NMR Spectroscopy

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Direct Detection of $^{3h}J_{NC'}$ Hydrogen-Bond Scalar Couplings in Proteins by Solid-State NMR Spectroscopy**

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Hydrogen bonds (H-bonds) are important and ubiquitous interactions in chemistry and biology. They are a key element in proteins and nucleic acids for stabilizing the three-dimensional fold and are thus important for the functionality. The presence of an H-bond can be indirectly deduced from the local geometry as obtained from X-ray or NMR methods, and a variety of NMR parameters depend also on hydrogen bonding (e.g. chemical shifts induced by H-bonds or ^{2}H quadrupolar coupling constants). Direct evidence of hydrogen bonding, however, is provided by the presence of an H-bond-mediated scalar coupling. Experiments that directly measure *J* couplings across N−H···N[1–3] and N−H···O=C[4–6] bonds in nucleic acids and proteins, respectively, have been introduced for solution-state NMR spectroscopy and have received great interest. Such experiments allow the direct identification of the donor and acceptor side of each H-bond, as well as the determination of the size of the coupling—which is a very sensitive probe of the geometry around an H-bond.

Even though solid-state NMR experiments can also use the dipolar interaction to indirectly probe N−H···N hydrogen bonding in nucleic acids[7,8] or N−H···O=C bonds in proteins,[9] the intrinsic through-H-bond nature of the scalar coupling is appealing. *J*-based correlation spectroscopy[10,11] has been demonstrated for proteins in the solid state[12–15] and has been successful in measuring *J* couplings down to a few Hertz, including N−H···N H-bond couplings in crystals of organic molecules[16–18].

The small values for the scalar coupling constants of H-bonds in proteins, with average values in ubiquitin for N−H···O=C H-bond scalar coupling constants ($^{3h}J_{N,C'}$) of (-0.38 ± 0.12) Hz (α-helix) to (-0.65 ± 0.14) Hz (β-sheet),[4] make these experiments challenging in terms of sensitivity. In liquids, difficulties arise particularly for larger molecules, where the coherence decay owing to faster T_2 relaxation greatly attenuates the signal during the long time periods required for the polarization transfer mediated by the small *J* coupling. The situation is even more challenging in solids because of the presence of secular anisotropic interactions, which provide additional mechanisms of transverse dephasing.

The strategies employed to minimize this additional dephasing (and thus minimizes T_2') include the use of high magic-angle-spinning (MAS) frequencies[19] and the dilution of the ^{1}H spin system by extensive deuteration[15,20] to avoid the need for high-power proton decoupling over extended time periods. Herein, we demonstrate the use of a deuterated and partially backprotonated microcrystalline protein at high MAS frequencies, to directly measure sub-Hertz *trans*-H-bond scalar coupling constants.

Figure 1 shows strips from a "long-range" 3D HNCO correlation experiment optimized for the detection of $^{3h}J_{NC'}$ couplings, and were recorded on a microcrystalline sample of ^{2}H,^{13}C,^{15}N-labeled ubiquitin, which was protonated at 20% of the backbone amides and other exchangeable sites. The pulse sequence used to obtain these data is a purely *J*-based HNCO experiment, akin to an experiment proposed by Cordier and Grzesiek[4] for measurement of $^{3h}J_{NC'}$ in the solution state. The details of the pulse sequence are shown in Figure S1 of the Supporting Information. The key features of this ^{1}H-detected experiment are long out-and-back N−C′ INEPT blocks for a duration of $2T = 66.6$ ms to transfer coherence from the H-bond donor ^{15}N to the acceptor ^{13}C′, and was carried out in the presence of low-power (3.1 kHz) ^{1}H decoupling. The evolution resulting from the large $^1J_{NC'}$ coupling (approximately 15 Hz = 1/(2T)) vanishes at multiples of 2T. Therefore, the small $^{3h}J_{NC'}$ couplings become detectable and lead to coherence transfer over H-bonds. The data in Figure 1 unambiguously show cross-peaks that arise from transfer over N−H···O=C hydrogen bonds involving the amides of residues L15, V17, I44, K6, and L50 (the latter two have overlapping cross-peaks) with the carbonyl groups of I3, M1, H68, L67, and L43. The size of the couplings involved is known to be 0.5–0.6 Hz (see below), which is almost an order of magnitude smaller than the smallest couplings measured to date using solid-state NMR spectroscopy.[18]

