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Comparison of Solution Conformations and Stabilities of Modified Helix 69 rRNA Analogues from Bacteria and Human†

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Abstract

The helix 69 (H69) region of the large subunit (28S) rRNA of *H. sapiens* contains five pseudouridine (Ψ) residues out of 19 total nucleotides, three of which are highly conserved. In this study, the effects of this abundant modified nucleotide on the structure and stability of H69 were compared with those of uridine in double-stranded (stem) regions. These results were compared with previous hairpin (stem plus single-stranded loop) studies in order to understand the contributions of the loop sequences to H69 structure and stability. The role of a loop nucleotide substitution from an A in bacteria (position 1918 in *E. coli* 23S rRNA) to a G in eukaryotes (position 3734 in *H. sapiens* 28S rRNA) was examined. Thermodynamic parameters for the duplex RNAs were obtained through UV melting studies, and differences in the modified and unmodified RNA structures were examined by circular dichroism spectroscopy. The overall folded structure of human H69 appears to be similar to the bacterial RNA, consistent with the idea that ribosome structure and function are highly conserved; however, our results reveal subtle differences in structure and stability between the bacterial and human H69 RNAs in both the stem and loop regions. These findings may be significant with respect to H69 as a potential drug target site.

INTRODUCTION

The helix 69 hairpin (H69, 19 nucleotides, Figure 1), which has a highly conserved sequence across a wide range of organisms,¹ is located in domain IV of the large subunit ribosomal RNA (rRNA). Helix 69 is the major component of the intersubunit bridge B2a, which connects the peptidyl-transferase center and decoding region of the ribosome.^{2–6} Several reports in the literature suggested that H69 behaves dynamically.^{2,7–9} The function of H69 is still not completely understood; however, previous studies revealed key roles for H69 in subunit association,^{10–11} termination of peptide synthesis,^{12–14} and ribosome recycling.^{15–17}

Helix 69 contains pseudouridine (Ψ), which is the most common modified nucleotide in nature (Figure 1).^{18–20} Pseudouridine has a non-standard glycosidic bond with a C1'-C5 linkage rather than a typical C1'-N1 bond. Due to isomerization of its base moiety, Ψ has a second imino nitrogen (N1) available for hydrogen bonding. The N1 imino proton can form water-mediated hydrogen bonds with the 5' side of the RNA-phosphate backbone.^{20–23} The presence of Ψ also increases stacking interactions with neighboring bases relative to uridine.²¹ The function of Ψ might be to stabilize RNA structure as observed in duplex RNAs;^{24–25} however, this is not always the case.²⁶

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SUPPORTING INFORMATION AVAILABLE

Figure S1 shows UV melting curves of H69 duplex analogues.

Three highly conserved Ψ s (Ψ_{1911} , Ψ_{1915} , and Ψ_{1917} using the *E. coli* numbering system) that are found in both *E. coli* and *H. sapiens* H69 are important, although not essential, for ribosome assembly and cell growth.^{14,27–29} Two additional Ψ s (Ψ_{3737} and Ψ_{3739} using the *H. sapiens* numbering system) found in the *H. sapiens* H69 stem region are also common in rRNAs of higher organisms, but not in bacterial RNAs.^{30,31}

Previous mutation and deletion studies suggested that the loop region of H69 has important functions for 70S ribosome formation and protein synthesis.^{16–17,28,32} Mutations ΔA_{1916} (deletion), insertion of two A residues between A_{1916} and Ψ_{1917} , and $C_{1914}U$ (all residues numbers are based on the *E. coli* 23S rRNA sequence) increased +1 and –1 frameshifting and read-through of all three stop codons.³² Translation was inhibited by the loop mutations $A_{1912}G$, $A_{1919}G$, and $\Psi_{1917}C$, and 70S ribosome formation was inhibited by the $A_{1916}G$ mutation.²⁸ In addition, N1 modifications of A_{1912} and A_{1918} with dimethylsulfate interfered with formation of 70S ribosomes.³³ Recent studies showed that A_{1919} , A_{1912} , and $U(\Psi)_{1917}$ are the most important residues in the H69 loop for ribosomal functions such as translation initiation, translational processivity, and other factor-dependent activities.^{17,34}

In contrast to loop mutations, there are only a few suppressor mutations reported for the stem region of H69. Mutations $G_{1921}C$ and $G_{1922}A$ in the *E. coli* sequence caused a slightly slowed growth rate,^{32,35} whereas the growth rate of the $G_{1922}U$ mutant was extremely slow.³⁵ There are two additional Ψ s in the *H. sapiens* H69 stem region (Ψ_{3737} and Ψ_{3739}) compared to the *E. coli* stem (G_{1921} and U_{1923}) (Figure 1), whereas one Ψ is located at the same position of *E. coli* (Ψ_{1911}) and *H. sapiens* (Ψ_{3727}) H69. A recent guide snoRNA depletion study showed that the lack of modifications at positions 2264 and 2266 (3737 and 3739 in the human rRNA) increased the sensitivity to neomycin.³⁶

Previous studies revealed that Ψ residues have effects on structure and stability in *E. coli* and *H. sapiens* H69 hairpin analogues.^{26,31,37–38} Circular dichroism (CD) and nuclear magnetic resonance (NMR) spectra showed that bacterial and human H69 hairpin RNAs have similar global conformations but contain subtle structural differences. These differences are challenging to identify in the dynamic loop region of H69; therefore, high-resolution structures of loop variants and human ribosomes still need to be determined in order to discover the specific effects of modification or sequence changes in H69. The CD spectrum of the *H. sapiens* Ψ -containing H69 RNA analogue (referred to as $\Psi 5$) showed a peak centered at 285 nm, which was not present in the spectrum of the corresponding unmodified, U-containing RNA (referred to as U5).³¹ These differences may result from the presence of the two Ψ s in the double-stranded stem region of the *H. sapiens* H69 RNA. The UV melting data described previously for the modified *H. sapiens* H69 hairpin ($\Psi 5$) indicated that it is ~1 kcal/mol more stable than the corresponding unmodified RNA sequence (U5).³¹ In contrast, there was no significant difference in stability between the unmodified and modified H69 hairpin RNAs (referred to as UUU and $\Psi\Psi\Psi$, respectively) from *E. coli*.²⁶

The goal of the work presented here was to increase our understanding of the structural and stabilizing effects of Ψ s in double-stranded regions of H69. These results were then compared to hairpin studies in order to also understand the contributions of loop Ψ s to H69 structure and stability. Seven different RNA sequences were chemically synthesized and used in various combinations to form six different H69 stem analogues from *E. coli* and *H. sapiens* (Figure 1). These analogues were used to examine the structural and stabilizing effects of Ψ s in individual strands of the H69 stem. Together, these solution studies provide important information about localized differences (e.g. stem vs. loop of H69) between human and bacterial ribosomes in the absence of high-resolution crystal structures.

