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Stabilization of RNA stacking by pseudouridine

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ABSTRACT

The effect of the modified nucleoside pseudouridine (Ψ) on RNA structure was compared with uridine. The extent of base stacking in model RNA oligonucleotides was measured by ^{1}H NMR, UV, and CD spectroscopy. The UV and CD results indicate that the model single-stranded oligoribonucleotides AAUA and AAΨA form stacked structures in solution and the CD results for AAΨA are consistent with a general A-form helical conformation. The AAΨA oligomer exhibits a greater degree of UV hypochromicity over the temperature range 5–55°C, consistent with a better stacked, more A-form structure compared with AAUA. The extent of stacking for each nucleotide residue was inferred from the percent 3′-endo sugar conformation as indicated by the H1′-H2′ NMR scalar coupling. This indirect indication of stacking was confirmed by sequential NOE connectivity patterns obtained from 2D ROESY NMR experiments. NMR measurements as a function of temperature indicate that pseudouridine forms a more stable base stacking arrangement than uridine, an effect that is propagated throughout the helix to stabilize stacking of neighboring purine nucleosides. The N1-H imino proton in AAΨA exchanges slowly with solvent, suggesting a role for the extra imino proton in stabilizing the conformation of pseudouridine. These results show that the conformational stabilization is an intrinsic property of pseudouridine occurring at the nucleotide level. The characteristics of pseudouridine in these models are consistent with earlier studies on intact tRNA, indicating that pseudouridine probably performs the same stabilizing function in most structural contexts.

INTRODUCTION

Despite the wide distribution throughout biology of modified nucleosides in RNA, the function of RNA modification remains elusive. Pseudouridine (Ψ) is the most common modified nucleoside in RNA, occurring ubiquitously in tRNA and with high frequency in ribosomal RNAs and small nuclear RNAs (1–3). The positions of Ψ have been determined for a large number of tRNA isosacceptors, and the observation that the positions are highly conserved suggests a common function for Ψ among tRNAs. Evidence that Ψ plays an important role in rRNA and tRNA function comes from the recent placement of Ψ residues at the pepitdyltransferase center of 23S rRNA (4,5), for tRNA from the studies of polypeptide synthesis in *his*T bacterial mutants (6) and also of a Ψ requirement for codon-anticodon recognition in certain suppressor tRNAs (7). In yeast 26S RNA, Ofengand and co-workers have shown that a remarkable 3.5% of the uridine residues are modified to Ψ and as was found for *Escherichia coli* 23S, they are clustered within the peptidyltransferase center (4,5). Involvement in aminoacyl transfer has been proposed for pseudouridine in large subunit RNAs (8), but all direct evidence to date suggests an intrinsic structural function.

Pseudouridine is unique compared with uridine in that there are two NH imino protons available to serve as hydrogen bond donors. NMR studies on ^{15}N-labeled tRNAs established that the N1-H proton of Ψ is involved in a stable hydrogen bond, even within putative single-stranded regions (9,10). Published NMR data for tRNAs, where the pseudouridine N1-H resonances were assigned, indicate that the local structure of Ψ is basically the same for $tRNA_f^{Met}$, $tRNA^{Glu}$, $tRNA^{Phe}$ and $tRNA^{Tyr}$, regardless of its placement within the tRNA molecule (10). The conformation of the A31-Ψ39 base pair was established as *anti* for $tRNA^{Phe}$ and $tRNA^{Tyr}$. These studies also established that the N1-H proton exchanges slowly enough with solvent to be observed by NMR. The Ψ N1-H proton is invariably observed to exchange slowly, regardless of the location of the Ψ residue within the tRNA molecule. This strongly suggests that there is an intrinsic, sequence independent mechanism to protect the N1-H proton from rapid exchange with bulk water. Furthermore, the presence of Ψ-39 in the anticodon of *E.coli* $tRNA^{Phe}$ stabilizes the local structure compared with an otherwise identical tRNA containing U-39 (11). Davis and Poulter proposed that the NMR data and the observed structural stabilization within tRNA could be explained by an intramolecular water bridge. Recent studies of oligonucleotides containing Ψ indicated that sequences unique to tRNA were not required to observe the N1-H NMR resonance, demonstrating that N1-H stabilization was not unique to tRNA (12,13). An X-ray crystallographic study of $tRNA^{Gln}$ complexed with Gln-tRNA synthetase identified a hydrogen bond interaction between the Ψ N1-H proton and a water molecule coordinated to the phosphate backbone (14). No comment was made about the potential for similar interactions in solution, but the crystallographic model provides a mechanism for slowing exchange of the N1-H imino proton and is consistent with the model proposed in the earlier NMR studies.

