Comparison of H5 and H8 relaxation rates of a $^{2}H/^{13}C/^{15}N$ labeled RNA oligonucleotide with selective protonation at C5 and C8

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Abstract Uniformly $^{13}C/^{15}N$ enriched ribonucleotide monophosphates have been prepared with extensive deuterium enrichment of the non-exchangeable positions. The purine C8 and pyrimidine C5 base positions were selectively protonated prior to incorporation of the individual nucleotide triphosphates into an RNA oligonucleotide. The longitudinal and transverse relaxation rates of the H8 and H5 resonances of this deuterated molecule were compared with the relaxation rates of the corresponding protonated, $^{13}C/^{15}N$ enriched RNA molecule. Deuteration disrupts the efficiency of ^{1}H–^{1}H dipolar relaxation and reduces the longitudinal and transverse magnetization relaxation rates on average to 25% and 68%, respectively, of the values measured for the non-deuterated RNA molecule. Importantly, the longitudinal relaxation rates remain sufficiently rapid ($> 1s^{-1}$) to permit the use of short recovery delays in multidimensional NMR experiments without significant loss of sensitivity.

Key words: S8 rRNA; Deuterium enrichment; NMR; Resonance assignment; RNA structure

1. Introduction

Heteronuclear multidimensional NMR methods have extended the size and complexity of proteins and nucleic acids that can be studied in solution by improving spectral resolution and by providing the means to correlate resonances through scalar coupled pathways. However, the sensitivity of these experiments can be dramatically reduced by unfavorable relaxation characteristics associated with ^{1}H dipolar relaxation. These effects generally become more pronounced as the molecular correlation time increases and as the number of proximal ^{1}H relaxation partners increase.

Deuterium enrichment at the non-exchangeable sites of proteins and nucleic acids is an effective labeling strategy to lessen the ill effects associated with dipolar relaxation. Investigations using protein systems have demonstrated that amide proton line broadening associated with ^{1}H dipolar relaxation is reduced when the number of ^{1}H relaxation partners is decreased through perdeuteration and ultimately results in improved spectral sensitivity [1–4]. It has also been shown that selective deuteration within the sugar moieties of DNA [5] and RNA [6] oligonucleotides decreases the transverse relaxation rates of the remaining non-exchangeable protons and results in decreased linewidths and improved spectral resolution of those proton resonances. Further, perdeuteration of the non-exchangeable sites of ^{15}N enriched RNA oligonucleotides simplifies and improves the sensitivity of spectra involving the nitrogen bound imino and amino protons [7].

Here we report on the preparation of a $^{2}H/^{13}C/^{15}N$ enriched RNA oligonucleotide that is protonated at the pyrimidine base C5 and purine base C8 positions. The RNA oligonucleotide, which requires Mg^{2+} for proper folding, contains the binding site for ribosomal protein S8 and forms a hairpin that tumbles through solution slowly (Fig. 1) [8]. The longitudinal and transverse relaxation rates of the H5 and H8 nuclei of the deuterated RNA hairpin are slower than the relaxation rates of the corresponding nuclei of the fully protonated RNA hairpin. The presence of the imino and amino protons increases the longitudinal relaxation rates of the H8 nuclei but has no effect on their transverse relaxation rates. Finally, the proton enrichment at the pyrimidine C5 positions in the deuterated RNA hairpin facilitated assignment of several H5 resonances through the use of a filtered NOESY experiment.

2. Materials and methods

All enzymes were purchased from Sigma chemical (St. Louis, MO) with the exception of the T7 RNA polymerase which was prepared as described [9]. Deoxyribonuclease I Type II, pyruvate kinase, adenylate kinase, and 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. The 90% $^{2}H_2O$, ^{13}C enriched sodium acetate, and ^{15}N enriched ammonium sulfate were purchased from Cambridge Isotope Labs.

