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Properties of pseudouridine N1 imino protons located in the major groove of an A-form RNA duplex

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ABSTRACT

The exchangeable N1 imino protons of two pseudouridine (ψ) bases located at adjacent internal positions within an undecamer RNA duplex (5'AUACψψACCUG/3'UAUGAAUGGUC) can report on the environment of the major groove of an A-form double-stranded nucleic acid. The ψN1 imino protons of these residues (which are not involved in interstrand Watson-Crick hydrogen bonding) are protected from chemical exchange with the solvent water and thus are observable in the proton NMR spectrum in H_2O (1). These protons will exchange readily at increased pH values or upon thermal denaturation of the duplex. The longitudinal (T_1) relaxation times of the ψN1 imino protons in 100 mM NaCl or in 10 mM $MgCl_2$ and 100 mM NaCl are approximately two-fold faster than those of the ψN3 imino protons which are involved in Watson-Crick base pairing. With the addition of spermidine, the ψN1 imino protons become readily exchangeable at a temperature some 20°C below the melting temperature of the duplex.

INTRODUCTION

Pseudouridine (ψ) is a uridine analogue (Figure 1), and is one of the most common modified nucleotides found in a wide variety of RNAs from tRNAs (2) to small nuclear RNAs (snRNAs). In particular, human U1 snRNA contains two pseudouridine residues located near the 5-terminus of the 165 nucleotide sequence (3). In the proposed structure of the U1 snRNA, approximately twelve 5-terminal nucleoside residues, including the cap structure and two adjacent pseudouridine residues, are single stranded and available to pair with the 5-splice site of the messenger RNA. The putative duplex formed between the U1 snRNA and the pre-mRNA contains eleven base pairs including two A-ψ base pairs (4,5,6).

We have previously described thermodynamic properties of this eleven base pair duplex, 5'AUACψψACCUG/3'UAUGAAUGGAC, and those of the corresponding 'control' sequence containing two A-U base pairs in place of the A-ψ base pairs, and also assigned the imino proton resonances in the NMR spectra of the duplexes. Ten resonances between 12.4 and 13.6 ppm result from those protons hydrogen-bonded in Watson-Crick base-pairs. In the NMR spectrum of the pseudouridine duplex, two additional imino proton resonances were observed at 10.64 and 10.93 ppm. These resonances were assigned to the N1 imino protons of the two pseudouridine residues (1). While it is expected that the hydrogen-bonded ψN3 imino protons would be present in the ^{1}H-NMR spectrum, the presence of the ψN1 protons, which are presumably nonbonded in the major groove of the duplex structure, was unanticipated. Non-hydrogen bonded imino protons have been observed previously in NMR experiments, most notably in DNA loop structures (7,8) and RNA pseudoknots (9). In addition, the N1 imino protons of ψ_{32}, ψ_{39} and ψ_{55} have been observed in the ^{1}H/^{15}N NMR spectra of various tRNAs (10,11,12). The slow exchange of such imino protons has been explained by either a structure that restricts access by water molecules to the sites of the imino protons, or by the presence of an unidentified hydrogen bond acceptor.

In the RNA 11-mer duplex, the two adjacent pseudouridine N1 imino protons are located in the major groove of an apparently normal A-form RNA duplex. In the present report, we have examined the exchange properties of these imino protons in the presence of magnesium, spermidine and under a variety of pH and temperature conditions. The results suggest that the ψN1 imino protons exchange independently of the ψN3 hydrogen-bonded imino protons, and that although they are located in the deep and narrow major groove, they are accessible to specific ions.

EXPERIMENTAL

Materials

Thin layer chromatography (TLC) was performed on 5×10 cm silica gel $60F_{254}$ glass-backed plates, (E. Merck, Darmstadt, Germany). For flash chromatography silica gel 60 (particle size less than 0.063 mm, E. Merck, Darmstadt, Germany) was used under positive pressure from nitrogen gas. High performance liquid chromatography (HPLC) was carried out on ODS-Hypersil (4.6×250 mm) or MOS-Hypersil (9.4×250 mm), (Shandon Southern, England) using a Beckman HPLC system. FPLC employed a 0.5×5 cm or 1.0×10 cm column of mono Q using a Pharmacia FPLC system. ^{31}P and ^{1}H NMR spectra were obtained with a Varian XL-300 multinuclear spectrometer at 121 and 300 MHz, respectively. Absorption spectra were recorded by Perkin-Elmer Lambda 3B UV/Vis spectrophotometer.

Oligonucleotides were synthesized using nucleoside phosphoramidites on an Applied Biosystems 381A DNA synthesizer. 5'-O-(4,4'-dimethoxytrityl)-2'-O-(*t*butyldimethylsilyl) protected nucleoside phosphoramidites, containing aryl- or isobutyrylamides were purchased from Milligen-Biosearch (Bedford, MA). The controlled pore glass support containing the 3'-terminal nucleoside was a product of Peninsula Laboratories (Belmont, CA). 5-β-D-Ribofuranosyluracil (pseudouridine) was obtained from the Sigma Chemical Co. (St. Louis, MO).

