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^{13}C Direct Detected NMR Increases the Detectability of Residual Dipolar Couplings

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^{13}C direct detected *protonless* NMR spectroscopy[1] is a powerful tool to characterize systems where ^{1}H signals are difficult to analyze, such as paramagnetic systems,[2−4] unfolded proteins,[5] systems where H^N signals cannot be observed due to exchange broadening,[6] and possibly molecules with large size. In these cases, structural restraints such as NOEs and dihedral angles may be limited. Residual dipolar couplings (*rdc*) have been shown to be precious as structural restraints and as parameters to investigate dynamics.[7,8] Usually, *rdc* are measured through $^{1}H^N$ detected double and triple resonance experiments.[9−11] Such experiments require relatively long acquisition/evolution/coherence transfer delays, and therefore, when efficient relaxation mechanisms are operative and $^{1}H^N$ NMR lines are increasingly broad, *rdc* may become undetectable. Here, we address the determination of *rdc* through ^{13}C direct detection, which we propose for all or almost all systems mentioned above. *Rdc* of $C^\alpha-C^\beta$ and $C^\alpha-C'$, already described through ^{1}H detection,[10] are a straightforward result of our approach of $^{13}C-^{13}C$ spin decoupling.[1] Furthermore, we show that $H^\alpha-C^\alpha$ and H^N-N *rdc* can be obtained through ^{13}C detection even when the proton lines are very broad, or even not detected. This set of four *rdc* is particularly useful, as it provides the angles around C^α and the orientation of the peptide planes.

The homonuclear $^{1}J_{C\alpha C'}$ and $^{1}J_{C\alpha C\beta}$ coupling constants are large enough to be easily measured from the doublet splitting, e.g., using IPAP schemes.[9] In the direct dimension, *protonless* ^{13}C detected NMR experiments such as CACO-IPAP[1] in C′ detection (for $^{1}J_{C\alpha C'}$ measurements) and COCA-DIPAP[1] in C^α detection ($^{1}J_{C\alpha C\beta}$) can be used. When ^{13}C detected experiments are designed to measure large ^{1}J couplings, the intrinsically lower sensitivity of ^{13}C detection is more than compensated by the robustness of the experiments with respect to signal loss due to fast relaxation.

$^{1}J_{HN}$ and $^{1}J_{H\alpha C\alpha}$ are even larger than homonuclear C−C couplings and therefore would be the most useful if ^{1}H signals were not broad beyond *rdc* detectability. However, the corresponding ^{13}C signals are not as broad.[12] We show here that several $^{1}J_{HN}$ and $^{1}J_{H\alpha C\alpha}$ not visible in ^{1}H experiments can be recovered by heteronuclear detection and proton-recoupled ^{13}C detected experiments. To this end, two novel pulse sequences have been developed to obtain proton-recoupled ^{13}C detected NMR experiments, where the coupling to hydrogen nuclei is involved only during t_1 evolution.

The pulse sequences shown in Figure 1 use ^{13}C detection to measure $^{1}J_{H\alpha C\alpha}$ and $^{1}J_{HN}$ couplings. For $^{1}J_{H\alpha C\alpha}$ the experiment is based on a variant of the CACO-IPAP pulse sequence.[1] Proton broadband decoupling is switched off during t_1 in order to let the $H^\alpha-C^\alpha$ coupling develop during C^α evolution. A selective $^{13}C^\beta$ 180° pulse is applied after the $^{13}C'$ pulse to refocus both $^{1}J_{C\alpha C\beta}$ and $^{1}J_{C\alpha C'}$ couplings.[13] Observed signals are not affected by ^{1}H transverse relaxation, because ^{1}H are reintroduced as passive spins and are never excited. Longitudinal ^{1}H relaxation is operative only during the relatively short evolution period while signal decays during the longer preparation, mixing and detection periods are driven by $^{13}C'$ transverse relaxation rates. As this is a variant of a *protonless* experiment in which HC couplings are reintroduced, we called this experiment ReCACO.

