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Protein solid-state NMR resonance assignments from (^{13}C,^{13}C) correlation spectroscopy

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It is demonstrated that sequential resonance assignments can be obtained from (^{13}C,^{13}C) correlation spectroscopy on a uniformly labeled protein under magic angle spinning. The experiment relies on weak (C′,Cα) coupling conditions using a defined range of MAS rates and can be employed at arbitrary magnetic field strength.

Magic angle spinning[1] (MAS) solid-state NMR is rapidly developing into a tool to study biomolecular 3D structure,[2–5] protein aggregation[6–9] and ligand-binding to membrane receptors.[10] For many structural studies, near-complete sequential resonance assignments are a prerequisite and have now been obtained for several (membrane) proteins.[11–17] MAS-based spectral assignment methods have thus far relied on a collection of (^{15}N,^{13}C) and (^{13}C,^{13}C) one-bond dipolar or scalar polarization transfer schemes (see, for example, ref. 18) to determine inter-residue connectivities in the polypeptide of interest. While side-chain information may suffice to identify ^{13}C resonances in small peptides,[10] (^{13}C,^{15}N) isotope labeling and a triple channel (^{1}H,^{13}C,^{15}N) NMR setup is mandatory in larger systems. In particular, several polarization transfer steps are necessary if sequential (Cα,Cα)[17] or side chain–side chain interactions are to be investigated in a single 2D experiment.

In principle, a reduction in the number of transfer units and hence an increase in efficiency of a sequential assignment protocol could be achieved if inter-residue side chain – side chain assignments are established using polarization transfer among local (C′,Cα) moieties only.

For this purpose, polarization transfer must be mediated by a weak (inter-residue) coupling in the presence of two strong intra-residue (C′,Cα) interactions (Fig. 1). At the same time, the mixing scheme of choice should be applicable at arbitrary magnetic field strength $\boldsymbol{B}_0$. While the latter aspect suggests a transfer unit that is insensitive to isotropic or anisotropic chemical shielding interactions, broadband polarization transfer schemes have been shown to be unfavorable if weak couplings are to be studied in the presence of larger dipolar interactions.[19–23] Indeed, a series of quantum-mechanical (QM) model calculations assuming the four-spin topology given in Fig. 1 reveals that broadband zero or double-quantum transfer schemes fail to generate sizable inter-residue polarization exchange.

Instead, we consider in the following the phenomenon originally termed 'nuclear cross relaxation induced by specimen rotation' by Andrew and co-workers.[24,25] Here, the rate of polarization exchange in a dipolar coupled spin pair is enhanced if a multiple n of the MAS rate ω_R (divided by the Larmor frequency ω_0) is equal to the chemical shift difference $\Delta\Omega$, *i.e.* $\Delta\Omega = n\omega_R/\omega_0$. Under proton decoupling conditions, this 'rotational resonance' (RR) mechanism has been successfully used to determine structural constraints in a variety of pair-wise (see, for example,[6,26,27]) and, more recently, uniformly labeled amino acids and polypeptides.[28–32] Unlike broadband mixing, the transfer dynamics are in this case explicitly dependent on isotropic resonance offsets and the magnitude and orientation of anisotropic dipolar and chemical shielding (CSA) interactions. While analytical solutions exist for the case of a dipolar-coupled two-spin system under exact RR conditions,[33,34] a theoretical treatment of dipolar polarization transfer under off-RR conditions in a four-spin system is more complicated.

We hence resort to QM (four-spin) calculations that probe the signal intensity of the inter-residue transfer C′($i-1$) → Cα(i) in the presence of two strongly coupled intra-residue (C′,Cα) spin pairs. Our simulations were conducted for a range of isotropic chemical shift differences $\Delta\Omega$(C′,Cα) (Fig. 1) assuming standard dipolar and CSA[35,36] interactions under variable molecular orientations.

