 15780912]
- Kay LE, Nicholson LK, Delaglio F, Bax A, Torchia DA. Pulse Sequences for Removal of the Effects of Cross Correlation between Dipolar and Chemical-Shift Anisotropy Relaxation Mechanisms on the Measurement of Heteronuclear T1 and T2 Values in Proteins. *J Magn Reson.* 1992; 97:359–375.
- Kay LE, Torchia DA, Bax A. Backbone dynamics of proteins as studied by nitrogen-15 inverse detected heteronuclear NMR spectroscopy: application to staphylococcal nuclease. *Biochemistry.* 1989; 28:8972–8979. [PubMed: 2690953]
- Korzhnev DM, Tischenko EV, Arseniev AS. Off-resonance effects in N-15 T-2 CPMG measurements. *J Biomol NMR.* 2000; 17:231–237. [PubMed: 10959630]
- Li Z, Raychaudhuri S, Wand AJ. Insights into the local residual entropy of proteins provided by NMR relaxation. *Protein Sci.* 1996; 5:2647–2650. [PubMed: 8976574]
- Lipari G, Szabo A. Model-free approach to the interpretation of nuclear magnetic resonance relaxation in macromolecules. 1. Theory and range of validity. *J Am Chem Soc.* 1982a; 104:4546–4559.
- Lipari G, Szabo A. Model-free approach to the interpretation of nuclear magnetic resonance relaxation in macromolecules. 2. Analysis of experimental results. *J Am Chem Soc.* 1982b; 104:4559–4570.
- Long D, Liu M, Yang D. Accurately probing slow motions on millisecond timescales with a robust NMR relaxation experiment. *J Am Chem Soc.* 2008; 130:2432–2433. [PubMed: 18247615]
- Loria JP, Rance M, Palmer AG III. A relaxation-compensated Carr-Purcell-Meiboom-Gill sequence for characterizing chemical exchange by NMR spectroscopy. *Journal of the American Chemical Society.* 1999; 121:2331–2332.

