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Preparation and characterization of a uniformly $^{2}H/^{15}N$-labeled RNA oligonucleotide for NMR studies

E. P. Nikonowicz*, M. Michnicka, K. Kalurachchi and E. DeJong

Department of Biochemistry and Cell Biology, Rice University, Houston, TX 77005, USA

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ABSTRACT

An RNA oligonucleotide that contains the binding site for *Escherichia coli* ribosomal protein S8 was prepared with uniform ^{15}N isotopic enrichment and uniform deuterium enrichment at all non-exchangeable sites using enzymatic methods. The RNA binding site, which contains 44 nt, forms a hairpin in solution and requires Mg^{2+} for proper folding. The longitudinal magnetization recovery rates of the exchangeable protons were compared for the [^{2}H,^{15}N]-enriched RNA molecule and for the corresponding fully [^{2}H,^{15}N]-enriched RNA hairpin. It was found that ^{1}H–^{1}H dipolar relaxation significantly contributes to the recovery of exchangeable proton longitudinal magnetization. The exchangeable proton resonance line widths were less affected by deuteration, indicating that chemical exchange with H_2O remains the dominant mechanism of transverse magnetization relaxation. Nevertheless, deuteration of this RNA hairpin was found to enhance the sensitivity of NOE-based experiments relative to the fully protonated hairpin and to simplify 2D NMR spectra. The increased signal-to-noise ratio facilitated the assignment of the cytidine amino resonances and several of the purine nucleotide amino resonances and permitted the identification of NOE crosspeaks that could not be observed in spectra of the fully protonated RNA hairpin.

INTRODUCTION

Heteronuclear multi-dimensional NMR methods have extended the size and complexity of proteins and nucleic acids that can be studied in solution by improving spectral resolution and permitting the correlation of resonances through scalar coupled pathways. In particular, NOE-based experiments have significantly benefited from the enhanced resolution that is provided through ^{13}C and ^{15}N isotopic enrichment. The ^{13}C and/or ^{15}N separated NOESY experiments provide much structural information and are routinely applied to obtain high resolution structures of proteins up to ~22 kDa and are beginning to be applied to RNA molecules as large as 12–15 kDa (1,2). However, the sensitivity of NOE-based experiments can be dramatically reduced by line broadening associated with ^{1}H dipolar relaxation, thus limiting the amount of structural information that can be obtained from these experiments.

The ill effects of dipolar relaxation generally become more pronounced as the effective correlation time of a nucleus increases and as the number of proximal relaxation partners of a proton increase. These effects are not restricted to large molecules, however. Even moderately sized proteins and oligonucleotides that exhibit long correlation times can suffer the problems associated with dipolar relaxation. Proteins and oligonucleotides that are components of protein–nucleic acid complexes exhibit correlation times characteristic of the complexes which can be significantly longer than the correlation times of either of the individual components free in solution. Mg^{2+}, which is often required for the proper folding of RNA molecules (3,4), may also act to slow the correlation time of RNA oligonucleotides. Free Mg^{2+} can promote non-specific intermolecular interactions among RNA molecules which result in an increase in the molecular correlation time and a decrease in spectral sensitivity.

Several investigations have shown that deuteration can be used to reduce the adverse effects associated with ^{1}H dipolar relaxation (5–7). Recently, Venters and co-workers (8) and Grzesiek *et al.* (9) have demonstrated that perdeuteration of the non-exchangeable sites in ^{15}N-labeled proteins improves the sensitivity and resolution of ^{15}N separated NOESY experiments, permitting structure analysis of slowly tumbling proteins. The line broadening effects associated with dipolar relaxation exhibited by the amide protons are significantly reduced when the number of relaxation partners surrounding them is decreased. The narrower resonances ultimately result in improved sensitivity of experiments that employ deuterated proteins. It has recently been shown that selective deuteration within the sugar moieties of DNA (10) and RNA (11) oligonucleotides decreases the transverse relaxation rates of the remaining non-exchangeable protons. The more favorable relaxation rates and the elimination of ^{1}H scalar coupling result in decreased line widths and improved spectral resolution for the remaining proton resonances (10–12).

