Solid-State Protein NMR Spectroscopy

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Backbone Assignment of Fully Protonated Solid Proteins by ^{1}H Detection and Ultrafast Magic-Angle-Spinning NMR Spectroscopy**

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In the past decade solid-state magic-angle-spinning (MAS) NMR spectroscopy has emerged as a unique technique for obtaining information on the atomic-level structure and dynamics of complex biological macromolecules, which owing to their properties or size are accessible neither to X-ray crystallography nor to solution NMR spectroscopy. A small number of structures has been determined by solid-state NMR spectroscopy to date, ranging from microcrystalline samples to fibrils and membrane-associated systems.[1] Despite rapid acceptance in the biomolecular field, however, these determinations are far from being routine.

One of the main barriers to structural investigation is the complex but essential step of resonance assignment, which associates each signal in the spectrum to a particular nucleus in the protein. This assignment is typically achieved by a series of 3D experiments, correlating the resonance frequencies of nuclei belonging to the same (intra-residue connectivity) or to neighboring (sequential correlations) residues.[2] Solution-state structural studies of proteins accomplish this process through the detection of protons, which usually provide the best possible sensitivity owing to their high gyromagnetic ratio. In the solid state, however, the strong network of dipolar couplings connecting the different protons produces extremely broad ^{1}H NMR linewidths. The assignment process is then usually performed by direct detection of the ^{13}C nucleus, which provides significantly higher resolution despite the smaller gyromagnetic ratio but results in intrinsically lower sensitivity.

The use of perdeuterated proteins is a possible avenue to highly sensitive proton-detected solid-state NMR spectroscopy experiments by weakening the dipolar interactions between protons.[3] At moderate MAS rates, amide sites must be only partially reprotonated (typically using back-exchange levels of 10–30%) to accomplish acceptable dilution of the proton bath and yield well-resolved ^{1}H spectra.[4] The potential advantage of higher sensitivity is thus compromised to gain resolution, and thus the determination of internuclear distances becomes impractical, with few exceptions.[5] We have recently shown how this problem can be completely overcome by using 100% reprotonation of exchangeable sites, without loss of resolution, if perdeuteration is combined with ultrafast MAS (60 kHz) at high magnetic fields. Well-resolved "fingerprint" spectra may then be acquired rapidly, thereby enabling faster resonance assignment and the detection of a range of structurally important parameters.[6]

Complete deuteration of proteins, however, requires expression protocols that often affect the production yields and are not viable at all for some expression systems.[7] Moreover, the subsequent reprotonation of exchangeable sites in the interior of the protein depends on transient unfolding of the protein; this approach is thus not feasible for proteins that unfold irreversibly.

Clearly, protonated proteins are the ideal targets to improve sensitivity, provide a generally viable assignment strategy, and measure ^{1}H–^{1}H distances. Measuring ^{1}H-detected spectra of protonated proteins in the solid state has only been reported for one small model protein (GB1, 56 residues) in microcrystalline form.[8] In this pioneering work, Rienstra and co-workers used ^{1}H-detection schemes to obtain fast assignments of backbone amide and H^{α} protons with the prior knowledge of ^{15}N and ^{13}C backbone shifts.

Herein we show that the use of high magnetic fields and ultrafast MAS allows narrow ^{1}H linewidths to be achieved from significantly larger fully protonated proteins even in a noncrystalline precipitate, without any need for reduction of the proton background signals by deuteration. We demonstrate that this approach provides a route to the extensive, robust, and expeditious assignment of the backbone ^{1}H, ^{15}N,

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^{13}CA (C^{α}), and ^{13}CO (C') resonances based on ^{1}H detection of fully protonated medium-sized proteins.

Figure 1 shows the ^{1}H-detected dipolar HN correlation of two [^{13}C,^{15}N]-labeled and fully protonated protein domains from the *E. coli* DNA replisome;[9] the spectra were acquired at high magnetic field (800 MHz) under ultrafast MAS (60 kHz). The first sample is a microcrystalline form of the N-terminal proofreading exonuclease domain of the ε subunit (ε_{186}, 20 kDa) of DNA polymerase III,[10] and the second is a gel-like precipitate of the tetrameric single-stranded DNA-binding protein (SSB, 4 × 18 kDa).[11] These two samples were chosen to illustrate the applicability of ^{1}H detection to different classes of biological substrates, featuring different levels of local order. In particular, the gel-like sample is likely to be representative of a broad range of noncrystalline samples of protein and protein–protein complexes.

