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Article

Characterisation of hydrogen bonding networks in RNAs via magic angle spinning solid state NMR spectroscopy

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Abstract

It is demonstrated that the spatial proximity of ^{1}H nuclei in hydrogen bonded base-pairs in RNAs can be conveniently mapped via magic angle spinning solid state NMR experiments involving proton spin diffusion driven chemical shift correlation of low gamma nuclei such as the imino and amino nitrogens of nucleic acid bases. As different canonical and non-canonical base-pairing schemes encountered in nucleic acids are characterised by topologically different networks of proton dipolar couplings, different base-pairing schemes lead to characteristic cross-peak intensity patterns in such correlation spectra. The method was employed in a study of a 100 kDa RNA composed of 97 CUG repeats, or $(CUG)_{97}$ that has been implicated in the neuromuscular disease myotonic dystrophy. $^{15}N-^{15}N$ chemical shift correlation studies confirm the presence of Watson–Crick GC base pairs in $(CUG)_{97}$.

Introduction

RNA plays a central role in many biological processes and exhibits a variety of secondary and tertiary structural features that are stabilised via hydrogen bonding (Gesteland et al., 1999). The development of efficient methods for the synthesis of isotopically labelled RNAs has enabled their structural investigation via multidimensional solution state NMR. However, scalar coupling based techniques employed in the solution state, e.g. for resonance assignments, often involve radio-frequency pulse trains with large inter-pulse delays (Wijmenga and van Buuren, 1998). This generally precludes the study of large RNA systems that typically have short transverse spin relaxation times. On the other hand, structural studies in the solid state have no such size limitations and our recent studies clearly indicate that MAS solid state NMR based investigations of large RNAs are feasible (Leppert et al., 2004; Riedel et al., 2004, 2005). Among the different RNA secondary structural elements, duplex regions arising from consecutive formation of hydrogen bonded base pairs are of common occurrence. In addition to canonical Watson–Crick base pairs, non-canonical base pairs play critical roles in defining the structure and function of nucleic acids (Gesteland et al., 1999). Figure 1 shows some possible base pairing schemes. The identification of the hydrogen bonded base-pairs and the characterisation of the underlying hydrogen bonding patterns is of critical importance in the study of RNA, both in the context of resonance assignments and for obtaining structural constraints.

Recently we have demonstrated an approach for the identification of NH...N hydrogen bonds

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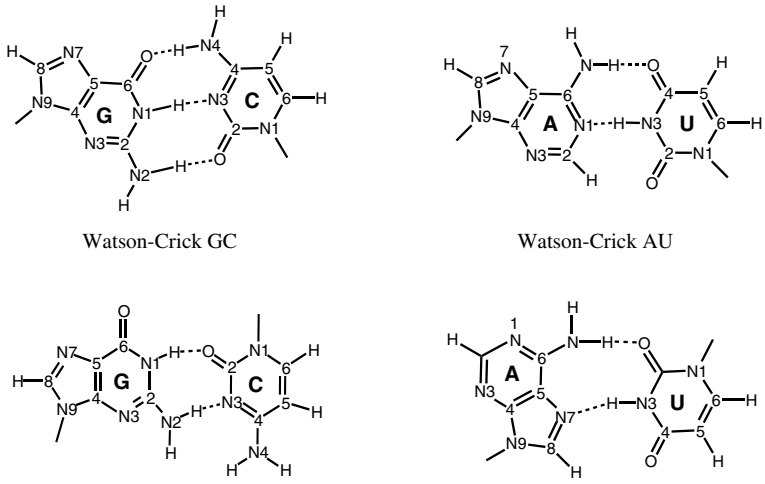

Figure 1. Possible hydrogen bonded GC and AU base-pairing schemes.

