Remarkable Stability of Hairpins Containing 2′,5′-Linked RNA Loops

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Abstract: We report here the results of a comparative study of hairpin loops that differ in the connectivity of phosphodiester linkages (3′,5′- versus 2′,5′-linkages). In addition, we have studied the effect of changing the stem composition on the thermodynamic stability of hairpin loops. Specifically, we constructed hairpins containing one of six stem duplex combinations, i.e., DNA:DNA ("DD"), RNA:RNA ("RR"), DNA:RNA ("DR"), 2′,5′-RNA:RNA ("$\underline{R}$R"), 2′,5′-RNA:DNA ("$\underline{R}$D"), and 2′,5′-RNA:2′,5′-RNA ("$\underline{RR}$"), and one of three tetraloop compositions, i.e., 2′,5′-RNA ("$\underline{R}$"), RNA ("R"), and DNA ("D"). All hairpins contained the conserved and well-studied loop sequence 5′-...C(UUCG)G...-3′ [Cheong et al. *Nature* **1990**, *346*, 680−682]. We show that the 2′,5′-linked loop C($\underline{UUCG}$)G, i.e., ...$C_{3'p5'}U_{2'p5'}U_{2'p5'}C_{2'p5'}G_{2'p5'}G_{3'p5'}$..., like its "normal" RNA counterpart, forms an unusually stable tetraloop structure. We also show that the stability imparted by 2′,5′-RNA loops is dependent on base sequence, a property that is shared with the regioisomeric 3′,5′-RNA loops. Remarkably, we find that the stability of the $\underline{UUCG}$ tetraloop is virtually independent of the hairpin stem composition (DD, RR, $\underline{RR}$, etc.), whereas the native RNA tetraloop exerts extra stability only when the stem is duplex RNA (R:R). As a result, the relative stabilities of hairpins with a 2′,5′-linked tetraloop, e.g. ggac($\underline{UUCG}$)gtcc (T_m = 61.4 °C), are often superior to those with RNA tetraloops, e.g. ggac(UUCG)gtcc (T_m = 54.6 °C). In fact, it has been possible to observe the formation of a 2′,5′-RNA:DNA hybrid duplex by linking the hybrid's strands to a ($\underline{UUCG}$) loop. These duplexes ($\underline{R}$D), which are not stable enough to form in an intermolecular complex [Wasner et al. *Biochemistry* **1998**, *37*, 7478−7486], were stable at room temperature ($T_m \sim 50$ °C). Thus, 2′,5′-loops have potentially important implications in the study of nucleic acid complexes where structural data are not yet available. Furthermore, they may be particularly useful as structural motifs for synthetic ribozymes and nucleic acid "aptamers".

Introduction

RNA hairpins are prominent structural motifs that are associated with a wide variety of biological functions. They are of immense importance for recognition of RNA by proteins[2] as well as for folding[3] and stabilizing RNA in vivo.[4] Hairpins having the tetranucleotide loop sequence UUCG commonly occur in ribosomal RNA.[5] Tuerk et al.[3] found that messenger RNAs with the sequence 5′...C(UUCG)...3′ prevented reverse transcriptase from reading through, and that hairpin loops with this particular sequence are significantly more stable relative to those with homopolymeric tetraloops. Pioneering studies by Tinoco and co-workers revealed that the tetraloop is stabilized by extended base stacking, a reverse wobble U_5(anti):G_8(syn) base pair, and an electrostatic interaction between C_7 and the phosphate group linking U_5 and U_6 (Figure 1).[6] The NMR structure revealed that the U_6 residue is not involved in any specific contact with other bases in the loop, and protrudes out into the solvent. These observations are consistent with chemical modification experiments in that the U_6 residue could be mutated to any base without loss of stability.[7] Furthermore, mutation of C_7 to a uracil residue destabilized the hairpin by 1.5 kcal/mol,[7] consistent with the proposed specific C_7-phosphate contact.

Slight perturbations in the sugar−phosphate backbone have a profound effect on hairpin stability. For example, Sakata[8] and Tinoco[7,9] demonstrated that stability drops substantially when the loop riboses are replaced by 2′-deoxyriboses. Thus, 5′-UGAGC(UUCG)GCUC-3′ forms a more stable folded structure compared to 5′-UGAGC(uucg)GCUC-3′, 5′-ggac(ttcg)gtcc-3′ or 5′-ggac(uucg)gucc-3′.[7−9] Although there is general agreement that the loop 2′-hydroxyl groups impart stability, the molecular basis for this stabilization remains unclear. Molecular dynamics studies[10] revealed favorable electrostatic interactions between

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(1) Abbreviations: DNA, 3′,5′-linked deoxyribonucleic acid (represented by small letters); RNA, 3′,5′-linked ribonucleic acid (represented by capital letters); 2′,5′-RNA, 2′,5′-linked ribonucleic acid (represented by capital underlined letters); gu = $dG_{3'p5'}dU_{3'p}$; GU = $rG_{3'p5'}rU_{3'p}$; $\underline{GU}$ = $rG_{2'p5'}rU_{2'p}$; $\underline{UUCG}$ = $U_{2'p5'}U_{2'p5'}C_{2'p5'}G_{2'p5'}$; T_m, melting temperature in degrees Celsius; CD, circular dichroism; eu, entropy units in cal K^{-1} mol^{-1}; % H, percentage hypochromicity.