The published NMR and crystallographic data suggest that the local structure of Ψ is similar in different sequence contexts. In order to understand the function of Ψ, we decided to investigate whether Ψ in simple single-stranded oligonucleotides formed structural interactions similar to those observed in tRNA. Furthermore, we wished to determine the effect on RNA structure of replacing a uridine with a Ψ in a model RNA oligonucleotide system that was simple enough to characterize rigorously. Finally,

we thought it was important to determine the minimal sequence element necessary to observe the slowly exchanging N1-H imino proton since this NMR property is the one consistent and unique feature of Ψ modification determined to date.

To test our hypothesis that Ψ is capable of stabilizing A-form RNA structure in single-stranded regions, three RNA oligonucleotides were chosen. Single-stranded models were chosen rather than duplexes since Ψ modified sites are common in single-stranded regions. The sequence AΨA was chosen as the minimal model for Ψ in a single-stranded region of RNA; we thought at least one nucleotide 5′ to the Ψ would be necessary to model accurately interactions found in RNA. The sequence AAΨA was also investigated since the work of Arnez and Steitz indicated that a stabilizing water molecule could be coordinated to both the n–1 phosphate as well as the Ψ(n) phosphate (14). The sequence AAUA was used as a comparison control for Ψ containing single-stranded RNA since it became apparent that we should focus on tetramers rather than trimers as more appropriate RNA models.

MATERIALS AND METHODS

Oligoribonucleotide synthesis and purification

The Ψ phosphoramidite was synthesized as described (13) then dissolved in acetonitrile at 0.1 M concentration for oligonucleotide synthesis. RNA oligonucleotides were synthesized using solid phase phosphoramidite chemistry on an Applied Biosystems 394 synthesizer using Perseptive Biosearch Expedite phosphoramidites. The oligonucleotides were cleaved from the CPG support and the base protecting groups removed using 3:1 NH_3:ethanol at 55°C for 3 h. The 2′-*tert*-butyldimethylsilyl protecting groups were removed with triethylamine trihydrofluoride (Aldrich) and the RNA recovered by butanol precipitation as described in Applied Biosystems Bulletin no. 79 (15). The oligonucleotides were purified by PRP-1 HPLC (16).

UV and CD spectroscopy

Samples for UV and CD spectroscopy were prepared at a concentration of ~1.7 OD_{260} in 25 mM phosphate buffer, pH 7.0, containing 100 mM NaCl and 0.2 mM EDTA. The UV monitored T_m experiments were done on an HP 8452A spectrophotometer equipped with a 890090 Peltier temperature control unit; the temperature was increased in 2°C increments with a 1 min equilibration time. CD experiments were done on a JASCO J-720 CD spectrophotometer. The temperature was controlled with a Neslab RTE-110 refrigerated water bath and a flow-through CD cell. The temperature was allowed to equilibrate for 10 min at each temperature point.

NMR spectroscopy

All NMR experiments were done using a Varian Unity 500 MHz NMR spectrometer and a Nalorac ID500 indirect detection probe. The NMR samples contained 1 mM RNA in 0.5 ml of 25 mM phosphate buffer, pH 7.0, containing 25 mM NaCl, 10 mM $MgCl_2$ and 0.2 mM EDTA. Samples in D_2O were prepared by twice lyophilizing then redissolving the aqueous samples in 99.96% D_2O and finally redissolving in 0.5 ml of 99.996% D_2O. 2D ROESY, PECOSY and heteronuclear COSY experiments were collected as hypercomplex data sets using TPPI-States phase cycling (17) and processed with Varian VNMR software. The ROESY experiments were collected using a time-shared spinlock field of ~3500 Hz (18,19). The F_2 data were collected with 4096 total data points and 400 t_1 increments were zero-filled to 2048 total points in F_1. PECOSY (20) spectra were collected with WALTZ-16 ^{31}P decoupling and a decoupler field strength of ~500 Hz to minimize sample heating. The PECOSY data were collected with a spectral width of 2300 Hz, centered at the water frequency, with 4096 data points collected in the F_2 domain and 400 t_1 increments zero-filled to 2048 data points in F_1. The proton-detected heteronuclear COSY (21) data were collected with 144 transients per increment, spectral widths of 4300 and 800 Hz in F_2 and F_1 respectively and 64 t_1 increments; the t_1 data was zero-filled to 1024 points.

RESULTS

UV and CD spectroscopy

Optical spectroscopy was used to provide a qualitative assessment of the extent of base stacking for AAUA and AAΨA, and from the results it was also possible to draw some conclusions about the helical conformation. The UV absorbance change as a function of temperature demonstrated a classic decrease in hypochromicity as the bases unstacked with increasing temperature (22,23). The 'melting' of the single-stranded oligonucleotides is non-cooperative, but the relative amount of base stacking at the limiting temperatures of 5 and 55°C can be determined by comparing the UV spectra where the high temperature absorbance is normalized for the two molecules as shown in Figure 1. As the temperature decreases, the absorbance decrease reflects the average base stacking for the oligonucleotide and is clearly greater for AAΨA.

