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ARTICLE

Artifacts can emerge in spectra recorded with even the simplest of pulse schemes: an HMQC case study

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Abstract
With the development of sophisticated pulsed field gradient- and phase cycling-approaches for suppressing certain coherence transfer pathways and selecting for others it is sometimes easy to forget that the process is not flawless. In some cases artifacts can emerge because unwanted transfers are immune to the phase cycle or the application of gradients. We consider here a simple $^{1}H,^{13}C$ HMQC pulse scheme and show that imperfections in the single ^{1}H 180° refocusing pulse can give rise to small artifacts in methyl spectra that cannot be eliminated through extensive phase cycling or the use of gradients, but that are easily removed when the pulse is of the composite variety.

Keywords HMQC · Pulse imperfections · Methyl groups · Methyl-TROSY

Introduction

Pulse sequences that are used for biomolecular NMR applications have become sophisticated, with a large number of different approaches available for optimizing the transfer of magnetization between spins (Cavanagh et al. 1996; Sattler et al. 1999). This transfer is often directed through phase cycling or via the use of pulsed field gradients that minimize artifact levels in spectra (Cavanagh et al. 1996), enabling the observation of weak correlations that can be critical to the analysis and interpretation of the data. Herein I examine one of the simplest pulse sequences, the HMQC, that has a 'built-in' methyl-TROSY effect (Tugarinov et al. 2003), and that, therefore, has assumed an important role in solution NMR studies of high molecular weight protein systems (Rosenzweig and Kay 2014). Despite the inherent simplicity of the experiment, with a total of only 4 pulses in its most popular implementation (Bax et al. 1983), small but noticeable artifacts can be observed in spectra associated with high sensitivity cross-peaks even in applications that involve an extensive phase cycle of the single 180° pulse in the sequence and that use pulsed field gradients (see below). In what follows I describe the origin of the artifacts and how they can be removed. But I must admit that the motivation for this study goes beyond spin physics because it brings me back to my roots, to a wonderful time when I had the great pleasure of working with a pair of post-doctoral mentors whom I know share my passion for the wonders of spin. In particular I would like to dedicate this work to one of them, Dennis A. Torchia, on the occasion of his 80th birthday.

I first met Dennis in 1987 when I interviewed with Ad Bax for a post-doctoral position in the Bax laboratory at the National Institutes of Health in Bethesda, MD. Dennis, Ad and I were literally huddled in a broom closet that functioned as Dennis's office discussing scalar relaxation. Aside from the intimacy of the surroundings what impressed me the most was the collegiality between the two NIH scientists and the high level of spin physics that was discussed. I decided right then that I would have to work with both of them during my post-doc. In many respects the discussion that ensued during that half hour was a harbinger of many subsequent discussions over the following three and a half years where we would present spin physics problems to each other in the hopes of gleaning insight into why an experiment worked or didn't, or attempt to understand nuances in spin relaxation that were both perplexing and frustrating (at least for me). These were exciting times, with the development of

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multi-dimensional NMR, including triple resonance (Bax 1994; Montelione and Wagner 1990), the application of spin relaxation measurements across the complete backbone of a protein to quantify pico- to nano-second timescale dynamics (Kay et al. 1989), and the continuation of my foray into methyl-groups (Kay et al. 1992) that had initially begun during my graduate school days in Jim Prestegard's laboratory (Kay and Prestegard 1987). I want to present here a throwback to those 'good old days' where the emphasis was on Science the practice and not Science the journal and where we worked on problems in some cases simply for amusement, although practical applications almost always ensued. I describe one such amusing problem here that was puzzling initially to me, but trivial when one thinks about it for a few minutes, and that reminded me of the many fine discussions that I had with my two NIH mentors during a remarkable period of my scientific career.

Results and discussion

The problem

Figure 1 highlights an HMQC pulse scheme based on sequences originally proposed by Mueller (1979) and subsequently developed and applied further (Bax et al. 1983). The ^{1}H–^{13}C version of the experiment is particularly useful for studies of high molecular weight proteins using methyl group probes because equal amounts of methyl magnetization traverse two pathways, one of which is almost completely immune to the intra-methyl ^{1}H–^{1}H and ^{1}H–^{13}C dipolar interactions