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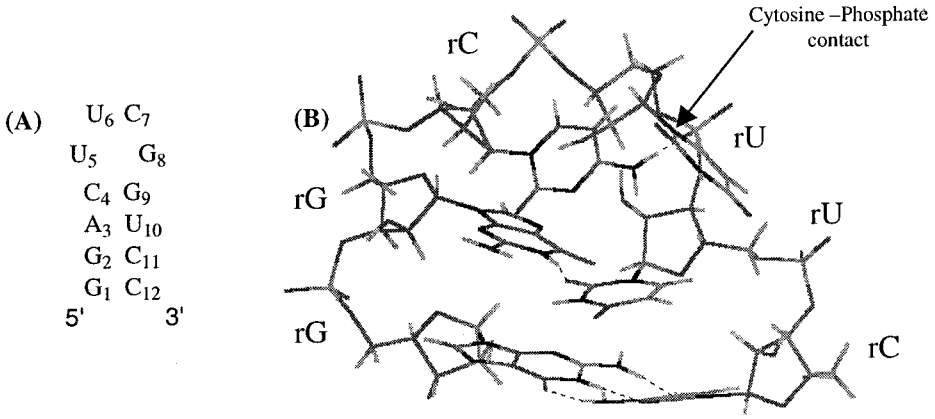

Figure 1. (A) Base sequence of hairpin structure studied consisting of (1) the loop region formed by U_5-G_8 residues and (2) the hairpin stem defined by G_1-C_4 and G_9-C_{12} residues. (B) Energy minimized structure of the 3′,5′-linked r(UpUpCpGp) tetra loop from NMR data.[6c]

the 2′-hydroxyl group and O3′, which would rigidify both the stem and loop regions; this "pre-organization" would make the riboses more compact and hence more stable than deoxyribose-based loops. Specific tertiary interactions involving 2′-hydroxyl groups have also been proposed.[11]

Very little is known about structural RNA motifs containing 2′,5′-linked internucleotide linkages. In fact, the hybridization properties of single-stranded 2′,5′-DNA[12] and 2′,5′-RNA[13] are only beginning to be explored. These studies have shown that both single and multiple 2′,5′-phosphodiester substitutions within RNA and DNA helices result in lower melting temperatures of duplexes. In addition, RNA:2′,5′-RNA hybrids and pure 2′,5′-RNA duplexes have lower melting temperatures than RNA duplexes, whereas DNA:2′,5′-RNA hybrids are too unstable to be observed. Braich and Damha recently showed that branched pentaloops of the type rA*-[d(CC)]$_2$ (where rA* is joined to both d(CC) units via *vicinal* 2′,5′ and 3′,5′-phosphodiester linkages) induce the formation of DNA hairpins that are slightly thermally more stable than those with the native d(CCCC) loop.[14] The domains I, II, and III within a hammerhead ribozyme have been replaced by 2′,5′-linkages without impairing catalytic activity.[15] To our knowledge, contiguous 2′,5′-substitutions and the impact that these have on the structure and thermodynamic stability of RNA tetraloops have not been characterized.

In this study we explore the effects of multiple 2′,5′-internucleotide substitutions in the loop region of RNA hairpins.[16] Specifically, we wished to learn if 2′,5′-linked RNA ("R"), like the native RNA ("R"), can adopt unusually stable tetraloop structures, and if so, whether the same sequences are extra stable. Remarkably, we find that 2′,5′-linked RNA (UUCG) tetraloops show a pattern of relative stability similar to that observed for the native RNA hairpins with identical base sequence. We also find that while the stability of both loops is highly dependent on the nature of the C_4:G_9 loop-closing base pair and how they stack in the overall context of the stem, the 2′,5′-loop analogue (UUCG) remains extra stable with various stem hybrids whether the stem is either RD, RR or RR. These observations not only advance our knowledge on the molecular origin of tetraloop stabilization, but also have implications for the use of 2′,5′-RNA as novel structural motifs (e.g. RNA-based "aptamers").

Experimental Section

Materials. 5′-*O*-Dimethoxytrityl-2′-*O*-*tert*-butyldimethylsilylribonucleoside-3′-*O*-(2-cyanoethyl) *N*,*N*′-diisopropyl phosphoramidite monomers for normal RNA synthesis were purchased from Dalton Chemical Laboratories (Ontario, Canada). The corresponding ribonucleoside 2′-*O*-phosphoramidites (2′,5′-RNA synthesis) were obtained from ChemGenes Corp. (Watham, MA). These reagents were stored at −20 °C and dried in vacuo (under P_2O_5) for 24 h prior to use.

Oligoribonucleotide Synthesis. Hairpins were synthesized on an Applied Biosystems (381A) synthesizer following slight modifications of our published procedures.[17] Monomer coupling times were 10 min (RNA or 2′,5′-RNA) and 2 min (DNA). Extended coupling times were used for riboG 2′- or 3′-O-phosphoramidite (15 min) and dG monomers (3 min). Concentrations of monomers were 0.15−0.17 M (RNA) and 0.1 M (DNA). The activator solutions consisted of 0.1 M 1*H*-tetrazole in acetonitrile for 3′,5′-RNA synthesis, and 0.65 M 5-ethylthio-1*H*-tetrazole/acetonitrile for 2′,5′-RNA assembly.[18,19] Assembly of sequences was also carried out on an Expedite 8909 PE synthesizer with standard DNA/RNA protocols supplied by the manufacturer (PerSeptive Biosystems, Inc). Except for the phosphoramidite solutions (RNA monomers, 51−58 mM; DNA monomers, 56−66 mM), the same reagent concentrations were used as those employed for the 381A synthesizer. The sequences were purified on denaturing polyacrylamide gels (24%, 7 M urea), and desalted by gel filtration on a Sephadex G-25 column.[17] The purity of all sequences was assessed by using 24% analytical gels and the structures were confirmed by MALDI-TOF mass spectrometry.