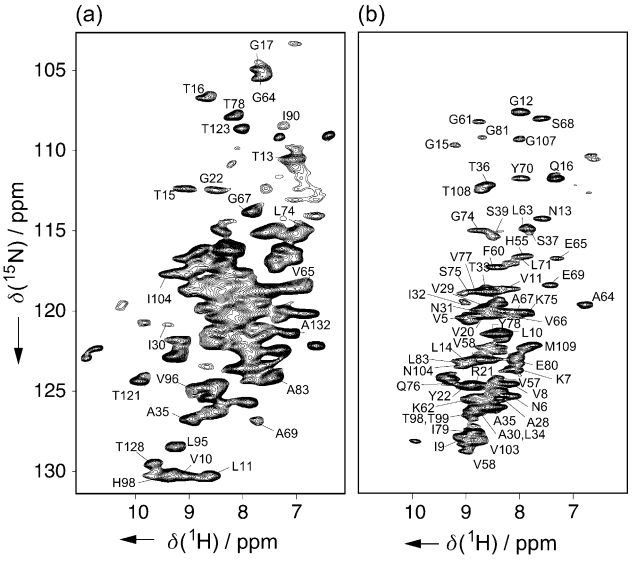

Figure 1. a, b) Proton-detected HN correlation spectra of a) [^{13}C,^{15}N]-ε_{186} from *E. coli* DNA polymerase III and b) [^{13}C,^{15}N]-SSB from *E. coli*, at an ^{1}H NMR frequency of 800 MHz and an MAS rate of 60 kHz. The assigned signals are labeled with the number and the one-letter code of the corresponding amino acid.

While the ^{1}H resonances have, as expected, significantly shorter coherence lifetimes (T_2') than in deuterated analogues (T_2' values of 1.5 versus 10 ms), the actual apparent linewidths are typically only about twofold larger (225 ± 75 Hz). Notably, both spectra display similar ^{1}H NMR linewidths, despite their different molecular weight and aggregation state.

Figure 2 illustrates in more detail the dependence of the ^{1}H NMR linewidths on the MAS rate and the B_0 field. These data indicate a substantial improvement in ^{1}H resolution and coherence lifetimes with increasing spinning rates; an increase of the spinning rate from 40 to 60 kHz results in a 40% reduction in linewidths (Figure 2a). This gain in resolution now makes much larger biological systems amenable to the approach outlined by Rienstra and co-workers.[8] Figure 2b,c and Figure S3 in the Supporting Information illustrate the additional improvement in resolution obtained