MATERIALS AND METHODS

Preparation of RNAs

The six- (3' side or 'lower' strand) or seven-nucleotide (5' side or 'upper' strand) RNAs used in this study were obtained from Dharmacon Research (Lafayette, CO). These RNAs were chemically synthesized on a 0.2 μ mol scale and deprotected under mild acidic conditions.³⁹ The sequences of the RNAs are as follows (ss is used to represent single-stranded RNAs):

5'-G₁₉₀₆GCCGUA₁₉₁₂-3' (EssU, upper)

5'-G₁₉₀₆GCCGΨA₁₉₁₂-3' (EssΨ, upper)

3'-C₁₉₂₄UGGCA₁₉₁₉-5' (EssU, lower)

5'-G₃₇₂₂GGAGUA₃₇₂₈-3' (HssU, upper)

5'-G₃₇₂₂GGAGΨA₃₇₂₈-3' (HssΨ, upper)

3'-C₃₇₄₀UCUCA₃₇₃₅-5' (HssU, lower)

3'-C₃₇₄₀ΨCΨCA₃₇₃₅-5' (HssΨ, lower)

The numbering for these *E. coli* (E)- and *H. sapiens* (H)-based oligomers is based on the full-length *E. coli* 23S rRNA and *H. sapiens* 28S rRNA sequences. The RNAs were purified by HPLC on an XTerra MS C18 column (2.5 μ m, 10 $\times$ 50 mm, Waters, Milford, MA) in which the eluent was 0.1 M triethylammonium acetate (TEAA) buffer, pH 7.0, with a 5–9% linear gradient of acetonitrile over 15 minutes at a flow rate of 4.0 mL/min. Alternatively, 25 mM TEAA at pH 6.5 was used as the mobile phase with a 4–7% linear gradient of acetonitrile over 30 min. After HPLC purification, the RNA was desalted by ethanol precipitation. For UV melting, the RNAs were further desalted using Sep-Pak columns (Waters, Milford, MA). All purified RNAs were characterized by MALDI-TOF mass spectrometry.

RNA concentrations were calculated using Beer's law and single-stranded extinction coefficients (at 260 nm)⁴⁰ of 69,500 M⁻¹cm⁻¹ for EssU and EssΨ upper strands, 56,100 M⁻¹cm⁻¹ for EssU lower strands, 79,400 M⁻¹cm⁻¹ for HssU and HssΨ upper strands, and 53,600 M⁻¹cm⁻¹ for HssU and HssΨ lower strands. These extinction coefficients are obtained using the nearest-neighbor approximation, and the same extinction coefficient was used for uridine and Ψ (1.0 $\times$ 10⁴ M⁻¹cm⁻¹ at pH 7.0).⁴⁰ Equal amounts of each RNA strand were combined and annealed in 10 mM Tris-HCl, pH 7.5, by heating to 90 °C for 2 min followed by slow cooling to room temperature.

Circular dichroism spectroscopy

The CD spectra were obtained on a Jasco J600 spectropolarimeter (220 to 320 nm) in 15 mM NaCl, 20 mM sodium cacodylate, and 0.5 mM Na₂EDTA at pH 7.0. The concentrations were 6.0 to 6.4 μ M for all duplex RNAs and 5.0 to 7.5 μ M for all single-stranded RNAs. The temperature-dependent CD spectra were obtained from 15 to 85 °C, and the temperature was controlled by a water bath connected to a cylindrical CD cell. The average of the extinction coefficients for two corresponding single strands (62,800 M⁻¹cm⁻¹ for *E. coli* upper and lower strands and 66,500 M⁻¹cm⁻¹ for *H. sapiens* upper and lower strands) was used to calculate total strand concentration of the corresponding duplex RNA. All CD measurements were done in duplicate or triplicate.

UV melting

The absorbance vs. temperature changes were monitored on an Aviv 14DS UV-visible spectrophotometer with a five-cuvette thermoelectric controller. Microcuvettes with four

different path lengths (0.1, 0.2, 0.4, and 0.8 cm; volumes of 60, 120, 300, and 480 μL , respectively) were employed. The buffer used for these experiments contained 15 mM NaCl, 20 mM sodium cacodylate, and 0.5 mM Na_2EDTA , pH 7.0. The RNA concentration of each sample was determined from the UV absorbance value (260 nm) at 90 °C. The stem analogues are non-self-complementary; therefore, the average extinction coefficients of the corresponding single strands ($62,800 \text{ M}^{-1}\text{cm}^{-1}$ for *E. coli*-based stem analogues and $66,500 \text{ M}^{-1}\text{cm}^{-1}$ for *H. sapiens*-based stem analogues) were used to calculate the total RNA strand concentrations.⁴¹ The melting curves of samples (20 to 400 μM) were obtained from 5 to 95 °C at 280 nm. The melting curves of 10 μM samples were obtained from 0 to 90 °C at 260 nm. The melting curve for HdsU at 394 μM was obtained from 5 to 95 °C at 280 nm and from 0 to 90 °C at 260 nm for all other concentrations. The measurements were done at 260 or 280 nm to maximize the sensitivity and have appropriate absorption values over the entire concentration range. The thermodynamic parameters were calculated by using MeltWin 3.5⁴² and linear fit with a van't Hoff equation assuming a two-state model.⁴² The error-weighted average was calculated as reported previously.^{43–44}

RESULTS AND DISCUSSION

A set of modified RNAs representing modified, unmodified, or partially modified H69 was designed and synthesized (Figure 1). Hairpin and duplex RNAs containing the bacterial or human H69 sequences were generated. The individual strands derived from the 5' end of the hairpin are referred to as the 'upper strands' and those from the 3' end will be referred to as the 'lower strands'. All upper-strand sequences of the hairpins are seven nucleotides in length with a dangling A at the 3' end. We were unable to place Ψ at the starting position (3' end) in the chemical synthesis^{26,38} due to lack of a suitable Ψ -modified solid support. The EdsU and HdsU duplex (ds = double stranded) RNAs were unmodified (U = uridine-containing), stem-only analogues of *E. coli* (E) and *H. sapiens* (H) H69, respectively. The Eds Ψ and Hds Ψ duplexes were the corresponding modified (Ψ = pseudouridine-containing) stem analogues of H69. Two additional *H. sapiens* H69 stem analogues were generated in which the unmodified and modified single strands were shuffled. The Hds Ψ /U stem contained a Ψ at position 3727 in the upper strand, and the HdsU/ Ψ stem contained two Ψ s in the lower strand at positions 3737 and 3739 (*H. sapiens* 28S rRNA numbering).

