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Off-resonance effects in ^{15}N T_2 CPMG measurements

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Abstract

The systematic difference between T_2 values obtained from CPMG and $T_{1\rho}$ experiments was observed for backbone ^{15}N nuclei of bacterial ribonuclease barnase. Theoretical consideration suggests that the observed difference is caused by off-resonance effects of 180° pulses of the CPMG pulse train. Namely, at off-resonance conditions T_1-dependent secondary echo coherence pathways considerably contribute to the signal decay in the CPMG experiment and result in systematic (up to 10%) offset-dependent overestimation of ^{15}N T_2 measured by the CPMG technique. Under certain circumstances off-resonance effects result in dependence of ^{15}N T_2 on CPMG frequency, which might be erroneously interpreted as conformational exchange on the millisecond time-scale. A procedure for numerical correction of ^{15}N T_2 (CPMG) data is proposed.

Introduction

Data on transverse relaxation time T_2 for backbone ^{15}N nuclei are essential to study pico–nanosecond as well as micro–millisecond dynamics of proteins (Palmer et al., 1996). Measurements of ^{15}N T_2 are usually based on the Carr–Purcell–Meiboom–Gill (CPMG) sequence (see e.g. Farrow et al., 1994). Alternatively, ^{15}N T_2 might be obtained by performing $T_{1\rho}$ experiments (Peng et al., 1991; Zinn-Justin et al., 1997; Mulder et al., 1998). Both methods appear to be useful in studies of protein internal mobility. For example, it was shown that $T_{1\rho}$ and T_2 (CPMG) data complement each other when considering motions on the micro–millisecond time-scale (Mulder et al., 1999).

For accurate characterization of protein dynamics one needs ^{15}N T_2 measured at high precision and free of any systematic error. Special care is taken to get rid of undesirable effects leading to the systematic deviation of experimental ^{15}N T_2 from the actual values. A high ^{15}N pulse repetition rate in the CPMG pulse train is used to remove the effects of scalar coupling (Palmer et al., 1992). The effects of cross-correlated cross-relaxation are suppressed by application of ^{1}H 180° pulses during the relaxation period (Palmer et al., 1992). It was recently shown that oscillations due to off-resonance effects of 180° pulses might result in substantial errors in T_2 measured by the CPMG technique (Ross et al., 1997). Apart from oscillations, due to off-resonance effects magnetization during the CPMG sequence precesses out of the XY plane and decays with the effective relaxation time depending both on T_1 and T_2. In other words, off-resonance effects populate the coherence pathways where the magnetization spends some time along the Z direction, allowing multiple stimulated echoes (Simbrunner and Stollberger, 1995). In consequence of $T_1 > T_2$ this effect will lead to overestimation of the measured T_2 value.

Here we report on a systematic difference between ^{15}N T_2 obtained from CPMG and $T_{1\rho}$ experiments for small extracellular ribonuclease barnase from *Bacillus amyloliquefaciens*. Theoretical consideration suggests that the observed difference is accounted for by offset-dependent overestimation of T_2 (CPMG) due to off-resonance effects of 180° pulses of the CPMG pulse train.

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Methods

The measurements of T_1, off-resonance $T_{1\rho}$ and T_2 (CPMG) for ^{15}N nuclei of barnase were carried out on a 600 MHz (^{1}H) Varian *Unity* spectrometer. Spectra were acquired at 30 °C on a 1.0 mM sample of uniformly ^{15}N labeled protein dissolved in 90% H_2O/10% D_2O 10 mM potassium phosphate buffer at pH 6.5.

The pulse sequences of Farrow et al. (1994) were used for ^{15}N T_1 and T_2 (CPMG) measurements. The 90° ^{15}N pulse length was 54 μs. Delays Δ of the Δ-$180^{\circ}_{N(x)}$-Δ CPMG block were 250, 300, 400, 500 and 600 μs. The T_2 (CPMG) experiment with $\Delta = 500$ μs was repeated with three different ^{15}N carrier frequencies. The values of T_1 and T_2 (CPMG) were obtained by fitting of an exponent to the decay of signal intensities in spectra recorded with 12 relaxation delays ranging from 10 to 1000 ms in T_1 and from 0 to 200 ms in T_2 (CPMG) experiments. The measured ^{15}N T_1 and T_2 (CPMG) values appear to be quite uniform over the sequence of barnase with T_1 ranging from 480 to 560 ms and T_2 ranging from 120 to 140 ms.

