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Resolution of the ^{1}H-^{1}H NOE spectrum of RNA into three dimensions using ^{15}N-^{1}H two-bond couplings

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Abstract

The feasibility of using two-bond ^{15}N-^{1}H couplings to resolve the ^{1}H-^{1}H nuclear Overhauser effect spectrum of RNA into a third dimension was investigated, using the 36-nucleotide gene *32* messenger RNA pseudoknot of bacteriophage T2 as an example. The two-bond ^{15}N-^{1}H couplings present in adenosine and guanosine were found to be suitable for generating a three-dimensional ^{1}H-^{1}H-^{15}N NOESY-HSQC spectrum with reasonably good sensitivity, as well as favorable chemical shift dispersion in the nitrogen dimension. The described NMR experiment provides a tool that can be used to complement other heteronuclear methods in the analysis of RNA structure.

Introduction

The nuclear Overhauser effect is the primary source of inter-proton distance information used in determining RNA structure by NMR methods. Resonance overlap within two-dimensional ^{1}H-^{1}H NOE spectra tends to be particularly severe in the case of RNA, due to the narrow chemical shift ranges in which the ribose and base protons usually occur. One useful solution to this overlap problem is to resolve the ^{1}H-^{1}H NOE spectrum into a third dimension using a through-bond coupling to a third nucleus. This third nucleus may be another proton, as in the 3D NOESY-TOCSY spectrum of RNA (Wijmenga et al., 1994; Hoffman and White, 1995), or a heteronucleus such as ^{13}C or ^{15}N (c.f. Nikonowicz and Pardi, 1992; Aboul-ela et al., 1995; Dieckmann and Feigon, 1997; Holland et al., 1999). Methods that exploit couplings to ^{13}C or ^{15}N have in the past focused almost exclusively on those nuclei that are directly bonded to protons, due to the high efficiency of magnetization transfer via the single bond coupling.

In the present study, the possibility of using two-bond proton-nitrogen correlations to non-exchangeable protons to resolve the ^{1}H-^{1}H NOE spectrum of RNA was investigated, using a ^{15}N-enriched sample of the 36-nucleotide pseudoknot from the T2 bacteriophage gene *32* messenger RNA. This particular RNA pseudoknot has been structurally characterized using proton NMR methods (Du et al., 1996), combined with mutational analysis (Du and Hoffman, 1997), and more recently standard heteronuclear NMR methods (Holland et al., 1999). The RNA pseudoknot contains two A-form helical stems, with a single nucleotide (loop 1) crossing the major groove of stem 2, and seven nucleotides (loop 2) crossing the minor groove of stem 1. The two stems are coaxial or very nearly coaxial, forming a quasi-continuous helix with the C7-G16 base pair of stem 1 stacked upon the A15-U28 base pair of stem 2 (Figure 1). Coordinates for the pseudoknot are available from the Protein Data Bank (PDB entry number 2tpk).

Materials and methods

Preparation of the ^{15}N-enriched RNA

Labeled ribonucleotides were isolated from *Escherichia coli* grown on minimal media using ^{15}N ammonium chloride as the nitrogen source. Total cel-

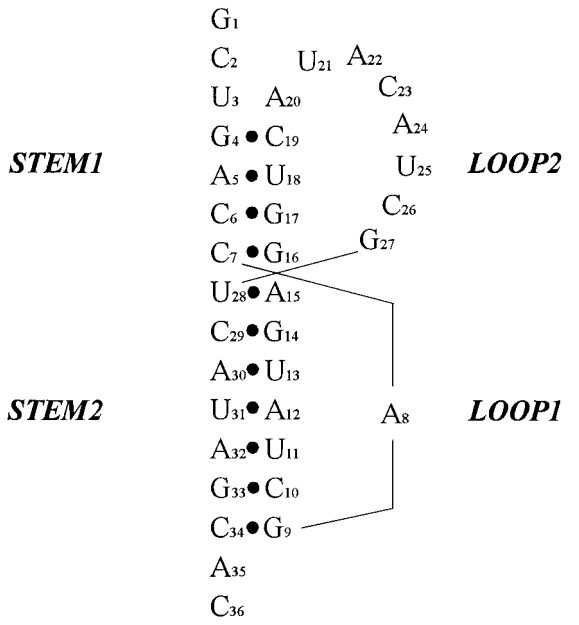

Figure 1. The nucleotide sequence of the bacteriophage T2 gene *32* mRNA pseudoknot. Dark circles indicate pairings that are confirmed by slowly exchanging imino and amino protons and NOE patterns that are characteristic of Watson–Crick base pairs (Du et al., 1996).

