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Thermal Stability of RNA Hairpins Containing a Four-Membered Loop and a Bulge Nucleotide†

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ABSTRACT: Fourteen RNA hairpins containing a four-membered loop and a bulge nucleotide were synthesized and their thermal stabilities determined. The combined contribution of a four-membered loop and bulge A to the free energy of a hairpin is calculated to be 9.3 kcal/mol at 37 °C and successfully predicts the stability of an independent RNA hairpin. The introduction of a bulge nucleotide to the helical stem of an RNA hairpin destabilizes the molecule in a sequence-dependent manner. The individual thermodynamic contributions of a four-membered loop and bulge A, G, and U residues to the stability of an RNA hairpin loop are presented.

Since they were first published, the thermodynamic parameters for RNA secondary structure stability (Tinoco et al., 1971, 1973) have been improved and expanded. The contributions of RNA secondary structure features such as nearest-neighbor stacking interactions, nucleotide mismatches, and terminal unpaired nucleotides to the stability of RNA have now been well characterized (Hickey & Turner, 1985; Sugimoto et al., 1986; Freier et al., 1986) and can successfully predict the stability of a number of RNA duplexes.

Bulge nucleotides and hairpins are also common elements of RNA secondary structure. For example, bulge nucleotides appear in 12 locations in the current *Escherichia coli* 16S RNA secondary structure (Gutell et al., 1985). The same molecule contains 31 hairpins ranging in size from 3 to 11 nucleotides. Both of these structural features have been associated with the binding of proteins (Peattie et al., 1981; Mougel et al., 1986). It is quite likely that each individual hairpin and bulge has a defined conformation. Nucleotides in a loop can form defined structures as a result of stacking interactions and intraloop hydrogen bonds as seen in the anticodon loop of yeast $tRNA^{Phe}$ (Quigley & Rich, 1976). Bulge nucleotides can assume intercalated (Patel et al., 1982) and extrahelical (Morden et al., 1983) states or even an equilibrium between the two. Since the contribution of loops and bulges to RNA stability may be very sequence and neighbor dependent, it is important to determine if these structures can even be considered independent thermodynamic entities for the purpose of predicting RNA secondary structure stability.

Early attempts to characterize the stability of RNA hairpins were hampered by limitations in synthetic capabilities such that only a few simple sequences could be studied (Uhlenbeck et al., 1973; Gralla & Crothers, 1973). Although RNA hairpins have been purified from larger RNA molecules (Coutts, 1971; Gralla et al., 1974; Baan et al., 1977), their isolation depends upon the fortuitous cleavage properties of each RNA source and thus limits the range of sequences available for study. Thus, the current thermodynamic parameters for RNA hairpin loop and bulge nucleotides (Tinoco et al., 1973; Freier et al., 1986) do not reliably represent RNA sequences and structures that occur naturally.

Recent advances in the enzymatic synthesis of RNA make possible the synthesis of virtually any RNA for physical studies (Lowary et al., 1986; Milligan et al., 1987). In this work, we have synthesized 10 RNA hairpins that share the same 4-membered loop sequence and have a bulge A residue at the same nucleotide position. The helix–coil transition of these molecules was analyzed to determine the combined contribution of the four-membered loop and bulge A to the stability of a hairpin. Three additional hairpins were studied to de-

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termine the individual thermodynamic contribution of a four-membered loop and bulge A, G, and U structures. Finally, the stability of one more hairpin was determined and its value compared to that predicted by the parameters derived in this work.

Materials and Methods

Oligonucleotide Synthesis. DNA promoter and template strands were chemically synthesized on an Applied Biosystems 380B DNA synthesizer (Caruthers, 1985). The DNA was deprotected and gel purified for use in RNA transcription reactions.

