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Long-Observation-Window Band-Selective Homonuclear Decoupling: Increased Sensitivity and Resolution in Solid-State NMR Spectroscopy of Proteins

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Abstract

Sensitivity and resolution are the two fundamental obstacles to extending solid-state nuclear magnetic resonance to even larger protein systems. Here, a novel long-observation-window band-selective homonuclear decoupling (LOW BASHD) scheme is introduced that increases resolution up to a factor of 3 and sensitivity up to 1.8 by decoupling backbone alpha-carbon (C^α) and carbonyl (C') nuclei in U- ^{13}C -labeled proteins during direct ^{13}C acquisition. This approach introduces short (<200 μ s) pulse breaks into much longer (~8 ms) sampling windows to efficiently refocus the J -coupling interaction during detection while avoiding the deleterious effects on sensitivity inherent in rapid stroboscopic band-selective homonuclear decoupling techniques. A significant advantage of LOW BASHD detection is that it can be directly incorporated into existing correlation methods, as illustrated here for 2D CACO, NCO, and NCA correlation spectroscopy applied to the β 1 immunoglobulin binding domain of protein G and 3D CBCACO correlation spectroscopy applied to the α -subunit of tryptophan synthase.

1. Introduction

The application of solid-state nuclear magnetic resonance (NMR) spectroscopy to protein systems has benefited from a steady progression in resolution, often with a corresponding gain in sensitivity. Indeed, advances in sample preparation, hardware design, and pulse-sequence methodologies have progressed to the point where it is common in solid-state NMR spectroscopy to resolve J -couplings between ^{13}C nuclei in U- ^{13}C , ^{15}N -labeled proteins (1–9). In many cases, homonuclear J -coupling interactions (such as $^1J_{C^\alpha C'}$ and $^1J_{C^\alpha C^\beta}$) are

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now the dominant sources of inhomogeneous (10) broadening and the effective limit to achievable resolution in uniformly isotopically-enriched proteins (11).

In cases where the nuclei can be decoupled, the collapse of the multiplet structure offers an increase in resolution and, most often, sensitivity. A number of highly-effective methods have been put forward to decouple homonuclear J -couplings in the indirectly observed dimension(s) of multidimensional solid-state NMR spectroscopy experiments, including the use of selective pulses (4; 5; 12; 13), evolution under multiple quantum coherences (14; 15), and constant-time experiments (7–9; 16). Removing the J -coupling interaction during direct detection offers similar benefits, which has inspired the development of spin-state-selective techniques that concentrate the signal intensity into just one of the multiplet components (2; 3; 6; 17–19). In their most general form, spin-state-selective techniques append additional delay periods before detection, which in quickly relaxing systems can moderate the sensitivity gains to less than the theoretical 2 gain for C' detection. In special cases, such as double-quantum – single-quantum based correlation experiments, where these delays can be folded into the sequence, the sensitivity gains can exceed this value (2; 20–22). A second approach is direct homonuclear decoupling during detection; but this is more challenging as a balance must be struck between the time allocated for the application of radio-frequency decoupling pulses (which are within the bandwidth of the receiver) and the detection of the free induction decay (FID) (23; 24). Nonetheless, rapid stroboscopic techniques for homonuclear decoupling of ^{13}C nuclei in both solution (14; 25) and solid-state NMR (1) have been shown to be quite effective at increasing resolution, although the potential gains in sensitivity have typically been offset by the lower duty cycle of detection.

Here we provide an alternative, high-sensitivity method for band-selective homonuclear decoupling of backbone alpha-carbon (C^α) and carbonyl (C') nuclear spins during direct ^{13}C acquisition that is based upon a novel *long-observation-window bandselective homonuclear decoupling* (LOW BASHD) scheme. LOW-BASHD detection avoids the detrimental effect of low detection duty cycle on sensitivity inherent in rapid, stroboscopic BASHD techniques and requires no additional delay periods be incorporated into the sequence. We have found both increased resolution and sensitivity under LOW-BASHD detection, with signal-to-noise ratio (S/N) improvements up to 1.8 for C' detection. LOW BASHD detection can be incorporated into standard NMR experiments that detect on ^{13}C , as illustrated below for CACO and NCA correlation spectroscopy applied to the $\beta 1$ immunoglobulin binding domain of protein G (GB1) and 3D CBCACO correlation spectroscopy applied to the α -subunit of tryptophan synthase (α -TS).