Methods

Synthesis of the Pseudouridine Building Block

5'-O-(4,4'-Dimethoxytrityl)pseudouridine **1**. To 1.25 g (5.12 mmol) of pseudouridine in an anhydrous mixture of 45 ml pyridine and 45 ml dimethylsulfoxide was added 2.1 g (6.19 mmol) of 4,4'-dimethoxytrityl chloride and the reaction mixture stirred overnight at ambient temperature. The reaction was stopped by the addition of 5 ml of methanol, the solvents were partially evaporated and the resulting solution was dissolved in methylene chloride and extracted with saturated sodium bicarbonate. The organic phase was washed a number of times with water plus dried $MgSO_4$, evaporated to dryness and the residue was chromatographed on silica gel. The fractions containing product (eluting at 4% methanol/methylene dichloride) were pooled and evaporated to dryness. Yield: 2.05 g (3.74 mmol) 73%.

R_f (CH_2Cl_2/CH_3OH, 9/1): 0.51

U.V. (CH_3OH): λ_{max}=229, 261 nm

^{1}H-NMR ($DMSO_{d6}$ + trace D_2O) δ = 2.45 (m, DMSO), 3.02−3.19 (m, 3H, $H_{4'}$, $H_{5'}$, $H_{5''}$), 3.58, (HOD), 3.83 (m, 1H, $H_{3'}$), 3.91 (m, 1H, $H_{2'}$), 4.52 (d, 1H, $H_{1'}$), 6.85−7.23 (m, 14H, Ar-H, H_6).

2'-O-(t-butyldimethylsilyl)-5'-O-(4,4'-dimethoxytrityl)pseudouridine **2**. Compound **2** was prepared using procedures developed by Ogilvie (13) and Narang (14). To 2.0 g (3.65 mmol) of **1** in 25 ml anhydrous pyridine was added 0.77 g (5.11 mmol) t-butyldimethylsilyl chloride and 0.7 g (10.22 mmol) imidazole and the reaction mixture stirred overnight. After a standard work-up of the reaction mixture, tlc analysis (methylene dichloride/methanol, 95/5) indicated the presence of three spots. The two major products appeared to be the 2' and 3' silyl derivatives, while the third spot (running just ahead of the 2' silyl derivative) appeared to be the 2',3'-bissilyl compound. After resolution by silica gel chromatography, 0.65 g of pure 2' isomer was obtained. The 3' silyl derivative was isomerized to a mixture of 2' and 3' derivatives by stirring overnight in methanol containing a few drops of triethylamine. After chromatographic isolation an additional 0.46 g of pure 2' silyl derivative was obtained. Yields: **2**, 1.11 g (1.68 mmol) 46%, mixture of 2' and 3' silyl derivatives, 0.69 g (1.04 mmol) 28%, the 2',3'-bissilyl compound (containing some 2' derivative) 0.45 g (~0.58 mmol) 16%.

R_f (CH_2Cl_2/CH_3OH, 95/5): 0.38

U.V. (CH_3OH): λ_{max}=229, 260 nm

^{1}H-NMR ($DMSO_{d6}$ + trace D_2O) δ = 0.19 (s, 3H, CH_3), 0.20 (s, 3H, CH_3), 0.84 (s, 9H, *t*-butyl), 2.45 (m, DMSO), 3.05−3.16 (m, 3H, $H_{4'}$, $H_{5'}$, $H_{5''}$), 3.58, (HOD), 3.84 (m, 1H, $H_{3'}$), 4.10 (m, 1H, $H_{2'}$), 4.57 (m, 1H, $H_{1'}$), 6.82−7.47 (m, 14H, Ar-H, H_6).

2'-O-(t-butyldimethylsilyl)-3'-O-[(N,N-diisopropylamino)(β-cyanoethoxy)phosphinyl]-5'-O-(4,4'-dimethoxytrityl)pseudouridine **3**. Compound **3** was prepared from **2** employing the procedure described by Usman (15) using 150 mg (0.23 mmol) **2**, 325 mg (2.7 mmol) 2,4,6-collidine, 9.5 mg (0.11 mmol) N-methylimidazole and 250 ml (1.15 mmol) 2-cyanoethyl N,N-diisopropylchlorophosphoramidite. After purification by silica gel chromatography (dichloromethane, 1% triethylamine and a gradient of methanol), the pooled fractions containing the product were precipitated into pet. ether. Yield: 120 mg (0.14 mmol) 61%.

R_f (Ethylacetate/hexane/triethylamine, 49.5/49.5/1): 0.65

^{31}P-NMR ($CDCl_3$) δ = 145.5, 147.4 (H_3PO_4 ext).

