Optimization of band-selective homonuclear dipolar recoupling in solid-state NMR by a numerical phase search

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ABSTRACT

Spin polarization transfers among aliphatic ^{13}C nuclei, especially ^{13}Ca – $^{13}\text{C}\beta$ transfers, permit correlations of their nuclear magnetic resonance (NMR) frequencies that are essential for signal assignments in multidimensional solid-state NMR of proteins. We derive and demonstrate a new radio-frequency (RF) excitation sequence for homonuclear dipolar recoupling that enhances spin polarization transfers among aliphatic ^{13}C nuclei at moderate magic-angle spinning (MAS) frequencies. The phase-optimized recoupling sequence with five π pulses per MAS rotation period (denoted as PR5) is derived initially from systematic numerical simulations in which only the RF phases are varied. Subsequent theoretical analysis by average Hamiltonian theory explains the favorable properties of numerically optimized phase schemes. The high efficiency of spin polarization transfers in simulations is preserved in experiments, in part because the RF field amplitude in PR5 is only 2.5 times the MAS frequency so that relatively low ^1H decoupling powers are required. Experiments on a microcrystalline sample of the $\beta 1$ immunoglobulin binding domain of protein G demonstrate an average enhancement factor of 1.6 for $^{13}\text{Ca} \rightarrow ^{13}\text{C}\beta$ polarization transfers, compared to the standard ^{13}C – ^{13}C spin-diffusion method, implying a two-fold time saving in relevant 2D and 3D experiments.

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I. INTRODUCTION

In solid-state nuclear magnetic resonance (NMR) of proteins, assignment of ^{15}N , $^{13}\text{C}'$ (carbonyl), ^{13}Ca , and $^{13}\text{C}\beta$ NMR frequencies to specific sites is a prerequisite for molecular structure determination, for investigations of molecular motions, or for nearly any other type of study. Assignments are typically determined from multidimensional solid-state NMR spectra in which chemical shift correlations are revealed through nuclear spin polarization transfers, driven by nuclear magnetic dipolar couplings. Under magic-angle spinning (MAS), which is required for high spectral resolution and good sensitivity, radio-frequency (RF) pulse sequence

techniques that produce “dipolar recoupling” must be employed. ^{15}N – $^{13}\text{C}'$ and ^{15}N – ^{13}Ca chemical shift correlations along a protein backbone can be established by techniques such as double cross-polarization (DCP), ^{RESPIRATION}CP, and “dual back-to-back pulses” (DBP).^{1–4} ^{13}Ca – $^{13}\text{C}\beta$ correlations, which require transfers of nuclear spin polarization from the backbone to amino acid side-chains, are particularly important to identify amino acid types.^{5,6} Although many homonuclear recoupling methods to restore ^{13}C – ^{13}C dipolar couplings have been developed, few are optimized for ^{13}Ca – $^{13}\text{C}\beta$ correlations under moderate MAS frequencies (defined as approximately 10 kHz), where many solid-state NMR experiments are performed.

For uniformly ^{13}C -labeled proteins, band-selectivity is a central concern when transferring ^{13}Ca polarization to ^{13}Cb in the presence of a third spin ^{13}C . Currently, ^{13}Ca - ^{13}Cb correlations are mostly established by spin diffusion methods or radio-frequency-driven recoupling (RFDR) sequences.^{7–17} Although these methods are robust and simple to implement, their lack of selectivity impairs the $^{13}\text{Ca} \rightarrow ^{13}\text{Cb}$ transfer efficiency. For band-selective ^{13}C - ^{13}C recoupling in uniformly labeled samples, a simple approach is to use sequences with low RF fields, such as the “dipolar recoupling enhanced by amplitude modulation” (DREAM) technique,^{18,19} which is particularly suitable at high MAS frequencies and in high magnetic fields.²⁰ RN- and CN-symmetry-based sequences can be designed for homonuclear dipolar recoupling under slow to ultrafast spinning conditions, with performance controlled by basic pulse elements and supercycles.^{21–30} These symmetry-based methods provide various options for the ratio of ^{13}C RF field amplitudes (ω_{rf}) to MAS frequencies (ω_{R}), such as R18₈⁵ ($\omega_{\text{rf}}/\omega_{\text{R}} = 2.25$),^{24,30,31} C3₁C3₁¹ ($\omega_{\text{rf}}/\omega_{\text{R}} = 3$),²⁷ and SPC5₃¹ ($\omega_{\text{rf}}/\omega_{\text{R}} = 3.33$).²⁸

A second important issue for experiments on nondeuterated protein samples is proton decoupling during ^{13}C - ^{13}C homonuclear recoupling. Although recoupling sequences with strong ^{13}C RF fields and no ^1H decoupling have been reported,^{15,32–36} their selectivity and efficiency for $^{13}\text{Ca} \rightarrow ^{13}\text{Cb}$ polarization transfer remain to be explored. For most applications in the slow to

moderate MAS regime, recoupling sequences are accompanied by high power ^1H decoupling. At low MAS frequencies and with strong ^1H decoupling fields, five-fold or seven-fold symmetric homonuclear dipolar recoupling methods perform well,^{37–40} but they are seldom applied at MAS frequencies above 10 kHz, because of the requirements that the ^1H decoupling field amplitude be 2.5–3 times the ^{13}C RF field amplitudes.^{16,41} Courtney and Rienstra have recently summarized these issues related to homonuclear ^{13}C - ^{13}C dipolar recoupling.³⁵

Since the first demonstration of ^{13}C - ^{13}C dipolar recoupling by MAS-synchronized pulse sequences,⁴² homonuclear dipolar recoupling methods have been pursued continually.^{43,44} The performance of symmetry-based recoupling sequences^{21–33,35,37–40,45,46} may be improved by exponential modulation of amplitudes and phases.^{27,47} Phase modulation, matching, or incrementation generates new categories of sequences, including cosine modulated rotary resonance (CMpRR), constant phase increment (CPI), four pulse recoupling (FPR), three pulse recoupling (TPR), and two phase modulated recoupling (TOPR).^{48–51} The optimization of RF phases using genetic algorithms^{52,53} or optimal control^{54–58} may also improve the performance of recoupling sequences.

In this paper, we report the development of useful RF pulse sequences for ^{13}C - ^{13}C recoupling by a simple numerical optimization of RF phases. Based on the idea that almost any pulse sequence

(A) PR5

(B) Selective Transfer

(C) 2D ^{13}C - ^{13}C Correlation

(D) 3D ^{15}N - ^{13}C - ^{13}C Correlation

FIG. 1. Pulse sequences for PR5 and its derivatives. The PR5 block (a) consists of ten π pulses in two rotor periods, which cycle according to the phase scheme ($\phi_1, \phi_2, \phi_3, \phi_4, \phi_5, \phi_6, \phi_7, \phi_8, \phi_9, \phi_{10}$). In this work, the final phase scheme is $(0, 2, 10, 5, 5, 17, 17, 11, 19, 1) \times 18^\circ$. PR5 is incorporated into pulse sequences for selective transfer (b), 2D ^{13}C - ^{13}C correlation (c), and 3D ^{15}N - ^{13}C - ^{13}C (d) experiments. In the selective transfer experiment (b), ^{13}Ca polarization is selected by a Gauss $\pi/2$ pulse and then transferred to other ^{13}C sites. Counterparts of the pulse sequences (b–d) are built by replacing PR5 (and ^1H decoupling) with DARR.⁹ Proton decoupling schemes are CW during PR5 mixing and SPINAL-64 during t_1, t_2 , or t_3 .

can be a recoupling sequence,⁵⁹ we start with a general form for the sequence in which a contiguous train of ten π pulses, all with the same amplitude, is applied within two MAS rotor periods so that $\omega_{rf}/\omega_R = 2.5$ [Fig. 1(a)]. Since the performance of a recoupling sequence is sensitive to the choice of RF phases,^{60–62} we vary the phases of all π pulses and assess the performance of all phase combinations through numerical simulations of $^{13}\text{C}\alpha \rightarrow ^{13}\text{C}\beta$ polarization transfer. We then use experiments to evaluate the performance of the top candidates from the simulations and to compare the new recoupling sequences with existing sequences. We call the resulting pulse sequences “phase-optimized recoupling with five π pulses per rotor period” (PR5). The low ^{13}C RF field in PR5 alleviates the requirement for impractically high ^1H decoupling fields. The use of π pulses in PR5 reduces the adverse effects of phase transients in real experiments.^{63–65} As demonstrated below, the simulated maximal polarization transfer efficiency of PR5 approaches the theoretical efficiency maximum (0.73) of the so-called γ -encoded recoupling methods.⁶⁶ In experiments, owing to the low ^{13}C RF field, PR5 requires relatively low ^1H decoupling power and realizes band-selective ^{13}C – ^{13}C dipolar recoupling. These two features make PR5 practical in aliphatic ^{13}C – ^{13}C correlation experiments on uniformly ^{13}C labeled proteins.