2.1. Preparation of the $^{2}H/^{13}C/^{15}N$ labeled RNA

The RNA oligonucleotides were prepared by in vitro transcription as described [10] using either $^{2}H/^{13}C/^{15}N$ labeled or $^{13}C/^{15}N$ labeled 5′-nucleoside triphosphates (5′-NTPs). The $^{2}H/^{13}C/^{15}N$ 5′-NTPs were obtained by growing *E. coli* on minimal media [11] containing 90% $^{2}H_2O$, ^{15}N ammonium sulfate as the sole nitrogen source, and $^{13}C_2$-sodium acetate as the sole carbon source. Starter cultures were prepared in LB media and transferred to 25 ml of minimal media containing M9 salts [11], ammonium sulfate (5 mM), sodium acetate (38 mM), $MgSO_4$ (1 mM), $FeCl_3$ (50 μM), $CaCl_2$ (10 μM), $MnCl_2$ (10 μM), and nicotinamide (1.5 mg/l). The cultures were passed sequentially through 25 ml of media containing 0%, 50%, 75%, and 92% $^{2}H_2O$ each time being allowed to reach an O.D. of $A_{600} = 0.8$ before being transferred. The final culture contained 55 ml of media and was used to inoculate five 330 ml cultures containing 90% $^{2}H_2O$ and 99% $^{13}C_2$-sodium acetate (38 mM). The 330 ml cultures were harvested when an O.D. of $A_{600} = 1.5$ was reached and yielded 8.2 g of wet cell paste. Ribosomal RNA was extracted and degraded to 5′-NMPs as described [12,13].

Protonation of the purine C8 and pyrimidine C5 positions was accomplished chemically using a method similar to that employed for deuteration of DNA oligonucleotides [14]. The 5′-NMPs were

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Abbreviations: NOE, nuclear Overhauser enhancement; NOESY, NOE spectroscopy; 2D, two-dimensional; HSQC, heteronuclear single quantum coherence

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diluted to a concentration of 30 mM in a 300 mM solution of ammonium sulfite, pH 8.0 and warmed to 65°C. After 60 h, ^{1}H and ^{13}C 1D NMR indicated >95% protonation of purine C8 and >90% protonation of pyrimidine C5 positions. The reaction was lyophilized and the 5′-NMPs purified by passage through a boronate affinity column. The labeled 5′-NTPs were prepared using standard procedures [12,13] and yielded 2800 A_{260} O.D. units.

Two 16 ml in vitro transcription reactions were performed to obtain the $^{13}C/^{15}N$ labeled (protonated) and the $^{2}H/^{13}C/^{15}N$ labeled (deuterated) RNA hairpins. Deuterium enrichment did not affect the yield of RNA obtained from the 14 h transcription reactions. 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. The samples were annealed and dialyzed against a buffer of 10 mM NaCl, 10 mM potassium phosphate, pH 6.8, and 12.5 mM $MgCl_2$ and then concentrated to a volume of 220 μl and lyophilized. The samples were resuspended in either 100% $^{2}H_2O$ or 90% H_2O/10% $^{2}H_2O$ to a concentration of 135 A_{260} O.D. units in 220 μl (≈1.2 mM).

2.2. NMR spectroscopy

Spectra were acquired on a Bruker AMX-500 spectrometer equipped with a ^{1}H-{$^{13}C/^{15}N$} triple resonance probe. Broadband decoupling of the base carbon and nitrogen resonances was achieved using GARP [15]. For relaxation spectra collected in 90% H_2O, solvent suppression was achieved using either spin lock pulses [16] or binomial 1$\bar{1}$ read pulses [17] with maximum excitation at 8.0 ppm. Quadrature detection was achieved using the States-TPPI method and acquisition was delayed by a half-dwell in the indirectly detected dimensions. ^{1}H longitudinal (R_1) and transverse (R_2) relaxation rates were determined at 28°C and were measured using constant time versions of 2D HSQC experiments as reported elsewhere [3]. For R_1 measurements, nine experiments with recovery delays ranging from 25–800 ms were acquired for each molecule and each experiment was repeated three times. The spectral widths were ω_1=1200 Hz and ω_2=6100 Hz and the system was allowed 3.5 s to recover between scans. For R_2 measurements of H8, eight experiments were recorded with recovery delays ranging from 4-40 ms using a spin lock field of 8.3 kHz with the carrier set at 7.60 ppm. This produces an effective tip angle of 86° for the most off-resonance H8 nuclei. A spin lock field of 2.9 kHz with the carrier set at 4.76 ppm was used for the H5 measurements to alleviate effects of possible Hartmann-Hahn transfer between H5 and the residual H6 nuclei of the deuterated molecule. This produces effective tip angles of 78° for the most downfield H5 resonance and 64° for the most upfield residual H6 resonance. An ω_1 ^{13}C half-filtered 2D NOESY experiment [18] was acquired in 90% $^{1}H_2O$ at 19°C using spectral widths ω_1=5600 Hz and ω_2=12 195 Hz and was optimized for detection of the imino proton resonances in ω_2. The filter delay was optimized for $^{1}J_{HC}$=175Hz. All data were processed using Felix 95 (Biosym/MSI, San Diego, CA).