Fourteen RNA fragments (Figure 1) were transcribed from synthetic DNA templates using T7 RNA polymerase either by the method of Lowary et al. (1986) or by the improved method of Milligan et al. (1987). A typical 10-mL transcription reaction contained 50 nM DNA template, 1 mM each ribonucleoside triphosphate, 1 mM spermidine, 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.01% (v/v) Triton X-100, 80 mg/mL poly(ethylene glycol) (M_r 8000), 40 mM Tris-HCl (pH 8.1 at 37 °C), and 20 μg/mL T7 RNA polymerase. After incubation for 4 h at 37 °C, the reactions were phenol extracted and ethanol precipitated. The RNA products were purified on a denaturing 20% polyacrylamide gel. In most cases, two major transcription products were obtained corresponding to the desired molecule and an oligomer one nucleotide longer (Milligan et al., 1987). The desired RNA fragments were located by UV-shadowing and excised from the gel. The gel slices were then crushed and soaked in 0.3 M K^+/0.5 M OAc^-, pH 5.0, overnight at 4 °C. After filtration to remove gel particles, the RNAs were ethanol precipitated. From 60 to 130 nmol of each RNA was recovered for thermal denaturation studies. The purity of each RNA was confirmed on a denaturing 20% polyacrylamide gel. Although the RNAs still contain small amounts of polyacrylamide, their further purification on DEAE-cellulose did not alter their melting behavior and therefore generally was not done. Oligonucleotide concentrations were calculated by the method of Freier et al. (1983).

High-Performance Liquid Chromatography. The approximate molecular weight of the full-length RNA transcripts was determined under native conditions by HPLC on a size-exclusion column. The HPLC system consisted of a Spectra-Physics 8700 solvent delivery system and 8750 organizer, a Hewlett-Packard 3390A integrator, and an ISCO V4 absorbance detector. The column was an Altex Spherogel-TSK 2000SW (7.5 mm i.d. × 30 cm). The HPLC solvent was 1 M NaCl, 10 mM Na_2HPO_4, and 0.1 mM EDTA, pH 7.0, with a flow rate of 0.5 mL/min. The sample size was 20 μL. The markers used for calibrating the column were $tRNA^{Phe}$ (M_r 26 000), a previously characterized RNA hairpin loop of 19 nucleotides (M_r 6270), and ATP (M_r 507).

Absorbance–Temperature Profiles. Thermal denaturation profiles were performed on a Gilford 2400 spectrophotometer interfaced to an IBM XT personal computer equipped with an analogue-to-digital Data Translation DT2801 I/O board. The acquisition and fitting programs for the analysis of thermal denaturation data developed by Petersheim and Turner (1983) were adapted for the personal computer. The data fitting program was further modified for the study of monophasic intramolecular helix–coil transitions. The temperature was controlled by a Gilford 2527 thermoprogrammer.

All thermal denaturation profiles were measured at 260 nm in 10 mM Na_2HPO_4/0.1 mM EDTA, pH 7.0, with and without 1 M NaCl. At least five profiles were measured for each RNA molecule over a 10-fold range of concentration. For each determination, the samples were heated to 99 °C for 5 min and then cooled to the starting temperature (usually 0 °C). The starting A_{260} was recorded, and the temperature was ramped at 1 °C/min. Four absorbance readings were determined per minute until the final temperature was reached (usually 90 °C). After the samples were cooled to the starting temperature, the A_{260} was recorded again, and if the value differed from the starting A_{260}, the data were not used. Absorbance–temperature profiles determined at slower heating rates or by decreasing the temperature at 1 °C/min gave identical results. Duplicate determinations were superimposable and gave T_m values within 0.5 °C. After the thermal analysis, each oligonucleotide was checked for degradation on a 20% denaturing gel. Degradation of samples used in later data analyses was not detected.

Assuming a two-state process, the helix–coil transition of a hairpin is represented by the equilibrium

$$\text{random coil} \rightleftarrows \text{hairpin}$$

The equilibrium constant, K, for this transition is

$$K = f/(1-f) = \exp(-\Delta H^\circ/RT + \Delta S^\circ/R)$$

where ΔH° and ΔS° are changes in enthalpy and entropy, respectively, and f is the fraction of molecules in the hairpin conformation. At the T_m of the transition, $f = 0.5$ and $T_m = \Delta H^\circ/\Delta S^\circ$. f is related to the absorbance at any temperature, $A(T)$, by

$$f(T) = \frac{A_s(T) - A(T)}{A_s(T) - A_d(T)}$$

where $A_s(T)$ and $A_d(T)$ are the absorbances of the random coil and hairpin, respectively, at temperature T (Martin et al., 1971) and are approximated by assuming linear upper and lower base lines:

