Thermodynamic examination of the pyrophosphate sensor helix in the thiamine pyrophosphate riboswitch

STEPHANIE FURNISS and NEENA GROVER

Department of Chemistry and Biochemistry, the Colorado College, Colorado Springs, Colorado 80903, USA

ABSTRACT

Riboswitches are functional mRNA that control gene expression. Thiamine pyrophosphate (TPP) binds to *thi*-box riboswitch RNA and allosterically inhibits genes that code for proteins involved in the biosynthesis and transport of thiamine. Thiamine binding to the pyrimidine sensor helix and pyrophosphate binding to the pyrophosphate sensor helix cause changes in RNA conformation that regulate gene expression. Here we examine the thermodynamic properties of the internal loop of the pyrophosphate binding domain by comparing the wild-type construct (RNA WT) with six modified 2×2 bulged RNA and one 2×2 bulged DNA. The wild-type construct retains five conserved bases of the pyrophosphate sensor domain, two of which are in the 2×2 bulge (C65 and G66). The RNA WT construct was among the most stable ($\Delta G_{37}^{\circ} = -7.7$ kcal/mol) in 1 M KCl at pH 7.5. Breaking the A•G mismatch of the bulge decreases the stability of the construct ~ 0.5 –1 kcal/mol, but does not affect magnesium binding to the RNA WT. Guanine at position 48 is important for RNA–Mg²⁺ interactions of the TPP-binding riboswitch at pH 7.5. In the presence of 9.5 mM magnesium at pH 5.5, the bulged RNA constructs gained an average of 1.1 kcal/mol relative to 1 M salt. Formation of a single A•C mismatch base pair contributes about 0.5 kcal/mol at pH 5.5, whereas two tandem A•C mismatch base pairs together contribute about 2 kcal/mol.

Keywords: bulge stabilities; divalent ion interactions; metal–RNA interactions; RNA thermodynamics

INTRODUCTION

Riboswitches are functional mRNA that can control gene expression by changing conformation upon binding target metabolites (Lai 2003; Sudarsan et al. 2003; Breaker 2009), such as thiamine pyrophosphate (TPP) (Miranda-Ríos et al. 2001; Winkler et al. 2002). TPP is an essential cofactor in bacteria, archaea, and eukaryotes (Sudarsan et al. 2003; Thore et al. 2006) that binds to the *thi*-box riboswitch to control the expression of genes that code for proteins involved in the biosynthesis and transport of thiamine (Winkler et al. 2002; Sudarsan et al. 2005). The metabolite–RNA complex inhibits translation by forming alternate structures and can discriminate between mono- and diphosphorylated analogs (Nahvi et al. 2002; Mandal and Breaker 2004). The binding of a TPP metabolite also stabilizes additional tertiary interactions that are distant from the TPP binding sites (Serganov et al. 2006; Lang et al. 2007; Kulshina et al. 2010).

Crystal structures of the TPP riboswitch reveal TPP binds to two parallel stems of the fork-like, or “h” shaped, riboswitch (Edwards and Ferré-D’Amaré 2006; Serganov et al. 2006; Kulshina et al. 2010). Thiamine binding to the pyrimidine sensor helix and pyrophosphate binding to the pyrophosphate sensor helix causes changes in RNA conformation that regulate gene expression through a variety of mechanisms in archae, bacteria, and eukaryotes (Thore et al. 2006). Crystal structures show that one or two Mg²⁺ ions are required for pyrophosphate binding (Edwards and Ferré-D’Amaré 2006; Serganov et al. 2006; Thore et al. 2006). TPP binds the riboswitch more tightly with increasing concentrations of Mg²⁺, as shown by a 40-fold increase in the association constant (K_a) for TPP in the presence of 1 mM Mg²⁺ (Yamauchi et al. 2005).

This study examines the pyrophosphate sensor helix region of the *thiC* riboswitch RNA derived from *Arabidopsis thaliana*. Five bases in the pyrophosphate sensor domain are conserved (Thore et al. 2006; Ontiveros-Palacios et al. 2008). Positions C45, G48, and G64 are conserved bases in the helical stems and positions 65 and 66 are conserved in the 2×2 internal bulge (Fig. 1). Nucleotides G64, C65, and G66 have been implicated in direct interactions with the pyrophosphate group in a deep cleft (Edwards and Ferré-D’Amaré 2006; Serganov et al. 2006; Thore et al. 2006),

Reprint requests to: Neena Grover, Department of Chemistry and Biochemistry, 14 E. Cache La Poudre Street, the Colorado College, Colorado Springs, CO 80903, USA; e-mail: ngrover@coloradocollege.edu; fax: (719) 389-6182.

Article published online ahead of print. Article and publication date are at <http://www.rnajournal.org/cgi/doi/10.1261/rna.2263211>.

