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Communication

Amino-acid selective experiments on uniformly ^{13}C and ^{15}N labeled proteins by MAS NMR: Filtering of lysines and arginines

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Abstract

Amino-acid selective magic-angle spinning (MAS) NMR experiments can aid the assignment of ambiguous cross-peaks in crowded spectra of solid proteins. In particular for larger proteins, data analysis can be hindered by severe resonance overlap. In such cases, filtering techniques may provide a good alternative to site-specific spin-labeling to obtain unambiguous assignments that can serve as starting points in the assignment procedure. In this paper we present a simple pulse sequence that allows selective excitation of arginine and lysine residues. To achieve this, we make use of a combination of specific cross-polarization for selective excitation [M. Baldus, A.T. Petkova, J. Herzfeld, R.G. Griffin, Cross polarization in the tilted frame: assignment and spectral simplification in heteronuclear spin systems, Mol. Phys. 95 (1998) 1197–1207.] and spin diffusion for transfer along the amino-acid side-chain. The selectivity of the filter is demonstrated with the excitation of lysine and arginine side-chain resonances in a uniformly ^{13}C and ^{15}N labeled protein preparation of the α-spectrin SH3 domain. It is shown that the filter can be applied as a building block in a ^{13}C–^{13}C lysine-only correlation experiment.

Keywords: Solid-state NMR; Spectral editing; Protein; Lysine filter

1. Introduction

Structure investigations with NMR rely on the successful assignment of chemical shifts. In solid-state magic-angle spinning (MAS) NMR, the resolution in spectra obtained from structurally homogeneous preparations of small proteins (up to ~15 kDa) is often high enough to achieve complete resonance assignments [1–4]. For larger proteins, however, spectra can quickly become congested due to the large number of overlapping resonances. In order to accomplish a full resonance assignment, the number of observed resonances needs to be reduced to alleviate spectral crowding.

In this communication, we focus on the selective excitation of lysine and arginine signals. Filtering of these residues can be helpful during the chemical shift assignment, since they strongly overlap with other residues (in particular with leucines). Selection of single types of amino acids can be done either by use of site-specific isotope labeling or by spectral editing. In our opinion, labeling of single amino acids can be laborious and expensive, and the information that can be collected from such preparation is often limited. In a more efficient approach, it was shown that separation of lysine from leucine signals can also be achieved by 'unlabeling' of the lysine signals, as done for example in the TEASE experiment, proposed by Hong and Jakes [5], or by reverse labeling of lysines, originally proposed by Bax and co-workers [6] and recently used in MAS NMR by Heise et al. [7].

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Here we suggest a different strategy and demonstrate that selection of lysine and arginine side-chains can be achieved from fully ^{13}C-enriched proteins by application of specific cross-polarization (sCP). Specific CP is commonly used as a building block in triple-resonance pulse sequences to selectively transfer magnetization from the amide nitrogen to the C^{α} or to the CO [8]. Specific CP is achieved by using low-power RF spin-lock pulses on the ^{15}N and ^{13}C channels which results in the excitation of narrow spectral regions for which the Hartmann–Hahn matching condition is fulfilled [8]. The criterion for a successful application of sCP is that the selected spin types resonate sufficiently far away from other spins. In the present communication we use sCP for selective magnetization transfer from N^{ζ} to C^{ε} in lysine and from N^{ε} to C^{δ} in arginine residues. The use of the lysine filter is demonstrated with a ^{13}C–^{13}C lysine sub-spectrum recorded from a fully ^{13}C, ^{15}N-enriched protein sample of α-spectrin SH3 domain.

2. Materials and methods

The preparation of uniformly ^{13}C, ^{15}N-labeled (U–^{13}C, ^{15}N) α-spectrin SH3 domain is described elsewhere [9]. Samples typically containing ~1.4 μmol (about 10 mg) of SH3 domain were used for the MAS NMR correlation experiments.

The ^{13}C–^{15}N and ^{13}C–^{13}C MAS NMR dipolar correlation spectra on (U–^{13}C, ^{15}N) α-spectrin SH3 domain were recorded at a MAS frequency of $\omega_R/2\pi = 8.0$ kHz. The experiments were performed at 280 K at a field of 9.4 T, using a wide-bore Avance-400 spectrometer equipped with a 4 mm triple-resonance CP/MAS probe (Bruker, Karlsruhe, Germany). A TPPM sequence with the ^{1}H RF power set to ~75 kHz was used for heteronuclear decoupling during all evolution and acquisition periods [10]. We found that TPPM decoupling during ^{15}N–^{13}C transfer performed slightly better (~10% more signal intensity) than continuous-wave decoupling. In general, however, we suggest testing both decoupling methods for best performance.

