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Communication

^{1}H detected ^{1}H,^{15}N correlation spectroscopy in rotating solids

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Abstract

We describe new correlation experiments suitable for determining long-range ^{1}H–^{1}H distances in ^{2}H,^{15}N-labeled peptides and proteins. The approach uses perdeuteration together with back substitution of exchangeable protons during sample preparation as a means of attenuating the strong ^{1}H–^{1}H dipolar couplings that broaden ^{1}H magic angle spinning (MAS) spectra of solids. In the approach described here, we retain 100% of the ^{1}H sensitivity by labeling and detecting *all* exchangeable sites. This is in contrast to homonuclear multiple pulse decoupling sequences that are applied during detection and that compromise sensitivity because of the requirement of sampling between pulses. As a result ^{1}H detection provides a gain in sensitivity of >5 compared to the ^{15}N detected version of the experiment (at a MAS frequency of 13.5 kHz). The pulse schemes make use of the favorable dispersion of the amide ^{15}Ns resonances in the protein backbone. The experiments are demonstrated on a sample of the uniformly ^{2}H,^{15}N-labeled dipeptide N-Ac-Val-Leu-OH and are analogous to the solution-state suite of HSQC-NOESY experiments. In this compound the ^{1}H amide linewidths at 750 MHz vary from ~0.67 ppm at $\omega_r/2\pi \sim 5$ kHz to ~0.20 ppm at $\omega_r/2\pi \sim 30$ kHz, indicating that useful resolution is available in the ^{1}H spectrum via this approach. Since the experiments circumvent the problem of dipolar truncation in the ^{1}H–^{1}H spin system, they should make it possible to measure long-range distances in a uniformly labeled environment. Thus, we expect the experiments to be useful in constraining the global fold of a protein.
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1. Introduction

In the past few years, interest in the application of high resolution, solid-state magic angle spinning (MAS) NMR experiments for structure determination of uniformly labeled peptides [1,2] and proteins [3,4] has grown rapidly, and the initial structures obtained using these techniques are beginning to appear [5,6]. However, applications of the techniques are and will be limited primarily because of the relatively low sensitivity of the experiment. This arises because of the necessity of detecting the spectra via low-γ nuclei such as ^{13}C, ^{15}N, etc. Efforts to increase the sensitivity of MAS experiments with the incorporation of dynamic nuclear polarization into the experiment appear promising [7]. However, any improvement in sensitivity is important since it will facilitate the spectroscopy and widen its applicability.

In principle, ^{1}Hs are ideally suited for detection of low-γ nuclei, an approach that was exploited in early solid-state experiments [8–12] for detection of low-γ nuclei. Some years later, this idea was introduced to solution-state NMR by Bodenhausen and Ruben [13]. Today, the liquid-state version of the indirect detection experiment, known as HSQC, is used routinely in almost all structural studies of biological molecules in solution. The impetus for using indirect detection is the factor of $(\gamma_I/\gamma_S)^{3/2}$ gain in sensitivity over direct observation. For $I = {}^{1}$H, the gain in sensitivity is a factor of 31 in signal intensities for ^{15}N and $N * 8$ for ^{13}C, where N is the number of attached ^{1}Hs. It is worth noting that the sensitivity available via indirect detection is the crucial feature that enables structural studies of proteins and nucleic acids in solution.

Currently, it is customary to perform MAS experiments on solids using low-γ detection, because of the favorable dispersion of ^{13}C or ^{15}N resonances. The relatively weak homonuclear dipolar couplings among

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low-γ nuclei and the large spectral dispersion result in an inhomogeneous dipolar Hamiltonian that yields narrow lines during MAS. In contrast, ^{1}H shifts are not sufficiently large to truncate the flip-flop terms of the dipolar Hamiltonian and the ^{1}H spectrum is homogeneously broadened. Thus, even MAS spinning frequencies of $\sim$35 kHz are not sufficient to average ^{1}H–^{1}H dipolar interactions. For this reason experiments involving ^{1}Hs have customarily employed multiple pulse sequences like MREV to narrow the ^{1}H spectrum. Since the magnetization is sampled between pulses in these experiments, it is necessary to open the audio filters of the spectrometer to avoid overload, lowering the signal-to-noise. In the approach described here, we retain 100% of the sensitivity by labeling and detecting all exchangeable sites.

