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Homonuclear decoupled ^{13}C chemical shift anisotropy in ^{13}C doubly labeled peptides by selective-pulse solid-state NMR

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Abstract

We describe a new experiment for measuring homonuclear-decoupled anisotropic chemical shift patterns in doubly ^{13}C-labeled compounds under magic-angle spinning. The experiment combines a pair of selective and non-selective 180° pulses to suppress the ^{13}C–^{13}C scalar and dipolar interactions. This is combined with the recently developed SUPER technique to recouple the chemical shift anisotropy. Demonstrations on $^{13}C\alpha$ and ^{13}CO-labeled amino acids and peptides show that accurate chemical shift powder patterns can be obtained. This permits the use of chemical shift anisotropy for conformational studies of suitably extensively ^{13}C-labeled peptides and proteins.

Keywords: Chemical shift anisotropy; Dante pulses; Homonuclear decoupling; Isotopic labeling; Magic-angle spinning; Selective excitation

1. Introduction

The chemical shift anisotropies (CSAs) of backbone ^{13}C sites in peptides and proteins are strongly influenced by conformation [1,2]. In particular, various solution and solid-state NMR experiments [3–7] and quantum chemical calculations [7,8] have shown that the $C\alpha$ chemical shift tensor is exquisitely sensitive to the ϕ and ψ torsion angles of the amino acid residues. To determine the chemical shift anisotropy with high accuracy and site resolution in unoriented samples, Schmidt-Rohr and coworkers [9] have recently developed a robust 2D CSA-recoupling technique under magic-angle spinning (MAS) called SUPER. The experiment utilizes phase-compensated and rotor-synchronized 2π pulses placed at specific time points in a rotation period to yield static-like powder patterns [10]. This technique has now been demonstrated on many crystalline and amorphous organic compounds, peptides, and polymers to derive conformation information and to identify chemical functionality [5,11,12].

Unfortunately, the original SUPER experiment cannot be directly applied to uniformly or extensively ^{13}C-labeled peptides and proteins, because homonuclear ^{13}C–^{13}C dipolar coupling is reintroduced along with the CSA interaction, and *J*-coupling is also present, both distorting the CSA lineshape. Yet extensive ^{13}C labeling is important for determining the structure of medium to large peptides and proteins efficiently [13–15]. Thus, a modified CSA-recoupling experiment that suppresses the ^{13}C–^{13}C dipolar and *J*-couplings is desirable.

In this paper, we present a modified SUPER experiment that utilizes selective and non-selective π pulses to average the ^{13}C–^{13}C couplings in doubly ^{13}C-labeled amino acids and peptides. These π pulses are incorporated into the original SUPER experiment to yield coupling-free, undistorted, CSA powder patterns. Similar homonuclear decoupling strategies have been demonstrated in other 2D experiments for protein resonance assignment [16] and for distance measurements [17]. Here, a rotor-synchronized DANTE sequence [18] is chosen to achieve selective inversion of one of the two coupled ^{13}C spins. We call this technique separation of undistorted powder-patterns by effortless recoupling without mixed anisotropies (SUPERWOMAN).

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2. Experimental

All NMR experiments were conducted on a Bruker DSX-400 spectrometer (Karlsruhe, Germany) at a field strength of 9.4 T. A triple-resonance MAS probe with a 4-mm spinning module was used. Typical 90° pulse lengths were 3.5 μs for ^{1}H and 5 μs for ^{13}C and ^{15}N. The ^{1}H decoupling field was 75 kHz during acquisition and increased to 95 kHz during the CSA evolution. A ^{15}N decoupling fields of 15 kHz were used. The ω_1 (CSA) dimension of the 2D experiments was incremented rotor-synchronously. The CSA scaling factor is 0.155 for the experiments reported here, as determined by the timing of the 2π pulses [9].