Although PR5 sequences with good performance are found by simulations and verified by experiments without a detailed theoretical analysis, as demonstrated in Sec. III, a theoretical analysis provides insight into factors that affect the properties of specific phase combinations. Section IV contains a general treatment of recoupling sequences that consist of contiguous π pulses with variable phases, based on average Hamiltonian theory (AHT). The lowest-order AHT analysis demonstrates that the optimized PR5 sequences are pure double-quantum (DQ) recoupling sequences with relatively large scaling factors for dipole-dipole couplings and that these sequences are inherently insensitive to chemical shift anisotropy (CSA), resonance offsets, and RF field inhomogeneity.

II. METHODS

A. Numerical simulations

Since the aim of this study is to construct an efficient band-selective homonuclear recoupling sequence requiring relatively low-power ^1H decoupling, we chose the general form for the PR5 sequence to be a pulse train with an RF amplitude (ω_{rf}) of $2.5\omega_R$ (ω_R is the MAS frequency). Choosing the ω_{rf}/ω_R ratio of 2.5 is an empirical trial based on ratios in current methods.^{23,24,28,30,38,39} We fixed each pulse in the train to be a π pulse with a length of $\tau_R/5$, where τ_R is the rotor period, to reduce the number of RF phase changes over the sequence and thus reduce the adverse effects of phase transients in experiments. It should also be noted that π pulses are inherently insensitive to symmetric phase transients, since $R_y(\epsilon)R_x(\pi)R_y(\epsilon) = R_x(\pi)$, where $R_x(\pi)$ is a rotation about the x axis by π and $R_y(\epsilon)$ is a rotation about the y axis by an angle ϵ that represents an RF phase transient.

As shown in Fig. 1(a), the PR5 block contains two rotor periods, each with five π pulses. The ten π pulse phases are marked as $\phi_1, \phi_2, \phi_3, \phi_4, \phi_5, \phi_6, \phi_7, \phi_8, \phi_9$, and ϕ_{10} . In the search for optimal phase schemes, ϕ_1 was fixed to be 0° , ϕ_2 was searched from 0° to 180° , and the other phases were searched from 0° to 342° , with

increments of 18° . To identify the interesting phase scheme candidates as quickly as possible, we used a two-step procedure in simulations. At the first step, we evaluated phase schemes according to their efficiency for polarization transfers (from z polarization of a $^{13}\text{C}\alpha$ spin to z polarization of a $^{13}\text{C}\beta$ spin) with the ideal RF amplitude of ω_{rf} . Small orientational angle sets were used for powder averaging to speed up these simulations. At the second step, we evaluated the most promising phase schemes according to the average polarization transfer efficiency with RF amplitudes of $0.9\omega_{rf}$, ω_{rf} , and $1.1\omega_{rf}$. Phase schemes that were relatively insensitive to variations in the RF amplitude were then tested in experiments.

Simulations of PR5 sequences were performed using Simpson software,⁶⁷ on a $^{13}\text{C}\alpha, ^{13}\text{C}\beta$ spin system at 14.1 T with chemical shifts, CSA, and dipolar couplings given in Table S1. The simulations were run on a personal computer with two Intel Xeon(R) E5-2670 at 2.60 GHz CPU cores (32 threads total). In simulations to optimize the phase schemes, the MAS frequency was 10.00 kHz and the evolution time for polarization transfer (i.e., the mixing time) was 1.2 ms ($12\tau_R$). In simulations presented in figures, the MAS frequency was 11.111 kHz and the mixing time was 1.26 ms ($14\tau_R$).

B. Samples

The procedure to prepare the microcrystalline U- ^{13}C , ^{15}N labeled $\beta 1$ immunoglobulin binding domain of protein G (GB1) was based on our previous work with slight modifications.⁶⁸ U- ^{13}C , ^{15}N labeled wild-type GB1 was expressed in *E. coli* BL21(DE3) in M9 medium containing 1 g/L $^{15}\text{NH}_4\text{Cl}$ and 2 g/L ^{13}C -glucose. Cells were grown to an OD600 of 0.9–1.0 at 37°C and induced with 0.5 mM IPTG for 4 h. The cells were collected by centrifugation at 6000 rpm for 10 min, resuspended in PBS buffer (50 mM phosphate, 150 mM NaCl, and pH 7.4) and disrupted in water at 80°C for 6 min. After a second centrifugation, GB1 protein in the supernatant was exchanged to 50 mM sodium phosphate buffer (pH 5.5) using a HiPrep 26/10 desalting column, further purified by gel exclusion chromatography with a Sephadex 200 column, and then concentrated to 30 mg/mL using Centrplus 3 kDa molecular weight cutoff (MWCO) filters. Microcrystalline protein was prepared from a mixture containing the GB1 solution, 2-methyl-2, 4-pentandiol (MPD), and isopropanol (IPA) in the volume ratio of 1:2:1, which was collected by centrifugation and then transferred into a 3.2 mm thin-wall zirconia rotor with 36 μL sample volume.

Uniformly ^{13}C -labeled alanine (U- ^{13}C -alanine) was purchased from Cambridge Isotope Laboratories (Andover, USA) and packed in a 3.2 mm rotor with 22 μL volume, without recrystallization. No attempt was made to limit the alanine and GB1 samples to the center part of rotors and to mimic the RF inhomogeneity in typical solid-state NMR experiments.

C. NMR experiments

All experiments used a wide-bore Varian 600 MHz solid-state NMR spectrometer with VNMRs software (Agilent Technologies, Santa Clara, USA). Spectra were acquired at a MAS frequency of 11.111 kHz using a 3.2 mm HX T3 probe (experiments on U- ^{13}C -alanine) or a 3.2 mm HXY T3 probe (experiments on U- ^{13}C , ^{15}N GB1). For alanine, the ^1H – ^{13}C cross-polarization (CP) contact time was 0.7 ms. For GB1, the ^1H – ^{13}C CP and ^1H – ^{15}N CP contact times were 1.2 ms, and the ^{15}N – ^{13}C CP contact time was 5.0 ms.

SPINAL-64 decoupling (70 kHz for alanine and 65 kHz for GB1) was used during indirect and direct time periods, and CW decoupling was applied during the selective Gauss $\pi/2$ pulse (90 kHz), ^{15}N - ^{13}C CP (80 kHz), and PR5.⁶⁹ The ^1H decoupling RF fields during PR5 were limited to 0–100 kHz, to focus on practical applications of PR5 in proteins. PR5 was compared to the frequently used dipolar-assisted rotational resonance (DARR) technique,⁹ using the pulse sequences shown in Figs. 1(b)–1(d) and their counterparts with DARR.