The measurements of off-resonance $T_{1\rho}$ were carried out using the pulse sequence described by Mulder et al. (1998). Alignment of ^{15}N magnetization along the effective field was performed using a 5 ms tanh/tan adiabatic pulse. Off-resonance $T_{1\rho}$ were measured for 16 offsets of the ^{15}N spin-lock field ranging from −5000 to 5000 Hz from the center of the spectrum. The spin-lock field strength was 1050 ± 30 Hz. For each spin-lock offset the value of $T_{1\rho}$ was obtained by fitting of an exponent to the decay of signal intensities in 12 spectra recorded with spin-lock lengths ranging from 10 to 250 ms. ^{15}N T_2 were obtained by least square fitting of experimental $T_{1\rho}$ data by theoretical $T_{1\rho}$ calculated using the equation (Peng et al., 1991):

$$\frac{1}{T_{1\rho}} = \frac{1}{T_1}\cos^2(\theta) + \frac{1}{T_2}\sin^2(\theta) \qquad (1)$$

where $\theta = \arctan(\omega/\Omega)$, ω is the spin-lock field strength, Ω is the resonance offset from the carrier, T_1 is the experimental ^{15}N longitudinal relaxation time.

Temperature calibration and control in all experiments were performed as described in Orekhov et al. (1999). A one-dimensional ^{1}H spectrum was recorded in one scan immediately after each experiment. The methyl resonance in this spectrum, which position with respect to the solvent (lock) is most temperature sensitive, was used as an indicator of the mean temperature of the sample. The sample heating was ca. 0.3–0.4 K in T_2 (CPMG), 0.1 K in $T_{1\rho}$ with the longest 250 ms spin-lock and less than 0.1 K in T_1 experiments. The appropriate temperature compensation was performed in T_2 (CPMG) experiments. Besides, in order to reduce temperature oscillations T_2 (CPMG) was recorded in an interleaved manner (Orekhov et al., 1999). Thus, the temperature drift within individual experiments and the temperature difference between different experiments did not exceed 0.1–0.2 K.

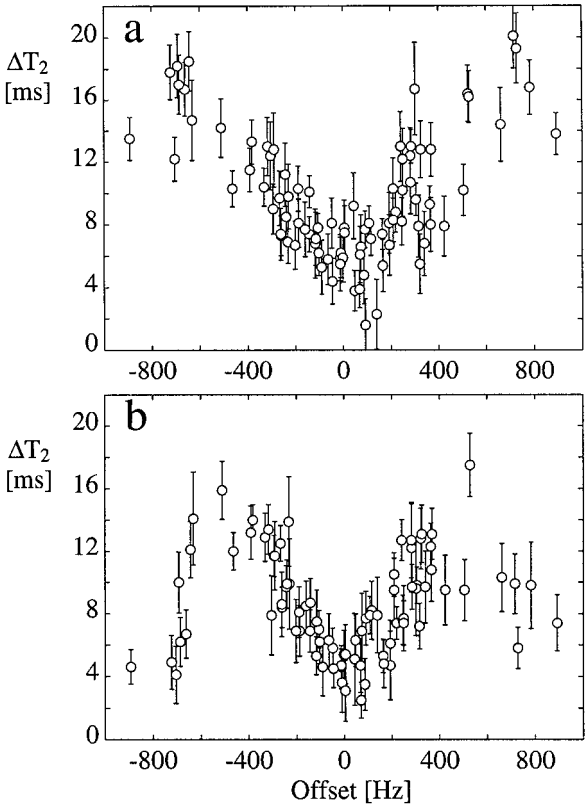

Figure 1. Difference ΔT_2 between T_2 (CPMG) and T_2 obtained from off-resonance $T_{1\rho}$ measurements for backbone ^{15}N nuclei of barnase plotted versus ^{15}N resonance offsets from the carrier frequency of 180° pulses of the CPMG pulse train. Delays Δ of the Δ-$180^{\circ}_{N(x)}$-Δ CPMG block are (a) 250 μs and (b) 500 μs.