The exchangeable imino and amino proton resonances of nucleic acids tend to be broad. Importantly, many of the ^{1}H–^{1}H NOE interactions that report on the secondary and tertiary structural features of these molecules involve the exchangeable protons (13,14). Although chemical exchange processes contribute significantly to the line widths of the exchangeable resonances (15,16), ^{1}H dipolar interactions can also contribute. Deuteration

* To whom correspondence should be addressed. Tel: +1 713 527 4912; Fax: +1 713 285 5154; Email: edn@bioc.rice.edu

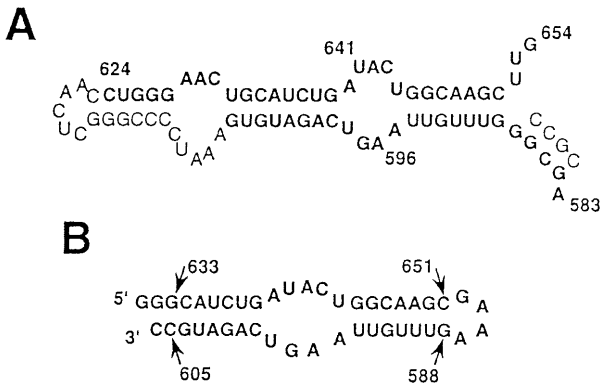

Figure 1. (**a**) Helix 21 of 16S rRNA from *E.coli* and (**b**) secondary structure of the deuterated and fully protonated RNA hairpins used in this study. The nucleotide numbering is based on that for *E.coli* 16S rRNA. The bold nucleotides in (a) are protected from enzymatic cleavage by ribosomal protein S8 (35).

of the non-exchangeable sites offers a method to eliminate several of the dipolar relaxation partners of the exchangeable protons, thus providing enhanced sensitivity and improved spectral quality.

In this report, we compare the NMR spectral properties of the exchangeable protons of a deuterated, ^{15}N-enriched RNA molecule and the corresponding fully protonated, ^{15}N-enriched molecule. The oligoribonucleotide used in this study forms a hairpin in solution and contains the binding site for ribosomal protein S8 (Fig. 1). The apparent longitudinal relaxation times of several of the imino protons of the deuterated RNA hairpin were found to be longer than the corresponding resonances of the protonated RNA hairpin. In addition, NOE-based spectra of the deuterated RNA hairpin exhibited increased sensitivity and a greater number of crosspeaks between exchangeable protons. Finally, spectra from the deuterated RNA hairpin were used to identify several NOE crosspeaks involving exchangeable proton resonances that could not be obtained from spectra of the fully protonated RNA hairpin.

MATERIALS AND METHODS

All enzymes were purchased from Sigma Chemical Co. (St Louis, MO) with the exception of the T7 RNA polymerase. The T7 RNA polymerase was prepared as previously described (17). Deoxyribonuclease I type II, pyruvate kinase, adenylate kinase and the nucleotide monophosphate kinase were obtained as powders and were dissolved in solutions of 15% glycerol, 1 mM dithiothreitol and 10 mM Tris–HCl, pH 7.4, and stored at –20°C. The guanylate kinase and nuclease P_1 were obtained as solutions and stored at –20°C. Phosphoenolpyruvate (potassium salt) was obtained from Bachem.

Preparation of the ^{2}H/^{15}N-labeled RNA

The labeled RNA hairpin was transcribed *in vitro* with T7 RNA polymerase using a synthetic DNA template as previously described (18,19) and ^{2}H/^{15}N-labeled 5′-nucleoside triphosphates (5′-NTPs). The 5′-NTPs were prepared by growing *Escherichia coli* on minimal medium containing 88% $^{2}H_2O$, [^{15}N]ammonium sulfate as the sole nitrogen source and [$^{2}H_3$]sodium acetate as the sole carbon source. Starter cultures were prepared in LB medium and transferred to 25 ml minimal medium containing ammonium sulfate (5 mM) and sodium acetate (40 mM). The cultures were passed sequentially through 25 ml medium containing 0, 50, 75 and 88% $^{2}H_2O$ and 90% $^{2}H_2O$/100% [^{2}H]sodium acetate, each time being allowed to reach an OD A_{600} of 0.8 before being transferred. The final culture contained 55 ml medium and was used to inoculate 11 cultures in 320 ml 88% $^{2}H_2O$/100% [^{2}H]sodium acetate. The 320 ml cultures were harvested when an OD of 2.0 (A_{600}) was reached and yielded 18.3 g wet cell paste. rRNA was extracted and the labeled 5′-NTPs were prepared as previously described (19); 7330 A_{260} OD units were obtained.