As an example, the maximum Cα(i) signal intensity is plotted in Fig. 2a as a function of the MAS rate for static magnetic fields $\boldsymbol{B}_0$ corresponding to 400, 600 and 800 MHz ^{1}H resonance frequency. Assuming an initial density operator that reflects longitudinal magnetization on the C′($i-1$) spin only, polarization transfer to Cα(i) was monitored in the initial rate regime (0.8 ms mixing time) setting $\Delta\Omega$(C′($i-1$),Cα(i)) = $\Delta\Omega$($i-1$,i) = 122 ppm. Intra-residue pairs with $\Delta\Omega$($i-1$, $i-1$) = 111 ppm and $\Delta\Omega$(i,i) = 112 ppm were assumed. As visible from Fig. 2a, inter-residue polarization transfer (including the complete four-spin system) is possible around the $n = 2$ (400–800 MHz) and $n = 1$ (400 MHz) RR conditions for the considered MAS rates. Similar results are obtained if the magnitude or relative orientation of dipolar and CSA tensors is varied. Even in the degenerate case, *i.e.* $\Delta\Omega(i-1,i) = \Delta\Omega(i,i) = \Delta\Omega(i-1,i-1)$, sizable polarization transfer is possible. Comparative calculations assuming a (C′($i-1$),Cα(i),C′(i)) three-spin topology show that the latter result is only found if two sequential (C′,Cα) spin pairs are considered. According to our QM four-spin study, the polarization transfer profile shown in Fig. 2a within the inter-residue (C′,Cα) pair hence largely follows the general transfer characteristics of an isolated two-spin system. Normalized to the initial polarization, efficiencies of up to 20% for inter-residue polarization transfer are predicted from the simulation.

Setting the MAS rate at exact RR conditions would result in severe line broadening effects and hence must be avoided. Furthermore, sequential resonance assignments should be possible at variable $\boldsymbol{B}_0$. A band-selective (C′($i-1$),Cα(i))

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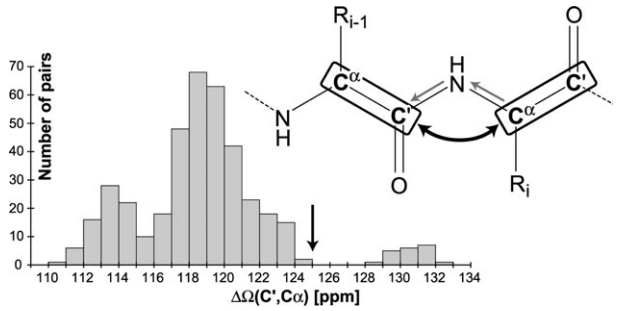

Fig. 1 Statistical analysis of (C′,Cα) chemical shift differences using average chemical shifts compiled by the BioMagResBank data bank. Polarization transfer pathways to establish sequential (^{13}C′,^{13}Cα) resonance assignments can invoke (N,C) polarization transfer (grey) or can be mediated by (C′,Cα) pairs (black). Numerical simulations shown in Fig. 2 were performed for the four-spin system indicated in bold. The arrow indicates the chemical shift difference (divided by two) equivalent to the MAS rates used in Figs. 3–5.

polarization transfer profile can be created by changing the MAS rate during mixing[38] or by incorporation of an additional radio frequency field on the ^{13}C channel.[13,31,39–42] While these methods permit the use of MAS rates away from RR conditions they also strongly modulate or remove the chemical-shift dependence of the polarization scheme. Preserving the general chemical shift selectivity and, at the same time, broadening the transfer profile can be achieved by removing proton decoupling during mixing. The influence of the dipolar coupled proton reservoir has been described using first order perturbation theory[24,43] and is, in the context of our experiment, commonly referred to as proton-driven spin diffusion (PDSD, ref. 44). PDSD mixing is possible for a variety of MAS rates and is characterized by an exponential rather than (damped) oscillatory (as in the two- or four-spin case) polarization transfer. On the other hand, the rate of polarization exchange depends on the zero-quantum line-shape function exhibiting a maximum at $\Delta\Omega = n\omega_R/\omega_0$ in line with the theoretical four-spin simulations shown in Fig. 2.