III. NUMERICAL AND EXPERIMENTAL RESULTS

A. Optimized PR5 sequence

A systematic search over RF phase schemes resulted in the identification of the scheme ($\phi_1, \phi_2, \phi_3, \phi_4, \phi_5, \phi_6, \phi_7, \phi_8, \phi_9, \phi_{10}$) = (0, 36° , 180° , 90° , 90° , 306° , 306° , 198° , 342° , 18°) as being an optimal choice, in terms of its simulated polarization transfer efficiency in a two-spin system with realistic NMR parameters and its insensitivity to variations in the RF pulse amplitude. Note that this numerically optimized PR5 sequence does not exhibit any obvious symmetry. As mentioned above, there are a number of symmetry-based RF excitation sequences that can cover a wide range of MAS frequencies. We compared PR5 with several of those methods using numerical simulations (see Figs. S1–S3). Compared with the $R18_8^5$ sequence ($\omega_{\text{rf}}/\omega_{\text{R}} = 2.25$),^{24,30,31} the $\text{C}3_1^1\text{C}3_1^1$ sequence ($\omega_{\text{rf}}/\omega_{\text{R}} = 3$),²⁷ and the $\text{SPC}5_3^1$ sequence ($\omega_{\text{rf}}/\omega_{\text{R}} = 3.33$),²⁸ PR5 has a similar maximum polarization transfer efficiency and a relatively short transfer time (Fig. S1A). Sensitivity of PR5 to variations in the ^{13}C RF field amplitude is greater than the sensitivity of $\text{SPC}5_3^1$, comparable to that of $R18_8^5$, and less than that of $\text{C}3_1^1\text{C}3_1^1$ (Fig. S1B). Demands on ^1H decoupling for PR5 are similar to those for $\text{C}3_1^1\text{C}3_1^1$ and $R18_8^5$ and somewhat less than those for $\text{SPC}5_3^1$ (Fig. S2). PR5 has greater NMR frequency band-selectivity than the other recoupling sequences (Fig. S3), which is advantageous for transfer efficiencies in many-spin systems.

B. Double-quantum filtering and polarization transfer efficiencies

For nondeuterated protein samples, dipolar recoupling efficiencies depend strongly on ^{13}C RF field amplitudes and in turn the required ^1H decoupling power. For example, the SPC-5 and POST-C7 sequences require ^{13}C RF fields of $5\omega_{\text{R}}$ and $7\omega_{\text{R}}$, respectively.³⁷ The double-quantum filtering (DQF) efficiency of SPC-5 is reported to be roughly 0.5 at 8.999 kHz MAS with 106 kHz ^1H decoupling⁷⁰ and would decrease at higher MAS frequencies.³⁷ At a MAS frequency of 11.111 kHz and with 100 kHz ^1H decoupling, we found the DQF efficiency of SPC-5 to be only 0.21 for U- ^{13}C -alanine. In contrast, under 90 kHz ^1H decoupling, the DQF efficiency of PR5, which requires a ^{13}C RF field of 2.5 times the MAS frequency, was up to 0.44 (Fig. 2).

Homonuclear dipolar recoupling methods are often applied to measure distances between specific ^{13}C -labeled carbon sites in selectively labeled samples^{42,66,71–73} and are mostly used for polarization transfer in 2D/3D experiments for uniformly labeled proteins.^{5,15,74,75} Figure 3(a) shows the buildup curve of polarization transfer from $^{13}\text{C}\alpha$ to $^{13}\text{C}\beta$ under PR5. The optimal mixing time is

FIG. 2. ^{13}C CP (a) and DQF spectra (b, c) of U- ^{13}C -alanine. The pulse sequences for DQF experiments resemble those in the literature,³⁷ with SPC-5 or PR5 blocks. For SPC-5 (b) and PR5 (c), DQ excitation and reconversion times were both 0.72 ms ($8\tau_{\text{R}}$). The DQF efficiencies of SPC-5 (0.21) and PR5 (0.44) were defined as the integrals of $^{13}\text{C}\beta$ peaks normalized to those in the CP spectrum. Each spectrum was acquired with 8 scans and a recycle delay of 3 s. The ^1H decoupling RF fields were 100 kHz during SPC-5 and 90 kHz during PR5.

1.26 ms ($14\tau_{\text{R}}$) in both simulations and experiments. The simulated maximal transfer efficiency is 0.67, close to the theoretical efficiency 0.73 of γ -encoded methods.⁶⁶ (The full γ -encoded polarization efficiency is achieved with PR5 when chemical shift differences and CSA are set to zero, as shown in Table I and discussed in Sec. IV H.) The experimental maximum efficiency is about 0.5. In this regard, PR5 surpasses the frequently used DARR technique as shown in Fig. 3(b). For U- ^{13}C -alanine, the transfer efficiency of PR5 (with a mixing time of 1.26 ms) is about 1.5 times that of DARR (with a mixing time of 15 ms). The enhancement in efficiency means that, compared to the DARR method, PR5 may reduce the experimental data acquisition time in common 2D/3D experiments, such as aliphatic 2D ^{13}C - ^{13}C , 3D NCACB, and CON(CA)CB experiment, by a factor of two or more.

C. Sensitivity to ^1H decoupling fields and ^{13}C RF fields

In the experiments shown in Figs. 2 and 3, the strength of the ^1H decoupling RF field was set to 90 kHz. As shown in Fig. 4, the polarization transfer efficiency in U- ^{13}C -alanine increased with ^1H decoupling field amplitude, reaching a plateau above 90 kHz. The efficiencies with 70 kHz, 80 kHz, and 90 kHz ^1H decoupling were 57%, 85%, and 97% relative to that with 100 kHz ^1H decoupling, respectively. Stronger ^1H decoupling fields have little effect, according to simulations (Fig. S2). Hohwy *et al.* have shown that the efficiencies of several recoupling schemes oscillate as a function of the ^1H decoupling field at different MAS frequencies.³⁷ Data in Fig. 4 show no such oscillation as long as ^1H decoupling fields are greater than or equal to 70 kHz. The lack of large oscillatory polarization

(A) Buildup Curves

(B) Spectra of $^{13}\text{C}\alpha \rightarrow ^{13}\text{C}\beta$

FIG. 3. Simulated and experimental PR5 buildup curves (a) and spectra (b) of $^{13}\text{C}\alpha \rightarrow ^{13}\text{C}\beta$ transfer. A Gaussian $\pi/2$ pulse of 1.8 ms was used to select $^{13}\text{C}\alpha$ polarization using the sequence shown in Fig. 1(b). The DARR mixing time was optimized to be 15 ms for $^{13}\text{C}\alpha \rightarrow ^{13}\text{C}\beta$ transfer, and the PR5 mixing time was 1.26 ms ($14\tau_R$). The transfer efficiencies of DARR (0.32) and PR5 (0.49) were defined as the integrals of $^{13}\text{C}\beta$ peaks normalized to those of $^{13}\text{C}\alpha$ peaks with no mixing. Each spectrum was acquired with 8 scans and a recycle delay of 3 s. The ^1H decoupling RF field during PR5 was 90 kHz.

transfer under PR5 is attributed to its low ^{13}C RF field ($2.5\omega_R$), which facilitates the setting of ^1H decoupling conditions. Although Fig. S2 shows sharp dips in polarization transfer at certain decoupling field amplitudes, these features are not seen in Fig. 4, presumably due to inhomogeneity of the ^1H RF field, which effectively averages the experimental polarization transfers over a small range of decoupling field amplitudes.

While the ^1H decoupling field is key for high recoupling efficiency, sensitivity to variations in the ^{13}C RF field amplitude is also an important consideration, since RF fields typically have $\pm 10\%$ variations over the sample volume in solid-state NMR experiments. Figure 5 shows the simulated and experimental ^{13}C RF mismatching curves, demonstrating that PR5 is robust toward RF variation or inhomogeneity. This robustness makes the PR5 sequence applicable in long-time experiments and quantitative measurements. In addition, the dependences on the RF field in simulated and experimental curves are in good agreement (apart from the lower absolute efficiency in experiments), confirming that our two-step procedure to numerically search the phase schemes of a robust sequence is reasonable.

