Residual Dipolar Coupling TOCSY for Direct Through Space Correlations of Base Protons and Phosphorus Nuclei in RNA

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Established residual dipolar coupling measurements in partially aligned molecules report on the relative orientation of ^{1}H−^{15}N and ^{1}H−^{13}C bond vectors of known lengths with respect to the molecular alignment tensor,[1] and are increasingly used in the structure determination of oligonucleotides by NMR.[2] Recently, methods for the measurement of homonuclear ^{1}H−^{1}H through space dipolar couplings in anisotropic media were introduced.[3] In addition, homonuclear TOCSY sequences can be used to transfer magnetization through dipolar couplings between protons that are not scalar coupled.[4]

Here we extend the observation of dipolar couplings in partially aligned molecules to heteronuclear through space interactions, specifically ^{1}H−^{31}P residual dipolar couplings, which directly link the phosphate backbone and base protons in oligonucleotides. We introduce the RADAR-TOCSY (*r*esidu*a*l *d*ipol*a*r coupling *t*ot*a*l *c*orrelation *s*pectroscop*y*) experiment for the direct observation of through space heteronuclear dipolar couplings in weakly aligned molecules. In analogy to the indirect scalar spin−spin coupling interaction $\hat{H}_J$, the dipolar coupling interaction between a given pair of unlike spin-$^1/_2$ nuclei I,S is expressed by the dipolar Hamiltonian $\hat{H}_D$

$$\hat{H}_D = 2\pi D_{IS}\{2I_zS_z - I_xS_x - I_yS_y\}$$

providing the physical basis for in-phase coherence transfer using heteronuclear Hartmann−Hahn sequences.[5] In the weak coupling limit, the truncated dipolar $\hat{H}_D$ and the truncated scalar coupling Hamiltonian $\hat{H}_J$ have an identical form. Here, we show for the first time that even in the absence of any isotropic scalar J_{IS} coupling, heteronuclear TOCSY in weakly aligned molecules is applicable for coherence transfer between unlike spins through sufficiently large dipolar couplings.

The RADAR-TOCSY sequence (see Figure S1 in the Supporting Information) is similar to the Hetero-TOCSY experiment proposed by Kellog for the correlation of ^{31}P and ^{1}H ribose resonances in RNA through heteronuclear *J* cross polarization in isotropic solution.[6] After presaturation of the ^{1}H resonances with a series of 120° pulses during the recycle delay, ^{31}P resonances are excited and subsequently evolve chemical shift during t_1, where heteronuclear H,P couplings are refocused by a 180° proton pulse in the middle of the t_1 period. After a *z*-filter, a band-selective heteronuclear planar effective coupling Hamiltonian is created by using DIPSI-2.[7] The proton carrier is placed in the middle of the aromatic region and a sufficiently low radio-frequency amplitude is used to achieve efficient in-phase coherence transfer between ^{31}P and base ^{1}H6,8 protons while avoiding magnetization leakage due to competing heteronuclear H,P and homonuclear H,H couplings. ^{1}H6,8 protons are detected during the acquisition period t_2.

One severe drawback of high-resolution NMR studies in partially aligned molecules is the increasing number of H,H dipolar couplings that leads to line broadening and complex spectra. However, the nonexchangeable proton spectrum of RNA exhibits two well-separated regions consisting of base ^{1}H2,6,8 and ribose ^{1}H1′,2′,3′,4′,5′,5″ resonances (including ^{1}H5), respectively. The significant line broadening of the observed ^{1}H6,8 proton resonances due to remote residual H,H dipolar couplings can be effectively removed by homonuclear band-selective time-shared adiabatic decoupling during the acquisition period t_2.[8]

