Proton assisted recoupling and protein structure determination

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We introduce a homonuclear version of third spin assisted recoupling, a second-order mechanism that can be used for polarization transfer between ^{13}C or ^{15}N spins in magic angle spinning (MAS) NMR experiments, particularly at high spinning frequencies employed in contemporary high field MAS experiments. The resulting sequence, which we refer to as proton assisted recoupling (PAR), relies on a cross-term between $^1\text{H}-^{13}\text{C}$ (or $^1\text{H}-^{15}\text{N}$) couplings to mediate zero quantum $^{13}\text{C}-^{13}\text{C}$ (or $^{15}\text{N}-^{15}\text{N}$ recoupling). In particular, using average Hamiltonian theory we derive an effective Hamiltonian for PAR and show that the transfer is mediated by trilinear terms of the form $C_1^\pm C_2^\mp H_Z$ for $^{13}\text{C}-^{13}\text{C}$ recoupling experiments (or $N_1^\pm N_2^\mp H_Z$ for $^{15}\text{N}-^{15}\text{N}$). We use analytical and numerical simulations to explain the structure of the PAR optimization maps and to delineate the PAR matching conditions. We also detail the PAR polarization transfer dependence with respect to the local molecular geometry and explain the observed reduction in dipolar truncation. Finally, we demonstrate the utility of PAR in structural studies of proteins with $^{13}\text{C}-^{13}\text{C}$ spectra of uniformly ^{13}C , ^{15}N labeled microcrystalline Crh, a 85 amino acid model protein that forms a domain swapped dimer (MW=2 × 10.4 kDa). The spectra, which were acquired at high MAS frequencies ($\omega_r/2\pi > 20$ kHz) and magnetic fields (750–900 MHz ^1H frequencies) using moderate rf fields, exhibit numerous cross peaks corresponding to long (up to 6–7 Å) $^{13}\text{C}-^{13}\text{C}$ distances which are particularly useful in protein structure determination. Using results from PAR spectra we calculate the structure of the Crh protein. © 2008 American Institute of Physics. [DOI: 10.1063/1.3036928]

I. INTRODUCTION

Solid-state NMR (SSNMR) structural studies of proteins are generally performed under high resolution conditions obtained via magic angle spinning (MAS), an approach which averages second rank tensor interactions such as the chemical shift anisotropy and the homonuclear dipolar interaction.¹ Thus, while MAS introduces high resolution by attenuating the shift anisotropy, it also suppresses the distance information that is the source of structural data in the spectra. This structural information can be reintroduced selectively in a manner consistent with the goal of high resolution by the application of a carefully chosen sequence of rotor synchronized rf pulses,^{2–8} an approach that was introduced approximately 20 years ago and is referred to as dipolar recoupling. Since the original experiments, homonuclear and heteronuclear recoupling sequences have been refined in many ways and now allow accurate measurement^{3,9–11} of distances and torsion angles¹² as well as distance estimates using spin diffusion based techniques.^{13–17}

Among the repertoire of dipolar recoupling techniques, homonuclear experiments, which recouple $^{13}\text{C}-^{13}\text{C}$ and $^{15}\text{N}-^{15}\text{N}$ spins, play an especially important role in biomolecular structural studies.

In particular, the favorable dispersion of ^{13}C and ^{15}N chemical shifts and the development of efficient methods for $^1\text{H}-^{13}\text{C}/^{15}\text{N}$ decoupling^{18–20} ensure high spectral resolution that is essential for spectral assignments. Moreover, $^{13}\text{C}-^{13}\text{C}$ spectra also provide structurally valuable restraints, and they are potentially more numerous than either $^{15}\text{N}-^{15}\text{N}$ or $^{15}\text{N}-^{13}\text{C}$ distance restraints. Thus, homonuclear $^{13}\text{C}-^{13}\text{C}$ recoupling sequences have been used extensively in SSNMR structural studies.^{14,16,21–23}

Despite their importance for structure determination, the measurement of long $^{13}\text{C}-^{13}\text{C}$ distances with nonselective techniques in uniformly labeled samples is complicated by a phenomenon known as dipolar truncation. In particular, the magnetization transfer due to weak dipolar couplings, characteristic of long distances, is strongly attenuated by the presence of larger noncommuting dipolar interaction characteristic of short distances. While this effect is much maligned and is usually discussed as the major bottleneck for structure determination, it is actually important for performing efficient, error-free resonance assignments. In fact, efficient truncation of medium- and long-range transfers by strong one-bond couplings induces a one-bond relayed transfer along the $^{13}\text{C}-^{13}\text{C}$ chains. This is illustrated below with broadband double quantum (DQ) cosine modulated rotary resonance (CM₅RR)^{8,24} spectra of

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FIG. 1. (Color) (a) PAR pulse sequence for obtaining 2D homonuclear correlation spectra. The PAR mixing consists of continuous wave (cw) irradiation on the ^1H and ^{13}C channels that induces a second-order cross term between ^1H – ^{13}C dipolar couplings [terms 2 and 3 of Eq. (6)] in order to transfer polarization from $^{13}\text{C}_1$ to $^{13}\text{C}_2$. (b) The PAR subspace can be seen as a coupled basis between a fictitious ZQ operator involving two ^{13}C 's and a ^1H spin. The red arrows indicate PAR recoupling axis and longitudinal tilting field resulting from autocross terms (see Sec. IV).

[U- ^{13}C , ^{15}N]-Crh protein²⁵ that contain exclusively one-bond cross peaks. At longer mixing times, additional cross peaks appear with alternating signs which is a signature of a DQ process that depends on the number of one-bond couplings involved in the transfer. While this spectral behavior is obviously a very attractive feature of the technique for performing resonance assignments, it, nevertheless, does not provide distance information that could be directly related to the fold of the protein, except for what is available through statistical analyses of secondary chemical shifts.²⁶

In order to utilize ^{13}C – ^{13}C recoupling for three-dimensional (3D) structure determination of proteins, we require methods that either attenuate or quench dipolar truncation. A number of different groups of techniques have been proposed to circumvent the problem,^{8,10,11,27–32} and three of them have led to structures of model proteins: (1) second-order techniques including Proton Driven Spin Diffusion (PDSD) (Ref. 33) and Dipolar Assisted Rotational Resonance RF Assisted Diffusion (DARR/RAD),^{13,34} (2) techniques combining ^1H – ^1H spin diffusion with indirect detection such as CHHC/NHHC,^{22,35,36} and (3) rotational resonance (R^2) based on selective first-order techniques.³⁷

Both PDSD and DARR/RAD rely on the ^1H – $^{13}\text{C} \times ^{13}\text{C}$ – ^{13}C and ^{13}C – $^{13}\text{C} \times ^{13}\text{C}$ – ^{13}C second-order cross terms, respectively, to promote ^{13}C – ^{13}C polarization transfer on a time scale of milliseconds to seconds.³⁸ To date, 3D structure determinations using PDSD and DARR have been demonstrated primarily on selectively labeled spin systems^{14,15,39} and very recently on uniformly labeled systems.^{16,23} A second approach—CHHC and NHHC—employs ^1H – ^1H contacts that are probed in the initial rate regime ($\tau_m \sim 10^2 \mu\text{s}$) and detected indirectly via rare spins.^{35,40–42} CHHC/NHHC based approaches were successfully used for structural studies of uniformly ^{13}C and ^{15}N labeled peptides/proteins^{36,43} including the 3D structure determination of Crh dimer.⁴² Finally, R^2 -based frequency selective techniques are well established as methods for measuring accurate ^{13}C – ^{13}C distances and were used extensively in the context of small peptide structure determination. Recently, it was demonstrated that combined restraints from

homogeneously broadened R^2W and PDSD are sufficient to calculate the structure of a uniformly ^{13}C and ^{15}N model protein GB1.³⁷

In this paper we introduce a homonuclear recoupling technique that exhibits substantially reduced dipolar truncation and concurrently functions well at the high spinning frequencies that are desirable for experiments performed at high magnetic fields. It is in this important regime (high B_0 and $\omega_r/2\pi$) that the performance of PDSD and DARR begins to degrade. This sequence, proton assisted recoupling (PAR), is based on the more general third spin assisted recoupling (TSAR) mechanism that was used recently to design experiments for ^{15}N to ^{13}C cross polarization⁴⁴ and to understand the spin dynamics underlying the beneficial effect of a weak ($<0.25 \omega_r$) ^1H irradiation on the DQ CmpRR ^{13}C – ^{13}C transfer efficiency.⁸ More generally, the B -[A]- C TSAR mechanism serves to connect two spins B and C via a cross term involving dipolar couplings with a third spin A (B - A and C - A dipolar couplings, respectively). In particular, the TSAR effective Hamiltonian contains trilinear terms of the form $B^\pm C^\mp A_Z$, which induce zero quantum (ZQ) polarization transfer between spins B and C . In the specific cases of ^{13}C – ^{13}C and ^{15}N – ^{15}N recouplings, the terms that mediate polarization transfer are of the form $C_1^\pm C_2^\mp H_Z$ or $N_1^\pm N_2^\mp H_Z$.

In proteins and nuclei acids, protons are ideal candidates to mediate second-order TSAR transfers. Thus, the homonuclear PAR experiment is used for ^{13}C –[^1H]- ^{13}C and ^{15}N –[^1H]- ^{15}N polarization transfers. In contrast to most recoupling sequences that require decoupling of ^1H from the ^{15}N – ^{13}C , ^{13}C – ^{13}C , and ^{15}N – ^{15}N spin dynamics, the TSAR effect described here uses the intrinsic properties of protons (high abundance, large γ) to facilitate the transfer process. As a consequence, and in spite of the fact that TSAR is a second-order effect, it significantly accelerates the polarization transfer between remote ^{13}C 's or ^{15}N 's when compared to mechanisms based on ^{13}C – ^{13}C or ^{15}N – ^{15}N dipolar coupling alone. Note that the homonuclear dipolar coupling is not involved in the TSAR mechanism, a fact that intrinsically differentiates it from the spin diffusion methods that involve ^{13}C – ^{13}C and ^{15}N – ^{15}N couplings. Moreover, even though the ^1H 's are involved in the PAR mechanism, the polarization is

not transferred through the proton network using ^1H – ^1H couplings. This is an essential difference with respect to CHHC-type experiments where the polarization is transferred from ^{13}C to ^1H , then between ^1H 's, and indirectly detected on ^{13}C . The TSAR mechanism also leads to attenuated dipolar truncation as we demonstrate below both theoretically and experimentally, allowing the detection of cross peaks corresponding to distances up to ~ 6 – 7 Å in the spectra of uniformly $[\text{U-}^{13}\text{C}, ^{15}\text{N}]$ -Crh protein.

The paper is organized into the following eight additional sections. In Sec. II we delineate the principles of PAR, the effective Hamiltonian [from average Hamiltonian theory (AHT)], and the associated ZQ-TSAR subspace. Section III compares analytical and numerical PAR simulations, describes the PAR optimization map, and details the dependence on local molecular geometry. Section IV discusses the effect of the spin system topology on the efficiency of PAR transfers. Using a simple model system, Sec. V describes the reduced dipolar truncation observed with PAR. Section VI highlights various aspects of the PAR polarization transfer in uniformly labeled proteins with a focus on dipolar truncation, the issue of relayed versus direct polarization transfer, as well as the effect of multiple protons mediating PAR. Section VII discusses using the PAR experiment for estimating distances. Section VIII highlights an important phenomenon, namely, that additional ZQ ^{13}C – $[\text{H}]$ – ^{13}C TSAR transfer may be present during most of the contemporary first-order recoupling sequences. Section IX demonstrates that the PAR technique is a suitable approach for protein structure determination and compares its features to other alternative methods such as PDS/DARR and CHHC/NHHC. Note that a detailed description of the materials and methods can be found in the Supporting Information (SI),⁶⁹ including Sec. IIB that describes the PAR optimization protocol.

II. PRINCIPLES OF PAR

Figure 1(a) illustrates the pulse sequence used throughout this paper to record homonuclear two-dimensional (2D) correlation spectra [except for the spectra in Figs. 9(a) and 12(a) for which the pulse sequences can be found in De Paep *et al.*⁸]. In what follows we cast the discussion in terms of ^{13}C – ^{13}C recoupling, but all of the theory and discussion are equally applicable to the case of ^{15}N – ^{15}N recoupling, which is specifically described in separate paper.⁴⁵ The initial ^1H to ^{13}C cross polarization (CP) step is followed by ^{13}C indirect t_1 evolution, and then the PAR recoupling block, consisting of cw irradiation, is applied to both the ^{13}C and ^1H channels. The final step is ^{13}C detection during t_2 in the presence of TPPM (Ref. 18) decoupling. As we shall see below, the PAR mechanism relies on second-order recoupling involving $^{13}\text{C}_1$ – ^1H and ^1H – $^{13}\text{C}_2$ dipolar interactions, or in other words the ^{13}C – $[\text{H}]$ – ^{13}C TSAR mechanism.

