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Thermodynamics and folding pathway of tetraloop receptor-mediated RNA helical packing

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Summary

Little is known about the thermodynamic forces that drive the folding pathways of higher order RNA structure. In this study, we employ calorimetric (ITC and DSC) and spectroscopic (NMR and UV) methods to characterize the thermodynamics of the GAAA tetraloop – receptor interaction, utilizing a previously described bivalent construct. ITC studies indicate that the bivalent interaction is enthalpy-driven and highly stable, with a binding constant (K_{obs}) of $5.5 \times 10^6 \text{ M}^{-1}$ and enthalpy ($\Delta H_{\text{obs}}^\circ$) of -33.8 kcal/mol at 45°C in 20 mM KCl and 2 mM MgCl_2 . Thus we derive the $\Delta H_{\text{obs}}^\circ$ for a single tetraloop-receptor interaction to be -16.9 kcal/mol at these conditions. UV absorbance data indicate that an increase in base stacking quality contributes to the enthalpy of complex formation. These highly favorable thermodynamics are consistent with the known critical role for the tetraloop-receptor motif in the folding of large RNAs. Additionally, a significant heat capacity change ($\Delta C_{p,\text{obs}}^\circ$) of $-0.24 \text{ kcal}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$ was determined by ITC. DSC and UV monitored thermal denaturation experiments indicate that the bivalent tetraloop-receptor construct follows a minimally 5-state unfolding pathway, and suggest the observed $\Delta C_{p,\text{obs}}^\circ$ for the interaction results from a temperature-dependent unbound receptor RNA structure.

Introduction

An understanding of the RNA folding problem, and the ability to predict RNA structure from sequence through free energy minimization, requires detailed knowledge of the thermodynamic forces that drive folding. At the secondary structure level, free energy parameters have been measured for many (but not all) helical sequences, and algorithms have been developed that can predict RNA secondary structure with reasonable accuracy (1). RNA folding is largely hierarchical, with secondary structure forming its final state prior to tertiary structure formation. However, strong tertiary interactions and ligand binding can remodel existing secondary structure (2–4).

The 11 nt GAAA tetraloop – receptor motif (hereafter referred to as the tetraloop – receptor) is an important and commonly occurring RNA tertiary structure motif that mediates helical packing, and has been found in the group I and group II self-splicing introns (5), as well as RNase P (6). In the folding of the *Tetrahymena thermophila* group I intron, the tetraloop –

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receptor interaction mediates the very earliest (100 ms – 1 s) (7–9) and most stable (10–13) specific global compaction event. Disruption of the tetraloop – receptor interaction alters the intron folding pathway (14) and destabilizes tertiary interactions in the final structure by 2–3 kcal/mol (13,15).

The tetraloop – receptor is modular, and can be studied through the use of bivalent constructs with C_2 -symmetry that associate either as heterodimers or homodimers (Figure 1A and B) (16). These constructs assemble into well-defined RNA quaternary structures, mediated solely by the tetraloop – receptor interaction. We solved the NMR structure of the homodimeric tetraloop receptor RNA construct (Figure 1B) (17–19). Additionally, we have studied the free receptor as a monomeric construct (Figure 1C) that contains a double mutation in the tetraloop (GAAA to GUGA), which abolishes binding while maintaining a stable helical fold (18). The crystal structure of the tetraloop – receptor interaction has been solved in larger RNAs (20–22). The crystal and NMR structures of the bound receptor are in excellent agreement, exhibiting the same stacking and hydrogen bonding interactions and similar patterns of ion localization (17).

The structure of the tetraloop – receptor interaction can be decomposed into several smaller motifs of RNA structure, including: a U-turn in the GAAA tetraloop (23), an adenine platform in the receptor that forms a stacking interaction with the 5' adenine of the tetraloop (24), 2 ribose zipper motifs, in which 2'-hydroxyl groups on the tetraloop and receptor form two interdigitated hydrogen bonds (20,25), a hydrogen bond between 2'-hydroxyl groups of Watson-Crick base pairs adjacent to the receptor and the tetraloop, and A-minor interactions (26) between the tetraloop adenines and the receptor that result in formation of a base triple and a base quadruplet (Figure 1D). In total, docking of the tetraloop into the receptor results in formation of 10 intermolecular hydrogen bonds and 1 purine-purine stacking interaction (the 5' tetraloop adenine on the A-platform). In addition, a uracil from the receptor is bulged into an extrahelical conformation upon docking of the tetraloop (Figure 1D).

The GAAA tetraloop appears to dock as a relatively rigid body (20,27), while the receptor undergoes induced fit upon docking. NMR studies revealed an alternative conformation for the free receptor (28), and even in 125 mM $MgCl_2$ the bound conformation of the receptor is not significantly populated in the absence of tetraloop (29). FRET measurements with a tethered tetraloop – receptor construct revealed a significant docking activation energy as large as 12.7 kcal/mol (30).