$$A_d(T) = m_d T + b_d$$

$$A_s(T) = m_s T + b_s$$

The data were fit to six adjustable parameters ΔH°, ΔS°, m_d, b_d, m_s, and b_s (Petersheim & Turner, 1983) using the Marquardt nonlinear least-squares method (Bevington, 1969). An alternate method for fitting the data involved independently determining the slope and intercept of the upper and lower base lines from the linear regions of the profile and then using the nonlinear least-squares program to fit the data to two parameters, ΔH° and ΔS°. In every case, the values for ΔH° and ΔS° from a two-parameter fit did not vary significantly from the values obtained from a six-parameter fit. However, a fitted curve calculated by using the results of the two-parameter fit did not approximate the real absorbance data as well as the results from a six-parameter fit.

Results

RNA Synthesis. The 14 RNA fragments in this study are shown in Figure 1. The first 10 molecules vary in stem sequence but maintain the same 4-membered loop and bulge A. **1–7** have base pair changes adjacent to the bulge A, and **8–10** have base pair changes further from the bulge A. All 10 of these RNAs were shown to bind R17 coat protein, suggesting that they all have similar solution structures (Wu & Uhlenbeck, 1987). Any differences, then, in the T_m of these RNA molecules are expected to be a result of the different nearest-neighbor stacking interactions whose stability is predictable (Freier et al., 1986). Consistent differences betwen the stability of the stems would indicate that the combined contribution of the 4-membered loop and bulge A residue to

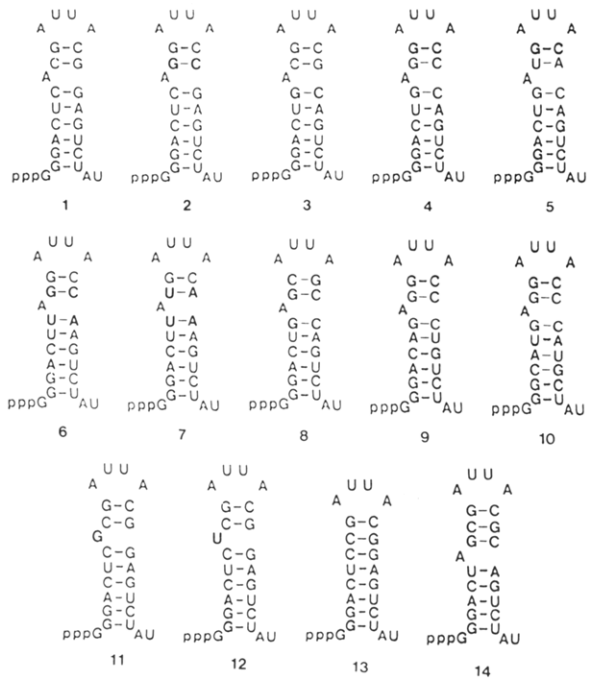

FIGURE 1: Fourteen RNA hairpins.

the stability of all 10 molecules is the same.

11–13 maintain the stem and loop sequence of **1**, but the bulge is changed to a G (**11**), a U (**12**), and eliminated entirely (**13**). These RNAs were designed to determine directly the independent contributions of the four-membered loop and the three bulge sequences to the stability of an RNA hairpin. A bulge C was not considered as alternate base pairing schemes are possible with this sequence and it is uncertain that it would have a single conformational form. **12** and **13** do not bind R17 coat protein due to the nature of the bulge (Wu & Uhlenbeck, 1987).

14 differs from **1–10** by having a different location of the bulge A in the helix and a slightly different stem sequence. It was designed to test whether or not the stability of an RNA hairpin containing the four-membered loop and bulge A can be predicted.