2. Experimental

2.1 Materials

$\text{U-}^{13}\text{C}$, ^{15}N -GB1 sample preparation: The 56 residue $\beta 1$ immunoglobulin binding domain of protein G was prepared uniformly- ^{13}C , ^{15}N -enriched through overexpression and purification as reported previously (26), with the addition of a final FPLC ion-exchange chromatography step. This final purification step used a Pharmacia Biotech Mono Q® HR5/5 column, 20 mM Tris-HCl buffer (pH 7.3), and a NaCl gradient; GB1 was eluted

between 11% and 13% NaCl and GB1 fractions were verified by SDS-PAGE then pooled, dialyzed against 50 mM sodium phosphate (pH 5.5) at 4°C, and concentrated.

$U\text{-}^{13}\text{C}, ^{15}\text{N}\text{-}\alpha\text{TS}$ sample preparation: Uniformly- $^{13}\text{C}, ^{15}\text{N}$ -labeled *Salmonella typhimurium* tryptophan synthase was expressed and purified as previously described (27). The α -subunit was separated from the holoenzyme by acid precipitation of the β -subunit to yield a suspension of α -subunit in 0.1 M Tris-HCl, pH 7.8 as outlined by Miles et al (28). To determine the conditions for crystal growth that would yield sufficient quantities of the *Salmonella typhimurium* tryptophan synthase α -subunit for solid-state NMR spectroscopy experiments, the procedure of Yamagata et al (29) (used to obtain X-ray quality crystals of the *Pyrococcus furiosus* tryptophan synthase α -subunit) was taken as a starting point. It was observed that crystallization of the α -subunit occurred at concentrations of 30% PEG-8000 at pH 6.5. To achieve slow growth of the crystals, a 0.1 M Tris-HCl suspension of α -subunit, pH 7.8, was diluted 1:1 with a crystallization buffer of 0.1 M MES and 30% PEG-8000, pH 6.5, then dialyzed against the crystallization buffer overnight at 4°C. The contents of the dialysis bag were removed by pipette and centrifuged to collect crystals of the α -subunit.

2.2 NMR Spectroscopy

NMR spectroscopy experiments were performed at 9.4 T (400.37 MHz ^1H , 100.69 MHz ^{13}C , 40.57 MHz ^{15}N) on a Bruker AVIII spectrometer equipped with an $^1\text{H}\text{-}^{13}\text{C}\text{-}^{15}\text{N}$ triple resonance 2.5 mm MAS probe. All experiments were performed at a MAS rate of 20 kHz. Cross-polarization from ^1H to ^{13}C (or ^{15}N for NCA correlation experiments) was accomplished at a ^1H spin-lock field of 73 kHz (tangent ramped ± 10 kHz) and ^{13}C (^{15}N) spin-lock of 53 kHz; 85 kHz XiX ^1H decoupling (30) ($\tau_{\text{pulse}} = 92.5 \mu\text{s}$) was used during direct acquisition. In all experiments, a digitally-oversampled FID (sampling dwell 0.025 μs) was acquired and subsequently digitally filtered to produce a time-domain signal before Fourier transformation. LOW-BASHD detection was implemented using the detection scheme shown in Figure 1 with experiment-specific parameters given below.

1D CP-MAS experiments were performed on $U\text{-}^{13}\text{C}, ^{15}\text{N}$ -labeled glycine with and without LOW BASHD. A digitally-filtered time-domain signal consisting of 128 complex-valued time-domain points spaced at 400 μs (spectral width 2.5 kHz, $t_{\text{acq}}=51.2$ ms) was acquired with the spectral window centered at 175 ppm. LOW BASHD detection was implemented using $\tau_{\text{dec}}=8$ ms and 200 μs Gaussian π -pulses, cosine modulated at 13.5 kHz.

For multidimensional protein solid-state NMR experiments, the temperature of the bearing gas was cooled to 263 K, resulting in an effective sample temperature of $\sim 5^\circ\text{C}$ due to frictional heating. Hard $\pi/2$ and π pulses at 83 kHz for ^{13}C were used throughout, along with 140 kHz XiX ^1H decoupling during indirect evolution, constant time intervals, and refocusing periods, and 85 kHz XiX decoupling during z-filters and direct detection. Chemical shifts were referenced on the DSS scale using adamantane as an external secondary chemical shift standard at 40.48 ppm (downfield peak).

2D CTUC COSY CACO correlation experiments were performed using the pulse sequence reported in reference (9). The mixing times for the CTUC COSY were set to $\tau_1=4.0$ ms and

$\tau_2=4.5$ ms. In the indirect dimension, 318 complex points with a dwell of 25 μ s (spectral width 40 kHz, total acquisition time 7.95 ms) were acquired. In the direct dimension, 128 complex points with a dwell of 400 μ s were acquired (spectral width 2.5 kHz, total acquisition time 51.2 ms) with and without LOW-BASHD decoupling ($\tau_{\text{dec}}=8$ ms and $\tau_{\text{pulse}}=200$ μ s; Gaussian π -pulse, cosine modulated at 13 kHz). 8 scans per transient were averaged with a relaxation delay of 4 s for a total experiment time of 5 hours, 48 minutes.