The thermodynamics of association between the GAAA tetraloop and its receptor, and for interactions mediating RNA helical packing in general, are not well understood. Isothermal titration calorimetry (ITC) and differential scanning calorimetry (DSC) are powerful and accurate approaches toward this goal. Calorimetric studies in RNA systems have been primarily limited to the analysis of secondary structure formation or the multistep unfolding of large molecules (31–34), although more recent work has directly investigated tertiary interactions (35–37). In this study, we have taken advantage of a well-defined RNA system, in which two helices associate specifically through non-Watson-Crick interactions, to investigate the thermodynamics of tetraloop – receptor mediated RNA helical packing. We find the association process occurs with large and negative ΔH_{obs}° and $\Delta C_{p,obs}^\circ$ values. The ΔH_{obs}° can be attributed in part to an increase in base stacking interactions in the tetraloop – receptor interface and potentially to the formation of intermolecular hydrogen bonds. We propose that the $\Delta C_{p,obs}^\circ$ is rooted in the temperature-dependent coupling of binding to an equilibrium between a low temperature ('native') and intermediate forms of the free RNA. Thermal denaturation experiments demonstrate good agreement between the heterodimeric and homodimeric constructs, indicating that the thermodynamic measurements are not

influenced by construct design. Finally, we find that thermally-induced unfolding of the bivalent tetraloop – receptor interaction is modeled best by a minimally 5-state pathway.

Results

Titration Calorimetry Study of Tetraloop – Receptor Driven Heterodimeric Construct Association

Because the isolated tetraloop – receptor does not form a highly stable complex (30,38), we have made use of engineered bivalent constructs (Figure 1) (16). These constructs more than double the magnitude of $\Delta G_{\text{obs}}^{\circ}$ for bimolecular association, making calorimetric and structural studies practical. Previously, we studied structural features of the interaction using the homodimeric bivalent construct (17,18).

Isothermal titration calorimetry (ITC) was used to probe the thermodynamic basis of stability of the tetraloop – receptor interaction. These experiments employed heterodimeric construct (Figure 1a) (16), whereby a molecule containing two GAAA tetraloops separated by a helical turn (the TT construct) binds in a bivalent fashion to a molecule containing two receptor motifs (RR). The sequences of the heterodimeric RNAs for this study were optimized to promote proper folding, and native gels verified formation of a single high-affinity dimeric complex (Supplemental Figure S1). The RR RNA is > 95 % conformationally homogenous, as determined by native gel electrophoresis. However, the dumbbell-shaped TT construct contains a minor misfolded population (Supplemental Figure S1). This sub-population does not bind RR, so it does not significantly interfere with measurement of the binding constant (K_{obs}). The ITC fitting equation considered only the active population of TT, which varied slightly depending upon the RNA preparation (see Materials and Methods).

Representative forward and reverse ITC titrations in solution conditions similar to those used in previous NMR experiments (20 mM KCl, 2 mM MgCl₂, 20mM HEPES, pH 7.0, 30 °C) reveals the formation of a highly stable complex (Figure 2). In a forward titration (Figure 2), TT is serially injected into a cell containing RR, and the roles are exchanged in a reverse titration. Titrations in both directions demonstrate a stoichiometry near 1:1, consistent with a bivalent association process. The sharp transition of the normalized heat signal in Figure 2 from the initial plateau value to baseline value indicates that essentially all of the injected TT is bound until the free population of RR is depleted. In this case, only a lower bound on K_{obs} can be determined ($K_{\text{obs}} \geq 2 \times 10^7 \text{ M}^{-1}$). The enthalpy of binding ($\Delta H_{\text{obs}}^{\circ}$) is equal to the normalized heat signal of an injection within the initial plateau region of the titration. Binding is highly exothermic, with $\Delta H_{\text{obs}}^{\circ} = -28.7 (\pm 1.0) \text{ kcal/mol}$ in the experiment shown in Figure 2. The complementary reverse titration gives a $\Delta H_{\text{obs}}^{\circ}$ of $-27.2 (\pm 0.9) \text{ kcal/mol}$ (Supplemental Table S1).

Representative sets of forward titrations spanning the temperature range of 10 °C to 47.5 °C are shown (Figure 3). All datasets exhibit negative normalized heat signals, indicating that binding is exothermic over the entire temperature range. $\Delta H_{\text{obs}}^{\circ}$ is least negative at 10 °C and decreases (increases in magnitude) as temperature is increased to 47.5 °C. As observed at 30 °C, titrations performed at 10 °C and 20 °C yield binding isotherms that transition sharply from the flat initial plateau region to the final baseline, so that the binding constant cannot be resolved. At 40 °C and above, binding is weaker as expected for an exothermic interaction, and K_{obs} is measurable. The average values of K_{obs} and $\Delta H_{\text{obs}}^{\circ}$ at each temperature are listed, along with the free energy change for binding ($\Delta G_{\text{obs}}^{\circ} = -RT \ln K_{\text{obs}}$) and its entropic contribution ($T\Delta S_{\text{obs}}^{\circ} = \Delta H_{\text{obs}}^{\circ} - \Delta G_{\text{obs}}^{\circ}$) (Table 1).

The ITC-determined $\Delta H_{\text{obs}}^\circ$ values are plotted against experimental temperature (Figure 4a). The binding enthalpy becomes more exothermic at higher temperature, varying linearly from an average value of $-26.0 (\pm 1.8)$ kcal/mol at 10 °C to $-37.2 (\pm 1.8)$ kcal/mol at 47.5 °C. The heat capacity change ($\Delta C_{p,\text{obs}}^\circ$) for binding can be determined directly from the temperature dependence of the enthalpy change according to

$$\Delta C_{p,\text{obs}}^\circ = \frac{\partial \Delta H_{\text{obs}}^\circ}{\partial T} \quad \text{Equation (1)}$$

A linear fit to the $\Delta H_{\text{obs}}^\circ$ measurements gives a slope and estimated $\Delta C_{p,\text{obs}}^\circ$ for the interaction of $-0.24 (\pm 0.04)$ kcal/(mol K) (solid line in Figure 4a). Below we propose a temperature-dependent free receptor structure as the source of the $\Delta C_{p,\text{obs}}^\circ$ (see Discussion).