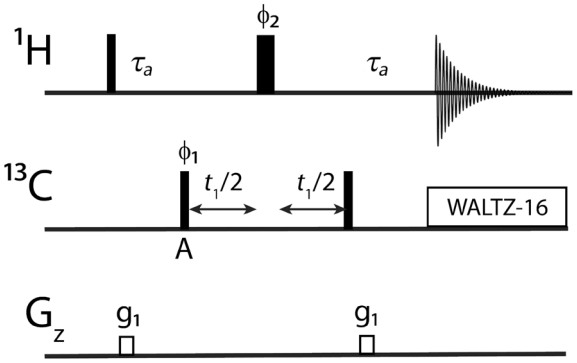

Fig. 1 Pulse scheme for the HMQC sequence. Narrow(wide) rectangular bars denote 90° (180°) pulses, applied with the highest possible B_1 fields. The wide ^{1}H bar denotes either a simple 180° pulse or a composite pulse of the form $90^{\circ}_{\phi 2-\pi/2}240^{\circ}_{\phi 2}90^{\circ}_{\phi 2-\pi/2}$ (Freeman et al. 1980) or $90^{\circ}_{\phi 2-\pi/2}180^{\circ}_{\phi 2}90^{\circ}_{\phi 2-\pi/2}$ (Levitt and Freeman 1978). A gradient pair (g_1) surrounds the central ^{1}H 180° pulse. Delays τ_a are set to $1/(2J_{CH})\sim 4$ ms. The phase cycle is: ϕ_1 = x, −x; ϕ_2 = 2(x), 2(y), 2(−x), 2(−y); receiver phase = x, −x, −x, x. In applications in our laboratory an additional $90^{\circ}_{x}90^{\circ}_{\phi 3}$ pair of ^{13}C pulses is applied immediately prior to acquisition, with ϕ_3 = 2(x), 2(−x) (Bax et al. 1990)

that would normally contribute to rapid signal decay (Tugarinov et al. 2003). Studies using this methyl-TROSY based experiment have extended the realm of solution NMR spectroscopy into the domain of molecular machines (Rosenzweig and Kay 2014), a current interest in my laboratory. In some of our initial applications close to 20 years ago we noticed that when the ^{1}H 180° refocusing pulse in the center of the t_1 interval was applied as a simple pulse (i.e., not a composite pulse) small artifacts were observed for very intense residues, located at $\pm J_{CH}/2$ Hz from the central resonance. Figure 2a shows such a spectrum, recorded on a 1 mM sample of a U–^{2}H, Met-[ε^{13}CH$_3$]-labeled α_7 proteasome ring (180 kDa) at 18.8 T and 45 °C. This construct contains 7 protomers of an approximately 25 kDa polypeptide chain with 5 Met residues whose ε-methyl groups serve as probes of molecular dynamics and structure in this system. We have replaced Met at position 1 with an Ile, as the Met methyl groups of residues −1 (M–1) and 1 (M1) are poorly resolved in spectra (Huang et al. 2017), complicating quantitation. The artifactual peaks described above are highlighted with red stars in Fig. 2a and are clearly observed for the intense M–1 correlation, although not for the other less intense Met cross-peaks. A cross-section at a proton chemical shift of 2.21 ppm is also included that clearly illustrates the unwanted doublets.

The presence of additional peaks at $\omega_C \pm \pi J_{CH}$ ($J_{CH}/2$ Hz from the central resonance) for M–1 might at first seem surprising, as the HMQC pulse sequence contains few pulses and the central ^{1}H 180° pulse is both surrounded by a pair of equal gradients and exorcycled (Bodenhausen et al. 1977) (Fig. 1). Yet when the simple 180° pulse is replaced by one that is composite, of the form $90^{\circ}_{\phi 2-\pi/2}240^{\circ}_{\phi 2}90^{\circ}_{\phi 2-\pi/2}$ (Freeman et al. 1980) (Fig. 2b) or $90^{\circ}_{\phi 2-\pi/2}180^{\circ}_{\phi 2}90^{\circ}_{\phi 2-\pi/2}$ (Levitt and Freeman 1978) (Fig. 2c), the artifacts are eliminated, strongly suggesting that the 180° pulse is the culprit. When the refocusing pulse is eliminated and the ^{1}H carrier placed on-resonance with the protons of M−1 so that there is no net ^{1}H chemical shift evolution during the pulse scheme, three well-phased multiplet components are observed for the ε-methyl of this residue, at frequencies of ω_C, $\omega_C \pm 2\pi J_{CH}$ (Fig. 2d). Additional peaks associated with M–1 are then not present.