Two 12 ml *in vitro* transcriptions were carried out as described (18,19) using either ^{15}N-labeled 5′-NTPs or ^{2}H/^{15}N-labeled 5′-NTPs. The PAGE purified RNA molecules were dissolved in 1.0 M NaCl, 20 mM potassium phosphate, pH 6.8, and 2.0 mM EDTA and dialyzed extensively against 10 mM NaCl and 10 mM potassium phosphate, pH 6.8, using a Centricon-3 concentrator (Amicon Inc.). The sample was diluted with buffer to a volume of 200 µl and annealed. $MgCl_2$ was added to a concentration of 30 mM and the samples were dialyzed twice against a buffer of 10 mM NaCl and 10 mM potassium phosphate, pH 6.8, and 12.5 mM $MgCl_2$. The RNA was diluted to a volume of 200 µl using the final dialysis buffer and lyophilized to a powder. The samples were resuspended in 90% H_2O/10% $^{2}H_2O$ to a concentration of 75 A_{260} OD units in 220 µl (~0.85 mM).

NMR spectroscopy

Spectra were acquired on a Bruker AMX-500 spectrometer equipped with a ^{1}H–{X} reverse detection probe. Broadband decoupling of the imino nitrogen resonances was achieved using GARP (20) (γB_2 = 1570 Hz). Solvent suppression was achieved using either spin lock pulses (21) or binomial $1\bar{1}$ or $1\bar{3}3\bar{1}$ read pulses (22) with maximum excitation at 12.5 p.p.m. and were acquired at 12°C. Quadrature detection was achieved using the States-TPPI method and acquisition was delayed by a half-dwell in all indirectly detected dimensions. ^{1}H longitudinal relaxation measurements were performed as 2D HSQC experiments as reported elsewhere (23), except that the final read pulse was of the binomial type and the H_2O resonance was not presaturated. Nine experiments with longitudinal magnetization recovery delays ranging from 20 to 800 ms were acquired for each molecule and each experiment was repeated five times. The spectral widths were ω_1 = 1500 Hz and ω_2 = 12 205 Hz and the system was allowed 3.5 s to recover between scans. The spectral widths for the 2D NOESY experiments were ω_1 = 5500 Hz and ω_2 = 12 205 Hz. The 2D ^{15}N ω_1 half-filtered NOESY experiments (24) were collected at a mixing time (τ_m) of 260 ms and the filter delay was set to 10.4 ms, which is equal to $1/J_{HN}$. For the 3D {^{15}N}-HSQC-NOESY experiments, τ_m = 240 ms, the ^{15}N–^{1}H anti-phase magnetization was allowed to develop for 5.0 ms and spectral widths were ω_1 = 1200 Hz, ω_2 = 5500 Hz and ω_3 = 12 205 Hz. ^{2}H decoupling was not employed in the 2D and 3D experiments.

RESULTS AND DISCUSSION

Problems associated with low signal-to-noise ratios and resonance overlap limit the analysis of NMR spectra of large or slowly tumbling molecules. Complete deuteration of the non-exchangeable

sites of proteins facilitates the structure determination of large proteins by reducing dipolar relaxation-associated line broadening of the amide proton resonances (8,9,25). An analogous isotopic enrichment scheme has been applied to the RNA binding site for ribosomal protein S8 (Fig. 1), leaving only the exchangeable imino and amino protons available for ^{1}H NMR studies. The exchangeable protons participate in hydrogen bonding networks that mediate base–base interactions in nucleic acids and are the source of NOEs for establishing RNA secondary structure. NOEs involving these protons can also provide more global structural information if tertiary interactions can be identified, such as those involving coaxial stacked helices (14). Thus, NOE-based experiments involving the exchangeable proton resonances of ribonucleic acids are of particular interest for RNA structure studies.

Preparation of perdeuterated RNA

The procedure for preparation of the perdeuterated RNA is similar to published procedures for preparation of ^{13}C- and ^{15}N-enriched RNAs (19,26,27). Uniformly ^{15}N/^{2}H-labeled 5′-NMPs were prepared from labeled rRNA that was extracted from *E.coli* cultured in medium of 90% ^2H_2O with [^{15}N]ammonium sulfate and [^{2}H]sodium acetate as the sole nitrogen and carbon sources. Cells were grown on 11.0 g [^{2}H]sodium acetate and yielded 7330 A_{260} absorbance units (~6.60×10^{-4} mol using an average extinction coefficient of ε_{260} = 11 100) of rRNA. This yield is ~65% less per g carbon source than we have obtained from cells cultured using glucose and H_2O, however, the reduced yield is offset by the lower per mol cost of [^{2}H]sodium acetate relative to [^{2}H]glucose. The fraction of ^{2}H enrichment was estimated from one-dimensional ^{13}C NMR spectra. All of the ribose carbon positions exhibited a 90–95% level of deuterium enrichment. The C2 (adenine) and C8 (purine) positions contained 85 and 90% deuterium respectively and the C6 and C5 positions of the pyrimidines contained 90 and 88% deuterium respectively.