3. Results and discussion

Deuteration alleviates effects resulting from rapid nuclear relaxation rates that limit the analysis of NMR spectra of large or slowly tumbling molecules. Perdeuteration of $^{13}C/^{15}N$ enriched proteins facilitates the resonance assignment by reducing the amide proton linewidths and by extending the T_2 relaxation times of the deuterated ^{13}C nuclei [3,12,19]. The

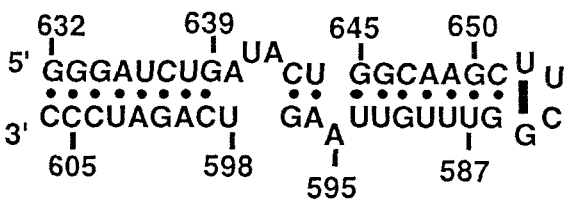

Fig. 1. Secondary structure of the deuterated and protonated RNA hairpins used in this study. The nucleotide numbering is based on that used for the *E. coli* 16S rRNA.

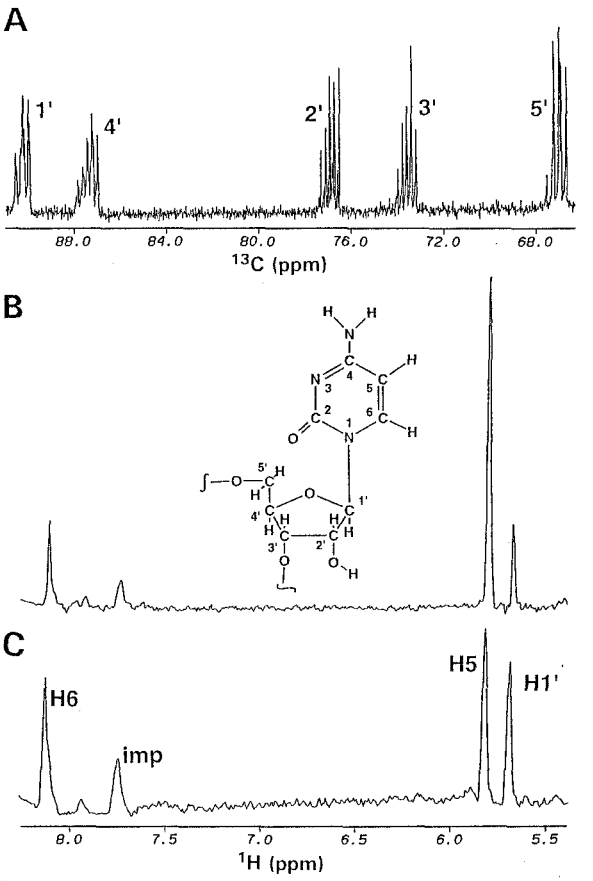

Fig. 2. (A) The ribose region of the $^{1}H/^{2}H$ decoupled ^{13}C spectrum of $^{2}H/^{13}C/^{15}N$ labeled 5′-CMP. The C1′ and C4′ peaks exhibit residual ^{15}N and ^{31}P coupling, respectively. Base and 1′ region of the $^{13}C/^{15}N$ decoupled proton spectrum of 5′-CMP (B) after the proton exchange reaction and (C) before the proton exchange reaction. An impurity is marked *imp*. There was no conversion of 5′-CMP to 5′-UMP as determined by HPLC.

NMR experiments that employ perdeuterated proteins achieve the highest possible sensitivity by beginning and ending with amide proton magnetization. An analogous isotopic enrichment scheme has been devised for application to RNA molecules. This scheme makes available only the purine H8 and pyrimidine H5 resonances for ^{1}H NMR studies in $^{2}H_2O$.