The PAR experiment can be analyzed with AHT (Ref. 46) that permits visualization and understanding of the spin dynamics in the PAR subspace. Accordingly, we consider a three spin system consisting of two dipolar coupled ^{13}C 's– $^{13}\text{C}_1$, $^{13}\text{C}_2$ –, and an assisting ^1H spin subject to two

cw rf fields of strengths $\omega_{1\text{C}}$ and $\omega_{1\text{H}}$ applied to the ^{13}C and ^1H spins, respectively. The internal Hamiltonian can therefore be written as

$$H = \Delta\omega_{\text{C}_1}C_{1z} + \Delta\omega_{\text{C}_2}C_{2z} + \Delta\omega_{\text{H}}H_z + \omega_{\text{C}_1\text{C}_2}[2C_{1z}C_{2z} - (C_{1x}C_{2x} + C_{1y}C_{2y})] + \omega_{\text{C}_1\text{H}}2C_{1z}H_z + \omega_{\text{H}\text{C}_2}2H_zC_{2z} + \omega_{1\text{C}}(C_{1x} + C_{2x}) + \omega_{1\text{H}}H_x, \quad (1)$$

where $\Delta\omega_{\text{C}_1}$, $\Delta\omega_{\text{C}_2}$, and $\Delta\omega_{\text{H}}$ denote the shift tensors and resonant offsets of the ^{13}C (or ^{15}N) and ^1H nuclei, respectively, and $\omega_{\text{C}_1\text{C}_2}$, $\omega_{\text{C}_1\text{H}}$, and $\omega_{\text{H}\text{C}_2}$ the homonuclear and heteronuclear dipolar couplings. Note that rotation at the magic angle induces a time dependence of the spatial anisotropy of the interactions.

For computational convenience, the Hamiltonian (1) is rewritten using spherical tensor notation,

$$H = \omega_{\text{C}_1\text{H}}2T_{10}^{\text{C}_1}T_{10}^{\text{H}} + \omega_{\text{H}\text{C}_2}2T_{10}^{\text{H}}T_{10}^{\text{C}_2} + \sqrt{6}\omega_{\text{C}_1\text{C}_2}T_{20}^{\text{C}_1}T_{20}^{\text{C}_2} + \Delta\omega_{\text{C}_1}T_{10}^{\text{C}_1} + \Delta\omega_{\text{C}_2}T_{10}^{\text{C}_2} + \Delta\omega_{\text{H}}T_{10}^{\text{H}} + \omega_{1\text{C}}\left\{\frac{-T_{11}^{\text{C}_1} + T_{1-1}^{\text{C}_1}}{\sqrt{2}}\right\} + \omega_{1\text{C}}\left\{\frac{-T_{11}^{\text{C}_2} + T_{1-1}^{\text{C}_2}}{\sqrt{2}}\right\} + \omega_{1\text{H}}\left\{\frac{-T_{11}^{\text{H}} + T_{1-1}^{\text{H}}}{\sqrt{2}}\right\}. \quad (2)$$

In order to appreciate the effect of the cw rf fields, it is convenient to further recast the Hamiltonian in the interaction frame defined by the two fields. Such a transformation can be decomposed into two rotations: $R_1(\alpha_1=0, \beta_1=\pi/2, \gamma_1=0)$ which rotates the z -axis to the x -axis, and $R_2(\alpha_2=\omega_{1\text{C}/1\text{H}}t, \beta_2=0, \gamma_2=0)$, which rotates the spin system around the new z -axis by angles $\omega_{1\text{C}}t$ and $\omega_{1\text{H}}t$. Each of the operators transforms as follows:

$$[T_{lq}^\lambda]^\dagger = \sum_q T_{lq}^\lambda d_{q0}^l(-\pi/2)e^{-iq\omega_{1\text{C}/1\text{H}}t}, \quad (3)$$

where $d_{q0}^l(\beta)$ is a reduced Wigner matrix element. We reduce the dependence on three different averaging frequencies ($\omega_r/2\pi$, the rotor frequency, and $\omega_{1\text{C}}/2\pi$ and $\omega_{1\text{H}}/2\pi$ the strength of the ^{13}C and ^1H cw fields, respectively) to a single frequency dependence by assuming that these frequencies are commensurate, implying that we can find indices p_{C} and p_{H} , and integers p_{C}^1 , p_{C}^2 , p_{H}^1 , and p_{H}^2 , such that

$$\omega_{1\text{C}}/2\pi = p_{\text{C}}(\omega_r/2\pi) = \frac{p_{\text{C}}^1}{p_{\text{C}}^2}(\omega_r/2\pi), \quad (4)$$

$$\omega_{1\text{H}}/2\pi = p_{\text{H}}(\omega_r/2\pi) = \frac{p_{\text{H}}^1}{p_{\text{H}}^2}(\omega_r/2\pi), \quad (5)$$

where $p_{\text{C}}^1/p_{\text{C}}^2, p_{\text{H}}^1/p_{\text{H}}^2$ denote irreducible ratios. Assuming that the frequencies are commensurate is not a demanding constraint and results in a Hamiltonian that is periodic modulo $n\tau_r$, to which AHT is applicable. Here $n = \text{lcm}\{p_{\text{C}}^2, p_{\text{H}}^2\}$, given that n is sufficiently small to ensure rapid convergence and τ_r is a rotor period. The expression for the Hamiltonian in the interaction frame can thus be obtained using Eqs. (2)–(5),

$$\begin{aligned}
\tilde{H} = & \overbrace{\sum_{m_1=-2}^2 \sum_{\substack{q_{1C}=-1 \\ q_{1C} \neq 0}}^1 \sum_{\substack{q_{1H}=-1 \\ q_{1H} \neq 0}}^1 \omega_{C_1H}^{m_1} \text{sgn}(q_{1C}) \text{sgn}(q_{1H}) T_{1q_{1C}}^{C_1} T_{1q_{1H}}^H \exp\left\{-i(nX_1) \frac{\omega_r t}{n}\right\}}^1 \\
& + \overbrace{\sum_{m_2=-2}^2 \sum_{\substack{q_{2C}=-1 \\ q_{2C} \neq 0}}^1 \sum_{\substack{q_{2H}=-1 \\ q_{2H} \neq 0}}^1 \omega_{HC_2}^{m_2} \text{sgn}(q_{2H}) \text{sgn}(q_{2C}) T_{1q_{2H}}^H T_{1q_{2C}}^{C_2} \exp\left\{-i(nX_2) \frac{\omega_r t}{n}\right\}}^2 \\
& + \overbrace{\sum_{m_3=-2}^2 \sum_{q_{3C}=-2}^2 \omega_{C_1C_2}^{m_3} d_{q_{3C}0}^2 (-\pi/2) T_{2q_{3C}}^{C_1C_2} \exp\left\{-i(nX_3) \frac{\omega_r t}{n}\right\}}^3 \\
& + \overbrace{\sum_{i=1}^2 \sum_{m_i=-2}^2 \sum_{\substack{q_{Ci}=-1 \\ q_{Ci} \neq 0}}^1 \omega_{C_i}^{m_i} (\text{sgn}(q_{Ci})/\sqrt{2}) T_{1q_{Ci}}^{C_i} \exp\left\{-i(nX_{C_i}) \frac{\omega_r t}{n}\right\}}^4 \\
& + \overbrace{\sum_{m=-2}^2 \sum_{\substack{q_H=-1 \\ q_H \neq 0}}^1 \omega_H^m (\text{sgn}(q_H)/\sqrt{2}) T_{1q_H}^H \exp\left\{-i(nX_H) \frac{\omega_r t}{n}\right\}}^5,
\end{aligned} \tag{6}$$

where we use the following expressions:

$$X_1 = m_1 + p_C q_{1C} + p_H q_{1H},$$

$$X_2 = m_2 + p_H q_{2H} + p_C q_{2C},$$

$$X_3 = m_3 + p_C q_{3C},$$

$$X_{C_i} = m_{C_i} + p_C q_{C_i},$$

$$X_H = m + p_H q_H, \tag{7}$$

and $\text{sgn}(q)$ is the sign function of q .

We assume that the rf fields are chosen so that neither Hartmann–Hahn⁴⁷ (HH) nor rotary resonance recoupling (R^3) (Ref. 48) conditions are matched, i.e., $X_1 \neq 0, X_2 \neq 0, X_{C_i} \neq 0, X_H \neq 0$, and that the ^{13}C – ^{13}C dipolar coupling is not recoupled to first order ($X_3 \neq 0$). Under these conditions, the first-order average Hamiltonian vanishes.

In order to describe the PAR recoupling mechanism, we calculate the cross term between terms 1 and 2 in Eq. (6),

$$\tilde{H}_{1 \times 2}^{(2)} = \sum_{\substack{m_1, q_{1C}, q_{1H}, \\ m_2, q_{2C}, q_{2H}}} \left[\frac{1}{2iT} \omega_{C_1H}^{m_1} \text{sgn}(q_{1C}) \text{sgn}(q_{1H}) \omega_{HC_2}^{m_2} \text{sgn}(q_{2H}) \text{sgn}(q_{2C}) T_{1q_{1C}}^{C_1} T_{1q_{2C}}^{C_2} [T_{1q_{1H}}^H, T_{1q_{2H}}^H] \right. \\
\left. \int_0^T dt_2 \int_0^{t_2} dt_1 (\exp\{-i\omega_r(X_1 t_2 + X_2 t_1)\} - \exp\{-i\omega_r(X_2 t_2 + X_1 t_1)\}) \right]. \tag{8}$$

The above expression is nonzero if and only if

$$(2) \quad X_1 = -X_2 \neq 0, \tag{10}$$

$$(1) \quad q_{1H} = -q_{2H} \Rightarrow [T_{1q_{1H}}^H, T_{1q_{2H}}^H] = -\text{sgn}(q_{1H}) T_{10}^H, \tag{9}$$

which implies that

$$(m_1 + m_2) + p_C(q_{1C} + q_{2C}) = 0. \quad (11)$$

Equation (11) has two solutions.

DQ solution: For $q_{1C}=q_{2C}=\pm 1$ and $p_C \in \{\pm 0, 0.5, 1, 1.5, 2\}$ with no restriction on p_H , which results in DQ terms of the form $T_{1\pm 1}^{C_1} T_{1\pm 1}^{C_2} T_{10}^H$.

ZQ solution: For $q_{1C}=-q_{2C}$ and $m_1=-m_2$ with no restriction on p_C and p_H , which results in ZQ terms of the form $T_{1\pm 1}^{C_1} T_{1\pm 1}^{C_2} T_{10}^H$. Note the change in sign of the subscript— $T_{1\pm 1}^{C_1}$ —yielding a ZQ Hamiltonian.

Thus, it appears that the ZQ solution, as opposed to the DQ solution, is much easier to fulfill, and ZQ TSAR recoupling occurs when the following conditions are satisfied:

$$q_{1H} = -q_{2H} = q_H, \quad q_{1C} = -q_{2C} = q_C, \quad m_1 = -m_2 = m, \quad (12)$$

and providing that we concurrently avoid the DQ conditions associated with $p_C = \{0, \pm 0.5, \pm 1, \pm 1.5, \pm 2\}$, HH conditions with $p_C = \pm p_H \pm \{1, 2\}$, and R^3 conditions with $p_C = \{1, 2\}$.

Using the identity $(\omega_{C_1H}^m \omega_{HC_2}^{-m})^* = \omega_{C_1H}^{-m} \omega_{HC_2}^m$, we obtain the following expression for the effective PAR term:

$$\begin{aligned} \bar{H}_{1 \times 2}^{(2)} &= 2\omega_{\text{PAR}}(T_{11}^{C_1} T_{1-1}^{C_2}) T_{10}^H + 2\omega_{\text{PAR}}^*(T_{1-1}^{C_1} T_{11}^{C_2}) T_{10}^H \\ &= 2\omega_{\text{PAR}} C_1^+ C_2^- H_Z + 2\omega_{\text{PAR}}^* C_1^- C_2^+ H_Z \\ &= \text{Re}(\omega_{\text{PAR}}) 2I_{C_1 C_2, X}^{(23)} I_{H, Z} + \text{Im}(\omega_{\text{PAR}}) 2I_{C_1 C_2, Y}^{(23)} I_{H, Z} \end{aligned} \quad (13)$$

and the PAR coupling

$$\begin{aligned} \omega_{\text{PAR}} &= \left(\frac{\text{Re}(\omega_{C_1H}^1 \omega_{HC_2}^{-1})}{\omega_r} \lambda(1, p_C, p_H) \right. \\ &\quad + \frac{\text{Re}(\omega_{C_1H}^2 \omega_{HC_2}^{-2})}{\omega_r} \lambda(2, p_C, p_H) \\ &\quad + i \left(\frac{\text{Im}(\omega_{C_1H}^1 \omega_{HC_2}^{-1})}{\omega_r} \sigma(1, p_C, p_H) \right. \\ &\quad \left. + \frac{\text{Im}(\omega_{C_1H}^2 \omega_{HC_2}^{-2})}{\omega_r} \sigma(2, p_C, p_H) \right), \end{aligned} \quad (14)$$

with

$$\begin{aligned} \lambda(m, p_C, p_H) &= \left(\frac{-(p_C + p_H)}{m^2 - (p_C + p_H)^2} + \frac{-(p_H - p_C)}{m^2 - (p_H - p_C)^2} \right), \\ \sigma(m, p_C, p_H) &= \left(\frac{m}{m^2 - (p_H + p_C)^2} - \frac{m}{m^2 - (p_H - p_C)^2} \right). \end{aligned} \quad (15)$$

The above expressions permit us to visualize the subspace in which the TSAR spin dynamics evolve. The PAR subspace [Fig. 1(b)] can be viewed as a coupled basis described extensively in the context of solution NMR (Ref. 49) between a fictitious ZQ operator involving the two ^{13}C 's and a ^1H . The proton spin is essentially a bystander, i.e., no magnetization is sent to this proton in the spin dynamics described by Eq. (13). In this process, the dipolar couplings to the ^1H are used to create an effective transverse PAR com-

ponent composed of trilinear terms of the form $C_1^+ C_2^- H_Z$ that can invert the z -component in the PAR subspace ($(C_{1Z} - C_{2Z})/2$) and thus induce polarization transfer between the two ^{13}C 's. The similar case that is encountered in solution NMR is described further in Supporting Information, Sec. I.