FIGURE 1. The RNA WT construct was designed based on the TPP-binding *thiC* riboswitch from *Arabidopsis thaliana* (Thore et al. 2006). The base numbering system is consistent with 2CKY pdb structure (Thore et al. 2006). The wild type (A), modified constructs (B–G), Core Helix (H), and DNA WT (I) were examined here. The modified bases that differentiate each construct from the RNA WT are represented in red. Pu represents purine substitution. Note that DNA WT construct has lengthened helical stems. Added nucleotides are shown in red.

whereas residues A49 (residue A61 in 2HOJ) (Edwards and Ferré-D'Amaré 2006) and C65 indirectly interact with the pyrophosphate group through a water molecule (Edwards and Ferré-D'Amaré 2006; Serganov et al. 2006). Residues G66 and G48 are shown to bind directly to a magnesium ion (Edwards and Ferré-D'Amaré 2006; Serganov et al. 2006; Thore et al. 2006), through which they indirectly interact with the pyrophosphate group (Edwards and Ferré-D'Amaré 2006; Serganov et al. 2006). U47 (residue U59 in 2HOJ; Edwards and Ferré-D'Amaré [2006] is not included in RNA wild-type [WT] construct), A49, and C65 indirectly interact with the same magnesium ion through a water molecule each (Serganov et al. 2006; Edwards et al. 2007). A63 (not included in RNA WT construct), G64, and C65 indirectly interact with a second magnesium ion through a water molecule each (Serganov et al. 2006; Edwards et al. 2007; Miranda-Ríos 2007).

In this study we are examining the pyrophosphate sensor helix to determine various contributors to its stability, the changes in stability in the presence of magnesium ions, and the potential role of various nucleotides in metal–RNA interactions.

RESULTS

The wild-type pyrophosphate sensor construct is the among the most stable at pH 7.5

At pH 7.5 and 1 M monovalent salt, the seven RNA bulge constructs (Fig. 1) range in stabilities from $\Delta G_{37}^{\circ} = -6.6$ to -8.0 kcal/mol (Fig. 2; Tables 1, 2). The RNA WT construct and C48–G67 construct have the highest stabilities.

Changing guanine at position 66 to purine (A•Pu/A•C construct) or cytosine (A•C/A•C construct) decreases the stability of the RNA by ~ 1 kcal/mol over the RNA WT construct (Fig. 2; Tables 1, 2). Changing the 5' closing base pair from G48–C67 to C48–G67 (C48–G67 construct) in-

creases the stability by 0.3 kcal/mol over the RNA WT (Fig. 2; Tables 1, 2). The construct without the bulge (Core Helix) has a $\Delta G_{37}^{\circ} = -11.0$ kcal/mol at pH 7.5, which is ~ 3 kcal/mol greater than the RNA WT (Fig. 2; Tables 1, 2). The DNA equivalent of RNA WT construct was relatively unstable. Hence the helical stems were extended for this study. DNA WT had a $\Delta G_{37}^{\circ} = -10.3$ kcal/mol at pH 7.5.

Examining the pH effect on RNA stability

Upon lowering the pH to 5.5, the stabilities of the bulged RNA constructs in the 1 M KCl range from -7.3 to -10.6 kcal/mol (Fig. 2; Table 2). The RNA WT construct has a free energy of -8.1 kcal/mol at pH 5.5, which is 0.4 kcal/mol more stable than at pH 7.5 (Fig. 2). The stability of the Core Helix and A•A/A•A constructs were not affected upon lowering the pH in 1 M KCl (Fig. 2; Table 2). The C•C/C•C construct gains the greatest stability, -3.3 kcal/mol, and is only 0.5 kcal less stable than the Core Helix at pH 5.5 (Fig.

FIGURE 2. The negative free energy ($-\Delta G_{37}^{\circ}$) of the RNA and DNA constructs in 1 M KCl at pH 7.5 (gray) and pH 5.5 (gray and black). The black segments represent the additional stability gained when the pH is lowered from 7.5 to 5.5.

TABLE 1. Thermodynamic parameters in 1 M salt at pH 7.5

Construct name	Duplex sequences	ΔH° (kcal/mol)	ΔS° (eu)	ΔG°_{37} (kcal/mol)	T_m ($^\circ\text{C}$, 1.0E–04 M)
RNA WT	5'CGAG ^{AA} CGAA ^{3'} 3'AGCUC _{GC} GCU _{5'}	–58.1	–162.6	–7.7	43.2
A•Pu/A•C	5'CGAG ^{AA} CGAA ^{3'} 3'AGCUC _{Pu} C _G CU _{5'}	–46.3	–128.0	–6.6	37.5
A•C/A•C	5'CGAG ^{AA} CGAA ^{3'} 3'AGCUC _{CC} GCU _{5'}	–56.7	–161.6	–6.6	37.1
C48-G67	5'CGAG ^{AA} CGAA ^{3'} 3'AGCUC _{GC} GCU _{5'}	–56.5	–156.6	–8.0	45.1
A•A/A•A	5'CGAG ^{AA} CGAA ^{3'} 3'AGCUC _{AA} GCU _{5'}	–51.8	–143.3	–7.3	41.8
C•A/C•A	5'CGAG ^{CC} CGAA ^{3'} 3'AGCUC _{AA} GCU _{5'}	–50.4	–139.7	–7.1	40.4
C•C/C•C	5'CGAG ^{CC} CGAA ^{3'} 3'AGCUC _{CC} GCU _{5'}	–46.0	–124.7	–7.3	42.4
Core Helix	5'CGAGCGAA ^{3'} 3'AGCUCGCU _{5'}	–60.8	–160.8	–11.0	61.4
DNA WT	5'GCGAG ^{AA} CGAGCA ^{3'} 3'ACGCTC _{GC} GCTCG _{5'}	–65.1	–176.7	–10.3	55.6

Thermodynamic parameters of the wild-type RNA construct (RNA WT), modified RNAs and DNA WT in 1 M salt at pH 7.5. The average errors in these measurements are 5% for ΔH° , 6% for ΔS° , and 1% for ΔG°_{37} . As the van't Hoff analysis underestimates the errors, these should be considered 15% for ΔH° and ΔS° , and 5% for ΔG°_{37} . To indicate this error, the font size has been decreased for the last digit.