'Weak coupling' conditions can hence be created if an MAS rate close to the (but not at the exact) RR $n = 2$ transfer condition (*i.e.* around 125 ppm/2, Fig. 1) is selected and PDSD is active. As a result, polarization transfer is significantly

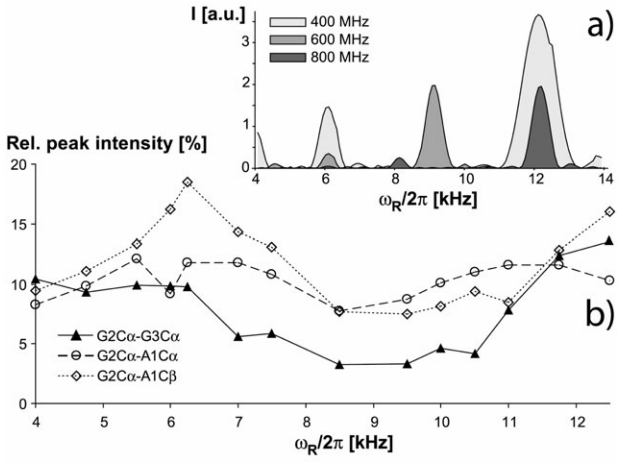

Fig. 2 (a) C′(i − 1) → Cα(i) polarization transfer (simulated in GAMMA[37]) as a function of MAS rate and magnetic field $\boldsymbol{B}_0$. Isotropic chemical shift differences as described in the text were assumed. While anisotropic CSA interactions of C′ and Cα and the orientation of the C′ CSA tensor were taken from the literature,[36,37] QM simulations were performed for different molecular orientations of the Cα CSA tensor. (b) Inter-residue cross peaks (integrated and normalized against the G2Cα diagonal peak intensity) obtained on U-[^{13}C,^{15}N]-labeled AGG for variable MAS rate. Data were recorded on a 400 MHz NMR instrument (Bruker Biospin).

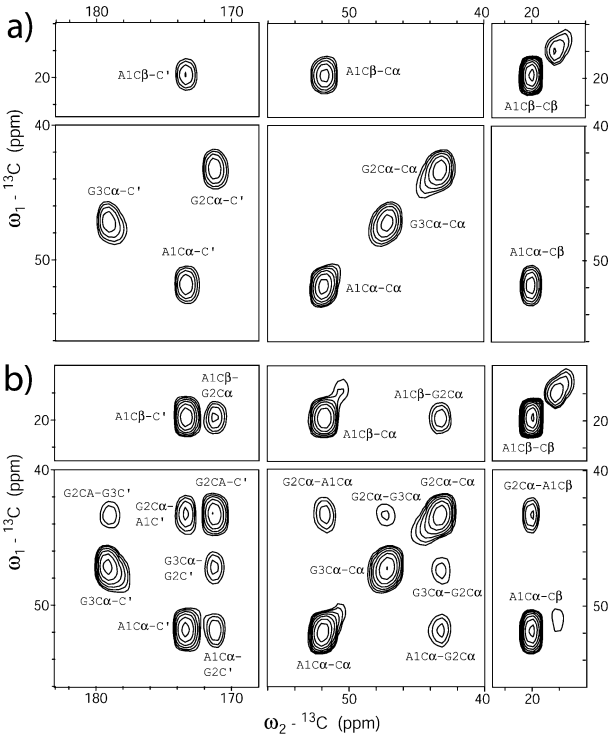

Fig. 3 2D (^{13}C,^{13}C) correlation experiments on AGG at 7 kHz MAS for a mixing time of 10 ms (a) and 50 ms (b). During evolution and detection periods, TPPM[46] was used at 90 kHz.

