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Communication

Cross-correlated spin relaxation effects in methyl ^{1}H CPMG-based relaxation dispersion experiments: Complications and a simple solution

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Abstract

Artifacts associated with the measurement of methyl ^{1}H single quantum CPMG-based relaxation dispersion profiles are described. These artifacts arise due to the combination of cross-correlated spin relaxation effects involving intra-methyl ^{1}H–^{1}H dipolar interactions and imperfections in ^{1}H refocusing pulses that are applied during CPMG intervals that quantify the effects of chemical exchange on measured transverse relaxation rates. As a result substantial errors in extracted exchange parameters can be obtained. A simple 'work-around' is presented where the ^{1}H chemical shift difference between the exchanging states is extracted from a combination of ^{13}C single quantum and ^{13}C–^{1}H multiple quantum dispersion profiles. The approach is demonstrated with an application to a folding/unfolding reaction involving a G48M mutant Fyn SH3 domain.

NMR spectroscopy is a powerful technique for the study of molecular exchange processes (Palmer et al., 2001). The sensitivity of solution NMR to chemical exchange was already apparent in the first reported high resolution investigation of a molecule, that of ethanol, showing evidence of exchange involving the hydroxyl proton (Arnold, 1956). In the intervening half century since this seminal work there have been many more studies of exchange, including applications involving complex systems such as biomolecules. The development of multi-dimensional NMR methods and isotope labeling schemes have significantly impacted on the types of exchange problems that can be investigated. Many of the experiments for the study of exchange dynamics in proteins focus on AX spin system probes (A=^{15}N or ^{13}C, X=^{1}H), such as backbone ^{15}N–^{1}HN (Loria et al., 1999; Tollinger et al., 2001; Ishima & Torchia, 2003; Dittmer & Bodenhausen, 2004; Korzhnev et al., 2004b; Massi et al., 2004; Orekhov et al., 2004) or $^{13}C^{\alpha}$–$^{1}H^{\alpha}$ (Hill et al., 2000) spin pairs, since the underlying spin physics is well understood (Palmer et al., 2001). It is therefore possible, with straightforward manipulations, to separate the effects of chemical exchange from those associated with other relaxation processes, such as spin flips, that manifest due to the interaction of the spin probe with external spins, for example (Loria et al., 1999). Complications can also arise from cross-correlated interactions from within the probe spin system. For example, in the case of studies involving ^{15}N relaxation, suppression of dipole–CSA interactions during CPMG pulse trains (Carr & Purcell, 1954; Meiboom & Gill, 1958) that

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monitor chemical exchange is accomplished through the insertion of elements that refocus such effects over the course of the relaxation interval(s) (Loria et al., 1999). In the case of more complicated spin systems such as AX_N groups (N > 1) constant-time approaches can be employed so that multi-exponential decay processes that derive from dipole–dipole relaxation interactions do not compromise extraction of exchange parameters from $A=^{15}N$ (Mulder et al., 2001) or ^{13}C (Skrynnikov et al., 2001) dispersion experiments. Unfortunately, the corresponding CPMG experiments that quantify exchange by measuring the relaxation of $X=^{1}H$ spins in AX_N moieties may still be subject to complications that severely limit the extraction of accurate chemical exchange information in the (general) case where X refocusing pulses are not perfect. Here we present an example of such a situation involving the use of ^{1}H single quantum CPMG-based dispersion profiles of $^{13}CH_3$ probes

