REPORT

NMR spectroscopy of RNA duplexes containing pseudouridine in supercooled water

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ABSTRACT

We have performed NMR experiments in supercooled water in order to decrease the temperature-dependent exchange of protons in RNA duplexes. NMR spectra of aqueous samples of RNA in bundles of narrow capillaries that were acquired at temperatures as low as -18°C reveal resonances of exchangeable protons not seen at higher temperatures. In particular, we detected the imino protons of terminal base pairs and the imino proton of a non-base-paired pseudouridine in a duplex representing the eukaryotic pre-mRNA branch site helix. Analysis of the temperature dependence of chemical shift changes (thermal coefficients) for imino protons corroborated hydrogen bonding patterns observed in the NMR-derived structural model of the branch site helix. The ability to observe non-base-paired imino protons of RNA is of significant value in structure determination of RNA motifs containing loop and bulge regions.

Keywords: pseudouridine; branch site; RNA; supercooled water; NMR; imino protons

INTRODUCTION

Determination of nucleic acid structures by NMR methodology relies upon numerous inter- and intramolecular distances and angular constraints. Interactions involving exchangeable imino and amino protons provide important information about hydrogen bonds and stabilizing interactions with water molecules, particularly for analysis of unusual structural motifs of folded RNA molecules. A sizable fraction of labile protons in RNA ($-\text{NH}$, $-\text{NH}_2$, and $-2'\text{OH}$), however, exchange rapidly with solvent at $T > 0^{\circ}\text{C}$. As a result, they are exchange broadened beyond detection, thus escaping use in structural studies. Sufficiently decreased temperature slows chemical exchange between these protons and water, allowing for their direct detection and recruitment for structural study. Borer and colleagues (Kerwood et al. 2001) detected resonance peaks of imino protons of terminal nucleotides at -6°C that were not observed at 0°C . In order to monitor non-base-paired

imino protons, however, a method to achieve temperatures as low as -20°C without freezing is desirable.

Exploiting the empirical observation that the freezing point of water decreases proportionally with volume (Angell 1982), Poppe and van Halbeek (1994) studied sucrose in glass capillary tubes at -17°C , allowing measurement of $-\text{OH}$ proton chemical shifts and $^3J_{\text{HH}}$ couplings. The capillary technique was extended to BPTI, ubiquitin, dATP, and dGTP in supercooled water at about -18°C (Skalicky et al. 2000, 2001).

In this study, we have investigated the structural role of exchangeable protons of pseudouridine (Ψ), a rotational isomer of uridine attached to its ribose through C5, in RNA duplexes. Ψ has two imino nitrogen atoms, $\Psi \text{ N}^1\text{H}$ and $\Psi \text{ N}^3\text{H}$, both of which are protonated at physiological pH (Hall and McLaughlin 1991). Presence of Ψ in RNA helices has been shown to increase thermal stability without altering structure (Davis and Poulter 1991; Hall and McLaughlin 1991; Arnez and Steitz 1994; Kintanar et al. 1994; Durant and Davis 1999; Yarian et al. 1999), postulated to be the result of a water-mediated hydrogen bond involving the $\Psi \text{ N}^1\text{H}$ (Arnez and Steitz 1994; Newby and Greenbaum 2002a) and/or improved base stacking (Yarian et al. 1999; Chui et al. 2002). In the case of the pre-mRNA branch site helix of the yeast spliceosome, the presence of a conserved Ψ induces a strikingly different structure than that observed with uridine (Newby and Greenbaum 2001,

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FIGURE 1. Sequences of the two RNA duplexes Ψ_{comp} (top) and Ψ_{BP} (bottom). Ψ_{BP} represents short sequences from the pairing of U2 snRNA (top strand) and the intron (bottom strand) from the yeast *S. cerevisiae*. The numbering scheme was that used in previous structure determination studies (Newby and Greenbaum, 2001, 2002a,b). The position of Ψ (Ψ_6 in this sequence) corresponds to a phylogenetically conserved Ψ residue in U2 snRNA (Ψ_{35} in yeast). Ψ_{comp} is a similar sequence without the bulged A.