The temperature dependence of the CD spectra can also be used to determine the stability of a particular conformation in solution and provide some information about the nature of the structure. However, it was difficult to use the CD data to compare the conformations of AAUA and AAΨA because Ψ substitution results in such an unusual CD spectrum. The CD phase change for Ψ-containing molecules has been described and arises from the rotation of the base, changing the orientation of the transition dipoles (24,25). As shown in Figure 1, we were able to assess structural change by plotting the change in the CD maximum near 270 nm as function of temperature for each oligonucleotide. The slopes are similar for each molecule with AAΨA appearing to be slightly more affected by temperature, again consistent with greater stacking. The CD spectra at the two limiting temperatures of 5 and 25°C shown in Figure 2 indicate AAUA adopts more of an A-form structure at 5 than at 25°C, although neither AAUA spectra have an ideal A-form appearance (26). The CD spectrum at 25°C for AAUA is characteristic of a more B-form or of an unstacked structure, an interpretation that is confirmed by the 2D NMR results described below.

1D NMR spectra of AAUA, AΨA, AAΨA

The ^1H NMR spectra of the three oligonucleotides in D_2O buffer at 20°C are shown in Figure 3. In each case the samples are free from failure sequences and other products of the oligonucleotide synthesis. For AAΨA and AAUA, the H1′ protons between 5.0 and 6.0 p.p.m. are mostly resolved and for AAΨA the adenosine H1′ protons remain resolved over the entire temperature range

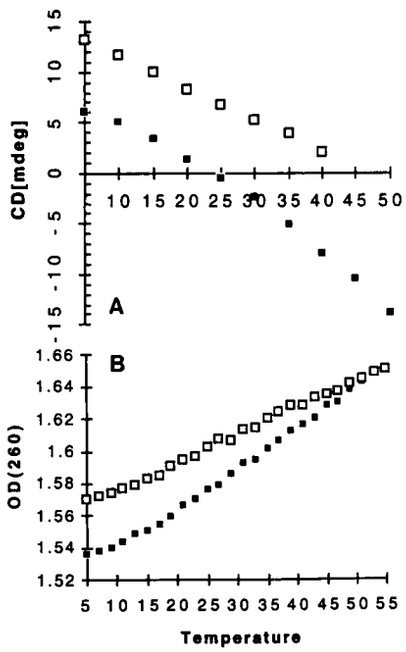

Figure 1. UV and CD T_m data showing change in hypochromicity or CD as a function of temperature; open squares are data points for AAUA, filled squares are for AAΨA . (**A**) The CD measured at the positive maximum near 270 nm as a function of temperature. The CD spectrum became seriously degraded for AAUA above 40°C. (**B**) The UV absorbance at 260 nm as the temperature was varied from 5 to 55°C. The experiments were repeated three times for each sample.

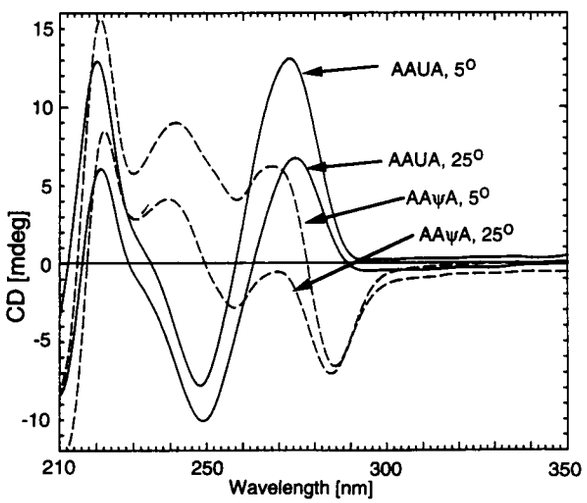

Figure 2. CD spectra for AAUA and AAΨA at 5 and 25°C.

from 5 to 40°C, allowing for straightforward measurement of the $^3J_{H1'-H2'}$ scalar coupling. The obvious differences between AAUA and AAΨA are the pyrimidine base protons for uridine H6 at 7.4 and H5 at 5.3 p.p.m., and the Ψ H6 proton at 6.9 p.p.m. The Ψ H1′ proton is visible as a doublet with a small J coupling at 4.3 p.p.m. The proton NMR assignments were made by a combination of 2D ROESY and PECOSY experiments.