3.1. Preparation of perdeuterated RNA

The procedure for preparation of the deuterated RNA is similar to published procedures for preparation of ^{13}C and ^{15}N enriched RNAs [12,13,20,21]. Uniformly $^{2}H/^{13}C/^{15}N$ labeled 5′ NMPs were prepared from labeled rRNA that was extracted from *E. coli* cultured in media of 90% $^{2}H_2O$ with ^{15}N ammonium sulfate and ^{13}C sodium acetate as the sole nitrogen and carbon sources. Cells were grown on 5.0 g of sodium acetate and yielded 2980 A_{260} absorbance units (~2.68×10^{-4} mol using an average extinction coefficient of ε_{260}=11,100) of rRNA. This yield is approximately 10% less per gram of carbon source than recently obtained using 90% $^{2}H_2O$ and deuterio-sodium acetate as the sole carbon source [7]. The fraction of ^{2}H enrichment of the 5′ NMPs was estimated from one-dimensional ^{13}C NMR spectra acquired with ^{1}H and ^{2}H decoupling and is listed in Table 1. The enrichment of the ribose methyne groups (C1′–C4′) is extensive with

> 68% deuteration at all positions. The ribose methylene carbon, the C5′ position, is approximately 56% enriched with one deuteron and exhibits a slight stereospecific selection for proton over deuteron at the pro-R position. This is indicated by the ^{1}H NMR spectra of the 5′-NMPs which show that the upfield shifted H5″ resonance, pro-R [22], is 12–15% more intense than the H5′ resonance. Finally, 38% of the ribose C5′ positions are enriched with two deuterons and 6% remain fully protonated. Fig. 2 shows the ribose region of the ^{13}C spectrum of 5′-CMP. The ^{2}H bound ^{13}C nuclei are shifted ~0.4 ppm upfield from the ^{1}H bound ^{13}C nuclei. Higher deuterium enrichment levels (> 90% at all ribose carbon positions) could have been achieved using deuterio-sodium acetate [7]. However, it should be noted that because the cost of the deuterated carbon source is three times greater than the cost of the protonated carbon source, the use of methylotrophic bacteria for the production of fully deuterated RNA molecules may be a less expensive alternative [23].

The ^{2}H nuclei at the purine C8 and pyrimidine C5 positions of the unseparated 5′-NMPs were exchanged for ^{1}H nuclei using a procedure similar to one that had previously been applied to exchange deuterons for protons in a DNA oligonucleotide [14]. The exchange reaction was tested using an unlabeled full length S8 RNA oligonucleotide and resulted in non-specific cleavage of > 75% of the RNA molecule within 24 h. Thus, the exchange was performed on the 5′-NMPs and purification was achieved by passage through a boronate affinity column before conversion to the 5′-NTPs. Fig. 2 compares the H6–H5 region of the ^{1}H spectrum of 5′-CMP before and after treatment with the ammonium bisulfite solution. A possible side product of the exchange reaction is the proton catalyzed deamination of cytidine to uridine [24]. However, partial cytidine deamination is not a serious concern since the exchange is performed on the NMPs and uridine is ~25% less abundant than cytidine in *E. coli* rRNA. The exchange at C8 and C5 resulted in > 95% and > 92% protonation at these two positions. No additional proton enrichment was observed at other carbon positions as determined by 1D ^{13}C NMR.

The cost of molecule preparation is an important consideration for the general applicability of triple isotopic enrichment of RNA. Although more expensive than $^{13}C/^{15}N$ double labeling schemes, the ^{13}C source remains the most expensive component for preparation of the triple labeled RNA molecule (> 60% of the cost). However, the costof preparation can be further reduced through the recovery of the media $^{2}H_2O$.

Table 1
Percent ^{2}H isotope enrichment at the ribose and non-exchangeable base positions of the ribonucleosides

Position	^{2}H enrichment
ribose C1′	75
ribose C2′	83
ribose C3′	78
ribose C4′	68
ribose C5′	56[a], 38[b]
Cyt, Ura C5	64
Cyt, Ura C6	77
Gua, Ade C8	65
Ade C2	80

[a]Single deuterium enrichment.
[b]Double deuterium enrichment.