All 14 fragments were transcribed from synthetic DNA templates using T7 RNA polymerase (Lowary et al., 1986; Milligan et al., 1987). Since this method also produces a transcript that is one nucleotide longer than the desired full-length product (Milligan et al., 1987), it was necessary to confirm that the lower product band was the correct full-length RNA. This was done by preparing [α-^{32}P]UTP-labeled transcripts under identical conditions as the preparative reactions, isolating the product bands, and subjecting them to specific cleavage with RNase A. The digestion products were identified by two-dimensional thin-layer chromatography (Nishimura, 1979). Since all of the RNAs terminate with a UpApU sequence, RNase A digestion of the correct full-length product releases [^{32}P]ApUp from the 3′ terminus (Figure 2). Due to the presence of an internal UpApU sequence in **7**, RNase A digestion of the correct full-length transcript of this RNA produces both [^{32}P]ApUp and [^{32}P]ApU while the transcript one nucleotide longer produces only [^{32}P]ApUp (data not shown). All the correct full-length RNAs were determined in this manner. Sequencing the full-length transcripts revealed that no misincorporation of nucleotides occurred during the transcription process (Milligan et al., 1987).

RNA Size and Conformation Analysis. The RNA molecules in this study are capable of forming a stable duplex in equilibrium with the monomolecular hairpin. HPLC sieving

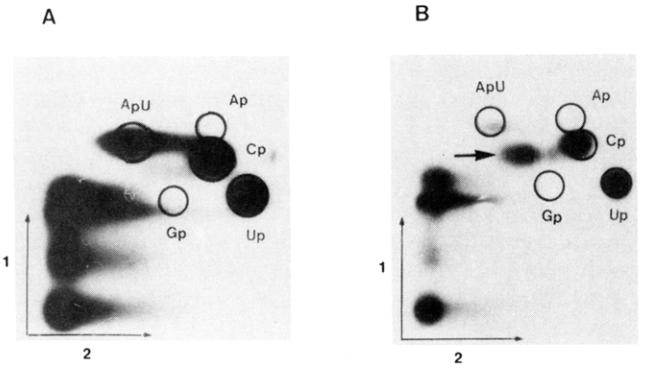

FIGURE 2: Thin-layer chromatography of an RNase A digestion of **6** (A) and the transcript one nucleotide longer (B). The arrow indicates ApUp in (B). The longer digestion products were not characterized.

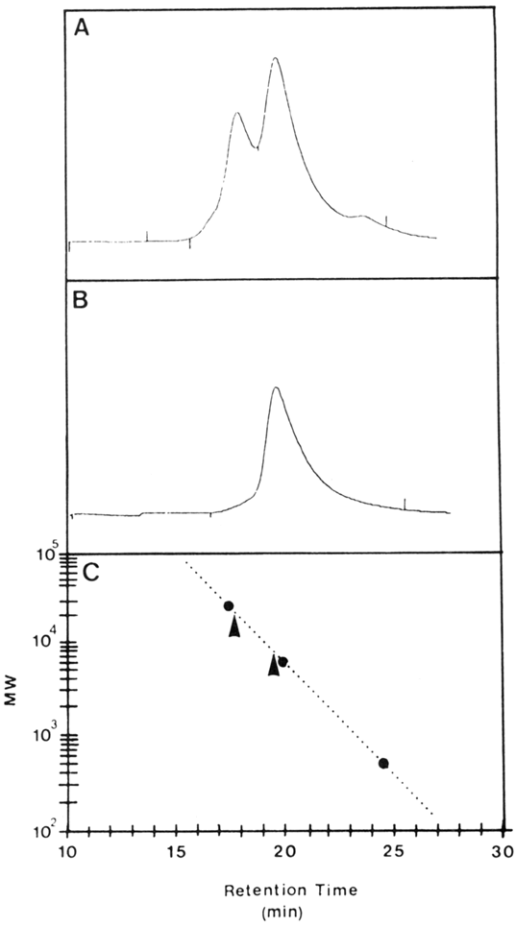

FIGURE 3: HPLC sieving column analysis of a 29 μM sample of **2** thawed at 25 °C (A) or heated to 99 °C (B) before injection. (C) Calibration curve.