- Meiboom S, Gill D. Modified Spin-Echo Method for Measuring Nuclear Relaxation Times. *Rev Sci Instrum.* 1958; 29:688–691.
- Mulder FA, van Tilborg PJ, Kaptein R, Boelens R. Microsecond time scale dynamics in the RXR DNA-binding domain from a combination of spin-echo and off-resonance rotating frame relaxation measurements. *J Biomol NMR.* 1999; 13:275–288. [PubMed: 10212986]
- Myint W, Gong Q, Ishima R. Practical aspects of ¹⁵N CPMG transverse relaxation experiments for proteins in solution. *Concepts Magn Reson.* 2009; 34A:63–75.
- Myint W, Ishima R. Chemical exchange effects during refocusing pulses in constant-time CPMG relaxation dispersion experiments. *J Biomol NMR.* 2009; 45:207–16. [PubMed: 19618276]
- Nalam MN, Peeters A, Jonckers TH, Dierynck I, Schiffer CA. Crystal structure of lysine sulfonamide inhibitor reveals the displacement of the conserved flap water molecule in human immunodeficiency virus type 1 protease. *J Virol.* 2007; 81:9512–8. [PubMed: 17596316]
- Nicholson LK, Kay LE, Baldisseri DM, Arango J, Young PR, Bax A, Torchia DA. Dynamics of methyl groups in proteins as studied by proton-detected ¹³C NMR spectroscopy. Application to the leucine residues of Staphylococcal nuclease. *Biochemistry.* 1992; 31:5253–63. [PubMed: 1606149]
- Nirmala NR, Wagner G. Measurement of C-13 relaxation-times in proteins by two-dimensional heteronuclear H1-C-13 correlation spectroscopy. *J Am Chem Soc.* 1988; 110:7557–7558.
- Orekhov VY, Pervushin KV, Arseniev AS. Backbone dynamics of (1-71)bacterioopsin studied by two-dimensional ¹H-¹⁵N NMR spectroscopy. *Eur J Biochem.* 1994; 219:887–896. [PubMed: 8112340]
- Palmer AG III. Nmr probes of molecular dynamics: overview and comparison with other. *Annu Rev Biophys Biomol Struct.* 2001; 30:129–55. [PubMed: 11340055]
- Palmer AG III, Rance M, Wright PE. Intramolecular motions of a zinc finger DNA-binding domain from Xfin characterized by proton-detected natural abundance ¹³C Heteronuclear NMR spectroscopy. *J Am Chem Soc.* 1991; 113:4371–4380.
- Palmer AG III, Skelton NJ, Chazin WJ, Wright PE, Rance M. Suppression of the Effects of Cross-Correlation between Dipolar and Anisotropic Chemical-Shift Relaxation Mechanisms in the Measurement of Spin Spin Relaxation Rates. *Mol Phys.* 1992; 75:699–711.
- Peng JW, Wagner G. Frequency spectrum of NH bonds in eglin c from spectral density mapping at multiple fields. *Biochemistry.* 1995; 34:16733–16752. [PubMed: 8527448]
- Redfield C. Using nuclear magnetic resonance spectroscopy to study molten globule states of proteins. *Methods Mol Biol.* 2004; 34:121–132.
- Ross A, Czisch M, King GC. Systematic errors associated with the CPMG pulse sequence and their effect on motional analysis of biomolecules. *J Magn Reson.* 1997; 124:355–365.
- Skelton NJ, Palmer AG III, Akke M, Kordel J, Rance M, Chazin WJ. Practical Aspects of 2-Dimensional Proton-Detected N-15 Spin Relaxation Measurements. *J Magn Reson Ser B.* 1993; 102:253–264.
- Smith LJ, Mark AE, Dobson CM, van Gunsteren WF. Comparison of MD simulations and NMR experiments for hen lysozyme. Analysis of local fluctuations, cooperative motions, and global changes. *Biochemistry.* 1995; 34:10918–31. [PubMed: 7662673]
- Szyperski S, Luginbühl P, Otting G, Güntert P, Wüthrich K. Protein dynamics studied by rotating frame. *J Biomol NMR.* 1993; 3:151–164. [PubMed: 7682879]
- Viles JH, Duggan BM, Zaborowski E, Schwarzingner S, Huntley JJ, Kroon GJ, Dyson HJ, Wright PE. Potential bias in NMR relaxation data introduced by peak intensity analysis and curve fitting methods. *J Biomol NMR.* 2001; 21:1–9. [PubMed: 11693564]
- Wrabl JO, Shortle D, Woolf TB. Correlation between changes in nuclear magnetic resonance order parameters and conformational entropy: molecular dynamics simulations of native and denatured staphylococcal nuclease. *Proteins.* 2000; 38:123–33. [PubMed: 10656260]
- Yamasaki K, Saito M, Oobatake M, Kanaya S. Characterization of the internal motions of Escherichia coli ribonuclease HI by a combination of ¹⁵N-NMR relaxation analysis and molecular dynamics simulation: examination of dynamic models. *Biochemistry.* 1995; 34:6587–601. [PubMed: 7756290]

- Yang DW, Kay LE. Contributions to conformational entropy arising from bond vector fluctuations measured from NMR-derived order parameters: Application to protein folding. *J Mol Biol.* 1996; 263:369–382. [PubMed: 8913313]
- Yip GN, Zuiderweg ER. A phase cycle scheme that significantly suppresses offset-dependent artifacts in the R2-CPMG 15N relaxation experiment. *J Magn Reson.* 2004; 171:25–36. [PubMed: 15504678]

Figure 1. Simulated time course of transverse magnetization simulated using (A) the [00-00] sequence (circles) and (B) the [00-13] sequences (diamonds). In each figure, calculations were performed with $\omega_{off}/2\pi$ set equal to +2400 Hz (open circles) and -2400 Hz (closed circles).

Figure 2.

(A) Transverse relaxation rate, R_2 , and (B) its error, $R_2^{\text{fit_err}}$, determined by simulation using the [00-00] (filled circles) and the [00-13] (open diamonds) sequences, plotted as a function of the absolute off-resonance frequency, $\omega_{\text{off}}/2\pi$. Simulation of the time dependence of magnetization was performed varying $\omega_{\text{off}}/2\pi$ from -3000 to 3000 Hz at a 200 Hz step. Note that there are two data points determined using each pulse scheme at each $\omega_{\text{off}}/2\pi$ point, i.e., at the positive and the negative frequencies (Here, the two data points are shown by the same symbols). The arrow in (A) indicates a data point at a frequency that is close to the inverse of the CPMG pulse duration, a condition in which magnetization undergoes almost 360° precession during the period between the two adjacent CPMG pulses (see text). In (A), the dashed line indicates the original R_2 determined only by the auto relaxation. R_2 values are all plotted with $R_2^{\text{fit_err}}$ as the error bars, while some of the error bars may not be notably large. $R_2^{\text{fit_err}}$ s were estimated from the residual of the fits (see Methods section).