Effects of deuteration on ^{1}H magnetization

The secondary structure of the RNA oligonucleotide used in this study is depicted in Figure 1. Stabilization of the RNA tertiary structure and complex formation between the RNA and ribosomal protein S8 require a Mg^{2+} concentration of ~15 mM. The combination of molecular size and divalent cation concentration extends the correlation time of the free RNA hairpin, resulting in broad imino proton resonances (~33 Hz) and limiting the sensitivity of the NMR experiments.

Recovery of longitudinal magnetization of the imino protons after inversion was measured for the deuterated and fully protonated RNA hairpins. Although the observed rates of recovery contain contributions from both proton dipolar interactions and from chemical exchange (15), the relative rates of recovery between corresponding protons within the deuterated and protonated hairpins reflect primarily differences originating from proton dipolar interactions. Figure 2 compares the distribution of imino proton recovery times for the two molecules at the same concentration and under the same buffer conditions. The recovery data were fitted to single exponential decays to provide a qualitative comparison between the two molecules. This method is supported by the high quality of fits achieved, as determined by calculated values of χ^2 (typical values were <0.5).

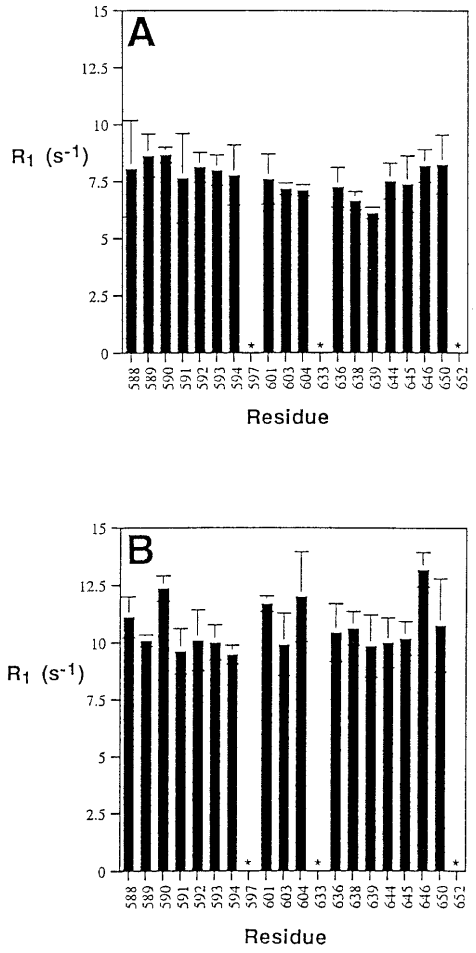

Figure 2. Graphical presentation of the longitudinal recovery rates, R_1, for (**a**) the deuterated RNA hairpin and (**b**) the protonated RNA hairpin. The recovery of magnetization from residues G597, G633 and G652 is dominated by exchange with the solvent. The recovery rates contain contributions from both dipolar relaxation and from chemical exchange with the solvent. Error bars indicate the standard deviations calculated from five sets of relaxation experiments.

The observed recovery times for protons of the deuterated hairpin ranged between 20 and 40% longer than those of the fully protonated RNA molecule (33% longer on average). Only ^{1}H dipolar interactions contribute significantly to the different relaxation rates, since deuteration is not expected to influence the chemical exchange processes. Deuteration of the non-exchangeable sites in proteins has similarly been shown to decrease the longitudinal relaxation rates of the amide protons by almost 35% relative to those of the fully protonated protein (25). Thus, deuteration of the non-exchangeable sites in RNA molecules reduces the number of dipolar interactions of the imino protons and results in a decrease in the recovery rate of longitudinal magnetization of the imino protons even in the presence of chemical exchange.