column analysis of a thawed purified sample of **2** reveals the presence of two conformational forms (Figure 3A). Heating the same RNA sample to 99 °C prior to HPLC sieving column analysis resulted in only the slower migrating peak (Figure 3B). The slower moving peak has an observed molecular weight of 7400 (Figure 3C), which is a good approximation of the calculated molecular weight of the hairpin conformation. The faster moving peak has an observed molecular weight of 24 000 (Figure 3C) which corresponds to a complex of three RNA monomers. However, considering that duplex formation has been observed from other hairpin sequences (Gralla & Crothers, 1973; Hilbers et al., 1985), it is quite possible that the faster migrating peak is a dimer migrating anomalously on the HPLC column. In any event, this faster moving peak

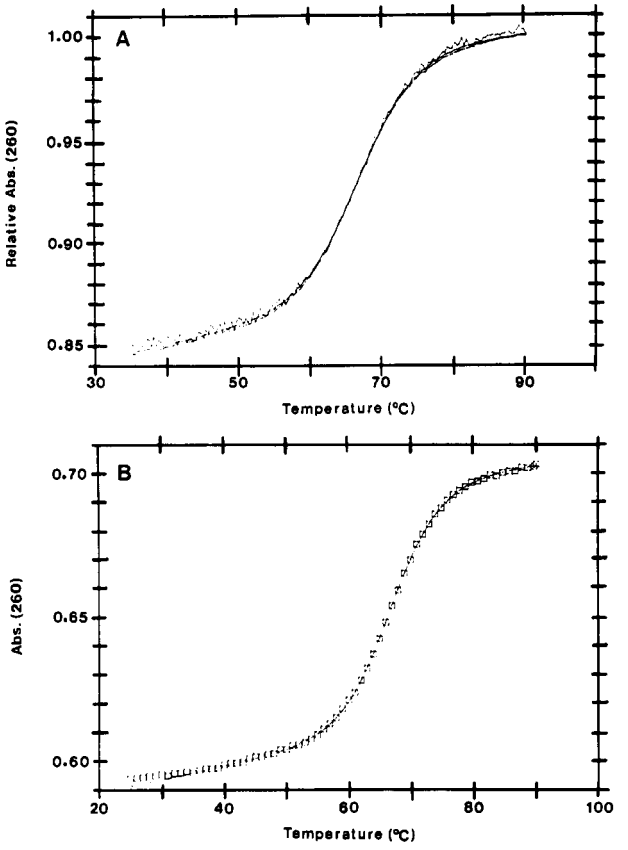

FIGURE 4: (A) Thermal denaturation profiles of **7** at 56.3, 40.5, and 6.8 μM. (B) Six-paramete fit (—) of the thermal denaturation profile of **7** (□).

is associated with the freezing process as it only appears in thawed samples and can be eliminated by heating. Heating of different RNA fragments at various oligonucleotide concentrations and buffer conditions has always resulted in only the monomolecular conformation as determined by HPLC. These results, when combined with those of Wu and Uhlenbeck (1987) which show that these loops bind R17 coat protein, establish that the RNAs form the hairpin conformation.

Thermodynamic Analysis of RNA Stability. Absorbance–temperature profiles of each hairpin were determined over a 10-fold range of oligonucleotide concentration and, as shown in Figure 4A for **7**, are reproducible and identical. This is expected for an intramolecular helix–coil transition. The thermal denaturation data were fit to a six-parameter equation describing the $\Delta H°$, $\Delta S°$, and the upper and lower base lines of a two-state helix–coil transition. As shown in Figure 4B, a curve calculated from the parameters fits the thermal denaturation data precisely, consistent with the assumption of a two-state transition used in the fitting program. Albergo et al. (1981) also obtain an excellent agreement using a similar analysis for a DNA duplex.

Table I lists the thermodynamic parameters of RNA hairpin formation obtained after averaging the fitted thermodynamic values from 5 or more thermal denaturation profiles for each of the 14 RNA hairpins. The standard deviation in the fitted thermodynamic parameters for the stability of a given RNA hairpin loop was no greater than ±2.3 kcal/mol for $\Delta H°$ and ±7.0 cal/(mol·deg) for $\Delta S°$. These errors are consistent with the errors observed in the fitted thermodynamic parameters for the helix–coil transition of RNA duplexes (Petersheim & Turner, 1983).