The chemical shift anisotropy is reported as the span Ω, which is defined as $\Omega = \delta_{11} - \delta_{33}$, where the principal values follow the convention $\delta_{11} \geqslant \delta_{22} \geqslant \delta_{33}$. The asymmetry parameter η is $(\delta_{11} - \delta_{22})/(\delta_{iso} - \delta_{33})$ when δ_{11} is closer to the isotropic shift, and $(\delta_{22} - \delta_{33})/(\delta_{11} - \delta_{iso})$ when δ_{33} is closer to the isotropic shift. The ^{13}C chemical shifts were referenced externally to the glycine carbonyl peak at 176.4 ppm.

3. Pulse sequence and implementation

Fig. 1a shows the pulse sequence for the homonuclear decoupled SUPER experiment. Compared to the original experiment, the main difference is that the CSA evolution period is divided into two halves by a constant period, $t_{cc,dec}$, that includes a train of Dante pulses and a non-selective π pulse in the middle. The Dante pulses, applied on every rotational echo, have a small flip angle δ and a combined flip angle of 180°. A weak ω_1 field, about 12.5 kHz in our experiments, is used for these δ pulses. The long duration of the Dante period and its synchronization with sample rotation ensure the selective inversion of the phase of the on-resonance magnetization and its sidebands [18]. The weak field strength of the pulses, while not absolutely necessary, further suppresses the magnetization that is far-off resonance by limiting the excitation bandwidth. In the experiments shown below, the Cα peak is put on resonance, thus its centerband and sidebands exclusively experience the Dante pulse. In contrast, the non-selective π pulse, typically implemented with a field strength of ~65 kHz, affects all ^{13}C spins, such as the ^{13}CO spin that is coupled to Cα. Thus, the net effect of this $t_{cc,dec}$ period is to invert the phase of the CO or Cβ spin while leaving the Cα site unaffected. Since most Cα–CO chemical shift differences are significantly larger than the one-bond ^{13}C–^{13}C couplings, the homonuclear dipolar and *J*-coupling Hamiltonian can be considered to be in the weak coupling limit. Thus, it is truncated by the chemical shift difference Hamiltonian to $H_{cc} = \omega_d \cdot I_z S_z + 2\pi J \cdot I_z S_z$. Such a bilinear Hamiltonian is inverted when one spin is inverted by a single 180° pulse while the other is unperturbed by two 180° pulses. Therefore, at the end of the symmetric period, $t_{cc,dec} + t_1$, the effect of the dipolar coupling and *J*-coupling is refocused. Meanwhile, since the Cα spin experiences no net inversion, it has evolved under the chemical shift interaction for a period t_1. During this CSA evolution period, the ^{13}C–^{15}N dipolar coupling is suppressed by continuous-wave ^{15}N decoupling.

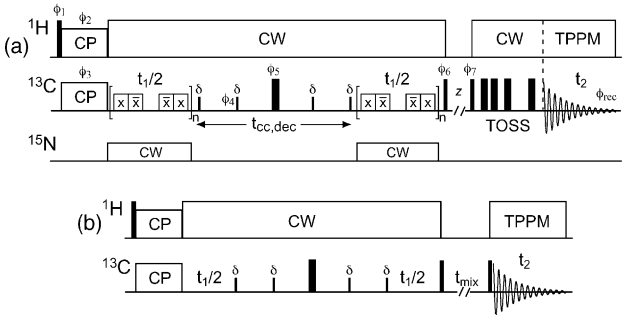

Fig. 1. (a) Homonuclear-decoupled ^{13}C CSA-recoupling experiment. The CSA evolution during t_1 is broken into two halves by a rotor-synchronized Dante pulse train that contains a non-selective 180° pulse in the middle. Narrow and wide rectangles represent 90° and 180° pulses, respectively. After $t_{cc,dec} + t_1$, evolution under the ^{13}C–^{13}C dipolar and *J*-couplings between on-resonance and off-resonance spins is refocused. The phase cycles are: $\phi_1 = 31$, $\phi_2 = 0$, $\phi_3 = 00112233$, $\phi_4 = 2 \times (11223300)\ 2 \times (00112233)\ 2 \times (33001122)\ 2 \times (22330011)$, $\phi_5 = -\phi_4$, $\phi_6 = 11223300$, $\phi_7 = -\phi_6$, $\phi_{rec} = 02132031$, where $0 = +x$, $1 = +y$, $2 = -x$, $3 = -y$. (b) ^{1}H-driven ^{13}C spin diffusion experiment with ^{13}C–^{13}C homonuclear decoupling.