UV Melting Studies. UV thermal denaturation curves were acquired on a Varian CARY 1 UV−Vis spectrophotometer equipped with a multicell holder and a Peltier temperature controller. All thermal measurements were conducted in 0.01 M Na_2HPO_4, 0.1 mM Na_2EDTA buffer, at pH 7.00 ± 0.02. Absorbance versus temperature spectra were

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Table 1. Thermodynamic Parameters[a] of Hairpins with RNA Stems

code	5′-hairpin-2′/3′ [b]	T_m[c] (°C)	% H	$\Delta H°$ (kcal/mol)	$\Delta S°$ (eu)	$\Delta G°_{37}$ (kcal/mol)
RRR	GGAC(UUCG)GUCC	71.8	8.5	−53.4	−154.8	−5.4
RR̲R	GGAC(U̲U̲C̲G)GUCC	69.3	9.6	−55.6	−162.1	−5.3
RD̲R	GGAC(uucg)GUCC	63.4	7.3	−47.3	−140.1	−3.7
RR^{1}R	GGAC(U**A**CG)GUCC	62.3	8.1	−45.5	−135.6	−3.4
RR̲^{2}R	GGAC(U̲U̲**U**G)GUCC	66.9	8.6	−47.3	−139.2	−4.2
RR̲^{3}R	GGAC(U̲U̲**U**U̲)GUCC	60.5	7.3	−46.7	−139.9	−3.3
RR$_U$R	GGAC(U̲UCG)GUCC	60.2	7.3	−52.0	−155.8	−3.7
R$_C$R̲R	GGAC̲(UUCG)GUCC	62.6	8.2	−52.1	−155.0	−4.0
RRR$_G$	GGAC(UUCG)G̲UCC	58.0	6.4	−45.9	−138.7	−2.9
R$_C$RR	GGAC̲(U̲U̲C̲G)GUCC	61.5	8.2	−47.2	−141.0	−3.5

[a] Measurements were made in 0.01 M Na_2HPO_4, 0.1 mM Na_2EDTA buffer, at pH 7.00 ± 0.02; oligonucleotide concentration ~4.5 μM. Values represent the average of at least five independent measurements. Errors in thermodynamic parameters are within ±7.5% for $\Delta H°$ and $\Delta S°$, and ±0.20 kcal/mol for $\Delta G°_{37}$. For a more accurate calculation, $\Delta G°_{37}$ was determined from $\Delta H°$ and $\Delta S°$ before rounding off. [b] Capital letters represent RNA residues; underlined letters are 2′,5′-RNA residues (e.g. U̲C̲ = $U_{2'p5'}C_{2'p}$); DNA residues are represented by small letters; bold letters are base mutations in the loop. [c] The melting curves show a single cooperative and completely reversible transition that is independent of oligonucleotide concentration over at least a 30-fold range

collected at 260 nm over a range from 5 to 90 °C with 0.1 °C increments and a heating rate of 0.5 °C/min. Samples (~4.5 μM) were annealed by heating rapidly to 95 °C for 10−15 min, followed by cooling slowly to room temperature, and cooling (5 °C) overnight. Before data acquisition, oligonucleotides were degassed in an ultrasound bath for 3 min and consequently equilibrated at 5 °C for at least 10 min prior to the melting run. Single-strand molar extinction coefficients were calculated from those of mononucleotides and dinucleotides using the nearest-neighbor approximation method.[20] 2′,5′-RNA and RNA:2′,5′-RNA chimeras were assumed to have the same molar extinction coefficient as RNA. Single-strand concentration was determined from UV absorbance at high temperature. Percentage hypochromicity (%H) was calculated from UV absorbances of the hairpin and fully denatured species by using the following equation: $\% H = (A_f - A_0)/A_f$.

Thermodynamic Calculations and Data Analysis. Melting temperatures and thermodynamic parameters for hairpin formation were computed with Cary WinUV version 2 software (Varian Ltd.). Absorbance versus temperature profiles were fit to a two-state (all-or-none) model and the software was adapted for a unimolecular transition. Sloping baselines was achieved by constructing linear least-squares lines for associated and dissociated parts and extrapolating to both ends of the melting curve. Consequently, a plot of the fraction of single strands in the hairpin state (α) versus temperature was constructed and used to calculate the T_m value by interpolating to $\alpha = 0.5$. The transition equilibrium constant, K, was calculated at various temperatures by using the equation:

$$K = \alpha/(1 - \alpha) \quad (1)$$

A van't Hoff plot of ln K versus $1/T$ was generated, and the slope and intercept of the calculated line yielded the enthalpy ($\Delta H°$) and entropy ($\Delta S°$) of hairpin formation, respectively, from which Gibbs free energy at 37 °C ($\Delta G°_{37}$) was calculated according to the equation:

$$\ln K = -[\Delta H°/R]/T + \Delta S°/R \quad (2)$$

where R is the gas constant and T the temperature in Kelvin. The data obtained represent the average of at least five independent measurements for each hairpin. To ensure that only hairpin species formed in solution, the samples were melted over at least 30-fold nucleic acid concentration with no detectable change in melting temperature, thus confirming an *intramolecular* transition.

The calculated error limits in the thermodynamic data represent standard deviations with all values weighted equally.[21] The variations in T_m values for all hairpin structures are within ±1 °C. The variations in $\Delta H°$ and $\Delta S°$ are within ±7.5% while those for $\Delta G°_{37}$ are within ±0.2 kcal/mol.