The logarithms of the measured binding constants are also plotted against temperature (Figure 4b). $\log K_{\text{obs}}$ decreases monotonically with temperature, from $7.0 (\pm 0.2)$ at 40 °C to $6.6 (\pm 0.2)$ at 47.5 °C (Table 1). The decrease in complex stability with increasing temperature follows from the exothermicity according to the van't Hoff relationship:

$$\frac{\partial \log K_{\text{obs}}}{\partial T} = \frac{\Delta H_{\text{obs}}^\circ}{2.303RT^2} \quad \text{Equation (2)}$$

Over a temperature range where $\Delta C_{p,\text{obs}}^\circ$ is independent of temperature (i.e., at least 10 – 47.5 °C), integration of equation (2) yields equation (3) for $\log K_{\text{obs}}$ at any temperature in terms of $\log K_{\text{obs}}$ and $\Delta H_{\text{obs}}^\circ$ at an arbitrary reference temperature T_{ref}

$$\log K_{\text{obs}} = \log K_{\text{ref}} - \frac{\Delta H_{\text{ref}}^\circ}{2.303RT} \left(1 - \frac{T}{T_{\text{ref}}} \right) + \frac{\Delta C_{p,\text{obs}}^\circ}{2.303R} \left(\frac{T_{\text{ref}}}{T} - 1 + \ln \left(\frac{T}{T_{\text{ref}}} \right) \right) \quad \text{Equation (3)}$$

The dotted curve in Figure 4b plots equation (3) using a reference temperature of 40 °C, where the heat capacity change ($\Delta C_{p,\text{obs}}^\circ = -0.24$ kcal/(mol · K)) and enthalpy change at 40 °C ($\Delta H_{40\text{C}}^\circ = -33.0$ kcal/mol) were set equal to the values determined by the abovementioned linear fitting of $\Delta H_{\text{obs}}^\circ$ versus temperature, and the reference $\log K_{\text{obs}}$ ($\log K_{40\text{C}} = 7.1$) was determined through least squares fitting. The temperature-dependence of the experimental $\log K_{\text{obs}}$ agrees well with both the direction and magnitude of this prediction, demonstrating excellent consistency among all of the measured thermodynamics.

Thermodynamic Stability of the Homodimeric Tetraloop – Receptor Complex

A detailed examination of the temperature-induced unfolding of an RNA molecule containing both secondary and higher order structure can be conducted with these constructs. The quaternary structure in this bimolecular system mimics the long-range tertiary interactions involved in helical packing of large RNAs. The homodimeric construct design (the TR construct; Figure 1b) (16–18) *per se* is amenable to denaturation studies because of its homogeneous unfolding profile. As in the heterodimeric system, two C₂-symmetry-related tetraloop – receptor interactions drive dimerization, so the bimolecular association thermodynamics are expected to be similar. In order to unambiguously assign unfolding

transitions to the underlying structural events, a monomeric negative control was also utilized (T_{GUGAR} ; Figure 1c).

Mechanistic features of the unfolding process could be identified using NMR. The chemical shift of the imino proton of G9, a guanine residue within the receptor that forms an intermolecular hydrogen bond to a tetraloop adenine as part of a base quadruplet, is a robust marker for the formation of the tetraloop – receptor complex (Figure 5). Below 50 °C, the RNA is fully in the dimeric form and exhibits a G9 imino proton chemical shift at 12.60 ppm (18). At 50 °C, a small amount of dissociation becomes detectable as indicated by the presence of the monomeric G9* peak at 12.15 ppm (Figure 5). Half dissociation of TR occurs between 68 and 72 °C under these conditions (80 μM RNA, 20 mM KCl, 2 mM MgCl_2). The RNA is nearly fully dissociated into monomers by 76 °C, and a further increase in temperature causes the long helix to unfold. The G9* monomer peak diminishes simultaneously with all base pairing chemical shifts in this region (Figure 5; data not shown).

To further examine structural aspects of the unfolding process, we performed UV absorbance thermal denaturation experiments with both TR and T_{GUGAR} (Figure 6). Figure 6 plots UV data for the two RNAs in terms of the fractional hyperchromicity (normalized increase in absorbance). Over the course of the melting experiment, the increase in absorbance at 260 nm approaches 30% for either construct, comparable to the increase expected for denaturation of a double helix. Closer inspection of the curves reveals two significant and useful differences in the profiles of the two RNA molecules. The TR construct elicits an overall hyperchromic effect of $30.4 (\pm 0.2)\%$, which is reproducibly larger than the $28.6 (\pm 0.5)\%$ hyperchromicity displayed by T_{GUGAR} (Figure 6). This finding suggests that disruption of the tetraloop – receptor interaction decreases the number and/or quality of base stacking interactions, providing a means to monitor the tertiary interaction. Correspondingly, the absorbance of the TR construct begins increasing gradually with temperature at approximately 45 °C, while the increase for the T_{GUGAR} construct begins at 55 °C, demonstrating that dissociation is the first unfolding event that occurs with accompanying hyperchromicity. First derivative plots of the UV data discern a small peak denoting a transition midpoint in the TR data at 51 °C, an attribute not present in T_{GUGAR} (Figure 6 inset).