Circular dichroism studies – stem regions of H69

The CD spectra of the H69 RNA stem regions were obtained in order to determine the effects of Ψ on the double-stranded regions and to later deduce influences on the loop structures. For all spectra, the data are reported in molar ellipticity ($\Delta\epsilon$) or molar circular dichroism ($[\theta]$) ($\Delta\epsilon = [\theta]/3300$; $[\theta] = 100*\theta/Cl$, in which concentration C is expressed in moles of RNA molecules per liter, the path length $l = 1.0$ cm, and θ is the observed circular dichroism).⁴⁵ The CD spectra of the stem or duplex (ds) analogue RNAs ($\Delta\epsilon_{\text{ds}}$) were first compared with the summation of the CD spectra of corresponding individual single-stranded stem oligomers ($\Delta\epsilon_{\text{sum_ss}} = \Delta\epsilon_{\text{upper_strand}} + \Delta\epsilon_{\text{lower_strand}}$) at low and high temperatures. Our goal was to determine which differences in the CD spectra were caused by differences between the structures of the RNAs and which were due to sequence or base-composition effects (U vs. Ψ) (Figure 2). One would expect that if certain CD peaks were derived from specific base-stacking interactions in the RNA duplex structure, then the summed spectrum ($\Delta\epsilon_{\text{sum_ss}}$) should be different from the stem (duplex) spectrum ($\Delta\epsilon_{\text{ds}}$). In contrast, if structural features of the RNA duplex did not contribute uniquely to the CD spectrum, and instead the sequence and single-strand features were dominant, then the summed spectrum would be equal to the duplex spectrum. Furthermore, the difference spectra between unmodified and modified RNAs ($\Delta\epsilon_{\text{ds_U}} - \Delta\epsilon_{\text{ds_}\Psi}$) at high temperature were expected to indicate contributions of electronic distribution differences between uridine and Ψ to

specific CD features. Finally, the difference spectra between unmodified and modified RNAs ($\Delta\epsilon_{ds_U} - \Delta\epsilon_{ds_Psi}$) at low temperature were anticipated to reveal not only the effects of base composition, but also structural effects (base stacking, etc.) of Ψ vs. U.

The CD spectra of the stem (duplex) RNAs ($\Delta\epsilon_{ds}$) and sums of the individual strands ($\Delta\epsilon_{sum_ss}$) at high temperature (85 °C) exhibit only subtle differences in the peak maxima, peak minima, and crossover points (Figure 2, compare lower intensity, gray solid and gray dotted lines in panels A–F). The stems are expected to be denatured to form individual random coils at high temperature. In contrast, the peak maxima and crossover points of the CD spectra for the duplex RNAs ($\Delta\epsilon_{ds}$) at low temperature (22 or 15 °C for the *E. coli* and *H. sapiens* RNA stems, respectively) are shifted to shorter wavelengths when compared to the summation of the CD spectra of their corresponding single strands ($\Delta\epsilon_{sum_ss}$) (Figure 2, compare higher intensity, black solid and black dotted lines in panels A–F). In general, the stem spectra ($\Delta\epsilon_{ds}$) at low temperature reveal considerable variability from one analogue to the next (see comparison in Figure 3A); whereas, the summed spectra ($\Delta\epsilon_{sum_ss}$) at low temperature show much less variability between RNA analogues (Figure 3B).

The summed spectra ($\Delta\epsilon_{sum_ss}$) for Esum_ss Ψ , Esum_ssU, Hsum_ss Ψ /U, and Hsum_ssU show similar features with peak maxima at 270 nm and minima at 238 nm (Figure 3B). The spectra for Hsum_ss Ψ and Hsum_ssU/ Ψ differ from the other analogues, but are similar to one another with peak maxima at 260 nm and peak minima at 240 nm. These results suggest a specific influence of the two modified nucleotides, Ψ_{3737} and Ψ_{3739} , which is unique to Hds Ψ and HdsU/ Ψ on the summed CD spectra. There is little difference between the spectra of HdsU/ Ψ and Hsum_ssU/ Ψ (Figure 2E), revealing that little change in the RNA structure occurs when the duplex is formed. This result implies that the modified single-stranded H69 RNA component itself, in the absence of base pairing, has significant secondary structure features (e.g., base-stacking interactions), consistent with prior studies on other single-stranded RNAs containing Ψ .²¹

The differences in CD spectra of the duplex RNAs ($\Delta\epsilon_{ds}$) (Figure 3A) likely reflect differences in their structures, such as alterations in base stacking due to the absence or presence of Ψ residues. For example, EdsU and Eds Ψ duplex spectra ($\Delta\epsilon_{ds}$) at low temperature have identical peak maxima (260 nm), but different peak shapes (Figure 4A); whereas, the summed CD spectra ($\Delta\epsilon_{sum_ss}$) have similar peak maxima shifted by 10 nm (270 nm) and similar peak shapes (Figure 4B). The HdsU/ Ψ and HdsU spectra at 15 °C show only small differences with the corresponding summed spectra (Figures 2C and 2E), suggesting that their structures are not significantly altered upon duplex formation. In contrast, the Hds Ψ /U and Hds Ψ spectra at 15 °C both reveal significant differences with the corresponding summed spectra (Figures 2D and 2F, 4C, and 4D). Furthermore, significant secondary peaks with maxima at 286, 288, and 290 nm in the Eds Ψ , Hds Ψ /U, and Hds Ψ duplex ($\Delta\epsilon_{ds}$) spectra, respectively, are not observed in the summed spectra ($\Delta\epsilon_{sum_ss}$) of the corresponding single strands at low temperature (Figures 2B, 2D, and 2F, compare the solid black and black dotted line spectra). These extra peaks indicate differences in the RNA duplex structures compared to the individual single strands. A common feature of those RNAs is a Ψ at position 1911/3727. Of note, the same peak was observed in CD spectra of the H69 hairpins representing *E. coli* and *H. sapiens* RNAs.^{26,31,37–38} Therefore, the secondary peaks with maxima between 286 and 290 nm observed in the modified RNA stem spectra are believed to result from structural effects of Ψ_{1911}/Ψ_{3727} .

Overall, a comparison of the CD spectra from *E. coli* and *H. sapiens* H69 stems indicates that the structural effects of Ψ depend on the sequence context. The CD summation spectra reveal clear structural differences between RNAs with a Ψ at the terminal base-pair position ($\Psi_{1911/3727}$) and Ψ s in the middle of the duplex region (Ψ_{3737} and Ψ_{3739}) (Figure 3B). The

two internal stem Ψ s in the *H. sapiens* analogue lead to structured single-stranded RNAs that do not change much upon duplex formation; whereas, a Ψ at the end of a duplex region induces a noticeable conformation change in the RNA. Thus, these results reveal an important role for Ψ positioning within the stem region.