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Circular Dichroism (CD) Spectroscopy. CD spectra were measured on a JASCO J710 spectropolarimeter at ambient temperature (unless otherwise indicated) as previously described.[13d] Each spectrum represented the average of five 220−340 nm scans obtained at a rate of 0.5 nm/min (bandwidth, 1 nm; sampling wavelength, 0.2 nm). The buffer used was 0.01 M Na_2HPO_4, 0.1 mM Na_2EDTA, at pH 7.00 ± 0.02. The data were processed on a PC computer with Windows based software supplied by the manufacturer (JASCO, Inc.). The obtained CD spectra were normalized by subtraction of the background scan with buffer.

Results and Discussion

The compounds studied in this work are hairpins containing the conserved 5′ (UUCG) 3′ loop sequence (Figure 1A). Synthesis and characterization of all oligomers described in this work were carried out following slight modifications of our published procedures.[13d,17] Thermal dissociation data are also summarized in Tables 1, 2, and 3, along with comparative data for unmodified (all-DNA and all-RNA) hairpin sequences. Hairpins RRR, RDR, and RR̲R (where "R̲" represents a 2′,5′-linked RNA segment) were designed to test the effect of the tetraloop structure on the stability of RNA hairpins. Similarly, compounds DRD, DR̲D, and DDD were designed to assess the stabilizing effect of the various tetraloops on duplex DNA. Moreover, comparison of all-DNA, all-RNA, and all-2′,5′-RNA hairpins provides a direct means to compare the properties of 2′,5′-RNA with the more common nucleic acids.

RNA Hairpins: R versus R̲ Loops. The key properties of hairpins containing 2′,5′-RNA tetraloops are brought out in Figures 2 and 3 and Table 1. The melting curves show a single cooperative transition (T_m) that is independent of oligonucleotide concentration over at least a 30-fold range (0.01 M Na_2HPO_4, 0.1 mM Na_2EDTA, pH 7.0) (Figure 2B). This result supports the view that these oligonucleotides form hydrogen-bonded, base-stacked structures reversibly in solution by folding *intramolecularly* to a hairpin conformation.

The top section of Tables 1 and 2 shows the T_m and thermodynamic parameters of RNA and DNA hairpins differing only in the loop composition. Their base sequence is identical with that reported by Tinoco and co-workers,[7] thus providing a good model system with which the thermodynamic effects can readily be obtained. The calculated $\Delta G°_{37}$ and T_m values we measured for the C(UUCG)G hairpin [i.e., RRR] are very similar to what has been obtained by Tinoco. Its melting temperature is ca. 20 °C higher than a hairpin of purportedly normal thermodynamic stability, such as the hairpin with the C(UUUU)G loop.[7] Table 1 also shows that RDR had intermediate

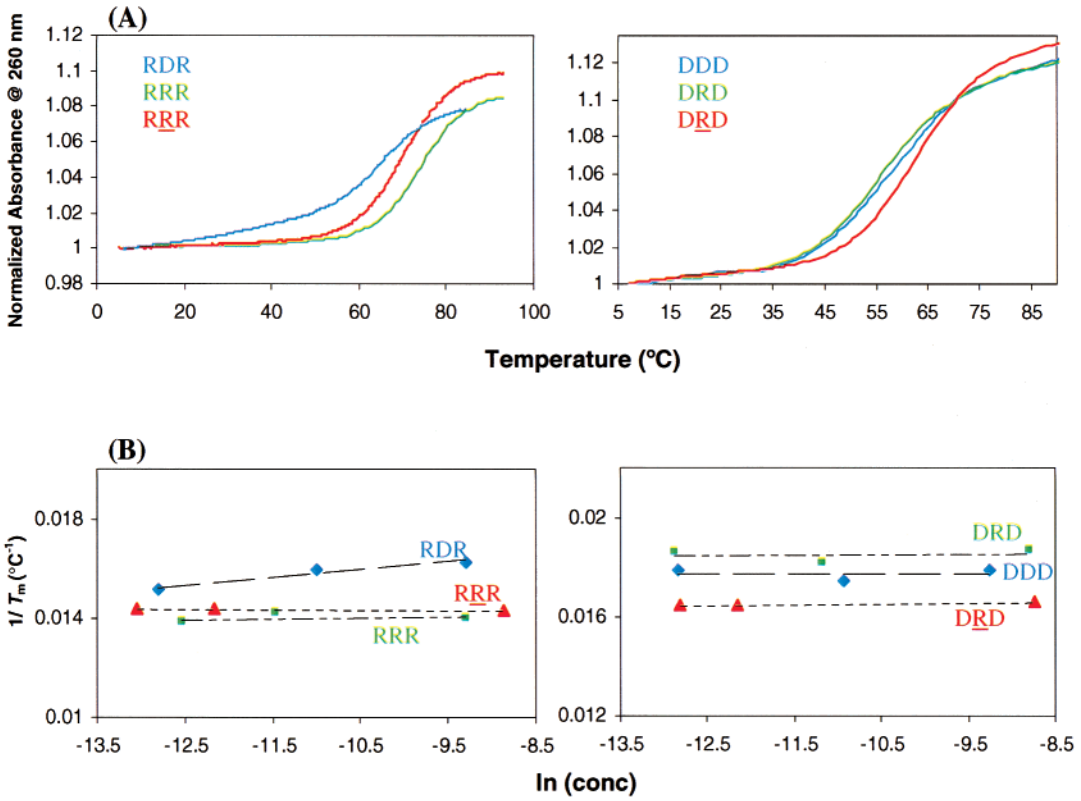

Figure 2. (A) Normalized melting temperature profiles of representative hairpin loops in 0.01 M Na_2HPO_4, 0.1 mM Na_2EDTA buffer, pH 7.00 ± 0.02, and an oligonucleotide concentration ~4.5 μM. (B) Van't Hoff plots of representative hairpins showing concentration independence over a 50-fold range.

