

High Resolution ^1H -Detected Solid-State NMR Spectroscopy of Protein Aliphatic Resonances: Access to Tertiary Structure Information

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Abstract: Biological magic angle spinning (MAS) solid-state nuclear magnetic resonance spectroscopy has developed rapidly over the past two decades. For the structure determination of a protein by solid-state NMR, routinely ^{13}C , ^{13}C distance restraints as well as dihedral restraints are employed. In protonated samples, this is achieved by growing the bacterium on a medium which contains [1,3]- ^{13}C glycerol or [2]- ^{13}C glycerol to dilute the ^{13}C spin system. Labeling schemes, which rely on heteronuclei, are insensitive both for detection and in terms of quantification of distances, since they are relying on low- γ nuclei. Proton detection can in principle provide a gain in sensitivity by a factor of 8 and 31, compared to the ^{13}C or ^{15}N detected version of the experiment. We report here a new labeling scheme, which enables ^1H -detection of aliphatic resonances with high resolution in MAS solid-state NMR spectroscopy. We prepared microcrystals of the SH3 domain of chicken α -spectrin with 5% protonation at nonexchangeable sites and obtained line widths on the order of 25 Hz for aliphatic ^1H resonances. We show further that ^{13}C resolved 3D- ^1H , ^1H correlation experiments yield access to long-range proton–proton distances in the protein.

Magic-angle spinning (MAS) solid-state nuclear magnetic resonance spectroscopy has rapidly progressed over the past few years, allowing the structure determination of several uniformly isotopically enriched crystalline and noncrystalline proteins.^{1–5} For structure calculation, routinely ^{13}C – ^{13}C distance restraints² as well as dihedral restraints are employed.^{6,7} Thereby, the precision of the calculated tertiary protein structure benefits in particular from long-range restraints involving side chains. Due to their peripheral localization, protons are ideally suited to deliver nontrivial distance restraints. ^1H – ^1H distance restraints can be obtained in XHHX (X = C/N) type experiments.^{8–10} These experiments suffer, however, from a low signal-to-noise level due to the detection of low- γ nuclei. Furthermore, high resolution ^1H spectra of nonexchangeable protons are difficult to achieve in the solid state, even when homonuclear decoupling schemes are employed,¹¹ which is due to the large intrinsic ^1H , ^1H dipolar couplings.

A reduction of the proton dipolar network and accordingly a resolution enhancement can be accomplished by deuteration.¹² In order to reduce the proton density of nonexchangeable protons in a protein, we prepared highly deuterated, uniformly ^{13}C , ^{15}N isotopically enriched samples of the SH3 domain of chicken α -spectrin, using u -[^2H , ^{13}C] glucose and 5–15% H_2O (95–85% D_2O) in the M9 bacterial growth medium (for details see the

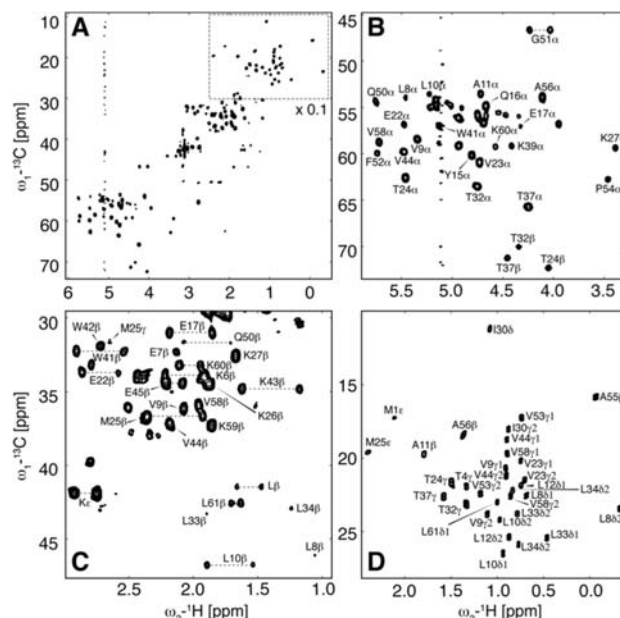


Figure 1. MAS solid-state ^1H -detected ^1H , ^{13}C -HMQC spectrum of the SH3 domain of α -spectrin. The sample was produced by growing bacteria in a minimal medium that contains 5% H_2O and 95% D_2O . The spectrum was recorded at 600 MHz, setting the MAS frequency to 20 kHz. The effective temperature was adjusted to 17 °C. The acquisition times in the direct and indirect dimension were set to 52 and 21 ms, respectively. The full spectrum (A) is represented enlarged in (B), (C), and (D) displaying the $^1\text{H}\alpha$ – $^{13}\text{C}\alpha$ region, $^1\text{H}\beta$ – $^{13}\text{C}\beta$ region, and the methyl region, respectively. The methyl region in (A) has been scaled by a factor of 0.1. Details on experimental parameters are given in the Supporting Information.

