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High Resolution ^{1}H Detected ^{1}H,^{13}C Correlation Spectra in MAS Solid-State NMR using Deuterated Proteins with Selective ^{1}H,^{2}H Isotopic Labeling of Methyl Groups

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Magic angle spinning (MAS) solid-state nuclear magnetic resonance (ssNMR) has developed rapidly over the past few years. This development led to the first structural models of amyloidogenic peptides[1−3] and membrane proteins.[4] A major obstacle in the determination of these structures is the low signal-to-noise ratio associated with ssNMR experiments. So far, all standard ssNMR experiments have relied on heteronucleus detection (^{13}C/^{15}N). Protons would, in principle, be better suited due to their large gyromagnetic ratio (γ_H). However, a large γ_H implicates large ^{1}H,^{1}H dipolar couplings even in the presence of line narrowing techniques, such as magic angle spinning (MAS) and homonuclear dipolar decoupling, because of the homogeneous nature of the proton coupling network.[5] Deuteration of a protein and back-substitution of exchangeable deuterons by protons allows one to decrease the effective ^{1}H,^{1}H dipolar couplings and facilitates ssNMR pulse sequence development.[6−8] We could show recently[9] that a further decrease in the proton spin density—employing 90% D_2O in the crystallization buffer—resulted in a 4-fold reduction in the ^{1}H line width (compared to a sample recrystallized from 100% H_2O). The obtained H^N line width was on the order of 20 Hz at a MAS frequency of 24 kHz. However, this approach induces a loss in sensitivity due to the decreased number of protons. The approach which is presented in this pilot study avoids this pitfall by labeling of methyl groups as originally proposed by Kay and co-workers.[10] The labeling protocol involves growing cells in 90% D_2O, using 3-[66%-^{2}H,^{13}C]-labeled pyruvate as the sole carbon source. Methyl groups of alanine, valine, leucine, and γ-isoleucine are expressed as isotopomeric mixtures of CD_3, CHD_2, CH_2D, and CH_3 with relative concentrations of approximately 30, 44, 22, and 4%, respectively. The degree of enrichment of individual isotopomers can be monitored using solution-state NMR. Figure S1 represents a typical ^{2}H-coupled (along t_1) ^{1}H−^{13}C correlation pattern indicating the isotopic distribution. We show in this communication that, out of all expected isotopomers, one can be selectively observed by MAS solid-state NMR.

Figure 1 represents ^{13}C 1D spectra recorded under various preparation and decoupling conditions. In the absence of ^{2}H decoupling, proton decoupling alone is not sufficient to obtain a well resolved spectrum (Figure 1A). Introducing ^{2}H,^{13}C scalar decoupling yields a major improvement in the spectral quality (Figure 1B). Typical ^{13}C line widths are on the order of 18 Hz (at a MAS frequency of 25 kHz). Focusing on Ala55-Cβ, the ^{2}H,^{13}C CP experiment yields intensity for only one of the methyl isotopomers (Figure 1C). We assign this resonance to the CD_3 isotopomer. Introduction of a deuterium nucleus in a methyl group yields an isotope induced upfield shift of approximately −0.3 ppm in the carbon dimension (see Figure S1).[11] Therefore, subsequent