Differential scanning calorimetry (DSC) was employed to characterize the unfolding mechanism of the homodimer and the thermodynamics of the tetraloop – receptor interaction. Here DSC directly reports thermodynamic information by measuring C_p^{ex} , the excess heat capacity of the RNA solution relative to a buffer control, as a function of temperature. When expressed on a molar basis, this quantity relates directly to $C_{p,\text{obs}}$, the partial molar heat capacity of the RNA in this solution (Materials and Methods). In general, the positive enthalpy change associated with a thermally-induced cooperative unfolding transition causes an increase in C_p^{ex} ; in the absence of additional transitions, the resulting peak occurs with a maximum near the T_m (melting temperature) and an area equal to the transition $\Delta H_{\text{obs}}^\circ$.

A representative raw experimental C_p^{ex} trace for 430 μM TR demonstrates multi-step unfolding for the homodimer (Figure 7a). As the temperature is raised from 8 °C, C_p^{ex} initially increases gradually with a small constant slope of $\partial C_p^{\text{ex}}/\partial T = 23 (\pm 1) \text{ cal} \cdot \text{mol}^{-1} \cdot \text{K}^{-2}$ (i.e., $1.6 (\pm 0.1) \times 10^{-3} \text{ cal} \cdot \text{g}^{-1} \cdot \text{K}^{-2}$). This temperature-dependence is comparable to that observed for stable globular proteins (39), but is significantly smaller than that observed for short duplexes or polymeric single-stranded nucleic acids, for which temperature-dependent noncooperative processes (end fraying, stacking) comprise a significant fraction of the molecule (40–42). This suggests a relatively stable folded complex; notably, $\partial C_p^{\text{ex}}/\partial T$ for the monomeric T_{GUGAR} construct is 30% larger on a molar or weight scale (see below). Above 55 °C, the presence of

two distinct but overlapping peak regions indicate multi-state unfolding. The first peak region is broad with a maximum near 76 °C and an integrated area of approximately 70 kcal/mol, and the second is much sharper and larger with a maximum at 86 °C and an integrated area of approximately 120 kcal/mol. A large $\Delta C_{p,obs}^{\circ}$ of +1 – +2 kcal/mol is discerned from the shift in the baseline C_p^{ex} in these transitions (Figure 7a). The bulk of this $\Delta C_{p,obs}^{\circ}$ likely arises from melting of regions of duplex, and is consistent with recent measurements (31, 40, 41, 43–46).

In order to unambiguously characterize the unfolding mechanism, DSC experiments were carried out over a range of concentrations with both bound (TR) and free (T_{GUGAR}) RNA (Figures 7b and c). Figure 7b shows molar-normalized C_p^{ex} data for the TR construct over a concentration range of 60 μM to 570 μM, which have been offset to reflect the RNA partial molar heat capacity ($C_{p,obs}$). Increasing [RNA] both delays the onset and increases the height of the low temperature transition region. The DSC experiments with T_{GUGAR} also demonstrate greater than 2-state unfolding, with a small low-temperature peak preceding a larger and narrower peak with a maximum at 86 °C (Figure 7c). The low temperature peak is unquestionably a separate transition (or set of transitions) since fitting to the overall progress of T_{GUGAR} DSC or UV melts (see below) yields a van't Hoff $\Delta H_{obs}^{\circ} \approx 35$ kcal/mol, much smaller than the quantity measured directly through integration of the DSC data. Figure 7c also reveals a complete lack of [RNA] dependence in T_{GUGAR} melting, in contrast to the TR construct. These results indicate that dissociation of TR occurs within or below the low-temperature peak, and demonstrate that T_{GUGAR} does not form higher order structure. Thus, T_{GUGAR} unfolding is minimally a 3-state process and TR, which possesses an additional [RNA]-dependent step, is minimally 4-state. Below we show that the data are more accurately modeled as respective 4- and 5-state unfolding processes.

Discussion

Analysis of the unfolding pathway

The simplest thermodynamic mechanism capable of describing the unfolding data is shown (Figure 8). In this scheme, the first unfolding event is dissociation of the TR homodimeric complex (C) into native monomers (N), which is followed by three isomerization steps that are independent of [RNA]. Implicit in this scheme is the I₁ intermediate, which is modeled as a monomer with a partially unfolded receptor. The proposed unfolding pathway is minimally a 5-state process for the TR complex ($C \leftrightarrow 2N \leftrightarrow 2I_1 \leftrightarrow 2I_2 \leftrightarrow 2U$) and a 4-state process for monomeric T_{GUGAR} ($N \leftrightarrow I_1 \leftrightarrow I_2 \leftrightarrow U$). Data supporting the scheme in Figure 8 are highlighted below and detailed further in the accompanying Supplemental Material. (The insufficiency of a 4-state TR unfolding model is also demonstrated in the supplemental section).