2D NCA correlation experiments were performed using SPECIFIC CP transfer (31) from ^{15}N to ^{13}C at a ^{15}N field of 7.25 kHz and ^{13}C spin-lock field of 12.75 kHz (tangent ramped ± 2 kHz). In the indirect dimension, 64 complex points with a dwell of 250 μ s (spectral width 4 kHz, total acquisition time 16 ms) were acquired, with the spectral window centered at 120 ppm. In the direct dimension, 128 complex points with a dwell of 300 μ s were acquired (spectral width 3.333 kHz, total acquisition time 38.4 ms) with and without LOW-BASHD decoupling ($\tau_{\text{dec}}=8$ ms and $\tau_{\text{pulse}}=200$ μ s; Gaussian π -pulse, cosine modulated at 12 kHz), as described above. 64 scans per transient with a relaxation delay of 4 s were averaged for a total experiment time of 9 hours, 20 minutes.

3D Constant-time *J*-MAS CBCACO correlation experiments were performed using the pulse sequence in reference (27). A selective 225 μ s r-SNOB pulse was used for the aliphatic-selective π pulse in t_1 and the *J*-transfer delays were set to $\tau_1 = 7.0$ ms, $\tau_2 = 5.0$ ms, and $\tau_4 = 5.0$ ms. As described in references (8; 9), setting $\tau_1 = 7.0$ ms selects against residues with an aliphatic C' , making this experiment semi-selective for alanine, cysteine, serine, asparagine, and aspartic acid residues; aromatic residues are also substantially suppressed due to the bandwidth of the selective pulse. During t_1 and t_2 , the carrier frequency for carbon was placed near the center of the aliphatic carbon resonances (39 ppm), while during t_3 it was moved to 177 ppm for $^{13}\text{C}'$ detection. In both indirect dimensions, 64 complex points with a dwell of 150 μ s (spectral width 6666 kHz, total acquisition time 9.6 ms) were acquired. In the direct dimension, 64 complex points with a dwell of 400 μ s were acquired (spectral width 2.5 kHz, total acquisition time 25.65 ms) with and without LOW-BASHD decoupling ($\tau_{\text{dec}}=8$ ms and $\tau_{\text{pulse}}=200$ μ s; Gaussian π -pulse, cosine modulated at 12 kHz). 8 scans per transient were averaged with a relaxation delay of 3 s for a total experiment time of 4 days, 21 hours, 5 minutes.

3. Results and Discussion

The goal of band-selective homonuclear decoupling is to observe one partner of a *J*-coupled pair while refocusing the scalar-coupling interaction between them. BASHD experiments in uniformly ^{13}C labeled proteins can take advantage of the ~ 120 ppm separation between C' and C^α spins and the relatively consistent value of $^1J_{\text{C}^\alpha\text{C}'}$ of 55 Hz to achieve band-selective homonuclear decoupling using long-observation windows. The general scheme for LOW BASHD detection is shown in Figure 1, where long detection windows (~ 4 –8 ms) are punctuated by short (~ 200 μ s) pulse breaks, during which selective refocusing pulses are applied to one partner of a *J*-coupled pair; this is illustrated in Figure 1 for the case of C' detection with decoupling of C^α , but would be equally applicable to C^α detection with decoupling pulses on C' . The application of the selective π pulses refocuses the *J*-coupling interaction at integer multiples of the decoupling period, τ_{dec} . The length of the detection

and pulse windows are chosen to balance the efficacy of decoupling with breaks in the free induction decay that lower the effective detection duty cycle and that can, if not compensated for, introduce line shape distortions (*vide infra*).