Origin of artifacts

In order to understand the origin of the artifacts we first briefly review why when the ^{1}H 180° pulse is removed from the HMQC sequence of Fig. 1 a 1:2:1 triplet is obtained in F_1 in the absence of relaxation, Fig. 2d. This can be understood by noting that at point A in the scheme of Fig. 1, and neglecting both relaxation and ^{1}H chemical shift evolution, the coherences of interest are given by

$$2I_xC_y = \sum_{j=1-3} 2I_x^jC_y \qquad (1)$$

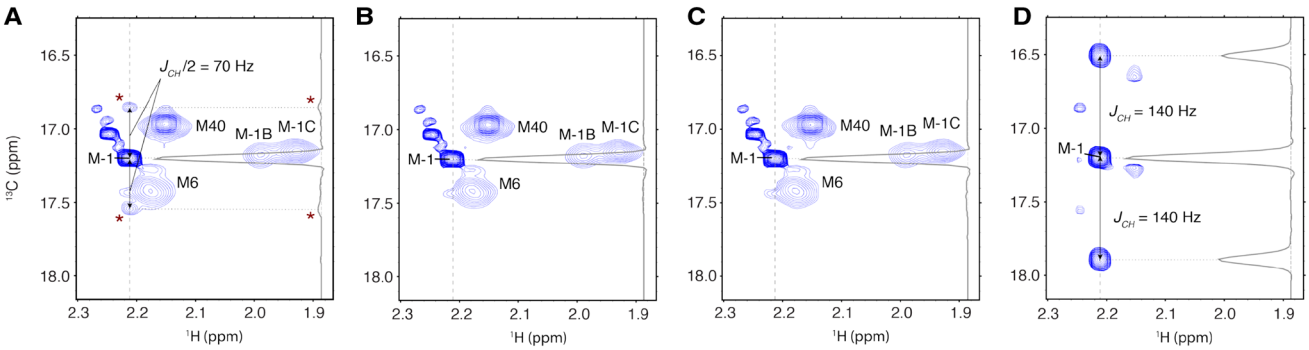

Fig. 2 ^{1}H, ^{13}C HMQC correlation maps of a U–^{2}H, Met-[ε^{13}CH$_3$]-labeled α_7 proteasome ring (180 kDa) recorded at 18.8 T and 45 °C. The sample was 1 mM in concentration, prepared as described previously (Religa et al. 2010). **a** Spectrum obtained using the scheme of Fig. 1 with a single (non-composite) central ^{1}H refocusing pulse, highlighting Met cross-peaks. Several conformations for M–1 are observed (Religa et al. 2010), with the corresponding peaks denoted by M–1, M–1B, M–1C. In addition, weak artifacts are noted at $\pm J_{CH}/2$ from the central M–1 peak (red asterisks). Application of $90^{\circ}_{\phi 2-\pi/2}240^{\circ}_{\phi 2}90^{\circ}_{\phi 2-\pi/2}$ (**b**) or $90^{\circ}_{\phi 2-\pi/2}180^{\circ}_{\phi 2}90^{\circ}_{\phi 2-\pi/2}$ (**c**) composite pulses eliminates these artifacts. **d** Spectrum recorded using the scheme of Fig. 1, with the central ^{1}H 180° pulse eliminated, the second gradient of the g_1 pair inverted, the ϕ_2 phase cycle eliminated, and the receiver phase adjusted accordingly (receiver = x, −x). The ^{1}H carrier was set on-resonance for M–1. Although pure absorptive lineshapes are obtained for multiplet components of M–1, mixed-mode lineshapes are generated for other (off-resonance) Met signals. An additional ^{1}H 90° pulse is, therefore, applied immediately prior to acquisition (t_2) so that lineshapes of all peaks are absorptive in the F_2 dimension (Kay and Bax 1990). Peaks for M–1B, M–1C, M6 and M40 are very weak because of the severe distortion in lineshape in F_1. Only positive contours are plotted. In **a**–**d** a trace at $\delta_H = 2.21$ ppm is highlighted to emphasize the F_1 multiplet structure for M–1

where A_q is the q-component of A magnetization and the summation is over all three ^{1}H spins in the methyl group. In order to explicitly include all proton spins, that will be useful in what follows, Eq. (1) can be recast as

$$\sum_{j=1-3} 2I_x^j C_y = \sum_{j=1-3} 2I_x^j C_y \{|\alpha\alpha><\alpha\alpha| + |\alpha\beta><\alpha\beta| + |\beta\alpha><\beta\alpha| + |\beta\beta><\beta\beta|\}^{kl} \quad (2)$$