2; Table 2). Similar to the RNA WT, the DNA WT construct gains 0.3 kcal/mol in stability at pH 5.5 (Fig. 2; Table 2).

Most RNA constructs are stabilized by magnesium ions

In pH 7.5, the RNA WT construct gained 0.4 kcal/mol upon the addition of 9.5 mM magnesium over 1 M salt (Fig. 3). As shown in Table 2 and Figure 3, six of the seven bulged RNA constructs examined here are slightly more stable in 9.5 Mg^{2+} than in 1 M monovalent salt at pH 7.5, with an average gain of 0.5 kcal/mol. The C48–G67 construct was the only bulged RNA construct that was less stable than RNA WT in 9.5 mM Mg^{2+} over 1 M KCl (0.4 kcal/mol), making the C48–G67 construct 0.8 kcal/mol less stable than the RNA WT in 9.5 mM magnesium at pH 7.5 (Fig. 3, yellow bars).

In pH 5.5, all RNA constructs are stabilized in 9.5 mM magnesium relative to 1 M monovalent ions, with the RNA WT gaining 1.2 kcal/mol over 1 M KCl (Fig. 3; Table 2). At pH 5.5, the bulged constructs gained an average of 1.1 kcal/mol in stability in the presence of 9.5 mM Mg^{2+} relative to 1 M KCl (Fig. 3, blue bars). The Core Helix construct gained ~ 2 kcal/mol in stability in 9.5 mM Mg^{2+} over 1 M KCl at pH 5.5 (Fig. 3; Table 2). The DNA WT construct was not additionally stabilized by divalent ions at either pH relative to 1 M KCl (Fig. 3; Table 2).

DISCUSSION

We examined the pyrophosphate sensor helix region of the *thiC* riboswitch RNA derived from *Arabidopsis thaliana* through a thermodynamic analysis of a wild-type (RNA WT) and six modified RNA constructs. The RNA WT construct studied here maintains five conserved bases that have been identified in the pyrophosphate sensor helix of the TPP-binding riboswitch, as well as the A•G/A•C 2×2 bulge sequence (Edwards and Ferré-D'Amaré 2006; Serganov et al. 2006; Thore et al. 2006). The RNA WT construct has a stability of -7.7 kcal/mol at pH 7.5 (Fig. 2; Tables 1, 2). It retains the conserved bases C45, G48, G64, C65, and G66 of the pyrophosphate sensor helix and has an A•G mismatch pair, which is expected to form at pH 7. When guanine at position 66 was modified (breaking the A•G base pair) to purine (A•Pu/A•C construct) or cytosine (A•C/A•C construct), about 1.0 kcal/mol decrease in free energy is seen relative to the RNA WT at pH 7.5 and 1 M salt (Fig. 2; Tables 1, 2). The duplex is, on average, 4 kcal/mol more stable at pH 7.5, which is likely due to breaking the helical structure and distortion of backbone required to accommodate mismatch base pairs of different sizes (Xia et al. 1997).

If the A•G mismatch base pair is contributing about 1.0 kcal/mol in stability to the RNA WT, then disrupting this base pair in an all pyrimidine or all purine bulge is expected to cause a similar decrease in stability. Both the C•C/C•C

TABLE 2. Thermodynamic parameters in varying monovalent and divalent ion concentrations, and at pH 7.5 and 5.5

Construct name	Duplex sequences	10 mM KCl ΔG_{37}° (kcal/mol)	1 M KCl ΔG_{37}° (kcal/mol)	9.5 mM Mg^{2+} ΔG_{37}° (kcal/mol)
RNA WT	5'CGAG ^{AA} CGAA3'	−4.6	−7.7	−8.1
	3'AGCUC _{GC} GCU5'	−6.2	−8.1	−9.3
		−1.6	−0.4	−1.2
A•Pu/A•C	5'CGAG ^{AA} CGAA3'	−3.1	−6.6	−7.1
	3'AGCUC _{PuC} GCU5'	−5.7	−7.3	−8.3
		−2.6	−0.7	−1.2
A•C/A•C	5'CGAG ^{AA} CGAA3'	−4.8	−6.6	−7.5
	3'AGCUC _{CC} GCU5'	−6.9	−8.7	−10.0
		−2.1 (2 × −1.05)	−2.1 (2 × −1.05)	−2.5 (2 × −1.25)
C48–G67	5'CGAG ^{AA} CGAA3'	−4.8	−8.0	−7.6
	3'AGCU _G G _{GC} GCU5'	−5.7	−8.4	−9.4
		−0.9	−0.4	−1.8
A•A/A•A	5'CGAG ^{AA} CGAA3'	−3.5	−7.3	−7.6
	3'AGCUC _{AA} GCU5'	−5.1	−7.4	−8.8
		−1.6 (2 × −0.8)	−0.1 (2 × −0.05)	−1.2 (2 × −0.6)
C•A/C•A	5'CGAG ^{CC} CGAA3'	−6.4	−7.1	−7.6
	3'AGCUC _{AA} GCU5'	−9.2	−9.3	−10.2
		−2.8 (2 × −1.4)	−2.2 (2 × −1.1)	−2.6 (2 × −1.3)
C•C/C•C	5'CGAG ^{CC} CGAA3'	−6.6	−7.3	−7.9
	3'AGCUC _{CC} GCU5'	−9.1	−10.6	−11.5
		−2.5 (2 × −1.25)	−3.3 (2 × −1.65)	−3.6 (2 × −1.8)
Core Helix	5'CGAGCGAA3'	−8.3	−11.0	−11.9
	3'AGCUCGCU5'	−7.4	−11.1	−13.0
		0.9	−0.1	−1.1
DNA WT	5'GCGAG ^{AA} CGAGCA3'	−6.2	−10.3	−10.3
	3'ACGCTC _{GC} GCTCG5'		−10.6	−10.5
			−0.3	−0.2