RNA Synthesis

The two RNA fragments, one containing two pseudouridine residues, ^{5}'AUAC$\psi\psi$ACCUG, and its complement, ^{5}'C-AGGUAAGUAU, were synthesized from 10 μmol of CPG-bound guanosine (or uridine). For each elongation cycle we employed 97, 99, 97, 86, and 86 mg of phosphoramidite dissolved in 1.0 ml of anhydrous acetonitrile (generating a 0.1 M solution) for the incorporation of guanosine, adenosine, cytidine, uridine and pseudouridine, respectively. The CPG beads were contained in a glass column 13 mm in diameter and approximately 25 mm long. The flow of reagents to the column was controlled by setting the argon pressure at 4.2 bar. Under this pressure, the flow of acetonitrile to the column was 2.7 ml/min. Three 0.33 ml aliquots of each phosphoramidite solution (with a corresponding amount of tetrazole) was added to the CPG beads. The addition of each aliquot was followed by a reaction time of 2 min, for a total reaction time of 6 min for each elongation. After completion of the synthesis, and removal of the final 5'-terminal DMT group, each fragment was deprotected using the following procedures: The glass beads containing the fully protected RNA, minus the 5'-terminal DMT residue, were treated with concentrated aqueous ammonia/ethanol (3/1) for 6h. The glass beads were filtered and nitrogen was passed through the resulting solution to remove most of the ammonia prior to evaporation of the solution to dryness. The residue was dried by evaporation from anhydrous pyridine (×3) and the residual pyridine was removed by evaporation once from toluene. To the residue was added 4 ml of 1.0 M tetrabutylammonium fluoride, and this solution was gently shaken in the dark at ambient temperature for 16 h. To this solution, 0.5 ml of 0.5 M sodium cacodylate, pH 6.0 was added and the solution was evaporated

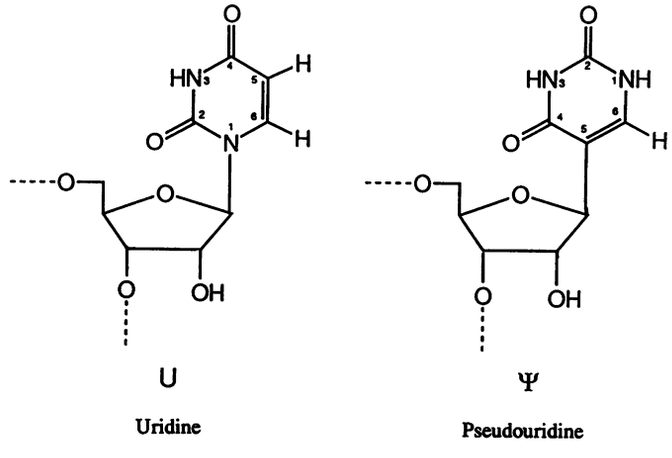

Figure 1. Comparison of the chemical structures of uridine and pseudouridine.

to remove most of the tetrahydrofuran. Enough water was added to dissolve the residual salts and the crude RNA sample was desalted on a 2.5×40 cm column of Sephadex G10. The collected material was lyophilized to dryness, dissolved in 2 ml of distilled water and purified by ion-exchange chromatography using multiple injections on a 0.5×5 cm or 1.0×10 cm mono Q column. The material collected from the mono Q column was desalted and lyophilized to dryness. The yields of purified undecamers varied from 200−240 A_{260} units (~10−12 mg).

NMR Experiments

Samples for NMR experiments were dissolved in 350 μl of 100 mM NaCl, 10 mM sodium cacodylate, pH 6, with 5% D_2O at a concentration of approximately 2 mM. The pH of this solution was measured with a microelectrode (Microelectrodes Inc., Londonderry, NH) and adjusted with 0.1 M NaOH or 0.1 M HCl. After a series of pH experiments, the solution was readjusted to pH 6. Stock solutions of 1 M $MgCl_2$ or 1 M spermidine were added directly to the RNA solution. Addition of spermidine did not alter the pH value of the solution. The temperature of the NMR sample was controlled with the Varian VT unit to within ±1°C.

Imino proton spectra were recorded on the Varian Unity 500 spectrometer fitted with a Nalorac 5 mm indirect detection probe (Nalorac Cryogenics Corp, Martinez, CA) at Washington University. Water suppression was accomplished with a 1-3-3-1 pulse sequence. A relaxation delay of 0.5 msec was used. For measurement of the imino proton T_1 relaxation times, all imino protons were nonselectively inverted; an array of relaxation delay times prior to observation allowed accumulation of a series of spectra. Measurements were made at various temperatures and as a function of the solution conditions. Spectra were processed, then the arrayed spectra were fit to a single exponential using VNMR T_1 analysis software.

RESULTS

Pseudouridine was converted to the corresponding protected phosphoramidite using procedures that were only slightly modified from those employed for uridine. Pseudouridine exhibits poor solubility in anhydrous pyridine, and the reaction of a suspension of the nucleoside in pyridine with the dimethoxytrityl chloride gave reduced yields with a significant amount of pseudouridine containing two DMT groups. However, this problem was easily addressed by performing this reaction in an anhydrous solution of pyridine/DMSO (1/1).