D. Frequency band-selectivity

Figure 6(a) shows the simulated dependence of polarization transfer efficiencies on frequency offsets for PR5. Efficient polarization transfer occurs within a bandwidth of roughly 9 kHz, which

covers the frequency ranges of $^{13}\text{C}\alpha$, $^{13}\text{C}\beta$, and other aliphatic ^{13}C chemical shifts in a 14.1 T field (600 MHz ^1H NMR frequency) while avoiding polarization transfers from $^{13}\text{C}\alpha$ to $^{13}\text{C}'$ or from $^{13}\text{C}\beta$ to aromatic ^{13}C . Figure 6(b) shows the experimental polarization transfer as a function of ^{13}C transmitter offsets in U- ^{13}C -alanine, revealing that $^{13}\text{C}\alpha \rightarrow ^{13}\text{C}\beta$ polarization transfer improves with the ^{13}C carrier frequency set closer to the $^{13}\text{C}\beta$ chemical shift. Herein, we focus on the application of PR5 at moderate MAS frequencies. PR5 may also be efficient at higher MAS frequencies (e.g., 40 kHz), although its bandwidth would increase along with the ^{13}C RF fields as simulated in Fig. S4.

Figure 7 shows extracted regions of 2D ^{13}C - ^{13}C correlation spectra of U- ^{13}C , ^{15}N labeled GB1 using the sequence shown in Fig. 1(c). Since the chemical shifts of our sample were very similar to those reported by Frank *et al.*, peaks were assigned accordingly.⁷⁶ In the PR5 spectrum, the red and blue contours indicate that crosspeaks arising from one-step polarization transfers have the opposite sign relative to those arising from auto-correlation or two-step polarization transfers.⁷⁷ This feature of DQ recoupling sequences helps to identify $^{13}\text{C}\beta$, $^{13}\text{C}\gamma$, and/or $^{13}\text{C}\delta$, e.g., Ile6, as shown in Fig. 7. The absence of obvious polarization transfer from $^{13}\text{C}\alpha$ to $^{13}\text{C}'$ confirms the selectivity of PR5. This helps to enhance the transfer to $^{13}\text{C}\beta$. To demonstrate the effectiveness of PR5 on specific residues, we measured two 3D ^{15}N - ^{13}C - ^{13}C spectra using the sequence shown in Fig. 1(d) (DARR for NCACX and PR5 for NCACB). As shown in Fig. 8, the comparison of 1D slices clearly demonstrates the

TABLE I. Effects of phase schemes on the RMS values of tensor coefficients in average Hamiltonians and on the simulated polarization transfer efficiencies. The overall rotation angle (Φ_T) and coefficients ($c_{d,1}$, $c_{d,2}$, $c_{CSA,1}$, $c_{iso,1}$, and $c_{rfi,1}$) were calculated for two rotor periods (upper row for each phase scheme, italicized) and four rotor periods (lower row) as described in Sec. IV G. Changes of phases in the phase scheme used in experiments were highlighted in bold. Simulations of polarization transfer efficiencies were performed for a system of two ^{13}C nuclei without chemical shifts, with three values of the RF field amplitude ($0.9\omega_{rf}$, ω_{rf} , and $1.1\omega_{rf}$, with $\omega_{rf} = 2.5\omega_R$). The MAS frequency was 10.00 kHz, and the mixing time was 1.2 ms.

Phase scheme		RMS values of tensor coefficients					Transfer efficiency		
$\phi_k/18^\circ$ ($k=1, \dots, 10$)	Φ_T (deg)	$c_{d,1}$	$c_{d,2}$	$c_{CSA,1}$	$c_{iso,1}$	$c_{rfi,1}$	$0.9\omega_{rf}$	ω_{rf}	$1.1\omega_{rf}$
0, 2, 10, 5, 5, 17, 17, 11, 19, 1	<i>180</i> 0	<i>0.081</i> 0	<i>0.160</i> 0.160	<i>0.027</i> 0	<i>0.016</i> 0	<i>0.099</i> 0	0.63	0.73	0.68
0, 2, 10, 5, 5, 17, 17, 11, 0, 2	<i>180</i> 0	<i>0.080</i> 0	<i>0.158</i> 0.158	<i>0.034</i> 0	<i>0.027</i> 0	<i>0.146</i> 0	0.65	0.73	0.68
0, 2, 10, 5, 5, 17, 17, 12 , 19, 0	<i>180</i> 0	<i>0.080</i> 0	<i>0.141</i> 0.141	<i>0.026</i> 0	<i>0.030</i> 0	<i>0.027</i> 0	0.51	0.72	0.56
0, 2, 10, 5, 5, 17, 18 , 11, 19, 2	<i>180</i> 0	<i>0.079</i> 0	<i>0.141</i> 0.141	<i>0.043</i> 0	<i>0.094</i> 0	<i>0.135</i> 0	0.64	0.71	0.63
0, 2, 10, 5, 17 , 17, 5 , 11, 19, 1	<i>180</i> 0	<i>0.069</i> 0	<i>0.116</i> 0.116	<i>0.096</i> 0	<i>0.016</i> 0	<i>0.146</i> 0	0.12	0.62	0.10
0, 2, 10, 6, 6 , 17, 17, 11, 19, 1	<i>180</i> 0	<i>0.080</i> 0	<i>0.145</i> 0.145	<i>0.035</i> 0	<i>0.016</i> 0	<i>0.040</i> 0	0.60	0.71	0.60
0, 2, 10, 5, 5, 17, 17, 11, 19, 2	<i>216</i> 72	<i>0.082</i> 0.025	<i>0.155</i> 0.126	<i>0.030</i> 0.009	<i>0.025</i> 0.008	<i>0.069</i> 0.021	0.05	0.10	0.07
0, 2, 10, 5, 5, 17, 17, 12 , 19, 2	<i>252</i> 144	<i>0.079</i> 0.046	<i>0.127</i> 0.039	<i>0.036</i> 0.020	<i>0.041</i> 0.024	<i>0.036</i> 0.021	0.04	0	0.02
0, 2, 10, 5, 5, 17, 11 , 19 , 17, 1	<i>36</i> 72	<i>0.061</i> 0.058	<i>0.135</i> 0.109	<i>0.080</i> 0.074	<i>0.148</i> 0.141	<i>0.151</i> 0.143	0	0.07	0
0, 2, 10, 5, 5, 17, 17, 19 , 11 , 1	<i>36</i> 72	<i>0.056</i> 0.053	<i>0.112</i> 0.090	<i>0.068</i> 0.063	<i>0.251</i> 0.239	<i>0.268</i> 0.255	0.04	0.05	0.03

advantage of PR5 to discriminate $^{13}\text{C}\beta$, $^{13}\text{C}\gamma$, and/or $^{13}\text{C}\delta$. For example, we can easily assign the $^{13}\text{C}\beta$ and $^{13}\text{C}\gamma$ of Glu56, according to the negative and positive signs of peaks relative to $^{13}\text{C}\alpha$, respectively [Fig. 8(a)].