Figure 1 shows RADAR-TOCSY spectra of 1.5 mM unlabeled HIV-2 TAR 30-mer RNA partially aligned in a Pf1 filamentous bacteriophage (ASLA Ltd., Riga, Latvia) solution of ∼70 mg/mL (observed ^{2}H doublet splitting due to incomplete averaging of ^{2}H quadrupolar coupling: 67 Hz).[11] Another sample was prepared with a lower degree of alignment by adding ∼25 mg/mL (observed ^{2}H doublet splitting: 23 Hz) Pf1 phage. The comparison of a RADAR-TOCSY spectrum without (A) and with (B) homonuclear decoupling verifies the ability to observe direct through space correlations between base ^{1}H6,8 proton and ^{31}P nuclei in RNA and the efficiency of the band selective homonuclear decoupling during t_2. It should be noted that all observed ^{1}H6,8 proton resonances in Figure 1B experience Bloch−Siegert shifts due to the off-resonant homonuclear decoupling during the acquisition period. Observable quadrupolar splittings as well as dipolar couplings scale approximately linear with the phage concentration.[12] Through space correlations between ^{1}H6,8 protons and ^{31}P nuclei were observable at lower phage concentrations, though the effective reduction of residual D_{PH} dipolar couplings by roughly a factor of 3 resulted in spectra of poor quality (see Figure S2 in the Supporting Information).

Dipolar couplings depend on both the orientation of the internuclear ^{31}P−^{1}H vector with respect to the molecular alignment tensor and the time averaged internuclear distance r_{PH}. RNA structures exhibit a wide range of ^{31}P−^{1}H6,8 distances that can potentially give rise to observable correlations. The HIV-2 TAR RNA structure in the absence of ligands is defined by two A-form helical stems, with a 50° bend due to a two-nucleotide bulge, and capped by a hexanucleotide loop.[13] Intranucleotide ^{1}H6,8(i)−^{31}P(i) distances taken from the average solution structure of the

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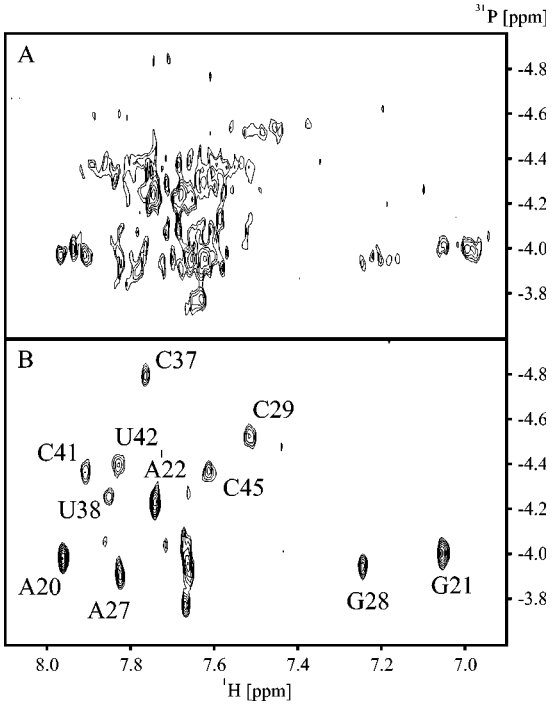

Figure 1. Expansion of RADAR TOCSY spectra showing ^{1}H6,8 proton to ^{31}P cross-peaks. Spectra were recorded on 1.5 mM unlabeled partially aligned HIV-2 TAR RNA (A) without and (B) with HS homonuclear band selective time-shared adiabatic decoupling during t_2. Unambiguous assignments are given with the one-letter code followed by the residue number. Adiabatic HS hyperbolic secant inversion[9] pulses of duration 6.67 ms covering a bandwidth of 1400 Hz were applied at 4.88 ppm with a 10% duty cycle (pulse/delay ratio of 1/9). Pulses were expanded according to a 20-step supercycle. Otherwise, experiments were collected under identical conditions. Carrier positions in the present work were −3.64 ppm for ^{31}P and 7.64 ppm for^{1}H, respectively. High-power proton pulses were applied with a field strength of 27.47 kHz, while high-power ^{31}P pulses were applied with a field strength of 6.41 kHz. The low-power selective DIPSI-2 mixing sequence[7] was applied for $\tau_m = 73.9$ ms, using a $\gamma B_1/2\pi = 1.17$ kHz field strength. Thirty-two complex points were recorded with an acquisition time of 32.0 ms for ^{31}P (ω_1), and 1024 complex points with an acquisition time of 171.5 ms for ^{1}H (ω_2). A presaturation sequence of 600 120° pulses evenly separated by 5 ms between transients was used, with 160 scans per complex increment (total measuring time 9.5 h). All spectra were recorded on a three-channel Varian Inova 600 MHz spectrometer equipped with an actively shielded *z*-gradient triple-resonance probe, at a temperature of 298 K. Spectra were processed and analyzed with FELIX 2000 (MSI, San Diego, USA) and NMRPipe program packages.[10] The sample buffer contained 10 mM phosphate buffer, pH 6.4, 50 mM sodium chloride, and 0.1 mM EDTA in 500 mL of 99.9% D_2O.