where the superscripts j, k, l distinguish the three protons, and (α, β) are ^{1}H spin states. In what follows, and without loss in generality, we will focus on the I_+C_+ component of $2I_xC_y$ ($A_+ = A_x + iA_y$). Assuming that the proton and carbon spins are on-resonance, and neglecting relaxation, the evolution of I_+C_+ during the complete t_1 interval in the case where the central ^{1}H 180° pulse is eliminated is given by

$$I_+C_+ \xrightarrow{t_1} I_+C_+\{|\alpha\alpha><\alpha\alpha|e^{-i2\pi J_{CH}t_1} + |\alpha\beta><\alpha\beta| + |\beta\alpha><\beta\alpha| + |\beta\beta><\beta\beta|e^{i2\pi J_{CH}t_1}\} \quad (3)$$

and a 1:2:1 triplet is obtained with each peak separated from its neighbor by J_{CH} Hz. In contrast, when a 'perfect' 180° ^{1}H pulse is applied after the first $t_1/2$ period, interchanging α and β spin states, coherences evolving according to $e^{-i\zeta}$ during the first $t_1/2$ element evolve as $e^{i\zeta}$ for the second $t_1/2$ delay. Thus, as an echo occurs at t_1,

$$\begin{aligned} &I_-C_+\{|\beta\beta><\beta\beta|e^{-i2\pi J_{CH}t_1/2}e^{i2\pi J_{CH}t_1/2} + |\beta\alpha><\beta\alpha| + |\alpha\beta><\alpha\beta| \\ &+ |\alpha\alpha><\alpha\alpha|e^{i2\pi J_{CH}t_1/2}e^{-i2\pi J_{CH}t_1/2}\} \\ &= I_-C_+\{|\beta\beta><\beta\beta| + |\beta\alpha><\beta\alpha| + |\alpha\beta><\alpha\beta| + |\alpha\alpha><\alpha\alpha|\} \end{aligned} \quad (4)$$

as the ^{1}H–^{13}C scalar coupled evolution is refocused. In this case a 2D spectrum would be generated, consisting of a single peak centered at (ω_C, ω_H) with no multiplet structure.

The situation is more complex (interesting) if the ^{1}H refocusing pulse is imperfect. In what follows we consider the case where an on-resonance ^{1}H pulse is applied along the x-axis with flip angle θ in the middle of the t_1 period, neglecting, as before, ^{1}H and ^{13}C chemical shift evolution and relaxation. Recalling that (Cavanagh et al. 1996; Ernst et al. 1987)

$$\begin{aligned} &\exp(-i\theta I_x)|\alpha> = \cos(\vartheta)|\alpha> - i\sin(\vartheta)|\beta> \\ &\exp(-i\theta I_x)|\beta> = \cos(\vartheta)|\beta> - i\sin(\vartheta)|\alpha>,\ \vartheta = \theta/2 \end{aligned} \quad (5)$$

and considering, for example, the evolution of the $I_+C_+|\alpha\alpha><\alpha\alpha|$ term during the $\frac{t_1}{2} - \theta_x - \frac{t_1}{2}$ portion of the sequence of Fig. 1, it can be shown that

$$\begin{aligned} I_+C_+|\alpha\alpha><\alpha\alpha| \xrightarrow{t_1/2\ \theta_x\ t_1/2} &3S^2C^4e^{-i\gamma}I_-C_+|\alpha\alpha><\alpha\alpha| \\ &+ (2S^4C^2 - S^2C^4)e^{-i\gamma/2}I_-C_+|\alpha\beta><\alpha\beta| \\ &+ (2S^4C^2 - S^2C^4)e^{-i\gamma/2}I_-C_+|\beta\alpha><\beta\alpha| \\ &+ (S^6 - 2S^4C^2)e^{0}I_-C_+|\beta\beta><\beta\beta| \end{aligned} \quad (6)$$