The ^{31}P NMR spectra shown in Figure 4 were assigned starting with the proton H3′ assignments and subsequent correlation to the

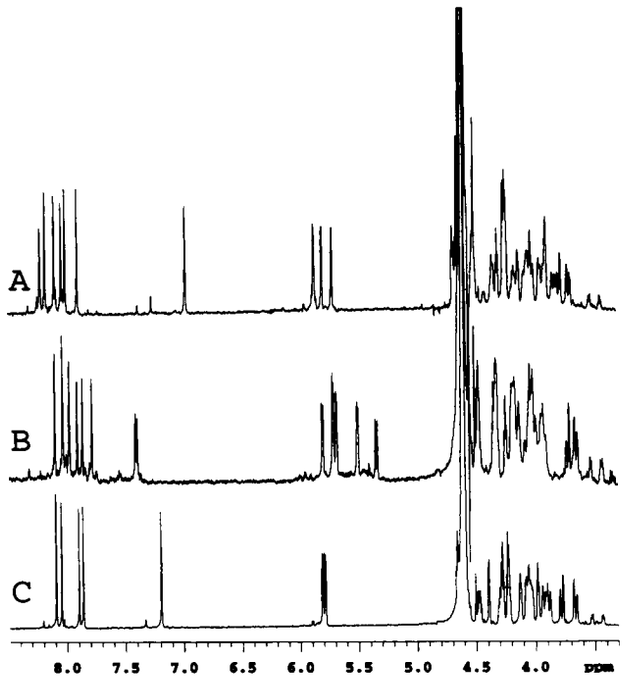

Figure 3. Proton NMR spectra at 20°C for (**A**) AAΨA, (**B**) AAUA, (**C**) AΨA.

^{31}P resonances using a ^{1}H-^{31}P proton detected 2D heteronuclear COSY experiment. The ^{31}P spectra indicate either that there are differences in the backbone conformation of AAUA and AAΨA at the pyrimidine phosphate or that, despite a similar conformation, there is some deshielding of the Ψ phosphate compared to the U phosphate (27). However, the differences in ^{31}P shifts for U and Ψ are not as large as would be expected if there was a dramatic change in the backbone conformation, for example from B_I to B_{II} (28). A 17 nucleotide stem–loop model of the anticodon of tRNA^{Lys} with a single Ψ at the base of the stem shows a similar downfield shift for the phosphate 5′ to Ψ, indicating that the phosphate environment of AAΨA may be similar to that for Ψ in tRNA (Davis,D.R. and Durant,P.C., unpublished data).

The ^{1}H NMR spectra acquired in H_2O at 5°C for AΨA and AAΨA are shown in Figure 5; the corresponding imino spectrum of AAUA is not shown since there were no detectable downfield resonances. For AΨA, a broad hump is seen centered at 10.6 p.p.m., but it is impossible to make a conclusive assignment as to the origin of this resonance. For AAΨA, an imino resonance is seen at 10.4 p.p.m., assigned to N1-H of Ψ by virtue of an NOE to the H6 proton of Ψ (data not shown). The imino resonance is temperature labile, broadens significantly even at 10°C and is not detected once the temperature is raised above 15°C. The chemical shift of this resonance is characteristic of N1-H resonances assigned in spectra of tRNA (10) and also similar to that reported for Ψ in RNA duplexes (12,13). The two spectra shown in Figure 5 indicate that the additional adenosine of AAΨA provides stabilization of the imino proton from solvent exchange. The additional phosphate between A1 and A2 could provide an extra coordination site for a water molecule as suggested by the crystallographic results, or additional stacking stabilization by the extra adenosine may lower the energy of the conformational form required to protect the N1-H imino proton from exchange. The

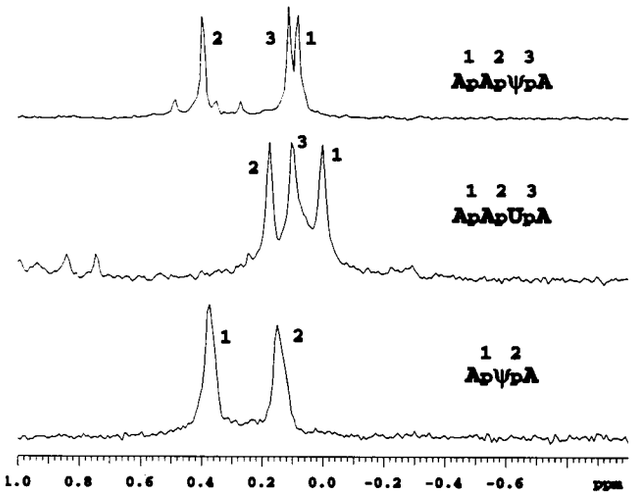

Figure 4. ^{31}P NMR spectra of (**A**) AAΨA, (**B**) AAUA, (**C**) AΨA, referenced to 85% H_3PO_4. The same samples were used as for the spectra in Figure 3.