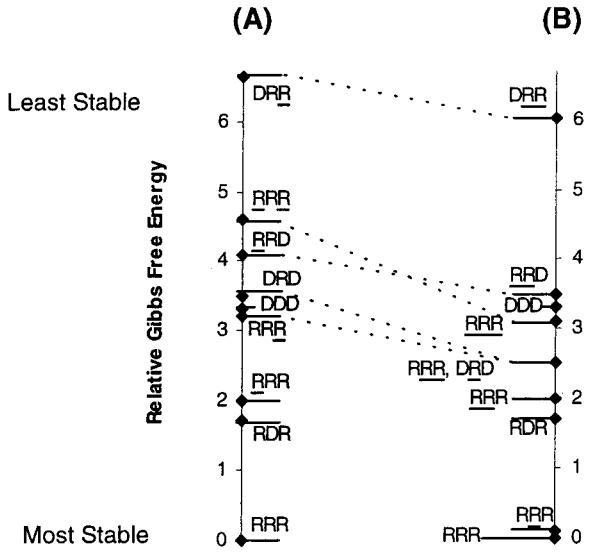

Figure 3. Relative Gibbs free energies of formation ($\Delta\Delta G°_{37}$) in kcal/mol for hairpins with different stem combinations but with (A) 3′,5′-RNA loops versus (B) 2′,5′-RNA loops. The control hairpin sequences (DDD, RDR, and RRR) are also shown for comparison purposes. The dashed lines represent the drop in Gibbs free energy upon substitution of 2′,5′-RNA in the loop for 3′,5′-RNA. All values are referenced relative to the RRR hairpin exhibiting the lowest (most negative) free energy value.

stability whereas RRR [i.e., C(UUCG)G loop] is unusually stable. To determine if the apparent stabilization imparted by the 2′,5′-tetraloop was dependent on loop sequence, we synthesized hairpins containing mutations within the loop. We found that the hairpin with the C(UUCG)G loop (RRR; T_m = 69.3 °C) was more stable than the RR^{2}R hairpin with the C(UU**U**G)G loop (T_m = 66.9 °C), which in turn was significantly more stable than the RR^{3}R hairpin with the C(UU**U**U)G loop (T_m = 60.5 °C). This parallels perfectly well the effects observed by Tinoco for the native RNA system,[7] and raises the interesting possibility that the 2′,5′-loop bears global structural similarity to the native 3′,5′-loop. As shown in Figure 4B, the negative CD band at ca. 220 nm was reduced for the RRR hairpin in comparison to the same band in the spectra of RRR; however, the overall CD spectrum of RRR is very similar to that of RRR, indicating that they share conformational features. However remarkably, the somewhat large decrease in T_m on changing the loop from C(UUCG)G to C(U**A**CG)G is not in agreement with this notion (RRR *versus* RR^{1}R; $\Delta\Delta G°_{37}$ = 1.9 kcal/mol), since according to the native loop structure, the second U in the loop is not involved in any interaction and can be mutated to any base without loss of hairpin stability.[7] These results indicate that the second U residue in the 2′,5′-RNA loop contributes significantly to the *intramolecular* loop stabilizing interactions and yet the 2′,5′-RNA loop still retains a high degree of stability compared to 3′,5′-RNA loops. Clearly, high-field NMR analysis and/or crystallographic work on an RRR hairpin will be required to elucidate the finer structural features of 2′,5′-loops that are responsible for the apparent anomalous behavior with this system.

To further assess the effect of 2′,5′-substitutions on loop stability, we also prepared hairpins containing one 2′,5′-linkage at, or near, the loop region, namely C(UUCG)G, C(UUCG)G, and C(UUCG)G (Table 1, section 3). The T_m data for the ...C(UUCG)G... hairpin (RR$_U$R; T_m = 60.2 °C) indicate that a major disturbance of the native loop structure occurs as a result of a single modification (i.e., U/G wobble pairing is disrupted). Also, we noted a 7−12 °C drop in T_m and a 1.4−2.5 kcal/mol increase in free energy at 37 °C when the 2′,5′-phosphodiester

Table 2. Thermodynamic Parameters[a] of Hairpins with DNA Stems

code	5′-hairpin-2′/3′ [b]	T_m (°C)	% H	$\Delta H°$ (kcal/mol)	$\Delta S°$ (eu)	$\Delta G°_{37}$ (kcal/mol)
DTD	ggac(tttt)gtcc	54.7	9.6	−37.9	−115.7	−2.0
DUD	ggac(UUUU)gtcc	52.8	7.1	−38.7	−118.8	−1.9
DDD	ggac(uucg)gtcc	56.2	11.3	−36.6	−111.1	−2.1
DRD	ggac(UUCG)gtcc	54.6	11.5	−36.0	−109.8	−1.9
D$\underline{R}$D	ggac($\underline{UUCG}$)gtcc	61.4	12.6	−39.9	−119.4	−2.9
DR^{1}D	ggac(U**A**CG)gtcc	56.7	10.7	−37.4	−113.5	−2.2
DR^{2}D	ggac(UU**U**G)gtcc	62.0	9.8	−42.6	−127.0	−3.2
DR^{3}D	ggac(UU**UU**)gtcc	54.5	9.7	−40.7	−124.2	−2.2

[a] See footnotes *a* and *c* from Table 1. [b] Capital letters represent RNA residues; underlined letters are 2′,5′-RNA residues (e.g. $\underline{UC}$ = $U_{2'p5'}C_{2'p}$); DNA residues are represented by small letters; bold letters are base mutations in the loop.