The PAR recoupling term [Eq. (13)] can be decomposed into two terms which we refer to as the $m=1$ and $m=2$ contributions, where m denotes for the spatial component index of the dipolar tensor during MAS. The dependence of the PAR term on the local geometry and the powder Euler angles is detailed in Sec. IV. Note that the PAR mechanism usually involves multiple protons: any proton is potentially a candidate to participate in such a process, but only the closest ones will contribute significantly to the polarization transfer. This issue is discussed further in Sec. VI.

The three spin term derived above drives the polarization transfer in a PAR experiment. However, the PAR term is not the only possible cross term present during double cw irradiation, and a complete picture of the spin dynamics of the PAR experiment requires that we evaluate the other second-order cross terms. For example, an important contribution, which we refer to as an autocross term, arises from a cross term of term 1 in Eq. (6) with itself (i.e., $^1\text{H}-^{13}\text{C}_1$ term crossed with $^1\text{H}-^{13}\text{C}_1$). These autocross terms yield nonzero contributions that can be expressed as a function of p_C and p_H , the rf field strengths in units of the MAS frequency.

The autocross term of term 1 in Eq. (6) (i.e., the term involving the $^1\text{H}-^{13}\text{C}_1$ dipolar coupling crossed with the $^1\text{H}-^{13}\text{C}_1$ dipolar coupling) can be written as follows:

$$\begin{aligned} \bar{H}_{1 \times 1}^{(2)} &= \frac{1}{\omega_r} [\omega_{C_1H}^1 \omega_{HC_1}^{-1} \chi(1, p_C, p_H) \\ &\quad + \omega_{C_1H}^2 \omega_{HC_1}^{-2} \chi(2, p_C, p_H)] T_{10}^{C_1} \\ &\quad + \frac{1}{\omega_r} [\omega_{C_1H}^1 \omega_{HC_1}^{-1} \kappa(1, p_C, p_H) \\ &\quad + \omega_{C_1H}^2 \omega_{HC_1}^{-2} \kappa(2, p_C, p_H)] T_{10}^H, \end{aligned} \quad (16)$$

with

$$\begin{aligned} \chi(m, p_C, p_H) &= -\frac{1}{2} \left(\frac{(p_H + p_C)}{m^2 - (p_H + p_C)^2} - \frac{(p_H - p_C)}{m^2 - (p_H - p_C)^2} \right), \\ \kappa(m, p_C, p_H) &= -\frac{1}{2} \left(\frac{(p_H + p_C)}{m^2 - (p_H + p_C)^2} + \frac{(p_H - p_C)}{m^2 - (p_H - p_C)^2} \right). \end{aligned} \quad (17)$$

Similarly, one can derive $\bar{H}_{2 \times 2}^{(2)}$ —the second-order contribution of term 2 in Eq. (9) with itself (i.e., the term involving $^1\text{H}-^{13}\text{C}_2$ dipolar coupling cross $^1\text{H}-^{13}\text{C}_2$ dipolar coupling).

In order to obtain a more complete expression of the longitudinal contribution, one should also evaluate the cross term of the chemical shift tensors of the ^{13}C 's and ^1H 's with themselves:

$$\begin{aligned}
& \tilde{H}_{4 \times 4}^{(2)} + \tilde{H}_{5 \times 5}^{(2)} \\
&= \frac{1}{\omega_r} \left[\frac{(\omega_{C_1}^0)^2}{2p_C} + \xi(1, p_C) \omega_{C_1}^1 \omega_{C_1}^{-1} + \xi(2, p_C) \omega_{C_1}^2 \omega_{C_1}^{-2} \right] T_{10}^{C_1} \\
&+ \frac{1}{\omega_r} \left[\frac{(\omega_{C_2}^0)^2}{2p_C} + \xi(1, p_C) \omega_{C_2}^1 \omega_{C_2}^{-1} + \xi(2, p_C) \omega_{C_2}^2 \omega_{C_2}^{-2} \right] T_{10}^{C_2} \\
&+ \frac{1}{\omega_r} \left[\frac{(\omega_H^0)^2}{2p_H} + \xi(1, p_H) \omega_H^1 \omega_H^{-1} + \xi(2, p_H) \omega_H^2 \omega_H^{-2} \right] T_{10}^H,
\end{aligned} \tag{18}$$

where

$$\xi(m, p) = \frac{p}{(p^2 - m^2)}. \tag{19}$$

The autocross term arising from J coupling may also be present, but is generally negligible compared to the $\tilde{H}_{k \times k}^{(2)}$ -type terms considered above. Finally, the $\tilde{H}_{k \times k}^{(2)}$ cross terms yield longitudinal T_{10} operators [along the irradiation axis, see Fig. 1(b)] that directly interfere with the ZQ PAR polarization transfer described in Eq. (13). The relevant Hamiltonian in the ZQ- ^{13}C subspace can hence be described by the following equation:

$$\begin{aligned}
\tilde{H}_{ZQ}^{(2)} = & \text{Re}(\omega_{\text{PAR}}) 2I_{C_1 C_2, X}^{(23)} I_{H, Z} \\
& + \text{Im}(\omega_{\text{PAR}}) 2I_{C_1 C_2, Y}^{(23)} I_{H, Z} + \omega_{\text{auto}} I_{C_1 C_2, Z}^{(23)},
\end{aligned} \tag{20}$$

where

$$\omega_{\text{auto}} = \frac{1}{\omega_r} \left[\frac{(\omega_{C_1}^1 \omega_{H C_1}^{-1} \omega_{C_2}^1 \omega_{H C_2}^{-1}) \chi(1, p_C, p_H) + (\omega_{C_1}^2 \omega_{H C_1}^{-2} - \omega_{C_2}^2 \omega_{H C_2}^{-2}) \chi(2, p_C, p_H)}{(\omega_{C_1}^0)^2 - (\omega_{C_2}^0)^2} + \xi(1, p_C) (\omega_{C_1}^1 \omega_{C_1}^{-1} - \omega_{C_2}^1 \omega_{C_2}^{-1}) + \xi(2, p_C) (\omega_{C_1}^2 \omega_{C_1}^{-2} - \omega_{C_2}^2 \omega_{C_2}^{-2}) \right]. \tag{21}$$

To summarize, we have learned that the ZQ-PAR mechanism can potentially be active for all (p_C, p_H) combinations except when $p_H = \pm p_C \pm \{0, 1, 2, 3, 4\}$ or $p_C = \{0.5, 1, 1.5, 2\}$. Moreover, autocross terms from both the heteronuclear dipolar interactions and the chemical shift tensors yield contributions that are depicted in the PAR subspace introduced above as longitudinal contributions [see Fig. 1(b)]. The importance of these longitudinal terms is discussed in Sec. IV.

III. NUMERICAL AND ANALYTICAL SIMULATIONS OF THE PAR MECHANISM

Even though the expressions derived in Sec. II provide considerable insight into the spin dynamics in the PAR subspace, we believe it is instructive to examine numerical simulations that account for the influence of other interactions and higher-order terms on the PAR spin dynamics. As we will see the salient features of the numerical simulations are in excellent agreement with the analytical expressions derived above.

Figure 2 shows simulations illustrating the PAR recoupling mechanism for a simple spin system consisting of two directly bonded ^{13}C 's (C_1 and C_2) and a single ^1H directly bonded to C_1 . The magnetization is initially present on C_1 . The usual CH (1.12 Å) and CC (1.54 Å) bond lengths and a 109.5 H- C_1 - C_2 angle lead to a C_1 -H- C_2 angle of $\sim 42^\circ$. The three contour plots in Figs. 2(a)–2(c) represent the magnetization on C_1 , C_2 , and ^1H , respectively, after 3 ms of simultaneous $^{13}\text{C}/^1\text{H}$ PAR irradiation. The x - and y -axes of the contour plots indicate the rf field strengths applied in

units of spinning frequency ω_r for the ^{13}C ($p_C = \omega_{1C}/\omega_r$) and ^1H ($p_H = \omega_{1H}/\omega_r$) channels, respectively. The black dashed lines indicate the ^1H - ^{13}C HH matching conditions and its sidebands (i.e., $p_H = p_C \pm n$ where $n=0, 1, 2$).

As expected, we observe magnetization transfer from $^{13}\text{C}_1$ to the ^1H at the HH conditions. However, we transfer a substantial amount of magnetization to C_2 for rf fields that depart from the HH condition and match the PAR conditions in two regions of the optimization map that are illustrated in Fig. 2(b) and denoted with red X's. The first lies just below the line corresponding to the $n=0$ matching condition ($\omega_{1H} < \omega_{1C}$ but with $p_C > 2$), and since this region uses high power cw irradiation, it corresponds to relative broadband recoupling. In contrast, the second area ($p_C < 1$, $p_H > 3$) employs lower power cw ^{13}C irradiation and results in more selective recoupling.

As we will see below the TSAR term alone, $C_1^\pm C_2^\mp H_Z$ [Eqs. (13)–(15)], is not sufficient to fully explain the features of the PAR optimization map. Thus, in order to obtain a more complete understanding of the underlying spin dynamics, we performed analytical simulations of the magnetization exchange. The spin dynamics in the TSAR subspace are described by Eq. (20) [and depicted in Fig. 1(b)]. If the magnetization starts on the first carbon (C_{1z}) and is detected on the second carbon (C_{2z}), the polarization transfer efficiency for a given crystallite orientation can be written $\frac{1}{2} \cos^2(\theta_{\text{eff}}) \times [1 - \cos(\omega_{\text{eff}} t)]$, where ω_{eff} represents the recoupling frequency along the effective tilted axis and θ_{eff} is the angle between the transverse PAR component and the effective

FIG. 2. (Color) PAR optimization map simulated using SPINEVOLUTION (Ref. 50). The initial magnetization is on the C_1 spin ($C_{2,X}$ operator) and is detected after 3 ms PAR mixing on the three spins: (a) C_1 spin, (b) C_2 spin, and (c) H spin ($C_{1,X}$, $C_{2,X}$, H_X operators, respectively). The spin system is composed of two directly bonded ^{13}C 's and one ^1H bonded to the $^{13}\text{C}_1$ spin (see the inset of the figure). The distance between the $^{13}\text{C}_2$ spin and the ^1H is 2.15 Å. The angle between the two CH dipolar vectors is 42° and arises from the usual tetrahedral geometry. Simulations include typical chemical shift tensor values (see SI for details) (Ref. 69). The black dashed lines indicate the locations of the $n=0$ and ± 1 and ± 2 HH matching conditions.

tilted component when longitudinal cross terms are considered. Both parameters can be expressed as a function of ω_{PAR} (the PAR recoupling frequency) and ω_{auto} (the autocross-term contribution) using the following expressions: $\tan(\theta_{\text{eff}}) = \omega_{\text{auto}}/\omega_{\text{PAR}}$ and $\omega_{\text{eff}}^2 = \omega_{\text{PAR}}^2 + \omega_{\text{auto}}^2$. The scaling factor $\cos^2(\theta_{\text{eff}})$ accounts for the fact that the effective recoupling axis is not perpendicular the z -axis of the PAR subspace (which stands as both the initial magnetization axis and detection axis). After averaging over the Euler angles, the polarization transfer efficiency can be thus be written as

$$E = \frac{1}{8\pi^2} \int_{\alpha=0}^{2\pi} \int_{\beta=0}^{\pi} \int_{\gamma=0}^{2\pi} \cos^2(\theta_{\text{eff}}) \times \sin^2(|\omega_{\text{eff}}|t/2) \sin(\beta) d\alpha d\beta d\gamma. \quad (22)$$

Figure 3 compares analytical and numerical simulations performed on the same spin system as in Fig. 2. Note that these simulations do not include chemical shift tensors in order to isolate the effect of the ^1H – ^{13}C autocross terms on the TSAR spin dynamics. Shift tensor effects can be incorporated into the discussion using Eq. (21). The resulting analytical simulations are in excellent agreement with numerical simulations shown in Fig. 2 (data not shown).