slower than under exact RR conditions (Fig. 2a) but the desired mixing characteristics are preserved. In addition to the C′(i − 1) → Cα(i) transfer, proton-driven spin diffusion also allows for subsequent polarization transfer from Cα(i) to side chain resonances of residue i. A standard two-dimensional PDSD experiment (see, for example, ref. 11 for further experimental details) hence should not only contain intra-residue correlations but also inter-residue cross peaks such as (C′(i − 1),Cα(i)), (Cα(i − 1),Cα(i)) and (Cα(i − 1),Cβ(i)). In Fig. 3, this effect is demonstrated on the uniformly (^{13}C,^{15}N) labeled tripeptide Ala–Gly–Gly for an MAS rate of 7 kHz on a 400 MHz instrument. For a mixing time of 10 ms (Fig. 3a), only intra-residue (^{13}C,^{13}C) correlations are detected in agreement with the spectral assignments reported previously[45] and in line with the general PDSD transfer profile. Increasing the mixing time to 50 ms (Fig. 3b) leads, in addition, to the occurrence of sequential (C′,Cα) (left), (Cα,Cα) (middle) and (Cα,Cβ) cross peaks. Inter-residue polarization transfer is enhanced if MAS rates close to the RR conditions are selected (Fig. 2b) in accordance with the QM model calculations (Fig. 2a). Line-broadening effects can be detected that are strongest at $n = 1$ conditions but range between 0–20% (compared to the ^{13}C line width far off RR conditions) if MAS rates close the $n =$ 2 condition are selected. Further experimental evidence that inter-residue polarization transfer indeed results from indirect (Cα ↔ C′ ↔ Cα) interactions comes from a 2D control experiment, in which C′ resonances are decoupled by a weak on-resonance rf field during mixing (data not shown). In this case, both C′ and Cα peaks are strongly attenuated indicative of a coupled spin state. Additional experiments using variable mixing times reveal that the maximum intensity of the sequential (C′,Cα) and (Cα,Cα) cross peaks is detected for mixing times in the order of 50 ms and 100 ms, respectively. For reasons of spectral resolution and dispersion, the latter interactions are, along with additional side-chain correlations, most informative for sequential (^{13}C,^{13}C) resonance assignments.

To investigate the use of weakly coupled (C′,Cα) correlation spectroscopy in larger systems and at higher magnetic fields, we conducted experiments on two uniformly labeled proteins.

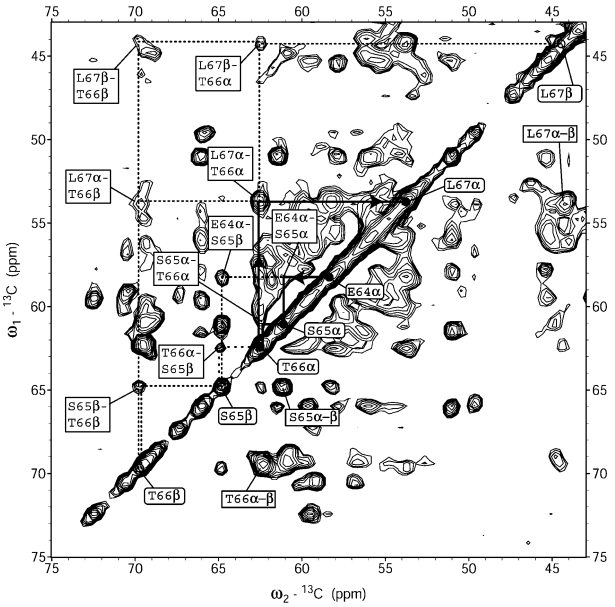

Fig. 4 (^{13}C,^{13}C) correlation experiment conducted under weak (C′,Cα) coupling conditions described in the text on U-[^{13}C,^{15}N]-labeled ubiquitin. Sequential (Cα,Cα) (Cα,Cβ) or (Cβ,Cβ) correlations are indicated on the left of the diagonal. Intra-residue assignments are included in grey on the right side of the diagonal. During evolution and detection periods, TPPM[46] decoupling was used at 90 kHz. Following procedures described in refs. 14 and 49, 8 mg of ubiquitin were precipitated from MPEG.