The line width of a resonance is often determined by its transverse relaxation rate, but other factors, including chemical exchange, may also have a significant effect. The line widths of the imino proton resonances of the deuterated RNA hairpin are 10–15% narrower than the corresponding resonances of the

protonated RNA hairpin (~28 and ~33 Hz respectively). This indicates only a small decrease in the effective ^{1}H transverse relaxation rates in the deuterated RNA hairpin. The small degree of resonance narrowing upon deuteration suggests that chemical exchange significantly contributes to the imino proton resonance line width relative to the contribution of proton cross-relaxation. The relative crosspeak heights in HSQC and HMQC spectra of the two RNA hairpins are also consistent with a line width that is dominated by chemical exchange. An HMQC experiment was optimized for detection of the imino resonances and HSQC experiments were optimized for separate detection of the imino and amino resonances. An increase in the signal-to-noise ratio upon deuteration is expected if proton cross-relaxation significantly contributes to the line width of the exchangeable protons. In the case of the imino proton resonances, there is only a small difference in the signal-to-noise ratio between corresponding resonances of the deuterated and protonated RNA hairpins in either the HMQC or HSQC spectra. However, the cytidine amino resonances of the deuterated hairpin exhibit a greater range of sensitivity enhancement (10–60% signal-to-noise ratio increase) over the corresponding resonances of the protonated hairpin. Nevertheless, this sensitivity gain is modest compared with the 2.5-fold sensitivity increase observed for the amide protons of proteins (8). Relaxation measurements of the amide proton resonances of proteins indicate that transverse relaxation rates can be reduced by 50% when the side chain positions are deuterated, resulting in narrower amide resonances and improved signal-to-noise ratios (25). Thus, the large sensitivity gains that are expected from decreased transverse relaxation rates appear to be lost to varying degrees to chemical exchange in this RNA molecule.

Consequences of deuteration for NOE-based spectra

Several NOE-based experiments were performed using the S8 RNA hairpin to investigate the effects of complete deuteration on the spectroscopic properties of medium sized RNA molecules. Figure 3a–d compares the imino regions of 260 ms mixing time NOESY spectra of the deuterated, ^{15}N-enriched and fully protonated, ^{15}N-enriched RNA hairpins. Both crosspeaks and diagonal peaks in the spectrum of the deuterated molecule exhibit 2- to 5-fold higher signal-to-noise ratios than the corresponding peaks of the fully protonated RNA molecule. However, the line widths of the peaks are similar in the spectra of both RNA molecules, indicating that the increased signal-to-noise ratio exhibited by the deuterated RNA hairpin results from a reduced number of cross-relaxation pathways, rather than decreased transverse relaxation rates of the protons in the deuterated molecule. Figure 4 compares one-dimensional vectors along ω_1 of the 260 ms NOESY spectra at the imino proton frequency of U591H3. In order to ensure that the sensitivity differences observed between the two spectra at 260 ms were not significantly influenced by the NOE build-up rate, 240 and 280 ms mixing time NOESY spectra of the fully protonated RNA hairpin were also collected. The difference in the crosspeak intensities is greater between the latter spectra of the fully protonated RNA molecule and the 260 ms NOESY spectrum of the deuterated RNA molecule. This suggests that the sensitivity improvement results from the reduced number of cross-relaxation pathways available to the imino protons in the deuterated molecule rather than a more rapid decay of the NOEs in the fully protonated