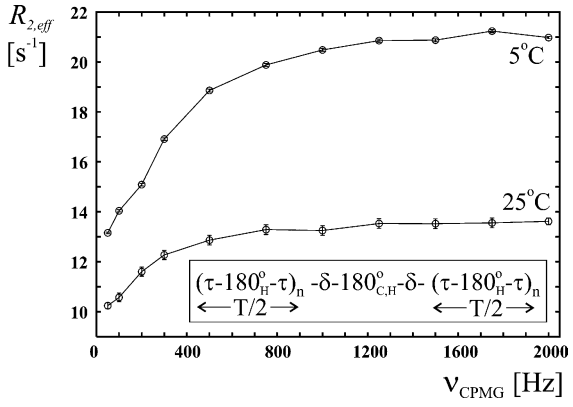

Figure 1. $R_{2,eff}$ vs.ν_{CPMG} ^{1}H single quantum CPMG dispersion profile for Val 49γ2 of U-[^{2}H] Ileδ1-[$^{13}CH_3$] Leu,Val-[$^{13}CH_3$,$^{12}CD_3$] labeled protein L, 100% D_2O, at 25 (correlation time $\tau_c = 5$ ns) and 5°C ($\tau_c = 10$ ns), 11.4*T*. The sample of protein L was prepared as described previously (Korzhnev et al., 2004a). Dispersion profiles for all Ile, Leu and Val methyls in protein L have features very similar to those of Val 49γ2 (with the exception of Leu8, see text). Values of $R_{2,eff}$ and associated errors were calculated as described previously (Korzhnev et al., 2004b). Error bars are indicated with vertical lines. The inset shows the constant-time element with variable numbers of ^{1}H refocusing pulses used in the pulse scheme. This element consists of two periods of duration *T*/2 with a refocusing interval to convert in-phase ^{1}H magnetization to anti-phase magnetization with respect to the attached ^{13}C as described by Loria et al. (1999) ($\delta = 1/(4J_{CH})$). A value of $T = 40$ ms was employed. The scheme used is similar to an experiment developed by Ishima and Torchia for the measurement of ^{1}H single quantum dispersions in ^{15}N–^{1}HN amide spin systems (2003).

of exchange in a protein. We show that exchange parameters that would normally be obtained from the ^{1}H-based dispersion experiments can be significantly affected by intra-methyl dipole–dipole cross-correlated relaxation unless ^{1}H refocusing pulses during the CPMG train are perfect. An alternative approach is suggested that involves recording ^{13}C–^{1}H multiple quantum (Korzhnev et al., 2004a) and ^{13}C single quantum (Skrynnikov et al., 2001) dispersions, to obtain exchange information that is 'unique' to the ^{1}H single-quantum experiment.

The relaxation of both ^{1}H and ^{13}C coherences in $^{13}CH_3$ methyl groups has been the subject of both theoretical and experimental studies over the past several decades (Werbelow & Marshall, 1973; Werbelow & Grant, 1977; Vold & Vold, 1978; Kay & Prestegard, 1987; Muller et al., 1987; Kay & Torchia, 1991). A complete description of transverse relaxation in the general case involves consideration of all 10 single quantum ^{1}H and 8 single quantum ^{13}C transitions, as well as a large number of higher order coherences. Because of the degeneracies in the spin system many of the transitions can cross relax efficiently with each other (see Figure 2 of Tugarinov et al. (2003) for an energy level diagram), leading to a complex network of relaxation. The situation is simplified, however, in the macromolecular limit, and assuming that the methyl is isolated from other proton spins and that it rotates rapidly about its three-fold axis. In this case the relaxation of the individual single quantum transitions is single exponential and, further, the decay of each transition is either fast due to the constructive addition of local dipolar fields or much slower, resulting from the cancellation of intra-methyl dipolar interactions (Kay & Torchia, 1991; Tugarinov et al., 2003). Thus, after a 90° excitation pulse, the decay of ^{1}H magnetization from a rapidly rotating methyl group tumbling in the macromolecular limit can be expressed as follows,

$$S(T) = 0.5\,\exp(-T/T_{2,f}) + 0.5\,\exp(-T/T_{2,s}), \quad (1)$$

where $T_{2,f}$ and $T_{2,s}$ are decay constants for the rapidly and slowly relaxing proton single quantum transitions and chemical shift evolution has been neglected. Expressions for $T_{2,f}$ and $T_{2,s}$ are given elsewhere (Tugarinov et al., 2003); $T_{2,f}$ contains

contributions from both ^{1}H–^{1}H and ^{13}C–^{1}H intra-methyl dipole interactions, while only ^{13}C–^{1}H terms are important for $T_{2,s}$.