Results and discussion

The differences ΔT_2 between the values of T_2 (CPMG) and T_2 calculated from T_1 and $T_{1\rho}$ data are plotted versus resonance offset from the carrier frequency of 180° pulses of the CPMG sequence in Figure 1. As can be seen from the figure the values of ^{15}N T_2 obtained from $T_{1\rho}$ data are systematically lower

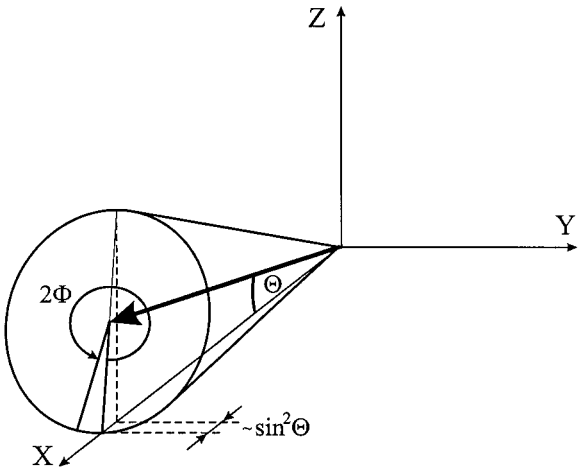

Figure 2. Schematic representation of the effective rotation of magnetization during the CPMG sequence. The angles Θ and Φ are given by Equation 2.

than those measured by the CPMG technique. Besides that, the difference ΔT_2 strongly depends on resonance offset from the ^{15}N carrier frequency in CPMG experiments: i.e., ΔT_2 has a clearly defined minimum at zero offset and wings symmetric with respect to zero offset. Similar dependencies were observed with shifted ^{15}N carrier frequency in T_2 (CPMG) experiments (data not shown), which clearly suggests that the observed difference is caused by off-resonance effects in the CPMG sequence. Larger ^{15}N T_2 (CPMG) values as compared to T_2 obtained from $T_{1\rho}$ data were also noted by Lee et al. (1998) for HIV-1 nucleocapsid protein and by Lee and Wand (1999) for ubiquitin, but remain unexplained.

Off-resonance effects in the CPMG sequence were theoretically considered by Ross et al. (1997). It was shown that the $(\Delta\text{-}180^\circ_{(x)}\text{-}\Delta)_{2n}$ CPMG block might be regarded as a single rotation of magnetization by an angle $2n\Phi$ about an effective axis in the XZ plane of the rotating frame tilted to the X axis by an angle Θ (Figure 2). The angles Φ and Θ (Figures 2 and 3) depend on resonance offset from the carrier (Ω), interpulse delay (2Δ), and field strength of the pulse (ω; in frequency units):

$$\tan(\Theta) = \cos(\lambda)\cot(\theta) + \sin(\lambda)\sin^{-1}(\theta)\cot(\phi/2) \qquad (2)$$

$$\cos(\Phi/2) = \cos(\lambda)\cos(\phi/2) - \sin(\lambda)\cos(\theta)\sin(\phi/2)$$

where $\lambda = \Omega\Delta$, $\theta = \arctan(\omega/\Omega)$, $\phi = k\pi\times\sqrt{1+(\omega/\Omega)^2}$, k is the factor accounting for pulse imperfection ($k = 1$ for a perfectly calibrated pulse).

After 2n repetitions of the $\Delta\text{-}180^\circ_{(x)}\text{-}\Delta$ CPMG block, magnetization is not aligned along the X axis, but precesses on the surface of a cone determined by the angles Φ and Θ (Figure 2). Thus, offset-dependent oscillations of amplitude $\sin^2(\Theta)$ are superimposed to the exponential decay of the experimentally observed X-component of the magnetization. These oscillations might lead to deviation of ^{15}N T_2 (CPMG) from the actual values.

The above consideration (Ross et al., 1997) is valid, however, only in the case of $T_1 = T_2$. An accurate analysis of relaxation during the CPMG sequence is available through numerical simulations. Rigorously, evolution of 16 components of magnetization corresponding to product operators of a two-spin system should be considered (see, e.g., Allard et al., 1998). However, the numerical analysis showed that at standard CPMG settings used in ^{15}N T_2 experiments the simulations can be safely carried out using the master equation for the ^{15}N nucleus alone:

$$\frac{d}{dt}\begin{bmatrix}\langle E/2\rangle\\ \langle N_x\rangle\\ \langle N_y\rangle\\ \langle N_z\rangle\end{bmatrix} = -\begin{bmatrix}0 & 0 & 0 & 0\\ 0 & 1/T_2 & \Omega & -\omega_y\\ 0 & -\Omega & 1/T_2 & \omega_x\\ -2F & \omega_y & -\omega_x & 1/T_1\end{bmatrix}\begin{bmatrix}\langle E/2\rangle\\ \langle N_x\rangle\\ \langle N_y\rangle\\ \langle N_z\rangle\end{bmatrix} \qquad (3)$$

where $\langle N_x\rangle$, $\langle N_y\rangle$ and $\langle N_z\rangle$ are X, Y and Z-components of ^{15}N magnetization, E is the unity operator, ω_x and ω_y are the X and Y components of the radio-frequency field (in frequency units), Ω is the ^{15}N offset from the carrier, $F = M_0/T_1$, M_0 is equilibrium ^{15}N magnetization. The solution of Equation 3 is written as:

$$\mathbf{B}(t) = \exp[-\mathbf{R}_n\Delta t_n]..\exp[-\mathbf{R}_1\Delta t_1]\mathbf{B}(0) \qquad (4)$$

where $\mathbf{B}(0)$ corresponds to the initial conditions, $\mathbf{R}_i$ is a 4×4 matrix (Equation 3) at the Δt_i period corresponding to a radio-frequency pulse or chemical shift evolution. If ^{15}N T_1, T_2 values, resonance offset from the carrier (Ω) and pulse field strength (ω) are known one might generate the trace of the X-component of ^{15}N magnetization sampled at a different number of repetitions of a single CPMG block (single CPMG 'block' means two repetitions of the $\Delta\text{-}180^\circ_{N(x)}\text{-}\Delta$ fragment). An apparent relaxation time T_{2app} is then obtained by least square fitting of an exponent to the generated decay of the X-component of ^{15}N magnetization.

We have carried out the numerical simulation of the CPMG sequence for a ^{15}N nucleus with $T_1 = 600$ ms and $T_2 = 100$ ms (these relaxation times correspond to $S^2 = 0.936$, $\tau_e = 0$, $\tau_R = 7.141$ ns calculated using Lipari and Szabo (1982) spectral density