A second problem encountered in MAS structural studies involves the attenuation of weak dipolar couplings by much stronger couplings—an effect referred to as *dipolar truncation* [14]. This effect was initially recognized in uniformly ^{13}C-labeled systems where directly bonded ^{13}C–^{13}C couplings of $\sim$2 kHz prevent the measurement of structurally important ^{13}C–^{13}C distances ($\sim$4–6 Å, $\sim$50–100 Hz) which are crucial for the determination of the global fold of a protein. Recently, techniques have been introduced which are suitable for structure determination in uniformly labeled peptides or proteins [2,15–17], but a method to measure ^{1}H–^{1}H distances as in solution-state NOESY experiments [18,19] is not generally available.

The approach described in this paper addresses the problem of sensitivity and *dipolar truncation* (and thus measurement of structurally significant distances in a uniformly labeled environment) by utilizing perdeuteration together with ^{15}N labeling. Currently, deuteration is used frequently in solution-state NMR [20–23] and poses few difficulties in sample preparation. Since the ^{1}Hs in these samples are magnetically dilute, the Hamiltonian behaves inhomogeneously and the spectrum narrows with MAS. Further, because of the magnetic dilution, it is in principle possible to measure ^{1}H–^{1}H distances in the range of 5–10 Å. Thus, it is possible that the approach could be useful for constraining the global fold of a protein. The experiments are demonstrated on a sample of a uniformly ^{2}H,^{15}N-labeled dipeptide, N-Ac-VL.

2. Results

Recently, we introduced an experiment to determine the distance between two H^N protons in a perdeuterated peptide that relies on ^{15}N detection [17]. In the peptide, exchangeable H^N sites were back substituted by ^{1}Hs in the process of sample preparation. This allows 100% sensitivity compared to other spin dilution techniques [24,25]. However, the experiment suffers from several drawbacks. First, the experiment is relatively insensitive due to ^{15}N detection. As is discussed below, a factor of $>$5.0 and $>$9.0 can be regained by making use of ^{1}H detection, adjusting the MAS frequency to 13.5 and 33.3 kHz, respectively. Second, the experiment relies on the dispersion of the ^{1}H resonances in the indirect dimension, and thus has intrinsically low resolution. Last, the evolution of double quantum coherences in the indirect dimension leads to an approximate doubling of the linewidth in this dimension. The approaches described in this communication circumvent these problems by utilizing ^{1}H detection, and making use of the more favorable dispersion of the resonances in the ^{15}N dimension. In contrast to other applications that rely on ^{1}H detection for sensitivity improvement [26–30], the experiments presented here are performed at moderate spinning frequencies of about 13 kHz that are suitable for applications where spinning at very high rotation frequencies is problematic.

Fig. 1a illustrates the first pulse sequence that we employ for the determination of long-range correlations in a perdeuterated peptide or protein. The experiment begins with a period of ^{15}N chemical shift evolution, that is followed by POST-C7-DQF{^{1}H,^{1}H} mixing, and ends with detection on ^{1}H. The solution-state analogue of this experiment would be a 2D-HSQC-NOESY [31,32] with chemical shift evolution in the ^{15}N indirect dimension. Instead of choosing a mixing period where cross-relaxation can occur via the nuclear Overhauser effect, a dipolar recoupling sequence is employed to actively recouple the ^{1}H–^{1}H spins. In particular, the POST-C7 element [33] was chosen for DQF{^{1}H,^{1}H} mixing because of its favorable RF bandwidth and lack of sensitivity to frequency offsets. In contrast to spin diffusion, a defined recoupling sequence permits the quantitative determination of distances as has been shown previously [17]. A quantitative interpretation of cross-peak build-up rates in the solid-state is not easily possible using a spin diffusion mixing scheme.