Figure 3(a) shows the polarization transfer from the $^{13}\text{C}_1$ to $^{13}\text{C}_2$ after 1.5 ms of simultaneous cw $^{13}\text{C}/^1\text{H}$ irradiation when only the PAR term is included. As expected from Eq. (13), we observe an efficient polarization transfer for a wide range of p_C and p_H values. The influence of the inclusion of the autocross term [see Eq. (20)] on the overall spin dynamics is illustrated in the analytical simulation in Fig. 3(b). The structure of the resulting optimization map is very different than in Fig. 3(a), but very similar to the numerical SPINEVOLUTION simulation in Fig. 3(c).

Note that the PAR transfer is maximized for points close to the diagonal of the map ($p_C = p_H$, $p_C > 2.25$) and for points where $p_C < 1$ and $p_H > 3$. The optimal rf settings yield a minimal tilt angle θ_{eff} (i.e., corresponding to points where the PAR recoupling term dominates the longitudinal T_{10}

component). This can easily be seen for the central recoupling condition (near the $n=0$ matching condition). The two white lines displayed on the contour plots in Figs. 3(b) and 3(c) represent points where $\chi(1, p_C, p_H) = 0$ and $\chi(2, p_C, p_H) = 0$ (i.e., autocross terms for spatial components $m=1$ and $m=2$ are equal to 0), described by equations $p_H = \sqrt{p_C^2 - 1}$ and $p_H = \sqrt{p_C^2 - 4}$, respectively. If only one of the two spatial components is taken into account, we clearly observe that either the first or the second conditions maximize the polarization transfer [see Fig. SI 3 (Ref. 69)]. In the case where both $m=1$ and $m=2$ components are used, the maximized polarization transfer appears closer to the $m=1$ matching condition, which is consistent with the fact that the PAR scaling factor is higher for the $m=1$ component. Practically, this means that the optimal transfer will utilize a ^1H rf field slightly smaller than the ^{13}C rf field (with ^{13}C rf $> 2.25\omega_r$).

The remarkable agreement between Figs. 3(b) and 3(c) demonstrates that second-order AHT provides considerable insight into the TSAR process. The TSAR transfer is active as long as the longitudinal off-resonance contribution (autocross terms) is sufficiently small, and first-order recoupling (HH, R^3) is avoided. The agreement between the numerical and analytical plots could be further improved by including higher-order corrections to the AHT expansion. This does not, however, appear necessary to understand the fundamentals of the TSAR mechanism.

From the discussion and results above, it is clear that a knowledge of the longitudinal cross terms is of primary importance for implementing the TSAR mechanism and that the maximum TSAR polarization transfer occurs when this longitudinal component is minimized. The next step is to understand the influence of the various other interactions present in the spin Hamiltonian during a TSAR polarization transfer. Figure 4 illustrates polarization buildup curves corresponding to $p_C = 2.6$ and $p_H = 2.35$ (the area indicated with a red X on the C_2 optimization map in Fig. 2). Figure 4(a) shows that there is a substantial polarization transfer from C_1 to C_2 with all couplings included in the simulation. Figure

FIG. 3. (Color) PAR optimization maps where the polarization transfer between the ^{13}C is monitored as a function of ^{13}C and ^1H irradiation strengths (in units of ω_r) and a 1.5 ms mixing period. The spin system is identical to that in Fig. 2. No chemical shift interactions were included in these simulations. The three panels represent (a) analytical simulations of the ^{13}C polarization transfer from $^{13}\text{C}_1$ to $^{13}\text{C}_2$ arising from only the TSAR term, (b) ^{13}C signal intensity showing the analytical simulation obtained with the TSAR term and the longitudinal autocross-term contributions, and (c) ^{13}C signal intensity depicting the numerical simulations performed with SPINEVOLUTION. The two white lines displayed on the contour plots in panels (b) and (c) represent points where $\chi(1, p_C, p_H)=0$ and $\chi(2, p_C, p_H)=0$ (i.e., autocross terms for spatial components $m=1$ and $m=2$ are equal to 0), described by equations $p_H=\sqrt{p_C^2-1}$ and $p_H=\sqrt{p_C^2-4}$, respectively.

4(b) depicts the same simulation as in (a) except that the $\text{C}_1\text{--C}_2$ coupling is absent from the spin system. We note that identical transfer curves are observed Figs. 4(a) and 4(b), illustrating that the polarization transfer does not rely on the C_1C_2 coupling. Since the TSAR mechanism is based on the cross term involving the C_1H and C_2H couplings, we would therefore predict, based on Eqs. (13)–(15), that removing one or both of the CH couplings would either attenuate or quench polarization transfer from $^{13}\text{C}_1$ to $^{13}\text{C}_2$. This point is illustrated in Figs. 4(c), 4(d), and 4(f). The small polarization transfer to the H [Figs. 4(d) and 4(e)] is due to the cross terms involving the C_1H interactions and through higher-

FIG. 4. (Color) Simulations of polarization transfer in the PAR experiment for a HC_1C_2 spin system. The chemical shift is not included in the simulations. The PAR ^{13}C and ^1H cw rf field strengths correspond to $p_C=2.6$ and $p_H=2.35$, respectively [see Fig. 3(b)]. The panels illustrate simulations that include the following couplings: (a) all dipolar couplings; (b) couplings C_1H and C_2H but not C_1C_2 ; (c) couplings C_2H and C_1C_2 , but not C_1H (note that the polarization remains on C_1); (d) couplings C_1H and C_1C_2 but not C_2H ; (e) coupling C_1H but not C_2H or C_1C_2 ; (f) C_1C_2 but not C_1H and C_2H .

order AHT analysis. The proton polarization buildup is indeed almost unchanged when the C_2H coupling and both the $\text{C}_2\text{H}/\text{C}_1\text{C}_2$ couplings are removed [Figs. 4(d) and 4(e)]. However, the ^1H buildup collapses when the C_1H coupling is removed [Fig. 4(c)]. The same explanation should also hold for the C_2H interactions via symmetry arguments, however,

FIG. 5. (Color) Dependence of PAR polarization transfer on the local geometry of the spin system. (a) The three-spin system geometry used in the simulations: the first ^{13}C and the ^1H are fixed in space, whereas the position of the second ^{13}C is defined by θ and ϕ , the spherical coordinates with the origin at the ^1H . The distances between the ^{13}C 's and the ^1H are constant and equal to 1.1 and 2.6 Å, respectively. The spherical map represents the $^{13}\text{C}\text{--}^{13}\text{C}$ polarization transfer efficiency for a PAR mixing time of 4.2 ms using $p_C=2.75$ and $p_H=2.5$. Polarization transfer for $\theta=0$ orientation (aligned geometry) as a function of time is presented in panels (b) and (c). The buildup curves labeled (1)–(5) represent analytical simulations performed with the following contributions: (1) both $m=1$ and $m=2$ components without autocross terms, (2) both $m=1$ and $m=2$ with autocross terms, [(3) and (4)] only $m=1$ without and with autocross terms, [(5) and (6)] only $m=2$ without and with autocross terms.

its effect is not appreciable since the C_2H interaction is much smaller than the C_1H for the particular spin geometry. Thus, only the cross term involving the C_1H dipolar interaction is able to induce polarization transfer from C_1 to 1H on a time-scale under 20 ms. Finally, a comparison of Figs. 4(d) and 4(e) shows that the residual slowly rising C_2 polarization transfer present in Fig. 4(d) does rely on the ^{13}C - ^{13}C couplings. In this case the C_2 polarization is driven by the cross terms involving terms 1 and 3 from Eq. (6). Note, however, that simulation, Fig. 4(b), indicates that this contribution is negligible compared to the TSAR term.

IV. SPIN SYSTEM GEOMETRY AND PAR TRANSFER EFFICIENCY

In the discussion above we assumed a model spin system with a particular geometry to analyze the TSAR transfer mechanism. Although this is a useful point of departure, it is clear that the details of the spin system geometry and the averages over the powder Euler angles will influence the TSAR polarization transfer process. We now discuss these two factors in more detail.

As demonstrated above, the TSAR recoupling term is described by Eq. (13), and the effective TSAR recoupling frequency contains terms of the form $\omega_{C_iH}^m \omega_{HC_j}^{-m}$ representing the product of the dipolar coupling components. The m -component of the $^{13}C_i$ - 1H dipolar coupling interaction can be expressed by the following equation:

$$\begin{aligned} \omega_{C_iH}^m &= \frac{1}{\sqrt{6}} d_{m0}^2(\theta_M) e^{-im\gamma} \sum_{m'} d_{0m'}^2(b_{C_iH}) d_{m'm}^2(\beta) e^{-im'(\alpha+c_{C_iH})} \rho_{20}^{C_iH} \\ &= d_{m0}^2(\theta_M) e^{-im\gamma} \sum_{m'} d_{0m'}^2(b_{C_iH}) d_{m'm}^2(\beta) e^{-im'(\alpha+c_{C_iH})} d_{C_iH}, \end{aligned} \quad (23)$$

where $(0, b_{C_iH}, c_{C_iH})$ represents the Euler angles between the

principal axis system (PAS) of the dipolar $^{13}C_i$ - 1H interaction and the crystal frame (CF) and (α, β, χ) represent the Euler angles between the CF and the rotor frame. Here the angle between the rotor axis and the laboratory magnetic field B_0 is set to the magic angle, i.e., $\theta_M = 54.7^\circ$. d_{C_iH} , the dipolar coupling constant between the $^{13}C_i$ and 1H spins, and is related to the internuclear distance by

$$d_{C_iH} = - \frac{\mu_0 \gamma_{C_i} \gamma_H \hbar}{4 \pi r_{C_iH}^3} \quad (24)$$

where γ is the gyromagnetic ratio of the spins and r_{C_iH} the distance between the two spins.

In order to simplify the derivation of the expression for $\omega_{C_iH}^m \omega_{HC_j}^{-m}$, one can arbitrarily choose the CF to match the PAS of the $^{13}C_i$ - 1H dipolar interaction which implies $(0, b_{C_iH}, c_{C_iH}) = (0, 0, 0)$. We can also rewrite $(0, b_{HC_j}, c_{HC_j}) = (0, \theta, \phi)$, where θ and ϕ represent the spherical coordinates of a system centered on the 1H spin with the C_iH vector aligned with the z -axis, as shown in Fig. 5(a).

With the simplifications above,

$$\omega_{C_iH}^m = d_{C_iH} e^{-im\gamma} d_{m0}^2(\theta_M) d_{0m}^2(\beta) \quad (25)$$

and

$$\omega_{HC_j}^{-m} = d_{HC_j} e^{-im\gamma} d_{m0}^2(\theta_M) \sum_{m'} d_{0m'}^2(\theta) d_{m'm}^2(\beta) e^{-im'(\alpha+\phi)}, \quad (26)$$

we finally obtain the following two equations for the $m=1$ and $m=2$ components, respectively:

$$\omega_{C_iH}^1 \omega_{HC_j}^{-1} = -d_{C_iH} d_{HC_j} \frac{(\sin(2\beta))}{3} \sqrt{\frac{3}{8}} \begin{pmatrix} \sqrt{\frac{3}{8}} \sin^2(\theta) \left(\frac{1 + \cos(\beta)}{2} \sin(\beta) \right) e^{+2i(\alpha+\phi)} \\ - \sqrt{\frac{3}{8}} \sin(2\theta) \left(\cos^2(\beta) - \frac{1 - \cos(\beta)}{2} \right) e^{i(\alpha+\phi)} \\ - \frac{3 \cos^2(\theta) - 1}{2} \sqrt{\frac{3}{8}} \sin(2\beta) \\ + \sqrt{\frac{3}{8}} \sin(2\theta) \left(\frac{1 + \cos(\beta)}{2} - \cos^2(\beta) \right) e^{-i(\alpha+\phi)} \\ - \sqrt{\frac{3}{8}} \sin^2(\theta) \left(\frac{1 - \cos(\beta)}{2} \sin(\beta) \right) e^{-2i(\alpha+\phi)} \end{pmatrix}, \quad (27)$$

$$\omega_{C_H}^2 \omega_{HC_H}^{-2} = d_{C_H} d_{HC_H} \frac{\sin^2(\beta)}{6} \sqrt{\frac{3}{8}} \left(\begin{aligned} & \sqrt{\frac{3}{8}} \sin^2(\theta) \left(\frac{1 + \cos(\beta)}{2} \right)^2 e^{+2i(\alpha+\phi)} \\ & + \sqrt{\frac{3}{8}} \sin(2\theta) \left(\frac{1 + \cos(\beta)}{2} \right) \sin(\beta) e^{i(\alpha+\phi)} \\ & + \frac{3 \cos^2(\theta) - 1}{2} \sqrt{\frac{3}{8}} \sin^2(\beta) \\ & - \sqrt{\frac{3}{8}} \sin(2\theta) \left(\frac{1 - \cos(\beta)}{2} \right) \sin(\beta) e^{-i(\alpha+\phi)} \\ & + \sqrt{\frac{3}{8}} \sin^2(\theta) \left(\frac{1 - \cos(\beta)}{2} \right)^2 e^{-2i(\alpha+\phi)} \end{aligned} \right). \quad (28)$$

Equations (27) and (28) show the angular dependence of the PAR process with respect to the two powder Euler angles α , β and the two polar angles θ , ϕ . Note that the Euler angle γ is absent from the expression for the PAR recoupling frequency. Thus, the PAR mechanism appears as a γ -compensated process (note that it is distinct from a γ -encoded mechanisms where γ -angle appears as a phase factor).