Central to the single quantum ^{1}H CPMG relaxation dispersion experiment is a pair of constant-time elements of duration $T/2$, during which a variable number of ^{1}H 180° pulses are applied to refocus proton magnetization, separated by a - δ 180°_{CH}-δ block, Figure 1 inset. Ishima and Torchia have described such a scheme for the study of exchange at backbone amide proton positions in ^{15}N labeled, deuterated proteins (2003) and the basic experiment is very similar for methyl probes, with ^{15}N pulses replaced by ^{13}C pulses and the δ delays optimized to account for the differences in one bond ^{15}N–^{1}HN and ^{13}C–^{1}H scalar couplings. Briefly, in systems that show exchange, the effective decay of magnetization during this constant-time interval, $R_{2,\mathrm{eff}}$, can depend on the frequency of pulse repetition, $\nu_{\mathrm{CPMG}} = 1/(4\tau)$, where 2τ is the time between application of pulses and $R_{2,\mathrm{eff}} = -1/T \ln(I/I_0)$, with I and I_0 the intensities of signal in the presence and absence of the constant-time element (Mulder et al., 2001). The dependence of $R_{2,\mathrm{eff}}$ on ν_{CPMG} is a function of the parameters describing the exchange process, such as the exchange rates, populations of interconverting states and their chemical shift differences (Palmer et al., 2001).

Figure 1 shows the $R_{2,\mathrm{eff}}$ vs ν_{CPMG} ^{1}H single quantum CPMG dispersion profile for Val 49γ2 of U-[^{2}H] Ileδ1-[$^{13}CH_3$] Leu,Val-[$^{13}CH_3$,$^{12}CD_3$] labeled protein L, 100% D_2O, at 25 (rotational correlation time $\tau_c = 5$ ns) and 5°C ($\tau_c = 10$ ns), 11.4T. A highly deuterated sample has been employed in this study to eliminate ^{1}H–^{1}H scalar couplings and to minimize cross-relaxation pathways that would otherwise interfere with the dispersion profiles in a manner which is ν_{CPMG} dependent (Ishima & Torchia, 2003). Essentially all but a pair of the methyl ^{1}H curves have the same features (the only exception are those for Leu 8, see below). Namely, $R_{2,\mathrm{eff}}$ increases with pulse repetition rate, with (concave) dispersions growing in size with molecular correlation time (compare curves at 25 and 5°C). It is noteworthy that ^{13}C single quantum or ^{13}C–^{1}H multiple quantum dispersions recorded on the same sample are all flat (data not shown), indicating that there are no millisecond exchange processes at the level of methyl groups in this protein.

The concave profiles can be explained by noting that magnetization (in the absence of exchange) evolves during the constant-time element, T, as in Equation (1) and since $T_{2,f} < T_{2,s}$ there is an 'imbalance' in the amount of fast and slowly relaxing magnetization. If the ^{1}H 180° pulses that are applied during T perfectly refocus magnetization there is no cross-talk between fast and slowly relaxing coherences, assuming that the methyl is in a highly deuterated background (i.e., so that ^{1}H–^{1}H spin flips do not exchange components). In contrast, pulse imperfections interconvert fast and slowly decaying coherences from within the same manifold (as well as creating other coherences) and because the amount of slowly decaying magnetization very quickly exceeds that associated with the fast relaxing transitions there is a net transfer of magnetization from slow to fast decaying coherences. This leads to an increase in $R_{2,\mathrm{eff}}$ with ν_{CPMG}, at least for low to moderate pulse repetition rates, even in the absence of exchange. Clearly if $T_{2,f} = T_{2,s}$ the effect will be negligible and it will increase as the relaxation times become progressively different. For Val 49γ2 (Figure 1) $T_{2,f} \sim 35$ (15) ms and $T_{2,s} \sim 400$ (170) ms at 25°C(5°C) so that for $T = 40$ ms the relative amounts of slow vs fast relaxing ^{1}H magnetization are much more skewed at the low temperature, leading to the larger profile. Notably, the dispersion curves for Leu 8 (both δ1 and δ2) are much more flat than for the other residues. The order parameters characterizing the amplitudes of motion of the methyls of Leu 8 are the smallest in the protein, with S^2 values of 0.30 (δ1,δ2) at 25°C and 0.31, 0.37 (δ1,δ2) at 5°C (Skrynnikov et al., 2002); for low order parameters $T_{2,f}$ and $T_{2,s}$ become similar and the concave dispersions decrease.