We propose that the first non-dissociative unfolding event ($N \leftrightarrow I_1$) is detected as an extremely broad, low temperature transition and is evidenced in the elevated low temperature slope $\partial C_p^{\text{ex}} / \partial T$ in the T_{GUGAR} DSC data (Supplemental Figure 3). In TR, isomerization is suppressed to higher temperatures due to stable complex formation. Additional data argues for the free receptor as the transition locus, as modeled in Figure 8 (the physical location is however unessential to the unfolding data analysis). NMR studies at similar temperatures demonstrate a highly dynamic free receptor (unpublished results), and previous thermodynamic studies found the free receptor motif to be enthalpically-stabilized but overall destabilizing in the context of a short RNA duplex (47). $N \leftrightarrow I_1$ would probably be more accurately described as the result of noncooperative unstacking and base pair fraying within the receptor, but for modeling simplicity it is described as a single transition. The next transition ($I_1 \leftrightarrow I_2$) is sharper and minimally composed of denaturation of the lower helix. The final step ($I_2 \leftrightarrow U$) consists of unfolding of the 12 bp tetraloop-capped helix.

A unique unfolding parameter set cannot be fully determined through global fitting, due to correlation between the dissociative and first isomerization steps. We present a semi-quantitatively optimized set of parameters in Table 2, which successfully reproduce the full set of denaturation data (Figure 9). The simulated 5-state DSC melting profiles for TR predict the experimentally-observed [RNA]-dependent sharpening and increasing height of the low temperature peak, as well as the increasing temperature for dissociation-initiated unfolding (Figure 9a; Supplemental Figure S7). The simulations also recapitulate the T_{GUGAR} DSC data (Figure 9b). The first T_{GUGAR} transition is broad and only half-complete by 50 °C, so it contributes positively to the low temperature slope (see light gray lines); in contrast, for TR at 15 μM or greater, this transition is largely suppressed to temperatures above 50 °C by the TR dimer equilibrium. The unfolding model simultaneously describes the UV experiments with TR and T_{GUGAR}, which were conducted at > 10-fold lower RNA concentrations (Figure 9c).

The 5-state model is reasonably successful in predicting the experimentally-determined complex stability. At the apparent T_m, the apparent dissociation constant (K_{d,app}) is equal to the total RNA concentration, so each melting experiment with TR yields this stability measurement. K_{d,app} was directly determinable from UV and NMR TR data as 1.5 μM at 51 °C and 80 μM at 70 °C, respectively. Using difference thermograms, K_{d,app} values could also be determined from each DSC experiment with TR (Supplemental Figure S4). Figure 10 plots all of these values, along with the apparent K_d determinations by ITC. The overall level of agreement among data gathered with two different constructs and using multiple assays is remarkable, even while visual extrapolation of the ITC datapoints suggests that the heterodimeric complex is slightly more stable than the homodimer. The K_{d,app} function determined from the optimized 5-state model is also plotted in the figure according to

$$K_{d,app} = K_d(1 + K_{I_1}(1 + K_{I_2}(1 + K_U)))^2 \approx K_d(1 + K_{I_1}(1 + K_{I_2}))^2 \quad \text{Equation (4)}$$

The approximation in the second half of equation (4) is possible because dissociation always occurs well below 86 °C, so that (1 + K_U) ≈ 1. The temperature-dependence of K_{d,app} is reproduced well by the 5-state model, although it overestimates K_{d,app}. This may relate to the underlying noncooperative or multi-state nature of the first isomerization step, which would reduce K_{d,app}. The simulated ΔH_{obs}° values are also consistent with the proposed molecular mechanism. The dissociation (C ↔ 2N) and first isomerization (N ↔ I₁) transitions exhibit the smallest ΔH_{obs}° values (10.7 and 21.9 kcal/mol at 45 °C), the sum of which matches the ITC-determined ΔH_{obs}° (−33.8 kcal/mol at 45 °C). The ΔH_{obs}° for the dissociation step alone is consistent with the model-independent value determined by either comparison of the integrated T_{GUGAR} and TR endotherms (ΔH_{dissociation}° ≤ 23 kcal/mol) or by integration of the difference thermograms (ΔH_{dissociation}° = 5 ± 6 kcal/mol) (Supplemental Figure S6 and Figure S7). The ΔH_{obs}° for N ↔ I₁ also approaches the previously-determined free receptor enthalpic contribution to duplex unfolding (13.3 ± 7.0 kcal/mol) (47). The final two steps are well-constrained by fitting and the enthalpy values agree with nearest neighbor model predictions (48–50) (Table 2).

Analysis of Binding Thermodynamics

We have characterized the thermodynamics of the GAAA tetraloop – receptor motif and report the enthalpy of association for this common RNA tertiary interaction. Experimentally-measured ΔH_{obs}° values above contain the sum of two tetraloop – receptor site binding enthalpies (ΔH_{site}°) and any construct-specific additional contributions (ΔH_{other}°) so that

$\Delta H_{\text{obs}}^{\circ} = 2\Delta H_{\text{site}}^{\circ} + \Delta H_{\text{other}}^{\circ}$. We expect that $\Delta H_{\text{other}}^{\circ}$ is minimal because its primary contribution probably comes from RNA helical packing. The homodimeric NMR structure indicates that both helices remain hydrated outside of the tetraloop – receptor interface (no H-bond pairs within 3.5 Å and the closest interhelical phosphate oxygen distance is 5.5 Å (18,19)), so the enthalpic component of helical packing is probably small. $\Delta H_{\text{other}}^{\circ}$ might also include a small positive contribution due to the helical strain induced by alignment of the partner motifs. The backbone discontinuity within the helical section of the TT construct may reduce this penalty; in fact the heterodimeric $\Delta G_{\text{obs}}^{\circ}$ is only 0.6 kcal/mol less than the homodimeric value (Figure 10). Thus the $\Delta H_{\text{other}}^{\circ}$ contribution, which might regardless be viewed as intrinsic to the tetraloop – receptor interaction in natural contexts, is not large and allows the approximation

$$\Delta H_{\text{obs}}^{\circ} = 2\Delta H_{\text{site}}^{\circ} \quad \text{Equation (5)}$$