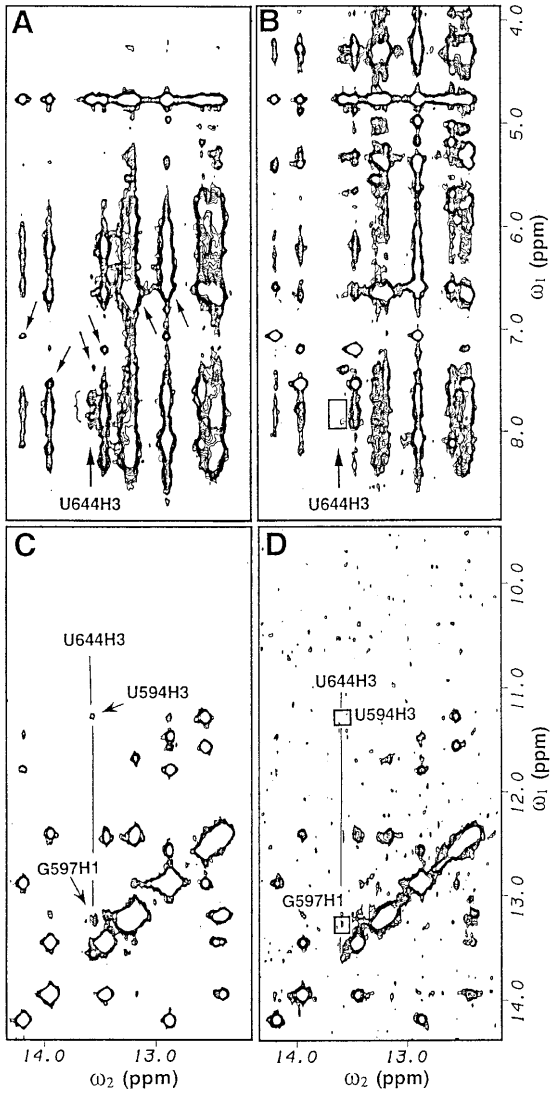

Figure 3. Downfield regions of the 260 ms mixing time 2D NOESY spectra. (**a**) The amino–imino region (**c**) and imino–imino region of the deuterated S8 RNA hairpin and (**b**) the amino–imino region and (**d**) imino–imino region of the fully protonated S8 RNA hairpin. The arrows in (a) indicate the H2–H3 crosspeaks corresponding to the six A·U base pairs of the deuterated RNA hairpin. The corresponding crosspeaks are considerably more intense in the spectrum of the fully protonated RNA hairpin. Positions of crosspeaks that are absent from the spectrum of the fully protonated RNA hairpin and are referred to in the text are indicated by boxes in (b) and (d). Crosspeaks between both amino protons of A596 and U644H3 are present in (a) but only one of the two crosspeaks is present in (b).

molecule. Thus, deuteration of the non-exchangeable sites limits cross-relaxation to a few pathways and enhances the sensitivity of the exchangeable proton NOESY spectrum.

Since deuteration results in a decrease in both longitudinal and transverse relaxation rates, the time required for the protons in the molecule to return to thermal equilibrium should increase. However, longer recovery delays become impractical in multi-dimensional NOE-based experiments and thus decrease the maximum sensitivity enhancement that can be realized from deuteration. Indeed, a 15–20% loss of sensitivity enhancement

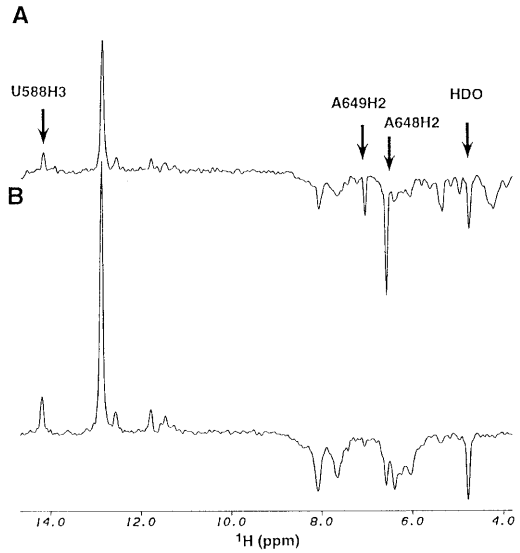

Figure 4. ω_1 vectors from the 2D NOESY spectra at ω_2 = 12.88 p.p.m. for (**a**) the fully protonated and (**b**) the deuterated RNA hairpins. The spectra are plotted to show the same level of noise. Peaks downfield of 11.3 p.p.m. in ω_1 are folded one time and therefore appear to be of opposite intensity relative to peaks upfield of 11.3 p.p.m.

was reported for a deuterated protein (8). However, by limiting excitation of the solvent resonance, sensitivity loss resulting from incomplete relaxation of the imino and amino protons can be avoided. The exchange of imino and amino protons with solvent is sufficiently rapid that the steady-state ^{1}H magnetization of a deuterated nucleic acid is largely dependent upon recovery of the solvent protons and not longitudinal relaxation of the exchangeable protons. Thus, the majority of imino and amino ^{1}H magnetization can be replenished during the relatively short recovery periods typically used in multi-dimensional NMR experiments and the sensitivity improvement provided by deuteration will not be diminished by incomplete magnetization recovery.

Spectral simplification via deuteration

The exchangeable protons can provide valuable information for a variety of structural features, including non-standard base pairs (28), base triples (29,30) and coaxial stacked helices (14). However, the crosspeaks between imino and amino protons can be difficult to interpret because of resonance overlap with non-exchangeable proton resonances. The NOESY spectrum of the deuterated RNA hairpin contains crosspeaks involving only the ^{15}N-bound exchangeable protons, which markedly simplifies the imino region of the spectrum (Fig. 3a and b). An ω_1-filtered NOESY experiment must be employed to achieve comparable spectral simplification using the fully protonated RNA hairpin. However, the sensitivity of this experiment is considerably reduced relative to a standard NOESY experiment, due to the effects of chemical exchange and relaxation during the filter delay periods, and results in the loss of most crosspeaks in this molecule (data not shown).

