Triple-Resonance Experiments for Assignment of Adenine Base Resonances in $^{13}C/^{15}N$-Labeled RNA

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The first step in a solution structure determination of a biomolecule involves assignment of the NMR spectrum. Through-bond experiments have become the dominant method for resonance assignment of $^{13}C/^{15}N$-labeled proteins.[1] Isotopic labeling of RNA[2,3] and DNA[4] has led to analogous through-bond assignment techniques in nucleic acids including (i) correlation of the sugar spin systems, (ii) intranucleotide correlation of sugar and base resonances, and (iii) correlation sugar and phosphate resonances on neighboring residues.[5] Here we present novel 2D and 3D HNC-TOCSY-CH NMR experiments for correlating the amino nitrogen resonances to the H8-(C8)−H2(C2) resonances in adenines bases. We recently demonstrated improved solution structure determinations of RNA by detection of NOEs involving exchange-broadened amino protons on adenine and guanine bases.[6] To make use of these new NOE data, one must assign the amino nitrogens. The through-bond experiments presented here not only allow unambiguous resonance assignment of adenine amino nitrogens but also represent a superior method for correlating adenine H8 and H2 resonances than previously published techniques.[7,8] High-sensitivity HNC-TOCSY-CH spectra are presented for two isotopically labeled RNAs, a 30-nucleotide lead-dependent ribozyme known as the leadzyme[9] and a 48-nucleotide hammerhead ribozyme−substrate complex.[10,11]

The HNC-TOCSY-CH experiment employs the magnetization transfer pathway schematically illustrated in Figure 1a using the pulse sequence given in Figure 2. The experiment starts on the amino protons, and magnetization is transferred to the amino nitrogen by a $^1H-^{15}N$ cross-polarization sequence.[12−14] In most nucleic acids the amino proton resonances in adenines have very large line widths (>80 Hz) because of rotation of the amino group. This exchange broadening leads to inefficient transfer ^1H magnetization to the covalently bound ^{15}N by a standard INEPT procedure. However, as previously discussed,

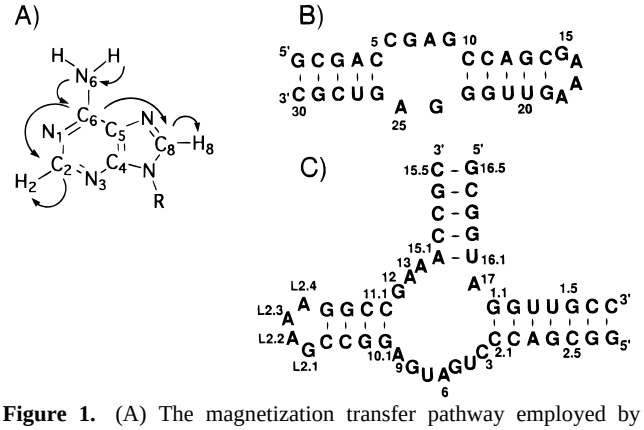

Figure 1. (A) The magnetization transfer pathway employed by the HNC-TOCSY-CH experiment. (B) Sequence and secondary structure of the $^{13}C/^{15}N$-labeled leadzyme.[9] (C) The hammerhead complex consisting of a $^{13}C/^{15}N$-labeled ribozyme and a ^{15}N-labeled substrate.[10,11]