To demonstrate the SUPERWOMAN technique, we used two amino acids, Val and Leu, each labeled at ^{13}Cα, ^{13}CO, and ^{15}N (Spectra Stable Isotopes, Columbia, MD). Neither sample contains any sidechain ^{13}C labels, thus providing simple homonuclear two-spin systems to test the experiment. We designate these samples as U-backbone Val and Leu. An ^{15}N-labeled but ^{13}C unlabeled Val sample (Cambridge Isotope Laboratories, Andover, MA) was used to compare with the homonuclear-decoupled CSA spectra of U-backbone Val. Unfortunately, an ^{15}N-labeled Leu sample had a different crystal modification and we were unable to recrystallize it into the same form as the U-backbone Leu sample. Thus, it cannot be used to compare with the spectra of U-backbone Leu. We also applied the SUPERWOMAN experiment to a pentadecapeptide,

The Dante π pulse and the non-selective π pulse in the SUPERWOMAN sequence are phase-cycled using the EXORCYCLE scheme to minimize the effect of pulse imperfection [19]. The EXORCYCLE inverts the magnetization every other scan, while the receiver phase is kept constant. As a result, the off-resonance magnetization that experiences a single π pulse is suppressed. On the other hand, the on-resonance Cα magnetization is unaffected since the soft and hard π pulses have exactly opposite phases.

$(VPGVG)_3$, which was synthesized as a model system for an elastic protein $[(VPGVG)_4\ (VPGVG)]_{39}$ [20]. The pentadecapeptide shows ^{13}C isotropic and anisotropic chemical shifts that are indistinguishable from those of the protein, indicating that its structure represents the protein conformation well [21]. The pentadecapeptide is uniformly labeled with ^{13}C and ^{15}N at Gly-8 and ^{15}N labeled at Val-9, and the Gly-8 Cα chemical shift powder pattern after homonuclear ^{13}C–^{13}C decoupling was extracted.

4. Results and discussions

Fig. 2 compares the recoupled Cα powder patterns of Val with and without homonuclear decoupling. The spectrum of the ^{13}C unlabeled Val sample (Fig. 2a) exhibits a narrow pattern with a span of $\Omega = 14 \pm 1$ ppm and an asymmetry parameter of $\eta = 0.79$. This anisotropy value already took into account the scaling factor of 0.155 for the CSA-recoupling sequence. This agrees well with the literature chemical shift anisotropy of Val obtained from sideband analysis of slow MAS spectra [22]. This spectrum serves as a reference for the U-backbone Val sample. When the original SUPER experiment without ^{13}C–^{13}C decoupling was applied directly to U-backbone Val, a broad pattern with a full width at half-maximum (FWHM) of 32 ppm was obtained (Fig. 2b). Both the *J*-coupling and residual dipolar coupling contribute to the broad lineshape: the $^{13}C\alpha$–^{13}CO *J*-coupling is 53.4 Hz, while the scaled one-bond dipolar coupling is $2\,\text{kHz} \times 0.155 \times 0.5 = 162$ Hz. Thus the total homonuclear coupling is 215 Hz. These combine with the scaled Cα CSA of $14\,\text{ppm} \times 0.155 \times 100\,\text{MHz} = 217$ Hz to yield a total scaled frequency of 432 Hz. This estimate agrees roughly with the observed linewidth of 496 Hz for the U-backbone Val sample.