Figure 5(a) illustrates the PAR polarization transfer for a three spin system similar to the one in Fig. 2. More specifically, the system is composed of a directly bonded $^{13}\text{C}_1$ –H

spin pair and a $^{13}\text{C}_2$ located on a sphere of constant radius centered on the proton spin [see Fig. 5(a)]. The polarization transfer from C_1 to C_2 after 4.2 ms is illustrated on the spherical map shown in Fig. 5(a). A substantial polarization transfer is present over the entire sphere with maxima occurring around the poles and the equator. Note that the polarization transfer efficiency does not depend on the ϕ -angle.

The angular dependence of the PAR recoupling sequence mechanism is given in Eq. (14), and the recoupling frequency is composed of four terms that can be rewritten as

$$\omega_{\text{PAR}} = \frac{1}{\omega_r} \left[\underbrace{\text{Re}(\omega_{C_1H}^1 \omega_{HC_2}^{-1}) \lambda(1, p_C, p_H) + i \text{Im}(\omega_{C_1H}^1 \omega_{HC_2}^{-1}) \sigma(1, p_C, p_H)}_{\omega_{\text{PAR}}^1} + \underbrace{\text{Re}(\omega_{C_1H}^2 \omega_{HC_2}^{-2}) \lambda(2, p_C, p_H) + i \text{Im}(\omega_{C_1H}^2 \omega_{HC_2}^{-2}) \sigma(2, p_C, p_H)}_{\omega_{\text{PAR}}^2} \right]. \quad (29)$$

The first and second terms, denoted ω_{PAR}^1 and ω_{PAR}^2 , of Eq. (29) correspond to the $m=1$ and $m=2$ components, respectively. We note that all of the analytical simulations shown in Fig. 5 were performed assuming $p_C=2.75$ and $p_H=2.5$, and that these values were chosen based on numerical simulations of the same system. In particular, they maximize the polarization transfer after 4.2 ms, but maintain the rf strengths within experimentally reasonable limits (≤ 55 kHz in this case). The rf field strengths (in units of the MAS frequency) almost fulfill the matching condition $p_H = \sqrt{p_C^2 - 1}$ that corresponds to points when the $m=1$ component of the autocross term is canceled. This reduction in the longitudinal contribution results, for example, in about 50% maximum polarization transfer in 4.2 ms for the geometry corresponding to the spins close to the poles of the sphere (see Fig. 5).

In order to gain additional insight into the TSAR process (or more specifically to elucidate the maximum theoretical efficiency of the TSAR process), we can choose $\theta=0$ and simulate the TSAR polarization transfer with the three spins aligned in a linear array. In this case, Eqs. (27) and (28) simplify yielding a $\sin^2(2\beta)$ and $\sin^4(\beta)$ dependence for the $m=1$ and $m=2$ component, respectively. Figure 5(b) shows the corresponding analytical magnetization buildup curves [(1)–(6)] where, in the case without the autocross terms, the contributions of both the $m=1$ and $m=2$ terms reach a theoretical maximum of $\sim 58\%$ [(3) and (4)]. The difference in buildup times, i.e., slower for the $m=2$ component, is also consistent with the $\sin^2(2\beta)$ and $\sin^4(\beta)$ dependences. Interestingly, when both components simultaneously contribute to the transfer, the maximum polarization is enhanced by constructive interference of the two-component process. Note

FIG. 6. (Color) Analytical contour plots of the PAR polarization transfer arising from the $m=1$ and $m=2$ components as a function of θ angle for a three spin system described in Fig. 5(a) with the mixing time and irradiation settings used in Fig. 5 with $\phi=0$. (a) $m=1$ PAR component, (b) $m=1$ PAR term plus $m=1$ autocross-term components, (c) $m=2$ PAR component included, (d) $m=2$ PAR term and $m=2$ autocross-term components, (e) $m=1$ and $m=2$ PAR components, and (f) $m=1$ and $m=2$ PAR plus $m=1$ and $m=2$ autocross-term components.

that in the case of first-order γ -encoded recoupling sequences, the Euler angle dependences are $\sin(2\beta)$ and $\sin^2(\beta)$, yielding a theoretical maximum of $\sim 73\%$.^{6,8,51} Including longitudinal autocross terms [see Eq. (21)] leads to an attenuation of the magnetization transfer in the three spin case mentioned above. The attenuation is more significant for the $m=2$ component due largely to the choice of rf power levels and consequently the scaling factor of the spin part. Indeed, as mentioned above, p_C and p_H were chosen (in this simulation) in order to minimize the $m=1$ component of the autocross terms. When both the $m=1$ and $m=2$ components are included, the maximum transfer efficiency can still approach 50%.

The PAR transfer should thus be seen as a superposition of two simultaneous recoupling pathways involving the $m=1$ and $m=2$ components, and we can decompose the contributions of the different components arising from the two cross terms and the autocross terms. Figure 6 shows maps of polarization transfer efficiency as a function of the θ -angle and the mixing time (with $\phi=0$ since as we have shown above ϕ does not influence the transfer). The $m=1$ pathway [Figs. 6(a) and 6(b)] has a better scaling factor than the $m=2$ component [Figs. 6(c) and 6(d)]. This can easily be understood by examining Eqs. (27) and (28). The comparison between Fig. 6(a) and Fig. 6(b) shows the influence of the autocross terms for the $m=1$ component. The overall features of Fig. 6(a) are preserved with some attenuation, except

around $\theta=0^\circ$ and $\theta=180^\circ$, where the autocross terms quench the TSAR polarization transfer. This effect can be explained by noting that the orientation dependence of the $m=1$ PAR frequency and the $m=1$ autocross terms are identical for $\theta=0^\circ$ or 180° and limited to the β -angle. Even if the choice of p_C and p_H minimizes the importance of this longitudinal contribution, its effect is further enhanced when the orientation dependence is identical: i.e., both the $m=1$ PAR and autocross term are minimized for $\beta=0, \pi/2$ and maximized for $\beta=\pi/4, 3\pi/4$. For the $m=2$ component [Figs. 6(c) and 6(d)], the effect of the autocross terms is larger. Again this is related to the choice of p_C and p_H that strongly attenuates the $m=1$ autocross terms compared to the $m=2$ condition. For similar reasons as with $m=1$, the area around $\theta=0^\circ$ and $\theta=180^\circ$ is the most affected. The last two panels—Figs. 6(e) and 6(f)—show similar simulations as the previous panels but with both the $m=1$ and $m=2$ components included. In this case, the two components constructively interfere yielding 50%–70% transfer with and without inclusion of the autocross terms.

Note that the rf settings that optimize the transfer for a particular spin system can be understood as a compromise between minimizing the autocross terms and maximizing the constructive interference of the two TSAR pathways ($m=1$ and $m=2$), given that they do have a different dependence on θ and the Euler angles, and thus different scaling factors.

The spin system that we have studied thus far is composed of a directly bonded $^{13}\text{C}_1$ – ^1H spin pair and a third $^{13}\text{C}_2$. As we have seen, the optimal rf fields correspond to points that minimize the size of the autocross terms. From Eq. (21), we also surmise that the TSAR autocross terms disappear when the assisting spin is equidistant from $^{13}\text{C}_1$ and $^{13}\text{C}_2$. This situation is encountered for the spin system in Fig. SI5 (Ref. 69) where the three spins are aligned and the ^1H is at 3 Å from each of the ^{13}C 's ($r_{\text{CC}}=6$ Å). The analytical simulation in Fig. SI5 (Ref. 69) shows that efficient polarization transfers can be achieved over a wider range of rf levels. Since the heteronuclear autocross term is absent in Fig. SI5a,⁶⁹ the optimal rf fields correspond only to the maximum interference between the $m=1$ and $m=2$ components of the TSAR recoupling frequency. Note that in this case over 85% of the polarization is transferred over a 6 Å distance in 60 ms.

Other examples illustrating various levels of autocross-term compensation with different spin system geometries can be found in the Supporting Information. Figure SI4 (Ref. 69) shows the PAR polarization transfer map in the case of a nonaligned three spin system where the ^1H spin is equidistant from the two ^{13}C 's. In such a situation, the off-resonance contribution from the autocross terms vanishes in the ZQ PAR subspace. Figure SI6 (Ref. 69) illustrates that in the case of a nonsymmetric spin system (i.e., two different couplings corresponding to a case where the proton is directly bonded to C_1 and at 4.9 Å from C_2), the polarization transfer occurs only along the white line corresponding to the cancellation of the $m=1$ off-resonance autocross terms. In this case the influence of the autocross terms is significant because of the very different ^1H – ^{13}C interactions involved.

The autocross-term compensation can be generalized as

FIG. 7. (Color) Illustration of dipolar truncation in the CM₅RR and PAR homonuclear recoupling schemes. The spin system 1 is composed of a directly bonded $C_\alpha H_\alpha$ pair and C_{remote} spins of 4.5 and 3.56 Å distant from the C_α and H_α spins. In the spin system 2, a C_β spin, directly bonded C_α , is also present. (a) The black dashed line depicts the polarization transfer ($\sim 30\%$) from C_α to C_{remote} ($r_{C-C}=4.5$ Å) using the broadband DQ CM₅RR in the three spin system 1. When a directly bonded C_β spin is added to the spin system ($r_{C-C}=1.5$ Å), the polarization transfer to the C_{remote} (red dash-dot line) is quenched for CM₅RR with most of the polarization being transferred to the directly bonded C_β (blue solid line), thus demonstrating the phenomenon of dipolar truncation. (b) In the PAR simulation the presence of a third strongly coupled spin leads to a partial decrease in polarization transfer to C_{remote} (red dash-dot line) showing that dipolar truncation is attenuated in the TSAR transfer mechanism. Simulations were performed with SPINEVOLUTION (Ref. 50) $\omega_r/2\pi=20$ kHz, $\omega_{0H}/2\pi=750$ MHz ^1H frequency and do not include chemical shifts.

soon as each of the two ^{13}C 's is coupled equally to the ^1H . This is illustrated in Fig. SI7 (Ref. 69) with the example of a four spin system consisting of two ^{13}C 's, each with a directly bonded ^1H . In Fig. SI7a (Ref. 69) the symmetry of the spin system ensures that the ZQ-TSAR off-resonant contributions vanish [see Eq. (24)]. The effect of the geometrical autocross-term compensation can be seen in the map shown in Fig. SI7a (Ref. 69) since the TSAR mechanism is active over a large part of the map and does not need to be close to the two lines defined by $\chi(1, p_C, p_H)=0$ and $\chi(2, p_C, p_H)=0$. If the spin system symmetry is broken (as in Fig. SI7b),⁶⁹ the ZQ off-resonance contribution is no longer zero, but still sufficiently small so that Fig. SI7b (Ref. 69) looks closer to Fig. SI7a (Ref. 69) compared to the case where only one proton is considered (Fig. SI7c) (Ref. 69).

In practice, this means that the ZQ off-resonant autocross term is intrinsically reduced when considering a TSAR transfer between ^{13}C 's arising from the same types of groups (e.g., CH and CH, CH₂ and CH₂, and CH₃ and CH₃) and that the TSAR effect is much easier to fulfill in this case (which is manifest as broader range of rf powers leading to appreciable TSAR effect).

V. DIPOLAR TRUNCATION AND LONG DISTANCE TRANSFER

Dipolar truncation is frequently alluded to in the literature and refers to the difficulty of detecting cross peaks corresponding to long distances arising from weak couplings in the presence of strong dipolar couplings.^{52,53} This phenomenon is illustrated in Fig. 7(a) with the example of a first-order broadband CM₅RR where, in the absence of a directly bonded C_β spin, we achieve significant polarization transfer from the C_α spin to the C_{remote} spin (dashed black line) with a ~ 25 ms buildup time for a 4.5 Å distance. However, in the presence of C_β spin, the polarization transferred from C_α to the C_{remote} (dash-dot red line) is severely attenuated. Note

that in the latter case most of the magnetization is directed to the directly bonded C_β spin. Thus, for example, γ -encoded dipolar recoupling sequences^{6–8,51,54} exhibit spectra that are dominated by a one-bond relayed transfer mechanism and thus provide excellent tools for assigning ^{13}C – ^{13}C spectra of proteins.⁵⁵

However, in order to determine 3D structures, it is necessary to measure weak couplings corresponding to structurally important long distances in the presence of the strong couplings from directly bonded neighbors. Thus, the design of new polarization transfer methods that suppress ^{13}C – ^{13}C dipolar truncation in uniformly ^{13}C labeled compounds is an area of active research. The initial solutions proposed for this problem were based on frequency selective ^{13}C – ^{13}C recoupling methods such as rotational resonance (R^2) and its variants— R^2 tickling,²⁷ R^2 width,^{10,28} and R^2 in the tilted rotating frame ($R^2\text{TR}$).^{11,29} More recently, two additional experimental approaches were introduced and tested on model compounds with the aim of circumventing truncation and providing more accurate ^{13}C – ^{13}C distance restraints.