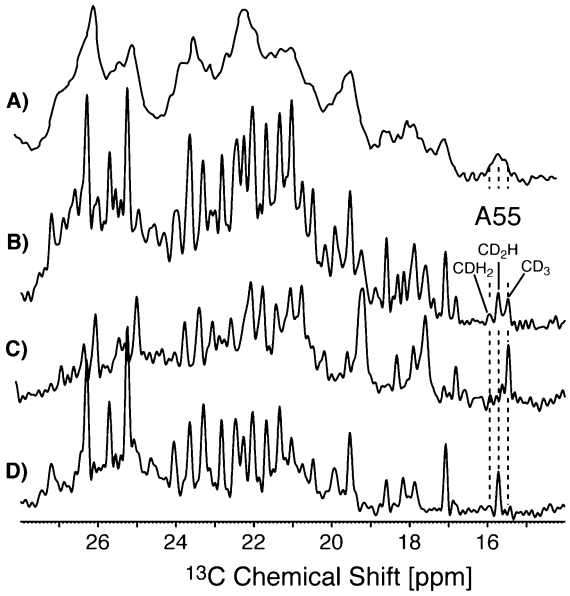

Figure 1. ^{13}C spectrum of the SH3 domain from chicken α-spectrin grown on 3-[66%-^{2}H,^{13}C]-labeled pyruvate. (A) ^{1}H,^{13}C CP (τ_{CP} = 1.8 ms) with only ^{1}H decoupling during acquisition using WALTZ-16. (B) ^{1}H,^{13}C CP (τ_{CP} = 1.8 ms) with ^{1}H and ^{2}H decoupling. (C) ^{2}H,^{13}C CP (τ_{CP} = 2.5 ms) with ^{1}H and ^{2}H decoupling. (D) ^{1}H,^{13}C INEPT with ^{1}H and ^{2}H decoupling during acquisition only.

downfield resonances can be assigned to CHD_2 and CH_2D isotopomers, respectively. Using INEPT for ^{1}H,^{13}C magnetization transfer, only one of the resonances is retained in the spectrum (Figure 1D). Apparently, the resonance of the CH_2D isotopomer is broadened in the course of the ^{1}H,^{13}C scalar coupling evolution period due to the presence of the ^{1}H,^{1}H dipolar coupling. The fourth isotopomer, CH_3, is not observable in any of the MAS solid-state NMR spectra.

This interpretation is corroborated by a 2D *J*-resolved correlation experiment which shows that the ^{13}C resonances associated with the CD_3 and the CD_2H moieties evolve as a singlet and as a doublet, respectively, with a coupling of 127 Hz (Figure S2). The observed splitting is independent of the rotation frequency. Interference effects between MAS, the scalar and dipolar coupling, as well as chemical shift anisotropy (CSA) as reported previously[12] can therefore be ruled out as contributing factors. We note that the ^{1}H,^{13}C CP experiment yields, as well, correlations to CD_3 groups. A possible reason for this might be the long CP time required to maximize the CP efficiency in this experiment, which may facilitate the magnetization transfer from not directly bonded protons, including those of residual H_2O in the protein sample.

Figure 2 represents the pulse sequence employed to record the ^{1}H detected 2D ^{1}H−^{13}C correlation experiment. A constant time

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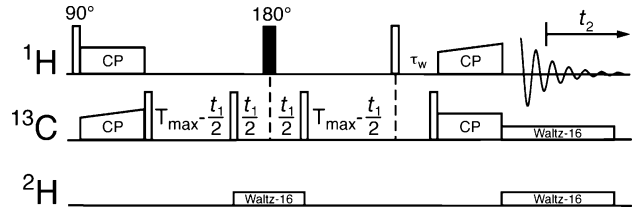

Figure 2. ^{1}H detected ^{1}H,^{13}C correlation experiment. Solid and open bars represent $\pi/2$ and π pulses, respectively. The $\pi/2$ pulse lengths were 3.8 and 6.1 μs on the ^{1}H and ^{13}C channel, respectively. Duration of CP was set to $\tau_{CP} = 1.8$ ms. A radio frequency field of 2−2.5 kHz for both ^{13}C and ^{2}H channels was applied for WALTZ-16 decoupling. The ^{1}H carrier was positioned on the water resonance. All NMR experiments were carried out using a 14.1 T wide-bore spectrometer (^{1}H frequency = 600 MHz) equipped with a 3.2 mm triple-resonance MAS probe.