Thermodynamic parameters of wild-type and modified constructs in various monovalent and divalent ion concentrations at pH 7.5 and 5.5. Data in the first rows are from pH 7.5 conditions. The pH 5.5 data are shown in the second rows (gray shading). The third rows list the change in free energy of the constructs upon lowering the pH ($\Delta\Delta G_{37, pH}^{\circ}$) (Fig. 2). The parentheses show the change in free energy per A•A, A•C, or C•C mismatch when present in tandem. Average error in these measurements is 2% for ΔG_{37}° . As the van't Hoff analysis underestimates the errors, it should be considered 5% for ΔG_{37}° .

and A•A/A•A constructs are 0.4 kcal/mol less stable than the RNA WT (Tables 1, 2). If A•C, C•C, and A•A base pairs behave similarly at pH 7.5, then the ~ 0.4 kcal/mol decrease in stability is attributed to the breaking of the A•G pair in the C•C/C•C and A•A/A•A constructs. This result indicates that at least 0.5 kcal/mol of change in free energy may be due to differences in interactions in and among various mismatch base pairs. This 0.5 kcal/mol difference could also be attributed to experimental error.

A•G mismatches are expected to be pH independent between pH 7.0 and 5.5 (SantaLucia et al. 1990). Upon lowering the pH to 5.5, the A+•C base pair is expected to form (due to protonation of the adenine) in the 2 × 2 bulge of the RNA WT construct, leading to a 0.4 kcal/mol increase in stability (Fig. 2). One or two A•C base pairs are present in A•Pu/A•C, A•C/A•C, C48–G67, and C•A/C•A constructs;

hence, these constructs should gain additional stability upon lowering the pH. The C48–G67 and A•Pu/A•C constructs show 0.4 and 0.7 kcal/mol increases in stability in 1 M KCl, respectively. The small difference between the A•Pu/A•C, RNA WT, and C48–G67 constructs is either due to differences in interactions with neighboring bases or can be attributed to experimental error. A 1 kcal/mol bonus is expected for the pH effect for symmetric tandem and single A•C mismatches (Chen et al. 2009). Our data show that an A•C base pair, located between a G–C closing base pair and one other mismatch base pair, contributes an additional ~ 0.5 kcal/mol to stability upon protonation of adenine. The C•A/C•A and A•C/A•C constructs each gain ~ 2.1 kcal/mol in stability upon lowering the pH in 1 M salt. Thus, an A+•C mismatch contributes greater stability when flanked by another A+•C mismatch.

FIGURE 3. The effect of Mg^{2+} ions on RNA stability in pH 7.5 and 5.5. The thermodynamic parameters were compared for 9.5 mM Mg^{2+} and 1 M KCl ($\Delta\Delta G^{\circ}_{37}$ relative to 1 M KCl). A positive $\Delta\Delta G^{\circ}_{37}$ indicates a greater stability in the presence of Mg^{2+} over 1 M KCl.

Crystal structure analyses suggest one or two Mg^{2+} ions are required for pyrophosphate binding, where positions 48, 64, 65, and 66 are potentially interacting with magnesium ions (Edwards and Ferré-D'Amaré 2006; Serganov et al. 2006; Thore et al. 2006). TPP binds the riboswitch more tightly with increasing concentrations of Mg^{2+} , with K_d increasing about 40-fold in the presence of 1 mM Mg^{2+} (Yamauchi et al. 2005). At pH 7.5, interactions with magnesium ions slightly increase the stability of the RNA WT construct relative to 1 M monovalent ions (0.4 kcal/mol). A purine modification at position 66 (A•Pu/A•C construct) decreases the construct's stability by ~ 1 kcal/mol relative to RNA WT in both 1 M KCl and 9.5 mM Mg^{2+} conditions (Table 2), indicating that both the RNA WT and A•Pu/A•C constructs are similarly stabilized by magnesium over 1 M KCl at pH 7.5 (Fig. 3, yellow bars). The role of guanine at position 66 may not be in stabilizing magnesium–pyrophosphate interactions at physiological pH. In the presence of 9.5 mM Mg^{2+} at pH 7.5, the A•A/A•A construct gained 0.3 kcal/mol, similar to the stability gained by the RNA WT construct. This suggests that the slight gain in stability upon interactions with magnesium is not dependent on the conserved base C65. The RNA–ion interactions seen in the crystal structures between the conserved bases of the bulge, C65 and G66, and magnesium may not contribute to the stabilization of the pyrophosphate domain itself, but may be important for stabilizing the interactions of a negatively charged ligand with this domain.