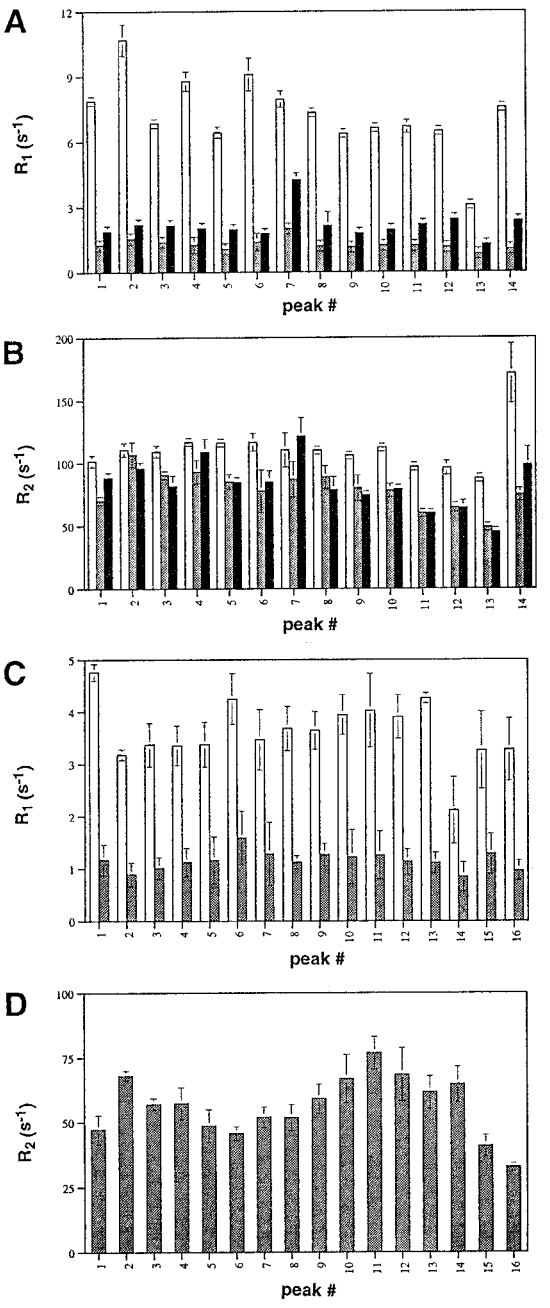

Fig. 3. Graphical presentation of the longitudinal, R_1, and transverse, R_2, relaxation rates of the RNA hairpins. (a) The H8 R_1 values for the deuterated (▩) and protonated (□) hairpins in $^{2}H_2O$ and the deuterated hairpin in 90% $^{1}H_2O$ (■). (b) The H8 R_2 values for the deuterated (▩) and protonated (□) hairpins in $^{2}H_2O$ and the deuterated hairpin in 90% $^{1}H_2O$ (■). (c) The H5 R_1 values for the deuterated (▩) and protonated (□) hairpins in $^{2}H_2O$ and (d) the H5 R_2 values for the deuterated hairpin in $^{2}H_2O$. Error bars indicate the standard deviations calculated from three sets of relaxation experiments.

3.2. *Effects of deuteration on ^{1}H magnetization relaxation*

The recovery of the H5 and H8 longitudinal magnetization after inversion was measured for the deuterated and fully protonated RNA hairpins in $^{2}H_2O$. Differences in the longitudinal relaxation rates between corresponding protons within

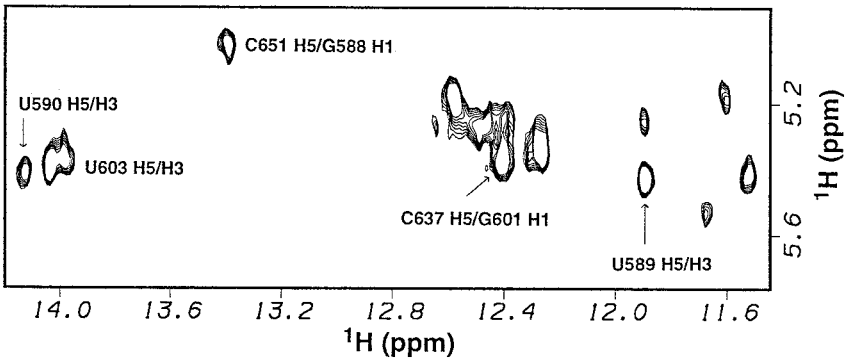

Fig. 4. Imino region of the ^{13}C ω_1-selected 2D NOESY spectrum. Cross peaks in this region correspond to NOE interactions between pyrimidine H5 and imino protons within the same base pair.