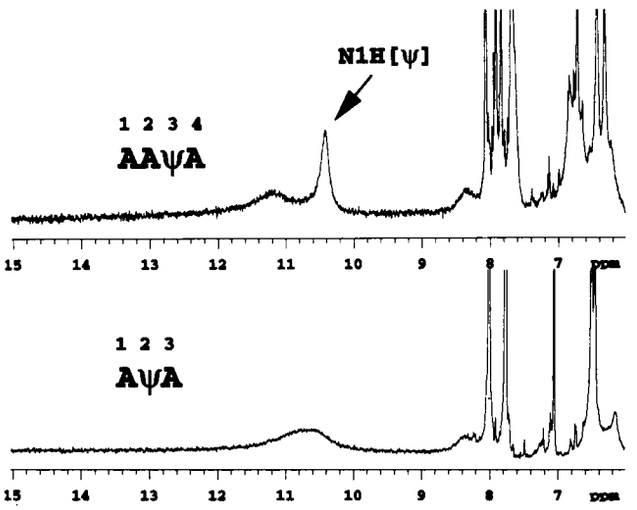

Figure 5. Imino proton region of the NMR spectra at 5°C for AAΨA and AΨA acquired in 90:10 H_2O:D_2O.

^{31}P NMR data suggest that the Ψ 5′ phosphate is involved in some interaction unique to the modified tetramer, but the resonance for the phosphate between A1 and A2 is not shifted downfield.

Measurement of $^3J_{H1'-H2'}$ scalar couplings

The sugar conformation for an A-form RNA helix is 3′-endo. For RNA oligonucleotides in general, it has been established that the percent 3′-endo conformation at the nucleoside level is an indication of the extent of base stacking at that nucleoside position (29,30). We measured the $^3J_{H1'-H2'}$ couplings for AΨA, AAUA, and AAΨA as a function of temperature from 5 to 40°C and converted the couplings into a percentage of 3′-endo conformation. Couplings used to calculate the sugar conformations were measured entirely from the 1D proton spectra. The sum of the $^3J_{H1'-H2'}$ and the $^3J_{H3'-H4'}$ couplings was shown to be equal to 10 by measuring both couplings at several temperature points where both cross-peaks were clearly resolved in a ^{31}P-decoupled PECOSY experiment. Table 1 shows the percentage 3′-endo (N) conformer at 5 and 25°C along with an equilibrium constant for the N and S conformational interconversion. The AΨA data provide a reference point to published data that help us to better understand the correlation between 3′-endo preference, base stacking and imino proton exchange stabilization. The stacking preferences for many RNA trimers have been reported, and we find that AΨA is stacked to about the same degree as reported for AAA, which is expected to be more stable than AUA (30).

Table 1. Percentage 3′-endo (N) conformer and equilibrium constants (N/S) at 25°C for oligonucleotides[a]

	%N, 25°C	%N, 5°C	K_{eq}(N/S), 25°C
AAUA	57	68	1.3
A**A**UA	57	68	1.3
AA**U**A	53	60	1.1
AAU**A**	50	53	1.0
AAΨA	64	100[b]	1.8
A**A**ΨA	75	100	3.0
AA**Ψ**A	100	100	>10
AAΨ**A**	55	75	1.2
AΨA	55	NA[c]	NA
A**Ψ**A	65	NA	NA
AΨ**A**	55	90	1.2

[a]The percentage of 3′-endo (N) conformer for the underlined nucleotide was calculated from %S = 100 × $J_{1',2'}/(J_{1',2'} + J_{3',4'})$ (42), %N = 100 – %S.
[b]$J_{1',2'}$ values <1 Hz were assumed to indicate sugars 100% in the N conformation.
[c]J values were not measured due to spectral overlap.

The 3′-endo conformation for the Ψ residue in AAΨA is dramatically stabilized compared with uridine in AAUA. Further more, the Ψ effect on the nucleoside sugar conformation is propagated throughout the sequence, resulting in a much more A-form helix for the entire molecule. The stabilization of the sugar conformation is more pronounced in the 5′ direction than the 3′ direction. While the adenosine after the Ψ is modestly more 3′-endo than the corresponding A in AAUA, the two adenosines preceding Ψ are 100% 3′-endo at 5°C compared with only 68% for both A1 and A2 in AAUA. This directional bias for stacking stabilization is consistent with the concept that the local structure 5′ to Ψ has to be constrained to protect the imino proton from exchange.