Lowering the pH to 5.5 increases the stability of all RNA constructs, including Core Helix (Fig. 3, blue bars). All bulged-RNA constructs are within 0.5 kcal/mol of the RNA WT at pH 5.5, regardless of possible pH-dependent base-pair interactions. The A•Pu/A•C and RNA WT constructs are similarly stabilized by magnesium at pH 5.5, as seen at pH 7.5. Although there is thermodynamic discrimination between the RNA WT and C48–G67 constructs in 9.5 mM Mg^{2+} at pH

7.5, these constructs have similar stabilities at pH 5.5 (-9.3 and -9.4 kcal/mol, respectively). The Core Helix gains an additional ~ 1 kcal/mol in stability upon lowering the pH.

Although protonation of adenine–cytosine and cytosine–cytosine base pairs stabilized RNA constructs over RNA WT in 1 M KCl at pH 5.5, these same constructs do not necessarily show the greatest change upon magnesium binding (Fig. 3, blue bars). For example, the A•C/A•C, C•A/C•A, and C•C/C•C constructs only gain an additional ~ 0.4 kcal/mol in magnesium binding upon lowering the pH to 5.5, whereas the A•A/A•A construct gains ~ 1 kcal/mol in magnesium binding upon lowering the pH. The change in RNA stability at lower pH needs to be further explored.

Guanine at position 48 is modified to cytosine in the C48–G67 construct. The modified 5' C48–G67 closing base pair is more stable by 0.3 kcal/mol in pH 7.5 over the wild-type construct's 5' G48–C67 closing base pair. This nearest-neighbor effect is not consistent with previous studies of G•A and A•G symmetric tandem mismatches, where a G–C closing base pair is more stable than a C–G base pair (Walter et al. 1994; Schroeder et al. 1999; Xia et al. 1999).

In the presence of magnesium, the C48–G67 construct is 0.8 kcal/mol less stable than the RNA WT construct at pH 7.5 (Fig. 3, yellow bars; Table 2). Although the C48–G67 closing base pair is thermodynamically favored at 1 M salt, our data suggest the 5' G48–C67 closing base pair, seen in the wild type, favors the interaction of magnesium with the RNA at pH 7.5. Consistent with our results, Ontiveros-Palacios et al. (2008) showed that TPP binding is threefold weaker when guanine at position 48 is modified to cytosine (Ontiveros-Palacios et al. 2008). Upon lowering the pH to 5.5, magnesium binding to the C48–G67 construct is restored to the level of the RNA WT (Fig. 3, blue bars); the relevance of this result is unclear for the riboswitch functions under physiological conditions.

When the conserved wild-type bulge sequence is changed to C•A/C•A (C•A/C•A construct), the modified construct is 0.6 kcal/mol less stable than the RNA WT at pH 7.5 (Fig. 2; Tables 1, 2). Comparing the C•A/C•A construct to the A•C/A•C construct, which has identical nearest neighbors, there is a significant difference in stability in 1 M salt at pH 7.5 and 5.5 (Fig. 2; Table 2); the C•A/C•A construct is ~ 0.6 kcal/mol more stable than the A•C/A•C construct in both conditions. These differences may be indicative of differing interactions with the two helical stems and are likely indicative of nonnearest neighbor interactions (Kierzek et al. 1999; Siegfried et al. 2007; Chen et al. 2009). The helical context of the internal loop has been seen to have an effect on RNA stability in previous studies, indicating that other nonnearest neighbor interactions contribute to internal loop stability (Schroeder and Turner 2000).

A pH-dependent protonation of cytosine in C•C mismatch base pairs forms C+•C base pairs, upon lowering the pH to 5.5 (SantaLucia et al. 1991; Boulard et al. 1997; Yu et al. 1997). The 3.3 kcal/mol increase in construct stability

suggests strong stabilizing interactions between the two bulge cytosine pairs. The free energy of the C•C/C•C construct is close to the predicted value, with a 1.0 kcal/mol C•C/C•C bulge destabilizing effect (Wu et al. 1995; Xia et al. 1998) and a 2.5 kcal/mol pH-dependent stabilizing effect ($\Delta\Delta G_{37, \text{pH}}^\circ$) (SantaLucia et al. 1991). Similarly, the free energies obtained here for the A•A/A•A construct are consistent with the nearest-neighbor prediction, with a 1.5 kcal/mol destabilizing effect next to G–C closing base pairs (Xia et al. 1998). Based on measured stabilities of 2×2 bulges, our data are consistent with prediction values for symmetric tandem mismatches where the exact bulge sequence and nearest-neighbor pairs have been previously studied and quantified. The nearest-neighbor prediction values from 2×2 symmetric tandem mismatches are not consistent for bulged constructs with other (nonsymmetric) tandem mismatches, as seen in the RNA WT construct examined here. Prediction values derived from a study of 3×3 internal bulges were not effective at predicting the stabilities of the 2×2 bulges examined in this study (Chen et al. 2009).

The pyrophosphate or TPP ligand binding to the RNA WT does not have a significant impact on the secondary structure (Supplemental Material), as would be expected in the absence of the entire riboswitch. The tight interactions between TPP metabolite and RNA involve additional tertiary interactions at sites that are distant from the TPP binding sites (Lang et al. 2007; Kulshina et al. 2010).

The DNA WT construct has similar stability in 1 M KCl as in the presence of 9.5 mM divalent ions. Similar results have been seen in thermodynamic studies of nonbulged DNA constructs in NaCl and magnesium (Williams et al. 1989).