The first category includes the SEASHORE and COMICS experiments, which use the chemical shift interactions to dephase unwanted DQ coherences. In the SEASHORE approach DQ excitation periods are alternated with delay windows where DQ coherences are dephased except if the carrier frequency matches the mean offset of the two carbons.³⁰ COMICS is based on the generalization of the second averaging principle and consists of the application of two successive averaging fields equal to $0.25\omega_r$ that simultaneously reintroduce the isotropic chemical shift interaction and the DQ dipolar terms leading to an extremely narrow-band sequence.⁸

The second category relies on the active truncation of a ZQ dipolar Hamiltonian by chemical shift tensors,³¹ an intellectually elegant approach recently introduced by Marin-Montesinos and employed in the triple oscillating field tech-

FIG. 8. (Color) (a) 2D ^{13}C – ^{13}C correlation spectrum of $[\text{U-}^{13}\text{C}, ^{15}\text{N}]\text{N-f-MLF-OH}$ diluted to 10% in a natural abundance lattice. The spectrum was recorded at $\omega_{\text{OH}}/2\pi=750$ MHz and $\omega_r/2\pi=20$ kHz with $\tau_{\text{mix}}=7.5$ ms, $\omega_{1\text{C}}/2\pi\approx 50$ kHz, $\omega_{1\text{H}}/2\pi\approx 47$ kHz, and the ^{13}C offset at 101 ppm. The circled cross peaks in (a) correspond to ≥ 4 Å ^{13}C – ^{13}C distances. (b) Numerical simulations of PAR polarization transfer between MC_β and LC_α (highlighted 4.3 Å distance) using rf power levels specified in (a). The spin system includes nearby protons ($2x\text{MH}_\beta$, MC_α , LH_α , LH) (back solid line) and nearby protons plus MC_α and MC' (red dash line). The dotted blue line represents simulation on a spin system including nearby protons plus MC_α and MC' with the ^1H – ^1H couplings removed from the calculation. Simulations include typical chemical shift tensor values (see SI for details) (Ref. 69). The plot illustrates that the contribution of the polarization relayed through MC_α and MC' to the polarization transfer between MC_β and LC_α is negligible compared to the direct polarization transfer.

nique rotor assisted dipolar refocusing (TOFU-RADAR) experiment.³² Whereas COMICS relies on a two step averaging at $0.25\omega_r$, TOFU-RADAR adds a third averaging step that produces the recoupling of the ZQ dipolar interaction and the chemical shift tensor. To date these sequences have been demonstrated on uniformly labeled model systems and allow measurement of ^{13}C – ^{13}C distances from sites selected using a Gaussian inversion pulse.

The PAR experiment differs significantly from the methods presented above since the spin dynamics do not depend on the ^{13}C – ^{13}C couplings, but involve couplings to the surrounding protons. An important consequence of this mechanism is the attenuation of dipolar truncation when compared to first-order ^{13}C – ^{13}C recoupling sequences. This is illustrated in Fig. 7(b) with numerical simulations where in the presence of the C_β spin, and in contrast to broadband DQ recoupling, we can still transfer a significant amount of polarization between the C_α and the C_{remote} spin. This prediction is confirmed experimentally with 2D spectra obtained with DQ CM_5RR where at a short mixing time (0.8 ms) only one-bond ^{13}C – ^{13}C dipolar transfer is observed (*vide infra*).

VI. POLARIZATION TRANSFER IN UNIFORMLY LABELED SYSTEMS

With the simulations in Sec. III we theoretically demonstrated the PAR mechanism for ^{13}C – ^{13}C polarization transfer. To confirm these predictions with experimental data we have studied two systems—the tripeptide $[\text{U-}^{13}\text{C}, ^{15}\text{N}]\text{N-f-MLF-OH}$ and $[\text{U-}^{13}\text{C}, ^{15}\text{N}]\text{-Crh}$ protein—and ^{13}C – ^{13}C spectra of these two molecules are shown in Figs. 8 and 9, respectively. The spectra permit us to illustrate the features of the PAR experiment and importantly permits

us to measure distances >4 Å at high spinning frequencies (>20 kHz) and subsequently, in the case of the Crh protein, to calculate the structure of a monomer.

In Fig. 8(a) we display a 2D ^{13}C – ^{13}C PAR correlation spectrum obtained from the $[\text{U-}^{13}\text{C}, ^{15}\text{N}]\text{-N-f-MLF-OH}$ diluted to 10% in a natural abundance lattice which insures that the observed contacts are due to the intramolecular interactions. In this 2D spectrum the circled cross peaks correspond to ^{13}C – ^{13}C distances ≥ 4 Å. Thus, this simple spectrum clearly demonstrates polarization transfer between ^{13}C 's separated by medium to long distances. Concurrently, the presence of these cross peaks raises important questions about the mechanism of the polarization transfer: (1) how can we extend the description of the PAR mechanism from a model three spin process involving a single ^1H to a real lattice where multiple assisting ^1H 's spins can contribute; (2) what is the nature of the polarization transfer between distant spins, i.e., is the polarization transfer directly through space or is it relayed via a chain of ^{13}C 's and which ^1H 's are involved in the TSAR mechanism? As shown below the answer to these questions depends strongly on the topology of the spin system together with the ^{13}C – ^{13}C distances. Nevertheless, it is possible to predict some qualitative general trends that are useful for evaluating the reliability of the distance estimates for various cases.

As mentioned above, only the ^1H 's in close proximity to the ^{13}C 's contribute significantly to PAR polarization transfer. With simulations we have considered PAR polarization transfer as a function of a ^1H – ^{13}C distance for several different three spin topologies [Fig. SI8 (Ref. 69)]. From these simulations it is apparent that the main contribution to the PAR polarization transfer involves directly bonded ^1H – ^{13}C spin pairs (since they result in the stronger TSAR couplings). At the same time, it is also clear that, in order to accurately

simulate the polarization transfer, ^1H 's other than those that are directly bonded to ^{13}C 's also have to be considered. Indeed the simulations suggest that, in general, the inclusion of the ^1H 's within 2.5 Å of the ^{13}C 's should lead to an adequate description of the spin dynamics. The contributions from more remote protons have TSAR couplings that are small and do not significantly alter the spin dynamics on the time-scale of ~ 20 – 30 ms. For example, even in the very favorable case where the remote proton is at an equal distance of 3 Å from the ^{13}C 's, the PAR polarization transfer requires more than 100 ms to achieve a maximum [see Fig. SI5 (Ref. 69)].

The relative contribution of ^{13}C relayed versus direct polarization transfer is illustrated below for three typical spin systems encountered in proteins. In all cases, the PAR polarization transfers correspond to structurally valuable ^{13}C – ^{13}C distances (>4 Å). Let us first examine the case of a sequential *inter-residue polarization transfer* between Met- C_β and Leu- C_α ($r_{\text{C-C}}=4.3$ Å) in N-*f*-MLF-OH [one of the cross peaks highlighted in Fig. 8(a)]. The SPINEVOLUTION simulations of the polarization transfer are displayed in Fig. 8(b) and highlight the influence of other nearby spins. The black solid line corresponds to the case with only neighboring ^1H 's included in the simulation, the red dashed line includes adjacent ^1H 's, Met- C_α , and Met- C' . The fact that the simulations from these three cases overlap indicates that the Met- C_α and Met- C' (i.e., ^{13}C 's in between Met- C_β and Leu- C_α) do not really alter the polarization transfer between Met- C_β and Leu- C_α . The polarization transfer is dominated by the Met- C_β – ^1H –Leu- C_α TSAR coupling, and thus the ^{13}C relayed magnetization is a negligible fraction of the overall magnetization transferred.

It is, however, possible to find spin systems where the situation is more complicated and where nearby ^{13}C 's do influence a particular magnetization transfer. This is illustrated in Fig. SI9 (Ref. 69) for an *intraresidue polarization transfer* along the leucine sidechain. Here we consider two cases: from Leu- C_α to Leu- C_δ and to Leu- $\text{C}_{\delta'}$ in $[\text{U-}^{13}\text{C}, ^{15}\text{N}]$ -N-*f*-MLF-OH. These very interesting cases illustrate that the nearby carbons (here C_β and C_γ) can influence the polarization transfer in different ways. In the case of the Leu- C_α to Leu- C_δ transfer, the polarization transfer maximum is significantly decreased, but the buildup time is very similar with C_β and C_γ included and without C_β and C_γ . In this case, the decrease in overall efficiency is explained by the fact that the initial magnetization present on the Leu- C_α is now distributed over three spins. However, because the buildup time is essentially constant, the polarization transfer is mainly direct. In the case the Leu- C_α to Leu- $\text{C}_{\delta'}$ transfer, the buildup time is changed when we include C_β and C_γ in the simulation, accounting for the fact that a substantial part of the polarization transfer in this case is relayed by the C_β and C_γ carbons. This is consistent with the fact that the C_β and C_γ carbons are located in the path between the C_α and $\text{C}_{\delta'}$ spins. This is illustrated in Fig. SI9 (Ref. 69).

Note that the three-bond PAR polarization transfers from LC_α to LC_δ and $\text{LC}_{\delta'}$ with C_β and C_γ included in the simulation are almost identical even though the ^{13}C – ^{13}C distances differ by almost 1 Å. This means that *intraresidue*

contacts generally lead to less accurate ^{13}C – ^{13}C distance estimates.

In summary, the ^{13}C relayed TSAR mechanism between two distant ^{13}C spins is likely to be significant for spin topologies where additional ^{13}C 's are located in between them. Cases with larger TSAR relayed couplings than direct TSAR coupling will be encountered primarily for intraresidue transfers and sequential contacts (except for $\text{C}_{\alpha,i}$ – $\text{C}_{\alpha,i+1}$ and $\text{C}_{\alpha,i}$ – $\text{C}_{\beta,i+1}$). For the rest of the inter-residue transfer, as demonstrated in Sec. VII, it is possible to relate the PAR experimental data to ^{13}C – ^{13}C distance estimates and to use them for protein structure determination in an approach analogous to Nuclear Overhauser Effect (NOE)-based methods in solution NMR.

VII. ^{13}C – ^{13}C Crh DISTANCE ESTIMATES FROM ^{13}C – ^1H – ^{13}C PAR DATA

In Sec. III we discussed the TSAR mechanism for the case of three spins, involving a single proton, and have seen that the PAR polarization transfer depends on the local geometry in this case. It is important to determine how this translates to situations where multiple ^1H 's are involved in the mechanism and if the polarization transfer can be used to estimate ^1H – ^{13}C or ^{13}C – ^{13}C distances in spin topologies where the contribution of relayed transfers is negligible.

Figure 9 shows ^{13}C – ^{13}C PAR spectra obtained from $[\text{U-}^{13}\text{C}, ^{15}\text{N}]$ -Crh protein, and Figs. 10(a)–10(d) experimental polarization buildup curves for a number of isolated unambiguously assigned ^{13}C – ^{13}C pairs obtained using the PAR technique at $\omega_r/2\pi=20$ kHz. The polarization transfer curves are sorted into different classes depending on the corresponding ^{13}C – ^{13}C distance extracted from the x-ray structure [1MU4 (Ref. 56)]: Fig. 10(a) corresponds to one-bond distances, (b) to two-bond distances, (c) to medium (4–5.5 Å), and (d) to long (5.5–7 Å) distance classes. Thus, the first two panels (a) and (b) represent intraresidue contacts, whereas the last two panels (c) and (d) show inter-residue distance ranging from 4 to 7 Å.

Buildup curves corresponding to one-bond distances are easily distinguishable from other polarization transfers: the maximum signal occurs at around 2 ms, and the intensity is larger than any other distance transfer. In this case, directly bonded ^1H 's are primarily responsible for the transfer since their TSAR couplings are dominant. Figure 10(b) shows PAR polarization transfer corresponding to two-bond distances and the optimal buildup time is about 5 ms. Figure 10(c) shows *medium distance inter-residue transfer* corresponding to the 4–5.5 Å distances, and the buildup time is around 5–10 ms with the maximum efficiency is comparable to the two-bond distance case. Note that it does not seem possible to differentiate between the distance classes in Figs. 10(b) and 10(c) based on the polarization profiles alone. For longer *nonsequential inter-residue* distances (>5.5 Å), the PAR buildups are quite distinguishable with the optimal polarization transfer occurring around $\tau_{\text{mix}}=15$ ms. Note that in this case, the polarization transfer efficiency is set to zero for PAR mixing times equal to 2 and 5 ms because the cross peak intensities were below the noise level.