Figure 2 shows the application of LOW BASHD during C' detection for $U-^{13}C, ^{15}N$ -glycine; for comparison, a reference spectrum without LOW BASHD is shown. In the LOW-BASHD detected spectrum, the J -coupling to C^α has been removed, with a corresponding increase in intensity by a factor of 1.70. Decoupling sidebands about the C' resonance have been introduced, but are less than 5% the intensity of the main resonance. These sidebands result from partial evolution under the J -coupling interaction during the relatively long J -refocusing periods. The spacing and intensity of the decoupling sidebands are modulated by the decoupling cycle time (τ_{dec}), with more rapid application of decoupling pulses resulting in less intense, more widely-spaced sidebands. Rapid pulsing, however, leads to an overall lower duty cycle for detection (the receiver must be turned off during the application of the decoupling pulses), as well as the accumulation of off-resonance pulse effects (32) within the spectral window of the observe spins. Both of these lower the intensity of the main resonance. Figure 3 shows the experimental optimization of the spectrum versus decoupling period; 8 ms was chosen above to maximize the main peak intensity. This long period between decoupling pulses means that only 6 pulses are necessary to effect efficient homonuclear decoupling over the 50 ms acquisition time of the FID. Note that to help compensate for first-order, off-resonance pulse effects, a cosine-modulated refocusing pulse is used, which gives two lobes of effective excitation at $\pm$ the modulation frequency, with one lobe centered on the desired decoupling region.

Because the receiver must be gated off during the time that the pulses are applied, there will be brief periods under LOW BASHD during which the FID cannot be observed. There are two options for dealing with this complication while recording the FID. The first is to suspend the digitizer (and its clock) during the decoupling pulses; the second is to leave the digitizer running with the receiver gated off. The former “cut-and-stitch” method effectively excises the gaps in the FID and this was the method used to acquire the spectrum in Figure 2. However, the cut-and-stitch procedure suffers frequency-dependent line shape distortions (Supplemental Material, Figure S1) due to signal discontinuities that appear at the stitch points and become progressively larger with frequency offset; there is also a slight frequency scaling of the spectrum. The second “crevasse” method digitizes the zeroed output of the receiver, leading to periodic holes in the digitized FID (Figure 4a) that give rise to a series of frequency-domain “sampling” sidebands as shown in Figure 4b. The effect of the pulse breaks on the FID are relatively minor because the digital filtration already applied to the oversampled data convolutes a time-domain filter function (33) to produce a signal that does not have sufficient time to fully decay to zero during the gap periods. Here the data were acquired with a sampling dwell of 400 μ s (spectral window of 2500 Hz), corresponding to a relatively broad associated time-domain filter function. At shorter dwell times (larger spectral window, narrower time-domain convolution partner), the signal does indeed fall to zero during the decoupling-pulse periods (Figure S2). While the sampling sideband intensities are small enough to be negligible in some cases, it is desirable in most situations to remove them. There are several ways that this can be accomplished, including linear

prediction and interpolation across the gaps in the time domain signal, and deconvolution in the frequency domain by the application of a suitable time-domain filter (34). We choose the latter method as the more general – once an appropriate LOW-BASHD filter is found, it can be used to process any directly detected signal acquired with the same sampling parameters (digitizer dwell time, frequency-domain filter, and LOW-BASHD sampling schedule). Although the LOW-BASHD filter is in principle calculable, it can also be determined experimentally. Figure 4 shows the time-domain signal for a constant amplitude input (in this case, the DC offset of the receiver) digitized using standard (d) and LOW-BASHD (c) sampling. The LOW-BASHD filter is their respective quotient (with the values during the initial group delay period (35) set to 1) shown in Figure 4e and applied to the FID (Figure 4a) to give the LOW-BASHD corrected FID in Figure 4f, with the corresponding homonuclear decoupled spectrum shown in Figure 4g. We make the approximation that this same filter function can be applied to spectral lines at any offset frequency within the spectral window (a very good approximation, as shown in Figure S3) and use it below for pre-processing the directly detected dimension in 2D and 3D correlation spectroscopy experiments acquired under LOW BASHD.

A major advantage of LOW-BASHD detection is that it can be incorporated into any experiment that detects on ^{13}C . Figure 5 shows three examples: the through-bond, CTUC COSY CACO correlation spectrum (9) of GB1, the through-space, SPECIFIC CP NCA correlation spectrum (31) of GB1, and the through-bond, constant-time *J*-MAS CBCACO 3D correlation spectrum (27) of α -TS. The through-bond CACO correlation spectrum of GB1 shows S/N enhancements in the range of 1.67–1.82 for LOW-BASHD compared to standard detection, with a corresponding improvement of 3 in resolution as the ~55 Hz coupling to C^{α} is removed. Similar gains are found in the NCO correlation spectrum of GB1 shown in Figure S4. LOW-BASHD detection on C^{α} , used to acquire the NCA correlation spectrum of GB1 shown in Figure 5b, increases sensitivity only at glycine residues, but still offers improved resolution as the near 1:2:1 intensity triplets (which result from couplings to both C' and C^{β}) are condensed to narrower 2:2 doublets. LOW BASHD is designed to remove only the coupling between C^{α} and C' ; coupling between C^{α} and C^{β} can be removed by fast stroboscopic BASHD techniques (1), but only with reduced S/N. Finally, Figure 5c shows the alanine region of the 3D CBCACO through-bond correlation spectrum of the 28.5 kDa α -TS. There are 40 alanine resonances in this region, many of which are now resolved. Here again, S/N enhancements in the range of 1.61–1.81 are observed and, even with a more modest acquisition time of 26 ms in the direct dimension, improvements in resolution of a factor of 2 are seen.