RNA Synthesis

Although solid phase RNA synthetic procedures have been reported for small quantitities of material (~1 μmol), there are few reports for larger syntheses (16). We have attempted to optimize the amount of phosphoramidite necessary to efficiently elongate 10 μmol of CPG-bound nucleoside. We have maintained the concentration of the phosphoramidite solutions at 0.1 M such that after mixing with an equivalent volume of tetrazole solution, the concentration of phosphoramidite present in the reaction column remained at 0.05 M. Under these conditions, it was necessary to add three equivalents of the phosphoramidite solution (total volume of 1.0 ml, and 85−100 mg of phosphoramidite) with a 2 min reaction time after each addition. These conditions produced high yield nucleoside elongations based upon the absorption of the solution of the DMT cation obtained after each reaction.

After complete deprotection of each sequence, HPLC analysis using an ion-exchange column indicated that the desired 11-mer was the major product (Fig. 2). The analysis of the pseudouridine-containing sequence also indicated the presence of significant amounts of smaller sequences (Fig. 2). While these shorter products may result from inefficient coupling reactions, at this time we think it is more likely that these peaks reflect some hydrolysis of the intact sequence during the ammonia deprotection step at 50°C for 6 h. It is likely that phosphoramidite derivatives containing aryloxyamides (e.g., phenoxyacetyl protection of the exocyclic amines) will result in higher yields of the desired product since the aryloxy derivatives can be hydrolyzed in ammonia at ambient temperature (16). HPLC purification of the 11-mers synthesized as described provided sufficient material for the NMR studies.

ψN1 imino protons

The resonances of the two N1 imino protons from the two pseudouridine bases have relatively narrow line widths in the NMR spectrum (Figure 3) indicating that the rate of exchange of these protons with water is reduced. The slow exchange rates for these imino protons permitted NOEs to be observed both between ψN1 protons on adjacent ψ_5 and ψ_6 bases as well as between each ψN1 H and its corresponding intrabase ψC6 proton

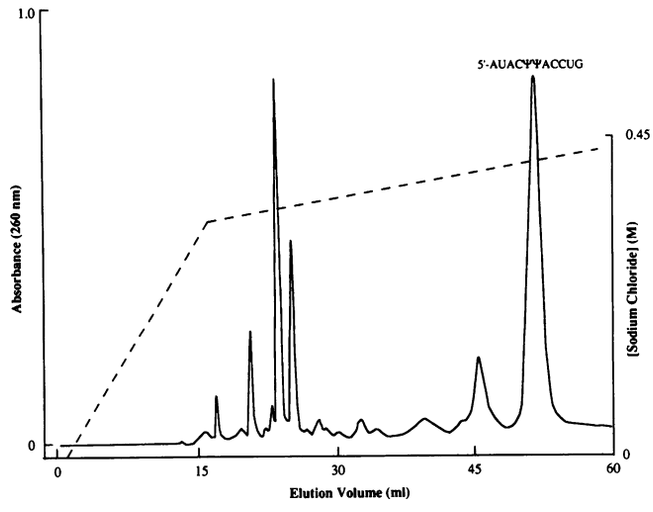

Figure 2. HPLC elution profile of the AUACψψACCUG 11-mer following deprotection.

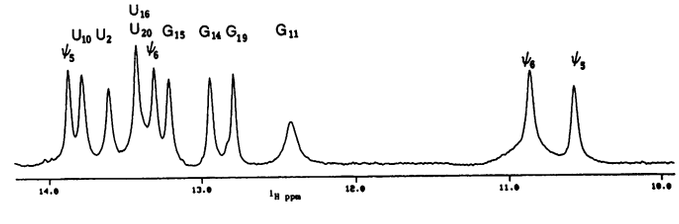

Figure 3. Imino proton NMR spectrum of the undecamer duplex at 25°C. Assignments are shown: $^{5'}A_1U_2A_3C_4\psi_5\psi_6A_7C_8C_9U_{10}G_{11}{}^{3'}/{}^{5'}C_{12}A_{13}G_{14}G_{15}U_{16}A_{17}A_{18}G_{19}U_{20}A_{21}U_{22}{}^{3'}$. The ψ5 and ψ6 resonances at 10.6 and 10.9 ppm are from the N1 protons.