2D NMR spectra of AAUA and AAΨA

The results of the UV and CD experiments gave a qualitative view of the effects of Ψ modification, and along with the determination of the sugar conformations from J couplings, are an indirect indication that pseudouridylation improves RNA stacking. Experiments using 2D NOESY or ROESY can give a direct indication of the proximity of functional groups within an RNA oligonucleotide, and the relative intensity of certain cross-peaks will establish whether an RNA is in fact helical. The most diagnostic NOE patterns involve the base protons and sugar

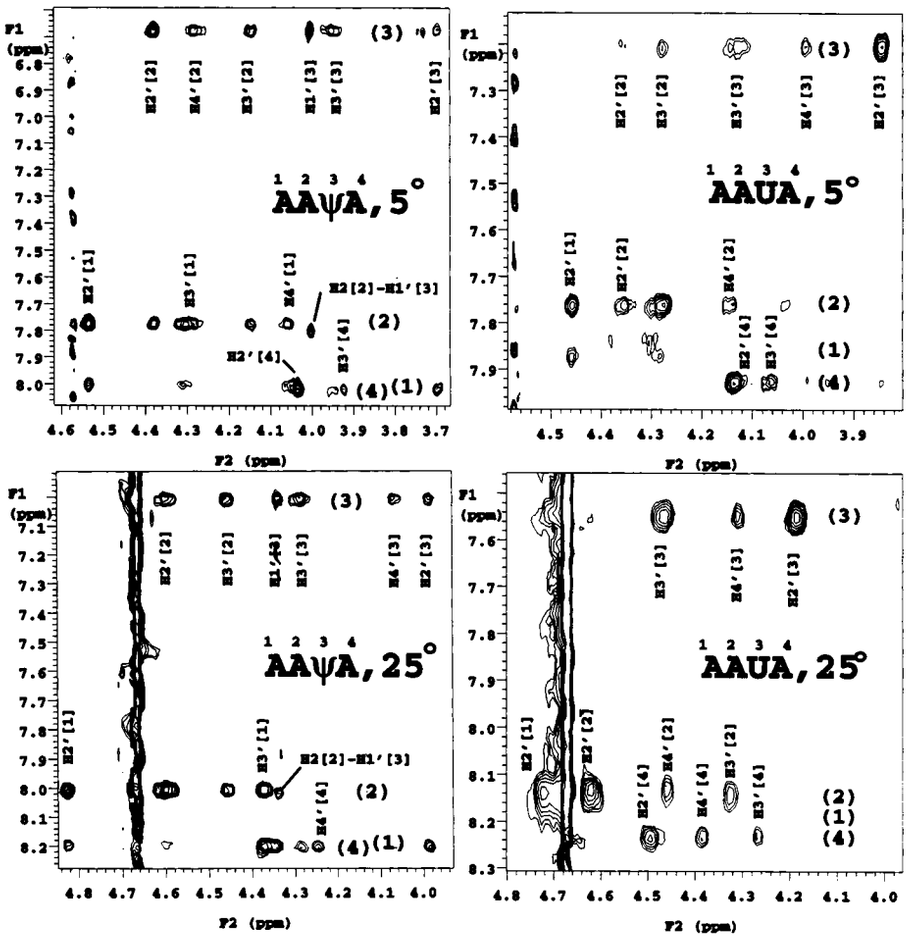

Figure 6. Base to sugar region of the 2D ROESY spectra for AAUA and AAΨA, a 300 ms mixing time was used. Assignments for the sugar protons are indicated with the residue number in brackets. The positions of the base protons are indicated in parenthesis.

protons within a nucleoside and also the NOEs between base protons and the sugar protons of the preceding nucleoside (31). We used the ROESY experiment almost exclusively since the oligonucleotides have correlation times near where the NOE goes to zero, and ROESY has the additional advantage that spin diffusion is less of a concern than for NOESY.

The 2D ROESY spectra in Figure 6 were assigned using the standard sequential assignment strategy and were found to agree qualitatively with the patterns expected for right-handed, helical RNA (31). In addition to the ROESY data, PECOSY experiments were used to assist in assigning the sugar protons via the scalar coupling network. Sequential assignments were straightforward for such a simple molecule, enabling us to make complete assignments, including the 5′,5″ protons.

All nucleosides were in the *anti* conformation as indicated by weak H8/H6-H1′ NOEs. The 2D ROESY spectra of AAΨA and AAUA both have very weak sequential NOEs for H8/H6-H1′ protons compared with the intraresidue H8/H6-H1′ NOEs, consistent with either A-form, or weakly stacked structures. The H8/H6-H1′ NOEs were therefore not particularly useful for comparisons between molecules, or even for comparing the same molecule at different temperatures. The H8/H6-H2′,H3′,H4′, H5′,H5″ region shown in Figure 6 is sensitive to small changes in the RNA structure and a comparison of intensities for the H6/H8-H2′ and H6/H8-H3′ NOEs was quite informative.