E. Residue-specific sensitivity enhancements

The sensitivity enhancements of PR5 compared to DARR depend on the types of amino acids, more specifically, the amino acid side-chains [Fig. 8(b)]. Based on the topologies and ^{13}C chemical shifts of side-chains, we can classify residues into four groups. (I) Ile and Val, in which $^{13}\text{C}\beta$ directly bonds to two $^{13}\text{C}\gamma$ nuclei. During the recoupling mixing, the $^{13}\text{C}\beta$ polarization coming from $^{13}\text{C}\alpha$ will simultaneously leak to two bonded ^{13}C spins. The average enhancement for Ile and Val in GB1 was 1.14 ± 0.24 , which is small compared to that of U- ^{13}C -alanine. (II) Glu, Gln, Leu, Lys, Met, and Thr, in which $^{13}\text{C}\beta$ bonds to one $^{13}\text{C}\gamma$ nucleus that bonds to an aliphatic $^{13}\text{C}\delta$. The average enhancement of Glu, Gln, Lys, and Met increased to 1.42 ± 0.24 . The enhancement of Thr was expected to be

similar to or greater than that of Leu and Lys, based on the topologies. However, we observed the average enhancement for Thr [Fig. 8(b), green] to be only 1.17 ± 0.23 , due to an unfavorable ^{13}C frequency offset during PR5. The ^{13}C carrier frequency was set at 40 ppm in PR5, ~ 3 kHz and ~ 4.5 kHz away from Thr $^{13}\text{C}\alpha$ (~ 60 ppm) and $^{13}\text{C}\beta$ peaks (~ 70 ppm), respectively. This ^{13}C carrier setting would compromise the transfer efficiency of Thr, according to the matching profile of frequency offsets [Fig. 6(a)]. We observed larger enhancements of Thr in a separate 2D ^{13}C - ^{13}C spectrum by moving the ^{13}C carrier frequency to 60 ppm (data not shown). (III) Ala. The average enhancement of Ala increased to $\sim 1.79 \pm 0.08$, benefiting from the absence of leakage of $^{13}\text{C}\beta$ polarization. (IV) Asp, Asn, Phe, Trp, and Tyr. For Asp and Asn, $^{13}\text{C}\beta$ polarization will leak to carbonyl $^{13}\text{C}\gamma$ in DARR experiments, whereas no such leakage occurs in PR5 experiments since their $^{13}\text{C}\gamma$ chemical shifts (~ 180 ppm) are outside of the effective region of polarization transfer [Fig. 6(a)]. This made the enhancement of Asp and Asn increase to 1.93 ± 0.17 . For Phe, Trp, and Tyr, the leakage of $^{13}\text{C}\beta$ polarization to the aromatic ring will be more than

FIG. 4. Experimental $^{13}\text{C}\alpha \rightarrow ^{13}\text{C}\beta$ polarization transfer as a function of ^1H decoupling RF fields. Polarization transfer percentages are relative to the polarization transfer with a 100 kHz decoupling RF field.

that of Asp and Asn in DARR experiments. Hence, their average enhancement of 2.48 ± 0.22 is the largest among all four groups of residues in proteins. Residues that are not present in GB1 can also be classified based on their topologies and ^{13}C chemical shifts of side chains, such as Arg and Pro in group II, Cys and Ser in group III, and His in group IV. The average enhancement for all the grouped residues in GB1 was about 1.6, meaning that we can reduce the experimental time to about 40% by replacing DARR with PR5 in experiments that involve $^{13}\text{C}\alpha \rightarrow ^{13}\text{C}\beta$ polarization transfer.

FIG. 5. Simulated and experimental $^{13}\text{C}\alpha \rightarrow ^{13}\text{C}\beta$ transfer efficiency as a function of ^{13}C RF fields. In experiments, ^{13}C RF fields varied within 0.9–1.1 times the optimal RF value of $2.5\omega_R$, and the mixing time was 1.62 ms ($18\tau_R$).

In a uniformly ^{13}C -labeled sample, such as the GB1 sample in Figs. 7 and 8, it is not possible to measure long-range ^{13}C – ^{13}C distances, due to interferences between noncommuting pairwise dipole-dipole couplings (i.e., “dipolar truncation”) and due to the fact that one-bond polarization transfers are inherently faster than long-range transfers. In selectively ^{13}C -labeled samples, PR5 may be used for quantitative or semi-quantitative distance measurements, although we have not demonstrated this experimentally.

IV. AVERAGE HAMILTONIAN THEORY OF π PULSE SEQUENCES

A. General formalism

In this section, we derive expressions for average Hamiltonians under recoupling sequences that consist of N contiguous π pulses in M MAS rotation periods τ_R , with radio-frequency (RF) phases ϕ_k ($k = 1, \dots, N$) and a constant RF amplitude ω_{rf} . We consider Hamiltonian terms for the magnetic dipole-dipole coupling $H_d(t)$, the anisotropic chemical shift $H_{CSA}(t)$, the isotropic chemical shift (or resonance offset from the RF carrier frequency) H_{iso} , and the RF inhomogeneity (or deviation from the nominal amplitude ω_{rf}) H_{rfi} . Under MAS at frequency $\omega_R = 2\pi/\tau_R$, these terms can be written as

$$H_d(t) = \frac{\gamma_I^2 \hbar}{R^3} \sum_{m=\pm 1, \pm 2} a_m(\beta) e^{im(\omega_R t + \gamma)} T_{20}, \quad (1)$$

$$H_{CSA}(t) = \gamma_I B_0 \delta_3 \sum_{m=\pm 1, \pm 2} b_m(\beta) e^{im(\omega_R t + \gamma)} T_{10}, \quad (2)$$

$$H_{iso} = \sqrt{2} \gamma_I \delta_{iso} B_0 T_{10}, \quad (3)$$

$$H_{rfi}(t) = \varepsilon_{rf} (I_x \cos \phi_k + I_y \sin \phi_k), \quad (k-1)\tau_p < t < k\tau_p \\ = \varepsilon_{rf} (T_{11} e^{-i\phi_k} - T_{1-1} e^{i\phi_k}), \quad (k-1)\tau_p < t < k\tau_p. \quad (4)$$

In Eqs. (1)–(4), γ_I is the gyromagnetic ratio for nuclear spins I , R is the internuclear distance for spin pairs I_1 and I_2 , B_0 is the static magnetic field of the NMR spectrometer, δ_3 is the third principal

FIG. 6. Simulated (a) and experimental (b) polarization transfer efficiencies as functions of ^{13}C frequency offsets. In experiments, the zero point of the ^{13}C transmitter offset is at 37.7 ppm, between the $^{13}\text{C}\alpha$ and $^{13}\text{C}\beta$ resonance peaks of U- ^{13}C -alanine.

value of the traceless chemical shift anisotropy (CSA) tensor (with $\delta_1 + \delta_2 + \delta_3 = 0$), δ_{iso} is the isotropic chemical shift or resonance offset, ε_{rf} is the deviation of the RF field amplitude from ω_{rf} , and $\tau_p = \pi/\omega_{rf}$ is the π pulse length. Irreducible tensor operator components are defined as

$$T_{20} = (3I_{z1}I_{z2} - \mathbf{I}_1 \cdot \mathbf{I}_2)/\sqrt{6},$$

$$T_{2\pm 1} = \mp (I_{z1}I_{\pm 2} + I_{\pm 1}I_{z2})/2, \quad (5)$$

$$T_{2\pm 2} = I_{\pm 1}I_{\pm 2}/2,$$

$$T_{10} = I_z/\sqrt{2},$$

$$T_{1\pm 1} = \pm I_{\pm}/2. \quad (6)$$

The Euler angles α, β, γ describe the orientation of the internuclear direction [for $H_d(t)$] or the CSA principal axis system [for $H_{CSA}(t)$] relative to an axis system that is fixed in the MAS rotor, with its z axis being the axis of sample rotation, which makes the angle $\theta_m = \cos^{-1}(1/\sqrt{3})$ with the static magnetic field. In Eqs. (1) and (2),

$$a_{\pm 1} = -\frac{\sqrt{3}}{2} \sin 2\beta,$$

$$a_{\pm 2} = \frac{\sqrt{6}}{4} \sin^2 \beta, \quad (7)$$

FIG. 7. Extracted regions from the 2D ^{13}C - ^{13}C correlation spectra of U- ^{13}C , ^{15}N labeled GB1, using 15 ms DARR mixing (gray) or 1.26 ms PR5 mixing (red and blue). In the PR5 spectrum, the red and blue contours indicate negative and positive cross-peaks, arising from one-bond correlations (red) and auto-correlations or sequential two-bond correlations (blue). Each spectrum was acquired with 8 scans and a recycle delay of 2.5 s, requiring 4.5 h of data acquisition. The ^{13}C carrier transmitter was set at 100.1 ppm, except during the PR5 mixing, where it was shifted to 40.0 ppm. The ^1H decoupling RF field during PR5 was 90 kHz.