Supporting Information), based on approaches introduced before in solution-state NMR.^{13–16} Figure 1 shows the MAS solid-state NMR ^1H -detected ^1H , ^{13}C correlation spectrum that is obtained for a sample that was prepared using 5% H_2O in the M9 medium. We obtain high-resolution spectra for all aliphatic proton–carbon pairs. The experimental proton line widths vary between 25 Hz for T37 γ and 60 Hz for T37 α . The carbon line width is determined by ^{13}C , ^{13}C scalar couplings and is on the order of 110 Hz (T37 α). The presented labeling scheme allows avoiding high-power decoupling schemes due to the dilution of the proton network. 2.5 kHz WALTZ-16¹⁷ decoupling is sufficient to achieve ^{13}C decoupling during ^1H -detection. For comparison, the proton line width for a uniformly protonated SH3 sample is about 170 Hz.¹⁸ The sensitivity and resolution of resonances in the methyl region of the spectrum are not compromised, even though the amount of protons has been increased dramatically in comparison to the previously presented approaches.^{19–21}

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Statistically, the isotope distribution for the methyl isotopomers CH_3 , CH_2D , CHD_2 , and CD_3 is $p^3/3p^2q/3pq^2/q^3 = 0.0001:0.007:0.14:0.86$ (using $p = 0.05$, $q = 1 - p$), disregarding the remaining 3% protonation, originating from the utilized 97% deuterated ^{13}C -glucose.²⁰ For methylene isotopomers CH_2 , CHD , and CD_2 , the statistical distribution is $p^2/2pq/q^2 = 0.002:0.1:0.9$. For methyls, the signal intensities, which arise from these distributions, have to be principally rescaled according to the number of bound protons. Experimentally, CH_3 and CH_2D are, however, not observable as the dipolar coupling among the protons induces a broadening of the resonance beyond detection.

In order to experimentally determine the degree of protonation in the different sites, we carried out solution-state NMR $\text{HN}(\text{CO})\text{CA}$ and ^1H , ^{13}C constant-time HSQC experiments, employing a sample that was prepared using an M9 medium containing 5% (and 15%) H_2O (for details see the Supporting Information). At the α -position, we find an incorporation of protons of $7 \pm 2\%$ ($17 \pm 2\%$), which correlates rather well with the expected value of 5% (15%). For methylene and methyl groups, we find a proton concentration of $3.2\% \pm 1.1\%$ ($13.8\% \pm 3.0\%$) and $1.1\% \pm 0.5\%$ ($11.8\% \pm 1.0\%$), respectively.

Recently, we have reported high-resolution $\text{DQ-}^2\text{H}$, ^{13}C correlation spectra using the perdeuterated SH3 domain.²² The observed resonances in the $^1\text{H}^\alpha$, $^{13}\text{C}^\alpha$ correlation experiment match rather well the $^2\text{H}^\alpha$, $^{13}\text{C}^\alpha$ correlation spectra. The experimental ^2H line width for the $\text{D}\alpha$ resonances was (30 ± 9) Hz. The $\text{H}\alpha$ line width in the presented HMQC spectrum in Figure 1A amounts to (50 ± 9) Hz. With the 6.5 times smaller gyromagnetic ratio of deuterium $\gamma(^2\text{H})$ to protons $\gamma(^1\text{H})$ taken into account, the resolution in the ^1H -detected spectrum is improved by a factor of about 2. At the same time, the signal-to-noise is increased by a factor of ca. $0.05 \times \gamma(^1\text{H})^{5/2} / \gamma(^2\text{H})\gamma(^{13}\text{C})^{3/2} \approx 0.05 \times 52 \approx 3$.