The example of Figure 1 illustrates the difficulty with recording ^{1}H single quantum dispersions of $^{13}CH_3$ groups. It is possible to get exchange information that is unique to ^{1}H dispersions (namely the differences in ^{1}H chemical shifts between exchanging states) in an indirect manner, however, by recording both ^{13}C single quantum (Skrynnikov et al., 2001) and ^{13}C–^{1}H multiple quantum (Korzhnev et al., 2004a) dispersion profiles, using existing pulse sequences in which variable numbers of ^{13}C refocusing pulses are applied. Such schemes, unlike those involving the application of ^{1}H pulses, cannot interconvert between differentially relaxing components that

are distinguished by different ^{1}H spin states, such as the fast and slowly relaxing ^{1}H single quantum coherences of a methyl group. Multiple quantum dispersions are sensitive to differences in ^{1}H ($\Delta\varpi_H$) and ^{13}C ($\Delta\varpi_C$) chemical shifts between interconverting states, and, in principle, both $\Delta\varpi_H$ and $\Delta\varpi_C$ can be extracted in some cases from such data (Korzhnev et al., 2004a). In contrast, ^{13}C single quantum profiles depend only on $\Delta\varpi_C$; accurate values of both $\Delta\varpi_H$ and $\Delta\varpi_C$ can be obtained, therefore, by simultaneous fits of both multiple quantum and single quantum data sets. Such an approach has already been described in the literature in connection with backbone amide ^{15}N–^{1}HN spin pairs (Korzhnev et al., 2004b), although in this case ^{1}HN single quantum dispersions free of artifacts can also be recorded, at least for perdeuterated proteins (Ishima & Torchia, 2003).

Figure 2 shows ^{13}C single quantum (bottom two profiles in each panel, filled circles) and ^{13}C–^{1}H multiple quantum dispersions (open circles), recorded at 14.1 (green) and 18.8T (red), 25°C, for a number of methyl groups from a U-[^{2}H,^{15}N] Ileδ1-[^{13}CH$_3$] Leu,Val-[^{13}CH$_3$,^{13}CH$_3$] G48M mutant of the Fyn SH3 domain. In a series of previous publications we have shown that this mutant interconverts between folded and unfolded states, with the exchange process giving rise to large dispersions in CPMG (Korzhnev et al., 2004b; Korzhnev et al., 2004c; Orekhov et al., 2004) and $R_{1\rho}$ (Korzhnev et al., 2005) experiments. The single and multiple-quantum data profiles from all residues with (single quantum) dispersions that exceed 5 s^{-1} have been fit simultaneously, with values of 418 ± 6 s^{-1} and 4.2 ± 0.1% obtained for the exchange rate, k_{ex}, and the population of the minor (unfolded) state, p_B, respectively. The data fit well to a two-site model of exchange with $\chi^2 = 408$, 646 degrees of freedom. Table 1 lists per-methyl values of $\Delta\varpi_C$ and $\Delta\varpi_H$, with the sign of $\Delta\varpi_C$ determined following the approach of Skrynnikov et al. (2002). The sign of $\Delta\varpi_H$ is not available from the ^{13}C–^{1}H multiple-quantum experiment. It is worth noting that only a single proton refocusing pulse is applied in the center of the T period in the multiple quantum scheme that has been employed along with a variable number of ^{13}C pulses (Figure 1 of Korzhnev et al. (2004), with the purge element). Sequences that make use of variable numbers of ^{1}H refocusing pulses suffer