Circular dichroism studies – loop structure

The effects of modification on the H69 loop structures were then deduced by comparing the H69 stem CD spectra ($\Delta\epsilon_{ds}$) obtained in this study with hairpin CD spectra ($\Delta\epsilon_{hp}$) obtained previously.^{31,38} The *E. coli* unmodified and modified H69 hairpin RNAs are referred to here as Ehpu and Ehp Ψ , respectively (previously referred to as UUU and $\Psi\P\P\P/\Psi m^3\P\P\P$, in which $m^3\P$ is 3-methylpseudouridine).³⁸ Similarly, the *H. sapiens* unmodified and modified H69 hairpin RNAs are referred to here as HhpU and Hhp Ψ , respectively (previously referred to as U5 and Ψ 5).³¹ Compared with the CD spectra of the H69 duplex analogues (Figure 2), the peak maxima, peak minima, and crossover points in the CD spectra of H69 hairpin analogues^{31,38} are shifted (Table 1). Unique features in the CD difference spectra ($\Delta\epsilon_{lp} = \Delta\epsilon_{hp} - \Delta\epsilon_{ds}$) (Figures 5 and 6) are therefore attributed to different loop (lp) structures.

As expected, the difference spectra ($\Delta\epsilon_{lp}$) for various analogues at high temperature (Figure 5B) are for the most part featureless. In addition, these spectra are all similar, showing that the effects of sequence and modification (U vs. Ψ and A vs. G) are minimal for the melted RNAs. The most noticeable differences are between the CD spectra of Elp Ψ and HlpU, which are the most and least stable analogues, respectively.⁴⁶

The peak shifts and shape changes in the difference spectra ($\Delta\epsilon_{lp}$, Figures 5A and 6A-D) at low temperature reflect structural effects in the loop regions from varying sequence and levels of modification. The CD spectra of Ehp Ψ with three Ψ s (referred to previously as $\Psi\P\P\P$ due to modifications Ψ_{1911} , Ψ_{1915} , Ψ_{1917}) or the natural sequence with 3-methylpseudouridine ($m^3\P$) at position 1915 ($\Psi m^3\P\P\P$) are identical,^{31,47} revealing that methylation does not alter the loop structure. Therefore, as expected, the CD difference spectra ($\Delta\epsilon_{lp}$) are identical using Ehp Ψ ($\Psi\P\P\P$) or $\Psi m^3\P\P\P$ hairpin spectra. In contrast, the CD difference spectra ($\Delta\epsilon_{lp}$) reveal new features at low temperature (22 °C for the *E. coli* RNA analogues and 15 °C for the *H. sapiens* RNA analogues) (Figure 5A).

The sequences of the H69 analogues are different, such as U vs. Ψ (Figure 6A and 6B) in the loop, or A vs. G (Figure 6C and 6D) on the 3' side of the loop. The CD spectra ($\Delta\epsilon_{lp}$) for these analogues are clearly different, and these differences are attributed to structural variations between the loops of the H69 analogues (Figure 6A). The region between 240 and 260 nm shows the largest differences between the Elp Ψ and ElpU RNAs; whereas, the Hlp Ψ , HlpU and E(A₁₉₁₈G)lpU spectra are similar in this region. The data show that the A vs. G difference affects the loop structure. The CD region between 260 and 285 nm shows a different trend, in which HlpU, ElpU and E(A₁₉₁₈G)lpU are similar and Hlp Ψ (mod) and Elp Ψ are similar. These data show that the Ψ s in the loops ($\Psi_{1915/3731}$ and $\Psi_{1917/3733}$) have specific structural effects on the H69 RNAs. In addition, the CD difference spectra of E(A₁₉₁₈G)lpU and HlpU (Figure 6C) are not overlapping, even though they have the same loop sequence. Thus, this combined set of CD data for the H69 RNAs indicates that the loop structures are also affected by the adjoining stem sequences, modifications, and/or structures.

UV melting studies on H69 stem analogues

Thermal melting studies of the H69 stem RNA analogues were carried out to determine the effects of Ψ modification on stem stability. All of the H69 stem RNA analogues used in this study are non-self-complementary duplexes. For sample preparation, equal amounts of

single strands were mixed to form the duplexes. The possibility of error in the single-strand ratio exists; however, these small errors are not expected to affect the results significantly unless one strand is more than 50% in excess over the other strand.⁴⁸ The upper strands (5' side) of the H69 stem oligomers are seven nucleotides in length, even though the natural hairpin stems only contain six base pairs. The reason for the longer oligomer is that RNA chemical synthesis proceeds in the 3' to 5' direction, and a Ψ solid support for the 3' starting nucleotide was not available. Thus, the stem duplexes contain a dangling A adjacent to $\Psi_{1911/3727}$ at the 3' end of the upper strand. Of the four standard RNA bases, a dangling A is known to provide the most stability to RNA duplexes ($\Delta G^\circ_{37} = -2.1$ kcal/mol compared with -1.0 , -1.5 , and -1.7 kcal/mol for C, U, and G dangling ends, respectively).^{49–50} Typically, there are no hydrogen bonds between dangling A residues and other bases; therefore, the stabilizing effect by the dangling end is most likely from base stacking. Although Ψ is generally believed to stack better than uridine in single-stranded RNAs, Davis showed that the stabilizing effects of Ψ are more pronounced on the 5' side.²¹ For this reason, the stabilizing effects from the dangling A were assumed to be similar between unmodified (U-containing) and modified (Ψ -containing) RNAs.

The plots of T_m^{-1} vs. natural logarithm of $C_t/4$ are shown in Figure 7, in which T_m is the melting temperature and C_t is total single-strand concentration. The fitted lines have negative slopes, which indicate that the T_m s are concentration dependent. The thermodynamic parameters for the H69 stem analogues are summarized in Table 2. The thermodynamic parameters were calculated by using two different methods, the melting curve analysis (average of curve fits)⁵¹ and the T_m -dependence model (T_m^{-1} vs. $\ln C_t/4$ plots) for each RNA analogue.⁵² The ΔH° values obtained by the two different methods agree within 10%, which is typical for a two-state transition from duplex to random coil.⁴⁹ In our previous stability studies of H69 hairpins,^{26,31} low salt conditions were used to avoid duplex formation; thus, using low salt conditions in this study was necessary for direct comparison between the hairpin and duplex RNAs. Table 2 also shows the average thermodynamic parameters from the two methods.