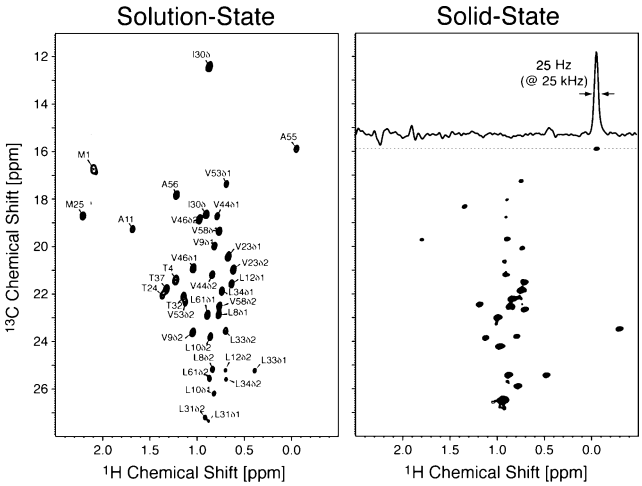

Figure 3. ^{1}H detected ^{13}C,^{1}H correlation experiment, displaying the methyl resonances of the SH3 domain of α-spectrin. Left: Solution-state spectrum. The sample used to record the spectrum was prepared by expressing the protein using glucose as the carbon source. Additional correlations in the solution-state spectrum are due to methionines, threonines, and isoleucine-δ which are not labeled in the pyruvate preparation. A version of a constant time HSQC pulse sequence was used to acquire the spectrum. Right: Solid-state NMR spectrum. In the experiment, $T_{max} = 65$ ms and $\tau_w = 29$ ms were employed; 400 complex t_1 points were acquired, with acquisition times of 40 and 100 ms in t_1 and t_2, respectively. The total experimental time was 12.5 h.

element is employed to yield optimal water suppression.[8] After CP, carbon magnetization is flipped along the *z*-axis and stored for a duration $T_{max} - t_1/2$. During the ^{13}C evolution period, ^{1}H,^{13}C scalar couplings are refocused by application of a ^{1}H 180° pulse in the center of t_1. WALTZ-16 decoupling was found to be well-suited to remove ^{2}H−^{13}C scalar couplings in t_1 and t_2.[8] An additional T_2 filter of duration τ_w is applied to dephase the residual water signal.

Figure 3a and b shows a comparison of the solution- and solid-state ^{1}H,^{13}C correlation spectra. Resolution in the solid state is very favorable given the fact that no care was taken to select for specific isotopomers. The observed ^{1}H line widths in the solid state are on the order of 25 Hz at a MAS frequency of 25 kHz. In comparison, line widths of ca. 300−450 Hz are obtained typically for protonated samples if protons are evolved in an indirect dimension, and ^{1}H,^{1}H dipolar interactions are decoupled using, for example, PMLG.[13] High MAS frequencies are required to achieve high resolution. Recently, Rienstra[14] and Hodgkinson[15] demonstrated that the slope of the dependence of the ^{1}H line width on the MAS frequency is a function of the overall proton density. The slope of this curve is related to the residual homonuclear ^{1}H,^{1}H dipolar interactions, as inhomogeneous interactions (in the sense of Maricq and Waugh[5]) would result in a MAS independent line width. In the nomenclature of Rienstra and Hodgkinson, the *y*-intercept *b* of the extrapolated curve refers to the ^{1}H line width at infinite rotation frequency. In our study, we find a slope $k = 440$ Hz/ms and a *y*-intercept $b = 7.5$ Hz. In contrast to the previously suggested labeling scheme,[9] methyl group labeling allows one to retain the full sensitivity associated with proton excitation and detection and permits at the same time sample preparation using 100% D_2O to avoid resonance broadening. Experimentally, we observe an increase in sensitivity by a factor of 4.71 compared to the ^{13}C detected version of the experiment assuming a CP efficiency of 60%. This factor might be increased further by optimization of the resonance circuit for proton detection.[16] In contrast to ^{1}H,^{15}N correlations,[17] values closer to the theoretical expected maximum value of $(\gamma_H/\gamma_X)^{3/2}$ are experimentally observed in this study due to the decreased proton line width.

In conclusion, we have shown that ^{1}H detected experiments are feasible in the solid state involving samples with extensively deuterated background and incorporation of protons in methyl groups. The obtained resolution is comparable to that in a solution-state ^{1}H,^{13}C correlation experiment. In addition, renunciation of high power decoupling reduces sample heating and is beneficial for sample stability. We expect that an additional increase in the signal-to-noise ratio by a factor of 2−3 can be obtained using precursors which are uniformly enriched with respect to the $^{13}CD_2H$ methyl isotopomer. The proposed labeling scheme has been employed recently for the study of side chain dynamics in the solid state.[18,19]

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Supporting Information Available: Figures S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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