$\Delta H_{\text{site}}^{\circ}$ for formation of a single tetraloop – receptor interaction as observed directly by ITC is therefore large and negative, ranging from $-13.0 (\pm 1.0)$ kcal/mol at 10 °C to $-18.6 (\pm 0.9)$ kcal/mol at 47.5 °C. This result agrees extremely well with a recent single molecule study by Nesbitt and co-workers, who obtained a binding enthalpy of $-17.2 (\pm 1.6)$ for a single tetraloop – receptor interaction in very similar solution conditions (1 mM MgCl₂ and 100 mM NaCl) according to the temperature-dependence of association from 21 to 47.4 °C (Fiore et al, 2008). This result dovetails with that of Cech and co-workers, who found that formation of tertiary structure in the P4–P6 domain of the *Tetrahymena* group I intron is enthalpy-driven with $\Delta H_{\text{obs}}^{\circ} = -28$ kcal/mol in 0.9 mM MgCl₂ at 39 °C (51). In addition to the P5b tetraloop – P6a receptor contact, the expansive P4–P6 fold is largely stabilized by the interaction between the P5a A-rich bulge and P4, so the enthalpic driving force may contain significant contributions from both motifs.

Favorable enthalpy changes in nucleic acid helix formation and folding generally derive from increases in base stacking and hydrogen bonding, and may also include contributions from coupled processes like protonation or hydration of a released coordinated ion (25,52–54). The hyperchromic effect revealed by UV melting experiments argues strongly for a stacking component. The dissociative hyperchromic effect is 6% as large as the combined remaining unfolding steps, which essentially amount to the unfolding of a 17 bp stem-loop. We can estimate that an increase equivalent to approximately 2 base stacking interactions occurs with binding, and could contribute approximately -10 kcal/mol (40,46,48). Structural inspection provides clues to the location of these stacking interactions. Clearly, intermolecular base stacking between the tetraloop adenine and the A-platform of the receptor contributes to the hyperchromic effect, although at the expense of extrahelical bulging of receptor uracil (18, 20). Perhaps more importantly, tetraloop docking may also increase the quality of base stacking within the receptor. Binding induces a significant receptor conformational change, whereby formation of the adenine platform and an A•U reverse Hoogsteen pair generates a continuous helical stack that is propagated from the receptor helix into the tetraloop. Structural quantification of the net increase in base stacking is difficult, due to the dynamic disorder exhibited by the free receptor (28)(manuscript in preparation). At the same time, the dynamics make plausible a decrease in the quantity of free receptor base stacking with increasing temperature (in addition to intra-receptor hydrogen bonding) as the source of the $\Delta C_{p,\text{obs}}^{\circ}$. The change in stacking upon binding within the tetraloop is probably minimal (18,20,23,27). Hydrogen bonding may also contribute to the exothermicity, and there are ca. 10 intermolecular hydrogen bonding interactions in the interface (Figure 1d). Interdigitated 2'-hydroxyl groups have been demonstrated to be net stabilizing (25), so it is reasonable to also hypothesize an

exothermic contribution by hydrogen bond formation. An increase in ion hydration can be highly exothermic as well (52,55,56). However, this is unlikely here and in fact an endothermic contribution appears more justified, based on the proposed coordination of potassium in the binding interface (57) and preliminary studies of the $[\text{Mg}^{2+}]$ -dependence of binding (data not shown). Coupled protonation also does not appear to have a large effect on the enthalpy, as indicated by the similar $\Delta H_{\text{obs}}^{\circ}$ values obtained using either Tris or HEPES buffer (Table 1).

The negative $\Delta H_{\text{obs}}^{\circ}$ for tetraloop – receptor binding is consistent with previous measurements of RNA tertiary interactions. Where the enthalpy of stabilizing tertiary interactions has been determined, it has been exothermic (33,34,58–62), although we note these studies have primarily employed thermal denaturation techniques, so an endothermic tertiary interaction would be difficult to identify. Helical junctions form another class of ‘tertiary interaction’, which form as a consequence of optimized secondary structure. These junctions are net destabilizing, and the $\Delta H_{\text{obs}}^{\circ}$ is construct- and reference state-dependent (35–37)

Hypothesized origin of the $\Delta C_{p,\text{obs}}^{\circ}$

As for the enthalpy, $\Delta C_{p,\text{other}}^{\circ}$ is probably negligible; $\Delta C_{p,\text{site}}^{\circ}$ is well-determined in our studies as one-half the measured value

$$\Delta C_{p,\text{obs}}^{\circ} = 2\Delta C_{p,\text{site}}^{\circ} \quad \text{Equation (6)}$$

For the tetraloop – receptor interaction, analysis of ITC data yields

$$\Delta C_{p,\text{site}}^{\circ} = -0.12 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}.$$

What is the origin of this significant negative $\Delta C_{p,\text{site}}^{\circ}$? From the structure of the complex, assuming a rigid body interaction without coupled conformational changes, we calculate that 800 Å² of RNA surface area (531 Å² of polar surface and 239 Å² of nonpolar surface) is dehydrated and buried in the interaction of one tetraloop with its receptor. Burial of nonpolar surface makes a negative contribution to $\Delta C_{p,\text{obs}}^{\circ}$ estimated from aliphatic and aromatic hydrocarbon solubility data to be approximately $-0.32 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$ per Å²; burial of polar surface makes a positive contribution to the observed $\Delta C_{p,\text{obs}}^{\circ}$, estimated from amide solubility data to be approximately $+0.14 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$ per Å² (63–65). Here the predicted contributions to the observed $\Delta C_{p,\text{site}}^{\circ}$ from changes in nonpolar and polar surface are of opposite sign and similar magnitude, and so compensate almost completely. A similar situation exists for nucleic acid duplex formation from stacked strands; here also the $\Delta C_{p,\text{obs}}^{\circ}$ is significantly negative, but the predicted net contribution from burial of polar and nonpolar surface is negligibly small (40).