The amino-acid selective experiments started with a ramped CP (75–100%) from ^{1}H to ^{15}N. Selective ^{15}N–^{13}C heteronuclear exchange was achieved by a specific CP step [8]. For the sCP transfer it is important that the effective fields of the RF irradiation (ω_C, ω_N) and chemical shift offsets (Ω_C, Ω_N) of the ^{13}C and ^{15}N spin fulfil the following modified Hartmann–Hahn matching condition [11,12], with $n = +1, +2$

$$\sqrt{\omega_C^2 + \Omega_C^2} - \sqrt{\omega_N^2 + \Omega_N^2} = \pm n\omega_R$$

In the case of the lysine-selective experiments, the sCP was optimized for transfer between $^{15}N^{\zeta}$ and $^{13}C^{\varepsilon}$, while for the arginine-selective experiments, it was optimized for $^{15}N^{\varepsilon}$ to $^{13}C^{\delta}$ transfer. For both cases, a shallow 100–90% ramp was applied to the carbon spin-lock pulse. For lysine, RF powers of typically ~10 and ~20 kHz were used for the ^{15}N and ^{13}C spin-lock pulses, respectively, during a 4 ms sCP. The mean ^{13}C RF strength was lower due to the ramp; Hartmann–Hahn matching was fine-tuned by applying a small resonance offset to the ^{13}C carrier relative to the C^{ε} frequency, whilst the ^{15}N were irradiated on resonance with the N^{ζ}. For arginine, lower RF powers of typically ~5 and ~3 kHz for ^{15}N and ^{13}C, respectively, were used, during a 3 ms specific CP. Hartmann–Hahn matching was tuned by setting the offset of the ^{13}C RF irradiation about 10 kHz off-resonance relative to the C^{δ} signal, whilst the N^{ε} was spin-locked on resonance.

For the 2D ^{13}C–^{13}C lysine-filtered experiment, a 150 ms proton-driven spin-diffusion (PDSD) [13] mixing time was introduced after specific CP, to transfer ^{13}C magnetization over the side chain (Fig. 1). Following the preparation period, the ^{13}C spins were allowed to evolve during t_1. After the evolution period, the ^{13}C spins were correlated using a second short PDSD mixing time of 25 ms followed by data acquisition. The 2D ^{13}C–^{13}C lysine-selective homonuclear correlation spectrum of the SH3 domain was recorded with 96 t_1-increments and 1600 scans. The total experimental time amounted to four days. In the indirect dimension, a spectral width of 130 ppm was chosen so as to reduce experimental time. As a result, carbonyl resonances were deliberately folded back from 175 ppm into the spectral window around 80 ppm.

All spectra were processed using the XWINNMR software (Bruker, Karlsruhe Germany) version 3.5 and further analyzed using the software Sparky, version 3.110 (T.D. Goddard and D.G. Kneller, University of California, San Francisco).

3. Results and discussion

In the sequence of the α-spectrin SH3 domain from chicken brain, eight of the 62 residues are lysines, making up more than 10%. Although lysines have a characteristic C^{ε} signal around 42 ppm, both C^{ε} and C^{δ} show little dispersion; in addition, the lysine C^{ε}–C^{δ} and C^{ε}–C^{γ} correlations overlap with other resonances, in particular with the C^{β}–$C^{\delta 1}$ and C^{β}–$C^{\delta 2}$ signals from leucines. The shifts of the α-, β- and γ-carbons are more dispersed but their cross peaks are obscured by overlap with other aliphatic resonances. Hence, lysines are difficult to assign.

The shift of 42 ppm for the C^{ε}, and that of 35 ppm for the triply protonated N^{ζ} (Biological Magnetic Resonance Data Bank [14]), renders the lysine N^{ζ}–C^{ε} pair distinct from any other N–C combination. This allows one to select them via narrow banded CP. Fig. 2A shows a 2D N^{ζ}–C^{ε} spectrum. The pulse sequence used for generating the data in Fig. 2 was similar to that for standard 2D NCA or NCO experiments [12], except that the matching conditions were optimized for the amino-acid side-chain of lysine. The spectrum in Fig. 2A shows the expected correlation at 42 ppm (^{13}C) and 35 ppm (^{15}N) in F2 and F1, respectively. This cross peak is the only observed correlation in the spectrum,