The spectra of AAΨA at 5 and 25°C have a similar pattern of intensities and several key NOE connectivities are seen that show the molecule has an A-form helical structure. The Ψ H6 proton has a more intense NOE to its own H3′ proton than to its H2′ proton, indicating the sugar is 3′-endo. The Ψ residue forms an A-form stack with respect to A2 as indicated by the strong NOE from H6[Ψ] to H2′[A2] while the corresponding H6[Ψ]-H3′[A2] NOE cross-peak is relatively weaker. The NOEs involving the H8 proton of A2 show that the A-form geometry is maintained at this residue even at 25°C. There is a strong NOE from H8[A2] to H2′[A1], a weaker NOE from H8[A2] to H3′[A2] and the NOEs from H8[A2] to its own H2′ and H3′ protons are again indicative of a 3′-endo nucleoside conformation. The intraresidue pattern is particularly obvious in the 25°C spectrum. The structure of the stacked conformer is also validated by the NOE from H2[A2] to H1′[Ψ], further constraining the helical stacking at the AΨ dinucleotide step.

The residues on the ends tend to deviate from an ideal A-form pattern, particularly at 25°C; A4 shows a stronger H8[A4] to H2′[A4] NOE than H8[A4] to H3′[A4] NOE, and the relative

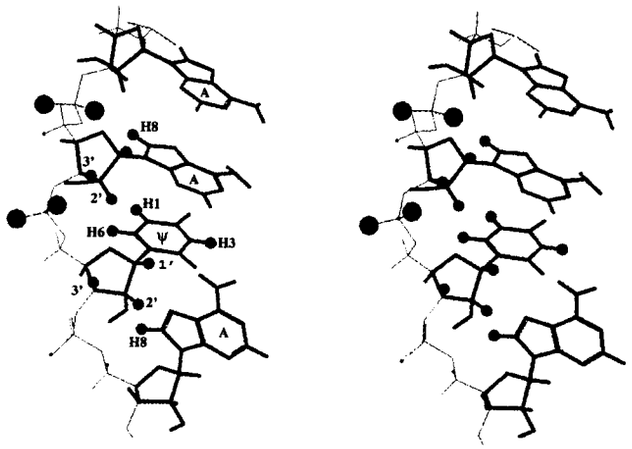

Figure 7. Cross-eyed stereo drawing of AAΨA shown as an A-form helix. The protons involved in key NOEs near the Ψ are highlighted, as well as the phosphate oxygens.

intensities for H8[A1] to H2′,H3′[A1] are about equal. For A4, the correlation between sugar conformation and NOEs from the base proton is clearly demonstrated. The significant H1′–H2′ scalar coupling at 25°C indicates some amount of 2′-endo sugar conformer for both A1 and A4, and this is accompanied by intraresidue NOE patterns from the H8 proton to H2′ and H3′ protons, confirming that there are appreciable 2′-endo contributions to the sugar conformation. A three-dimensional stereo drawing of AAΨA in the A-form helical conformation is shown in Figure 7.

While the basic NOE cross-peak pattern is unchanged for AAΨA at 5 and 25°C, the patterns for AAUA are quite different at the two temperatures. Even at 5°C where one would expect the two molecules to be optimally stacked, AAUA is clearly less in the A-form than AAΨA. At 5°C, the U H6 proton gives a strong NOE to its own H2′ proton at 3.84 p.p.m., a weaker NOE to its own H3′ and a very weak sequential NOE to H2′[A2]. For A2, there is a strong sequential NOE to H2′[A1], suggesting that in fact the A1/A2 step is better stacked than A2/U3 as might be expected for an AA versus an AU dinucleotide. When the temperature is raised to 25°C, there is no indication of stable stacking interactions between nucleosides, as can be seen by the lack of sequential NOEs. However, the base conformations are still *anti* as shown by the clear intranucleotide NOE patterns between the base and sugar protons, giving a clean pattern of three cross-peaks for each H6/H8 to its own H2′, H3′ and H4′ protons.

2D NOESY and ROESY experiments in H_2O

We attempted to obtain direct evidence for a water molecule coordinated to N1-H of Ψ that would provide a mechanism for the structural stabilization described above. NOESY and ROESY experiments were conducted in water (32,33) and NOE cross-peaks were observed from protons on AAΨA to the water resonance (data not shown). In particular, there was a clean cross-peak between H6-Ψ and water that was clearly distinguishable from exchange cross-peaks involving the adenosine NH_2 protons. None of the other base protons gave strong NOEs to the water resonance. However, the structure of AAΨA makes it impossible to distinguish between a direct water NOE and other relaxation pathways. The N1-H imino proton can exchange with water and then cross-relax to the adjacent H6 proton. Another facile relaxation path from H6 to water involves cross-relaxation from H6 to the 2′-OH of A2 which can then chemically exchange with water. The structure determined from the ROESY experiments in D_2O places the 2′-OH proximal to the H6 proton. To resolve this question, we are investigating the possibility of constructing other molecules containing Ψ where the structure might allow unambiguous NOE detection of a water molecule. We favor a structure that involves a water molecule, but at present we only have circumstantial evidence to support this hypothesis.