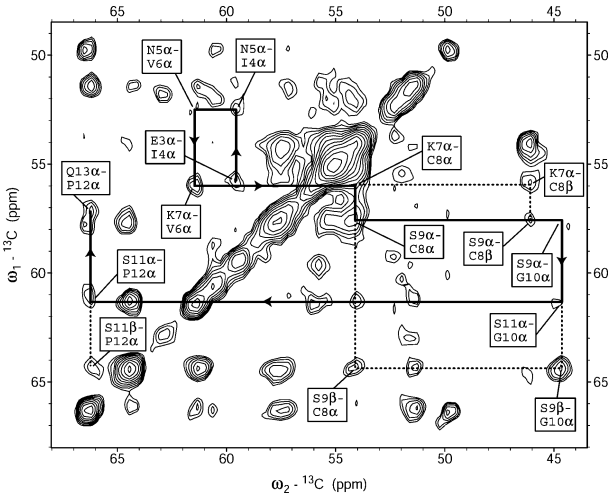

Fig. 5 800 MHz 2D (^{13}C,^{13}C) correlation experiment conducted on U-[^{13}C,^{15}N]-labeled Kaliotoxin, under weak (C′,Cα) coupling conditions. A longitudinal mixing time of 150 ms was used. Temperature and MAS rate were set to −15 °C and 12.5 kHz, respectively. During evolution and detection periods, spinal 64[50] decoupling was used at 83 kHz. 6 mg of U-[^{13}C,^{15}N] labeled KTX were measured after rehydration.

In Fig. 4, results are shown for the 76-residue protein ubiquitin obtained on a 600 MHz WB instrument (Bruker Biospin Germany) using an MAS rate of 9.375 kHz (*i.e.* 125 ppm/2, see Fig. 1) and a mixing time of 100 ms. Line broadening effects can be neglected and a variety of inter-residue Cα–Cα and Cα–Cβ cross peaks are readily observed. These dependencies are highlighted in Fig. 4 for the 4 amino-acid segment E64-L67. Inter and intra-residue correlations are indicated on the left and right side of the diagonal, respectively. The backbone Cα–Cα 'walk' is facilitated by the occurrence of sequential (Cα,Cβ) and (Cβ,Cβ) correlations that can be used to cross validate the assignment procedure. We note that the resulting spectral assignments have been confirmed by additional NC correlation experiments[47] and largely agree with chemical shift values reported recently by McDermott *et al.*[16]

Fig. 5 contains results obtained on an 800 MHz narrow-bore instrument (Bruker Biospin, Germany) on a uniformly (^{13}C,^{15}N) labeled sample of the 38-residue protein Kaliotoxin.[48] Again, the MAS rate was placed close to the $n = 2$ condition (12.5 kHz) and a mixing time of 150 ms was employed. A variety of sequential (Cα,Cα) correlations can be readily identified. Cross validation using a series of NCACB and NCOCA experiments reveals that these correlations again correspond to $(i,i-1)$ connectivities, for example of the eleven-residue stretch E3-Q13 highlighted in Fig. 5.

In conclusion, we have described a set of experimental conditions that permit establishing inter-residue correlations under MAS conditions from a single (^{13}C,^{13}C) correlation experiment. For a given $\boldsymbol{B}_0$ field, the technique relies on establishing weak (C′,Cα) coupling conditions using a defined range of MAS rates. Once sequential (C′,Cα) transfer is achieved, additional correlations (mediated by proton-driven spin diffusion) between side chain moieties are possible. As confirmed by further test experiments, simply increasing the selected mixing time leads to the occurrence of additional inter-residue correlations, including (i,i + 2) peaks.

Such extensions can greatly facilitate the spectral assignment process and are only limited by T_1 relaxation. Since no ^{15}N evolution and mixing periods are required, the overall signal to noise ratio can be significantly improved. Experiments can be performed on uniformly labeled protein samples at various magnetic fields. In the presented applications, the MAS rate was selected based on statistical considerations. In the presence of (C′,Cα) chemical shift variations, for example, due to the influence of secondary structure or hydrogen bonding, adjustments of MAS rate and mixing time may be necessary. Such conditions were successfully applied to the proteins considered here and reveal that weak (C′,Cα) coupling conditions can be established for a variety of experimental setups. Compared to sequential resonance assignments obtained from chemical-shift selective (N,C) correlation spectroscopy,[47] 2D (^{13}C,^{13}C) data recorded under weak coupling conditions contain both intra- and inter-residue correlations. However, both contributions can be easily separated by recording an additional 2D spectrum for short mixing times (see Fig. 3) or by using MAS rates $\omega_R/\omega_0 \gg \Delta\Omega$.

The transfer scheme can be easily incorporated into other, multi-dimensional correlation schemes without loss in spectral resolution. In particular, these experiments may provide a useful reference for the analysis of proton-mediated (^{13}C,^{13}C) correlation experiments.[5,51] In addition to the applications discussed here, weak coupling conditions may also be employed to monitor correlations between C′ spins and methyl or aromatic side-chain carbons.

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