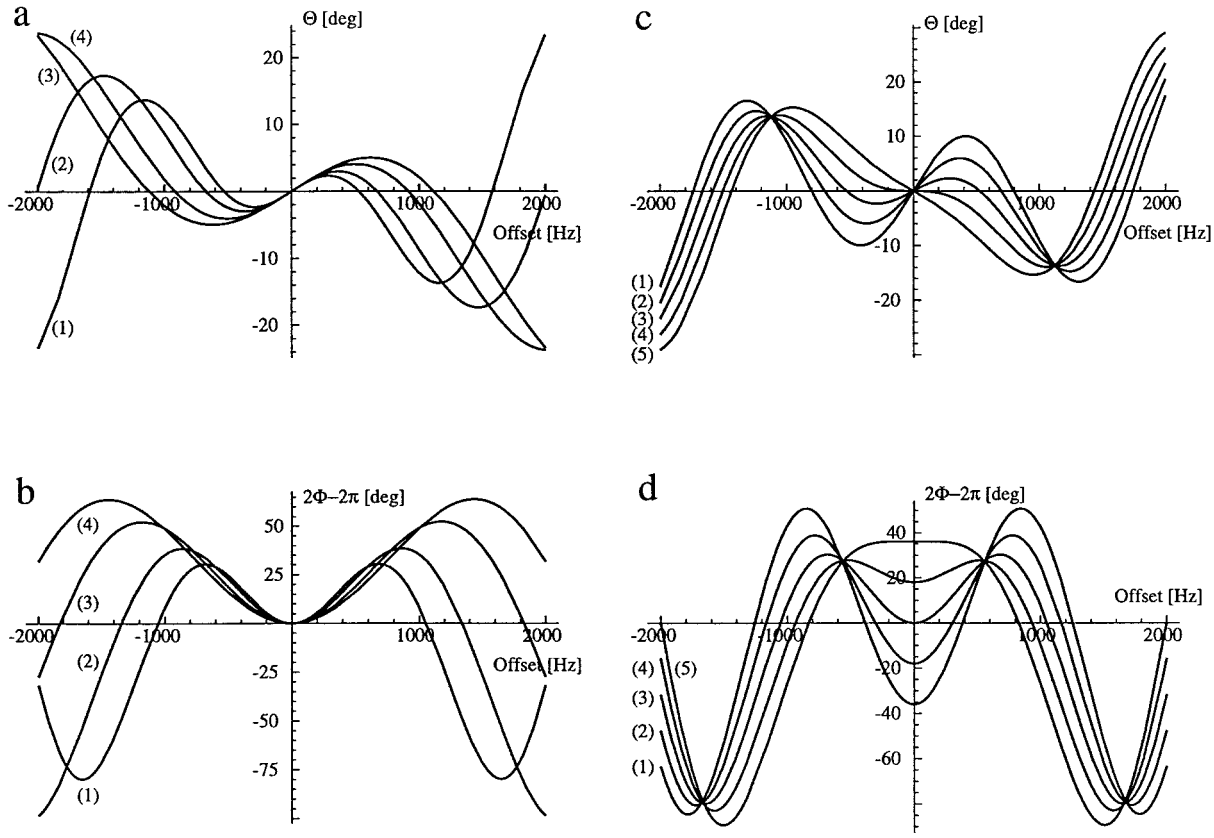

Figure 3. Angles Φ and Θ (Figure 2; Equation 2) plotted versus resonance offset from the carrier frequency of 180° pulses of the CPMG sequence. (a,b) Curves 1, 2, 3 and 4 correspond to delays Δ of 500, 400, 300 and 250 μs, respectively. (c,d) Delay Δ is 500 μs, curves 1 and 2 correspond to 10% and 5% underestimated ^{15}N pulse length, curve 3 is the exact pulse, curves 4 and 5 are 5% and 10% overestimated ^{15}N pulse length. The exact 90° ^{15}N pulse length is always 54 μs.

for the backbone NH vector at 600 MHz (^{1}H) spectrometer frequency). The simulation was performed for ^{15}N offsets Ω ranging from −2000 to 2000 Hz, for different 90° ^{15}N pulse lengths (40, 54 and 70 μs) and different delays Δ of the Δ-180°$_{N(x)}$-Δ CPMG block (250, 300, 400 and 500 μs). The results of the numerical simulation (Figure 4) show that the apparent relaxation time T_{2app} is always overestimated with respect to the actual value. The maximal difference ΔT_2 between T_{2app} and the actual T_2 value reaches 3–10%, depending on CPMG settings, for ^{15}N offsets Ω typical for proteins (e.g. ranging from −1000 to 1000 Hz at 600 MHz (^{1}H) spectrometer frequency). In particular, T_{2app} increases with increasing 90° ^{15}N pulse length (Figure 4a) and depends on delay Δ of the Δ-180°$_{N(x)}$-Δ CPMG block (Figure 4b). The effect of oscillations, predicted by Ross et al. (1997), becomes observable near the extreme values of $\sin^2(\Theta)$ (Equation 2; Figures 3 and 4). However, for Δ < 500 μs and a ^{15}N spectral width typical for proteins the oscillations negligibly affect the calculated effective relaxation time T_{2app}. Also, until off-resonance oscillations are negligible the relaxation time T_{2app} is almost independent of both the number of sampling points in the generated CPMG decay and the particular sampling scheme. The overestimation of T_{2app} correlates with the angle Φ (Equation 2; Figures 3 and 4) – the maximal deviation of 2Φ from 2π corresponds to the maxima of $\Delta T_2 = T_{2app} - T_2$.

The simulation of magnetization decay during the CPMG sequence provides the means for numerical correction of experimental T_2 (CPMG) data. The correction is performed as follows: first, the apparent relaxation time T_{2app} is calculated as described above on the basis of experimental T_1 and T_2 (CPMG) values, resonance offset from the carrier Ω and pulse field strength ω; then, the difference ΔT_2 between simulated T_{2app} and experimental T_2 (CPMG) is subtracted

from the experimental T_2 (CPMG) value. It is notable that for small ΔT_2 the relative difference $\Delta T_2/T_2$ corresponds to $-\Delta R_2/R_2$ for relaxation rates.

The proposed procedure for T_2 (CPMG) correction requires perfectly calibrated 180° ^{15}N pulses. The simulations show that overestimation of T_{2app} considerably depends on settings of the 180° ^{15}N pulse length in the CPMG sequence (Figure 4c; see also Figure 3c,d). In some cases over- or underestimation of pulse length results in oscillations in T_{2app} for ^{15}N resonance offsets typical for proteins (Figure 4c). It is clear that radio-frequency (RF) field inhomogeneity will result in a different pulse length for different parts of the sample. Thus, Figure 4c might be regarded as an illustration of the expected accuracy of the correction procedure in the case of *in amplitude* RF field inhomogeneity – for an RF field varying within ±5% under the conditions of Figure 4c one should expect ca. 2% uncertainty in the corrected T_2 value.