lular RNA was isolated from the *E. coli* by phenol and chloroform extraction, concentrated by ethanol precipitation, and hydrolyzed to mononucleotides with RNAse P1. Mononucleotides were purified using an Affigel 601 *cis*-diol affinity column (BioRad), and enzymatically converted to ribonucleotide triphosphates as described by Batey et al. (1992) and Nikonowicz et al. (1992). The 36-nucleotide RNA pseudoknot with the sequence shown in Figure 1 was prepared using T7 RNA polymerase, a synthetic DNA template and ^{15}N-enriched nucleotide triphosphates in an in vitro transcription reaction (Milligan and Uhlenbeck, 1989). The RNA product was separated from transcripts of incorrect size by electrophoresis on 20% polyacrylamide gels under denaturing conditions in 8 M urea, removed from the gel by electroelution, ethanol precipitated, lyophilized, and dissolved in a buffer containing 10 mM sodium phosphate, 100 mM sodium chloride, 1 mM sodium azide, and 0.1 mM EDTA, at pH 6.5, in a solvent of 90% H_2O and 10% D_2O.

NMR experiments

NMR spectra of the ^{15}N-enriched RNA pseudoknot were collected at 500 MHz using a Varian Inova spectrometer. The sample contained 240 OD_{260} units (approximately 1.3 mM) of RNA in a volume of 0.5 ml. The 3D spectrum was obtained using a ^{1}H-^{1}H-^{15}N NOESY-HSQC pulse sequence that employs z-axis gradient pulses for coherence selection and removal of unwanted transverse magnetization (Zhang et al., 1994). The NOESY part of the pulse sequence was performed first, with an NOE mixing time of 200 ms. The ^{15}N-^{1}H HSQC part of the pulse sequence was performed last, with the 1/4J delays set to 10 ms, which is optimum for 25 Hz ^{15}N-^{1}H couplings. This choice

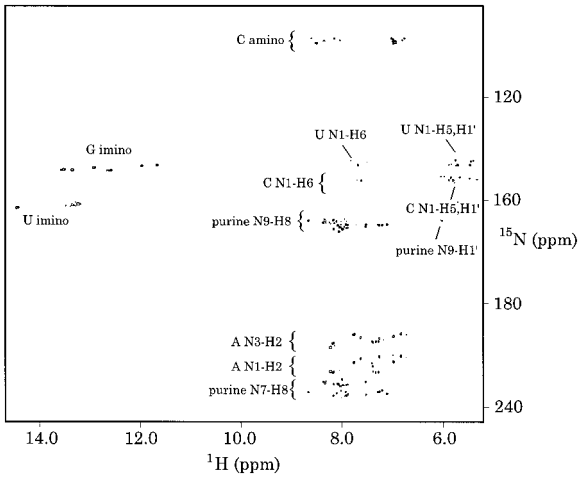

Figure 2. A section of the ^{15}N-^{1}H correlated spectrum of the bacteriophage T2 gene *32* mRNA pseudoknot. The spectrum was obtained with 1/4J delays optimum for 25 Hz coupling, permitting the one- and two-bond correlations to be detected within the same spectrum.

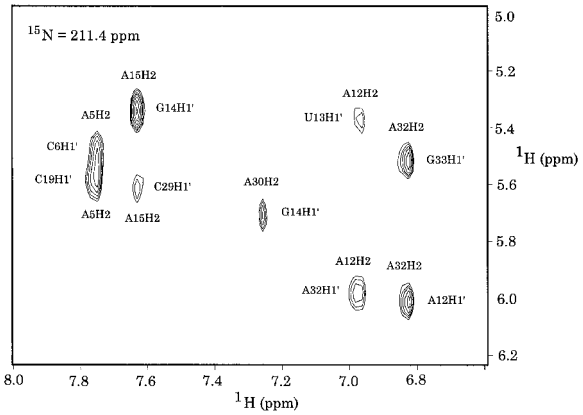

Figure 3. A section of the 3D ^{1}H-^{1}H-^{15}N NOESY-HSQC spectrum of the bacteriophage T2 gene *32* mRNA pseudoknot containing NOE cross peaks between adenosine H2 and ribose H1' protons, resolved by the adenosine N3 chemical shift. The ^{15}N chemical shift of the plane shown corresponds to the N3 nitrogens of A5, A15 and A32; other NOE cross peaks are better observed in adjacent ^{15}N planes.