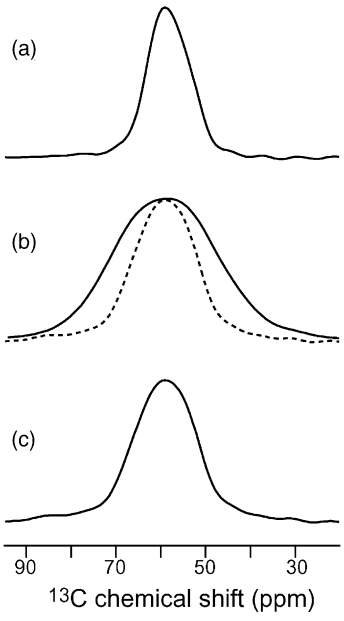

Fig. 2. Recoupled Cα CSA spectra of valine, obtained under a spinning speed of 2.5 kHz. (a) SUPER spectrum of ^{15}N-labeled Val. (b) SUPER spectrum (solid line) of U-backbone Val. Note the significant broadening of the pattern due to the $^{13}C\alpha$–^{13}CO dipolar and *J*-couplings. (c) Homonuclear decoupled CSA spectrum of U-backbone Val. The dashed line in (b) reproduces spectrum (c) for comparison.

The effect of the ^{13}C–^{13}C coupling in the Val spectrum is removed by the SUPERWOMAN experiment. The homonuclear decoupled spectrum of Val (Fig. 2c) is much narrower than the undecoupled spectrum, and is best fit with $\Omega = 16.5 \pm 2.5$ ppm and $\eta = 0.93$, in reasonable agreement with the result of the unlabeled Val (Fig. 2a). The somewhat larger anisotropy may partially result from the fact that the weak coupling approximation may not be well satisfied for some orientations of the crystallites under CSA recoupling.

To optimize the selective and non-selective π pulses in the SUPERWOMAN experiment, we first carried out a modified ^{13}C CP experiment that uses a rotor-synchronized Dante π pulse train to invert ^{13}C longitudinal magnetization. The critical parameter in the experiment is the flip angle δ of the Dante pulses, or equivalently, the duration of the Dante period. With the same ω_1 field strength and the same pulse interval, τ_r, a smaller flip angle or a longer Dante period makes the inversion more selective. For crystalline compounds with narrow linewidths, the flip angles can be as small as 18°, which correspond to a Dante period of 10 rotor periods. In contrast, for amorphous compounds with broad lines such as the elastin-mimetic peptide, flip angles as large as 45° or a Dante duration of only four rotor periods are used to ensure inversion of the entire Cα peak. This experiment yields negative intensities for the on-resonance peak and its sidebands but positive intensities elsewhere. This is demonstrated in Fig. 3a for U-backbone Val, where 10 pulses of flip angle $\delta = 18°$ were used in the Dante train. Subsequently, a second CP experiment containing both the Dante pulses and a non-selective π pulse was used to check the performance of the $t_{cc,dec}$ period of the SUPERWOMAN experiment. When the EXORCYCLE is not used for the π pulses, and when the same phase correction as the single-inversion experiment is used, this experiment should yield a spectrum with positive on-resonance peak and its sidebands but negative intensities elsewhere, as shown in Fig. 3b. When the EXORCYCLE is used, then the off-resonance CO signal and its sideband manifolds are suppressed (spectra not shown). In setting up these experiments, care must be taken to avoid the rotational resonance condition, under which the Dante pulses can also invert the undesired CO or Cβ signals.

The effectiveness of the double inversion sequence for removing homonuclear couplings is further tested by

