An Efficient Labelling Approach to Harness Backbone and Side-Chain Protons in \(^{1}H\)-Detected Solid-State NMR Spectroscopy

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Abstract: \(^{1}H\)-detection can greatly improve spectral sensitivity in biological solid-state NMR (ssNMR), thus allowing the study of larger and more complex proteins. However, the general requirement to perdeuterate proteins critically curtails the potential of \(^{1}H\)-detection by the loss of aliphatic side-chain protons, which are important probes for protein structure and function. Introduced herein is a labelling scheme for \(^{1}H\)-detected ssNMR, and it gives high quality spectra for both side-chain and backbone protons, and allows quantitative assignments and aids in probing interresidual contacts. Excellent \(^{1}H\) resolution in membrane proteins is obtained, the topology and dynamics of an ion channel were studied. This labelling scheme will open new avenues for the study of challenging proteins by ssNMR.

The recent advent of \(^{1}H\)-detection in biological solid-state NMR (ssNMR) spectroscopy can greatly increase spectral sensitivity,[1] and thereby bears the potential to critically broaden the scope of ssNMR spectroscopy. The prevailing method to detect protons in solid proteins is perdeuteration, that is, the complete deuteration and subsequent reintroduction of exchangeable protons in protonated buffers. This labelling scheme largely removes line-broadening \(^{1}H\)-\(^{1}H\) dipolar couplings and can provide spectra of extremely high quality.[2] Moreover, it allows automated backbone assignments[3] and probing contacts between backbone amino protons (H\(^{\beta}\)), which are important to define protein folds.[4]

However, the absence of aliphatic side-chain protons in perdeuterated proteins curtails the potential of \(^{1}H\)-detection, given that side chains are important factors for protein structure and function. In addition, the availability of side-chain protons could facilitate the assignment of complex proteins. In general, to assign side-chain protons and use them for structural studies has remained a major difficulty for \(^{1}H\)-detected ssNMR spectroscopy. In principle, fully protonated proteins in combination with magic-angle spinning (MAS) frequencies of higher than 100 kHz\[^{[2d]}\] could provide a future avenue to access side-chain protons, as suggested in recent studies with soluble and membrane proteins.[5] However, it can be envisaged that the residual \(^{1}H\) linewidths and spectral crowding will remain a challenge for larger proteins. Moreover, for proteins such as membrane proteins with a substantial inhomogeneous contribution to the \(^{1}H\) linewidth, MAS frequencies higher than 100 kHz may not compensate for the sensitivity loss resulting from comparably small sample volumes. Excellent resolution and assignments of aliphatic protons have been reported with residual adjoining protonation (RAP), which relies on the random incorporation of protons into a deuterated protein matrix.[6] Moreover, approaches such as ILV, proton cloud, or SAIL labelling can be used to probe contacts between side-chain protons of specific types of amino acids.[7] Yet, such approaches employ isolated labels which may be very difficult to assign de novo and only give access to a selection of side-chain protons. To assign side-chain protons and exploit them for structural studies, even in larger proteins, a labelling scheme which 1) provides a high global \(^{1}H\) density and 2) mitigates spectral crowding nonetheless, could be very advantageous. Thus we explored “fractional deuteration” in \(^{1}H\)-detected ssNMR spectroscopy. This labelling scheme, based on protonated \(^{13}C\)-glucose and D\(_{2}\)O in the growth medium, was previously proposed in solution NMR spectroscopy as an alternative to ILV labelling and in \(^{13}C\)-detected ssNMR spectroscopy for spectral editing.[8] These studies reported that certain carbon atoms, such as C\(_{\alpha}\), are highly deuterated in fractionally deuterated proteins, while many side-chain carbon atoms retain sizeable \(^{1}H\) levels.

Herein we demonstrate that fractional deuteration provides access to well-resolved \(^{1}H\) and side-chain protons of virtually all residues in one sample, and allows assignment and use of these protons for structural studies. Importantly, even though our approach works at much higher \(^{1}H\) levels, we observe an excellent resolution (0.07 ppm) for the H\(^{2}\) protons in the fractionally deuterated (FD) membrane-embedded K\(^{+}\) channel KcsA, and it rivals the resolution in the perdeuterated channel. We outline our approach on ubiquitin and then use it to study KcsA, including its membrane topology and dynamics, as well as important channel–water interactions.