Substantial differences in ΔH and ΔS are observed among the different RNA hairpins. In order to test whether these differences can be attributed solely to the different nearest-neighbor stacking interactions, it is necessary to subtract their contribution. We identify 10 structural features that can contribute to the stability of **1**–**10**. They are the four-membered loop, the bulge A, the seven nearest-neighbor stacking interactions (including the G·U base pair), and the terminal G-A mismatch (Figure 1). The contributions of the 3′-dangling U and the 5′-triphosphate to RNA hairpin stability are not expected to be large (Freier et al., 1984) and will be ignored. By use of the experimental parameters for the hairpins (Table I) and the existing thermodynamic data for the nearest-neighbor stacking interactions (Freier et al., 1986), the G·U base pair (Sugimoto et al., 1986), and the terminal G·A (Hickey & Turner, 1985), the combined $\Delta H°$ and $\Delta S°$ for the four-membered loop and the bulge A can be calculated for each hairpin. For example

$$\Delta\Delta H°_{\text{loop \& bulge}} = \Delta H°_{\text{hairpin}} - \Delta H°_{\text{stem}}$$

It is assumed that base stacking occurs between the base pairs on each side of the bulge A. This assumption may not be correct if the A is intercalated into the helical stem (Patel et al., 1982).

The thermodynamics parameters for the 4-membered loop and the bulge A are quite consistent among 8 of the 10 RNA hairpins (Table II). However, the $\Delta\Delta H$ and $\Delta\Delta S$ values for both **4** and **8** are substantially different than for the other RNA hairpins even though their $\Delta\Delta G$ values are not that different (Table II). If **4** and **8** are not included, the average of the four-membered loop and bulge A contribution is $\Delta\Delta H°$ = 18.1 ± 0.9 kcal/mol and $\Delta\Delta S°$ = 28.4 ± 2.7 cal/(mol·deg). The average destabilizing free energy for all 10 molecules is $\Delta\Delta G$ = 9.3 kcal/mol at 37 °C. Despite the sequence differences in the stems, the standard deviation of the average thermodynamic values for the four-membered loop and bulge

Table I: Thermodynamic Parameters of Hairpin Formation at 1 M NaCl

hairpin	$-\Delta H°$ (kcal/mol)	$-\Delta S°$ [cal/(mol·deg)]	$-\Delta G°_{37}$ (kcal/mol)	T_m (°C)
1	65.4	185.9	7.7	78.6
2	67.9	192.7	8.1	79.0
3	65.8	188.2	7.4	76.5
4	62.3	177.5	7.3	78.0
5	63.7	184.0	6.7	73.3
6	63.4	185.1	6.0	69.1
7	57.8	170.1	5.0	66.8
8	76.1	215.3	9.3	80.3
9	67.8	193.9	7.7	76.7
10	66.5	189.8	7.6	77.3
11	63.8	181.2	7.6	78.8
12	69.0	195.5	8.4	80.0
13	71.2	193.8	11.1	94.3
14	63.3	181.4	7.0	75.9

Table II: Combined Contribution of the Four-Membered Loop and Bulge A to RNA Hairpin Stability

hairpin	$-\Delta\Delta H°$ (kcal/mol)	$-\Delta\Delta S°$ [cal/(mol·deg)]	$-\Delta\Delta G°_{37}$ (kcal/mol)
1	−19.4	−32.6	−9.2
2	−16.9	−25.8	−8.9
3	−18.2	−27.8	−9.6
4	−23.9	−43.6	−10.4
5	−18.8	−31.7	−9.0
6	−17.2	−24.7	−9.5
7	−17.2	−28.4	−8.4
8	−12.1	−11.0	−8.7
9	−18.4	−27.2	−10.0
10	−19.0	−28.6	−10.1

Table III: Predicted and Experimental Stability of RNA Hairpin **14**

method	$-\Delta H°$ (kcal/mol)	$-\Delta S°$ [cal/(mol·deg)]	$-\Delta G°_{37}$ (kcal/mol)	T_m (°C)
predicted	65.9	187.9	7.7	77.6
experimental	63.3	181.4	7.0	75.9