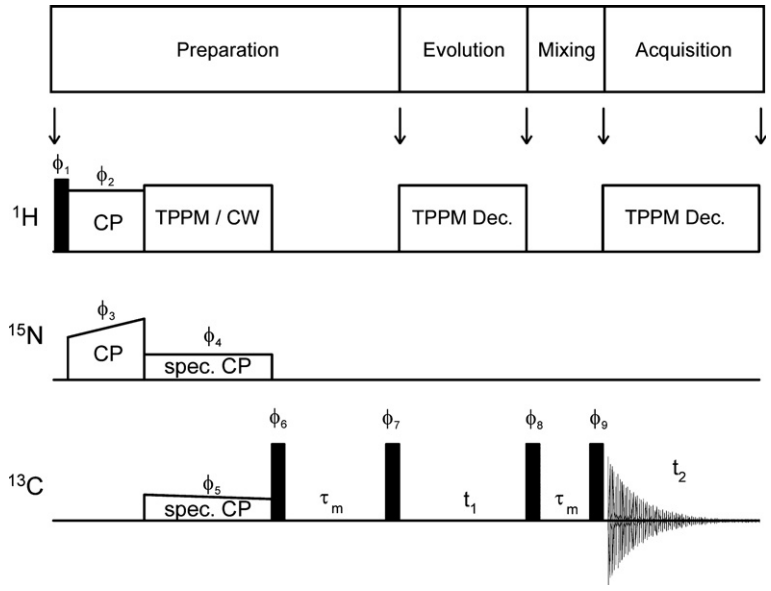

Fig. 1. Pulse sequence used for arginine- and lysine-specific ^{13}C–^{13}C spectroscopy. Specific cross-polarization (sCP) is used to select ^{15}N–^{13}C pairs upon their unique chemical shift. Following the sCP, the magnetization is transferred along the side-chain by a long proton-driven spin-diffusion (PDSD) mixing time (typically ~150 ms). After the preparation period, the carbon spins are allowed to evolve during t_1. The evolution is followed by a second (short) PDSD transfer time to correlate side-chain carbons, followed by acquisition under TPPM decoupling. For the first (^{1}H–^{15}N) CP, a 75–100% ramp is used on the ^{15}N channel. For the sCP, a 100–90% ramp is applied on the ^{13}C channel. In our studies, we found that TPPM decoupling performed slightly better during sCP than CW decoupling. This does not necessarily have to be the case in general, and both methods should be tested for optimal transfer efficiency. Pulses are cycled according to: $\phi_1 = 13$, $\phi_2 = 0$, $\phi_3 = 0022$, $\phi_4 = 02022020$ and $\phi_5 = 00002222$, $\phi_6 = 13$ and $\phi_7 = 3$, $\phi_8 = 1111111133333333$, and $\phi_9 = 1$, receiver phase = 0220022020022002; with 0123 = *xy*-*x*-*y*.

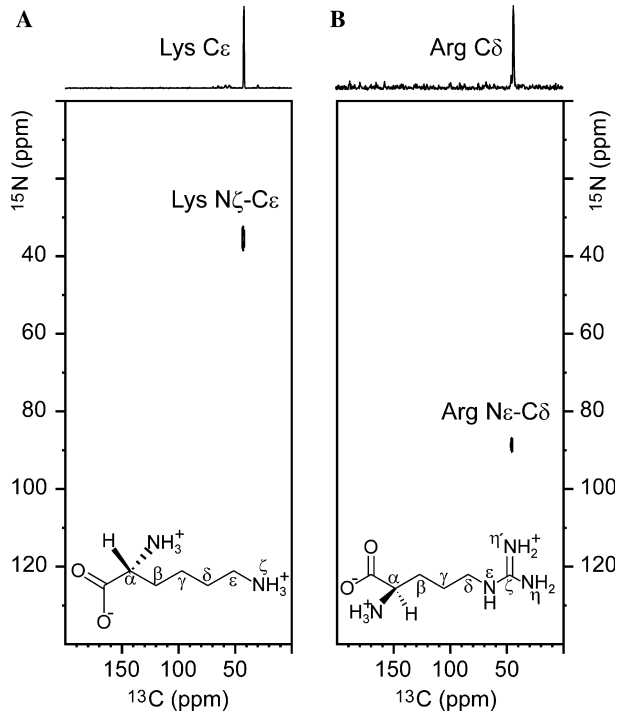

Fig. 2. Two-dimensional lysine N^{ζ}–C^{ε}-filtered (A) and arginine N^{ε}–C^{δ}-filtered spectrum (B), recorded from α-spectrum SH3 domain. The spectra were obtained at a field of 9.4 T and at a spinning frequency of 8 kHz. For the lysine N^{ζ}–C^{ε} correlation experiment, 64 scans per slice were used; for the arginine N^{ε}–C^{δ} experiment 32 scans per slice were used. The spectra are plotted over the full carbon and nitrogen spectral ranges to demonstrate the selectivity of the transfer.