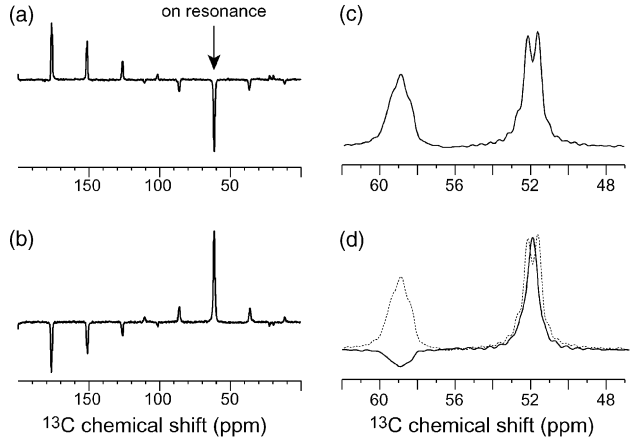

Fig. 3. ^{13}C MAS spectra of U-backbone Val after (a) a Dante π-pulse and (b) both a Dante π pulse and a non-selective π-pulse. The same phase correction was used for both spectra. Spectrum (b) was acquired without the EXORCYLE for the π pulses, so that the off-resonance CO magnetization shows negative intensities. (c, d) Cα cross-sections of U-backbone Leu from 2D ^{1}H-driven ^{13}C spin diffusion spectra. (c) Without ^{13}C–^{13}C decoupling. (d) With ^{13}C–^{13}C decoupling using Dante. The dotted line in (d) reproduces spectrum (c) for comparison. The peak at 59 ppm is the folded CO signal. The residual negative intensity of the CO peak in (d) results from incomplete suppression by the EXORCYCLE. Coherence transfer is achieved by 50 ms of mixing without ^{1}H decoupling.

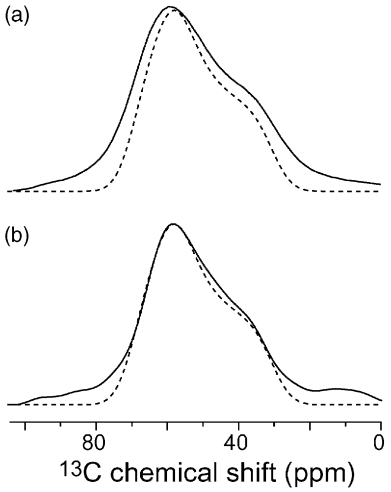

Fig. 4. Recoupled Cα CSA spectra of U-backbone Leu, obtained under a spinning speed of 3.5 kHz. (a) Without homonuclear decoupling (solid line). (b) With homonuclear decoupling (solid line). The best fit for the decoupled spectrum, with $\Omega = 39$ ppm and $\eta = 0.52$ (dashed line), is superimposed with both experimental spectra for comparison.

incorporating it into a 2D ^{13}C–^{13}C correlation experiment. As shown in the pulse sequence of Fig. 1b, the Dante train and the hard 180° pulse are inserted into the middle of the ^{13}C evolution period, so that a *J*-decoupled isotropic chemical shift spectrum is recorded in the indirect dimension of the 2D spectra. Figs. 3c and d compare the Cα cross-sections of two 2D spectra of U-backbone Leu, acquired without and with homonuclear decoupling. The experiment incorporating the 180° pulses showed a single Cα peak (Fig. 3d) without the ^{13}C–^{13}C *J*-splitting, and reduced the FWHM from 105 to 74 Hz.

Since the Val Cα powder pattern has a nearly symmetric lineshape with η close to 1, we applied the homonuclear-decoupled SUPER experiment to Leu, which has a more distinct and asymmetric CSA powder pattern. Fig. 4a shows the undecoupled CSA spectrum of Leu Cα. A broad wing that results from the residual ^{13}C–^{13}C couplings is observed on both sides of the powder pattern. Homonuclear decoupling yielded a CSA spectrum (Fig. 4b) with a reduced anisotropic span as well as a flatter baseline. Best-fit simulation yielded $\Omega = 39 \pm 2$ ppm and $\eta = 0.52$ (Fig. 4b).

The effectiveness of the SUPERWOMAN experiment on amorphous and biologically relevant systems is demonstrated on an elastin-mimetic pentadecapeptide, $(VPGVG)_3$. The ^{13}C linewidths of the labeled Gly-8 are 330 Hz and 425 Hz for Cα and CO, respectively, compared to an average linewidth of 120 Hz for the doubly ^{13}C-labeled crystalline amino acids. Thus, a less selective Dante condition, with a flip angle δ of 45°, was used. The undecoupled SUPER spectrum (Fig. 5a) shows a clearly distorted CSA pattern, while the decoupled CSA spectrum (Fig. 5b) is well fit with $\Omega = 50 \pm 3$ ppm and $\eta = 0.92$. These values agree well with the Gly-3 Cα CSA extracted previously from the polypeptide $[(VPGVG)_4(VPGVG)]_{39}$ (MW: 81 kDa) [23]. Moreover, comparison of the decoupled spectrum with the simulated spectra for β-sheet and α-helical conformations of Gly (Figs. 5c and d) indicates that Gly-8 in this peptide definitely does not adopt either of the two canonical secondary structures. This is again consistent with the previous polypeptide study, which concluded that the Gly residue following Pro is most likely in a β-turn conformation [23].