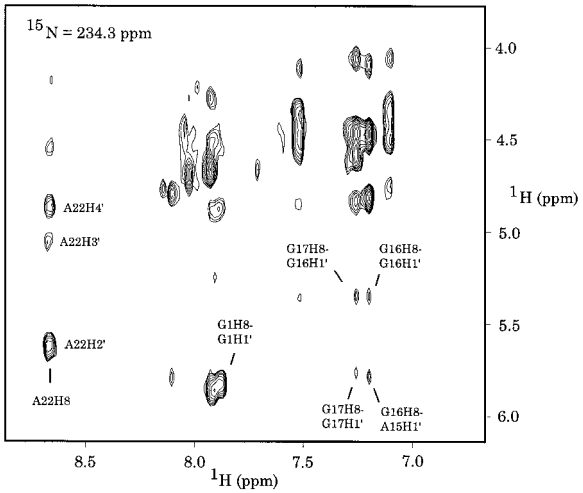

Figure 4. A section of the most crowded plane in the 3D ^{1}H-^{1}H-^{15}N NOESY-HSQC spectrum bacteriophage T2 gene *32* mRNA pseudoknot containing NOE cross peaks between purine H8 and ribose protons, resolved by the purine N7 chemical shift. Some of the H8 to H1' cross peaks are labeled. Unlabeled cross peaks arise from NOEs between purine H8 and other ribose protons. The ^{15}N chemical shift of the plane shown corresponds to the N7 nitrogen of G16, G17, A22 and G27; other NOE cross peaks are better observed in adjacent ^{15}N planes.

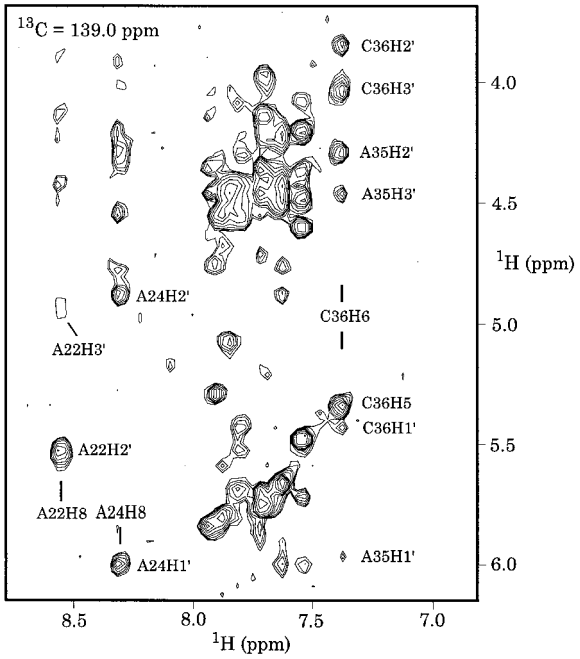

Figure 5. A section of the single most crowded plane in the 3D ^{1}H-^{1}H-^{13}C NOESY-HSQC spectrum bacteriophage T2 gene *32* mRNA pseudoknot, obtained using a sample that is enriched with both ^{15}N and ^{13}C (Holland et al., 1999). This ^{13}C-resolved NOE spectrum is shown so that the signal-to-noise and chemical shift dispersion can be compared with the most crowded section of the ^{15}N-resolved NOE spectrum in Figure 4. NOEs that arise from purine H8 and pyrimidine H6 protons appear in the spectrum, resolved by the ^{13}C chemical shifts of C8 and C6, respectively. Several of the best-resolved cross peaks are labeled.