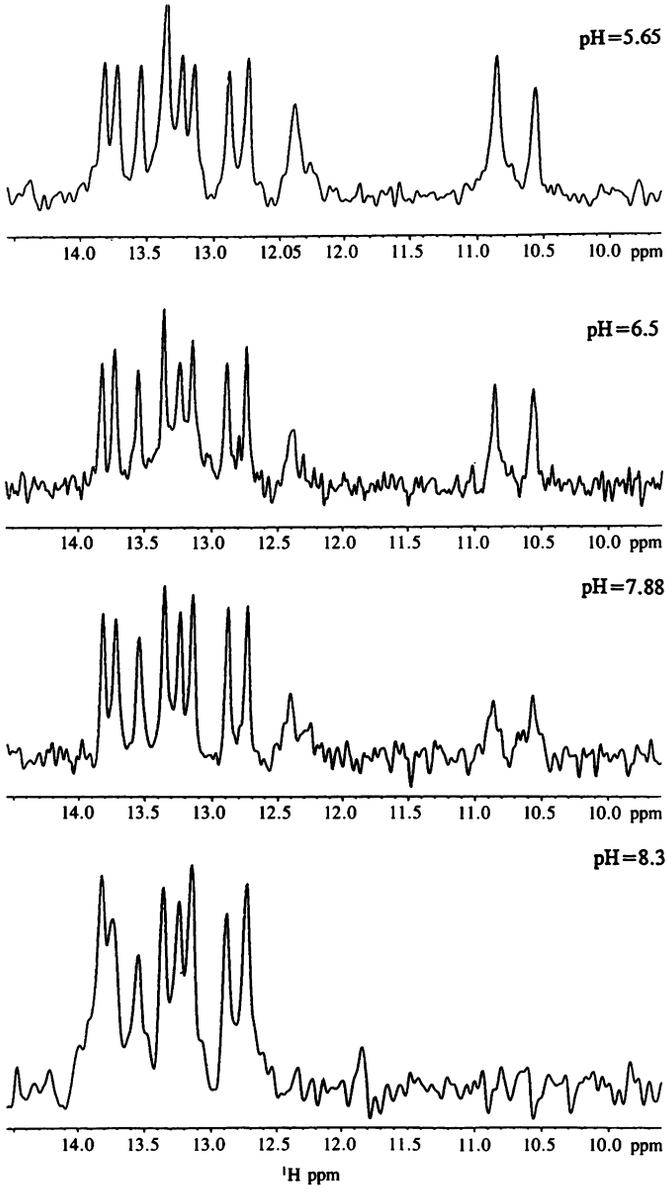

Figure 4. Imino proton spectra of the duplex at 25°C as a function of pH in 100 mM NaCl, 10 mM sodium cacodylate.

(1). These proton resonances were observed as long as the duplex remained intact, which suggested that exchange with water was rate limiting rather than the lifetime of the base pair (7). When exchange with solvent is rate limiting, the linewidth of the ψN1 proton resonances should be sensitive to the hydroxide ion concentration (pH value). As shown in Figure 4, raising the pH of the solution (increasing [OH^-]) increased the linewidth of the ψN1 proton resonances until at pH 8.3 and 25°C, they no longer appeared in the spectrum. At this pH value, the resonance from the hydrogen-bonded imino proton of the $C_{11}G_{12}$ base pair (at 12.4 ppm) had also disappeared, presumably because fraying of this terminal base pair results in rapid imino proton exchange with water. Other hydrogen-bonded imino proton resonances exhibit no change in chemical shift with pH, indicating that the structure of the duplex is unchanged.

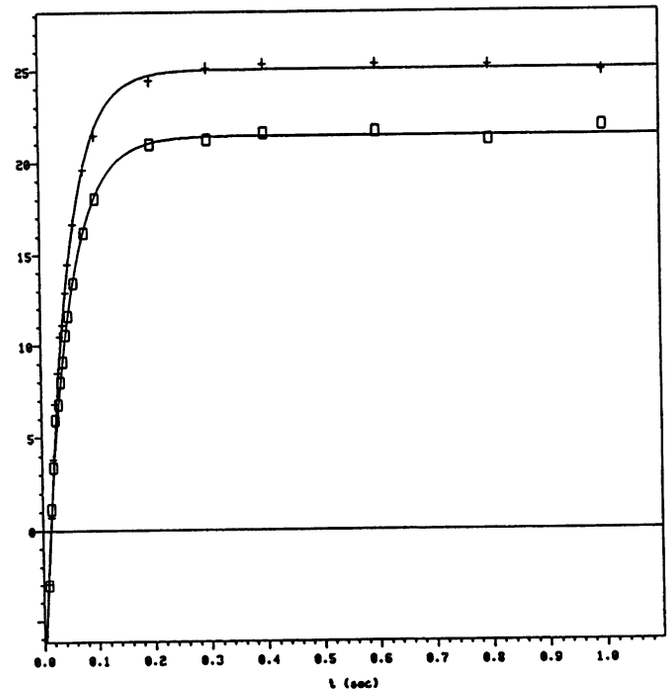

Figure 5. Data from inversion recovery experiments were fit to a single exponential. Data shown are fits of the ψN1 protons at 30°C in 100 mM NaCl, 10 mM $MgCl_2$, 10 mM sodium cacodylate, pH 6. + ψ_6N1 H; □ ψ_5N1 H.

Relaxation measurements

Longitudinal relaxation measurements were made using nonselective inversion recovery to compare the T_1 values of the ψN1 protons with those of hydrogen-bonded ψN3 protons. These values contain contributions from exchange and dipolar relaxation, which are not expressed separately. At the temperatures of these exerpiments, the dominant contribution is chemical exchange. These measurements were made as a function of temperature in the presence and absence of Mg^{2+}, as well as in the presence of spermidine. Values for the relaxation times were obtained from fitting the recovery curves to a single exponential (Figure 5 illustrates an example).