by MAS solid state NMR (Leppert et al., 2004) that makes use of the fact that the nitrogen nuclei involved in NH...N hydrogen bonds are spatially proximal with typical N–N distances being in the range of 2.9–2.6 Å (corresponding to a dipolar coupling strength $D_{NN} \sim 50$–70 Hz). Such spatial proximity of nitrogen nuclei and hence the presence of hydrogen bonded base pairs gets reflected as cross-peaks between the donor ^{15}N and acceptor ^{15}N nuclei in a 2D ^{15}N MAS dipolar chemical shift correlation spectrum. Here, we demonstrate that much stronger dipolar couplings between spatially proximal ^{1}H nuclei in hydrogen bonded base-pairs can be effectively used in proton spin diffusion (Schmidt-Rohr and Spiess, 1994) based chemical shift correlation experiments (Wilhelm et al., 1998; Wei and Ramamoorthy, 2001) for a characterisation of hydrogen bonding networks in RNAs. 2D proton spin diffusion based $^{15}N/^{13}C$ chemical shift correlation spectroscopy has been successfully employed recently for the extraction of structural constraints from MAS solid state NMR studies of peptides, proteins and other systems (de Boer et al., 2002; Lange et al., 2002; Lange et al., 2003; Reif et al., 2003; Tycko and Ishii, 2003). Here, the potential of this approach for monitoring internuclear distances between ^{1}H nuclei is exploited for the study of nucleic acids. The system investigated is a CUG triplet repeat expansion RNA $(CUG)_{97}$. Such expansions (>50 CUG) have been implicated in the neuromuscular disease myotonic dystrophy type 1 (DM1; Nykamp et al., 2004; Ranum and Day, 2004).

Experimental

The RF pulse sequence employed in this work is shown in Figure 2. The initial transverse ^{15}N magnetisation prepared by conventional cross-polarisation (CP) is allowed to evolve under ^{1}H decoupling during the t_1 period. The second CP step transfers the t_1 modulated ^{15}N magnetisation back to the protons. The proton magnetisation is then flipped to the z axis and longitudinal ^{1}H

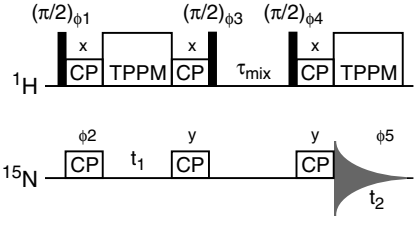

Figure 2. CPMAS pulse sequence employed for proton spin diffusion based isotropic chemical shift correlation of the protonated nitrogen sites in RNAs. The RF pulse and the receiver phases were cycled as: $\phi 1$ = (y, −y); $\phi 2$ = (−y, −y, y, y); $\phi 3$ = (4*y, 4*−y); $\phi 4$ = (8*y, 8*−y); $\phi 5$ = (x, −x, −x, x, −x, x, x, −x, −x, x, x, −x, x, −x, −x, x). High power TPPM decoupling was employed in t_1 and t_2 time periods and phase sensitive 2D spectra were generated via standard procedures.

magnetisation exchange mediated by proton–proton dipolar couplings takes place during the spin diffusion period τ_m. The proton magnetisation at the end of the mixing period is rotated back to the transverse plane and the final CP transfers the polarisation from the protons to the nitrogen nuclei for detection in t_2. The experiment is carried out with a very short CP contact time and proton spin diffusion mixing time τ_m of $\sim$100 μs. In this regime, with minimal proton spin diffusion during the CP contact time and relayed magnetisation transfers during the mixing time τ_m, cross-peaks with appreciable intensities are expected only between ^{15}N sites connected by short ^{1}H–^{1}H distances of less than $\sim$3 Å (Lange et al., 2002; Lange et al., 2003; Reif et al., 2003; Tycko and Ishii, 2003).

Experiments were carried out with an hydrated, undiluted ^{15}N, ^{13}C labelled RNA sample of $(CUG)_{97}$ (Leppert et al., 2004) at ~ -15 °C on a 500 MHz wide-bore Varian ^{UNITY} *INOVA* solid state NMR spectrometer equipped with a 5 mm DOTY supersonic triple resonance probe. The RNA sample was prepared using doubly labelled NTPs as described earlier (Leppert et al., 2004). Cross-polarisation under Hartmann–Hahn matching conditions was employed and all spectra, unless mentioned otherwise, were collected under high power ^{1}H decoupling ($\sim$90 kHz). 90° ^{1}H pulses with a duration of 3.0 μs were applied in these studies. Other details are given in the figure captions.