which proves the selectivity of the sCP transfer. Moreover, the complete lysine response appears as a single correlation, demonstrating the poor dispersion of the C^{ε} signals. The 1D spectrum at the top of Fig. 2 was recorded using the same pulse sequence, but without the nitrogen evolution period. The 1D version was indispensable for a quick optimization of the spin-lock power levels and resonance offset for maximal transfer efficiency. We have estimated the efficiency of the selective transfer by comparing the lysine C^{ε} signal intensity at 42 ppm in the 1D filtered experiment with a standard CP/MAS experiment. In this way, we estimate a transfer efficiency of approximately 20% for the lysine filtered experiment.

Arginine side-chains can be selected in a similar manner. Baldus et al. previously have shown specific transfer from N^{η}/N^{ε} to C^{ζ} in arginine side-chains [8]. To continue magnetization transfer along the arginine side-chain from C^{ζ} to C^{δ}, however, one needs to cross a second nitrogen. Therefore, we have chosen to transfer directly from N^{ε} to C^{δ}, by adjusting the sCP to the appropriate condition. In contrast to lysines, for which relatively broad spectral bandwidths could be used due to the upfield shift of the N^{ζ}, the N^{ε} of arginines resonate further downfield at around 87 ppm. For a B_0 field of 9.4 T, the N^{ε} are separated by less than 1 kHz from the amide region that runs from 105 to 130 ppm. Furthermore, the C^{δ} of arginine resonates at around 43 ppm, close to the C^{α}-region. Hence the Hartmann–Hahn matching condition for the arginines should be kept within a narrow region. For these reasons, low