of 1/4J delay was found to provide the best signal-to-noise in the 3D spectrum, and was found after trying a range of potential delay times. The selected 1/4J delay does represent a compromise, since the detected ^{15}N-^{1}H two-bond couplings are actually about 15 Hz. However, there is less decay of the desired magnetization if the shorter delay is used. The States-TPPI method was used to obtain phase-sensitive detection in both of the indirect dimensions. The sweep width was 5000 Hz in the acquisition (^{1}H) dimension, 5272 Hz in the ^{15}N dimension, centered at 192 ppm, and 6000 Hz in the indirectly detected ^{1}H dimension, centered at the water resonance frequency. NOE cross peaks to imino protons between 11.5 and 14.4 ppm were outside the set sweep width, but were detected as folded peaks in the indirect ^{1}H dimension. Sixteen scans of 512 complex points were collected for each FID, and 96 and 24 complex points were FIDs detected in the indirect ^{1}H and ^{15}N dimensions, respectively. A recycle time of 1.5 s was used between scans, for a total acquisition time of 63 h. Proton NMR spectra were referenced to internal DSS, and ^{15}N chemical shifts were referenced by multiplying the 0.0 ppm ^{1}H reference frequency by 0.101329118 to determine the ^{15}N 0.0 ppm frequency (Wishart et al., 1995). The 3D ^{1}H-^{1}H-^{15}N NOESY-HSQC experiment was also run in a 2D mode by keeping the ^{1}H frequency labeling period set to zero to obtain a ^{15}N-^{1}H correlated spectrum, or by keeping the ^{15}N frequency labeling period set to zero to obtain a ^{1}H-^{1}H NOE spectrum where one of the protons is two bonds removed from a ^{15}N nucleus.

Results and discussion

^{1}H-^{1}H NOE spectra have previously been resolved into a third dimension using the chemical shifts of imino or amino nitrogens (c.f. Nikonowicz and Pardi, 1992; Dieckmann and Feigon, 1997), since the strong 90 Hz one-bond couplings permit the most efficient magnetization transfer, and therefore the highest sensitivity in the 3D spectrum. A disadvantage of exploiting the single-bond ^{15}N-^{1}H coupling is that the ^{15}N chemical shift dispersion is typically quite poor for the imino and amino nitrogens (Figure 2). Furthermore, the imino and amino protons within RNA often exchange rapidly with the solvent and are not observable

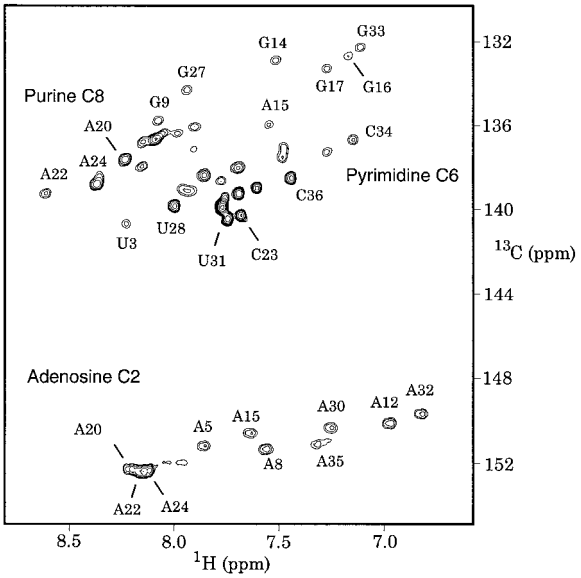

Figure 6. A section of the ^{13}C-^{1}H HSQC spectrum of the bacteriophage T2 gene *32* mRNA pseudoknot, shown so that the chemical shift dispersion can be compared with that of the ^{15}N-correlated spectrum. Several of the best resolved cross peaks are labeled; resonance assignments for the pseudoknot have been deposited in the BioMagResBank (entry number 4253).

in nucleotides that are not base paired, which frequently includes the most interesting parts of an RNA structure. A proton NOE spectrum resolved by ^{15}N chemical shift through two-bond couplings would potentially be advantageous, since detection would be through non-exchangeable protons. A large number of scalar coupled pairs are observable in the ^{15}N-^{1}H spectrum of the 36-nucleotide RNA pseudoknot (Figure 2), which contains both one- and two-bond ^{15}N-^{1}H correlations.

A NOESY-HSQC experiment was found to be effective at resolving the ^{1}H-^{1}H NOE spectrum into a third dimension using the 15 Hz two-bond ^{15}N-^{1}H couplings present in adenosine and guanosine (Figures 3 and 4). For NOE cross peaks involving the purine nucleotides, the sensitivity of the 3D ^{1}H-^{1}H-^{15}N NOESY-HSQC spectrum was found to be similar to that of an ordinary 3D ^{1}H-^{1}H-^{13}C NOESY-HSQC spectrum of the same sequence RNA labeled with ^{13}C (Figure 5), obtained using a similar concentration of sample in a similar amount of acquisition time (Holland et al., 1999). This result was unanticipated, since the ^{13}C spectrum exploited one-bond ^{13}C-^{1}H couplings which are an order of magnitude larger than the two-bond ^{15}N-^{1}H couplings. The favorable sensitivity of the ^{15}N two-bond experiment is likely due to the relatively long relaxation times present in the ^{15}N-enriched sample, compared to those present in a ^{13}C and ^{15}N doubly-enriched sample.