Results for the *E. coli*-based stem analogues show that the modified RNA (Eds Ψ) has a more favorable $\Delta G^\circ_{37}(\text{ds})$ (-9.1 kcal/mol) than the unmodified RNA (EdsU, -8.1 kcal/mol). The *H. sapiens*-based stem analogues show similar results. The modified stem RNA (Hds Ψ , $\Delta G^\circ_{37}(\text{ds}) = -5.7$ kcal/mol) is more stable than the unmodified stem RNA (HdsU, $\Delta G^\circ_{37}(\text{ds}) = -4.3$ kcal/mol). It is not surprising that the $\Delta G^\circ_{37}(\text{ds})$ values of the stem analogues with combinations of unmodified and modified single strands (HdsU/ Ψ and Hds Ψ /U) are more favorable than the HdsU stem and less favorable than the Hds Ψ stem (Table 2). The effects of Ψ were evaluated as the free energy, $\Delta G^\circ_{37}(\Psi_{\text{ds}})$ for the stem regions (in the upper and lower strands) and $\Delta G^\circ_{37}(\Psi_{\text{lp}})$ for the loop regions, calculated from the equations:

$$\Delta G^\circ_{37}(\Psi_{\text{ds}}) = \Delta G^\circ_{37}(\text{ds}\Psi) - \Delta G^\circ_{37}(\text{dsU}) \quad (1)$$

$$\Delta G^\circ_{37}(\text{U}/\Psi_{\text{ds}}) = \Delta G^\circ_{37}(\text{dsU}/\Psi) - \Delta G^\circ_{37}(\text{dsU}) \quad (2)$$

$$\Delta G^\circ_{37}(\Psi/\text{U}_{\text{ds}}) = \Delta G^\circ_{37}(\text{ds}\Psi/\text{U}) - \Delta G^\circ_{37}(\text{dsU}) \quad (3)$$

$$\Delta G^\circ_{37}(\text{lp}\Psi) = \Delta G^\circ_{37}(\text{hp}\Psi) - \Delta G^\circ_{37}(\text{ds}\Psi) \quad (4)$$

$$\Delta G^{\circ}_{37}(\text{lpU}) = \Delta G^{\circ}_{37}(\text{hpU}) - \Delta G^{\circ}_{37}(\text{dsU}) \quad (5)$$

$$\Delta G^{\circ}_{37}(\Psi_{\text{lp}}) = \Delta G^{\circ}_{37}(\text{lp}\Psi) - \Delta G^{\circ}_{37}(\text{lpU}) \quad (6)$$

in which $\Delta G^{\circ}_{37}(\text{ds}\Psi)$ is the free energy of formation of the H69 stem duplex containing Ψ (Eds Ψ , Hds Ψ), $\Delta G^{\circ}_{37}(\text{dsU}/\Psi$ or $\text{ds}\Psi/\text{U}$) is the free energy for HdsU/ Ψ or Hds Ψ /U, respectively, and $\Delta G^{\circ}_{37}(\text{dsU})$ is the free energy of formation of the corresponding unmodified H69 stem duplex (EdsU and HdsU). Furthermore, $\Delta G^{\circ}_{37}(\text{lp}\Psi)$ is the free energy difference between the modified hairpin (Ehp Ψ or Hhp Ψ), $\Delta G^{\circ}_{37}(\text{hp}\Psi)$, and stem (Eds Ψ or Hds Ψ) RNAs, and $\Delta G^{\circ}_{37}(\text{lpU})$ is the free energy difference between the unmodified H69 hairpin (EhpU or HhpU), $\Delta G^{\circ}_{37}(\text{hpU})$, and stem (EdsU or HdsU) RNAs.

The $\Delta G^{\circ}_{37}(\Psi_{\text{ds}})$ for the *E. coli* stem is -1.0 kcal/mol ($\Delta G^{\circ}_{37}(\text{Eds}\Psi) - \Delta G^{\circ}_{37}(\text{EdsU}) = -9.1 - (-8.1)$ kcal/mol). This value is in good agreement with the difference in ΔG°_{37} s between a singly modified H69 hairpin analogue (Ψ_{1911} at the closing base pair position, -5.9 kcal/mol) and corresponding unmodified RNA (U_{1911} , -4.9 kcal/mol).²⁶ Similarly, the *H. sapiens* $\Delta G^{\circ}_{37}(\Psi_{\text{ds}})$ for the fully modified analogue ($\Delta G^{\circ}_{37}(\text{Hds}\Psi) - \Delta G^{\circ}_{37}(\text{HdsU}) = -5.7 - (-4.3)$ kcal/mol) is -1.4 kcal/mol. The *H. sapiens* H69 RNA contains three Ψ s in the stem region, with Ψ_{3727} on the 5' side and Ψ_{3737} and Ψ_{3739} on the 3' side. The unmodified and modified stem oligomers were combined to form two unique stem analogues, HdsU/ Ψ with two Ψ s (Ψ_{3737} and Ψ_{3739}), and Hds Ψ /U with a single Ψ (Ψ_{3727}). Both stems have more favorable ΔG°_{37} s than the HdsU stem analogue, but less favorable values than the Hds Ψ stem analogues (-4.9 kcal/mol for Hds Ψ /U and -5.2 kcal/mol HdsU/ Ψ). These data indicate that Ψ s on both sides of the stem stabilize the duplex structure in human H69.

The $\Delta G^{\circ}_{37}(\Psi/\text{U}_{\text{ds}})$ for Hds Ψ /U is -0.6 kcal/mol ($-4.9 - (-4.3)$ kcal/mol), which indicates a subtle stabilizing effect of Ψ_{3727} . Similarly, the $\Delta G^{\circ}_{37}(\text{U}/\Psi_{\text{ds}})$ for HdsU/ Ψ is -0.9 kcal/mol ($-5.2 - (-4.3)$ kcal/mol), which shows slight stabilizing effects for the combination of Ψ_{3737} and Ψ_{3739} . These minor stabilizing effects of the 5' and 3' sides of stem are within 0.3 kcal/mol, even though the 3' side of the stem (lower strand) contains one more Ψ than the 5' side of the stem (upper strand). Thus, the effects of Ψ on G• Ψ mismatch stability differ from those involving A– Ψ pairs. In support of these results, a dimethylsulfate probing study in yeast showed that pseudouridylation at 2264 (3737 in the human sequence) enhances base pairing with A₂₂₅₂.³⁶ The individual stabilizing effects of Ψ s on the 3' side of the H69 stem (Ψ_{3737} and Ψ_{3739}) still need to be investigated. The sum of the stabilizing effects of the partially modified stems (sum of $\Delta G^{\circ}_{37}(\Psi_{\text{ds}})$ values for Hds Ψ /U and HdsU/ Ψ) is -1.5 kcal/mol ($-0.6 + (-0.9)$ kcal/mol), consistent with the stabilizing effects determined for all three Ψ s ($\Delta G^{\circ}_{37}(\Psi_{\text{ds}}) = \Delta G^{\circ}_{37}(\text{Hds}\Psi) - \Delta G^{\circ}_{37}(\text{HdsU}) = -5.7 - (-4.3)$ kcal/mol = -1.4 kcal/mol).