In the current implementation of the experiment, an ^{1}H RF field of 92.592 kHz was employed for the Rf pulses in the mixing sequence, corresponding to a 2.7 μs 90° pulse length, while the MAS frequency was adjusted to 13.227 kHz. The corresponding spectrum (using a mixing time of 2 rotor periods, or one complete POST-C7 cycle) is shown in Fig. 2. The cross-peak $[\omega_1, \omega_2] = [^{15}\text{N(Val)}, \text{OH(Leu)}] = [123$ ppm, 13 ppm] originates from excitation of double quantum coherences between Val-H^N and Leu-OH. All magnetization that originates from ^{1}Hs not bound to ^{15}N is suppressed by phase cycling the first CP transfer step (ϕ_2). The CP transfer time was adjusted to 150.0 μs in order to restrict heteronuclear magnetization transfer between directly bonded ^{1}Hs and ^{15}Ns. A double quantum filtered phase cycle of the first POST-C7 element (ϕ_3) selects for pairs

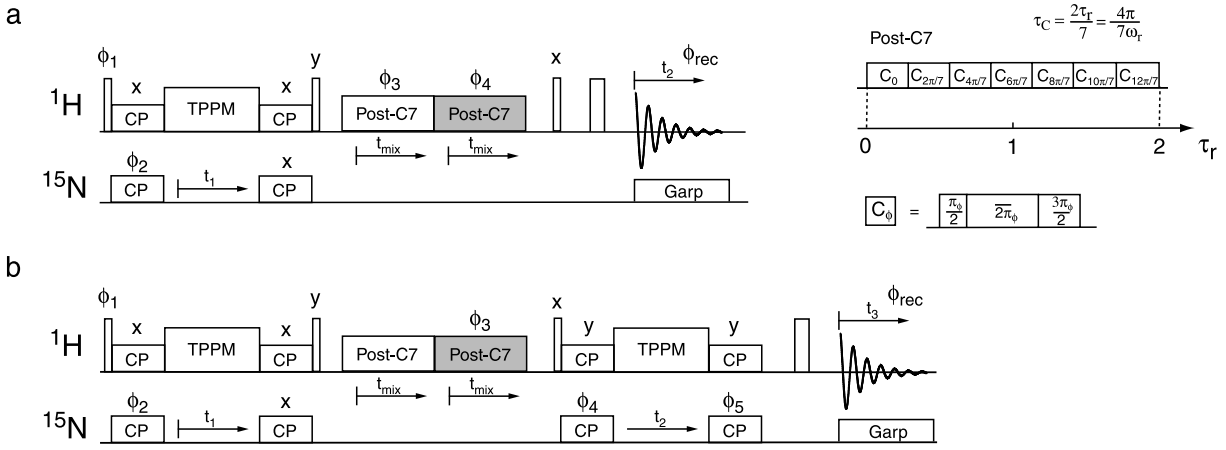

Fig. 1. The pulse experiments for ^{1}H detected ^{1}H,^{15}N correlation experiments for measurement of long-range ^{1}H–^{1}H interactions in the solid-state. In both experiments, POST-C7 [33] is used for DQ filtration. The details of the recoupling sequence are indicated on the right of the figure. In the experiments, the MAS frequency was adjusted to $\omega_r/2\pi = 13.227$ kHz. The mixing time was constant at two rotor periods of mixing ($t_{mix} = 0.151$ ms). The ^{1}H 90° pulse length in the POST-C7 element was set to 2.7 μs, to accommodate seven C-elements in two rotor periods. The recycle delay between each transient was set to 5.0 s. TPPM decoupling was used in the indirect evolution period, with a ^{1}H RF field of about 92.6 kHz. The CP time was set to 0.15 ms. (a) 2D ^{15}N-DQF{^{1}H,^{1}H}–^{1}H correlation. During ^{15}N evolution, 32 increments were recorded while t_1 was incremented in steps of 151.2 μs per experiment yielding a spectral width of 6.614 kHz. 64 scans were accumulated for each FID, yielding a total experimental time for the 2D experiment of 2.8 h. The phase cycle was: $\phi_1 = 16(+y), 16(-y)$; $\phi_2 = 32(+x), 32(-x)$; $\phi_3 = 4(+x), 4(+y), 4(-x), 4(-y)$; $\phi_4 = (+x,+y,-x,-y)$; $\phi^{rec} = 2(+x,-x,+x,-x,-x,+x,-x,+), 4(-x,+x,-x,+x,+x,-x,+x,-x), 2(+x,-x,+x,-x,-x,+x,-x,+x)$. (b) 3D ^{15}N-DQF{^{1}H,^{1}H}–^{15}N,^{1}H correlation. For the 3D experiment, 10 times 48 experiments were recorded in t_2 and t_1, using an increment of three and two rotor periods, respectively. For each FID, 32 scans were accumulated. The total experimental time was therefore 21 h. The phase cycle was: $\phi_1 = 16(+y), 16(-y)$; $\phi_2 = 32(+x), 32(-x)$; $\phi_3 = (+x,+y,-x,-y)$; $\phi_4 = 4(+x), 4(-x)$; $\phi_5 = 8(+x), 8(-x)$; $\phi^{rec} = 2(+x,-x,+x,-x,-x,+x,-x,+x), 4(-x,+x,-x,+x,+x,-x,+x,-x), 2(+x,-x,+x,-x,-x,+x,-x,+x)$.