HIV-2 TAR argininamide complex[14] range from 3.10 Å for G_{28} to 6.22 Å for U_{25}, located in the two-nucleotide bulge, with an average ^{1}H6,8(i)−^{31}P(i) distance ($n = 29$) of 3.9 ± 0.9 Å. The average intranucleotide distance for A-form helical segments is even shorter ($r_{HP} = 3.6 \pm 0.5$ Å, $n = 21$) and shows little variation with respect to differing base types. Based on observed one-bond residual D_{NH} dipolar couplings using a ^{15}N-labeled sample and neglecting effects arising from differing vector orientations and motional properties, the expected magnitude of residual D_{PH} dipolar couplings may be as large as 2 or 6 Hz for our weakly and moderately aligned system, respectively.

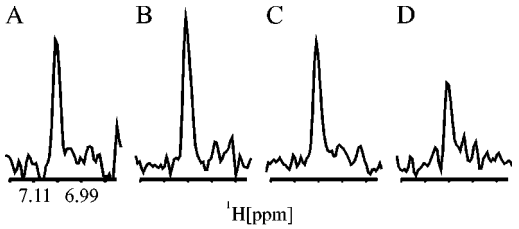

Figure 2. One-dimensional build ups taken along $\delta(^{31}P) = -4.01$ of the G_{21}H8,P resonance as a function of the heteronuclear DIPSI-2 mixing time τ_m in RADAR TOCSY. Experimental details and sample conditions are as given in the legend of Figure 1. The heteronuclear DIPSI-2 mixing time t_m was varied as a multiple n of the cycle length: (A) $n = 2$, $\tau_m =$ 49.3 ms, (B) $n = 3$, $\tau_m = 73.9$ ms, (C) $n = 4$, $\tau_m = 97.5$ ms, and (D) $n = 5$, $\tau_m = 121.2$ ms, respectively.

As a consequence of the scaled coupling constants a complete magnetization transfer in the RADAR-TOCSY is achieved by using a mixing time of $\tau_m = 1/D_{PH}$, assuming planar effective coupling tensors in a heteronuclear two-spin system.[15] However, transfer efficiencies under effective heteronuclear planar mixing conditions are complex functions of the relaxation of involved magnetization components, transfer functions, and radiofrequency inhomogenieties. A series of one-dimensional cross-sections of the G_{21}H8,P resonance as a function of the mixing time τ_m for the heteronuclear TOCSY is shown in Figure 2. Empirically derived optimal one-step transfer efficiency is obtained after 74 ms. Methods for quantification of D_{PH} are currently being worked out in our laboratory.

In summary, the proposed RADAR-TOCSY allows the first direct observation of through space correlations between ^{1}H6,8 protons and ^{31}P nuclei due to residual D_{PH} dipolar couplings. Heteronuclear Hartmann−Hahn sequences represent a novel building block for the correlation of unlike spins in partially aligned molecules through residual dipolar couplings. We applied a homonuclear decoupling scheme that greatly facilitates high-resolution NMR studies on partially aligned RNA. The experiment does not require any isotope enrichment, and detectable D_{PH} dipolar couplings rely on nonexchangeable protons that are uniformly distributed along an oligonucleotide sequence. The quantification of the D_{PH} dipolar couplings will provide orientation and distance information, and will have a substantial impact on the precision of oligonucleotide structure determination by NMR in terms of defining both global and local structural features.

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Supporting Information Available: Two figures showing the applied RADAR-TOCSY pulse sequence (S1) and a RADAR-TOCSY spectrum obtained in 25 mg/mL Pf1 phage (S2) (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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