FIG. 8. Slices (a) and site-specific enhancements (b) from 3D ^{15}N - ^{13}C - ^{13}C (NCACX or NCACB) spectra of U- ^{13}C , ^{15}N labeled GB1, using 15 ms DARR mixing or 1.26 ms PR5 mixing. The 3D spectra were acquired using the sequence shown in Fig. 1(d). (a) Slices (DARR in black and PR5 in red) were extracted from spectra processed by nmrPipe,⁷⁸ integrated within specific regions (marked in gray), and plotted with the same scale. (b) The enhancement factors for PR5 relative to DARR are defined as the ratios of peak integrals in 1D slices, and errors are estimated based on the integrals of noise regions. The enhancement factors are plotted against the residue number in the GB1 sequence. Filled triangles ($\blacktriangledown$) represent Ile and Val (group I), squares ($\square$) represent Glu, Gln, Leu, Lys, Met, and Thr (Thr in green) (group II), filled diamonds ($\blacklozenge$) represent Ala (group III), and circles ($\circ$) represent Asp, Asn, Phe, Trp, and Tyr (group IV). Leu7 and Leu12 share the same integral because their peaks are too close to be resolved in the 3D NCC spectra. Phe30 and Tyr45 also share the same integral for the same reason. Gly residues are not shown because they lack $^{13}\text{C}\beta$, and Thr25 is not shown because its $^{13}\text{C}\alpha$ and $^{13}\text{C}\beta$ have close chemical shifts that lead to cancellation of $^{13}\text{C}\alpha$ and $^{13}\text{C}\beta$ peaks in the PR5 spectrum. The ^{15}N carrier frequency was set at 119.0 ppm, and the ^{13}C carrier frequency was set at 55.0 ppm, except during the PR5 mixing, where it was shifted to 40.0 ppm. Maximum evolution times were 8.46 ms (48 real points, 180 μs increment) in the ^{15}N dimension (t_1), and 4.23 ms (48 complex points, 90 μs increment) in the $^{13}\text{C}\alpha$ dimension (t_2). Each spectrum was acquired with 4 scans per free-induction decay and a recycle delay of 3 s, requiring 15.5 h for data acquisition. The two 3D spectra were processed with the identical parameters in nmrPipe. The ^1H decoupling RF field during PR5 was 90 kHz.

$$b_{\pm 1} = -\frac{\sin 2\beta}{2} - \frac{\eta}{6} [e^{\pm 2i\alpha} \sin \beta (1 + \cos \beta) - e^{\mp 2i\alpha} \sin \beta (1 - \cos \beta)],$$

$$b_{\pm 2} = \frac{\sin^2 \beta}{2\sqrt{2}} + \frac{\eta}{12\sqrt{2}} [e^{\pm 2i\alpha} (1 + \cos \beta)^2 + e^{\mp 2i\alpha} (1 - \cos \beta)^2]$$

(8)

with asymmetry parameter $\eta = (\delta_1 - \delta_2)/\delta_3$.

According to the standard formulation of AHT,^{79,80} average Hamiltonians are calculated in the interaction representation with

respect to the Hamiltonian term $H_{rf}(t)$ that describes the applied RF pulse sequence

$$H_{rf}(t) = \omega_{rf}(I_x \cos \phi_k + I_y \sin \phi_k), \quad (k-1)\tau_p < t < k\tau_p. \quad (9)$$

The evolution operator $U_{rf}(t)$ produced by $H_{rf}(t)$ is a product of rotations, which can be expressed as

$$U_{rf}(t) = \exp[-i\omega_{rf}(I_x \cos \phi_k + I_y \sin \phi_k)t'] U_{rf}((k-1)\tau_p), \quad (k-1)\tau_p < t < k\tau_p$$

$$= R_z(\phi_k) R_x(\omega_{rf}t') R_z(-\phi_k) U_{rf}((k-1)\tau_p), \quad (k-1)\tau_p < t < k\tau_p, \quad (10)$$

$$U_{rf}((k-1)\tau_p) = R_z(\phi_{k-1}) R_x(\pi) R_z(-\phi_{k-1}) R_z(\phi_{k-2}) R_x(\pi) R_z(-\phi_{k-2}) \dots R_z(\phi_1) R_x(\pi) R_z(-\phi_1)$$

$$= R_z(2\phi_{k-1} - 2\phi_{k-2} + 2\phi_{k-3} - \dots + 2(-1)^k \phi_1) R_x((k-1)\pi)$$

$$= R_z(\Phi_k) R_x((k-1)\pi), \quad (11)$$

with $t' = t - (k-1)\tau_p$ and $\Phi_k = 2 \sum_{j=1}^{k-1} (-1)^{k+j-1} \phi_j$. $R_u(\xi)$ is the operator for a rotation of spin angular momentum about the u axis by angle ξ .

Equations (10) and (11) use the relations $R_x(\theta) = e^{-iI_x\theta}$, $R_z(\phi) = e^{-iI_z\phi}$, $R_z(\phi_a)R_z(\phi_b) = R_z(\phi_a + \phi_b)$, and $R_x(\pi)R_z(\phi) = R_z(-\phi)R_x(\pi)$.

In the interaction representation with respect to $H_{rf}(t)$, the other Hamiltonian terms $H_x(t)$ become

$$\tilde{H}_x(t) = U_{rf}(t)^{-1} H_x(t) U_{rf}(t)$$

$$= R_x((k-1)\pi) R_z(-\Phi_k) R_z(\phi_k) R_x(-\omega_{rf}t') R_z(-\phi_k) H_x(t) R_z(\phi_k)$$

$$\times R_x(\omega_{rf}t') R_z(-\phi_k) R_z(\Phi_k) R_x((k-1)\pi), \quad (k-1)\tau_p < t < k\tau_p. \quad (12)$$

Note that we take $R_x(-(k-1)\pi) = R_x((k-1)\pi)$, since rotations by π or $-\pi$ have the same effect on spin angular momentum operators. The contribution to the average Hamiltonian from the k th π pulse is then

$$\tilde{H}_{x,ave}^{(k)} = \frac{1}{\tau_p} \int_{(k-1)\tau_p}^{\tau_p} dt \tilde{H}_x(t)$$

$$= \frac{1}{\tau_p} \{ R_x((k-1)\pi) R_z(\phi_k - \Phi_k) \left[\int_0^{\tau_p} dt' R_x(-\omega_{rf}t') R_z(-\phi_k) H_x(t' + (k-1)\tau_p) R_z(\phi_k) R_x(\omega_{rf}t') \right]$$

$$\times R_z(\Phi_k - \phi_k) R_x((k-1)\pi) \}.$$

(13)

The total average Hamiltonian is $\tilde{H}_{x,ave} = \frac{1}{N} \sum_{k=1}^N \tilde{H}_{x,ave}^{(k)}$.

B. Average dipole-dipole coupling

To evaluate $\tilde{H}_{d,ave}^{(k)}$ with Eqs. (1) and (13), we use $R_z(\phi) T_{2m} R_z(-\phi) = e^{-im\phi} T_{2m}$ and $R_x(-\omega_{rf}t') T_{20} R_x(\omega_{rf}t') = R_z(\pi/2) R_y(\omega_{rf}t') T_{20} R_y(-\omega_{rf}t') R_z(-\pi/2)$

$$= R_z(\pi/2) \left[\frac{\sqrt{6}}{8} (1 - \cos 2\omega_{rf}t') (T_{22} + T_{2-2}) + \frac{\sqrt{6}}{4} \sin 2\omega_{rf}t' (T_{21} - T_{2-1}) + \frac{1}{4} (1 + 3 \cos 2\omega_{rf}t') T_{20} \right] R_z(-\pi/2)$$

$$\equiv R_z(\pi/2) \left[\sum_{q=-2}^2 f_q(t') T_{2q} \right] R_z(-\pi/2). \quad (14)$$