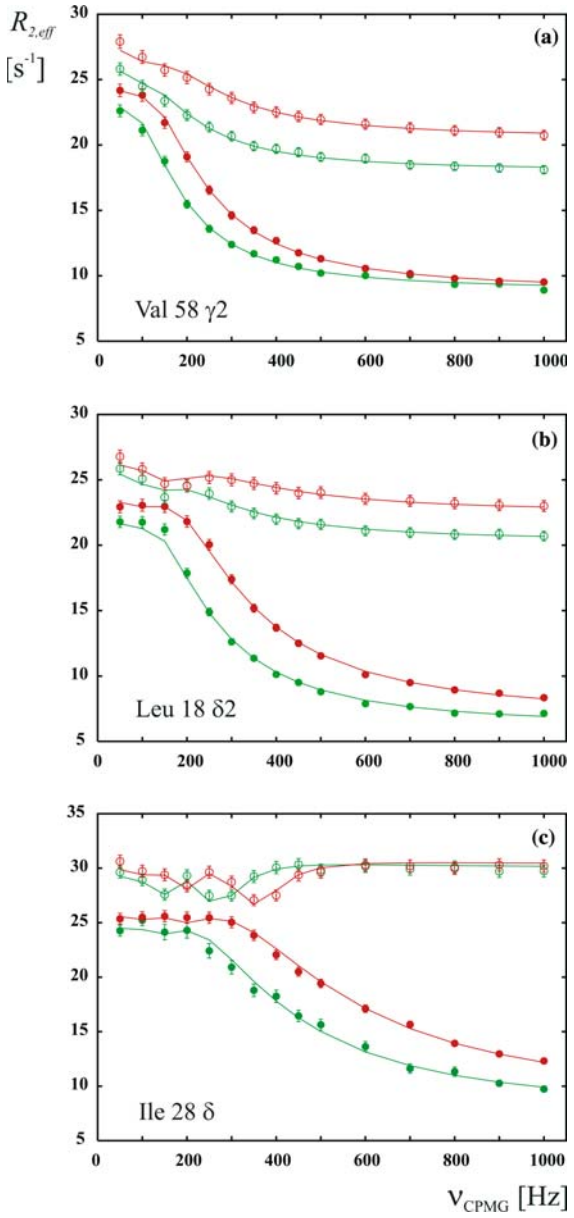

Figure 2. ^{13}C–^{1}H multiple-quantum dispersion profiles (upper two traces, open circles, recorded with the sequence of Figure 1 of Korzhnev et al. (2004), with the purge element) and ^{13}C single quantum curves (bottom pair, filled circles, recorded with the scheme of Skrynnikov et al. (2001)) for selected methyl groups of a U-[^{2}H,^{15}N,^{12}C] Ileδ1-[^{13}CH$_3$] Leu,Val-[^{13}CH$_3$,^{13}CH$_3$] G48M mutant of the Fyn SH3 domain, prepared using Ile, Leu, Val precursors similar to those described by Goto et al. (1999). The solid curves correspond to the dispersion profiles calculated from the parameters extracted from a global best fit of the data involving all methyl groups with $R_{ex} = R_{2,eff}(50\text{ Hz}) - R_{2,eff}(1000\text{ Hz}) > 5$ s^{-1} (evaluated from single quantum profiles) at both 14.1 (green data points) and 18.8T (red). A constant offset of 2 s^{-1} has been added to the multiple quantum data for ease of visualization.

Table 1. ^{15}C and ^{1}H chemical shift differences between exchanging states, $\Delta\varpi_C$ and $\Delta\varpi_H$ (in ^{13}C and ^{1}H ppm, respectively), extracted from a 'global' fit of ^{13}C single quantum and ^{13}C–^{1}H multiple-quantum dispersion profiles recorded at 14.1 and 18.8*T* for Ile, Leu and Val methyls of G48M Fyn SH3[a].