Elements of the RNA binding site for protein S8

The RNA binding site for ribosomal protein S8 consists of two helical regions that flank a core of 9 nt that are evolutionarily conserved (31). Core residues A596, G597, C643 and U644 are required for RNA–protein S8 association and have been proposed to form A·U and G·C base pairs (31,32). Crosspeaks between the imino protons of adjacent base pairs in helices facilitate the sequence-specific resonance assignment of the imino protons. The NOE between G597H1 and U644H3 is critical for identification of the G597·C643 base pair, but this interaction is too weak to be observed in the NOESY spectrum of the fully protonated RNA hairpin (Fig. 3). The G597H1–U644H3 NOE is present in the spectrum of the deuterated RNA hairpin and helps to confirm that G597 and C643 form a base pair. The signal-to-noise ratio of the NOESY spectrum of the fully protonated hairpin, and hence the possibility of identifying the G597–U644 NOE, can be improved by increasing the number of scans in the 2D experiment, but the time required to achieve the necessary improvement is impractical.

Comparative phylogenetic analysis studies predict that residues A595, A596 and U644 form an A·(A·U) base triple (31) and NMR spectral evidence supporting the base triple has recently been reported (33). The NOESY spectrum of the deuterated hairpin contains crosspeaks between the amino protons of A596 and the imino proton of U644. Importantly, both of the A596 amino protons resonate in the low field region of the spectrum, indicating that both protons are involved in base–base interactions. These NOEs cannot be identified in spectra of the fully protonated RNA hairpin. The NOESY spectrum of the deuterated RNA hairpin also contains a weak crosspeak between the imino resonance of U644 and the H2 resonance of A596 (Fig. 3a). This crosspeak is more easily identified in the spectrum of the fully protonated RNA hairpin. Together, these interactions are critical for confirming the presence of the A·(A·U) base triple in the RNA hairpins containing the complete binding site for protein S8.

Application of the ^{15}N separated NOESY experiment

^{15}N separated 2D and 3D NOE experiments provide a means to resolve crowded regions of the exchangeable proton spectrum. We have assigned the cytidine amino resonances of the deuterated RNA hairpin (Table 1) using a 3D HSQC-NOESY experiment that was optimized for detection of the imino protons. In this experiment, the imino–amino proton crosspeaks of the NOESY spectrum were labeled in the third dimension with their corresponding amino nitrogen chemical shifts. Figure 5a and b shows ω_2–ω_3 NOE planes of the 3D spectrum at the amino nitrogen chemical shifts corresponding to C637 and C651. The intra-base pair imino–amino crosspeaks of all G·C base pairs, except G604·C634 and G598·C643, which exhibit weak imino proton resonances, are present in the 3D spectrum. Many of the crosspeaks involving the adenine and guanine amino resonances are not readily observed in this experiment. However, the 2D version of this experiment permits identification of several intra-base pair and inter-base pair crosspeaks involving purine amino protons (Fig. 5c). The crosspeak between A596N6 and U644H3 helps to establish the participation of A596 in the A·(A·U) base triple by providing a means to assign NOEs that are observed between amino protons and U644H3 in the NOESY spectrum (Fig. 3a). These and other imino–amino interactions are not readily distinguished in the NOESY spectrum, but are