Table 3. Thermodynamic Parameters[a] of Hairpins with DNA:RNA, DNA:2′,5′-RNA, RNA:2′,5′-RNA, and 2′,5′-RNA:2′,5′-RNA Stem Hybrids

code	5′-hairpin-2′/3′ [b]	T_m (°C)	% H	$\Delta H°$ (kcal/mol)	$\Delta S°$ (eu)	$\Delta G°_{37}$ (kcal/mol)
DRR	ggac(UUCG)GUCC	56.5	7.4	−41.1	−124.7	−2.4
D$\underline{R}$R	ggac($\underline{UUCG}$)GUCC	56.7	8.0	−41.0	−124.4	−2.5
R$\underline{R}$D	$\underline{GGAC}$($\underline{UUCG}$)gtcc	48.1	3.1	−38.9	−121.1	−1.4
$\underline{R}$RD	$\underline{GGAC}$($\underline{UUCG}$)gtcc	52.8	4.6	−39.5	−121.3	−1.9
$\underline{DR}$R	ggac($\underline{UUCG}$)GUCC	24.1	5.8	−28.7	−96.6	+1.2 (−0.2)[c]
DR$\underline{R}$	ggac(UUCG)$\underline{GUCC}$	30.2	7.0	30.7	−101.1	+0.6 (−0.8)[c]
R$\underline{R}$R	$\underline{GGAC}$($\underline{UUCG}$)$\underline{GUCC}$	62.6	5.3	−44.2	−131.8	−3.4
$\underline{R}$RR	$\underline{GGAC}$($\underline{UUCG}$)GUCC	62.4	4.0	−44.7	−133.2	−3.4
$\underline{RR}$R	GGAC($\underline{UUCG}$)GUCC	54.1	7.1	−42.5	−122.2	−2.2
RR$\underline{R}$	GGAC($\underline{UUCG}$)$\underline{GUCC}$	58.1	7.4	−45.5	−137.2	−2.9
R$\underline{R}$R	$\underline{GGAC}$($\underline{UUCG}$)$\underline{GUCC}$	45.2[d]	3.9	−30.8	−98.2	−0.8
$\underline{RR}$R	$\underline{GGAC}$($\underline{UUCG}$)$\underline{GUCC}$	54.8	2.2	−42.1	−128.5	−2.3
TRT	tttt(UUCG)tttt	-[e]	-	-	-	-

[a] See footnotes *a* and *c* from Table 1. [b] See footnote *b* from Table 2. [c] Values in parentheses represent $\Delta G°$ calculated at 22 °C. [d] Very broad transition. [e] No melting transition observed

linkage is placed outside the tetraloop sequence [i.e., $\underline{C}$-(UUCG)G and C(UUCG)$\underline{G}$ *versus* C(UUCG)*G*]. In these cases, differences in stacking of the loop-closing base pair may be responsible for the drop in stability caused by introducing the 2′,5′-linkage. No destabilization is observed for C($\underline{UUCG}$)G when all 2′,5′-linkages are placed *within* the loop region.

Finally, we assessed the stability of a hairpin containing five consecutive 2′,5′-internucleotide linkages in the loop region, i.e., 5′-GGA$\underline{C}$($\underline{UUCG}$)GUCC-3′. The large decrease in T_m on adding an extra 2′,5′-linkage near the loop indicates a major disturbance of the overall structure.

D$\underline{R}$D *versus* DRD and DDD Hairpins. The stabilization conferred by 2′,5′-loops is not limited to hairpins containing RNA stems. Table 2 shows that the T_m value for the helix-to-coil transition is greater for D$\underline{R}$D than for DDD. A more significant observation is that D$\underline{R}$D is more stable than the DRD regioisomer by ca. 1.0 kcal/mol (ΔT_m = 6.8 °C). In fact, the T_m value of ggac(UUCG)gtcc (DRD) is virtually the same as that of ggac(UUUU)gtcc, ggac(uucg)gtcc, and ggac(tttt)gtcc, and thus we conclude that this particular hairpin (DRD) is not extra stable.

Interestingly, the loop of D$\underline{R}$D displays sequence dependence in a manner that is different from that seen for R$\underline{R}$R. Changing the loop from ggac($\underline{UUCG}$)gtcc to ggac($\underline{UU}$**U**$\underline{G}$)gtcc does not affect hairpin stability. This suggests that the cytosine loop residue in D$\underline{R}$D does not assume the same role as that seen in the (UUCG) and ($\underline{UUCG}$) loops of RRR and R$\underline{R}$R and is not involved in any stabilizing interaction. However, mutation of guanine G_8 to a uracil (i.e., D$\underline{R}^3$D) destabilized the hairpin by ca. 1.0 kcal/mol, which can be ascribed to the loss of wobble base pairing and/or base stacking. Similarly, hairpin ggac-($\underline{UUCG}$)gtcc is destabilized upon changing the second loop residue to ggac(U**A**CG)gtcc by ca. 0.7 kcal/mol and parallels the trend seen for R$\underline{R}$R versus R$\underline{R}^1$R. These observations evidently show that U_6 and G_8 residues participate in the loop stabilizing interactions but also indicate that the overall conformation of the 2′,5′-loop in the hairpin with DNA stem is different than that in the hairpin with RNA stem ($\underline{R}$ in D$\underline{R}$D versus R$\underline{R}$R).