The free energy differences between the hairpins and stems provide the values for free energy of the H69 loops from bacteria and human (Table 2). The energetic contributions of loop Ψ s relative to uridine were then determined. The *E. coli* $\Delta G^{\circ}_{37}(\Psi_{\text{lp}})$ value ($\Delta G^{\circ}_{37}(\text{Elp}\Psi) - \Delta G^{\circ}_{37}(\text{ElpU})$) is 0.8 kcal/mol. This result indicates that the two Ψ s in the *E. coli* H69 hairpin loop (Ψ_{1915} and Ψ_{1917}) have destabilizing effects, consistent with previous studies.²⁶ The *H. sapiens* $\Delta G^{\circ}_{37}(\Psi_{\text{lp}})$ ($\Delta G^{\circ}_{37}(\text{Hlp}\Psi) - \Delta G^{\circ}_{37}(\text{HlpU})$) ($1.9 - 1.6$ kcal/mol), is 0.3 kcal/mol, which is three-fold lower than the *E. coli* $\Delta G^{\circ}_{37}(\Psi_{\text{lp}})$. Thus, the effects of the two Ψ s in the *H. sapiens* H69 hairpin loop (Ψ_{3731} and Ψ_{3733}), which have not been determined previously, are smaller compared to the effects of Ψ s in the *E. coli* H69 hairpin

loop. The difference in stability between *E. coli* and *H. sapiens* H69 may be due to the base difference on the 3' side of the loop (A₁₉₁₈ in *E. coli* vs. G₃₇₃₄ in *H. sapiens*); however, previous studies showed that the $\Delta G^{\circ}_{37}(\text{hpU})$ values of EhpU and E(A₁₉₁₈G)hpU hairpins are essentially identical (−4.8 and −4.7 kcal/mol, respectively).³¹ Therefore, these differences may also be attributed to combined errors in the free energy calculations. Further comparison of the *E. coli* and *H. sapiens* H69 loops reveals that the A₁₉₁₈ residue is 0.6 kcal/mol more destabilizing in the modified RNAs than the unmodified RNAs ($\Delta G^{\circ}_{37}(\text{ElpU}) - \Delta G^{\circ}_{37}(\text{HlpU}) = 3.3 - 1.6 = 1.7$ kcal/mol; $\Delta G^{\circ}_{37}(\text{Elp}\Psi) - \Delta G^{\circ}_{37}(\text{Hlp}\Psi) = 4.1 - 1.9 = 2.2$ kcal/mol). Therefore, the data in this study indicate that the destabilizing or stabilizing effects of Ψ s in loop regions are influenced by the neighboring stem sequence and stability, which correlates well with the CD structural data.

Role of pseudouridine in H69

Many Ψ s are found in the functional regions of rRNA, such as the peptidyl-transferase center.⁵³ Helix 69 is located at the intersubunit bridge B2a, which connects the peptidyl-transferase center to the decoding region of the ribosome.² The functional roles of H69 are believed to be regulation of subunit association,^{10–11} ribosome recycling,^{15–16} translation termination,^{12–14} and factor-dependent translation initiation and translational processivity.¹⁷ Therefore, it is important to understand the similarities and differences in H69 structure and stability in order to compare ribosomes of different species. Furthermore, it is important to appreciate the varying structural and stabilizing effects of the individual modified (Ψ) residues in these regions to better understand their roles in the ribosome mechanism. These comparisons can be challenging to make in the absence of high-resolution structures, particularly in the case of eukaryotic ribosomes.

In this study, CD spectroscopy was useful for examining the contributions of different regions of the RNA hairpin to structure. Individual components of the H69 RNAs (stems vs. loops) were examined directly or through CD difference spectroscopy. A peak at 285 nm in the Hhp Ψ (human modified hairpin) CD spectrum was also observed in the CD spectra of the Ehp Ψ (*E. coli* modified hairpin), Eds Ψ (*E. coli* stem), Hds Ψ (human stem), and Hds Ψ /U (partially modified human stem) RNAs. This peak was not apparent at high temperatures and did not appear in the CD addition spectra of the corresponding single strands ($\Delta\epsilon_{\text{sum,ss}}$). This CD feature likely arises from structural effects of the Ψ residue at positions 1911/3727 (*E. coli*/*H. sapiens*). Thus, CD spectroscopy was a useful method for determining which Ψ residues impact the RNA structure. Furthermore, the CD difference spectra (hairpin minus stem CD spectra, Figure 5) are unique for all of the H69 RNA analogues. These data provide evidence that the loop solution structures of the bacterial and human H69 analogues are different, and that Ψ modification plays a role in these differences. This new information on human H69 loop structures was not available prior to this study due to lack of high-resolution structures of *H. sapiens* ribosomes.

The CD data presented here reveal that the single-stranded Ψ residues ($\Psi_{1915/3731}$ and $\Psi_{1917/3733}$) have different effects on the H69 loop structures. The base composition on the 3' side of the loop (A₁₉₁₈ and G₃₇₃₄) also influences the structure. Previous model studies revealed that the *E. coli* H69 conformation depends on pH of the system.³⁷ More recent chemical probing studies on 50S ribosomes indicated different conformational states of H69 that are dependent on the presence of Ψ residues and can be modulated by pH, temperature, and magnesium.⁵⁴ At neutral pH, the position of loop-closing Ψ_{1911} fluctuates, as revealed by NMR spectroscopy, and in turn causes changes within the loop region.³⁷ Crystal structures show that a neighboring residue, A₁₉₁₃, flips in and out of the H69 loop, which is important for interactions with other ribosome components such as the ribosome recycling factor (RRF) and the decoding region.^{3–5,8,55–57} Furthermore, studies with the metal complex [Rh(DIP)₃]³⁺, which has the ability to induce RNA strand scission in a structure-

dependent manner, showed that Ψ_{3727} in the human sequence is not cleaved, but the corresponding residue (Ψ_{1911}) in the *E. coli* H69 is targeted.⁴⁶ These results suggest that differences in the behavior of Ψ at the loop-closing position may lead to significant variations in the structure and loop dynamics of H69, as well as interactions with various protein factors in bacterial or human ribosomes.