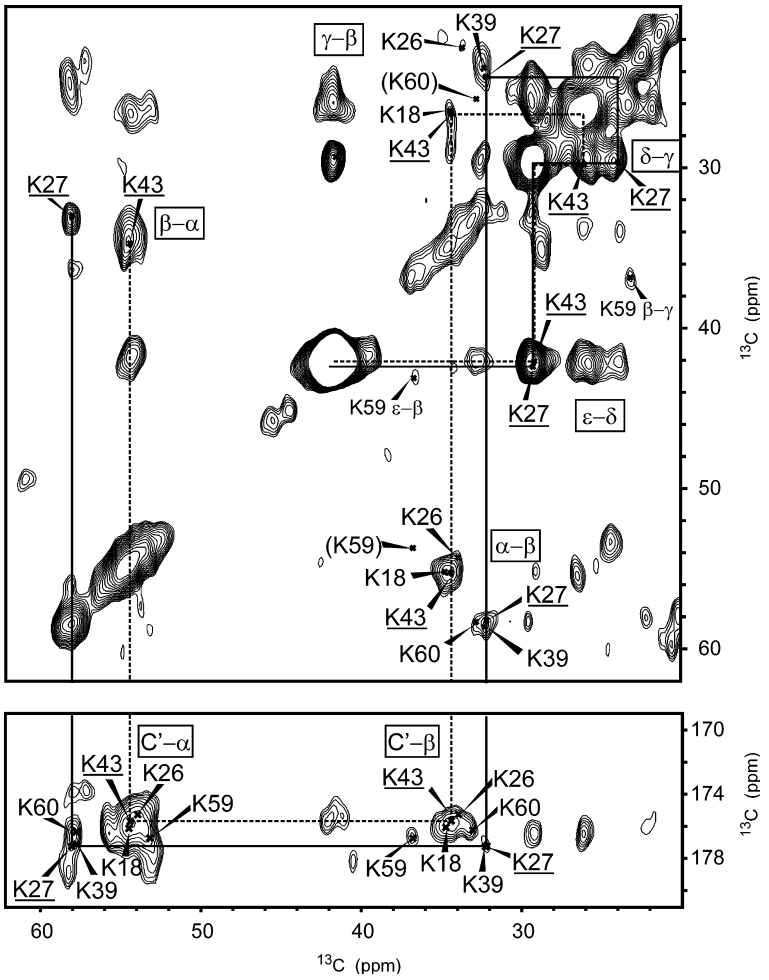

Fig. 3. Lysine-filtered spectrum of the α-spectrin SH3 domain sample, obtained with the pulse sequence shown in Fig. 1. The data were recorded at a field of 9.4 T, using a spinning frequency of 8 kHz. The proton-driven spin-diffusion (PDSD) transfer time in the preparation period was set to 150 ms, the PDSD transfer during the mixing unit was set to 25 ms. For the experiment 96 serials were collected, each with 1600 scans. To reduce experimental time the carbonyl region was folded into the spectral region at around 80 ppm. The C^{α}–C^{β} cross peak of K59 and the C^{γ}–C^{δ} cross peak of K60 appear at a lower contour level and are indicated within brackets.

power for arginine transfer had to be used to maintain selectivity, in particular to prevent excitation of glycine signals. In Fig. 2B, the 2D N^{ε}–C^{δ} experiment is shown, with the 1D version shown at the top. The strongest magnetization transfer was indeed observed for the C^{δ}'s of arginines.

The filters can be used to record arginine- or lysine-specific sub-spectra. This is illustrated in Fig. 3, that shows a 2D $(N^{\zeta})C^{\varepsilon}$–$C^{x}$ ^{13}C–^{13}C-correlation experiment for lysine residues (where C^{x} stands for 'any' carbon). The pulse program used for this spectrum is depicted in Fig. 1. It uses the specific CP transfer step from N^{ζ} to C^{ε} as a building block in the preparation period. After the selective transfer, a double PDSD step is introduced. An initial 150 ms PDSD step transfers magnetization from C^{ε} through the lysine side-chain. During the evolution period, lysine side-chain resonances evolve and are subsequently correlated using a second, short, 25 ms PDSD step. It is also possible to run the experiment as ^{15}N/^{13}C-correlation with evolution on the nitrogen and without a second PDSD-step. This is especially useful if the number of filtered residues is low, as it is the case for the two arginine residues in the SH3 domain (data not shown).

In the 2D ^{13}C–^{13}C correlation spectrum shown in Fig. 3, all expected intra-residual correlations, including the C^{α}–C^{β} cross peaks, are observed for seven of the eight lysine residues (K6 is on the flexible N-terminus) in the α-spectrin SH3 domain. The assignment of the lysines is further simplified by the observation of C′–C^{α} and C′–C^{β} cross peaks. The C′, C^{α}, and C^{β} have the highest dispersion for the different lysines, and help to differentiate between the different networks. As an example, for two lysines, K27 and K43, correlation walks are traced out in Fig. 3, using solid and dashed lines for K27 and K43, respectively. Other lysine patterns can be found in a similar manner and the assignments obtained here are consistent with the shifts published earlier [12]. For K59 and K60, complete networks are found at a lower contour level, which may indicate a higher degree of mobility for the C-terminal loop.

The double PDSD transfer is sufficiently efficient to transfer magnetization along the lysine side-chain. The polariza-

tion is distributed from a single ^{13}C spin, in this case the C^{ε}, over the entire lysine side-chain. Obviously this can put some limitations on the sensitivity of the experiment, which decreases with each additional step of transfer. However, the experimental time required to record the data shown in Fig. 3 amounted to four days, which is not excessively long and is compensated for by reducing ambiguity in the assignment process. Furthermore, an alternative approach would involve the preparation of proteins with isotope labeling of a specific residue, which is costly and time-consuming.

4. Conclusions

It has been shown that resonances of lysine and arginine residues can be filtered by adjusting specific CP for transfer between side-chain nitrogens and adjacent carbon spins. For lysine, it is demonstrated that 2D ^{13}C–^{13}C dipolar correlation experiments can be recorded by combining the specific CP with ^{13}C–^{13}C proton-driven spin-diffusion. Although lysine residues have long side-chains with characteristic C^{ε} signals around 42 ppm, the dispersion of the C^{ε} and C^{δ} is generally not high enough to be used for identification of different lysine correlation networks. Hence, it is difficult to assign lysine side-chains unambiguously without correlating the C^{ε} and C^{δ} to the C^{α}, C^{β}, and C' spins which show greater dispersion. For membrane-integrated proteins this technique may be helpful, since one does not need to resort to selective ^{13}C labeling of specific amino acids.

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