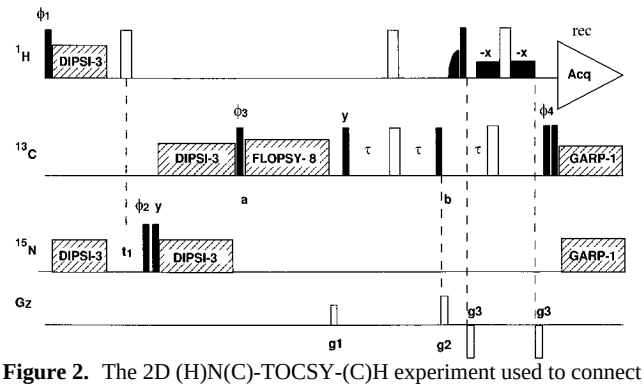

Figure 2. The 2D (H)N(C)-TOCSY-(C)H experiment used to connect amino nitrogen N6 to H2 and H8 resonances. The 90° and 180° pulses are indicated by filled and open rectangles, respectively. All pulses have phase = x unless otherwise indicated. The phase cycle is $\phi_1 = 2(y), 2(-y)$; $\phi_2 = y, -y$; $\phi_3 = 4(y), 4(-y)$; $\phi_4 = 8(x), 8(-x)$ and receiver = $x, 2(-x), x, -x, 2(x), -x$. A 7.48 ms DIPSI-3[14] (utilizing a R, −R half supercycle) at a RF field strength of 1.9 kHz was used for the $^1H-^{15}H$ hetero-TOCSY period. The $^{15}N-^{13}C$ transfer was performed with a 44.9 ms DIPSI-3 sequence at a RF strength of 1.9 kHz. A 37.8 ms FLOPSY-8 sequence[21] at a RF strength of 5 kHz was used during the ^{13}C homonuclear TOCSY period. The delay τ equals 1.25 ms. At point b, water flip back is achieved with a 2.9 ms selective E-BURP pulse.[18,22] A WATERGATE sequence[17] partially concatenated with the last INEPT period is used to suppress the residual water signal. Two 1.55 ms soft square pulses are applied at the water frequency during this WATERGATE sequence. During the detection period, ^{13}C and ^{15}N GARP1 decoupling is applied at RF fields of 1.6 and 1.14 kHz, respectively. The ^1H frequency is set to 7.2 ppm for the $^1H-^{15}N$ hetero-TOCSY period and shifted to 4.9 ppm at point a. The ^{15}N frequency is set to 81 ppm during the $^1H-^{15}N$ hetero-TOCSY period and shifted to 195 ppm at point a. The ^{13}C frequency is positioned at 161 ppm for the $^{15}N-^{13}C$ hetero-TOCSY period and is shifted to 145 ppm at point a for the ^{13}C-TOCSY period and to 142 ppm for the acquisition period. All gradients were applied along the z axis: g1 = 12 G/cm, g2 = 24 G/cm, and g3 = −32 G/cm. The gradient times for g1, g2, and g3 were 300, 300, and 450 μs, respectively. Each gradient was followed by a recovery time of 200 μs.

^1H magnetization for exchange-broadened resonances can be efficiently transferred to ^{15}N by either a cross-polarization or a CPMG-INEPT sequence.[6,15,16] Here, a $^1H-^{15}N$ cross-polarization sequence transfers magnetization from the amino exchange-broadened protons to the amino nitrogen. The nitrogen is then

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frequency labeled during the t_1 evolution period after which a $^{13}C-^{15}N$ cross-polarization sequence transfers magnetization from N6 to C6. A ^{13}C TOCSY sequence then transfers magnetization among all the carbons in the adenine ring. The carbon magnetization is transferred by INEPT to H8 and H2 for detection, with a flip-back WATERGATE sequence used as the final refocusing period to selectively suppress the H_2O signal.[6,17,18]

The 2D spectrum for the leadzyme (Figure 1B) is shown in Figure 3A, where the adenine amino nitrogen resonances show connectivities to both their H2 and H8 resonances. Six of seven adenine amino nitrogen were observed in the leadzyme. As previously reported, additional structural constraints were obtained in the leadzyme through observation of NOEs to exchange-broadened amino protons in a 2D ^{15}N-correlated NOESY spectrum.[6] This procedure requires assignment of the amino nitrogen resonances; therefore an important application of the HNC-TOCSY-CH experiment is unambiguous assignment of adenine amino nitrogens. As illustrated in Figure 3A, the amino nitrogens were unambiguously assigned because the H2 and H8 resonances have been previously assigned in the leadzyme.[7,11] The only adenine that was not observed in the leadzyme was A25. This adenine is involved in a protonated A^+-C base pair and exhibits dynamics on the microsecond timescale[11,19] which may lead to low sensitivity in the HNC-TOCSY-CH spectrum.

This HNC-TOCSY-CH experiment was designed to correlate the adenine N6 and H8 resonances. However as seen in Figure 3A each adenine N6 resonance shows cross peaks to both its H8 and H2 resonance, which means that this experiment also efficiently correlates adenine H2 and H8 resonances. HCCH-TOCSY experiments have been previously used to correlate adenine H2 and H8 resonances in ^{13}C-labeled RNAs.[7,8] However the sensitivity of the HCCH-TOCSY experiment is much lower than the HNC-TOCSY-CH experiment because the former requires magnetization transfer through two small $^2J_{CC}$ coupling constants whereas the latter transfer occurs via only one $^2J_{CC}$ coupling constant. To illustrate this improved sensitivity, Figure 3B shows the 2D (H)N(C)-TOCSY-(C)H spectrum on the hammerhead ribozyme−substrate complex (Figure 1C). We were unable to observe any H2−H8 correlations using a 2D HCCH-TOCSY experiment on the hammerhead due to fast ^{13}C relaxation. However many H2−H8 correlations are observed with the 2D (H)N(C)-TOCSY-(C)H experiment including all three adenines in the GAAA tetraloop. As seen in Figure 3B, the H2 resonances for $A_{L2.2}$ and $A_{L2.3}$ have similar chemical shifts in the hammerhead. To resolve this ambiguity we acquired a 3D (^{15}N, ^{13}C, ^1H) (H)N(C)-TOCSY-CH on the hammerhead. Figure 3C shows one 2D plane of this 3D spectrum which contains the C8 and C2 resonances for $A_{L2.2}$. These spectra on the hammerhead ribozyme show that the HNC-TOCSY-CH experiment can be successfully applied to larger RNAs.