2002b). Structural models of the branch site duplex suggested that Ψ did not appear to form a base pair with the opposing adenine (A23, adjacent to the bulged base, A24), and no resonance attributable to Ψ N³H was visible (Fig. 1). Identification of this resonance would contribute important information about the environment of this imino proton with respect to surrounding bases.

In order to slow the exchange of imino protons, we have acquired NMR spectra of two RNA duplexes containing Ψ (Fig. 1) in aqueous solutions at supercooled temperatures by implementation of the capillary technique (Poppe and van Halbeek 1994). In addition to detecting imino protons corresponding to terminal base pairs of an RNA duplex, we observed the upfield-shifted N³H imino proton of a non-base-paired Ψ in the branch site duplex. Determining the role of Ψ in stabilizing RNA structures may explain its phylogenetic conservation in the branch site helix and elsewhere in structural RNA molecules.

RESULTS AND DISCUSSION

We first acquired NMR spectra of pseudouridine monophosphate (Ψ MP) in order to characterize the resonances of its two imino protons, Ψ N¹H and Ψ N³H, in a non-base-paired environment. Spectra of imino protons (including line widths) were identical for samples in capillaries and as a bulk sample at 0°C and 5°C (data not shown). The Ψ N¹H and Ψ N³H proton chemical shifts were 10.6 and 10.8 ppm and line-widths at half-height ($\nu_{1/2}$) were ~ 3.8 and ~ 5.1 Hz at 0°C, respectively (Fig. 2A).

Upon cooling to -15°C , $\nu_{1/2}$ decreased monotonically (~ 2.0 and ~ 2.7 Hz for N¹H and N³H, respectively), consistent with line narrowing associated with slower solvent exchange. $\nu_{1/2}$ of N¹H was slightly larger than that of N³H, presumably as a result of dipolar broadening from the proximal H6 proton of Ψ (~ 2.5 and 4.8 Å between H6 and N¹H and N³H, respectively). We also observed a slight temperature dependence of chemical shift (thermal coefficient) for the imino protons. Positive thermal coefficients (i.e., more upfield chemical shift as a function of decreased temperature) correlate with rapid exchange, whereas negative thermal coefficients correlate with slower exchange, and are therefore consistent with hydrogen bonding (Nonin et al. 1995). We plotted the chemical shift with respect to temperature for each imino proton. The thermal coefficients for Ψ N¹H and Ψ N³H were 6.1 and 5.3 ppb/°C, respectively, consistent with a non-base-paired environment for each proton (data not shown).

By comparison, NMR spectra of a complementary duplex (Ψ_{comp}) (Fig. 1) indicate that all imino protons, including N³H of Ψ , participate in Watson-Crick base pairs (Fig. 2B). When Ψ is in the anti-conformation, its N³H

FIGURE 2. Imino proton spectra of Ψ -monophosphate (Ψ MP) (A), Ψ_{comp} (B), and Ψ_{BP} (C) acquired at different temperatures between 5°C and -17.5°C . (A) Ψ MP (10 mM in 10 mM Na phosphate at pH 6.4, 50 mM NaCl, 0.1 mM EDTA, in 90% $\text{H}_2\text{O}/10\%$ D_2O) was taken up into six capillary tubes, which were placed in a 5-mm NMR tube (fill factor of ~ 0.25). (B) Ψ_{comp} (2.7-mM duplex) was prepared as in A and was taken up into 10 capillary tubes (fill factor of ~ 0.4). (C) Ψ_{BP} (2.5 mM duplex) was prepared as in A and taken up into nine capillary tubes (fill factor of ~ 0.3). Data were collected using a jump-return-echo pulse sequence (Sklenár and Bax 1987) on a Varian 720-MHz and 600-MHz spectrometer at the National High Magnetic Field Laboratory.