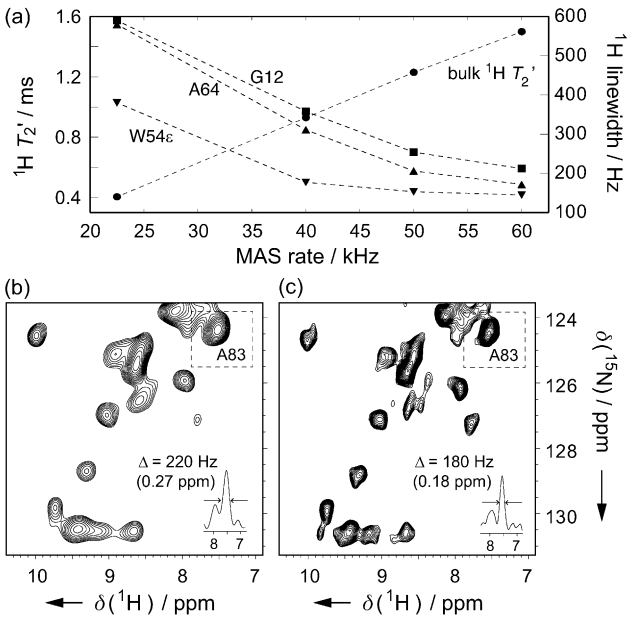

Figure 2. a) MAS-rate dependence of bulk coherence lifetimes (T_2' values, ●) and ^{1}H NMR linewidths for three signals (Gly12 H^N, ■; Ala64 H^N, ▲; and Trp54 H^{ε}, ▼) of [^{13}C,^{15}N]-SSB, at an ^{1}H NMR frequency of 800 MHz. b, c) Regions of proton-detected correlation spectra of [^{13}C,^{15}N]-ε_{186} recorded on 800 MHz (b) and 1 GHz (c) NMR spectrometers at an MAS rate of 60 kHz. The insets show cross-sections through the cross-peak of residue Ala83. Full spectra at different MAS rates and magnetic fields are reported in the Supporting Information.

by recording proton-detected experiments at increased magnetic field strength. Most notably, the ^{1}H resolution increases more than linearly with the field strength, thereby resulting in average ^{1}H NMR linewidths of 0.18 ppm (180 Hz) at 1 GHz. The combination of high MAS rates and high fields thus opens a new regime for the truncation of the ^{1}H dipolar interaction.

This level of resolution is however not sufficient to yield a fully resolved fingerprint spectrum in 2D. In the following, we show that all the protein resonances can be resolved when the ^{1}H–^{15}N correlation experiment is coupled to a third ^{13}C dimension, and that the larger ^{1}H linewidths do not render the 3D experiments unfeasible.

Figure 3a,b shows ^{15}N–^{13}CO and ^{15}N–^{13}CA projections of 3D (H)CONH and (H)CANH correlations of [^{13}C,^{15}N]-ε_{186} (projections from spectra recorded on [^{13}C,^{15}N]-SSB are reported in the Supporting Information). These spectra yield correlations where the amide ^{1}H and ^{15}N shifts of residue *i* are correlated to either the ^{13}CO of the preceding residue (*i*−1), or the ^{13}CA shifts of the same residue *i*. These 3D spectra combine favorable linewidths of ^{15}N and ^{13}C resonances in the indirect dimensions and sensitive ^{1}H detection, and can be acquired in remarkably short experimental times (17–34 h). 132 CANH and 133 CONH correlations were observed for ε_{186}, and 71 CANH and 66 CONH signals for SSB. In both samples, the detected signals represent more than 85% of residues located in the structured regions of the proteins. Conversely, no signals were observed