FIG. 9. (Color) 2D ^{13}C - ^{13}C correlation spectra of $[\text{U-}^{13}\text{C}, ^{15}\text{N}]$ -Crh protein comparing two advanced recoupling pulse sequences at $\omega_{0H}/2\pi = 750$ MHz and $\omega_r/2\pi = 20$ kHz: (a) Broadband CM₃R spectrum recorded with $\tau_m = 0.8$ ms $\omega_{1C}/2\pi \sim 100$ kHz and displaying only one-bond dipolar ^{13}C - ^{13}C cross peaks (see the gray monomer of the Crh dimer structure representation in the inset). Note that the spectrum was acquired in ~ 15 h without ^1H irradiation during the CMRR mixing time. (b) PAR spectrum corresponding to $\tau_m = 14$ ms with $\omega_{1C}/2\pi \sim 53$ kHz and $\omega_{1H}/2\pi \sim 50$ kHz cw fields displaying ^{13}C - ^{13}C cross peaks corresponding to medium (4.3 Å) and long (~ 5.4 Å) distances (acquired in ~ 40 h). Several illustrative examples are shown on the green monomer of the Crh dimer structure representation. A detailed description of the PAR optimization protocol can be found in the SI (Ref. 69).

Finally, we illustrate in Figs. 10(e)–10(g) the detailed data for the case of polarization transfer between G67C α and S31C α . The spin system is composed of ten spins [see Fig. 10(e) and Table SI3] and does not involve relaxation. The

magnetization is initially on the G67C α spin and the simulation use similar settings as for the experimental data shown in Fig. 9(b). The first striking observation is that the buildup curves corresponding to the G67C α -S31C α and G67C α

FIG. 10. (Color) PAR polarization transfer as a function of the ^{13}C - ^{13}C distance. [(a)–(d)] A sampling of experimental ^{13}C - ^{13}C PAR polarization transfer curves obtained on $[\text{U-}^{13}\text{C}, ^{15}\text{N}]$ -Crh protein with $\omega_r/2\pi = 20$ kHz, $\omega_{0H}/2\pi = 900$ MHz and a carrier frequency set to 38.9 ppm: (a) one-bond distance class; (b) 2.5–3.5 Å distance class; (c) 3.5–5 Å distance class; (d) > 5 Å distance class. (e) Spin system used in the PAR polarization transfer simulations [(f) and (g)]. Atom coordinates and chemical shift tensors used in the simulations can be found in the SI (Table SI3).

$-S31C_\beta$ cross peaks have similar characteristic times. This is a very satisfying result since the two distances are close to 4 Å. Moreover, the transfer to the carbonyls is small (even for the directly bonded G67C'). This behavior is expected since the carrier frequency was set in the middle of the aliphatic ^{13}C region. The PAR ^{13}C cw rf field is not large enough to compensate for the carbonyl chemical shift autocross term which induces a significant off-resonance component in the corresponding TSAR subspace. Figure 10(g) shows the G67C $_\alpha$ -S31C $_\alpha$ transfer in the presence or in the absence of the S31C $_\beta$ and S31C' spins. The maximum polarization transfer is higher in the latter case but the overall buildup time is preserved ensuring that the TSAR transfer is direct in this case.

Finally, we should mention that the influence of the network of ^1H - ^1H couplings on the TSAR mechanism is a complex question. As shown in Figs. 8(b), 10(f), and 10(g), the presence of ^1H - ^1H couplings can have different effects on the ^{13}C - $[^1\text{H}]$ - ^{13}C PAR polarization transfer. In the case of the sequential transfer from LC $_\alpha$ to MC $_\beta$ simulated in Fig. 8(b), the polarization transfer is only slightly affected by the removal of the ^1H - ^1H couplings. Concurrently, the long-range PAR transfer between S31C $_\alpha$ and G67C $_\alpha$ spins simulated in Fig. 10(g) is dependent on the presence and the absence of ^1H - ^1H couplings. The influence of ^1H - ^1H couplings on the ^{13}C - $[^1\text{H}]$ - ^{13}C TSAR spin dynamics depends on the spin system topology and the rf settings chosen. Moreover, the influence of the ^1H - ^1H couplings appears through second-order effects (i.e., $p_H \neq 0.5, 1$). However, in examples highlighted here, the presence or the absence of ^1H - ^1H couplings does not significantly change the ^{13}C - $[^1\text{H}]$ - ^{13}C buildup profiles, i.e., the maximum buildup time is preserved and the optimal polarization transfer is similar within 30%.

In summary, Figs. 10(a)-10(d) indicate that the homonuclear TSAR mechanism yields buildup profiles that can be used to estimate ^{13}C - ^{13}C distances in fully protonated protein samples (with the exception of intraresidue >2 -bond and sequential contacts different from $^i\text{C}_\alpha$ - $^{i+1}\text{C}_\alpha$, $^i\text{C}_\alpha$ - $^{i+1}\text{C}_\beta$). Furthermore, the effect of the ^1H - ^1H couplings does not appear as a limiting factor for using the PAR experiments to estimate ^{13}C - ^{13}C distances.

VIII. ^{13}C - $[^1\text{H}]$ - ^{13}C TSAR RECOUPLING DURING FIRST-ORDER ^{13}C - ^{13}C HOMONUCLEAR RECOUPLING

In Sec. II we demonstrated that ^{13}C - $[^1\text{H}]$ - ^{13}C TSAR transfers utilize two cw rf fields and that one of the matching conditions implies similar rf fields on the ^1H and ^{13}C channels. That is, appreciable PAR transfer is achieved when the mismatch between ^1H and ^{13}C rf field strengths is not sufficient to decouple the ^1H and ^{13}C baths. This in turn suggests the possibility of concurrent ^{13}C - $[^1\text{H}]$ - ^{13}C TSAR transfers during other homonuclear recoupling experiments, particularly those where the ^1H decoupling is nonideal. Potentially, this situation arises in first-order ^{13}C - ^{13}C dipolar recoupling experiments, where insufficient ^1H decoupling is known to interfere with the ^{13}C - ^{13}C recoupling spin dynamics and

produces intensity losses in DQ filtered experiments^{5,57} or ^{13}C - ^{13}C J -based transfer experiments. This interference becomes acute at high $\omega_r/2\pi$ and has stimulated the design of DQ recoupling sequences where no additional ^1H irradiation is required.^{7,53}

In the Supporting Information (Figs. SI10-SI12) (Ref. 69) we investigate with simulations the influence of the ZQ ^{13}C - $[^1\text{H}]$ - ^{13}C TSAR mechanism during the application of four ^{13}C - ^{13}C dipolar recoupling sequences [HORROR,⁶ CM $_p$ RR,⁸ RFDR,^{4,58} and SR6 $_2$ (Refs. 6 and 59)] as well as P9 $_{12}$ (Ref. 1) TOBSY (Ref. 60) through bond polarization transfer experiments. We show that the TSAR mechanism may become significant or even the dominant homonuclear polarization transfer mechanism for ^1H irradiation conditions considered as adequate ^1H decoupling fields (especially in applications on proteins). Note that this is in contrast to previous studies that have only considered the effects of inadequate ^1H decoupling fields in the context of ^{13}C - ^{13}C polarization transfer losses and not in terms of potential additional polarization transfer.

This is an important result as it shows that we should exercise caution when interpreting experimental data obtained on protonated samples on the basis of a ^{13}C - ^{13}C recoupling mechanism. For instance, if the goal is to assign the spectrum of an unknown molecule, it is crucial to be able to distinguish cross peaks due to covalently bonded nuclei from the cross peaks due to noncovalently bonded nuclei that are in close spatial proximity. In theory this can be achieved either using a broadband dipolar recoupling technique, in which dipolar truncation ensures one-bond relayed transfer, or using J coupling based through-bond methods.^{20,60,61} In practice, the presence of additional efficient through-space TSAR transfer is likely to jeopardize, or at least greatly complicate, the correct interpretation of the assignment data by producing additional cross peaks that are not distinguishable from the cross peaks resulting from the intended dipolar or J -based mechanism. We note that for techniques that recouple and decouple with ^{13}C irradiation alone (CM $_p$ RR,^{8,55} CMAR,⁷ radio-frequency-driven dipolar recoupling (RFDR),⁶² and some R and C sequences⁶³) that TSAR is suppressed.

In Fig. 11 we delineate these ideas with simulations using five spins (three ^{13}C 's and two ^1H 's) that illustrate the effect of a directly bonded spin (C3) on a long distance transfer from C1 to C2 (3.01 Å). As we will see the presence of the additional ^{13}C - $[^1\text{H}]$ - ^{13}C TSAR mechanism during first-order ^{13}C - ^{13}C recoupling pulse sequences may lead to an overall increase in long distance polarization transfer (C1-C2) which may be incorrectly interpreted as a reduction in dipolar truncation in the ^{13}C - ^{13}C recoupling mechanism.

If we consider the first set of simulations (dashed black lines) in Fig. 11 where only the ^{13}C spins are included, we confirm the general observation that most first-order ^{13}C - ^{13}C recoupling sequences lead to efficient dipolar truncation. The two other sets of curves (dashed red and blue lines) in Fig. 11 represent the same simulations with a larger spin system that includes three additional protons. More specifically, the second set of lines (dashed red) represents simulations where all the couplings are present, whereas the third

FIG. 11. (Color) Modification of the long distance polarization transfer in uniformly labeled systems when both first-order $^{13}\text{C}-^{13}\text{C}$ recoupling and second-order TSAR mechanism are simultaneously present. Note the different behavior for the simulations with only $^{13}\text{C}-^{13}\text{C}$ couplings included and the simulations also including ^1H 's. The chosen ^1H irradiations yield substantial TSAR mechanism contribution to overall polarization transfer (except CM_3RR where no ^1H irradiation was used in order to illustrate a case of pure second-order $^{13}\text{C}-^{13}\text{C}$ spin dynamics without TSAR contribution). (a) DQ CM_3RR with 100 kHz ^{13}C rf and no ^1H rf. (b) DQ HORROR with 15 kHz ^{13}C rf and 80 kHz ^1H rf. (c) ZQ PAR with 56 kHz ^{13}C rf and 54 kHz ^1H rf. (d) ZQ SR_{62} (Ref. 6) with 20 kHz ^{13}C rf and 82 kHz ^1H rf. (e) ZQ RFDR with 12.5 kHz pulses and 69 kHz ^1H rf. The simulations were performed at [(a), (c), (d), and (e)] $\omega_r/2\pi = 20$ kHz or (b) $\omega_r/2\pi = 30$ kHz and $\omega_0/2\pi = 700$ MHz and include isotropic chemical shift and CSA typical for the aliphatic sites (see SI) (Ref. 69). The spin system is based on the leucine sidechain in the structure of N-f-MLF-OH (Ref. 64). Note that all the pulse sequences except PAR [in (d)] are designed to reintroduce the $^{13}\text{C}-^{13}\text{C}$ dipolar coupling to the first order.

set of lines (blue) accounts only for the TSAR transfer as the $^{13}\text{C}-^{13}\text{C}$ and $^1\text{H}-^1\text{H}$ couplings are removed.

In the case of CM_3RR , no ^1H irradiation is present since the sequence was designed to efficiently recouple and decouple using only a ^{13}C irradiation field.^{7,8} In this case the spin dynamics is free from any TSAR contribution and dominated by first-order $^{13}\text{C}-^{13}\text{C}$ recoupling yielding a small polarization transfer to the remote spins (about 3% when all couplings are included). On the other hand, for PAR recoupling, the polarization transfer does not rely on $^{13}\text{C}-^{13}\text{C}$ couplings, and long-range polarization transfer can reach about 25% efficiency (all couplings included) solely based on $^{13}\text{C}-[^1\text{H}]-^{13}\text{C}$ TSAR mechanism. Note that the polarization transfer is only slightly affected by the $^1\text{H}-^1\text{H}$ couplings.

For the other examples, the long distance polarization transfers are substantially different between simulation performed with ^{13}C 's only (black dashed) and simulations in the presence of protons under ^1H decoupling (second set) and can even be larger in the latter case. This can be explained by the presence of a concurrent $^{13}\text{C}-[^1\text{H}]-^{13}\text{C}$ TSAR mechanism and is clearly demonstrated by comparing simulations without $^{13}\text{C}-^{13}\text{C}$ and $^1\text{H}-^1\text{H}$ couplings and simulations with all couplings included. The ^1H "decoupling" field in each case was chosen to obtain an appreciable (although not necessarily optimal) TSAR effect [see Fig. SI10 (Ref. 69)]. The ^1H field strengths are 80, 82, and 69 kHz for HORROR, SR_{62} , and RFDR, respectively. Note that these ^1H fields correspond to values often used in SSNMR protein studies. This is a very interesting observation as it explains why some first-order recoupling sequences under some conditions are

able to provide medium to long-range contacts. In all the cases studied here, the reason behind involves a second-order $^{13}\text{C}-[^1\text{H}]-^{13}\text{C}$ TSAR effect. This is a very satisfying observation as the dipolar truncation efficiency should not vary much between first-order recoupling sequences (either DQ or ZQ). Indeed the truncation efficiency primarily relies on the ratio between weak versus strong couplings and is thus independent of the pulse sequence scaling factor. For a given spin system geometry, the powder Euler angle dependence of the recoupling pulse sequence should be able to mainly account for the small variation in dipolar truncation efficiency.