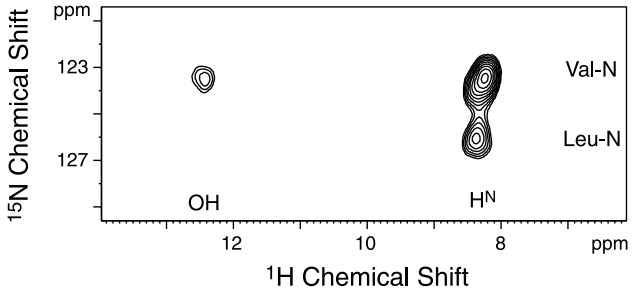

Fig. 2. Experimental spectra for U-[^{2}H,^{15}N]N-Ac-Val-Leu-OH obtained using the 2D ^{15}N-DQF{^{1}H,^{1}H} correlation experiment depicted in (a). The cross-peak $[\omega_1, \omega_2] = [^{15}\text{N(Val)}, \text{OH(Leu)}]$ corresponds to a ^{1}H–^{1}H distance of 2.78 Å in the crystal structure [36]. N, H^N cross-peaks originating from H^N, H^N correlations are not resolved in this experiment. The distance can be quantified by incrementing the mixing time t_{mix} and fitting the intensities of the cross-peaks assuming a γ-encoded transfer efficiency in the double quantum filtering elements [17].

of ^{1}Hs that are dipolar coupled. Cross peak intensity build-up curves are obtained by incrementing the mixing time, t_{mix}, and yield the same oscillatory behavior as we have observed previously [17] (data not shown).

For most applications the resolution in this experiment is not sufficient to resolve correlations between two different H^N protons. Therefore, we incorporated an additional ^{15}N chemical shift evolution period to improve the dispersion of the resonances of the remote amide. The sequence is shown in Fig. 1b. The corresponding 3D spectrum is illustrated in Fig. 3. As expected, no correlations between –OH and H^N protons are observed due to the ^{15}N filtering before and after the mixing step. There is, however, a strong cross-peak between H^N(Leu) and H^N(Val), corresponding to a 4.5 Å distance in the crystal structure. The symmetric cross-peak is missing due to the *truncation* [14] of a weak coupling (d[H^N(Val), H^N(Leu)] = 4.5 Å) in the presence of a very strong dipolar interaction (d[H^N(Val), OH(Leu)] = 2.8 Å). This is also consistent with previous observations [17]. In both experiments, GARP decoupling [34] was used during acquisition to decouple the ^{1}H–^{15}N heteronuclear scalar coupling. This yields an additional improvement in the linewidth as is illustrated in Fig. 4a.

Fig. 4b shows the 1D ^{1}H and ^{15}N spectra for U-^{2}H,^{15}N-labeled N-Ac-Val-Leu-OH recorded at a MAS frequency of 13.5 kHz. The signal-to-noise ratio for the Leu-H^N proton amounts to 300:1 and 560:1 for MAS frequencies of 13.5 and 33.3 kHz, respectively. After signal averaging for four scans, the FID was folded with a exponential function using 10 Hz of line broadening. The 1D ^{15}N spectrum displays a signal-to-noise ratio of 30:1 at a MAS frequency of 13.5 kHz after 16 scans. The cross-polarization step has an experimental transfer efficiency of ~50%, and thus, at a rotation frequency of only 13.5 kHz, we achieve a gain in sensitivity of 5.0 compared to the ^{15}N detected experiment. This is equivalent to a reduction in spectral acquisition time of