Residue	$\Delta\varpi_C$ [ppm]	$\lvert\Delta\varpi_H\rvert$ [ppm]
Leu 3 δ1	−0.48 ± 0.006	0.06 ± 0.002
Leu 3 δ2	−0.66 ± 0.008	0.09 ± 0.002
Leu 7 δ1	−0.47 ± 0.006	0.15 ± 0.006
Leu 7 δ2	1.49 ± 0.014	0.09 ± 0.002
Leu 18 δ1 *	−0.39 ± 0.007	0.11 ± 0.007
Leu 18 δ2	−1.58 ± 0.015	0.14 ± 0.003
Ile 28 δ	2.73 ± 0.032	0.43 ± 0.006
Leu 29 δ1	−0.78 ± 0.007	0.07 ± 0.001
Leu 29 δ2	1.07 ± 0.009	0.07 ± 0.001
Leu 42 δ1 *	−0.25 ± 0.005	0
Leu 42 δ2	−0.45 ± 0.004	0
Ile 50 δ	−0.89 ± 0.009	0.13 ± 0.003
Val 55 γ1 *	−0.37 ± 0.009	0.09 ± 0.008
Val 55 γ2	2.03 ± 0.025	0.17 ± 0.005
Val 58 γ1 *	−0.38 ± 0.008	0.06 ± 0.004
Val 58 γ2	−1.09 ± 0.011	0.09 ± 0.002

[a] The global data fit was performed for 12 methyl groups with $R_{ex} = R_{2,eff}(50\ Hz) - R_{2,eff}(1000\ Hz) > 5\ s^{-1}$ for single quantum profiles recorded at both 14.1 and 18.8*T*. Values of $\Delta\varpi_C$ and $\Delta\varpi_H$ were obtained for four additional methyl groups (marked by *) with k_{ex} and p_B fixed to the values obtained in the global fit. Signs of $\Delta\varpi_C$ (unfolded–folded) were determined following Skrynnikov et al. (2002). Errors were estimated by the covariance matrix method (Press et al., 1988).

from the same problems as the ^{1}H single quantum CPMG dispersion experiment described here, for reasons analogous to those mentioned above.

Figure 3 shows the correlation between ^{13}C methyl chemical shifts of the minor state determined jointly from the dispersion data and from a comparison of peak positions in HSQC data sets recorded at 11.4 and 18.8*T* and random coil chemical shifts tabulated by Wishart et al. (1995). The excellent correlation confirms that the minor state in the present example is indeed the unfolded form of the protein.

In summary, in this communication the importance of understanding the relaxation properties of the spin probe used to study exchange is highlighted with an example involving a $^{13}CH_3$ spin system. Robust ^{13}C single quantum experiments have been developed for such groups (Skrynnikov et al., 2001) because imperfections in ^{13}C pulses do not interconvert between different ^{13}C transverse multiplet components that relax

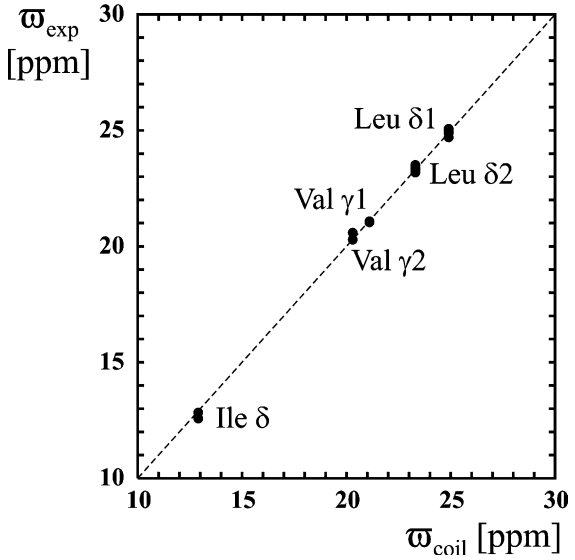

Figure 3. Correlation between ^{13}C Ile, Leu and Val methyl chemical shifts of the minor exchanging state in the G48M Fyn SH3 domain (25°C) and random coil ^{13}C chemical shifts. A value of 0.38 ppm was added to the experimental values to account for the small offset between the shifts measured and those reported by Wishart et al. (1995).

differently. In contrast, errors in ^{1}H refocusing pulses do lead to conversion between fast and slowly relaxing components that are distinguished on the basis of ^{1}H spin states, giving rise to artifacts in dispersion curves and potentially significant errors in extracted exchange parameters. In these cases, use of other experiments that circumvent the problem, such as the combination of ^{13}C single quantum and ^{13}C–^{1}H multiple quantum experiments in place of ^{1}H single quantum dispersions discussed here, facilitate the extraction of ^{1}H shift differences between exchanging states.

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