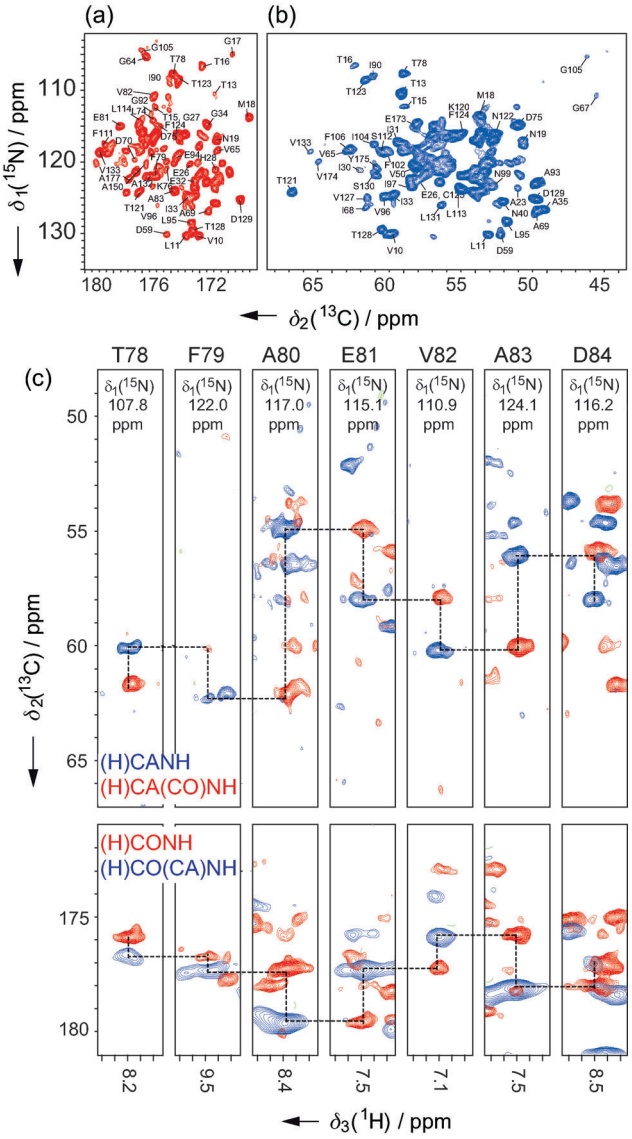

Figure 3. Proton-detected correlation spectra of [^{13}C,^{15}N]-ε_{186}, recorded on an 800 MHz NMR spectrometer at an MAS rate of 60 kHz. a, b) Assigned ^{13}C–^{15}N projections of 3D (H)CONH and (H)CANH spectra, respectively. c) Representative strips from (H)CANH and (H)CA(CO)NH (blue and red contours, respectively, in the upper panel), (H)CONH and (H)CO(CA)NH (red and blue contours, respectively, in the lower panel). Sequential correlations are marked by the dashed lines. Pulse sequences for the ^{1}H-detected correlations, as well as further experimental details, can be found in the Supporting Information.

for the intrinsically disordered C-terminal domain of SSB (residues 112–178),[11] or in loops with high *B* factors in the crystal structures (e.g. residues 1–8, 152–162 in ε_{186},[10] and 22–27, 40–49, 86–90 in SSB[11]).

Similar to the solution-state case, the resolving power of the ^{1}H dimension can be further exploited for sequential resonance assignment. Here, two additional 3D experiments were employed, which combine the HN dipolar correlation module with ^{13}C–^{13}C through-bond (scalar) transfers, providing complementary pairs of inter- and intra-residue CO/CANH correlations. These (H)CO(CA)NH and (H)CA(CO)NH sequences were developed in the context of resonance assignment of fully deuterated, 100% back-exchanged samples,[6b] and yield maps where the amide ^{1}H and ^{15}N shifts of residue *i* are correlated to either the ^{13}CO of the same residue or to the ^{13}CA of the preceding residue, respectively. Long coherence lifetimes are key to successful *J*-based correlation experiments.[12] Our present data show that, under suitable low-power ^{1}H decoupling,[13] fully protonated samples feature long ^{13}C and ^{15}N coherence lifetimes, of the order of 40 ms for ^{13}CO (comparable to those typically observed in deuterated, 100% back-exchanged preparations), and about 12.5 ms for ^{13}CA.[14] These long lifetimes correspond to linewidths that are significantly smaller than the $^{1}J_{CC}$ coupling constants, thus supporting high magnetization transfer efficiencies during the INEPT (insensitive nuclei enhanced by polarization transfer) blocks of the pulse sequences (about 30% for magnetization transfer between CA and CO spins in the examples of ε_{186} and SSB). In this regime, scalar-coupling-based techniques thus represent a competitive alternative to dipolar-coupling-based methods even for these fully protonated samples.

The alignment of strips from these spectra (Figure 3c) yields a sequential walk, from which backbone resonances can be readily assigned. Different from the corresponding ^{13}C-detected experiments, assignment ambiguities are removed by the use of two common chemical shifts of the ^{1}H(*i*) and ^{15}N(*i*) nuclei for strip alignment, in complete analogy to the strategies adopted in solution-state NMR spectroscopy. Notably, the measurement time to acquire the complete set of experiments for the backbone assignment was only about one week per sample, using about 3 mg of protein. ^{1}H, ^{15}N, ^{13}CO, and ^{13}CA shifts for a total of 132 residues (out of 168 non-proline residues) were assigned in this way for ε_{186}; these include residues in the central β sheet, in α helices, and in loops, thus demonstrating that the success of the assignment strategy is not dependent on local topology. Similarly, 83 out of 110 non-proline residues were assigned for the ssDNA-binding domain of SSB (see the Supporting Information). In this case, the procedure yielded a de novo assignment for a protein where no solution data were previously available.