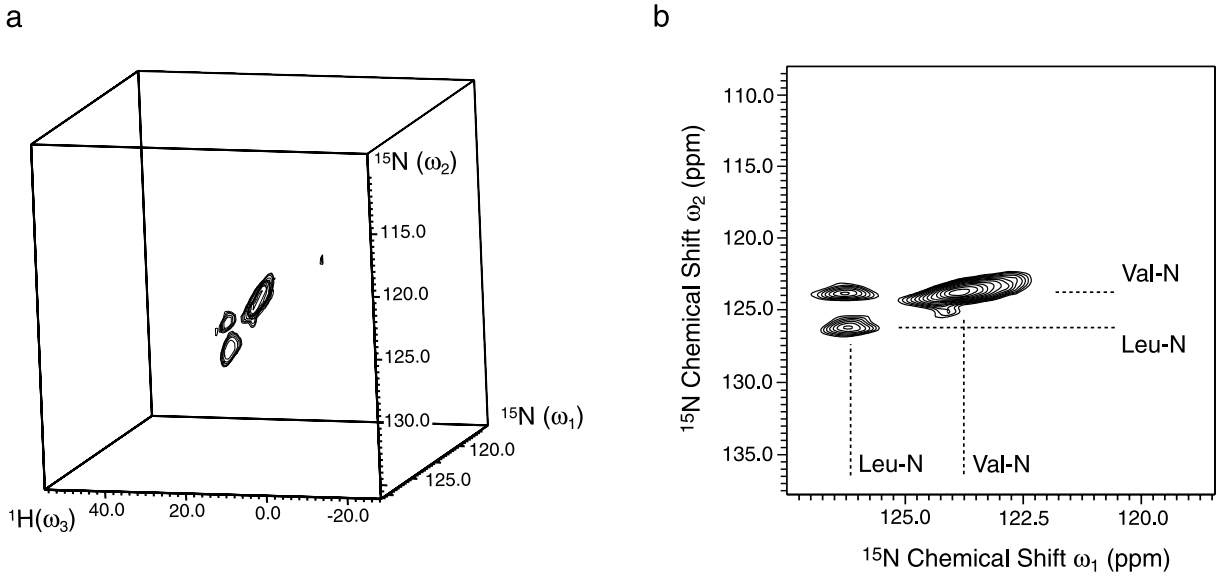

Fig. 3. Experimental spectra obtained with the 3D ^{15}N-DQF{^{1}H,^{1}H}–^{15}N,^{1}H correlation experiment from U-[^{2}H,^{15}N]N-Ac-Val-Leu-OH: (a) 3D cube representation of the experiment; (b) illustrates a 2D (ω_1, ω_2) slice taken at the chemical shift of the amide ^{1}Hs at $\omega_3 = 8.3$ ppm. The respective 2D ^{15}N,^{15}N correlation shows a strong correlation between $[\omega_1, \omega_2] = [^{15}\text{N(Val)}, ^{15}\text{N(Leu)}]$ corresponding to a 4.5 Å distance in the X-ray structure. The symmetric cross-peak $[\omega_1, \omega_2] = [^{15}\text{N(Leu)}, ^{15}\text{N(Val)}]$ is missing due to dipolar truncation effects.