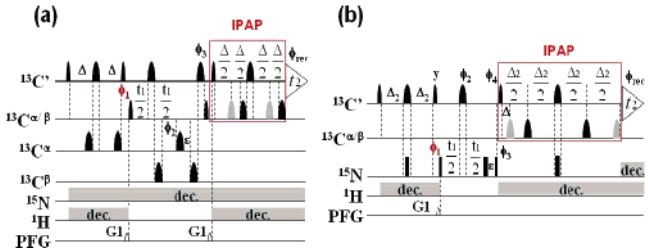

Figure 1. ReCACO (a), and ReCON (b). Narrow and wide round bars represent $\pi/2$ and π selective shaped pulse. $\Delta = 4.5$ ms, $\Delta_2 = 13$ ms, $\epsilon = t_1(0)$. Pulse field gradients were 0.8 ms long, with maximum intensities of 25 G/cm. Black and gray pulses in the IPAP building blocks indicate pulses to obtain in-phase term and anti-phase terms, respectively.[9,14] Phase cycles for ReCACO: $\phi_1 = x, -x$, $\phi_2 = 4x, 4y$, $\phi_3 = 2x, 2(-x)$ for IP, $\phi_3 = 2(-y)$, $2y$ for AP and $\phi_{rec} = x, -x, -x, x, -x, x, x, -x$; for ReCON: $\phi_1 = x, -x$, $\phi_2 = 2x, 2(-x)$, $\phi_3 = 4x, 4(-x)$, $\phi_4 = x$ for IP, $\phi_4 = -y$ for AP and $\phi_{rec} = x, -x, -x, x, -x, x, x, -x$. Experimental details are reported in the Supporting Information.

Likewise, the original CON-IPAP[4,15] was modified during the t_1 period to encode $^{1}J_{HN}$ couplings in the indirect dimension. Also in HNCA- and HSQC-type experiments, which are commonly used to measure $^{1}J_{H\alpha C\alpha}$ and $^{1}J_{HN}$,[9,11] the couplings are encoded during C^α and N evolutions. Therefore, ^{13}C direct detected experiments can be performed with the same resolution available in conventional ^{1}H detected experiments.

This approach is tested on the dicalcium protein calbindin D_{9k}, where the native Ca^{2+} ion at site II has been substituted with Tm^{3+} (CaTmCb). ^{1}H NMR signals may be already too broad at distances as large as 17 Å from the lanthanide center.[16] CaTmCb undergoes extensive self-orientation in high magnetic fields, inducing sizable *rdc*. This is an ideal testing sample representative of a number of cases in which ^{1}H relaxation is fast. In fact, the approach here described is general and holds for any molecules in any orienting device, including membrane proteins aligned via paramagnetic centers.[17] Even if the H^N is not detected because of exchange, the corresponding couplings can be detected.

Table 1 summarizes the number of peaks and couplings in CaTmCb that could be observed in ^{1}H and ^{13}C detected experiments, respectively. As witnessed by the number of observed peaks, paramagnetic relaxation prevents signal detection for about 50% of amino acids.[16] Recoupled experiments gave ca. 30% increase in the number of observed couplings. One hundred and thirty-six residual dipolar couplings could be obtained via ^{13}C detection experiments (see Table S1) vs 102 in ^{1}H detected experiments (152

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Table 1. Signals Observed and *rdc* Measured in ^{1}H and ^{13}C Experiments

	$^{1}J_{H\alpha C\alpha}$		$^{1}J_{HN}$		$^{1}J_{C\alpha C'}$		$^{1}J_{C\alpha C\beta}$		total	
	obs.	meas.	obs.	meas.	obs.	meas.	obs.	meas.	obs.	meas.
^{1}H	32	25	34	30	33	31	23	16	122	102
^{13}C	39	36	39	34	42	39	32	27	152	136

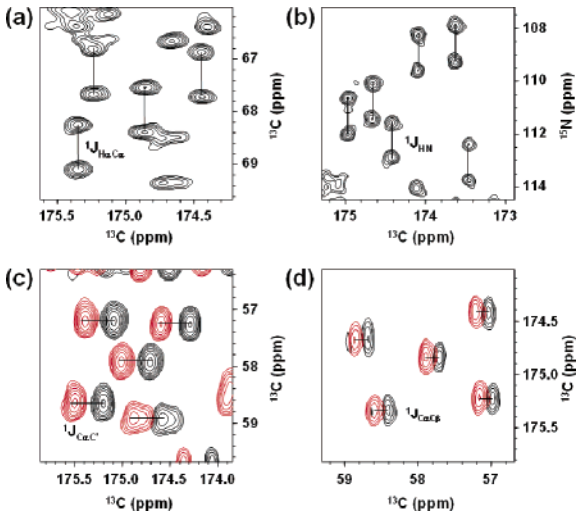

Figure 2. Selected regions of ReCACO (a), ReCON (b), CACO-IPAP (c), and COCA-DIPAP (d) spectra for CaTmCb sample (1 mM) at 300 K and 175 MHz.