In addition to correlation spectroscopy, the presented labeling scheme enables access to ^1H , ^1H distance restraints among side chains in a 3D-H(H)CH experiment as shown in Figure 2A. In the experiment, a first proton evolution period is followed by a ^1H , ^1H magnetization mixing step, utilizing a rotor synchronized adiabatic RFDR mixing scheme.²³ After mixing, magnetization is transferred to ^{13}C for chemical shift evolution and finally to ^1H for detection, using a scalar HMQC type sequence. Due to the fact that the proton spin system is sufficiently dilute, long-range interactions can be obtained without truncation of the dipolar coupling.

Figure 2B shows the experimental results, focusing on correlations involving A11 and M25 in the hydrophobic core of the α -spectrin SH3 domain. The ^{13}C resolved $^1\text{H}(f_1)$, $^1\text{H}(f_3)$ planes show all expected correlations between M25 ϵ , A11 β , V53 γ 2, and L10 δ 2. The structure of the protein is represented in Figure 2C. The shortest methyl–methyl proton distances are between 4.5 and 5.6 Å.

In contrast to the previous approach in which the exchangeable protons had to be partially replaced with deuterons,^{12,25} the presented labeling scheme does not require an H/D exchange step. This will be of particular importance for the investigation of membrane proteins, which have very stable amide protons that might not exchange within months.

In conclusion, we could demonstrate that the presented labeling scheme facilitates ^1H detection of aliphatic resonances and opens a new avenue for protein structure determination in MAS solid-state NMR. We presented further a 3D-H(H)CH correlation experiment which allows identification of ^1H , ^1H interactions among side chains which are close in space. We expect that this experiment will be crucial for the characterization of the tertiary structure of a protein in the future. The presented RAP (Reduced Adjoining Protonation) approach is easy to implement and allows bypassing

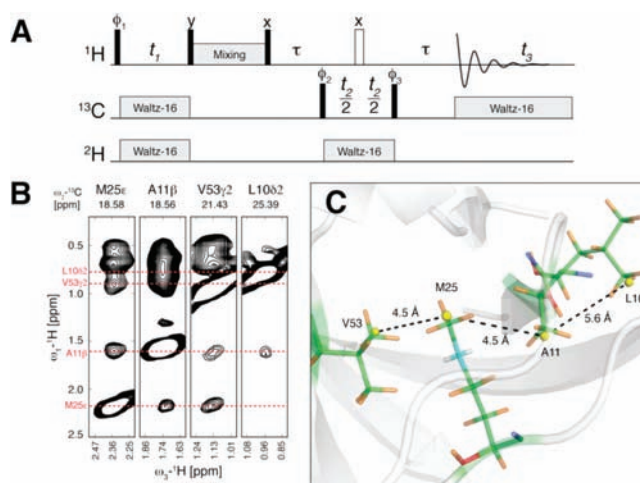


Figure 2. 3D-H(H)CH correlation experiment for the determination of long-range ^1H , ^1H distances in the solid state. For ^1H , ^1H mixing, a rotor synchronized adiabatic RFDR mixing scheme was used with a mixing time of 8 ms, employing an rf field of 55 kHz.²³ τ was set to 3.4 ms corresponding to $1/2J_{\text{CH}}$. The MAS rotation frequency was adjusted to 20 kHz. All experiments were carried out on a 600 MHz spectrometer at a sample temperature of 17 °C. The acquisition times were 57, 10, and 8 ms in the direct ^1H - and the indirect ^{13}C - and ^1H -dimension, respectively. Sixteen scans were accumulated for every increment. The total acquisition time amounted to 3 d. $\phi_1 = y, -y$; $\phi_2 = 2(x), 2(-x)$; $\phi_3 = 4(x), 4(-x)$; and $\phi_{\text{rec}} = y, -y, -y, y, -y, y, y, -y$. The ^1H carrier frequency was positioned on the HDO resonance. Quadrature detection in ω_1 and ω_2 was achieved using TPPI. (B) 2D stripes along the ω_2 - ^{13}C dimension of M25 ϵ , A11 β , V53 γ 2, and L10 δ 2. (C) The local proximity of those residues is illustrated using the crystal structure (PDB: 1U06).²⁴

more complicated labeling schemes, which rely on selectively labeled precursors, such as $[1,3]\text{-}^{13}\text{C}$ or $[2]\text{-}^{13}\text{C}$ glycerol,^{2,26,27} or which rely on dilution of exchangeable protons.¹²

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Supporting Information Available: Sample preparation; pulse scheme; quantification of protonation. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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