R and $\underline{R}$ Loops in Hairpins with DD and RR Stems. It is worth noting that while the 3′,5′-RNA loop behaves the same as the DNA loop in the hairpin with DD stem, the 2′,5′-RNA loop exerts remarkable stabilization (DRD and DDD *versus* D$\underline{R}$D). On the other hand, both UUCG and $\underline{UUCG}$ show the same degree of stability in the RR stem hairpin (RRR versus R$\underline{R}$R). This suggests that the overall hairpin stability is determined by an interplay of stem and loop structures as well as the degree of interdependence exhibited by the nucleobases upon stacking in these different contexts. The stem has an important role in modulating the loop structure and determining the influence of the loop on the overall hairpin stability. On the other hand, the structure of the loop stabilizes the loop-closing base pairs next to it leading to an overall stabilization in the stem and the hairpin structure.

Hairpins with Various Stem Hybrids: R *versus* $\underline{R}$ Loops. Hairpins with the stems DR, $\underline{R}$D, R$\underline{R}$, $\underline{RR}$ and containing UUCG and $\underline{UUCG}$ tetraloops were also synthesized (Table 3). Interestingly, the degree of stabilization provided by 2′,5′-RNA tetraloops is strongly dependent on the sugar composition of the helical stem. For example, stabilization by 2′,5′-RNA tetraloops is largest for the least stable stems, i.e. $\underline{RR}$ and $\underline{R}$D, but almost negligible for the most stable stems (RR and RD). This is evident from Figure 3, which compares the relative Gibbs free energy of association of hairpins ($\Delta\Delta G°_{37}$). Hence, dashed lines with a negative slope represent cases where stabilization of a 2′,5′-tetraloop is greater relative to a 3′,5′-linked tetraloop. It is clear that $\underline{UUCG}$ stabilizes a hairpin with either $\underline{R}$D or $\underline{RR}$ stems better than UUCG. For example, replacing the 3′,5′-