To confirm the results and to investigate the reproducibility of the measurement, we have performed an additional experiment under slightly different conditions and using a sample with a higher degree of protonation on the exchangeable sites (30% rather than 20%). The 2D long-range H(N)CO data set obtained on this sample is shown in Figure S2 of the Supporting Information. The spectrum reveals cross-peaks arising from H-bonds of residues F4 and E34, in addition to the ones observed in the 3D spectrum. In the 3D data set of Figure 1 these cross-peaks overlapped with residual one-bond cross-peaks and are resolved in the

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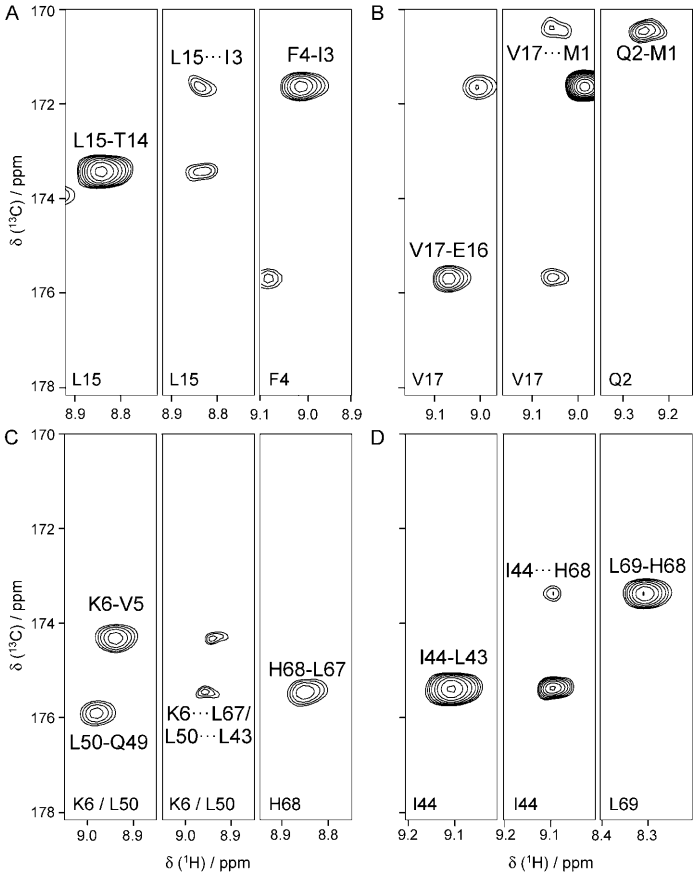

Figure 1. H–C′ strips of the reference and long-range HNCO 3D spectra ("20% amide-protonated" sample, 55 kHz MAS, sample temperature of 27 °C). In each of the four examples (A)–(D), the middle strip was extracted from the long-range experiment at the ^{15}N frequency for the H-bond donor, while the flanking strips show the reference spectrum at the H-bond donor (left) and acceptor (right) ^{15}N frequency. The total experimental time required for the long-range experiment was 112 h and the reference experiment took 8 h.

2D experiment resulting from slightly different chemical shift values because of a higher temperature. In addition to the $^{3h}J_{NC'}$ cross-peaks, two-bond intra-residue $^{2}J_{NC'}$ correlations are observed for residues E16, K48, and presumably E34 (the latter overlaps with the corresponding $^{3h}J_{NC'}$ cross-peak). Reported solution-state $^{2}J_{NC'}$ coupling constants in ubiquitin are on average (0.46 ± 0.24) Hz with the largest value of 1.13 Hz found for E34[4].

The magnitude of the observed long-range J couplings can be determined from the intensities found in the long-range experiment and the reference experiment[4] (see the Supporting Information for details). The extracted coupling constants (for resonances that do not have any resonance overlap) are obtained as $^{3h}J_{NC'}$ (V17···M1) $= (0.59 \pm 0.16)$ Hz (0.578 Hz), $^{3h}J_{NC'}$ (L15···I3) $= (0.605 \pm 0.16)$ Hz (0.616 Hz), $^{3h}J_{NC'}$ (I44···L68) $= (0.58 \pm 0.15)$ Hz (0.605 Hz), $^{2}J_{NC'}$ (E16) $= (0.46 \pm 0.3)$ Hz, where the values in brackets are values observed in the solution-state at 25 °C.[21] Solid- and solution-state coupling constants are thus identical (within error) for the available data.