vs 122 when we consider signals observed in reference experiments but for which *rdc* could not be measured due to overlaps). For well-isolated $^{13}C'$ signals, the $^{1}J_{C\alpha C'}$ could be measured also from IPAP versions of one-dimensional (1D) ^{13}C experiments. Overall, this has allowed us to measure *rdc* involving ^{1}H signals as large as 150 Hz. Such significant results represent further possibilities for investigating difficult systems. Noteworthy, the *rdc* dataset obtained from ^{1}H-based experiments is not co-incident with that obtained via ^{13}C detection. Therefore, both approaches can be used synergistically. We report in Figure 2 selected regions of 2D spectra. The selected columns of the 2D spectra are reported in Figure S1 to appreciate the difference in signal intensity for signals that are affected by substantial line broadening contributions. This is the case, for example, for Lys 29 and Gly 42, where $^{1}J_{H\alpha C\alpha}$ and $^{1}J_{HN}$ are not detectable in the standard ^{1}H detected experiments, while ReCON and ReCACO provide a reliable estimate of the coupling.

The four ^{1}J values that can be obtained through the protonless and proton-recoupled experiments described here (Table 1), taken together, efficiently restrain backbone dihedral angles. It is in fact well-known that three of the four *rdc* between C^{α} and its bound nuclei are in principle enough to provide the fold of the protein backbone, if measured with sufficient accuracy with two alignment tensors.[18,19] In addition, the $H^{N}-N$ *rdc* can fix the orientation of the peptide planes.

Sample calculations have been performed to analyze the impact of ^{13}C-derived *rdc* in the present protein using an upgraded version of the program PARAMAGNETIC CYANA[20] (see Supporting Information and Figures S1−S3). A structure calculated using ca. 1800 NOE (obtained for the Ce^{3+}-substituted derivative) has a backbone rmsd of 0.68 Å. The addition of the 136 *rdc* obtained via ^{13}C detection decreases the rmsd to 0.50 Å (Table S2). The improvement was most remarkable on the relative orientation of the four helices. Figure 3 shows the good agreement between observed and best-fit *rdc*, calculated from the refined structure, discussed in detail in the Supporting Information.

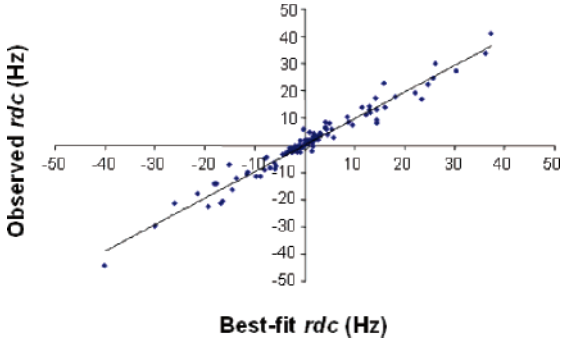

Figure 3. Correlation between calculated and observed $H^{\alpha}-C^{\alpha}$, $H^{N}-N$, $C^{\alpha}-C'$, and $C^{\alpha}-C^{\beta}$ residual dipolar couplings.

In summary, due to the smaller magnetogyric ratio, carbon nuclei suffer less than protons from line broadening. The availability of ^{13}C direct detected spectra provides an alternative method for the measurement of *rdc*. With the present approach, residual dipolar couplings can be obtained with a precision which is as good as that achieved with ^{1}H detection, but with the additional advantage that ^{1}J couplings can be precisely measured also for broad ^{1}H resonances. This is a further step in the general strategy of ^{13}C direct detection for structure and dynamics determination. Of course, in paramagnetic molecules these *rdc* values beautifully complement pseudocontact shifts and relaxation data.

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Supporting Information Available: Experimental details for ^{1}H and ^{13}C experiments, *rdc* measured for CaTmCb, structure calculation protocol, and structural statistics. This material is available free of charge via the Internet at http://pubs.acs.org.

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