CONCLUSIONS

Thermodynamic examination of the pyrophosphate sensor helix of the TPP-binding riboswitch suggests breaking the A•G mismatch of the RNA WT bulge decreased the stability of the construct by nearly 1 kcal/mol (for A•Pu/A•C and A•C/A•C constructs) or ~ 0.5 kcal/mol (for C•C/C•C and A•A/A•A constructs), but did not affect the stability gained from interactions with magnesium at pH 7.5 and 5.5. At physiological pH, the conserved guanine at position 66 is not important to interactions with magnesium as expected from the crystal structure. The A•C mismatch in the RNA WT bulge contributes about 0.5 kcal/mol in pH-dependent stability to the construct. Under physiological conditions, modifying guanine at position 48 decreases the RNA stability gained in the presence of magnesium ions indicating its importance for these interactions. Modification of position 48 is tolerated at pH 5.5. Our thermodynamic data indicate that interactions beyond the nearest neighbors influence the overall stability of the RNA constructs. Thus, studying functionally important RNA secondary structures is important for understanding the relationship between RNA structure and its functions.

MATERIALS AND METHODS

RNA design and purification

All constructs used in this study are shown in Figure 1. The RNA WT construct (Fig. 1A), is a double-stranded RNA structure that maintains the conserved bases of the P4 and P5 helical regions and the A49•G66 and A50•C65 mismatch base pairs in 2×2 internal bulge seen in J4/5 (Thore et al. 2006). The base numbering in our constructs is consistent with the *thiC* riboswitch RNA derived from *Arabidopsis thaliana* (Thore et al. 2006). The nomenclature followed here lists the first noncanonical base pair (positions 49–66) followed by the second noncanonical base pair (positions 50–65) in the 2×2 bulge of the RNA construct (thus, RNA WT construct can alternatively be labeled as A•G/A•C construct) (Fig. 1A).

To understand the effect of the conserved bases on the RNA WT construct's stability and free energy change, if any, upon interactions with divalent ions, single modifications at position 66 were designed by changing the guanine nucleotide to purine (A•Pu/A•C construct) (Fig. 1B) or pyrimidine (A•C/A•C construct) (Fig. 1C). The conserved guanine at position 48 is examined by changing the G48–C67 base pair to C4–G67 (Fig. 1D). The C•A/C•A construct (Fig. 1F) does not maintain the bases of the RNA WT bulge, but the helical stems are not affected. The C•A/C•A construct has the same bulge sequence at the A•C/A•C construct, but the cytosine–adenosine mismatch base pairs have different spatial relations to the two helical stems. The C•C/C•C construct maintains the conserved base C65 in the bulge, while modifying nucleotides at positions 49, 50, and 66 to cytosines (Fig. 1G). Although the A•A/A•A construct maintains adenosines at positions 49 and 50 in the bulge, it replaces the conserved bases C65 and G66 with adenosines (Fig. 1E). A Core Helix sequence, was designed by eliminating the bulge

sequence (Fig. 1H). RNA designed by base pairing the bulge sequence forms a structure that is too stable for these experiments. The DNA equivalent of the wild-type RNA construct was relatively unstable and hence, additional G–C pairs were added to stabilize the DNA construct (Fig. 1I).

All RNA and DNA constructs were designed to be non-self-complementary. RNA strands were ordered from Dharmacon, Inc. and DNA strands were ordered from Integrated DNA Technologies. RNA and DNA were purified with a thin-layer chromatography using 20 cm $\times$ 20 cm, 500- μ m-thick silica plates with a running buffer of 6:3:1 (v/v/v) 1-propanol, 30% ammonium hydroxide, and water. The samples were spin filtered to remove any excess silica and dried using SPD1010 SpeedVac System (Savant). RNA samples were deprotected in a 100-mM acetic acid buffer adjusted to pH 3.8 with TEMED, using manufacture's protocol. All samples were desalted using C18 Sep-Pak column (Waters). The loading buffer was 5 mM sodium bicarbonate at pH 6. Elution was performed twice using 2 mL of 30% acetonitrile, and once using 2 mL of 100% acetonitrile. The fractions containing RNA or DNA were dried, and the concentrations were determined using extinction coefficient derived from pairwise values for nearest neighbors.

Thermal melting experiments and data analysis

All thermodynamic data were collected on a Cary 100 Bio UV Visible Spectrophotometer fitted with 6×6 Peltier unit. Buffer conditions used were similar to those used for biochemical experiments. Samples were prepared with equimolar amounts of each RNA or DNA strand. All samples were run in a buffer containing 10 mM cacodylic acid, 10 mM KCl, and 0.5 mM EDTA at pH 7.5 (0 mM, pH 7.5 buffer) and pH 5.5 (0 mM, pH 5.5 buffer). For divalent metal ion experiments, appropriate amounts of magnesium chloride were added to the 0 mM buffer. The concentrations of divalent ions were established by complexometric titrations or atomic absorption spectroscopy (with the detection limit in low- to submicromolar range, respectively). The buffers were labeled based on the net concentration of divalent ions. For example, 10 mM cacodylic acid, 10 mM KCl, 0.5 mM EDTA, and 10 mM MgCl_2 buffer was labeled as 9.5 mM Mg^{2+} buffer. Thermodynamic parameters for each construct were also measured in 1 M KCl (added to the 0 mM buffer) and were used as reference measurement for divalent-specific interactions. Thermodynamic analyses performed here are similar to work published previously and allows for determining changes in RNA stabilities with respect to its own stability under different buffer conditions; thus, a comparative analysis is possible between diverse RNA (O'Connell et al. 2008).