Figure 1 shows \(^{1}H\)-detected two-dimensional (2D) CH and NH spectra of FD \(^{13}C\),[\(^{15}N\)]-ubiquitin in aqueous (100% H\(_{2}\)O) buffers, acquired at 52 kHz MAS and 800 MHz \(^{1}H\)-
These spectra are of remarkable quality and feature a resolution as high as 0.05 and 0.07 ppm for aliphatic and exchangeable protons, respectively. The absence of CαHα correlations, which typically appear around $\delta_{\text{Cα}} = 50–65$ ppm and $\delta_{\text{Hα}} = 3.0–5.0$ ppm, is readily visible in the CH spectrum. A quantitative analysis using solution NMR spectroscopy (see section S1 in the Supporting Information) revealed, next to the absence of Hα protons (< 2% population), an interesting pattern of H depletion for the side chains in FD ubiquitin (see Figure 1D and Table S1). The pyruvate-derived branched-chain amino acids (Ile, Leu, Val) exhibit very low (< 5%) H levels at the Cβ-position, which is the same for the amino acids (Arg, Gln, Glu, Pro) derived from α-ketoglutarate (< 8%). Amino acids that follow other pathways (such as Asn, Asp, His, Lys, Ser, Thr), however, retain much higher H levels (30–45%; 90% for Ser) at Cβ, with slightly reduced values for aromatic amino acids (Phe, Tyr). Most other carbon atoms, further away from the backbone, feature equally high H levels. Our data are in good agreement with the original solution NMR study, which also provides detailed biochemical explanations.\[9\] Hence, many sites remain robustly protonated in FD proteins, yet feature a narrow H linewidth because the H network is, on average, starkly diluted. However, we like to emphasize that the local and global H density in FD proteins are much higher than those in RAP-labelled proteins.\[8\] Broadening effects resulting from methylene isotopomers were not observed, probably because CH3 signals are broadened beyond detection. Methyl groups showed slight oval lineshapes because of isotopomers, but they did not significantly compromise the H resolution (0.05–0.08 ppm), presumably because CH2 signals are broader and less abundant than either CHD2 or CH3D signals (see Box I in Figure 1A and Figure S2). Prominent features of the CH spectrum of FD ubiquitin are the unusually intense CαHα signals. As it can be readily shown with simulations (see Box II in Figure 1A and section S2), this beneficial effect is caused by the absence of Hα protons in FD proteins.\[11\]

In Figure 2A we show assignments in FD ubiquitin, and they are based on dipolar transfer. Acquisition details can be found in section S2. Backbone connectivities were established with three-dimensional (3D) CαNH, Cα(CO)NH, and CCH experiments. In the 3D Cα(CO)NH, Cα polarization was prepared by a selective CP step (see Figure S4).\[10,12\] These experiments were sufficient for backbone assignments, given that extensive chemical-shift data are available for ubiquitin.\[13\] Side-chain assignments were performed with a 3D CCH experiment which included a 13C-13C DREAM\[14\] double-quantum mixing block, optimized for one-bond transfer. Importantly, the efficient transfer from Cα to Hβ in the CCH experiments, and hence the presence of intense CαHβ and CβHβ correlations, allow the use of Hβ as anchors to connect backbone and side chains, thereby greatly facilitating the assignment process. Moreover, the assignments of side-chain types are greatly simplified by the pattern of robustly protonated and deprotonated Cβ sites (see Table S1). CαHβ correlations were only detectable for residues with Hβ levels of greater than 20%, which much
Signals for signals, which all belonged to contacts, many interresidual backbone–backbone $\text{H}^\text{N}$–$\text{H}^\text{N}$ contacts, as well as backbone–side chain contacts between $\text{H}^\text{N}$ and aliphatic protons. Next to alar ge channel KcsA, $(0.13–0.18 \text{ ppm})$. This spectrum showed only around 25 signals, while KcsA features around 70 water-accessible residues, which, in particular, reduce the number of $\text{H}^\text{N}$–$\text{H}^\text{N}$ contacts, many interresidual backbone–side chain contacts, of up to an 8 Å distance, could be assigned or identified (based on the X-ray structure PDB: 1UBQ). These contacts demonstrate that the high $\text{H}$ density in FD proteins does not impede long-distance magnetization transfer. Unambiguous medium- and long-range $\text{H}^\text{N}$–$\text{H}^\text{N}$ contacts involved methyl groups and also methylene groups such as the CPHD and CyHD groups of Lys residues. Especially contacts are noteworthy, since they are complementary to ILV labelling.