Thirtyone *trans*-H-bond $^{3h}J_{NC'}$ couplings have been reported in ubiquitin by solution-state NMR spectroscopy,[4] whereas only seven *trans*-H-bond correlations (including two overlapping resonances) are observed in this work. We suspect that the missing correlations are too weak to be detected—the actual values of the $^{3h}J_{NC'}$ couplings of the missing residues is below the limit of detection. To further investigate this point, we analyzed the signal-to-noise ratio of one-bond correlation peaks in the reference experiment, and calculated from these data the minimal value $|J_{min}|$ of $^{3h}J_{NC'}$ that would give rise to an observable cross-peak. This value can be calculated from the sensitivity of the reference experiment and the number of scans applied in the reference and long-range experiment, respectively,[5] by using the equations summarized in the Supporting Information. Figure 2 compares the predicted $|J_{min}|$ values with the

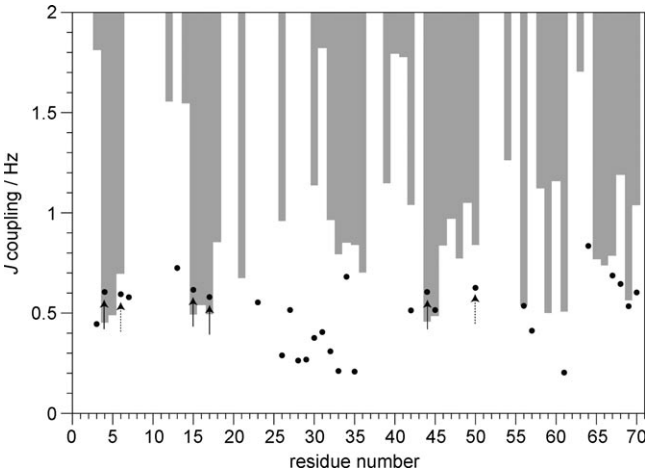

Figure 2. Residue-specific comparison of the range of ominimal bservable scalar coupling constants $|J_{min}|$ in the long-range 3D experiment for the values as calculated from the reference experiment with actual $|^{3h}J_{NC'}|$ coupling constants, which were obtained from liquid-state NMR spectroscopy at 25 °C.[18] The $|J_{min}|$ were calculated from the reference experiment. Circles inside gray areas should be observable scalar coupling constants. Actual observed H-bond correlations are indicated with arrows.

experimental $|^{3h}J_{NC'}|$ values reported from solution-state NMR spectroscopy.[21] Residues for which the predicted $|J_{min}|$ values are smaller than or equal to the values obtained through solution-state NMR $|^{3h}J_{NC'}|$, and should thus be detectable, are F4, L15, V17, I44, F45, and L56. Indeed, all these cross-peaks are detected, except for F45 and L56. In the case of L56 resonance overlap with the one-bond correlation peak makes detection of the *trans*-H-bond peak impossible. The fact that the $^{3h}J_{NC'}$ (F45···K48) cross-peak is not visible, although the predicted detection limit is below the solution-state value, may possibly be explained by a variation of the $^{3h}J_{NC'}$ value between solid and liquid states. Interestingly, both the donor nitrogen atom as well as the acceptor carbonyl group involved in intermolecular contacts within less than 4 Å in the crystal structure of our sample.[22] *Trans*-H-bond cross-peaks from K6 and L50 are not predicted to be visible, however, these peaks are observed in this data set because the

corresponding overlap of cross-peaks, thus increasing the signal intensity.

To better understand the large variation of J_{min} values found in Figure 2, we investigated the observed signal intensities in the reference spectrum. The signal intensity depends on two factors: 1) the T_2' relaxation time of ^{15}N, which dephases the magnetization during the long N–C′ INEPT transfer periods, and 2) the "'intrinsic" sensitivity with which a given correlation peak for an H–N amide bond can be detected. The latter not only depends on T_2' relaxation times for ^1H and ^{15}N, but also on heterogeneous broadening mechanisms. Figure 3 shows residue-specific T_2' relaxation rate constants for ^{15}N and Figure S4 in the Supporting Information shows residue-specific signal-to-noise ratios

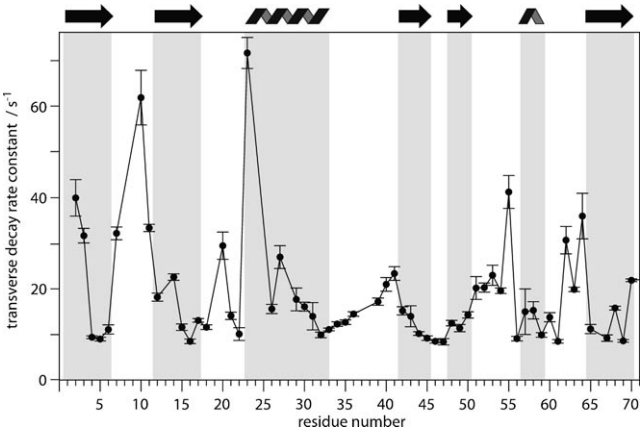

Figure 3. Residue-specific ^{15}N transverse decay rate constants $R_2' = 1/T_2'$ measured with the pulse scheme shown in Figure S5 under the same conditions as the data shown in Figure 1. Secondary structure elements are indicated on top of the figure. Examples of decay curves are plotted in Figure S3.