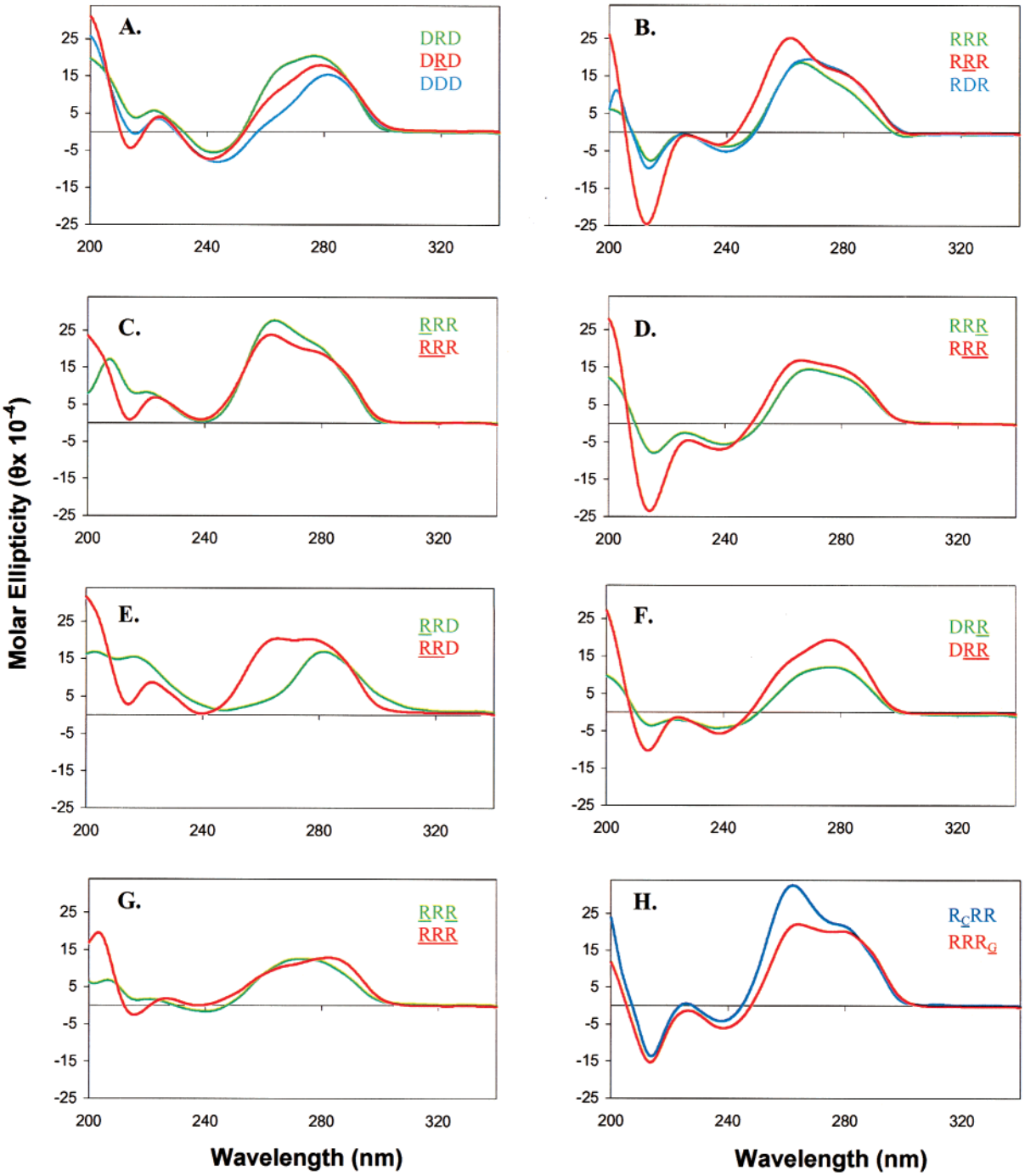

Figure 4. Circular dichroism spectra at 22 °C of DNA and RNA hairpins of various loop compositions: (1) 2′,5′-r(UpUpCpGp) loops; (2) 3′,5′-r(UpUpCpGp) loops; and (3) d(TTCG) loops. The CD spectra of DRR and DRR were recorded at 5 °C. All measurements were done in 0.01 M Na_2HPO_4, 0.1 mM Na_2EDTA buffer, at pH 7.00 ± 0.02. Molar ellipticities were normalized to strand concentration.

internucleotide linkages in RRR with 2′,5′-linkages (to obtain RRR) leads to a gain of 10 °C in melting temperature, or a $\Delta\Delta G°_{37}$ of −1.5 kcal/mol in free energy. Another example is the case of RRR, which is more thermodynamically stable than RRR ($\Delta\Delta G°_{37} = -0.7$ kcal/mol).

Substitution of 2′,5′-RNA in the loop regions of various hairpins does not seem to have any significant effect on the conformational freedom change ($\Delta S°$) associated with hairpin formation, suggesting that the stabilization brought about by 2′,5′-RNA loops is mainly a consequence of a more favorable enthalpy change ($\Delta H°$) of association.

Formation of 2′,5′-RNA:DNA Hybrids. We have observed for the first time the formation of a stable 2′,5′-RNA:DNA hybrid by linking the hybrid's strands to the UUCG loop (Table 3; RRD). RD duplexes, which are not stable enough to form in an intermolecular complex,[13d] were stable at room temperature (T_m = 52.8 °C; $\Delta G°_{37} = -1.9$ kcal/mol). The CD spectrum shows their structure is a mixture of both the pure RNA and DNA forms,[22] with a greater similarity to the B-type family (Figure 4E).

Effects on the Global Conformation/Structure of Hairpins. The CD spectra show that incorporation of 2′,5′-RNA in the loop region of hairpins with various stems does not perturb significantly the overall structure. However, there is a notable

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increase in the negative CD band at ~212 nm, and the long wavelength CD peak becomes a mixture of both pure RNA and pure DNA peaks (Figure 4). The increase in the depth of the CD peak at ~212 nm introduces A-like character in this region of the CD spectrum. For instance, DRD shows a B-form global conformation (Figure 4A) but with an increase in the short wavelength negative CD peak compared to DDD and DRD, and it shows a positive CD band at ca. 279 nm that is centered between that of DRD (ca. 277 nm) and DDD (ca. 282 nm). In a similar fashion, the CD spectrum of RRR (Figure 4B) shows a similarity to the classic A-form global conformation, but exhibits a substantial increase in the negative CD peak at ca. 213 nm relative to that of the wild-type RRR hairpin, and its 262 nm CD peak is a mixture of both pure A- and B-form bands with more A-character.

Comparison of all-DNA, all-RNA, and all-2′,5′-RNA Hairpins. The thermodynamic stability of RRR falls within the same order of magnitude as that of DDD, with RRR being the most stable (RRR > RRR ~ DDD). The all-2′,5′-RNA hairpin exhibits an entropy of formation that is intermediate between that of RRR and DDD. The hypochromicity data suggest that the DNA hairpin exhibits the best stacking interactions followed by the all-RNA hairpin and last by the all-2′,5′-RNA hairpin.

The global conformations of RRR and DDD exhibit characteristic A-form and B-form features,[22] respectively. The fully modified 2′,5′-RNA hairpin (RRR) does not fall into either a pure A-form or B-form pattern. The negative CD peak at ca. 216 nm resembles that of pure RNA, while the long wavelength positive CD band is a mixture of both DNA and RNA peaks with more B-like character. Based on the above, we conclude that the fully modified regioisomeric 2′,5′-RNA hairpin does not behave like the 3′,5′-RNA counterpart.

In conclusion, we have shown that the regioisomeric 2′,5′-linked RNA tetraloop UUCG, like the "natural" 3′,5′-RNA counterpart, can form superstable hairpin structures of comparable thermodynamic stabilities. Not only that, UUCG is virtually less dependent on stem composition and thus exerts higher stability in hairpins with certain stem constructs such as DNA:DNA, 2′,5′-RNA:2′,5′-RNA, DNA:2′,5′-RNA, and RNA:2′,5′-RNA compared to UUCG. The UUCG loop exhibits the same order of stability as UUCG in hairpins with RNA:RNA and DNA:RNA stems. The positive effects of 2′,5′-linkages on hairpin formation may be important for the design of novel nucleic acid enzymes[15] as well as antisense agents. These findings also support the hypothesis that 2′,5′-RNA (or RNA with both 2′,5′- and 3′,5′-linkages) may have been accessible during early RNA evolution.[23] High-resolution NMR in combination with molecular modeling experiments are in progress to resolve the unique structure of the 2′,5′-linked tetraloops.

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Supporting Information Available: A table of calculated extinction coefficients and mass spectral data (MALDI-TOF), and representative analytical denaturing gels (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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