the two RNA hairpins reflect differences originating from proton dipolar interactions. Fig. 3 depicts the distribution of H5 and H8 relaxation rates for the two RNA molecules at the same concentration and under the same buffer conditions. The H5 and H8 resonances are not uniquely identified in this report since only partial assignments have been determined for the base protons of the S8 RNA molecule. The resolution of the 2D HSQC spectrum permitted accurate rate measurements for 14 of 21 H8 and 16 of 22 H5 resonances. The relaxation data were fit to single exponential decays using the Levenberg-Marquardt non-linear least squares method [25]. The relaxation rates for the H8 nuclei of the deuterated hairpin were slower by a factor of 3–6 (3.9 on average) than those of the fully protonated RNA molecule. The relaxation rates of the H5 nuclei of the deuterated RNA molecule are 2–4 fold slower than the corresponding rates of the fully protonated molecule. The smaller R_1 reduction for the H5 resonances upon deuteration is consistent with the anticipated distribution of dipolar relaxation partners of H5 and H8. In canonical A-form helices, three to four non-exchangeable protons are located within 3.0Å of H8 while only one proton, H6, is within 3.0Å of H5. Substitution of these relaxation partners with deuterium dramatically reduces the efficiency of the R_1 process but results in a differential decrease of R_1 for the H5 and H8 nuclei.

In the absence of chemical exchange, the linewidths of the H5 and H8 resonances are dominated by their transverse relaxation rates. ^1H dipolar interactions are one of the factors that contribute to the mechanism of transverse relaxation of these nuclei. The R_2 values of the H5 and H8 nuclei of the deuterated molecule and the H8 nuclei of the protonated molecule are shown in Fig. 3. In this report, the decay of transverse magnetization is a measurement of $T_{1\rho}$ rather than T_2 since spin-lock experiments were employed. No attempt was made to measure the transverse relaxation rates of the pyrimidine H5 resonances of the protonated RNA hairpin due to the proximity of the through-bond coupled H6 resonances. The H8 resonances of the deuterated RNA hairpin exhibit on average a 35% reduction of R_2 and the linewidths of the H8 resonances of the deuterated RNA hairpin are 20–25% narrower than the corresponding resonances of the protonated RNA hairpin ($\sim$27 and $\sim$34 Hz respectively). Similarly, the linewidths of the H5 resonances of the deuterated RNA molecule are about 10–20% narrower than the corresponding resonances of the protonated RNA molecule, 26 Hz and 32 Hz respectively. These narrower resonances result in a modest (30–40%) signal-to-noise increase of H8 and H5 resonances in the HSQC spectrum upon deuteration.

The transverse relaxation rates of the amide proton resonances of proteins can be reduced by up to 50% when the side chain positions are deuterated resulting in narrower amide resonances and 2 fold gains in signal-to-noise [4]. The 10–20% linewidth reduction measured for the H5 and H8 resonances of this RNA molecule is not as dramatic as the 50% linewidth reduction that has been measured for the amide resonances of perdeuterated (80% actual enrichment) proteins [3]. Although the density of proximal ^1H neighbors may be different for amide and nucleotide base protons, it is also important to note that H5 and H8 are ^{13}C bound. The ^{13}C–^1H dipolar contribution to ^1H transverse relaxation is about twice as great as the ^{15}N–^1H dipolar contribution [26]. Studies on triple labeled proteins have suggested that heteronuclear dipolar relaxation mechanisms are relatively more efficient for R_2 than for R_1 [3]. This idea is consistent with the nearly 10 fold difference between the fractional decreases of R_1 and R_2 effected by deuteration (Fig. 3). Thus, the heteronuclear dipolar interaction reduces the effectiveness of deuteration in dramatically decreasing proton R_2 values in this molecule.