forms a hydrogen bond with the opposing adenine N¹, reflected in the downfield shift of the imino proton resonance to ~13.1 ppm (Fig. 2B; Hall and McLaughlin 1992; Durant and Davis 1999). Further confirmation of this assignment came from comparison of chemical shifts of a similar duplex in which Ψ were replaced by uridine (U). We assigned the resonance at 10.6 ppm to Ψ N¹H, which is on the major groove edge of the base when in an anti-conformation, based upon the chemical shift for this imino proton in Ψ MP and from observation of an NOE between it and the H6 proton (~2.5 Å away) (Hall and McLaughlin 1992; Newby and Greenbaum 2001). In order to determine how this proton is protected from rapid exchange with solvent, our previous studies made use of a CLEANEX-PM pulse sequence (Hwang et al. 1997) to characterize the interaction between Ψ N¹H and water molecules (Newby and Greenbaum 2002a). For protons undergoing chemical exchange, this experiment determines whether there is a component of cross-relaxation. The spectrum acquired using a CLEANEX-PM pulse sequence revealed a strong negative peak at the resonance location of Ψ N¹H (but no other imino proton), indicating that the interaction with water is characterized by a significant component of cross-relaxation. This observation is consistent with participation of Ψ N¹H in a water-mediated hydrogen bond with a phosphate oxygen atom of the same or a neighboring nucleotide. This hydrogen bonding status was predicted by Durant and Davis (1999), and visualized in a crystal structure of tRNA containing pseudouridine (Arnez and Steitz 1994).

At about -10°C, a broad peak appeared at ~12.2 ppm with a shoulder at ~12.5 ppm. All imino protons of Ψ_{comp} had previously been assigned except for those belonging to terminal base pairs (Fig. 2B); therefore, these new peaks were attributed to the imino protons of terminal residues. In contrast with the narrowing of peaks of the monophosphate at lower temperatures, broadening of peaks of the complementary duplex was observed below 0°C (line widths increased gradually from ~50 Hz to ~130 Hz). The exact reasons for this behavior may be a complex combination of slower tumbling of the larger molecule as a result of increased solvent viscosity, salt and buffer effects, or may represent the beginning of cold denaturation.

We then performed similar experiments on a minimal pre-mRNA branch site duplex of *Saccharomyces cerevisiae* (Ψ BP), which represents the pairing between a short consensus region of the U2 snRNA and the intron (Fig. 1). In contrast with the complementary duplex, presence of a Ψ residue in the U2 snRNA strand of Ψ BP (top strand in Fig. 1) in its conserved position (Yu et al. 1998; Ma et al. 2003) results in a very different conformation than in its unmodified counterpart (Newby and Greenbaum 2001, 2002b). In the novel Ψ BP motif, the unpaired adenosine is extruded from the helix and forms a base triple with the minor groove edge of A7 in the A7-U22 base pair. The 2'OH of this extrahelical

adenosine is the nucleophile in the first cleavage step of splicing. The structural motif preferred in the presence of this Ψ (Ψ 35 in the native yeast sequence) may explain the strong phylogenetic preservation of this modified base in this location. In Ψ BP, the chemical shift of Ψ N¹H is ~10.5 ppm (vs. ~10.6 ppm in Ψ MP and Ψ_{comp}). As was observed in Ψ_{comp} , previous NMR investigation of the interaction of Ψ N¹H of Ψ BP indicated that this proton undergoes cross-relaxation with water molecule(s) in the major groove of the duplex (Newby and Greenbaum 2002a). Unlike the case with Ψ_{comp} , proton spectra of Ψ BP revealed no resonance attributable to Ψ N³H, and structural models did not indicate formation of a base pair between Ψ and the opposing adenine (A23, adjacent to the branch site base A24).