NOE cross peaks between adenosine H2 and ribose H1′ in the helical stems of the pseudoknot (separated by about 4 Å) are clearly observed in the two-bond NOESY-HSQC spectrum (Figure 3), resolved by the chemical shift of the N3 nitrogen. NOE cross peaks arising from these same ^{1}H-^{1}H NOEs also appear a second time in the 3D spectrum, with equal clarity, resolved by the chemical shift of the adenosine N1 nitrogen, near 220 ppm. The NOE cross peaks involving H2 of adenosine 15 are particularly important in defining the pseudoknot structure, since they involve protons at the junction of the two stacked helical stems. Planes corresponding to ^{15}N chemical shifts between 232 and 238 ppm contain NOE cross peaks arising from adenosine and guanosine H8, resolved by the chemical shift of the N7 nitrogen (Figure 4); NOE cross peaks between H8 and H1′ protons are observed for all 18 of the purines, resolved by the chemical shift of the N7 nitrogen. These NOE cross peaks appear a second time in the 3D spectrum, resolved by the chemical shift of the purine N9 nitrogen near 168 ppm.

^{15}N-^{1}H two-bond correlations were also observed for the pyrimidine nucleotides (Figure 2), but were not strong enough to yield more than a few cross peaks in the 3D NOESY-HSQC spectrum (data not shown). However, the pyrimidine N3 and N1 chemical shifts were useful in distinguishing cytosine H5 resonances from those of uridine. The three-bond N1 to H5 correlation peaks in the pyrimidines are significantly stronger than the two-bond N1 to H6 correlations.

The distinctive chemical shifts of the purine N1 and N3 resonances, compared to those of N7 and N9, were useful in confirming the assignments of the H2 resonances of the adenosines, which cannot be distinguished from guanosine and adenosine H8 resonances on the basis of proton chemical shifts. In this respect, the ^{15}N-^{1}H spectrum serves the same purpose as using a ^{13}C-^{1}H single bond correlation spectrum to identify adenosine H2 resonances by the distinctive ^{13}C chemical shift of adenosine C2 (Varani and Tinoco, 1991). It was initially hoped that the chemical shifts of the N7 resonances would provide a reliable method of distinguishing adenosine from guanosine H8 resonances, since the adenosine N7 chemical shift is about 4 ppm upfield of the guanosine N7 in the free mononucleotides (Büchner et al., 1978). In the case of the RNA pseudoknot, most of the adenosine N7 resonances are upfield of guanosine N7; however,

the difference in chemical shift is probably not large enough to be an unambiguous indicator of nucleotide type.

It is interesting to compare the chemical shift dispersion of the purine C2 and C8 nuclei, which are used to resolve ^{1}H-^{1}H NOEs in a ^{1}H-^{1}H-^{13}C HSQC-NOE spectrum, with the chemical shift dispersion of the purine N1, N3, N7 and N9 nuclei, which are used to resolve the same NOEs via two-bond couplings in the present work. The chemical shifts of the N1 and N3 nuclei are uniformly spread over the range 211–227 ppm (or 810 Hz), and are significantly more disperse than the C2 nuclei, which have chemical shifts in the range 149.6–153 ppm (or 427 Hz). The chemical shifts of the purine N7, C8 and N9 nuclei are less disperse than those of N1 and N3. The most crowded ^{13}C plane of the ^{1}H-^{1}H-^{13}C HSQC-NOE spectrum (Figure 5) contains significantly more resonance overlap than the most crowded ^{15}N plane of the ^{1}H-^{1}H-^{15}N HSQC-NOE spectrum (Figure 4), primarily due to the fact that the pyridine C6 nuclei have resonance frequencies in a range that overlaps with the purine C8 (Figure 6).

Conclusions

In summary, the present work shows that a 3D ^{1}H-^{1}H-^{15}N NOESY-HSQC spectrum using two-bond ^{15}N-^{1}H couplings can provide valuable information in the study of RNA by NMR, obtained with reasonable sensitivity. These experiments can be used in a complementary manner with other heteronuclear NMR methods for analyzing RNA structure, such as those that use ^{13}C.

Acknowledgements

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