Figure 3.

(A, B, C) Transverse relaxation rate, R_2 , and (D, E, F) its error, $R_2^{fit_err}$, determined by simulation, plotted as a function of the off-resonance frequency, $\omega_{off}/2\pi$. Simulation was performed (A, D) using the [00-00] scheme, (B, E) using the [00-13] scheme, and (C, F) assuming the B_1 inhomogeneity for both. In all figures, circle and diamond symbols indicate for R_2 obtained using the [00-00] and the [00-13] schemes, respectively. In (A–E), simulation of the time dependence of magnetization was done at a correctly calibrated B_1 field strength of 5.56 kHz CPMG 180° pulse of 90 μ s (filled symbols), at a 5% stronger B_1 field strength (gray symbols), and at a 10% stronger B_1 field strength (open symbols). In (C) and (D), R_2 and $R_2^{fit_err}$ determination assuming B_1 inhomogeneity was performed for both of the [00-00] and the [00-13] schemes (see Methods section). In the calculation, $\omega_{off}/2\pi$ was varied from –3000 to 3000 Hz at a 200 Hz step. There are two data points determined using each condition at each $\omega_{off}/2\pi$, i.e., at the positive and the negative frequencies. In (A–C), R_2 values are all plotted with $R_2^{fit_err}$ as the error bars while some of the error bars may not be notably large.

Figure 4.

Transverse relaxation rate, R_2 , for ubiquitin experimentally determined using the [00-13] sequence was compared with (A) those obtained using the conventional [00-00] sequence and (B) those obtained using the [0013-0013] sequence using a 900 MHz NMR instrument. Difference of (C) the rates determined between the [00-00] and the [00-13] schemes, and (D) between the [0013-0013] and the [00-13] schemes are shown as a function of the off-resonance frequency, $\omega_{off}/2\pi$. Note that the number of relaxation data points corrected for the [0013-0013] sequence were smaller than those of [00-00] and [00-13] due to the longer cycle length. For this reason, a [00130031-00130031] sequence (Yip & Zuiderweg 2004) was not applied. Only the data points at $\omega_{off}/2\pi < 1500$ Hz were included because large error bars at larger resonance off-sets make it difficult to view the correlation plots. In (A) and (B), R_2 values are all plotted with $R_2^{\text{fit_err}}$ error bars but may not be notable when the errors are small.

Figure 5.

Uncertainties, R_2^{err} , of the transverse relaxation rates for ubiquitin experimentally determined using (A) [00-00], (B) [00-13], and (C) [0013-0013] sequences on a 900 MHz NMR instrument, shown as a function of the off-resonance frequency, $\omega_{\text{off}}/2\pi$. R_2 values that are shown in Figure 4 are used. An additional 16 sets of data that exhibited $R_2^{\text{err}} > 1 \text{ s}^{-1}$ were also included in the graphs in order to show how the uncertainty increases as a function of $\omega_{\text{off}}/2\pi$. R_2^{err} values were determined by two different Monte-Carlo error estimations in which the standard deviations of a Gaussian distribution function that generates synthetic data sets were given by rmsd of spectral noise, $R_2^{\text{noise_err}}$ (circle), and by rmsd of residuals of the initial fit intensities, $R_2^{\text{fit_err}}$ (x-symbols). Difference of the two uncertainties indicates errors other than experimental noise. R_2^{err} values above 2 s^{-1} are shown as the ceiling values.

Figure 6.

(A) Transverse relaxation rate, R_2 , and (B) its uncertainty, R_2^{err} , for HIV-1 protease experimentally determined using the [00-00] sequence on a 600 MHz NMR instrument with 117 ppm as ^{15}N pulse carrier frequency. In (A), R_2 values are all plotted with $R_2^{\text{fit_err}}$ error bars but may not be notable when the errors are small. In (B), R_2^{err} values were determined by two different Monte-Carlo error estimations in which the standard deviations of a Gaussian distribution function that generates synthetic data sets were given by rmsd of spectral noise, $R_2^{\text{noise_err}}$ (circle), and by rmsd of residuals of the initial fit intensities, $R_2^{\text{fit_err}}$ (x-symbols).