In order to identify the resonance location of the absent (and presumably exchange broadened) Ψ N³H in the branch site duplex at 0°C–5°C, we acquired spectra of Ψ BP in supercooled water (Fig. 2C). As in spectra of the complementary duplex, a broad new peak emerged at ~12.2 ppm below -5°C, corresponding to the G2 terminal base pair. The imino proton of the other terminal base pair may be degenerate with a resonance at ~13.3 ppm. Unique to the branch site duplex, a broad peak emerged at ~11.2 ppm at -15°C. Because assignments of all other imino protons of the branch site helix were made by systematic comparison with other duplexes (Newby and Greenbaum 2002b), we identified this upfield-shifted resonance as that of Ψ N³H. No NOEs were observed from this broad peak. The Ψ N³H chemical shift was close to that of the unpaired N³H of Ψ MP, which is not base paired (Fig. 2A) and very different from that of the Watson-Crick paired Ψ of Ψ_{comp} (Fig. 2B). The upfield location of Ψ N³H of Ψ BP (Fig. 2C) further supports our original finding that Ψ 6 does not form a canonical base pair with A23 (Newby and Greenbaum 2002b). As with Ψ_{comp} , line widths began to broaden below 0°C, increasing from ~50 Hz to ~200 Hz.

We noted that chemical shifts of each imino proton varied slightly with a decrease in temperature. We therefore generated a thermal coefficient for each imino proton resonance by plotting the chemical shifts as a function of temperature (Fig. 3). For Ψ_{comp} , negative thermal coefficients (more downfield shifts with respect to decreased temperature) were observed for all protons except the terminal imino proton (Fig. 3, top). The thermal coefficient for Ψ N¹H and Ψ N³H was -6.2 ppb/°C and -0.7 ppb/°C, respectively, suggesting that both form hydrogen bonds (Newby and Greenbaum 2001, 2002a,b). The thermal coefficient for G2 was positive, consistent with rapid exchange expected for a solvent-exposed terminal base pair.

In Ψ BP, protons with negative thermal coefficients belonged to G8, G5, G3, U4, and Ψ N¹H, and those with positive thermal coefficients were G2, U22, and Ψ N³H (Fig. 3, bottom). The negative thermal coefficient for Ψ

FIGURE 3. Thermal coefficients for Ψ_{comp} (top) and Ψ_{BP} (bottom). The chemical shift for each proton was plotted as a function of temperature at which the spectrum was acquired. The value (in ppb/ $^{\circ}\text{C}$) for each imino proton is shown next to its position in the sequence. Positive thermal coefficients correlate with rapid exchange and negative thermal coefficients correlate with hydrogen bonding (Nonin et al. 1995). See text for further details.

N^1H (-3.7 ppb/ $^{\circ}\text{C}$) reinforced the conclusion that this imino proton is involved in a hydrogen bond, as suggested by the results of earlier NMR studies (Newby and Greenbaum 2002a). The thermal coefficient of U22 was 1.03 ppb/ $^{\circ}\text{C}$ and that of Ψ N^3H was 0.08 ppb/ $^{\circ}\text{C}$. Although U22 forms a Watson-Crick base pair with A7, its involvement in the base triple with the branch site A and its position adjacent to the unpaired Ψ exposes U22 N^3H to solvent, which apparently increases its exchange rate. For Ψ N^3H , this positive value is in accord with rapid exchange, suggesting the proton is not involved in a hydrogen bond. This observation, combined with its upfield-shifted position and lack of NOEs to surrounding residues, especially the adjacent A^2H , is entirely consistent with our original conclusion about this Ψ 's non-base-paired status (Newby and Greenbaum 2002b) and assists in further refinement of the Ψ_{BP} structure.

In conclusion, NMR data at low temperatures allow us to identify imino protons that are undetected at higher temperatures due to rapid exchange. This technique may be helpful in structure determination of non-base-paired regions of novel RNA motifs and for extending the temperature range over which chemical shift data can be acquired. Applying the capillary technique appears to be promising for NMR in supercooled water using other interesting biomolecules.