DISCUSSION

Modified nucleosides are found to some extent in most functional RNA molecules in nature. The conservation of modification identity and position is very high in tRNAs and in the functional regions of larger RNA molecules such as the peptidyltransferase region of the large subunit RNAs. Along with sugar methylation, pseudouridylation is by far the most common nucleoside modification and occurs predominantly in putative single-stranded regions (1,2). Nucleoside modification provides a mechanism to introduce additional functional groups that may be directly involved in RNA–RNA or RNA–protein interactions, but studies to date on sugar methylation (34,35) and pyrimidine thiolation (36,37) suggest that for at least these 'simple' modifications, the function is a rather more mundane fine tuning of the RNA structure by stabilization of local nucleoside conformation.

Pseudouridine modification has been shown to affect the function of tRNAs by altering the ability to recognize correctly cognate codons in *his*T mutants (6). This effect was linked to an alteration in the anticodon structure of $tRNA^{Phe}$ as a result of destabilization of the terminal A31–Ψ39 base pair when replaced with an A–U base-pair (11). NMR studies of tRNA showed that Ψ was unique compared to uridine in that the imino N1-H proton was in slow exchange with water regardless of the structural context. Slowly exchanging imino protons are usually associated with specific structural environments; this is commonly in the form of base-pairs, but is occasionally due to an environment that simply protects the bases from access by solvent. The slow exchange of Ψ imino protons appears to be a more intrinsic property since pseudouridines in many different RNAs all have NMR resonances for their N1-H protons. The presence of the stable imino proton was linked to the structural stabilization observed for *E.coli* $tRNA^{Phe}$, but the proposed model involving a water molecule coordinated to the phosphate backbone could not be definitively proven. The crystallographic evidence of Arnez and Steitz (14) for a water molecule coordinated to Ψ reinforced the model of Davis and Poulter (11), but of course there are concerns about extrapolating from the crystal data to a model in solution. Prior to the work described here, two important issues remained unresolved: whether Ψ does have the intrinsic property of stabilizing RNA structure as seen in $tRNA^{Phe}$, and identification of the minimal RNA sequence necessary to manifest the conformational stabilization required to slow exchange of the N1-H imino proton.

The UV, CD and NMR experimental data presented here establish that a relatively simple RNA oligonucleotide containing Ψ is conformationaly stabilized compared to an analogous

oligonucleotide containing uridine. However, a substantial RNA sequence, AAΨA, is required for the effect to be manifest, in contrast with other nucleoside modifications that stabilize particular conformations at the nucleoside level (35,36). Pseudouridine nucleoside shows no preference for the 3′-endo conformer (38,39) while Ψ 5′ phosphate and the dinucleotide ApΨ show only a modest preference for the 3′-endo stacked conformer compared with the respective uridine nucleotides (25). In this paper, the correlation between the measured percentage of 3′-endo conformation and stacking between nucleosides within a strand of RNA was directly confirmed by ^{1}H NMR NOE measurements. The correlation between sugar conformation and stacking has been established for short oligonucleotides and is also valid for our RNA tetramers.

Substitution of pseudouridine for uridine has two distinct effects on RNA structure. The first is to promote the 3′-endo sugar conformation for the Ψ nucleoside compared with the U that it replaces. The second is that neighboring nucleosides, particularly those preceding the modified site, also experience stabilization of their conformation with a concomitant increase in base stacking. Stabilization of RNA structure is one of the few roles unequivocally demonstrated for RNA modification. Sugar methylation is the most well characterized example where stabilization of the 3′-endo sugar conformation results in an increase in duplex stability for 2′-*O*-methyl oligonucleotides (34). The importance of this strategy in nature is seen in the example of tRNAs from archaeal thermophiles where the extent of methylation directly correlates with growth temperature and tRNA stability (40).

Pseudouridylation can be seen as a complementary strategy to methylation for stabilization of local RNA structure. Both modifications are found throughout RNA sequences, but by far the highest frequency is found for loop regions. Pseudouridylation and sugar methylation are both 'simple' in that the enzymatic and energetic requirements for modification are modest. The pseudouridine synthases that have been characterized are small enzymes with no cofactor or ATP requirements (41); it is enzymatically and energetically cheap to stabilize RNA via pseudouridylation. Substitution of Ψ for uridine would decrease hydrophobicity while the addition of a methyl group to the sugar adds a hydrophobic group. Sugar methylated nucleosides and Ψ are often found together within a region of RNA and could be used in concert to fine tune the stability and hydrophobicity of RNA structure.

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