In the absence of significant intrinsic contribution from surface area burial, the negative $\Delta C_{p,\text{obs}}^{\circ}$ presumably results from a temperature-dependent coupled process (66). We propose that the temperature-dependence of the free receptor provides the bulk of the experimentally-observed $\Delta C_{p,\text{obs}}^{\circ}$. According to the parameters that generate the simulations in Figure 9, the monomeric form of the tetraloop – receptor construct (T_{GUGAR}) exists in at least two thermodynamic states at physiological temperatures (Figure 8). A transition within the tetraloop or its solvating environment is also possible, although the conformation appears to be relatively fixed (18,20,23,27). Thus we propose that complex formation most likely depletes

the receptor population of the relatively stacked and unfrayed N state, consequently driving the exothermic conversion $I_1 \rightarrow N$. According to this model, $\Delta H_{\text{obs}}^\circ$ contains a temperature-independent basal contribution for binding of the low temperature form of the receptor ($\Delta H_{\text{basal}}^\circ$) as well as a temperature-dependent contribution resulting from the coupled free receptor equilibrium ($\delta(T)_{\text{conversion}}$)

$$\Delta H_{\text{obs}}^\circ = \Delta H_{\text{basal}}^\circ + \delta(T)_{\text{conversion}} \quad \text{Equation (7)}$$

The 5-state model can quantitatively reproduce the experimental $\Delta C_{p,\text{obs}}^\circ$. Using the parameter set that generates the Figure 9 simulation, an increase in temperature from 10°C to 50°C increases the free receptor I_1 population from 1% to 50%. $\delta(T)_{\text{conversion}}$ accordingly increases from approximately -0.2 kcal/mol to -11.4 kcal/mol over this temperature range, yielding a bivalent $\Delta C_{p,\text{obs}}^\circ$ estimate of $-0.22 \text{ kcal}\cdot\text{mol}^{-1}\cdot\text{K}^{-1}$.

This proposal for the origin of $\Delta C_{p,\text{obs}}^\circ$ parallels previous proposals for secondary structure formation, where a negative $\Delta C_{p,\text{obs}}^\circ$ for duplex formation has been linked to a coupled temperature-dependent single-stranded stacking equilibrium (40,41,46). In fact, a stacking phenomenon within the free receptor may at least partially underlie the observed tetraloop – receptor $\Delta C_{p,\text{obs}}^\circ$. Batey and co-workers similarly proposed a temperature-dependence in a purine binding pocket conformation as the source of the observed binding $\Delta C_{p,\text{obs}}^\circ$ by the purine riboswitch aptamer domain (67).

Bimolecular $\Delta G_{\text{obs}}^\circ$ and quantification of the entropic contribution of helical packing

Because of construct symmetry, optimized motif spacing, and the absence of an entropic penalty for the loss of rotational and translational motion for one of the interactions, the magnitude of the binding free energy for these bivalent constructs is more than double that for a single interaction. The observed binding free energy can be decomposed as follows:

$$\Delta G_{\text{obs}}^\circ = 2\Delta G_{\text{site}}^\circ + \Delta G_{\text{other}}^\circ - \Delta G_{\text{rot-trans}}^\circ - RT\ln 2 \quad \text{Equation (8)}$$

where construct symmetry ($RT\ln 2$) and the absence of a rotational-translational penalty ($\Delta G_{\text{rot-trans}}^\circ$) both contribute favorably to the stability. As is the case for the association enthalpy, $\Delta G_{\text{other}}^\circ$ is composed of the thermodynamics of helical strain and helical packing, and parsing of $\Delta G_{\text{other}}^\circ$ between an intrinsic and construct-specific $\Delta G_{\text{obs}}^\circ$ is difficult both practically and theoretically. Unlike $\Delta H_{\text{other}}^\circ$ however, $\Delta G_{\text{other}}^\circ$ can be a significant term that is dominated by the entropy associated with formation of steep salt ion gradients resulting from packing of polyanionic helices (68). Thus $\Delta G_{\text{site}}^\circ$ is strongly dependent on the point of reference, which will be the subject of future work.