Temperature and Magnesium Effects

In Buffer A (100 mM NaCl, 10 mM sodium cacodylate, pH 6), the longitudinal relaxation times decreased as the temperature increased (Table I). The dramatic increase in T_1 values at 20°C is likely the result of duplex aggregation. The linewidth of the duplex imino protons increased slightly at 20°C and more noticably at 10°C (data not shown), suggesting that such aggregation was occurring. The ratios of the T_1 values (N1H/N3H) of each pseudouridine residue are relatively constant for the duplex from 20−45°C, despite apparent aggregation effects. The addition of Mg^{2+} lowered the T_1 values of both the hydrogen-bonded N3 protons and the N1 protons at 20°C, although no significant changes were observed at higher temperatures. At 20°C the addition of Mg^{2+} also decreased the linewidth of the imino proton resonances, suggesting that aggregation was reduced. Notably, the chemical shifts of these resonances did not change upon addition of $MgCl_2$, indicating that no conformational change of the RNA occurred. The

Table I. Longitudinal relaxation (T_1) Times of Pseudouridine Imino Protons

	20°C			30°C			45°C		
Buffer	A	B	C	A	B	C	A	B	C
ψ_5N3H	207±9	114±9	92±3	86±3	90±4	78±2	31±2	32±1	
ψ_5N1H	125±5	68±9	31±1	57±3	44±2	14±1	20±2	15±1	np
N1H/N3H	0.6	0.6	0.3	0.7	0.5	0.2	0.6	0.5	
ψ_6N3H	460±40	100±5	84±3	144±6	144±6	126±4	80±4	71±3	
ψ_6N1H	107±7	66±7	24±1	55±3	42±2	12±1	19±1	15±1	np
N1H/N3H	0.2	0.7	0.3	0.4	0.3	0.1	0.2	0.2	

T_1 values in msec. Errors calculated from the fit to the exponential recovery curve.
Buffer A: 100 mM NaCl, 10 mM sodium cacodylate, pH6.
Buffer B: 100 mM NaCl, 10 mM sodium cacodylate, 10 mM $MgCl_2$, pH6.
Buffer C: 100 mM NaCl, 10 mM sodium cacodylate, 10 mM $MgCl_2$, 10 mM spermidine, pH6.

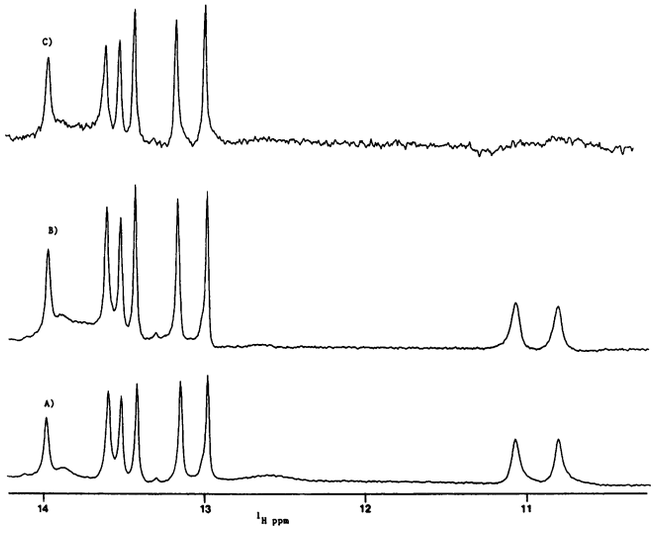

Figure 6. Comparison of the imino proton spectra of the duplex at 45°C in A) 100 mM NaCl, 10 mM sodium cacodylate, pH 6; B) with 10 mM $MgCl_2$, pH 6; C) with 10 mM $MgCl_2$, 10 mM spermidine, pH 6.

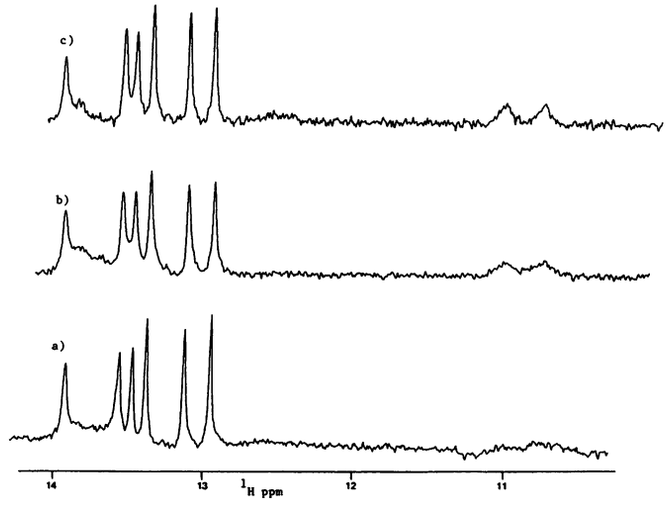

Figure 7. Comparison of the partial recovery of ψN1 imino proton resonances after addition of excess NaCl at 45°C. a)100 mM NaCl, 10 mM sodium cacodylate, 10 mM $MgCl_2$, 10 mM spermidine; b)with 500 mM NaCl; c) with 935 mM NaCl (dilution of the RNA and counterions changes their concentration by 10%).

N1H/N3H ratio of the T_1 values for the two pseudouridine imino protons do not appear to be Mg^{2+}-dependent, with the possible exception of those for ψ_6 at 20°C.