obtained in a 2D H–N correlation experiment, respectively. These data were measured under the same conditions as the data in Figure 1 (i.e. using the pulse sequences shown in Figure S5). A large variability is found in both the T_2' decay for ^{15}N and the "intrinsic" 2D H–N sensitivity as a function of the residues. Interestingly, the T_2' data suggest that a significant fraction of T_2' may arise as a consequence of internal dynamics, as the fastest decay is observed in loops or at the end of secondary structure elements. Future studies will investigate the role of internal dynamics and local disorder with respect to transverse dephasing and achievable sensitivity.

These sensitivity considerations demonstrate that an improved signal-to-noise ratio is required to detect additional H-bond scalar coupling constants in our model system. Possible ways to improve sensitivity include acquiring more scans, larger amounts of sample, optimization of the H/D ratio on amide sites, using a probe optimized for ^1H detection, or increasing the apparent T_2' time constants for ^{15}N (e.g. by faster MAS or by altering the dynamics by changing the temperature). This predicted influence of changes in sample amount, longer signal accumulation, and increased T_2' are shown in Figure S6 of the Supporting Information. Increasing the measurement time rapidly becomes impractical, as signal averaging for 18 days (four times the acquisition time of the 3D spectrum in Figure 1) is predicted to allow only four additional $^{3h}J_{NC'}$ couplings to be detected. Increasing the amount of sample may be a more promising route, and it is predicted that most of the H-bonds in the β-sheet, and three H-bonds in the α-helix could be detected by an increase in sample amount by five-fold (which could be achieved by using a 3.2 mm rotor instead of a 1.3 mm rotor used here). Decreased detection efficiency and more pronounced T_2' losses at the lower MAS frequency that are achievable with larger rotors may, however, outweigh the gains from increased sample amount. Current research is directed towards finding the optimum compromise between spinning frequency, heteronuclear decoupling efficiency, and the degree of deuteration with the goal of detecting more and smaller H-bond scalar coupling constants.

In summary, we have demonstrated the feasibility of directly measuring sub-Hertz scalar coupling constants mediated by H-bonds in solid-state NMR spectroscopy. Exciting future applications of this technique include the structural study of amyloid fibrils, like those involved in neurodegenerative diseases, which often consist of extended networks of β-sheet structure. The direct identification of the H-bond pattern in these structures will provide valuable and more reliable structural information than methods based on dipolar-coupling or indirect methods such as H/D exchange. Although the experiments are quite challenging at present, we expect that future advances in sample preparation, hardware and pulse sequence design will make the use of H-bond scalar coupling constants a routine technique for structural studies by solid-state NMR spectroscopy.

Experimental Section

^2H,^{13}C,^{15}N-labeled ubiquitin was produced by bacterial overexpression in D_2O-based M9 medium containing ^2H,^{13}C-glucose (2 g L^{-1}) as the sole carbon source and $^{15}NH_4Cl$ (1 g L^{-1}), and was purified using standard protocols. Microcrystals were obtained using $^2H_{12}$-2-methyl-2,4-pentanediol (MPD) as described.[23] In the solutions used for crystallization, 20% (30%) of the exchangeable hydrogen atoms (water and hydroxy groups in MPD) were of the ^1H isotope, while 80% (70%) were of the ^2H isotope, in the two respective samples. Protein crystals were packed into a 1.3 mm Bruker rotor by ultracentrifugation. The total increase in weight of the rotor upon filling was about 4 mg.

All NMR spectroscopy experiments were carried out on a Bruker Biospin AVANCE spectrometer operating at 850 MHz ^1H Larmor frequency using a 1.3 mm triple-resonance (^1H,^{13}C,^{15}N) probe (Bruker). Samples were spun at 55–57 kHz. Chemical shifts were referenced internally to 3-(trimethylsilyl)-1-propanesulfonic acid, sodium salt (DSS), and the sample temperature was determined from the chemical shift of the supernatant water. Data processing, using cosine-squared apodization (shifted by π/5 in the ^{13}C and ^{15}N dimensions), zero-filling, and baseline correction, was done using NMRPipe software.[24] Data were analyzed using nmrView (One-Moon Scientific, Inc.). Resonance assignment was done using 3D *J*-based triple-resonance assignment experiments (HNCA,

HN(CO)CA, HNCO, HNCACB).[15] Details will be presented elsewhere.

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