Table IV: Individual Contribution of a Bulge and the Four-Membered Loop to RNA Hairpin Stability

structure	$-\Delta\Delta H°$ (kcal/mol)	$-\Delta\Delta S°$ [cal/(mol·deg)]	$-\Delta\Delta G°_{37}$ (kcal/mol)
	1 M NaCl		
bulge A	−5.8	−7.9	−3.3
bulge G	−7.4	−12.6	−3.5
bulge U	−2.2	1.7	−2.7
AUUA loop	−12.3	−20.2	−6.0
	10 mM NaCl		
bulge A	−7.4	−10.5	−4.1
bulge G	−12.9	−28.1	−4.2
bulge U	−5.0	−4.4	−3.6

A (Table II) is close to the standard deviation in the thermodynamic values determined for the RNA hairpins (Table I). This suggests that the thermodynamic contribution of these two structural features can be considered as an independent unit.

In order to test whether the thermodynamic parameters for the four-membered loop and bulge A deduced in Table II can be applied to other molecules, an additional hairpin **14** was considered. As shown in Table III, its thermodynamic values calculated from Table II and the nearest-neighbor data compare favorably with the experimental data determined for **14**.

It is possible to separate the contribution of the bulge A from the loop through the use of hairpin **13** which is missing the bulge. For example

$$\Delta\Delta H°_{\text{bulge A}} = \Delta H°_{1} - \Delta H°_{13}$$

Similar values for the bulges U and G can be obtained by using hairpins **11** and **12**. As shown in Table IV, the destabilizing free energy of the bulges ranges from 2.7 to 3.5 kcal/mol at 37 °C and is sequence dependent. A bulge G is the most destabilizing and a bulge U the least destabilizing to an RNA hairpin.

The contribution of the four-membered loop to RNA hairpin stability can be calculated by subtracting the thermodynamic parameters for the bulge A in Table IV from the averaged thermodynamic parameters for the four-membered loop and bulge A in Table II. For example

$$\Delta\Delta H°_{\text{loop}} = \Delta\Delta H°_{\text{loop and bulge}} - \Delta\Delta H°_{\text{bulge A}}$$

The calculated $\Delta\Delta H°$ and $\Delta\Delta S°$ for the four-membered loop are 12.3 kcal/mol and 20.2 cal/(mol·deg), respectively (Table IV). This represents a destabilizing free energy of $\Delta\Delta G$ = 6.0 kcal/mol at 37 °C for the four-membered loop. Since the thermodynamic values for the different bulge sequences and the four-membered loop in Table IV are based upon a single molecule (**13**), they are less reliable than the values of the four-membered loop and bulge A combined (Table II). The study of additional RNA hairpins should better establish the contribution of these structures to RNA stability.

Thermal denaturation profiles were also performed at 10 mM Na^+ for each RNA hairpin (Table V). As expected, the stability of each molecular was considerably lower. Since the thermodynamic parameters for the nearest-neighbor stacking interactions as a function of ionic strength are not available, it is not possible to derive values for the four-membered loop and bulge A. However, the contribution of each bulge sequence to the stability of **13** at 10 mM Na^+ can be determined by directly comparing **1**, **11**, **12**, and **13**. At 10 mM Na^+, the destabilization free energy at 37 °C of the bulges ranges from 3.6 to 4.2 kcal/mol and shows a similar sequence dependence as in high salt (Table IV).

Table V: Thermodynamic Parameters of Hairpin Formation at 10 mM Na^+

hairpin	$-\Delta H°$ (kcal/mol)	$-\Delta S°$ [cal/(mol·deg)]	$-\Delta G°_{37}$ (kcal/mol)	T_m (°C)
1	67.7	205.2	4.1	57.0
2	71.0	215.0	4.3	56.9
3	66.2	202.1	3.5	54.5
4	73.2	222.1	4.3	56.6
5	64.9	200.9	2.6	50.1
6	64.7	201.6	2.2	47.9
7	55.9	176.8	1.1	42.8
8	73.5	220.1	5.2	60.8
9	72.8	221.0	4.3	56.2
10	70.7	214.4	4.2	56.7
11	62.2	187.6	4.0	58.4
12	70.1	211.3	4.6	58.7
13	75.1	215.7	8.2	75.1
14	64.9	197.6	3.6	55.1