The model calculations presented here were carried out for a protein of intermediate size (τ_R = 7.141 ns) assuming 600 MHz (^{1}H) spectrometer frequency. It is notable that an overestimation of ^{15}N T_2 (CPMG) is expected to increase for a protein of large size and for higher magnetic fields – i.e., with increase of the difference between ^{15}N transverse and longitudinal relaxation rates.

The observed offset-dependent difference between T_2 (CPMG) and T_2 obtained from T_1 and $T_{1\rho}$ data for ^{15}N nuclei of barnase (Figure 1) is well reproduced by numerical simulations (see Figure 4b). However, even at zero offset experimental T_2 (CPMG) systematically exceed the values obtained from T_1 and $T_{1\rho}$ data (Figure 1). This points to additional sources of systematic error in either T_2 (CPMG) or $T_{1\rho}$ experiments. Among these sources might be an inhomogeneous RF field of 180° pulses of the CPMG pulse train, spin-lock field inhomogeneity and power losses after RF irradiation in the $T_{1\rho}$ experiment (Guenneugues et al., 1999), inaccurate calibration of the spin-lock field strength affecting T_2 obtained by least-square fitting of $T_{1\rho}$ data (for our $T_{1\rho}$ data set a 50 Hz underestimated spin-lock field results in ca. 2% underestimated T_2).

Ross et al. (1997) considered transfer of the errors arising due to off-resonance oscillations in T_2 (CPMG) experiments to motional parameters obtained from the subsequent data analysis. It is clear that the offset-dependent overestimation of ^{15}N T_2 (CPMG) would also result in substantial errors in motional parameters. In particular, overestimation of T_2 leads to underestimation of the overall rotation correlation time τ_R

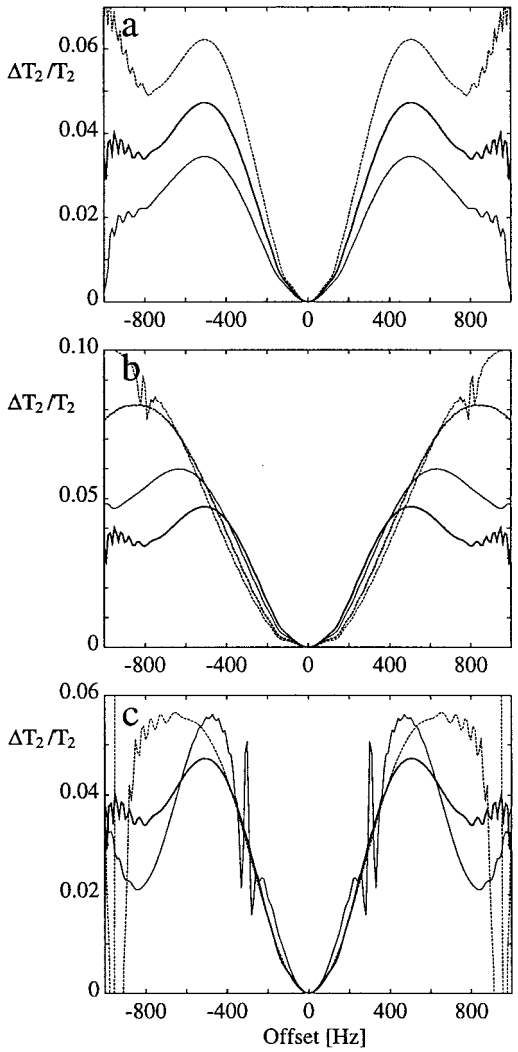

Figure 4. Relative difference $\Delta T_2/T_2$ ($\Delta T_2 = T_{2app} - T_2$) characterizing deviation of apparent relaxation time T_{2app} (CPMG) from the actual T_2 value versus resonance offset from the carrier frequency of ^{15}N 180° pulses of the CPMG sequence. T_{2app} (CPMG) was calculated by fitting of an exponent to the decay of transverse ^{15}N magnetization generated using Equations 3 and 4 for different CPMG settings (actual T_1 = 600 ms and T_2 = 100 ms). (a) Exact ^{15}N 90° pulse length = 70, 54 and 40 μs (from top to bottom), Δ = 500 μs. (b) Exact ^{15}N 90° pulse length = 54 μs, Δ = 250, 300, 400 and 500 μs (from top to bottom at 1000 Hz offset). (c) Δ = 500 μs and different ^{15}N pulse length: 5% underestimated: dashed line; exact (90° pulse of 54 μs): bold solid line; and 5% overestimated: thin solid line.