Results and discussion

Figure 3 shows 2D ^{15}N homonuclear isotropic chemical shift correlation spectra of $(CUG)_{97}$ obtained with the different proton spin diffusion mixing times indicated. The short CP contact time results in the detection of only the four proton attached imino and amino nitrogen resonances and the assignments indicated are based on our earlier studies on $(CUG)_{97}$ (Leppert et al., 2004; Riedel et al., 2004b). (The origin of the unlabelled weak peaks on the diagonal has not been examined in detail yet.) Most of the data we have obtained till date, including the ^{15}N–^{1}H HSQC spectrum obtained in the solution state (Leppert et al., 2004) indicate that we are not dealing with a system exhibiting significant conformational heterogeneity. We also do not see any cross peaks involving these minor peaks at any plot levels. A current model for DM1 pathogenesis suggests that such CUG repeats fold into large and stable double-stranded RNA hairpins with GC and UU base-pairs (Napierala et al., 1997; Michalowski et al., 1999). It is known that in helical RNAs with Watson–Crick GC base-pairs (Figure 1) the guanine imino proton (GN1) is spatially proximal ($\sim$2.5 Å) to one of the cytosine amino protons (CN4) of the opposite strand as well as to one of the guanine amino protons (GN2) ($\sim$2.2 Å). On the other hand, e.g. a reverse Watson–Crick GC base-pairing scheme (Figure 1) is characterised by the spatial proximity of the guanine and cytosine amino protons, with the guanine imino proton at a distance of >3 Å from the cytosine amino protons. Hence, at very short mixing times the WC GC base-pairing scheme should lead a correlation spectrum in which (1) the intensity of the cross-peak between the CN4 and GN1 nitrogens is much stronger than that between the CN4 and GN2 nitrogens, (2) the intensity of the cross-peak between the GN2 and GN1 nitrogens is much stronger than that between the GN2 and CN4 nitrogens and (3) the GN1 nitrogen should show cross-peaks of appreciable intensities with both the CN4 and GN2 nitrogens. However, a reverse WC GC base-pairing scheme would result in a correlation spectrum with cross-peak of appreciable intensity only between the GN1 and GN2 nitrogens and GN2 and CN4 nitrogens. In agreement with our earlier studies, the presence of canonical WC GC base-pairs in $(CUG)_{97}$ can be clearly deduced from the spectrum obtained with a mixing time of 100 μs. The cross-peak between the GN1 and CN4 nitrogens in $(CUG)_{97}$ could also arise from intrastrand proton–proton dipolar interactions between consecutive bases. As an A-helical RNA is characterised by an intrastrand distance of $>$3.5 Å between a guanine imino proton and an amino proton of an adjacent cytosine residue, it can be safely assumed that in the spectrum collected at a mixing time of 100 μs, the cross-peak between the GN1 and CN4 nitrogens arises only via interstrand proton–proton dipolar interactions. Although even at mixing times $>$100 μs the characteristic cross-peak intensity patterns expected for a canonical WC GC base-pair can be observed (Figure 3), with increasing values of the spin diffusion period τ_m additional correlation