Discussion

The study of RNA hairpins is potentially complicated by the formation of duplexes containing an internal loop (Gralla & Crothers, 1973). Since little is presently known about the effects of sequence, RNA concentration, and buffer conditions on the hairpin–duplex equilibrium, it was difficult to predict whether the RNAs prepared in this work would indeed form hairpins. Fortunately, at every condition studied, all 14 RNAs exclusively formed hairpins provided that frozen samples were heated to high temperatures before study. Presumably as a result of high RNA or salt concentrations occurring during the freezing process, thawed samples contained considerable amounts of a high molecular weight form. Although we did not determine whether this form was a duplex or a higher order aggregate, its presence emphasizes the propensity for RNA to take up alternate conformations.

A consistent contribution of the four-membered loop and bulge A to RNA hairpin stability is observed for 8 of 10 RNA hairpins studied. This consistency strongly suggests that the four-membered loop and bulge A have similar structures in these RNA hairpins. The fact that **1**–**10** bind to R17 coat protein (Wu & Uhlenbeck, 1987) also supports this presumption. The averaged thermodynamic values for the four-membered loop and bulge A can be used to accurately predict the stability of the quite different hairpin **14**; the four-membered loop and bulge A can be considered as an independent thermodynamic entity in the overall stability of RNA. Hairpins **4** and **8** display thermodynamic values for the four-membered loop and bulge A that deviate substantially from the average and are not clearly understood. A reexamination of the sequence and purity of both molecules as well as repeating their melting profiles did not alter the observations. Hairpin **4** only differs from **10** by a single internal base pair and shows exceptionally large values of $\Delta\Delta H$ and $\Delta\Delta S$. Hairpin **8** is unique in closing the loop with a C·G pair instead of a G·C pair and shows exceptionally small values of $\Delta\Delta H$ and $\Delta\Delta S$. While others have reported that hairpins closing with C·G pairs are exceptionally stable (Heus et al., 1983; Tuerk et al., 1988), we do not see this for **8**. Perhaps unique conformational intermediates occur during the melting of **4** and **8** that account for their unusual properties.

Hairpins **1**, **11**, **12**, and **13** differ only in the bulge nucleotide, and the contribution of the different bulge sequences can be directly determined from a simple comparison of the molecules. Our results show that the destabilization effect of a bulge purine is larger than for a bulge pyrimidine at both low and high ionic strengths. This is consistent with the observation that bulge purines destabilize RNA copolymers more than bulge pyrimidines (Lomant & Fresco, 1975). The thermodynamic values for the helix destabilization of a bulge A compare favorably with previous determinations (Fink & Crothers, 1972), but Freier et al. (1986) make no distinction in the destabilization from bulges of different sequences.

We have not been able to detect any effect of neighboring base pairs on the destabilization of the bulge A. Although this would appear to suggest that the bulge A is not interacting with its neighbors, the interpretation is complicated by a possible equilibrium between extrahelical and intercalated states of a bulge nucleotide (Patel et al., 1982; Morden et al., 1983) and the structural flexibility introduced into a helix by a bulge (White & Draper, 1987). More data are needed on the study of bulge nucleotides in RNA hairpin loops to better address this problem.

Using the averaged thermodynamic values in Table II and the thermodynamic parameters for a bulge A, we calculated parameters for the contribution of the AUUA loop. A relatively large $\Delta\Delta H$ was obtained which agrees with a previous evaluation of a four-membered loop (Uhlenbeck et al., 1973). However, such ΔH determinations are strongly dependent upon the assumption of a two-state model and the method of analyzing the upper and lower base lines. Calorimetric determinations of ΔH often differ substantially from those obtained by optical methods (Breslauer et al., 1975; Heus et al., 1983). The value of $\Delta\Delta G = +6.0$ kcal/mol for the AUUA loop is slightly less than that determined previously for a CCCC loop (Uhlenbeck et al., 1973; Gralla & Crothers, 1973) and virtually the same as assumed by Freier et al. (1986) for an arbitrary four-membered loop. Considering that the errors involved in subtracting the bulge contribution and calculating a ΔG at a temperature much lower than the T_m of the hairpin could be considerable, the agreement should be considered excellent.

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