Thermodynamic data were collected using a nine-step dilution scheme for thermal melting experiments as described previously (O'Connell et al. 2008). Briefly, the change in RNA absorbance was monitored at a wavelength of 260 nm. The sigmoidal curves generated by thermal melting experiments were analyzed using the two-state model (Xia et al. 1999; Matthews et al. 2004; Mathews and Turner 2006). Linear sloping baselines and temperature-independent enthalpy and entropy values were assumed to generate thermodynamic parameters using the Meltwin program to fit individual melting curves (McDowell and Turner 1996). Thermodynamic parameters generated by individual curve fit were compared with those generated by T_m^{-1} versus $\log(C_T/4)$ plot for non-self-complementary RNA using the following equations (where T_m is the melting temperature, C_T is the total concentration of RNA, and R is the gas constant):

$$T_m^{-1} = 2.303 \left(\frac{R}{\Delta H^\circ} \right) \log \left(\frac{C_T}{4} \right) + \left(\frac{\Delta S^\circ}{\Delta H^\circ} \right), \quad (1)$$

$$\Delta G^\circ = \Delta H^\circ - T \Delta S^\circ. \quad (2)$$

Thermodynamic data reported is from the van't Hoff analysis [T_m^{-1} versus $\log(C_T/4)$ plots]. The errors in our measurements are <11% for ΔH° , <15% for ΔS° , and <3% for ΔG°_{37} . The errors in the van't Hoff analysis are considered to be 15% for ΔH° and ΔS° , and 5% for ΔG°_{37} ; hence, we indicate this in our values by using a smaller font size for the last digit of the values reported in Table 1.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

ACKNOWLEDGMENTS

This work was funded through National Science Foundation Grant No. MCB-0950582 (N.G.), Venture Grants, and through

divisional and college-wide funding. We thank the Department of Chemistry and Biochemistry at the Colorado College for prioritizing undergraduate research.

Received May 14, 2010; accepted January 17, 2011.

REFERENCES

- Boulard Y, Cognet JAH, Fazakerley GV. 1997. Solution structure as a function of pH of two central mismatches, C•T and C•C, in the 29 to 39 K-ras gene sequence, by nuclear magnetic resonance and molecular dynamics. *J Mol Biol* **268**: 331–347.
- Breaker RR. 2009. Riboswitches: from ancient gene-control systems to modern drug targets. *Future Microbiol* **4**: 771–773.
- Chen G, Kennedy SD, Turner DH. 2009. A CA+ pair adjacent to a sheared GA or AA pair stabilizes size-symmetric RNA internal loops. *Biochemistry* **48**: 5738–5752.
- Edwards TE, Ferré-D'Amaré AR. 2006. Crystal structures of the *thi*-box riboswitch bound to thiamine pyrophosphate analogs reveal adaptive RNA-small molecule recognition. *Structure* **14**: 1459–1468.
- Edwards TE, Klein DJ, Ferré-D'Amaré AR. 2007. Riboswitches: small-molecule recognition by gene regulatory RNAs. *Curr Opin Struct Biol* **17**: 273–279.
- Kierzek R, Burkard ME, Turner DH. 1999. Thermodynamics of single mismatches in RNA duplexes. *Biochemistry* **38**: 14214–14223.
- Kulshina N, Edwards TE, Ferré-D'Amaré AR. 2010. Thermodynamic analysis of ligand binding and ligand binding-induced tertiary structure formation by the thiamine pyrophosphate riboswitch. *RNA* **16**: 186–196.
- Lai EC. 2003. RNA sensors and riboswitches: self-regulating messages. *Curr Opin Struct Biol* **13**: 285–291.
- Lang K, Rieder R, Micura R. 2007. Ligand-induced folding of the *thiM* TPP riboswitch investigated by a structure-based fluorescence spectroscopic approach. *Nucleic Acids Res* **35**: 5370–5378.
- Mandal M, Breaker RR. 2004. Gene regulation by riboswitches. *Nat Rev Mol Cell Biol* **5**: 451–463.
- Mathews DH, Turner DH. 2006. Prediction of RNA secondary structure by free energy minimization. *Curr Opin Struct Biol* **16**: 270–278.
- Mathews DH, Disney MD, Childs JL, Schroeder SJ, Zuker M, Turner DH. 2004. Incorporating chemical modification constraints into a dynamic programming algorithm for prediction of RNA secondary structure. *Proc Natl Acad Sci* **101**: 7287–7292.
- McDowell JA, Turner DH. 1996. Investigation of the structural basis for thermodynamic stabilities of tandem GU mismatches: Solution structure of (rGAGGUCUC)₂ by two-dimensional NMR and simulated annealing. *Biochemistry* **35**: 14077–14089.
- Miranda-Ríos J. 2007. The *THI*-box riboswitch, or how RNA binds thiamine pyrophosphate. *Structure* **15**: 259–265.
- Miranda-Ríos J, Navarro M, Soberón M. 2001. A conserved RNA structure (*thi* box) is involved in regulation of thiamin biosynthetic gene expression in bacteria. *Proc Natl Acad Sci* **98**: 9736–9741.
- Nahvi A, Sudarsan N, Ebert MS, Zou X, Brown KL, Breaker RR. 2002. Genetic control by a metabolite binding mRNA. *Chem Biol* **9**: 1043–1049.
- O'Connell IC, Booth D, Eason B, Grover N. 2008. Thermodynamic examination of trinucleotide bulged RNA in the context of HIV-1 TAR RNA. *RNA* **14**: 2550–2556.
- Ontiveros-Palacios N, Smith AM, Grundy FJ, Soberón M, Henkin TM, Miranda-Ríos J. 2008. Molecular basis of gene regulation by the *THI*-box riboswitch. *Mol Microbiol* **67**: 793–803.
- SantaLucia J Jr, Kierzek R, Turner DH. 1990. Effects of GA mismatches on the structure and thermodynamics of RNA internal loops. *Biochemistry* **29**: 8813–8819.