In Figures 3 and 4, we show the potential of $\text{H}$-detection in more complex FD proteins using the $K^+$ channel KcsA, a well-accepted model for ion-channel gating,[15] as an example. FD $[{^{13}\text{C}},^{15}\text{N}]$-KcsA in the closed-conductive state was reconstituted in E. coli lipids and aqueous (100% $\text{H}_2\text{O}$) buffers. Further details of the sample preparation are given in section S5. We acquired dipolar-based 2D NH and CH spectra of very high quality (Figure 3A and Figure 4A), featuring a resolution as high as 0.06 and 0.07 ppm for aliphatic and exchangeable protons, respectively. Remarkably, the $\text{H}^\text{N}$ resolution in FD KcsA is comparable to perdeuterated KcsA[15] (see Figure S11) and the perdeuterated membrane protein OmpG[3a] (0.13–0.18 ppm). This resolution strongly suggests that the availability of side-chain protons in many FD membrane proteins comes at either very low or no cost at fast (> 50 kHz) MAS, because the residual $\text{H}$ linewidth is dominated by inhomogeneous contributions. Hence, fractional deuteration is highly advantageous for $\text{H}$-detection in non-microcrystalline proteins.

FD KcsA was grown in $\text{D}_2\text{O}$ and only water-exposed residues are visible in the NH spectrum, which we used to study the membrane topology.[2c,4d] Intriguingly, the NH spectrum showed only around 25 signals, while KcsA features about 70 water-accessible residues, which, in particular, comprise the extracellular outer vestibule (residues 51–64 and 80–86) and the cytoplasmic domain (CPD; residues 118–160). To understand the composition of the NH spectrum, we performed 3D $\text{CuNH}$, 3D $\text{Cu(CO)}\text{NH}$, 3D $\text{NNH}$, 2D CH, and 2D $\text{C(OH)}$ experiments (see section S3 for a detailed discussion of the assignments), supported by $^{13}\text{C}$- and $^{15}\text{N}$-chemical-shift data.[15c-d] We validated our sequential assignments by $\text{H}^\text{N}$–$\text{H}^\text{N}$ contacts which we observed in a 3D NH experiment (see Figure S7). Moreover, by using a slightly longer $^{13}\text{C}$ to $\text{H}$ CP contact time (700 μs), we obtained many Cu$\text{H}^{2+}$ contacts in the 2D CH, which also allowed validation of sequential assignments (see Figure S8). Altogether, we could assign about 70% of the $\text{H}^\text{N}$ signals, which all belonged to the outer vestibule, thus demonstrating that the CPD is too dynamic for dipolar transfer (Figure 3B). Note that we did not observe marked sensitivity with scalar transfer, thus implying that CPD dynamics in lipid membranes are relatively slow (μs to ms). We also did not observe the CPD in open-inactivated KcsA (see Figure S10), where the CPD helices are loosely structured. The latter result, thus, excludes the possibility that the CPD is invisible in the 2D NH of the closed-conductive channel (Figure 3a) because of tight packing of CPD helices, which might possibly interfere with the reintroduction of protons. This finding is noteworthy given that the conformational flexibility of the CPD is important for KcsA activation gating.[10a] Furthermore, in Figures 3 C and D we used our $\text{H}$ assignments to study buried water behind the conductive selectivity filter, as it is important for the gating mode.[5c,12a] How this water is bound is not directly accessible in KcsA X-ray structures, since protons are not resolved. By transferring magnetization...
the use of expensive deuterated glucose, will significantly increase the impact of solid-state NMR spectroscopy, especially for membrane proteins or peptide assemblies such as fibrils, which usually cannot be obtained as microcrystalline preparations.

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