The ψ_5N1 proton exchanges about two times faster than the hydrogen-bonded ψ_5N3 proton both with and without Mg^{2+}. The exchange times of the ψ_6 N3 proton are slower than those of the adjacent ψ_5 N3 proton, but the exchange times of the ψ_6 N1 proton are virtually identical to those of the ψ_5 N1 proton. The addition of Mg^{2+} has no selective effect on either imino proton.

Spermidine effects

In the presence of 10 mM spermidine, 10 mM $MgCl_2$, 100 mM NaCl, and 10 mM sodium cacodylate pH 6, the exchange properties of the ψN1 proton resonances were altered. The temperature dependence of these resonances was more dramatic in the presence of spermidine, as illustrated in Figure 6, such that at 45°C, there were no visible peaks remaining from these protons. In contrast, six of the hydrogen-bonded imino protons were still visible at 65°C (data not shown), indicating that the inner hexamer duplex ^{5}'C$\psi\psi$ACC/^{3}'GAAUGG containing the two A-ψ base pairs remained intact at this temperature. No changes were observed in the chemical shift of any imino proton, suggesting that spermidine does not significantly alter the structure of the duplex.

Measurements of the longitudinal relaxation times also indicated a selective destabilization of the ψN1 proton resonances. At 30°C, the ψN1 T_1 values decreased to about 13 msec, roughly 10-fold less than that of the hydrogen-bonded ψN3 protons. This decrease in the N1 proton relaxation time should be unrelated to the lifetime of the base pair, since the T_1 value of the hydrogen-bonded ψN3 proton is independent of added spermidine. The selective change in the T_1 value for ψN1 more likely reflects an accelerated exchange of these protons mediated by spermidine from the intact base-pair.

Assuming that the association of spermidine with the duplex should be predominantly electrostatic (17), the sodium chloride concentration was increased in an attempt to displace the spermidine and recover the ψN1 imino proton resonances. Some recovery was observed at 45°C in 500 mM NaCl, and more signal intensity was observed in 900 mM NaCl (Figure 7a,b), but the recovery of these signals at high salt concentrations was not complete and the T_1 times did not return to their previous values.

DISCUSSION

Hydrogen-bonded imino proton exchange in nucleic acid duplexes has been shown to normally be limited by transfer to a proton acceptor, and occurs in the open state of the base pair (18,19). This exchange can be catalyzed, and the base-pair lifetime determined by extrapolation of the exchange time to infinite catalyst concentration (19). Amino protons are also exchangeable, with exchange times which usually differ from those of the imino protons. The two amino protons of adenosine and guanosine are normally not resolved in the NMR spectrum, due to rotation about the C-N bond; however, those of cytidine are usually resolved and can be assigned as external (major groove) or internal hydrogen-bonded protons. Exchange processes of adenosine amino protons apparently requires protonation of the N1 position, and thus exchange times of these protons are slower than those of the hydrogen-bonded imino protons (20). However, the exchange times of each of the two cytosine amino protons in a DNA duplex have been shown to be independent, for the external (non-hydrogen-bonded) proton is readily catalyst-exchanged when the internal one is not (19). Those results suggest that base-pair opening is unnecessary for exchange of external amino protons which are located in the major groove of DNA. It is now generally accepted that base-pair opening must occur for imino and some amino proton exchange, but that the imino proton exchange time is not equivalent to the base-pair lifetime since opening can occur many times before an exchange event, when the appropriate catalyst concentration is limiting. In contrast, cytidine amino protons in the major groove can exchange from an intact base-pair.

The mechanism of exchange of both hydrogen-bonded ψN3 imino proton and external ψN1 imino proton is undoubtedly similar to that of uridine and thymine (20). At pH 6, the conditions of the spermidine experiments, neither the ψN1 nor N3 position is likely to be deprotonated; the basic pK_a of the uracil ring has been estimated to be -3.4 (21,22) and the acidic pK_a of pseudouridine has been measured at 8.97 (23). Thus the exchange mechanism described for adenosine amino protons is unlikely to occur for pseudouridine imino protons. Exchange of the ψN3 hydrogen-bonded imino protons is certainly analogous to U, T, or G imino protons, and proceeds through the open state of the base-pair. Exchange of the ψN1 imino proton proceeds through a different mechanism, which must account for its normally slow exchange but also for its susceptability to accelerated exchange as a function of pH or spermidine. By analogy to the properties of the external cytidine amino protons (19), exchange of ψN1 proceeds from the intact base-pair. There are two likely mechanisms that could slow the exchange of the ψN1 imino protons. The first is the formation of a hydrogen bond to an unknown acceptor. The other is shielding of the exchangeable protons from bulk solvent through structural (steric and/or electrostatic) constraints of the RNA A-form major groove.

The formation of a stable hydrogen bond would assist in stabilizing the ψN1 imino proton from exchange (20). However, it seems unlikely that the geometry of a simple duplex RNA permits the formation of a direct hydrogen bond between the ψN1H and another base, carbohydrate or phosphate acceptor. To explain the exchange properties of pseudouridine N1 imino protons in tRNAs, Davis and Poulter (12) have suggested that the ψN1 imino proton could be involved in a water-mediated interaction to one of the phosphate residues. Crystallographic evidence for hydrogen bonding comes from the yeast $tRNA^{gln}$:Gln-tRNA synthetase co-crystal where the N1 imino proton of ψ38 is involved in hydrogen-bonding in a water network that includes bases of the anticodon stem and those of the distorted anticodon loop (24).