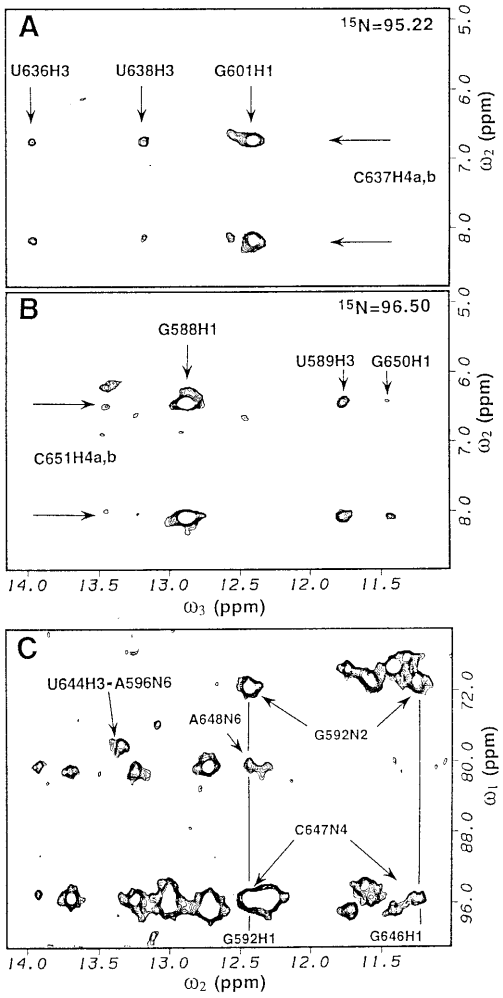

Figure 5. (**a** and **b**) ω_1–ω_3 planes extracted from the 3D HSQC-NOESY spectrum at the amino ^{15}N frequencies for C637 and C651 respectively. (**c**) Plot of the 2D HSQC-NOESY spectrum acquired on the deuterated RNA hairpin. The 2D spectrum acquired for the fully protonated RNA hairpin contained only a few intra-base pair crosspeaks. No imino–amino crosspeaks were observed in the 3D spectrum of the fully protonated RNA hairpin.

resolved and can be assigned using the ^{15}N separated NOESY spectrum. Analogous HSQC-NOESY experiments acquired using the fully protonated RNA hairpin exhibit poor sensitivity and could not be used to obtain these correlations.

Although the ^{15}N separated experiments facilitate crosspeak interpretation, the sensitivity of these experiments can be limited by chemical exchange in addition to relaxation. The amino protons of guanine and adenine bases are frequently broadened by chemical exchange of the amino group about the C–N bond. These chemical exchange processes are present in the S8 RNA hairpin and prohibit the efficient transfer of magnetization during the HSQC portion of the experiment. However, dipolar relaxation mechanisms are less severe for the deuterated molecule than the fully protonated molecule, resulting in improved sensitivity in the 2D and 3D experiments. Recently, experiments have been reported that facilitate the observation of NOEs involving the exchange-broadened amino proton resonances of guanine and adenine bases (34). These experiments make use of *xy*–16 pulse elements to overcome problems associated with C–N bond rotation and chemical exchange. Thus, even further sensitivity improvements during ^{1}H–^{15}N transfer steps should be realized using these methods, since the effects of amino proton chemical exchange are reduced.

Table 1. Amino resonance assignments for the deuterated RNA hairpin obtained from 2D NOESY and 2D and 3D HSQC-NOESY spectra

Residue	Ha, Hb	N4	N2	N6
C599	8.10, 6.78	93.89		
C605	8.37, 6.70	94.30		
C634	8.35, 6.72	95.15		
C637	8.19, 6.73	95.22		
C643	8.02, n.a.	99.46		
C647	8.15, 6.68	95.12		
C651	8.10, 6.45	96.50		
G592	6.60, 5.80		71.64	
G601	6.66, 5.93		71.40	
G604	n.a.		71.42	
G639	7.00, 5.43		71.10	
G645	5.38[a]		68.28	
G646	6.28[a]		71.01	
G650	6.24[a]		69.40	
A596	7.89, 7.69			78.18
A602	7.67, 6.06			81.09
A635	n.a.			81.14
A642	n.a.			75.80
A648	7.57, 6.03			80.55
A649	6.67, 6.02			80.47

^{1}H and ^{15}N chemical shifts are referenced to external standards of DSS and NH_4OH respectively, which are at 0.0 p.p.m. n.a., the resonance(s) has not been assigned.

[a]The amino proton chemical shifts are degenerate for these residues.

CONCLUSION

The variety and complexity of RNA systems amenable to high resolution NMR structural analysis has dramatically increased with the introduction of ^{13}C and ^{15}N heteronuclear NMR methods, in part by increasing the spectral dispersion. We have shown that perdeuteration of the non-exchangeable sites of RNA oligonucleotides can improve the quality of NOE-based spectra involving the exchangeable proton resonances. Perdeuteration decreases the longitudinal relaxation rates of the imino protons but has little effect on the exchangeable proton line widths, suggesting that chemical exchange dominates the apparent transverse relaxation rates. Nevertheless, deuteration results in an increased signal-to-noise ratio of NOE-based experiments and permits extraction of additional structural information. The incorporation of deuterium also simplifies the exchangeable region of the NOESY spectrum without the need to apply ^{15}N or ^{13}C filters, which can reduce sensitivity. Further, the residual protonation of adenine C2 permits the identification of A·U base pairs through the AH2–UH3 crosspeak in the deuterated molecule.

Although chemical exchange processes remain active in the deuterated molecule, the NOE crosspeaks contain fewer contributions from spin diffusion, which should provide the opportunity to extract more accurate inter-proton distances. We are beginning efforts to explore the utility of RNA deuteration in the protein S8–RNA complex, where the molecular correlation time is significantly increased.

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