- SantaLucia J Jr, Kierzek R, Turner DH. 1991. Stabilities of consecutive A•C, C•C, G•G, U•C, U•U mismatches in RNA internal loops: Evidence for stable hydrogen-bonded U•U and C•C+ pairs. *Biochemistry* **30**: 8242–8251.
- Schroeder SJ, Turner DH. 2000. Factors affecting the thermodynamic stability of small asymmetric internal loops in RNA. *Biochemistry* **39**: 9257–9274.
- Schroeder SJ, Burkard ME, Turner DH. 1999. The energetics of small internal loops in RNA. *Biopolymers* **52**: 157–167.
- Serganov A, Polonskaia A, Tuân Phan A, Breaker RR, Patel DJ. 2006. Structural basis for gene regulation by a thiamine pyrophosphate-sensing riboswitch. *Nature* **441**: 1167–1171.
- Siegfried NA, Metzger SL, Bevilacqua PC. 2007. Folding cooperativity in RNA and DNA is dependent on position in the helix. *Biochemistry* **46**: 172–181.
- Sudarsan N, Barrick JE, Breaker RR. 2003. Metabolite-binding RNA domains are present in the genes of eukaryotes. *RNA* **9**: 644–647.
- Sudarsan N, Cohen-Chalamish S, Nakamura S, Emilsson GM, Breaker RR. 2005. Thiamine pyrophosphate riboswitches are targets for the antimicrobial compound pyrithiamine. *Chem Biol* **12**: 1325–1335.
- Thore S, Leibundgut M, Ban N. 2006. Structure of the eukaryotic thiamine pyrophosphate riboswitch with its regulatory ligand. *Science* **312**: 1208–1211.
- Walter AE, Wu M, Turner DH. 1994. The stability and structure of tandem GA mismatches in RNA depend on closing base pairs. *Biochemistry* **33**: 11349–11354.
- Williams AP, Longfellow CE, Freier SM, Kierzek R, Turner DH. 1989. Laser temperature-jump, spectroscopic, and thermodynamic study of salt effects on duplex formation by dGCATGC. *Biochemistry* **28**: 4283–4291.
- Winkler W, Nahvi A, Breaker RR. 2002. Thiamine derivatives bind messenger RNAs directly to regulate bacterial gene expression. *Nature* **419**: 952–956.
- Wu M, McDowell JA, Turner DH. 1995. A periodic table of symmetric tandem mismatches in RNA. *Biochemistry* **34**: 3204–3211.
- Xia T, McDowell JA, Turner DH. 1997. Thermodynamics of non-symmetric tandem mismatches adjacent to G•C base pairs in RNA. *Biochemistry* **36**: 12486–12497.
- Xia T, SantaLucia J Jr, Burkard ME, Kierzek R, Schroeder SJ, Jiao X, Cox C, Turner DH. 1998. Thermodynamic parameters for an expanded nearest-neighbor model for formation of RNA duplexes with Watson-Crick base pairs. *Biochemistry* **37**: 14719–14735.
- Xia T, Mathews DH, Turner DH. 1999. Thermodynamics of RNA secondary structure formation. In *Prebiotic chemistry, molecular fossils, nucleosides, and RNA* (ed. DG Soll et al.), pp. 21–47. Elsevier, New York.
- Yamauchi T, Miyoshi D, Kubodera T, Nishimura A, Nakai S, Sugimoto N. 2005. Roles of Mg^{2+} in TPP-dependent riboswitch. *FEBS Lett* **579**: 2583–2588.
- Yu A, Barron MD, Romero RM, Christy M, Gold B, Dai J, Gray DM, Haworth IS, Mitas M. 1997. At physiological pH, d(CCG)₁₅ forms a hairpin containing protonated cytosines and a distorted helix. *Biochemistry* **36**: 3687–3699.

RNA

A PUBLICATION OF THE RNA SOCIETY

Thermodynamic examination of the pyrophosphate sensor helix in the thiamine pyrophosphate riboswitch

Stephanie Furniss and Neena Grover

RNA 2011 17: 710-717 originally published online March 2, 2011

Access the most recent version at doi:[10.1261/rna.2263211](https://doi.org/10.1261/rna.2263211)

Supplemental Material

<http://rnajournal.cshlp.org/content/suppl/2011/02/04/rna.2263211.DC1>

References

This article cites 35 articles, 6 of which can be accessed free at:
<http://rnajournal.cshlp.org/content/17/4/710.full.html#ref-list-1>

License

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).

To subscribe to *RNA* go to:
<http://rnajournal.cshlp.org/subscriptions>