LOW-BASHD detection offers higher gains in sensitivity than appended in-phase/anti-phase (IPAP) (17; 18; 36; 37) and related spin-state-selective (S^3) techniques (3) in which two independent spectra (pure or mixed combinations of in-phase and anti-phase components) are acquired. While these techniques are extremely robust, and in our hands give essentially the full theoretical improvement in S/N of $\sqrt{2}$ (see Supplementary Material), there is a distinct benefit to techniques such as LOW BASHD that collapse the multiplet structure directly during observation rather than combining independent multiplet components along with their associated noise from two frequency ranges – a necessary step to get the

maximum S/N increase in IPAP/S³ experiments. Indeed, compared to IPAP at its theoretical maximum enhancement, we see an additional 20%–40% increase in S/N for the LOW-BASHD detected experiments.

4. Conclusion

Sensitivity and resolution remain the two fundamental obstacles to extending solid-state nuclear magnetic resonance to even larger protein systems; LOW-BASHD detection can improve both at a very modest cost in experimental complexity. In LOW BASHD, a single selective refocusing pulse every 8 ms is sufficient to effectively decouple C^α and C' nuclei, allowing for long observation windows and high sensitivity during homonuclear decoupling. While detection using the crevasse method requires a separate LOW-BASHD filter be determined for each combination of LOW-BASHD sampling schedule and digital filter setting, these need only be measured once and can be derived experimentally quickly with very high signal-to-noise ratios. Finally, we note that although demonstrated here for solid-state NMR, LOW-BASHD detection would be equally applicable to ¹³C-detected NMR spectroscopy of proteins in solution (38).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version, at XXX

Highlights

1. A novel band-selective homonuclear decoupling scheme is introduced for ^{13}C
2. Long observation windows and short pulse breaks are used
3. It is specifically designed for ^{13}C acquisition in U- ^{13}C labeled proteins
4. Increases in resolution up to a factor of 3 and sensitivity up to 1.8 are observed
5. It is easily incorporated into existing correlation methods that detect on ^{13}C

Figure 1.

Detection scheme for long-observation window band-selective homonuclear decoupling (LOW BASHD) in which evolution under the J -coupling interaction between detect and partner spins (shown here as C' and C^α , respectively) is refocused at multiples of the decoupling period (τ_{dec}) by the application of short, selective τ pulses on the decoupled partner.

Figure 2. CP-MAS spectrum of U- ^{13}C , ^{15}N -glycine acquired with standard (right) and LOW-BASHD (left) detection. Partial evolution under the J -coupling interaction during the long refocusing periods leads to the decoupling sidebands observed in the LOW BASHD spectrum.

Figure 3.

LOW-BASHD detected CP-MAS spectrum of U- ^{13}C , ^{15}N -glycine acquired with varying refocusing periods, τ_{dec} . As the refocusing periods become shorter, the decoupling sidebands become less intense and push further out. At the same time, the more frequent application of refocusing pulses leads to intensity loss due to lower duty-cycle detection and the accumulation of off-resonance pulse effects. Under these experimental conditions, the optimum balance between these two effects is found at $\tau_{\text{dec}}=8$ ms.

Figure 4.

The LOW-BASHD filter. (a) The FID and (b) corresponding spectrum of U-¹³C, ¹⁵N-glycine acquired under LOW-BASHD detection using the crevasse method. (c) The time-domain signal for a constant DC voltage input (the DC offset of the receiver) digitized under LOW-BASHD and (d) standard detection. (e) The LOW-BASHD filter is the quotient of (d) and (c) (with the initial group delay period set to 1) and multiplies (a) to give the frequency-domain-deconvoluted (f) FID and (g) spectrum.

Figure 5.

Comparisons of LOW-BASHD (left) and standard (right) detection for multidimensional solid-state NMR correlation spectroscopy. (a) The CTUC CACO correlation spectrum of GB1 shows increased sensitivity and resolution for LOW BASHD detection on C' , while (b) the NCA correlation spectrum of GB1 shows increased sensitivity at glycine residues and improved resolution at all residues using LOW BASHD detection on C^α . (c) The alanine region of the constant-time, J-MAS 3D CBCACO correlation spectrum of α -TS shows increased sensitivity and resolution for LOW-BASHD detection on C' .