MATERIALS AND METHODS

Design and synthesis of samples

The solutions of RNA we used were the pseudouridine monophosphate (ΨMP), a complementary RNA duplex (Ψ_{comp} , $5'\text{-GGUG}\Psi\text{AGUA-3}'$ vs. $5'\text{-UACUACACC-3}'$) (shown in Fig. 1,

top), and an RNA duplex with a bulged adenosine, representing a minimal form of the pre-mRNA branch site from *S. cerevisiae* (Ψ_{BP} , $5'\text{-GGUG}\Psi\text{AGUA-3}'$ vs. $5'\text{-UACUACACC-3}'$; bulged A is underlined) (Fig. 1, bottom). Pseudouridine monophosphate was purchased from Berry and Associates (no. PYA11080). RNA oligomers were purchased from Dharmacon Research and deprotected according to company protocol (Wincott et al. 1995). Samples were precipitated with ethanol, partially desalted by three washes of the pelleted RNA with 80% ethanol, and lyophilized to dryness. The RNA pellets were then resuspended in diethyl pyrocarbonate-treated (DEPC) water, and strand concentrations were calculated from the absorbance at 260 nm. Equimolar amounts of the strands were combined to obtain duplexes of the Ψ_{comp} and Ψ_{BP} and verified by mobility on a non-denaturing gel. The duplexes were lyophilized to dryness and resuspended in $250\ \mu\text{L}$ of NMR buffer consisting of $10\ \text{mM}$ sodium phosphate (pH 6.4), $50\ \text{mM}$ sodium chloride, and $0.1\ \text{mM}$ EDTA in 90% $\text{H}_2\text{O}/10\%$ D_2O (99.96%; Cambridge Isotope Laboratories). Final concentration of RNA strands in NMR samples was $\sim 2.7\ \text{mM}$ for Ψ_{comp} and $\sim 2.5\ \text{mM}$ for Ψ_{BP} . NMR assignments of imino protons of Ψ_{BP} were reported previously (Newby and Greenbaum 2001, 2002b).

Sample preparation

Open-ended glass capillaries of $1.0\ \text{mm}$ OD were purchased from Fisher (no. 34500-99). The capillaries were prepared by soaking in 100% methanol overnight and then dried at 170°C . Solutions of RNA were centrifuged at $14,000\ \text{rpm}$ for $30\ \text{min}$ in order to remove particles that could nucleate ice crystals. The samples were then taken up by capillary action with $\sim 27\ \mu\text{L}$ in each capillary. The ends of the open capillaries were flamed sealed, and the capillaries were placed in a 5-mm NMR tube (Poppe and van Halbeek 1994; Skalicky et al. 2000, 2001). Bundles of 9–10 capillaries have a filling factor of 30%–40% to yield an effective concentration of $\sim 1\ \text{mM}$ for the two RNA duplexes; Ψ_{MP} , with a starting concentration of $10\ \text{mM}$ in six capillaries, had an effective concentration of $\sim 2.5\ \text{mM}$.

NMR spectroscopy

NMR spectra were acquired on a 720-MHz and 600-MHz Varian Unity Plus spectrometers (National High Magnetic Field Laboratory). Samples were cooled at a rate of $-1.5^{\circ}\text{C}/\text{h}$ to $-2.5^{\circ}\text{C}/\text{h}$ with an increase in rate as the temperature decreased. The jump-return echo pulse sequence (Sklenár and Bax 1987) was used to array the temperature decrease in order to observe the imino protons at different temperatures, and freezing of one or more capillaries was monitored by a proportional decrease in signal intensity, but no capillary tubes broke. We used 64 steady-state scans prior to each acquisition. In order to improve signal quality at lower temperatures, we doubled the number of acquisitions for each incremental temperature decrease of 2.5°C : 128 at -5°C , 512 scans for -10°C , and up to 4096 scans at -17.5°C . Processing of NMR data and simulation line-width at half height were accomplished using Felix 2.3 (Biosyn).

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