In Eq. (6) $S = \sin\vartheta$, $C = \cos\vartheta$ and $\gamma = 2\pi J_{CH}t_1$. We have included only those terms that ultimately can be observed, recognizing that the gradient pair flanking the central ^{1}H pulse (g_1 in Fig. 1) ensures that only ^{1}H magnetization proportional to I_+ prior to the pulse and transformed into I_- by the pulse (or vice versa) is detected. Equation (6) makes

it evident that imperfections in the central pulse 'spread' the starting $|\alpha\alpha><\alpha\alpha|$ density element (left hand side) into four separate, and detectable, terms (right hand side), that contribute to the intensities of the multiplet components that are obtained in Fig. 2a. In the case where a perfect refocusing pulse is applied in the center of the t_1 period, $\theta = 180°$, corresponding to $\vartheta = 90°$, $I_+C_+|\alpha\alpha><\alpha\alpha|$ is converted fully to $I_-C_+|\beta\beta><\beta\beta|$ (*i.e.*, $S = 1$, $C = 0$), while for $\theta = 0°$ $I_+C_+|\alpha\alpha><\alpha\alpha|$ remains unchanged and none of the terms proportional to I_- are excited. In the latter situation either the gradient pair or phase cycle would eliminate magnetization so that no signal would be observed.

Similar expressions can be derived starting from $I_+C_+|\alpha\beta><\alpha\beta|$, $I_+C_+|\beta\alpha><\beta\alpha|$, $I_+C_+|\beta\beta><\beta\beta|$ to generate a series of terms that evolve as $e^{\pm i\gamma}$, $e^{\pm i\gamma/2}$, and e^0 corresponding to multiplet components at frequencies of $\pm 2\pi J_{CH}$ rad/s, $\pm \pi J_{CH}$ rad/s and 0 rad/s, respectively, relative to the perfectly decoupled cross-peak. In total, therefore, 5 peaks are obtained with calculated multiplet component intensities of $3S^2C^4$ ($\pm J_{CH}$ Hz), $8S^4C^2 - 4S^2C^4$ ($\pm J_{CH}/2$ Hz) and $4S^6 - 8S^4C^2 + 6S^2C^4$ (0 Hz, central line) and in the limit $\theta = 180°$ so that $\vartheta = 90°$ it is clear that only the central line has intensity, as expected.

In cases where $\theta \sim 180°$ but not exactly 180°, all multiplet components will be non-zero although the central line will dominate. For example, for $\theta = 170°$ (175°) the outer lines (at $\pm J_{CH}$ Hz) are essentially unobservable, while the inner multiplet components ($\pm J_{CH}/2$ Hz) have calculated intensities close to 1.5% (0.4%) of the central line. Although small, such satellites can be observed for intense residues, such as for M–1 of the α_7 proteasome ring. In this specific case the components at $\pm J_{CH}/2$ Hz are measured to be approximately 1.5% of the intensity of the central peak (Fig. 2a), larger than what would be predicted given that it is possible to calibrate pulses accurately for concentrated samples. Notably, when the 90° ^{1}H pulse of the HMQC sequence is replaced by a $90°_x 90°_y 90°_{-x} 90°_{-y}$ excitation element that suppresses signals from regions of the sample where the B_1 field homogeneity is poor (Bax 1985) the artifacts decreased by about 30% to ~ 1% of the central line. When an identical sample was prepared in a Shigemi tube with a solution volume of 280 µL, as opposed to the 500 µL sample used in previous experiments, and an experiment recorded with a $90°_x 90°_y 90°_{-x} 90°_{-y}$ excitation element the intensity ratio decreased further to ~ 0.6%, suggesting that 'pick-up' from sample regions outside of the homogeneous excitation window is responsible for at least some of the intensity of the artifact peaks.

Notably, even a simple HMQC pulse scheme, with only a single 180° pulse, is not immune to artifacts, even when the refocusing pulse is exorcycled and surrounded by a pair of pulsed field gradients (Fig. 1). In the present case, the interchange of multiplet components, $I_+C_+|i><i| \rightarrow I_-C_+|j><j|$, $i,j \in (\alpha\alpha, \alpha\beta, \beta\alpha, \beta\beta)$, is not suppressed, so that undesired elements, such as those produced from transitions such as $I_+C_+|\alpha\alpha><\alpha\alpha| \rightarrow I_-C_+|\alpha\beta><\alpha\beta|$, for example, give rise to spurious correlations in methyl spectra. These peaks are typically very small and can only be observed in applications involving concentrated samples and when the peak of interest has high signal-to-noise. Further, they are easily eliminated using composite refocusing pulses (Fig. 2b, c). The artifacts observed here are closely related to those described in J-resolved spectra of methyl groups when ^{1}H 180° pulses are imperfect, and these could also be suppressed by the application of composite pulses (Freeman and Keller 1981). Artifacts such as those observed here serve as an important reminder that gradients and phase cycling cannot completely suppress the 'sins' of pulse imperfections, and that it is therefore always preferable to judiciously choose pulses such that these imperfections can be avoided in the first place.

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