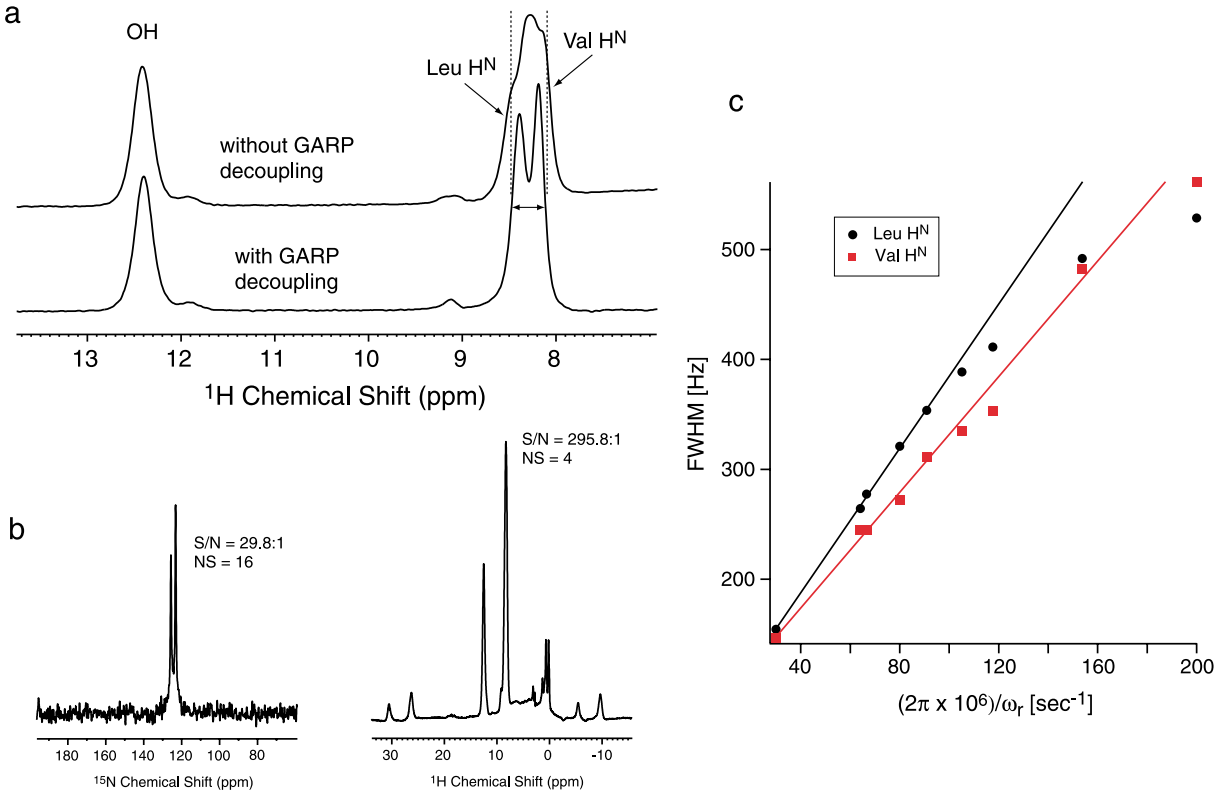

Fig. 4. (a) Amide region of the 1D ^{1}H spectrum of U-[^{2}H,^{15}N]N-Ac-Val-Leu-OH at $\omega_r/2\pi = 33.3$ kHz. Application of a GARP decoupling sequence (bottom spectrum) resolves the two amide ^{1}H signals, whereas it has no effect on the OH signal. The Rf field for GARP decoupling during acquisition was set to 2.5 kHz. The low power level was employed in order to avoid R^3 recoupling conditions. (b) Comparison of the signal-to-noise ratio (S/N) for the 1D ^{1}H and ^{15}N spectra in U-[^{2}H,^{15}N]N-Ac-Val-Leu-OH. The MAS frequency was adjusted to 13.5 kHz. The ^{1}H spectrum exhibits a S/N = 300:1, whereas the ^{15}N spectrum shows a reduced S/N = 30:1. Accounting for the different number of scans and a transfer efficiency of 50% in the CP step, a gain in sensitivity in the ^{1}H detected experiment of a factor of greater than 5.0 is observed. (c) Dependence of the ^{1}H linewidth on the MAS frequency. The ^{1}H linewidth was determined indirectly from ^{15}N detected ^{1}H,^{15}N HETCOR correlation spectra as described in [17]. Note that the linewidths vary from ~500 Hz at $\omega_r/2\pi = 5$ kHz to ~150 Hz at $\omega_r/2\pi = 33$ kHz.

a factor of 25. Fig. 4c shows the spinning frequency dependence of the ^{1}H linewidth for the two H^N protons in U-^{2}H,^{15}N-labeled N-Ac-Val-Leu-OH. The linewidths were extracted from ^{15}N detected ^{1}H,^{15}N HETCOR experiments [17], using no homonuclear decoupling in the indirect dimension. The H^N linewidth of Leu is systematically larger when compared to the H^N linewidth of Leu. However, both lines display a $1/\omega_r$ MAS dependence that is expected for dipolar coupled spins with vanishing isotropic chemical shift difference [35].