H69 is a recently identified target for antibiotics, including capreomycin and viomycin,⁵⁸ and many aminoglycosides such as gentamycin and paromomycin.^{5,59} Variants of *T. thermophilus* with mutations or deletions of A₁₉₁₃U, mU₁₉₁₅G, and Δ mU₁₉₁₅ resist capreomycin.⁶⁰ Thus, the loop structure and dynamics of H69 appear to be critical for normal protein synthesis to occur. *E. coli* ribosome crystal structures in the presence of RRF and aminoglycosides show rings I and II of gentamicin and neomycin interacting with the 3' side of the H69 stem (C₁₉₂₀–C₁₉₂₅),⁵ which is the location of the two additional Ψ s in the human sequence. These modification differences could also impact aminoglycoside affinity for H69. For example, the unmodified *H. sapiens* H69 has lower affinity to antibiotics than the unmodified *E. coli* H69 sequence.⁵⁹

Besides understanding the structural consequences of sequence or modification changes in the H69 loop, it is also important to quantify these differences in terms of thermodynamic stability. In this work, the thermal melting data for the H69 analogues indicate that the destabilizing effects of Ψ s in the *H. sapiens* H69 hairpin loop (Ψ_{3731} and Ψ_{3733}) are not as significant as the Ψ s in the *E. coli* H69 loop region (Ψ_{1915} and Ψ_{1917}).^{26,31,46} That difference may be caused by the base sequence change at the 3' end of the loop (A₁₉₁₈ in *E. coli* vs. G₃₇₃₄ in *H. sapiens*); however, unmodified EhpU and E(A₁₉₁₈G)hpU RNAs have the same ΔG°_{37} values.³¹ Therefore, G₃₇₃₄ may have a stabilizing effect in combination with the Ψ modifications. The loop-closing residue Ψ_{1911} has a stabilizing effect, as determined by differences in the unmodified and singly modified *E. coli* H69 RNA.²⁶ For the *E. coli* RNAs, Ψ_{1911} , Ψ_{1915} , and Ψ_{1917} each had different effects on the stability of *E. coli* H69, whereas the combination of all three Ψ s had little effect on the overall stability of the hairpin.²⁶ In *H. sapiens* H69 RNA, the stabilizing and/or destabilizing effects of Ψ residues are balanced differently, such that the modified and unmodified RNAs differ in overall stability.³¹

The individual stabilizing effects of Ψ_{3737} and Ψ_{3739} in human H69 are still unknown; however, these additional Ψ s that are not found in bacterial H69 may be necessary in order to compensate for sequence changes that would destabilize the stem of the human analogue relative to the *E. coli* analogue (the eukaryotic sequence has an A–U pair in place of a C–G pair found in the bacterial RNA). Such subtle changes in the ribosome structure and stability may be necessary to modulate its function and fidelity. In fact, Fahlman *et al.* have proposed such a strategy in aminoacyl-transfer RNAs binding to the ribosome.^{61–62} They suggest that different patterns of contact between the tRNA and ribosome sites have evolved through changes in tRNA sequence and levels of modification such that binding is uniform.

CONCLUSIONS

The structural and thermodynamic properties of bacterial and human H69 reported here are useful for explaining biochemical and functional data. More specifically, CD spectroscopy is a useful method for determining how various factors impact RNA structure. Moreover, CD difference spectroscopy reveals the cross talk of individual components in H69, which would be challenging to determine by using other biophysical methods. This information can be used to complement existing high-resolution structural data, or provide new insights when such high-resolution data are lacking. In this study, CD difference spectra revealed unique effects of pseudouridine in H69 RNA analogues from bacteria and human, which

have not been identified previously due to lack of *H. sapiens* structures. Such differences may lead to significant variations in the structure and loop dynamics of H69, such that interactions with other components of the bacterial or human ribosomes are impacted. Stability measurements reported here further support the conclusions that modifications have differing effects on H69 from bacteria and human. Our data indicate that the destabilizing or stabilizing effects of modifications in loop regions are influenced by the neighboring stem sequences. Such modification differences in human and bacteria could modulate affinity of antibiotics in functionally important regions of the ribosome. Further studies on site-specific ribosome modifications may lead to a greater understanding of their biological roles and be used to determine the impact of modification deletions or substitutions on protein synthesis. Studies beyond the model systems at the ribosome level will also be important to determine the roles of tertiary contacts between H69, tRNAs, and rRNA components such as the decoding region. Our future goal is to exploit the differences in H69 stability and structure between humans and bacteria for drug development and to understand how H69 loop mutations or modifications could lead to antibiotic resistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

The structure of pseudouridine and the helix 69 (H69) hairpin RNAs from *H. sapiens* (right side) and *E. coli* (left side) (in the boxed region) are shown. EhpΨ represents residues 1906–1924 of *E. coli* H69. HhpΨ represents residues 3722–3740 of *H. sapiens* H69. EdsU and EdsΨ represent the double-stranded stem residues 1906–1912 (upper strands) and 1919–1924 (lower strands) of *E. coli* H69. HdsU and HdsΨ, represent stem residues 3722–3728 (upper strands) and 3735–3740 (lower strands) of *H. sapiens* H69. HdsU/Ψ and HdsΨ/U strands are shuffled variants of HdsU and HdsΨ. The E(A₁₉₁₈G)hpU hairpin mutant (not shown) has a stem identical to that of EhpU and a loop identical to that of HhpU (unmod RNAs have U in place of Ψ). Ψ represents pseudouridine.

Figure 2.

CD spectra of EdsU (A), EdsΨ (B), HdsU (C), HdsΨ (D), HdsU/Ψ (E), and HdsΨ/U (F) stem (duplex, ds) analogues (solid lines, $\Delta\epsilon_{ds}$) overlaid with the summed CD spectra of the corresponding upper and lower single strands (dotted lines, $\Delta\epsilon_{sum_ss}$) at low temperature (spectra in black) (22 °C or 15 °C for *E. coli* or *H. sapiens* analogues, respectively) and high temperature (spectra in gray) (85 °C) are shown. The buffer for all CD experiments contained 15 mM NaCl, 20 mM sodium cacodylate, and 0.5 mM Na₂EDTA, pH 7.0. All CD measurements were done in duplicate and each spectrum is an average of four scans.

Figure 3.

An overlay of CD spectra of EdsU (navy blue), EdsΨ (green), HdsU (red), HdsΨ (orange), HdsΨ/U (turquoise), and HdsU/Ψ (grey) stem (duplex, ds) analogues ($\Delta\epsilon_{ds}$) (panel A, solid lines) or summed CD spectra of the corresponding upper and lower single strands ($\Delta\epsilon_{sum_ss}$) (panel B, dotted lines) at low temperature (22 °C or 15 °C for *E. coli* or *H. sapiens* analogues, respectively) are shown.