The SUPERWOMAN sequence described here works well only in the weak coupling limit, where the ^{13}C chemical shift differences are significantly larger than the homonuclear dipolar and *J*-couplings. This condition is mostly satisfied for Cα–CO spin pairs, as demonstrated here. However, the same is often not true for Cα–Cβ spin pairs, especially for certain amino acids such as Thr and Ser, which have small Cα and Cβ chemical shift differences. In these cases, the chemical shift Hamiltonian and homonuclear dipolar Hamiltonian do not commute, thus the homonuclear coupling is not refocused by the Dante pulse train. Further, the Cα–Cβ-labeled residues often encounter rotational resonance broadening [24] under typical magnetic field strengths and under the spinning speeds suitable for CSA recoupling. Therefore, an alternative method for homonuclear decoupling is necessary for sidechain ^{13}C-labeled residues.

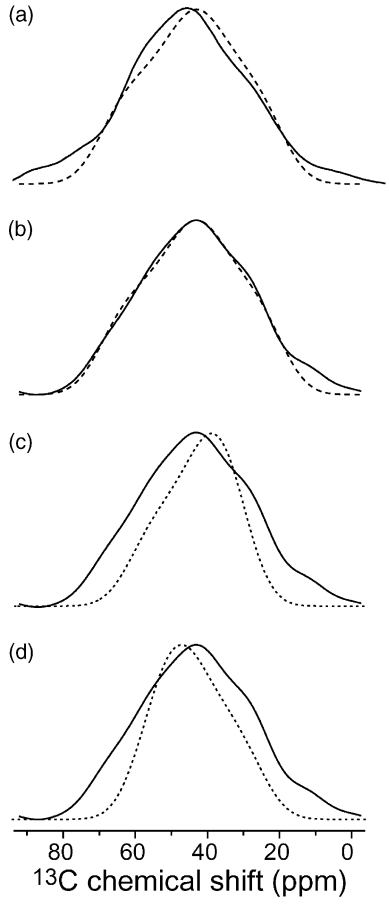

Fig. 5. Recoupled Cα CSA spectra of uniformly ^{13}C,^{15}N-labeled Gly-8 in $(VPGVG)_3$ obtained (a) without ^{13}C–^{13}C decoupling at 2.5 kHz and (b) with ^{13}C–^{13}C decoupling at 3 kHz. The best fit for the decoupled spectrum (dashed line), with $\Omega = 50$ ppm and $\eta = 0.92$, is superimposed with both experimental spectra. (c) Simulated spectrum (dotted lines) for β-sheet Gly, with $\Omega = 32$ ppm and $\eta = 0.47$. (d) Simulated spectrum (dotted line) for α-helical Gly, with $\Omega = 34$ ppm and $\eta = 0.61$. The experimental homonuclear-decoupled CSA spectrum (solid line) is superimposed with the simulations in (c) and (d) to highlight the deviations.

5. Conclusions

We have shown that the combination of selective and non-selective π pulses effectively removed the ^{13}C–^{13}C dipolar and *J*-couplings and, used in conjunction with the SUPER experiment, yields undistorted chemical shift anisotropy patterns in doubly ^{13}C-labeled compounds. This permits the experimental determination of ^{13}C CSAs for refining and potentially predicting the conformation of peptides and proteins in the solid state. The homonuclear-decoupled CSA experiment works quite well for Cα and CO doubly labeled compounds but is not effective for Cα- and Cβ-labeled residues or higher-spin systems. These limitations may be partially circumvented by using selectively ^{13}C-labeled proteins [13,25] to create backbone Cα–CO-labeled residues, for which the weak coupling limit holds and the rotational resonance phenomenon does not occur.

Acknowledgments

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