Structures have been observed to restrict access of water to normally exchangeable protons. For example, in DNA loops, the structure of the loop itself has been shown to hinder exchange of non-hydrogen-bonded imino protons (7,8). In RNA pseudoknots, imino protons from the loops are observable in NMR spectra (9). By analogy, the pseudouridine N1 imino proton may be reporting on the accessibility of the A-form major groove to water or specific counterions. The exchange properties of the cytidine external amino protons would presumably also reflect these interactions. Exchange is not limited by the opening of the base pair, but by exchange with water molecules, since the rate of ψN1 proton exchange is apparently uncorrelated to that of the associated ψN3 proton. Nonbonded ψN1 imino proton resonances have been previously observed in NMR experiments with the more structurally complex tRNAs (10,11,12,25,26) where exchange may be hindered by structure or hydrogen bonding.

Access to spermidine

The increased exchange of ψN1 protons in the presence of spermidine has been noted previously (27,28) for the ψ_{55} N1 proton in yeast $tRNA^{phe}$. ψ_{55} is in the T-loop of tRNA, where it is hydrogen-bonded through the C4 carbonyl to the G_{18} N1 proton, and through the N3 proton to phosphate 58. The N1 proton of ψ_{55} is observed in the NMR imino proton spectrum, although it does not appear to have a hydrogen-bond acceptor. In the NMR experiments of Tropp and Redfield (27), the addition of spermidine to yeast $tRNA^{phe}$ decreased the exchange rate of the ψ_{55} N3 proton, while that of the ψ_{55} N1 proton was increased. This selective protection of ψ_{55} N3 H could be due to a specific interaction which stabilized the tertiary folding; the destabilization of N1 H could be due to catalysis by the spermidine (28). Similarly, the non-hydrogen-bonded ψ39 N1 proton disappears from the NMR spectrum when spermidine is present (28), while the hydrogen-bonded ψ39 N3 proton is stable.

The results presented here, where the pseudouridine N1 protons are located in the major groove of a simple RNA duplex, suggest that spermidine enters the major groove and facilitates exchange of those protons. In the crystal structure of an A-form DNA, Jain et al. (29) found a spermine molecule located in the major groove, where it formed hydrogen bonds with O4 of T and N7 of G, and was within van der Waals contact of methyl groups and other atoms on the bases in the major groove. Several water molecules mediated these interactions. Spermidine is shorter than spermine, lacking one $(CH_2)_3$-NH_3^+, but shares its cationic properties. If the spermidine is able to enter the major groove, as suggested from the crystallographic data, then the exchange of the ψN1 protons observed in the NMR experiments could be accelerated either as the spermidine disrupted any hydrogen bonds, or through base catalysis by the spermidine itself.

Previous experiments examined the interaction between spermine or spermidine and DNA dodecamer duplexes, looking for NOEs between the the two molecules in D_2O (30) where exchangeable proton resonances are not present. No NOEs were observed, and no chemical shift changes of the DNA were reported upon addition of spermine; the results were interpreted to suggest that spermine showed either rapid diffusion along the DNA or diffuse nonspecific binding to the DNA backbone (30). These results may be specific for B-form helices (31) or may

reflect different solution conditions. These previous results together with the present pseudouridine data suggest that there may be (at least) two classes of binding sites for the spermidine molecules, only one of which affects the properties of the exchangeable protons in the major groove. One class of binding may be observed in crystal structures, such as the crystallographic data on B-form DNA/spermine complexes, which showed the spermine bound to the phosphate backbone spanning the major groove, reaching into the groove to contact guanine O-6 (32). Alternatively, the previous NMR experiments with spermidine:DNA may agree with the results reported here for RNA, if spermidine interacts in the major groove of B-form DNA but does not reside there long enough to allow measurable NOEs.

RNA: peptide interactions

Sequence-specific interactions of proteins with duplex DNA usually involve amino acid contacts with the bases in the major groove of a B-form structure (33,34). However, the geometry of the RNA A-form major groove does not permit easy access by protein side chains. Sequence specific interactions of proteins with duplex RNA structures may require that the amino acid side-chains be able to reach into the major groove of the A-form RNA. Such interactions may require proximity to an end of the helix, but since most helices in RNA are short, such geometry is common. The apparent interaction of spermidine with the major groove of the 11-mer RNA duplex suggests that amino acid side-chains may be able to reach into the major groove. Either arginine or lysine is similar to spermidine in the arrangement of methylenes and amino groups, and so are likely candidates for such interactions. One example already known involves the *tat* peptide which interacts binds to the TAR RNA hairpin bulge (35); these interactions may involve contacts in the major groove (36). Thus observing the properties of exchangeable protons in the major groove, whether it be cytidine amino protons or the pseudouridine imino proton, may provide a means to measure the interaction modes of model peptides.

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