Figure 4.

Overlays of the CD spectra (low temperature) of duplex analogues ($\Delta\epsilon_{ds}$) or summed CD spectra of the corresponding upper and lower single strands ($\Delta\epsilon_{sum_ss}$) are shown: A) Eds Ψ (solid), EdsU (dotted), B) Esum_ss Ψ (solid), Esum_ssU (dotted), C) Hds Ψ (solid), HdsU (dotted), D) Hsum_ss Ψ (solid), and Hsum_ssU (dotted).

Figure 5.

Overlays of the CD difference spectra ($\Delta\epsilon_{lp} = \Delta\epsilon_{hp} - \Delta\epsilon_{ds}$) of the H69 RNA analogues (ElpU (blue), Elp Ψ (green), HlpU (red), Hlp Ψ (orange), and E(A₁₉₁₈G)lpU (grey)) at low (22 °C or 15 °C for the *E. coli* and *H. sapiens* analogues, respectively) (panel A) and high (85 °C) (panel B) temperatures are shown.

Figure 6.

Overlays of the CD difference spectra ($\Delta\epsilon_{lp} = \Delta\epsilon_{hp} - \Delta\epsilon_{ds}$) of the H69 RNA analogues are shown: A) ElpΨ (solid), ElpU (dotted), B) HlpΨ (solid), HlpU (dotted), C) ElpU (red dotted), HlpU (black dotted), E(A₁₉₁₈G)lpU (blue dotted), D) ElpΨ (red solid), HlpΨ (black solid), and E(A₁₉₁₈G)lpU (blue solid).

Figure 7.

The reciprocal melting temperature (T_m^{-1}) vs. $\ln(\text{total strand concentration}/4)$ plots for the unmodified (grey diamonds, dashed line) and modified (black squares, solid line) H69 stem regions of *E. coli* (panel A) and *H. sapiens* (panel B) are shown. Panel B also shows the T_m^{-1} vs. $\ln(\text{total strand concentration}/4)$ plot for the duplexes formed between modified upper and unmodified lower (red circles, solid line) and for unmodified upper and modified lower (blue triangles, solid line) strands of *H. sapiens* RNA. The buffer conditions were 15 mM NaCl, 20 mM sodium cacodylate, and 0.5 mM Na_2EDTA , pH 7.0.

Table 1

Peak maxima, minima, and crossover points in the CD spectra

RNA	Peak Maxima, (nm)	Peak Minima, (nm)	Crossover Point, (nm)	Local Maxima, (nm)
EhpU	266	236	248	
<u>EhpΨ</u>	260	232	240	<u>282</u>
HhpU	264	238	248	
<u>HhpΨ</u>	256	214	238	<u>286</u>
EdsU	260	236	244	
<u>EdsΨ</u>	260	212	240	<u>284</u>
HdsU	268	238	246	
<u>HdsΨ</u>	254	216	224	<u>290</u>
HdsΨ/U	252	216	220	284
HdsU/Ψ	260	216	221	

Table 2

Thermodynamic parameters of helix 69 stem-region (ds) RNAs

RNA	$\Delta G_{37}^{\circ}(\text{ds})$ (kcal/mol)	$\Delta G_{37}^{\circ}(\text{lp})^a$ (kcal/mol)	ΔH° (kcal/mol)	ΔS° (e.u.)	T_m^b ($^{\circ}\text{C}$)
Average of curve fits					
EdsU	-8.1 ± 0.1		-45.6 ± 2.9	-120.7 ± 9.0	48.3
Eds Ψ	-9.2 ± 0.1		-55.3 ± 4.5	-148.7 ± 14.4	52.5
HdsU	-4.1 ± 0.2		-58.5 ± 3.1	-175.4 ± 10.4	24.8
Hds Ψ	-5.7 ± 0.1		-54.6 ± 3.2	-157.7 ± 10.3	32.5
Hds Ψ /U	-4.9 ± 0.1		-59.6 ± 2.4	-176.4 ± 7.6	28.5
HdsU/ Ψ	-5.1 ± 0.1		-51.3 ± 2.4	-148.8 ± 7.6	28.8
T_m^{-1} vs. $\ln C/4$ plots					
EdsU	-8.1 ± 0.2		-45.0 ± 4.1	-119.1 ± 12.9	48.0
Eds Ψ	-9.1 ± 0.1		-54.5 ± 1.3	-146.4 ± 4.0	52.3
HdsU	-4.4 ± 0.1		-53.4 ± 2.7	-157.9 ± 9.1	25.0
Hds Ψ	-5.8 ± 0.1		-53.3 ± 3.0	-153.3 ± 9.7	32.5
Hds Ψ /U	-4.9 ± 0.2		-56.8 ± 4.1	-167.4 ± 13.6	28.5
HdsU/ Ψ	-5.2 ± 0.1		-48.9 ± 1.8	-140.9 ± 5.9	28.7
Average of the two methods					
EdsU	-8.1 ± 0.1		-45.4 ± 2.4	-120.2 ± 7.4	48.2
Eds Ψ	-9.1 ± 0.1		-54.6 ± 1.2	-146.6 ± 3.8	52.4
HdsU	-4.3 ± 0.1		-55.6 ± 2.0	-165.5 ± 6.9	24.9
Hds Ψ	-5.7 ± 0.1		-53.9 ± 2.2	-155.4 ± 7.1	32.5
Hds Ψ /U	-4.9 ± 0.1		-58.9 ± 2.1	-174.3 ± 6.7	28.5
HdsU/ Ψ	-5.2 ± 0.1		-49.7 ± 1.4	-143.8 ± 4.6	28.8
Loops					
ElpU		3.3 ± 0.1			
Elp Ψ		4.1 ± 0.1			

RNA	$\Delta G^{\circ}_{37}(\text{ds})$ (kcal/mol)	$\Delta G^{\circ}_{37}(\text{lp})^a$ (kcal/mol)	ΔH° (kcal/mol)	ΔS° (e.u.)	T_m ($^{\circ}\text{C}$)
HlpU		1.6 ± 0.1			
HlpY		1.9 ± 0.1			

^a $\Delta G^{\circ}_{37}(\text{lp}) = \Delta G^{\circ}_{37}(\text{hp}) - \Delta G^{\circ}_{37}(\text{ds})$, in which lp = loop, hp = hairpin, and ds = stem/duplex

^bThe buffer contained 15 mM NaCl, 20 mM sodium cacodylate, and 0.5 mM Na₂EDTA, pH 7.0.