Moreover, at an MAS rate of 60 kHz, a similar resolution enhancement pertains for side-chain ^{1}H NMR resonances, and typical ^{1}H NMR linewidths of 140–160 Hz are observed for methyl and H^{α} protons (see the Supporting Information). This enhanced resolution in turn raises the possibility of monitoring proximities between amide (H^{N}) and side-chain protons, thus allowing the identification of secondary structure as well as tertiary contacts. This possibility is illustrated in Figure 4, which shows a plane of a 3D ^{15}N-edited ^{1}H–^{1}H dipolar correlation spectrum[5a,6b] (533 μs radio-frequency-driven dipolar recoupling (RFDR) mixing[15]) of SSB. Among several resolved ^{1}H–^{1}H cross-peaks, some medium-range contacts between H^{N} and aliphatic protons of adjacent residues can easily be assigned, as illustrated for the sequential cross-peak between the H^{N} proton of Ala67 and the H^{β} proton of Val66, both residing in helix α1. These preliminary data show the potential of recording ^{1}H–^{1}H distance restraints in fully protonated proteins.

In conclusion, we have shown that ^{1}H shifts can provide a resolving dimension in spectra of fully protonated crystalline and noncrystalline proteins of medium size recorded at high magnetic field and ultrafast MAS. By using a set of ^{1}H-detected correlation experiments, extensive, robust, and expeditious assignments were obtained for the backbone ^{1}H, ^{15}N, ^{13}CA, and ^{13}CO resonances of two proteins in different aggregation states, without the need of deuteration. The increased sensitivity afforded by ^{1}H spins at ultrafast MAS permitted acquisition of the complete set of experiments for backbone assignment using only 3 mg of [^{15}N,^{13}C]-labeled protein in less than a week in either study, which is considerably faster than conventional ^{13}C-detected experiments.[2]

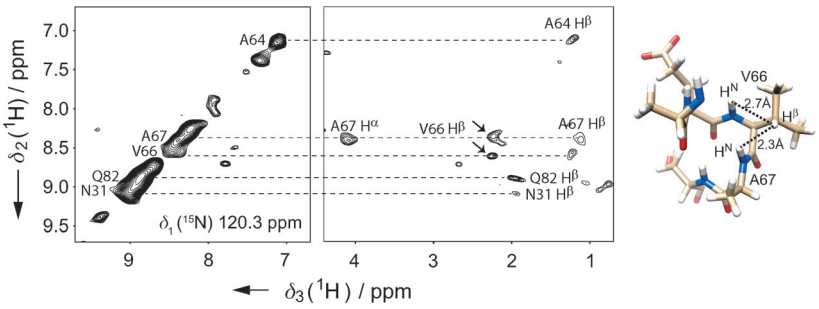

Figure 4. ^{15}N-plane from a 3D (H)NHH$_{RFDR}$ spectrum (533 μs ^{1}H–^{1}H mixing) of [^{13}C,^{15}N]-SSB. Dashed lines highlight cross-peaks between H^{N} and nearby side-chain protons. The two arrows in the spectrum identify the intra-residue and sequential H^{N}–H^{β} cross-peak observed for residues Ala67 and Val66 in helix α1, as depicted in the molecular model (H white; O red; N blue; C brown).

Labor-intensive and often unaffordable or unsuccessful strategies for deuteration and back-exchange of protons are thus no longer a prerequisite for useful ^{1}H-detected solid-state NMR spectra. This presents a major advance for biomolecular solid-state NMR spectroscopy, since constructive use of ^{1}H chemical shifts has to date been limited to samples where the ^{1}H spin bath was diluted through sparse ^{1}H labeling against a perdeuterated background. We believe that the approach outlined herein will significantly increase the impact of solid-state NMR spectroscopy in structural biology by extending ^{1}H-detected experiments to samples that cannot easily be deuterated and/or can only be obtained in small quantities. Furthermore, sensitive ^{1}H-detected NMR experiments with noncrystalline samples open new opportunities for the detection of ligand binding in solid-state samples that cannot be studied by X-ray crystallography or solution-state high-resolution NMR spectroscopy.

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