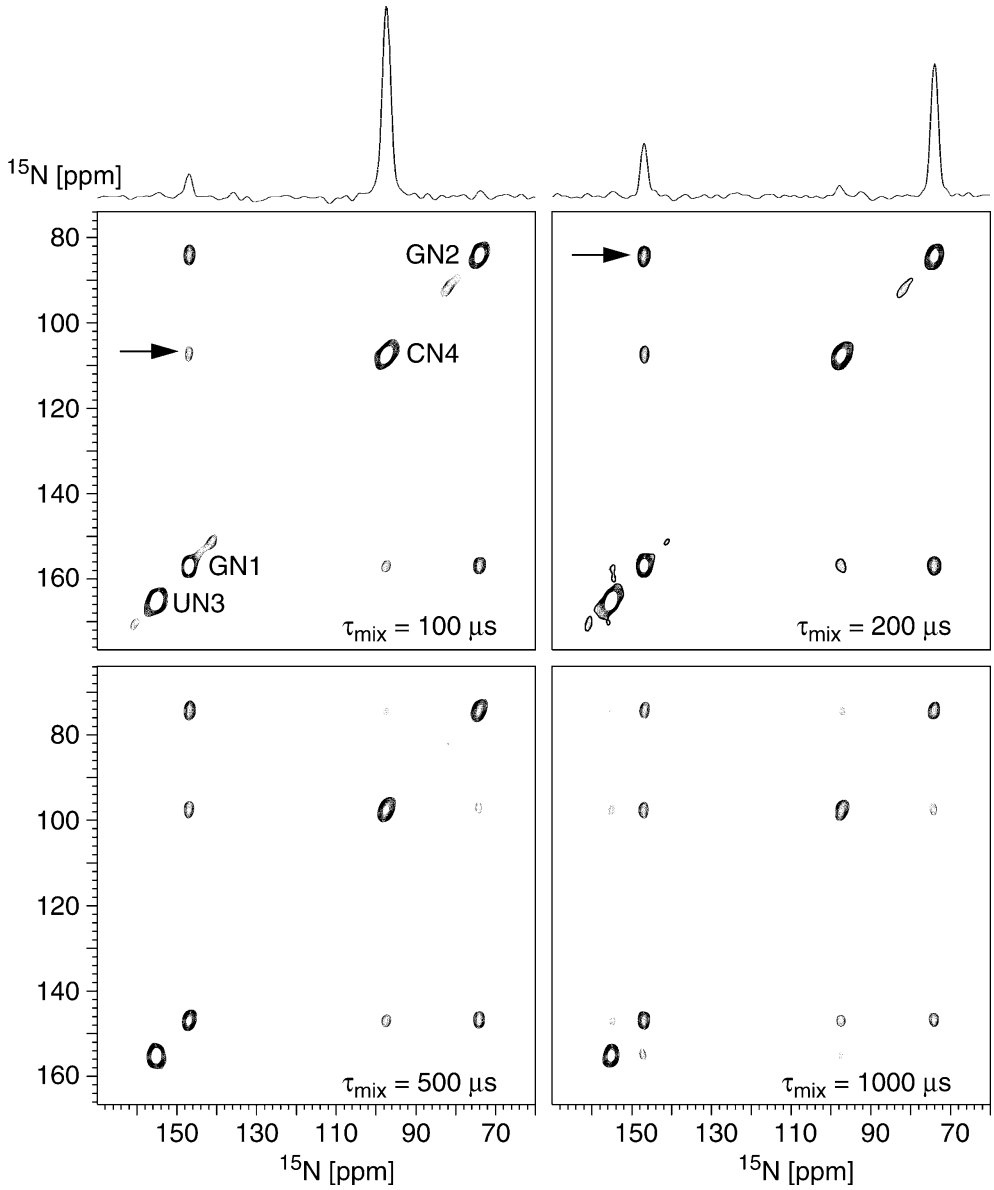

Figure 3. Experimental ^{15}N chemical shift correlation spectra of the hydrated RNA sample obtained at the mixing times indicated, employing a spinning speed of 10000 Hz, data acquisition in the direct dimension of 10 ms, CP contact times of 155 μs, recycle time of 2 s, ω_1 spectral width of 6000 Hz, 32 t_1 increments with 640 transients per t_1 increment. The RF carrier was kept at the centre of the spectral region. The chemical shifts are referenced to liquid NH_3 assuming that solid NH_4Cl has a chemical shift of 38.5 ppm. The assignments of the different resonances are indicated on the diagonal peaks in the data collected with τ_m of 100 μs. Spectral cross-sections taken at the indicated positions are also given at the top of the spectra obtained with mixing times of 100 μs and 200 μs.