The longitudinal and transverse relaxation rates of the H8 resonances of the deuterated RNA hairpin were also measured in 90% H_2O. Under these conditions, the imino and amino positions are protonated and therefore have the potential to facilitate relaxation of the non-exchangeable base protons. The R_1 values exhibit an average 25% increase in the presence of the amino and imino resonances while the R_2 values are largely unaffected (Fig. 3). The minimal R_1 effects observed for the H8 resonances are not unexpected since the H8-amino/imino proton distance is $\geq$4.5 Å in regular A-form RNA helices. The proximity of the H5 resonances to the water resonance prevented accurate determinations of the H5 R_1 and R_2 values in 90% ^1H_2O. Although the H5 relaxation rates were not determined, they are expected to increase due to the nearby amino protons found in G•C and A•U base pairs.

3.3. *Consequences of deuteration for RNA spectra*

The proton longitudinal relaxation rates of deuterium enriched proteins and nucleic acids have a significant impact on the experimental utility of these molecules. The maximum signal-to-noise gain expected from linewidth reduction will not be realized if the longitudinal magnetization returns to equilibrium *too* slowly ($1s^{-1} > R_1$), since long recovery delays

are impractical for many multidimensional experiments [4]. The recovery rates measured in ^2H_2O indicate that the H5 and H8 T_1 relaxation times are 1.0s which is comparable to the relaxation times observed in short, non-deuterated oligonucleotides without ^{13}C enrichment [3]. Thus the directly attached ^{13}C nuclei facilitate the relaxation of longitudinal magnetization and permit the use of efficient recovery delays without loss of sensitivity.

Deuterium enrichment of $^{13}C/^{15}N$ labeled RNA oligonucleotides can simplify and enhance the sensitivity of NOE based 2D and 3D experiments. The conventional sequence specific assignment strategy for the H5 resonances using the H6/H8-H1′ region of the NOESY spectrum is hindered by the broad lines resulting from slow molecular tumbling. Thus an alternative strategy employing a ^{13}C ω_1-selected half-filtered 2D NOESY spectrum was used to assign several of the H5 resonances through the imino-H5 cross peaks (Fig. 4). The intra-base UH5-UH3 and intra-base pair CH5-GH1 cross peaks in this spectrum are largely the products of spin diffusion through the amino protons (AN^6H_2 and CN^4H_2). The same spectrum acquired on the fully protonated RNA hairpin is complicated by the presence of both imino-H5 and imino-H1′ cross peaks. However, the relatively small degree of protonation at the 1′ ribose positions of the protonated RNA hairpin prevents the detection of the H1′-imino proton cross peaks and eliminates problems associated with H1′/H5 resonance overlap. Inter-base NOEs involving H5 and H8 resonances should also be enhanced by this deuteration scheme as the number of pathways that deplete observable magnetization is significantly reduced. These sequential H5 and H8 inter-atomic distances yield constraints that are fundamental to the RNA structure determination process.

An additional consequence of this isotopic enrichment scheme is the decreased relaxation efficiency of the deuterium attached ^{13}C nuclei. Deuteration of $^{13}C/^{15}N$ enriched proteins dramatically reduces the ^{13}C linewidths and has resulted in the application of structural NMR methods to systems ranging in size from 29–37 kDa [2,19]. The success of NMR techniques employing deuterium enriched proteins suggests strategies to enhance triple resonance scalar methods used to obtain correlations between base and ribose moieties in nucleic acids [27]. Decreasing the C1′ transverse relaxation rate by means of deuteration increases the efficiency of magnetization transfer through the glycosidic bond and can increase the sensitivity of the triple resonance base-ribose correlation experiments. Thus, the selective protonation of C5 and C8 combined with the favorable ^1H and ^{13}C relaxation properties imparted by deuteration should permit the development of improved scalar correlation methods for nucleic acids.

4. Conclusion

^{13}C and ^{15}N isotopic enrichment strategies have increased the variety and complexity of ribonucleic acid systems amenable to high resolution structural analysis using NMR. The inclusion of ^2H provides additional tools for the study of slowly tumbling nucleic acid systems. Although deuteration results in decreased longitudinal relaxation rates of the H5 and H8 nuclei, experimental sensitivity for this RNA molecule is not limited by H5 or H8 magnetization recovery. The ^1H–^{13}C dipolar coupling contributes to the proton transverse relaxation and limits the line narrowing advantages provided by deuteration. The isotopic enrichment scheme presented in this report should facilitate the investigation of sizable nucleic acid systems and can improve the quality of structures that can be achieved for large RNA molecules.

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