obtained from the T_1/T_2 ratio (Kay et al., 1989). In the subsequent 'model-free' analysis one should expect erroneous values of order parameters and correlation times of internal motions or even wrong selection of the model of spectral density function (Korzhnev et al., 1997).

The dependence of ^{15}N T_2 on pulse repetition rate in the CPMG sequence is often used for identification of conformational exchange on the micro–millisecond time scale (Orekhov et al., 1994). In some cases off-resonance effects might result in dependence of ^{15}N T_2 on the CPMG frequency, similar to those characteristic of millisecond conformational exchange. From Figure 4b it is clearly seen that for ^{15}N offsets $|\Omega|$ ranging from 500 to 1000 Hz T_{2app} increases with increasing CPMG pulse repetition rate, which might be erroneously interpreted as evidence of millisecond conformational exchange.

Using T_2 (CPMG) data without correction for off-resonance effects results in erroneous detection of conformational exchange for several residues of barnase. Two sets of ^{15}N T_2 data were considered: (i) raw experimental T_2 (CPMG) and (ii) T_2 (CPMG) corrected using the procedure described above. The ^{15}N T_2 data recorded with different delays Δ of the CPMG sequence were fitted by two models: (i) the 'simple' model (the data were approximated by constant T_2) and (ii) the model of two-state conformational exchange (Bloom et al., 1965; Orekhov et al., 1994). The selection of the appropriate model was carried out as described in Mandel et al. (1995): i.e., a more complex 'exchange' model is accepted if the 'simple' model is rejected based on the χ^2 criterion with 95% confidence and an F-test confirms that reduction of χ^2 loss function is meaningful with at least 80% confidence. With these criteria, on the basis of raw experimental T_2 (CPMG) data the amide groups of seven residues of barnase: T6, G9, G40, G53, F56, G61 and Y103, were found to be involved in millisecond conformational exchange. It is notable that the ^{15}N resonance offset from the carrier for all of these residues exceeds 600 Hz at 600 MHz (^{1}H) spectrometer frequency. For another two residues, N58 and E60, the 'exchange' model is accepted if 70% F-test confidence is assumed. The analysis of the corrected T_2 (CPMG) data showed that most of the observed conformational exchange is artificial. If the corrected data set is used, the 'exchange' model is accepted only for two residues, T6 and G9, with 80% F-test confidence and for three residues, N58, E60 and F82, with 70% F-test confidence.

Conclusions

It was shown that off-resonance effects of 180° pulses of the CPMG sequence lead to considerable offset-dependent overestimation of ^{15}N T_2 values. This overestimation is due to ^{15}N magnetization precessing out of the XY plane during the CPMG sequence and decays with a rate constant depending on T_1 and T_2. Several experimental schemes were proposed to ensure that the magnetization precesses in the XY plane for all considered nuclei. Czisch et al. (1997) proposed to apply field gradients during the CPMG pulse train. Zweckstetter and Holak (1998) proposed to use adiabatic instead of hard 180° pulses. However, the authors noted that the effects of diffusion, if field gradients are applied, or evolution of the magnetization during relatively long adiabatic pulses should be carefully accounted for when using these methods for T_2 measurements. Thus, to obtain accurate values of ^{15}N T_2 it seems to be reasonable to use $T_{1\rho}$ instead of T_2 (CPMG) measurements or perform the numerical correction of T_2 (CPMG) data. In fact, using $T_{1\rho}$ instead of T_2 (CPMG), due to errors associated with off-resonance effects in the CPMG experiment, was also suggested by Tjandra et al. (1996) and Ross et al. (1997). It should be noted, however, that the accuracy of T_2 obtained from $T_{1\rho}$ data might be substantially deteriorated by inhomogeneity of the spin-lock field and power losses after long RF irradiation (Guenneugues et al., 1999).

Acknowledgements

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