Then,

$$\tilde{H}_{d,ave}^{(k)} = \frac{\gamma_I^2 \hbar}{R^3} \sum_{m=\pm 1, \pm 2} \sum_{q=-2}^2 a_m(\beta) e^{-iq(\phi_k - \Phi_k + \pi/2)} e^{im[\gamma + (k-1)\omega_R \tau_p]} \times I_{q,m} R_x((k-1)\pi) T_{2q} R_x((k-1)\pi), \quad (15)$$

with $I_{q,m} \equiv \frac{1}{\tau_p} \int_0^{\tau_p} dt' e^{im\omega_R t'} f_q(t')$. Explicit expressions for $I_{q,m}$ are

$$\begin{aligned} I_{0,m} &= -\frac{Ni}{8M\pi} (e^{2imM\pi/N} - 1) \left[\frac{3}{2(m + \frac{N}{M})} + \frac{3}{2(m - \frac{N}{M})} + \frac{1}{m} \right], \\ I_{\pm 1,m} &= \pm \frac{\sqrt{6}N}{16M\pi} (e^{2imM\pi/N} - 1) \left[\frac{1}{(m - \frac{N}{M})} - \frac{1}{(m + \frac{N}{M})} \right], \\ I_{\pm 2,m} &= -\frac{\sqrt{6}Ni}{16M\pi} (e^{2imM\pi/N} - 1) \left[\frac{1}{2(m + \frac{N}{M})} + \frac{1}{2(m - \frac{N}{M})} - \frac{1}{m} \right] \end{aligned} \quad (16)$$

using $\omega_R \tau_p = 2M\pi/N$ and $\omega_{rf} = N\omega_R/(2M)$.

Since $R_x((k-1)\pi) T_{2q} R_x((k-1)\pi) = T_{2q'}$, with $q' = (-1)^{k-1}q$, the coefficient of T_{2q} in the total average Hamiltonian is

$$c_{d,q}(\beta, \gamma) = \frac{\gamma_I^2 \hbar}{NR^3} \sum_{k=1}^N \sum_{m=\pm 1, \pm 2} a_m(\beta) e^{(-1)^k i q (\phi_k - \Phi_k + \pi/2)} \times e^{im\{\gamma + [2(k-1)M\pi/N]\}} I_{(-1)^{k-1}q,m}. \quad (17)$$

For $q = 0$ and for a given value of m in Eq. (17), the sum over k is a sum over phase factors of the form $(e^{2imM\pi/N})^{k-1}$. In general, $\sum_{k=1}^N (e^{2imM\pi/N})^{k-1} = 0$ for any integer n , unless n is a multiple of N . Consequently, since $|m| = 1$ or 2 , $c_{d,0}(\beta, \gamma)$ must be zero if M/N is not a multiple of $1/2$.

C. Average chemical shift anisotropy

To evaluate $\tilde{H}_{CSA,ave}^{(k)}$ with Eqs. (2) and (13), we use

$$\begin{aligned} R_x(-\omega_{rf} t') T_{10} R_x(\omega_{rf} t') &= R_z(\pi/2) \left[\frac{\sin \omega_{rf} t'}{\sqrt{2}} (T_{11} - T_{1-1}) \right. \\ &\quad \left. + \cos \omega_{rf} t' T_{10} \right] R_z(-\pi/2) \\ &\equiv R_z(\pi/2) \left[\sum_{q=-1}^1 g_q(t') T_{1q} \right] R_z(-\pi/2). \end{aligned} \quad (18)$$

Then,

$$\begin{aligned} \tilde{H}_{CSA,ave}^{(k)} &= \gamma_I B_0 \delta_3 \\ &\times \sum_{m=\pm 1, \pm 2} \sum_{q=-1}^1 b_m(\alpha, \beta) e^{-iq(\phi_k - \Phi_k + \pi/2)} e^{im[\gamma + (k-1)\omega_R \tau_p]} \\ &\times I_{q,m} R_x((k-1)\pi) T_{1q} R_x((k-1)\pi), \end{aligned} \quad (19)$$

with $J_{q,m} \equiv \frac{1}{\tau_p} \int_0^{\tau_p} dt' e^{im\omega_R t'} g_q(t')$. Explicit expressions for $J_{q,m}$ are

$$\begin{aligned} J_{0,m} &= \frac{iN}{2M\pi} (e^{2imM\pi/N} + 1) \left[\frac{1}{(2m + \frac{N}{M})} + \frac{1}{(2m - \frac{N}{M})} \right], \\ J_{\pm 1,m} &= \pm \frac{N}{2\sqrt{2}M\pi} (e^{2imM\pi/N} + 1) \left[\frac{1}{(2m + \frac{N}{M})} - \frac{1}{(2m - \frac{N}{M})} \right]. \end{aligned} \quad (20)$$

Since $R_x((k-1)\pi) T_{1q} R_x((k-1)\pi) = (-1)^{k-1} T_{1q'}$, with $q' = (-1)^{k-1}q$, the coefficient of T_{1q} in the total average Hamiltonian is

$$c_{CSA,q}(\alpha, \beta, \gamma) = \frac{\gamma_I B_0 \delta_3}{N} \sum_{k=1}^N \sum_{m=\pm 1, \pm 2} (-1)^{k-1} b_m(\alpha, \beta) e^{(-1)^k i q (\phi_k - \Phi_k + \pi/2)} \times e^{im\{\gamma + [2(k-1)M\pi/N]\}} J_{(-1)^{k-1}q,m}. \quad (21)$$

For $q = 0$ and for a given value of m in Eq. (21), the sum over k is a sum over phase factors of the form $(-e^{2imM\pi/N})^{k-1}$. In general, for even N , $\sum_{k=1}^N (-e^{2imM\pi/N})^{k-1} = (1 - e^{2imM\pi/N}) \sum_{k=1}^{N/2} (e^{2imM\pi/N})^{k-1} = 0$ for any integer n , unless n is an odd multiple of $N/2$. Consequently, $c_{CSA,0}(\beta, \gamma)$ must be zero if M/N is neither an odd multiple of $1/4$ nor an odd multiple of $1/2$.

D. Average isotropic chemical shift

In the interaction representation with respect to $H_{rf}(t)$, the isotropic chemical shift term becomes

$$\begin{aligned} \tilde{H}_{iso}(t) &= (B_0 \delta_{iso}) U_{rf}((k-1)\tau_p)^{-1} R_z(\phi_k) \\ &\times [\sqrt{2} \cos \omega_{rf} t' T_{10} - i \sin \omega_{rf} t' (T_{11} + T_{1-1})] \\ &\times R_z(-\phi_k) U_{rf}((k-1)\tau_p), \quad (k-1)\tau_p < t < k\tau_p. \end{aligned} \quad (22)$$

Then,

$$\begin{aligned} \tilde{H}_{iso,ave}^{(k)} &= -\frac{2i}{\pi} R_x((k-1)\pi) \left(e^{i(\Phi_k - \phi_k)} T_{11} + e^{-i(\Phi_k - \phi_k)} T_{1-1} \right) \\ &\times R_x((k-1)\pi) \end{aligned} \quad (23)$$

and the coefficient of $T_{1\pm 1}$ in the total average Hamiltonian is

$$c_{iso,\pm 1} = -\frac{2i}{N\pi} \sum_{k=1}^N (-1)^{k-1} e^{\pm i(\Phi_k - \phi_k)}. \quad (24)$$

The coefficient of T_{10} is always zero.

E. Average RF inhomogeneity

In the interaction representation with respect to $H_{rf}(t)$, the RF inhomogeneity term becomes

$$\begin{aligned} \tilde{H}_{rfi}(t) &= \varepsilon_{rf} U_{rf}((k-1)\tau_p)^{-1} (e^{-i\phi_k} T_{11} - e^{i\phi_k} T_{1-1}) U_{rf}((k-1)\tau_p), \\ &\quad (k-1)\tau_p < t < k\tau_p. \end{aligned} \quad (25)$$

Then,

$$\begin{aligned} \tilde{H}_{rfi,ave}^{(k)} &= \varepsilon_{rf} R_x((k-1)\pi) \left(e^{i(\Phi_k - \phi_k)} T_{11} - e^{-i(\Phi_k - \phi_k)} T_{1-1} \right) \\ &\times R_x((k-1)\pi) \end{aligned} \quad (26)$$

and the coefficient of $T_{1\pm1}$ in the total average Hamiltonian is

$$c_{rfi,\pm1} = \pm \frac{\varepsilon_{rf}}{N} \sum_{k=1}^N e^{\pm(-1)^k i(\phi_k - \Phi_k)}. \quad (27)$$

Again, the coefficient of T_{10} is always zero.