All experiments were performed on a Bruker 750 standard bore spectrometer equipped with a 2.5 mm double resonance probe. The ^{2}H,^{15}N-labeled amino acids valine and leucine were purchased from Cambridge Isotope Laboratories (Andover, MA). The dipeptide was synthesized using standard FMOC peptide chemistry.

3. Discussion

The two ^{15}N, ^{1}H correlation experiments described here both rely on ^{1}H detection, and we therefore observe a gain in sensitivity compared to the ^{15}N detected version of the experiment that is significant even at a moderate spinning frequency of 13.5 kHz. The sensitivity enhancement factor can be described more quantitatively by the expression given in [27]

$$\xi = \left(\frac{\gamma_H}{\gamma_N}\right)^{3/2}\left(\frac{W_N}{W_H}\right)^{1/2}\left(\frac{Q_H}{Q_N}\right)^{1/2}\left(\frac{T_1^{prot}}{T_1^{deut}}\right)^{1/2}\frac{A_H}{A_N}\cdot f, \quad (1)$$

where f is the efficiency of the polarization transfer between ^{1}H and ^{15}N, γ corresponds to the gyromagnetic ratio of the corresponding nucleus, W is the effective linewidth, and Q the quality factor of the RF coil for ^{15}N and ^{1}H, respectively. T_1^{prot} and T_1^{deut} correspond to the different spin–lattice relaxation times for a protonated and a deuterated sample. A describes geometric factors which might be different for ^{1}H and ^{15}N. Typical values for the linewidth in the systems studied here are 40 Hz for ^{15}N and 350 Hz for ^{1}H, respectively, at a MAS frequency of 13.5 kHz. Typical values for the quality factors of a solid-state NMR probe are $Q_H = 300$, $Q_N = 60$–80 (at 500 MHz) or $Q_H = 170$, $Q_N = 60$ (at 750 Hz), respectively. With these assumptions, one obtains a theoretical enhancement factor $\xi = 6.4$, which corresponds well with the experimentally observed value of $\xi = 5.0$. In current probe designs, the circuit is optimized for ^{13}C detection. In principle, higher enhancements can be obtained, after optimization of the circuit for ^{1}H detection.

In contrast to the ^{1}H–^{1}H–^{1}H/^{13}C experiment described by Emsley and co-workers [30], where a spin diffusion mixing period is employed to obtain ^{1}H–^{1}H distance information, we use a defined recoupling sequence to reintroduce the ^{1}H–^{1}H dipolar interaction. Spin diffusion transfer depends strongly on the details of the spatial arrangement of the ^{1}Hs around the proton under investigation. On the other hand, POST-C7, CMR7 or SPC-5 recoupling is independent of the chemical shift difference and the differential chemical shift anisotropy, and recoupling occurs in a defined manner.

Upon inspection of Fig. 4c, it can be seen that the ^{1}H linewidth scales approximately as $1/\omega_r$. Therefore, we expect improved resolution at higher spinning frequencies and an additional factor of ca. 2.0 in signal-to-noise can be obtained, once recoupling sequences are available that have improved Rf characteristics at high MAS rotation frequencies (>30 kHz). At these frequencies, heteronuclear scalar decoupling can yield a significant improvement for the resolution of the respective H^N resonance line (Fig. 4a).

4. Conclusion

In this communication, we have described two solid-state NMR experiments for structure determination of uniformly ^{2}H-labeled peptides and proteins. The experiments rely on spin dilution by deuteration and on ^{1}H–^{1}H dipolar recoupling using the POST-C7 multiple pulse sequence. Using this approach, a significant increase of the signal-to-noise ratio can be achieved, even at moderate spinning frequencies of about 10 kHz. This allows the determination of structurally significant distances in the range of 4–5 Å which are otherwise difficult to obtain. We expect this approach to be useful to constrain the tertiary fold of larger biomolecules.

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