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Homonuclear decoupling for enhancing resolution and sensitivity in NOE and RDC measurements of peptides and proteins

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ABSTRACT

Application of band-selective homonuclear (BASH) ^{1}H decoupling pulses during acquisition of the ^{1}H free induction decay is shown to be an efficient procedure for removal of scalar and residual dipolar couplings between amide and aliphatic protons. BASH decoupling can be applied in both dimensions of a homonuclear 2D NMR experiment and is particularly useful for enhancing spectral resolution in the H^N–H^α region of NOESY spectra of peptides and proteins, which contain important information on the backbone torsion angles. The method then also prevents generation of zero quantum and H_z^N—H_z^α terms, thereby facilitating analysis of intraresidue interactions. Application to the NOESY spectrum of a hexapeptide fragment of the intrinsically disordered protein α-synuclein highlights the considerable diffusion anisotropy present in linear peptides. Removal of residual dipolar couplings between H^N and aliphatic protons in weakly aligned proteins increases resolution in the ^{1}H–^{15}N HSQC region of the spectrum and allows measurement of RDCs in samples that are relatively strongly aligned. The approach is demonstrated for measurement of RDCs in protonated ^{15}N/^{13}C-enriched ubiquitin, aligned in Pf1, yielding improved fitting to the ubiquitin structure.

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1. Introduction

It has long been recognized that removal of homonuclear ^{1}H–^{1}H or ^{13}C–^{13}C *J* couplings can significantly improve spectral resolution of 2D NMR spectra. In the indirectly detected t_1 dimension, this typically has been done either by using constant-time evolution [1–3] or, in the case of heteronuclear experiments, by application of a single BIRD pulse unit [4]. Alternatively, a combination of a non-selective 180° pulse and a frequency-selective pulse applied during a static gradient can be used to obtain broad-band ^{1}H–^{1}H decoupling [5–7]. This latter method, referred to as Zangger-Sterk or ZS, utilizes the non-selective/selective 180° pulse pair to invert all protons outside of the region inverted by the selective pulse [8]. Inside the selective-pulse-inverted region, the net rotation of the non-selective and selective 180° pulses is zero, and no net evolution of chemical shift or *J* couplings occurs during the selective pulse and its associated pulsed field gradients. The elegant ZS method is very effective at eliminating ^{1}H–^{1}H *J* couplings in a broad-band fashion, although the number of observed spins and thereby the intrinsic sensitivity of the experiment decreases in proportion to the thickness of the sample slice selected by the ZS pulse combination.

In the directly detected dimension, removal of the ^{1}H–^{1}H *J* couplings also can be achieved by bilinear rotation decoupling (BIRD), which relies on periodically inverting all ^{1}H spins that are not directly attached to ^{13}C [9], but limits detection to protons attached to ^{13}C, the latter being either at natural abundance or low levels of random enrichment. As was demonstrated very recently, ZS pulse/gradient combinations can also be applied during an interrupted FID, which then accomplishes homonuclear broad-band decoupling in the directly detected dimension [6]. This particularly elegant method results in dramatic spectral simplification, but again involves a significant cost in sensitivity related to slice selection. In favorable cases, the sensitivity loss can be minimized, however, by rapidly repeating the experiment on adjacent, non-perturbed regions of the sample [7].

For peptides and proteins, frequently much of the interest is focused on detection of amide protons. This includes the widely used ^{1}H–^{15}N HSQC experiment [10,11], and the large battery of amide-detected triple resonance experiments [12–14]. In particular when using protein samples in a weakly aligned state, necessary for the measurement of residual dipolar couplings, the presence of $^{1}H^N$–$^{1}H^\alpha$ as well as other $^{1}H^N$–$^{1}H^{sidechain}$ residual dipolar couplings can significantly limit the spectral resolution and sensitivity. As demonstrated by Vander Kooi et al. [15], band-selective decoupling of the aliphatic proton region can relieve this problem, and can be accomplished by alternately gating the ^{1}H homonuclear transmit channel and the ^{1}H receiver to periodically invert the aliphatic

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protons during acquisition of the FID. Analogous removal of $^3J_{HH}$ splittings in NOESY spectra by applying G3-shaped pulses through the homonuclear decoupling channel has been demonstrated by Hammerström and Otting [16]. Technically these methods are challenging, in part due to the difficulty in suppression of the water signal, and also due to the introduction of time-dependent Bloch–Siegert shifts caused by the decoupling RF irradiation. As a result, these methods have not yet become widely used.

Here, we describe an alternate method to achieve band-selective homonuclear (BASH) decoupling in peptides and proteins, using an approach that is essentially a hybrid of the Zangger-Sterk and Hammerström methods. BASH is particularly effective for gaining increased spectral resolution in 2D H^N–H^α NOE spectra, and then has the additional advantage of eliminating the generation of multi-quantum and *zz* spin states during the mixing period, which can adversely affect spectral quality. Much recent interest focuses on intrinsically unstructured proteins, where the spectral dispersion in both H^N and H^α regions is poor, and the additional resolution gained by homonuclear decoupling then is particularly valuable. Considerable variation in the ratio of the intraresidue over the sequential H^N–H^α NOE was recently reported for the intrinsically disordered protein α-synuclein [17]. This NOE ratio can be a sensitive reporter for the backbone torsion angle ψ, which is difficult to evaluate by alternate methods [18].