peaks arise both from direct and relayed magnetisation transfer processes, e.g. cross-peaks between (1) CN4 and GN2 and (2) uridine imino nitrogen (UN3) and GN1, GN2 and CN4 nitrogens. Hence, for long mixing times a reliable extraction of distance information is difficult. However, such cross-peaks e.g. between UN3 and GN1, GN2 and CN4 nitrogens arising from weak long range proton–proton dipolar couplings should be useful for sequential RNA resonance assignments in the solid state. This is similar to liquid state NMR studies of RNAs where imino–imino and imino–amino proton NOEs are often utilised for sequential resonance assignments (Moore, 1995).

As outlined above, proton spin diffusion induced ^{15}N chemical shift correlation experiments will be sufficient to distinguish WC GC from e.g. reverse WC GC base-pairing scheme. However, additional experiments would be required for the characterisation of the hydrogen bonding networks in other base-pairs like AU which, however, is not present in $(CUG)_{97}$. For example, in both the WC AU and reverse Hoogsteen AU base-pairing schemes (Figure 1) the uridine imino proton is spatially proximal to one of the adenine amino protons. In a proton spin diffusion based ^{15}N correlation experiment, these AU base-pairs will get reflected as a cross-peak between the UN3 and AN6 nitrogens. In order to distinguish between the WC and the reverse Hoogsteen situation, the spatial proximity of the uridine imino proton with respect to the adenine H2 and H8 protons in a WC AU and reverse Hoogsteen base-pair scheme, respectively, has to be assessed. For this purpose, a proton spin diffusion based ^{15}N,^{13}C heteronuclear correlation experiment could be employed to identify the type of AU base-pairing. In such an experiment the WC AU base-pair would lead to a cross-peak between the UN3 nitrogen and AC2 carbon and a reverse Hoogsteen AU base-pairing scheme would be reflected by a cross-peak between the UN3 nitrogen and AC8 carbon.

The data shown in Figure 3 demonstrate that strong dipolar interactions between spatially proximal ^{1}H nuclei can be effectively exploited for obtaining distance constraints from MAS solid state NMR spectroscopic studies of RNA, similar to the approach introduced for peptides and proteins (Lange et al., 2002; Lange et al., 2003; Reif et al., 2003; Tycko and Ishii, 2003). Although we have employed in this study a proton spin diffusion driven ^{15}N chemical shift correlation, it is worth mentioning that it is possible to generate proton-mediated rare-spin correlation data in the solid state employing other mixing schemes (Lange et al., 2003). The results presented here were obtained on a non-deuterated uniformly ^{15}N,^{13}C labelled $(CUG)_{97}$ sample. Deuterium labelling of protons attached to the carbons would further enhance the intensities of some of the cross-peaks seen in Figure 3 by eliminating magnetisation leakage pathways such as CN4H $\rightarrow$ CC5H, CC6H. Even though such samples would facilitate the extraction of more accurate internuclear proton–proton distances from an analysis of cross-peak intensity build-up curves, it is apparent that in situations where only a knowledge of approximate distance estimates is required, e.g. for the purpose of distance geometry based structure calculations, experimental measurements on non-deuterated samples, as demonstrated here, are sufficient. While the potential of proton spin diffusion based ^{15}N–^{15}N chemical shift correlation has been highlighted here for the characterisation of the hydrogen bonding networks, it is also possible to employ a similar approach for obtaining other structural constraints, e.g. the torsion angle χ in RNAs from conceptually similar ^{13}C based experiments. Such experiments are in progress in our laboratory and the results from these investigations will be reported elsewhere.

Conclusion

Significant differences of the inter-nuclear proton–proton distances in different canonical and non-canonical base-pairing schemes can be effectively exploited for probing hydrogen bonding networks in RNA via MAS solid state NMR spectroscopy.

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