Finally, it is important to highlight that the simultaneous reintroduction of first-order $^{13}\text{C}-^{13}\text{C}$ terms and second-order $^{13}\text{C}-[^1\text{H}]-^{13}\text{C}$ TSAR terms reduces the polarization transfer to the remote spin compared to the PAR case. As a consequence, if the objective is to record medium to long distance contacts in uniformly or extensively labeled systems, the obvious choice is to use a pulse sequence inducing a pure TSAR spin dynamics and not a mixture of first-order $^{13}\text{C}-^{13}\text{C}$ and TSAR spin dynamics.

To summarize, when a sufficiently large second-order $^{13}\text{C}-[^1\text{H}]-^{13}\text{C}$ TSAR effect is concurrently present during first-order $^{13}\text{C}-^{13}\text{C}$ recoupling experiments, then it manifests itself as an apparent reduction in dipolar truncation. Thus, in the case of first-order ZQ sequences such as RFDR, it is possible to observe polarization transfer over distances that are long compared to the situation where only $^{13}\text{C}-^{13}\text{C}$ couplings are present. This significant effect should be taken into account when interpreting experimental data both for assignments and structural studies.

FIG. 12. (Color) [(a) and (b)] Examples of ^{13}C - ^{13}C correlation spectra of $[\text{U-}^{13}\text{C}, ^{15}\text{N}]$ -Crh protein at $\omega_{\text{OH}}/2\pi=900$ MHz and $\omega_r/2\pi=20$ kHz. Expansion of the aliphatic region for (a) CM₅RR (0.8 ms) and (b) PAR (15 ms). The PAR spectrum contains numerous cross peaks corresponding to medium to long distances that involve methyl groups. As a comparison, the CM₅RR spectrum displays only one-bond cross peaks. A detailed description of the PAR optimization protocol can be found in the SI (Ref. 69). (c) Ensemble of structures of a Crh monomer (residues 12–85) calculated using a unique 2.5–6 Å distance class for all the unambiguous ^{13}C - ^{13}C cross peaks identified using the x-ray structure (Ref. 56) as a homology model. (e) Numerical simulations of the polarization transfer between CH₃ and CH illustrating the influence of the threefold methyl group hopping on the overall polarization transfer. The coordinates for spin system (d) used in the simulations were taken for A20Cα and I47Cδ1 from the x-ray structure (Ref. 56) of the Crh protein. Simulations do not include chemical shift.

IX. PROTEIN STRUCTURE CALCULATION

Finally, we illustrate the potential of the TSAR based techniques for 3D structure determination of proteins. More precisely, we show that the PAR data presented here contain information on a sufficient number of distances, including medium range (from contacts between sites that are between two to four residues apart) and long-range restraints (from contacts between the sites that are equal or more than five residues apart) to successfully calculate the structure of the Crh protein.

The ^{13}C - ^{13}C correlation spectra presented in Fig. 9 were recorded at 900 MHz and $\omega_r/2\pi=20$ kHz from a sample of microcrystalline $[\text{U-}^{13}\text{C}, ^{15}\text{N}]$ -Crh protein with mixing times of (a) 0.8 ms (CM₅RR) and (b) 15 ms (PAR). To reduce the experimental acquisition period, compared to the N-*f*-MLF-OH PAR spectra presented above, we focused on the aliphatic-aliphatic correlations in which case we need to sample only a ~ 14 kHz spectral width in the indirect dimension rather than ~ 40 kHz for the full bandwidth at 900 MHz ^1H frequency. Moreover, this approach allows us to work with rf fields of ~ 50 kHz without having to address bandwidth issues and concentrates the magnetization on the aliphatic sites, without distributing polarization to the carbonyl and aromatic nuclei.

The ^{13}C - ^{13}C PAR data shown in Fig. 9(b) contain ~ 800 cross peaks and assigning these unambiguously is a non-trivial task. Thus, we used the x-ray structure⁵⁶ as a homology model to facilitate in the assignment of ambiguous cross peaks present in the PAR data set. More precisely, we combined the chemical shift assignment data²⁵ and ^{13}C - ^{13}C dis-

tances extracted from the x-ray structure to simulate a ^{13}C - ^{13}C spectrum with a ^{13}C - ^{13}C distance cutoff of 7 Å. By allowing the chemical shifts to vary within ± 0.25 ppm and comparing this simulated spectrum with the experimental data, we were able to unambiguously assign 163 structurally meaningful ^{13}C - ^{13}C cross peaks. 92 of these correspond to long-range ^{13}C - ^{13}C contacts.

Using the unambiguously assigned cross peaks with a unique distance class of 2.2–6 Å, we then performed molecular dynamics simulated annealing in torsion angle space [CNS (Ref. 65)]. The backbone precision for the 10 best structures from the 100 calculated conformers was found to be 2.06 Å, and the accuracy (defined here as the backbone rmsd of the 10 best conformers with respect to the x-ray structure) is 2.31 Å. The calculated structure resulting from this simple procedure is presented in Fig. 12(c). Using only one ^{13}C - ^{13}C PAR data set and one distance class for all the contacts, we obtain the correct fold of the Crh monomer. Thus, this demonstrates that the PAR data presented here contain information on a sufficient number of distances to calculate a protein structure. It is worth mentioning that we did not use dihedral angle predictions from chemical shifts.²⁶

Note, that we have used only a single class of distances in the above calculation. However, as we have demonstrated in Sec. VII with polarization transfer curves, we can establish several distance classes that should enhance the precision and accuracy of the structure calculations using data from the TSAR based techniques. A *de novo* structure calculation utilizing ^{13}C - ^{13}C PAR and ^{15}N - ^{13}C PAIN-CP (Ref. 44) (i.e., ^{15}N - $[\text{H}]-^{13}\text{C}$ TSAR) restraints, that we have per-

formed in a separate study will be reported elsewhere.

In order to complete our discussion of the PAR experiment, we briefly contrast it with other techniques used for SSNMR 3D structure determination of proteins, primarily PDS³³ and DARR.^{13,34} Using these two approaches, structures of model proteins were determined using U-¹³C, ¹⁵N and alternately ¹³C labeled spin systems.^{14,16,23,66} The use of alternate labeling schemes⁶⁷ is motivated by excessive spectral crowding obtained at long mixing times on uniformly labeled systems, which complicates cross peak assignments. The extent of crowding in DARR spectra is dependent on several variables, such as the protein size, the magnetic field, and the MAS frequency. However, because the DARR mechanism relies on ¹³C-¹³C couplings, it efficiently relays magnetization through the ¹³C chain and thus leads to efficient polarization transfer for intraresidue (and also sequential) contacts, and this interferes with the observation of cross peaks arising from medium and long-range contacts.⁶⁸ Since the TSAR mechanism does not involve ¹³C-¹³C couplings, the same is not true for PAR. Indeed, if we compare cross peak intensities at $\tau_{\text{mix}}=20$ ms, we find that the ratio of cross peaks corresponding to long (>5 Å) as compared to short distances is between 1:1 and 1:2.

In contrast to PDS and DARR and similarly to the PAR method, CHHC/NHHC^{35,36} yields spectra with reduced spectral crowding. These experiments are used to extract structurally valuable cross peaks and therefore appear more suitable for application to uniformly labeled systems. This was recently illustrated with a structure determination of the [U-¹³C, ¹⁵N]-Crh dimer based on CHHC/NHHC data,⁴² and similar work is currently under progress using the PAR data.

Even though CHHC/NHHC and TSAR-based techniques appear as complementary approaches to extract relevant structural restraints, it is worth pointing out that they differ in terms of sensitivity. In particular, the CHHC/NHHC techniques utilize three polarization transfer steps, and as a consequence their sensitivity is low. In contrast, the PAR method uses a single, efficient transfer step and thus yields improved signal to noise. This point is illustrated in Fig. SI13 (Ref. 69) which compares a CHHC spectrum of Crh recorded with ~ 20 mg of sample at $\omega_{\text{OH}}/2\pi=500$ MHz in ~ 46 h with a ¹³C-¹³C PAR spectrum obtained with ~ 6 mg of sample at $\omega_{\text{OH}}/2\pi=750$ MHz in ~ 21.4 h. In both spectra, an intermediate mixing time was chosen and the lowest contour level was set at five times the noise level. These spectra illustrate the improved signal to noise of the PAR data obtained at high magnetic fields on small samples.

Another interesting observation is the significant attenuation or even the absence of cross peaks involving methyl groups in the CHHC spectra. This effect can be explained by the intrinsically inefficient polarization step from the ¹³C spin to methyl ¹H's and a reduction in ¹H-¹H polarization transfer efficiency due to threefold methyl group hopping.⁴⁰ In contrast, cross peaks involving methyl groups are featured prominently in the PAR spectra. Figure 12(b) illustrates this point with an expansion of the aliphatic region of the spectrum focusing on the cross peaks involving methyl groups.

The data suggest that the motion of the -CH₃'s has a positive effect on the polarization transfer in the PAR experi-

ment, providing a sensitive tool to probe (long-range) contacts involving methyl groups. This is of primarily importance for methyl-methyl side-chain contacts which are often buried in the hydrophobic core of the protein and thus particularly useful in structure calculations. This point is corroborated by the simulations shown in Fig. 12(d) that compare the 4.3 Å distance transfer between A20C _{α} and I47C _{δ 1} sites of Crh protein with and without methyl group rotation. The polarization transfer is greatly enhanced when the methyl protons are simulated under fast exchange conditions. It is worth noting that methyl groups have been recognized as very valuable probes of structure and dynamics in a wide range of biomolecular systems including membrane and amyloid proteins, and they were recently used to probe protein-ligand interaction in SSNMR.⁶⁶

The last feature of PAR that should be pointed out, especially in contrast to PDS and DARR sequences, is its flexibility with respect to the MAS regime. The PAR sequence was used in this work at $\omega_r/2\pi=20$ kHz, but its application at higher, as well as lower frequencies is also possible. This is depicted in Fig. SI14 (Ref. 69) with a numerical simulation of a ¹³C-¹³C PAR experiment at the lower and higher spinning frequencies, $\omega_r/2\pi=10$ and 70 kHz, respectively. In PDS the optimal buildup time for a fixed distance increases with $\omega_r/2\pi$, which limits the applicability of the technique to low and moderate spinning frequencies (<15 kHz). The DARR experiment, which relies on the same type of mechanism as PDS with an improved scaling factor, enables access to higher MAS frequencies. However, it is not practical to use DARR for obtaining long distance contacts ≥ 30 kHz MAS for two reasons. First, as in the case of PDS, the spin part of the DARR scaling factor is fixed, so the mixing time required to observe long distance contacts increases with $\omega_r/2\pi$; and second, the application of DARR for ~ 100 ms to observe long distances at >30 kHz MAS frequencies may induce a considerable amount of rf heating, which is not desirable for biological samples.

X. CONCLUSIONS

We have introduced and characterized the homonuclear version of the TSAR mechanism applicable to ¹³C-¹³C and ¹⁵N-¹⁵N polarization transfer in biomolecules at moderate (10–20 kHz) to high (70 kHz) spinning frequencies and high magnetic fields with moderate power requirements. The PAR sequence relies on a three spin process involving second-order cross terms between ¹H-¹³C₁ (¹H-¹⁵N₁) and ¹H-¹³C₂ (¹H-¹⁵N₂) that promotes polarization transfer between ¹³C₁ (¹⁵N₁) and ¹³C₂ (¹⁵N₂) via trilinear operators such as $C_1^{\pm}C_2^{\mp}H_Z$. The analytical expressions derived from AHT permit visualization of the subspace in which the TSAR spin dynamics evolves and indicates that the processes are influenced by the presence of an autocross term with major contributions from cross terms of the ¹H-¹³C coupling with itself. Moreover, in the context of analytical and numerical simulations, we discussed the dependence of PAR polarization transfer with respect to the local geometry of the spin system. We show that the autocross term may be compen-

sated to a large extent for specific combinations of ^{13}C and ^1H rf fields, as well as for some particular spin topologies. In addition, we demonstrate that dipolar truncation is significantly reduced in the TSAR mechanism, and that we can use the ^{13}C – ^{13}C PAR experiment to estimate ^{13}C – ^{13}C distances. With this concept in mind, we applied ^{13}C – ^{13}C PAR to the uniformly labeled Crh protein, which allowed us to obtain high quality spectra used to derive the structure of a Crh monomeric subunit. Moreover, we have discussed the possibility of inducing significant ^{13}C – $[^1\text{H}]$ – ^{13}C TSAR polarization transfer during the application of other ^{13}C – ^{13}C dipolar recoupling and J -based experiments that should be taken into account when interpreting the data. Interestingly, ^{13}C – $[^1\text{H}]$ – ^{13}C TSAR contributions are present during the first-order ^{13}C – ^{13}C recoupling experiments and may lead to the apparent reduction in dipolar truncation. In summary, TSAR based techniques—including ^{13}C – ^{13}C PAR, ^{15}N – ^{15}N PAR, and the previously introduced ^{15}N – ^{13}C PAIN-CP—constitute a new family of techniques for high resolution structural studies of large biomolecules at high magnetic fields and high spinning frequencies.

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