F. Effect of a nonzero overall rotation

The overall evolution operator for one repetition of the pulse sequence is $U(N\tau_p) \approx U_{rf}(N\tau_p) \exp(-iN\tilde{H}_{ave}\tau_p) = R_z(\Phi_T) \exp(-iN\tilde{H}_{ave}\tau_p)$, with $\Phi_T = 2\phi_N - \Phi_N$. If the overall rotation angle Φ_T is not a multiple of 2π , then the average Hamiltonian for n repetitions of the pulse sequence is not simply $\tilde{H}_{ave}$. Instead, $\tilde{H}_{ave}$ is averaged further by $U_{rf}(N\tau_p)$ according to

$$\begin{aligned} U(nN\tau_p) &\approx U_{rf}(N\tau_p) e^{-iN\tilde{H}_{ave}\tau_p} U_{rf}(N\tau_p) \\ &\times e^{-iN\tilde{H}_{ave}\tau_p} \dots U_{rf}(N\tau_p) e^{-iN\tilde{H}_{ave}\tau_p} \\ &= [U_{rf}(N\tau_p)]^n e^{-iN\tilde{H}_{ave}^{(n)}\tau_p} \\ &\times e^{-iN\tilde{H}_{ave}^{(n-1)}\tau_p} e^{-iN\tilde{H}_{ave}^{(n-2)}\tau_p} \dots e^{-iN\tilde{H}_{ave}^{(1)}\tau_p}, \end{aligned} \quad (28)$$

with

$$\begin{aligned} \tilde{H}_{ave}^{(j)} &= [U_{rf}(N\tau_p)^{-1}]^{j-1} \tilde{H}_{ave} [U_{rf}(N\tau_p)]^{j-1} \\ &= R_z(-(j-1)\Phi_T) \tilde{H}_{ave} R_z((j-1)\Phi_T). \end{aligned} \quad (29)$$

The average Hamiltonian for n repetitions of the pulse sequence becomes $\tilde{H}_{ave} = \frac{1}{n} \sum_{j=1}^n \tilde{H}_{ave}^{(j)}$. For example, if Φ_T is an odd multiple of π , irreducible tensor components $T_{2\pm1}$ and $T_{1\pm1}$ that may be present in $\tilde{H}_{ave}$ vanish from $\tilde{H}_{ave}$ when n is even. If $\Phi_T = 2\pi/3$, irreducible tensor components $T_{2\pm1}$, $T_{2\pm2}$, and $T_{1\pm1}$ vanish from $\tilde{H}_{ave}$ when n is a multiple of three.

G. Numerical calculations of average Hamiltonians

A Fortran95 program called `ave_ham1.f95` (available upon request from the authors at robertty@mail.nih.gov) was written to perform numerical calculations of the root-mean-squared (RMS) values of the coefficients of tensor operator components in the average Hamiltonians, averaged over orientations. This program uses the expressions in Eqs. (17), (21), (24), and (27). Results are calculated in units of $\gamma_I^2 \hbar / R^3$, $\gamma_I B_0 \delta_3$, $\gamma_I \delta_{iso} B_0$, and ε_{rf} , respectively. For the CSA interaction, an axially symmetric tensor (i.e., $\eta = 0$) is assumed so that orientational averaging involves only β and γ . The program allows arbitrary π pulse phases and arbitrary values of M and N , subject to the restriction that $|N/M| \neq 1, 2$, or 4 to avoid divergences in Eqs. (16) or (20). The program also calculates the net z rotation Φ_T discussed above.

H. Dependence of average Hamiltonians on the choice of phases

Table I shows the effects of changes in RF phases on the overall rotation angle (Φ_T), the RMS values of the coefficients of tensor operator components in the average Hamiltonians ($c_{d,1}$, $c_{d,2}$, $c_{CSA,1}$, $c_{iso,1}$, and $c_{rfi,1}$), and the simulated transfer efficiency. For the phase scheme (0, 2, 10, 5, 5, 17, 17, 11, 19, 1) $\times 18^\circ$ used in experiments, the RMS value of $c_{d,2}$ (for $T_{2\pm2}$) was 0.160. Values of other

coefficients were smaller in two rotor periods, suggesting that this phase scheme for PR5 is an approximate DQ recoupling method. Repeating the same phase scheme twice, in four rotor periods, results in a pure DQ recoupling sequence (i.e., all coefficients zero except for $c_{d,2}$), in agreement with experimental results described above.

Table I also shows that changes in the individual RF phases ϕ_k affect both Φ_T and the tensor component coefficients, resulting in different polarization transfer efficiencies. As expected, sequences with large RMS values of $c_{d,2}$ as well as zero values of other components (over four rotor periods) provide high transfer efficiencies. Minor changes in the RF phases (e.g., changing ϕ_{10} from 18° to 36° while keeping all other phases constant) can greatly reduce the polarization transfer efficiencies, primarily through effects on Φ_T .

Finally, Table I shows that the orientationally averaged polarization transfer efficiency for PR5 with an optimized phase scheme is 0.73, numerically equal to the efficiency of γ -encoded recoupling sequences,⁶⁶ when chemical shifts and CSA are zero. This is a surprising observation, since PR5 is not explicitly γ -encoded. More specifically, γ -encoded sequences have the property that only one value of m contributes to $c_{d,2}(\beta, \gamma)$ in Eq. (17) so that the magnitude of the average Hamiltonian (or $|c_{d,2}(\beta, \gamma)|$) is strictly independent of γ . The resulting dipolar powder pattern line shape for a two-spin system has sharp “horns,” approximating a simple doublet, which leads to a sinusoid-like oscillation in the time-dependence of the orientationally averaged polarization transfer and hence to large transfer efficiency. Numerical calculations within `ave_ham1.f95` indicate that this standard γ -encoding condition is not satisfied for PR5. Nonetheless, the same transfer efficiency is achieved.

V. CONCLUSION

We have demonstrated the feasibility of constructing useful dipolar recoupling sequences for solid state NMR through systematic optimization of only the RF phases (but not RF pulse lengths or amplitudes) within a pulse train. By systematic phase optimization of a train of ten π pulses with a constant RF amplitude (equal to $2.5\omega_R$), we have obtained the new band-selective homonuclear dipolar recoupling sequence PR5, which provides high polarization transfer efficiency for aliphatic ^{13}C sites in proteins under moderate MAS frequencies and high magnetic fields. Good performance is achieved without imposing symmetry on the pulse sequence.

Due to its high recoupling efficiency and the practicality of its ^{13}C RF field amplitude and related ^1H decoupling RF amplitude (<100 kHz at MAS frequencies below 13 kHz), PR5 is a promising tool for solid-state NMR studies of proteins and other organic materials. At higher MAS frequencies, PR5 may also be useful, especially for deuterated samples where low decoupling amplitudes can be used. Similar approaches to pulse sequence design may be useful for other purposes, such as homonuclear decoupling and heteronuclear recoupling.

SUPPLEMENTARY MATERIAL

See [supplementary material](#) for Fig. S1: simulated polarization transfer for PR5 and other recoupling sequences as a function of

time and RF amplitude; Fig. S2: simulated polarization transfer for PR5 and other recoupling sequences as a function of proton decoupling amplitude; Fig. S3: simulated polarization transfer for PR5 and other recoupling sequences as a function of frequency offsets; Fig. S4: simulated polarization transfer under PR5 as a function of frequency offsets at 40.0 kHz MAS frequency; Table S1: spin Hamiltonian parameters for two-spin simulations; and Table S2: spin Hamiltonian parameters for four-spin simulations.

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