The HNC-TOCSY-CH experiment represents an efficient procedure for identifying adenine resonances in isotopically labeled RNAs. This adenine-specific experiment complements recently reported uridine-specific and cytidine-specific HNC-CCH experiments.[20] These three base-specific experiments provide important starting points in the sequential resonance assignment of nucleic acids, and together they substantially simplify the resonance assignment of $^{13}C/^{15}N$-labeled RNAs.

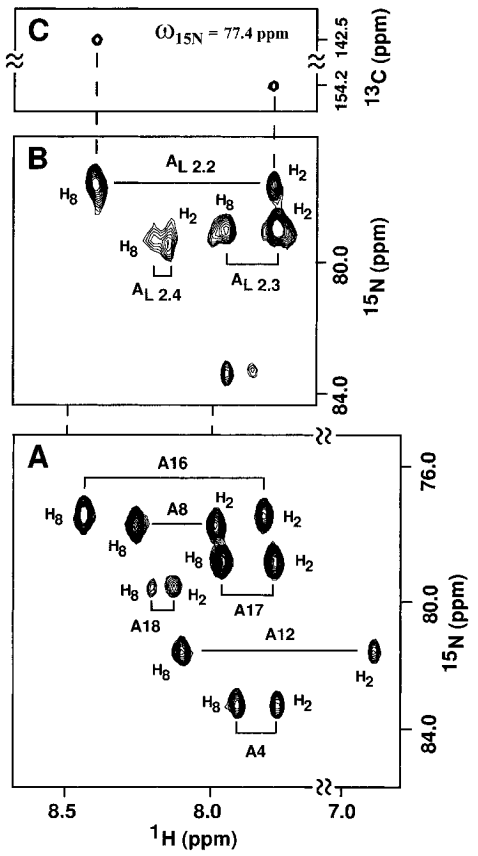

Figure 3. (A) The 2D (H)N(C)-TOCSY-(C)H spectrum of the 1.8 mM $^{13}C/^{15}N$-labeled leadzyme (Figure 1B). The H2 and H8 frequencies in ω_2 are correlated by their shared N6 frequency in ω_1 for all adenine nucleotides except A25 (see text). Spectral widths in the ^1H and ^{15}N dimensions were 6000 and 2750 Hz, respectively. Quadrature detection in ω_1 was obtained with the TPPI-States method.[23] The total experimental time was 19 h with 80 complex t_1 points, 512 complex t_2 points, 360 scans per FID, and a relaxation delay of 1 s. (B) The 2D (H)N-(C)-TOCSY-(C)H spectrum of a 1.5 mM hammerhead ribozyme−substrate complex (Figure 1C). The ribozyme is $^{13}C/^{15}N$-labeled, and the substrate is ^{15}N-labeled. The spectrum was acquired with the same parameters as the leadzyme except that 392 transients and 100 complex t_1 points were acquired for a total experimental time of 24 h. (C) A [$^{13}C(\omega 3)$, $^1H(\omega 3)$] plane at the $^{15}N(\omega 1)$ frequency of $A_{L2.1}$ in the 3D (H)N(C)-TOCSY-CH spectrum of the hammerhead. The experiment was acquired with 44 t_1 complex points, 44 t_2 complex points, and 512 t_3 points in 44 h. The pulse sequence is similar to that in Figure 2 except that a t_2 evolution period was included in the first INEPT following the FLOPSY-8 sequence (see Supporting Information). The ^{13}C carrier was set to 149.5 ppm just after the FLOPSY-8 sequence. The relaxation delay was 1.1 s, and 16 transients were acquired for each FID. The spectral width in the ^{15}N dimension was 1500 Hz, and the other parameters were the same as those for the 2D experiment. The $^{13}C/^{15}N$-labeled RNAs were synthesized as previously described.[2,11] All spectra were collected at 15 °C on a Varian Unityplus 500 MHz spectrometer and processed on a Silicon Graphics computer with the program FELIX 2.35 (Biosym Inc.).

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Supporting Information Available: Pulse sequence for the 3D (H)NC-TOCSY-CH experiment (2 pages). Ordering Information is given on any current masthead page.

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