A weakly ordered aqueous environment is commonly used for measurement of residual dipolar couplings (RDCs) in proteins [19]. The filamentous phage Pf1 is particularly widely used for this purpose as it is highly stable and commercially available [20]. Due to its large negative surface charge of ca 0.5 e/nm^2, some proteins align particularly strongly in this medium. It then can be challenging to generate conditions where the Pf1 remains liquid crystalline while the protein is aligned sufficiently weakly that homonuclear ^{1}H–^{1}H couplings do not dramatically decrease resolution and sensitivity of the ^{1}H–^{15}N HSQC spectrum. For example, for recording spectra on the widely studied protein ubiquitin, alignment in Pf1 tends to be quite strong, making it necessary to increase ionic strength to decrease protein alignment. This increased ionic strength, in turn, can lead to a paranematic phase or phase separation of the Pf1 [21]. As previously demonstrated by Vander Kooi et al. [15], homonuclear decoupling is an effective solution to remove the ^{1}H–^{1}H RDCs and thereby increase spectral resolution. We here demonstrate the BASH decoupling method for this purpose.

2. Results and discussion

2.1. The basic BASH scheme

The pulse sequence for recording the one-dimensional BASH-decoupled amide ^{1}H spectrum of a peptide or protein is shown in Fig. 1. Because selective and non-selective radiofrequency (RF) ^{1}H pulses are applied during the FID, and the spectra necessarily are recorded on samples dissolved in H_2O, adequate suppression of the intense water signal is important. To this extent, we find it necessary in homonuclear ^{1}H experiments to include weak (∼25 Hz RF field) presaturation of the water resonance, in addition to using an excitation scheme that does not excite any residual water magnetization. In Fig. 1, we use the 90_x–τ–90_{-x} jump-and-return sequence [22] for this purpose, or alternatively an EBURP pulse [23] can be used. This excitation is followed by an amide ^{1}H band-selective REBURP pulse [23], surrounded by gradients (G_2), to further suppress the water signal. The actual BASH decoupling is achieved by inserting a combination of a band-selective 180°_{-x} together with a non-selective 180°_x pulse, which flips the spin state of all protons outside of the selected amide band [8]. It is important that the two pulses have the exact same phase (or the exact opposite phase), as otherwise a jump in the phase of the transverse magnetization would occur each time this pulse combination is applied. Net evolution of the amide ^{1}H transverse magnetization is zero over the total interval used for application of the two pulses and the short gradient pulses surrounding each ^{1}H pulse. In order to minimize refocusing of water magnetization, excited by imperfection of e.g. the first 180° pulse pair, that will occur if a second subsequent gradient pulse pair is applied that uses the same set of pulsed field gradients, we use different gradient strengths for the second pulse pair. In principle it would be advantageous to ensure that all subsequent 180° pulse pairs are surrounded by gradients that differ from those applied previously, but in practice the occurrence of such echoes is difficult to suppress completely and we find that simply repeating this "double block" of (acquire *T*, pp1, acquire 2*T*, pp2, acquire *T*) suffices. Here, pp1 and pp2 refer to the first and second set of pulse pairs with associated gradient pulses, and 2*T* is the separation between the pulse pairs. Similarly to the X,X,-X,-X phase alternation used in heteronuclear decoupling schemes such as MLEV4 [24], phases of two consecutive 180° pulse pairs, within the bracketed loop, are inverted each time the loop is repeated.

As discussed by Aguilar et al. [25] and Meyer and Zangger [6], it is important to keep the duration of 2*T* much smaller than $1/J_{HH}$. If not, decoupling sidebands in the spectrum will occur at distances of $(4T)^{-1}$ from the decoupled resonances. These sidebands can become more significant if an antiphase term of the type $2H^N_xH^\alpha_z$ is present prior to application of the pulse train of Fig. 1A, and then appear as an antiphase doublet with a splitting of $(2T)^{-1}$, centered around the resonance of interest. In the limit where $J_{HH} \ll (2T)^{-1}$, the intensity of the antiphase decoupling sidebands approximately scales with $2T * J_{HH}$, whereas the amplitudes of the in-phase decoupling sidebands scale with $(2T * J_{HH})^2$, therefore presenting less of a problem.

The line width of the decoupled resonance is increased somewhat relative to its natural T_2-limited value for two reasons: First, relaxation losses during the pulse pair increase the apparent decay rate by a factor (2*T* + pp)/(2*T*), where pp is the duration of the pulse pair and its associated pulsed field gradients. Second, pulse imperfections in the 180° pulses result in a loss of magnetization each time such a pair is applied. If a fraction, α, of the magnetization is returned to its position immediately preceding that pulse pair, the apparent additional contribution to the decay rate equals -ln(α)/2T. Both relaxation losses and losses due to pulse imperfections are minimized when 2*T* is kept large, and this requirement therefore competes with the desire to keep the sidebands small. For random coil peptides, where $^3J_{HN-H\alpha} \approx 7$ Hz, we find a 2*T* duration of ∼10–25 ms to be a reasonable compromise between these separate requirements.

Fig. 2A/B compares the BASH spectrum of the amide region of the N-terminal acetylated, C-terminally amidated hexapeptide Ac-VAAAEK-NH_2 with the corresponding regular 1D spectrum. The spectrum, recorded at 747 MHz ^{1}H frequency, shows a clear collapse of the doublets, with only a modest, <1 Hz, increase in line width in the decoupled spectrum relative to the reference spectrum. The two spectra are displayed at the same scaling relative to the thermal noise, indicating an increase in true signal to thermal noise ratio upon BASH decoupling. However, small decoupling sidebands in the BASH spectrum (Fig. 2B) decrease the apparent S/N in this spectrum.

2.2. BASH-decoupled NOESY

Removal of $^3J_{HH}$ couplings is particularly useful when analyzing 2D (or 3D) NOESY spectra of peptides or intrinsically disordered proteins (IDPs). Whereas $^3J_{HH}$ provides an accurate constraint for