From the existing literature, we can however estimate an overall ‘cooperative effect’ for the bivalent construct ($\Delta G_{\text{other}}^\circ - \Delta G_{\text{rot-trans}}^\circ$), as determined elsewhere (12,13). A $\Delta G_{\text{obs}}^\circ$ of -4.6 kcal/mol was previously measured for the bimolecular association of a single tetraloop and receptor at 125 mM MgCl_2 and 20 °C (38); extrapolation to this temperature and $[\text{Mg}^{2+}]$ ($\partial \ln K_{\text{obs}} / \partial \ln [\text{MgCl}_2] = 2.5$ (unpublished results)) predicts a bivalent construct $\Delta G_{\text{obs}}^\circ$ of -17.5 kcal/mol. Using equation (8), we obtain $\Delta G_{\text{rot-trans}}^\circ - \Delta G_{\text{other}}^\circ = -7.9 \text{ kcal/mol}$ at 125 mM

MgCl₂ This value is significantly larger in magnitude than the $\Delta G_{\text{cooperative}}^{\circ}$ values between the distally located A-bulge and tetraloop–receptor tertiary contacts in the *Tetrahymena* P4–P6 domain according to a single molecule FRET study (–3.2 kcal/mol) and a global footprinting study (–2 to –3 kcal/mol) (12,13). Those experiments employed a single construct, so the additional ΔG of –4.5 kcal/mol here likely stems primarily from the absence of a rotational and translational motion penalty for one of the interactions.

The overall $\Delta G_{\text{obs}}^{\circ}$ for bivalent tetraloop – receptor association is surprisingly similar to that measured for bimolecular association of the two helical segments comprising the *Tetrahymena* P4–P6 domain (P5abc and P4–P6^{AP5abc}) (69). For that system $\Delta G_{\text{obs}}^{\circ}$ was –11.4 kcal/mol in 5 mM MgCl₂, 37 °C; at these conditions, we obtain –11.8 kcal/mol for the heterodimeric tetraloop–receptor construct. This suggests that the tetraloop – receptor interaction provides at least half of the stability for this domain’s tertiary structure, in agreement with a recent single molecule FRET study (12).

Materials and Methods

Isothermal Titration Calorimetry (ITC)

ITC Sample Preparation—ITC experiments were conducted using a VP-ITC (Microcal, Inc), and normalized heat signals were calculated using the bundled Origin software. In general, at least two forward titrations and two reverse titrations were performed at each temperature; replicate experiments in each direction always used different sample preparations.

Prior to each experiment, both the tetraloop-tetraloop and receptor-receptor constructs were dialyzed three times for ≥ 12 hours against 1 L of 2 mM MgCl₂, 20 mM KCl, 20 mM HEPES at the target pH. This procedure resulted in a nearly perfect match between the solutions and dialysate, so the heat of dilution was always small (< 1 kcal/mol for a typical injection) and essentially constant (see Figure 2). Control titrations (one each in the forward and reverse directions) were performed with each preparation of dialyzed samples in order to ensure that this level of buffer matching was attained.

In a typical experiment, ca. 100 μ M titrant was injected into a cell containing 5 – 10 μ M of the analyte, with an injection size of 5 – 10 μ L. For experiments conducted above 30 °C, an injection interval of 5 minutes was more than sufficient to allow for complete equilibration. Experiments at 10 °C – 30 °C used a larger injection interval of up to 8 minutes because of slow kinetics.

ITC experiments were carried out in 2 mM MgCl₂, 20 mM KCl, with either 20 mM HEPES or 20 mM Tris buffer. UV melts demonstrated that the highest ITC experimental temperature of 47.5 °C is well below the T_m for any helix denaturation for both RR (ca. 70 °C) and TT (ca. 90 °C). The buffer pH was adjusted at room temperature to account for the temperature-dependence of buffer ionization (70). For experiments in Tris, the solution was titrated to pH 7.00 at room temperature, so that the pH was 7.06 (20 °C) or 6.78 (30 °C). For experiments at 20 °C and 30 °C in HEPES, the solution was prepared to pH 7.05 so that the pH at the target temperature was 7.08 (20 °C) or 6.95 (30 °C). Experiments at higher temperatures were performed only with HEPES buffer titrated at room temperature to pH 7.28, so the target temperature pH ranged from 7.06 (40 °C) to 6.97 (47.5 °C).

ITC Data Analysis—All ITC data were analyzed and plotted in Igor Pro 6, using a 1:1 binding model that considered only the active RNA concentration by multiplying the measured TT and RR concentrations by empirical activity factors. This methodology was required because the TT preparation often includes a small fractional population of nonbinding fragments. Forward

and reverse titrations that possess an inactive TT subpopulation are shown (Supplemental Figure S2). In the experiments herein and in general, the TT activity typically ranges from 85% – 105% (an activity slightly greater than 100% was allowed to account for inaccurate concentration determination). Supplemental Table 1 and Supplemental Table 2 list the TT activity term used in fitting for all datasets. Relative to a simpler set of fitting equations that assume 100% titrant activity, the effect of this analysis method was almost entirely to increase the magnitude of $\Delta H_{\text{obs}}^{\circ}$ measured by forward titrations (Supplemental Material).

In order to unambiguously determine the TT activity parameter, forward and reverse titrations were performed on the same sample preparation whenever feasible (Supplemental Figure 2). These partnered forward and reverse titrations were then fit simultaneously using a common TT activity term, with all other parameters unlinked. The appropriate TT activity term was held during fitting to the value which yielded a binding stoichiometry nearest 1.0 in both directions. (The RR activity was allowed to differ in the two titrations to account for slight differences in stoichiometry; it was almost always $100 \pm 5\%$.)

Thermal Denaturation Experiments

Samples for UV and DSC thermal denaturation experiments were prepared by dialysis exchange into 2 mM MgCl₂, 20 mM KCl, 20 mM HEPES, which was titrated to pH 7.60 at room temperature, so that the pH was near 7.0 at the melting temperature. Thermal scan rates were generally 1 °C per minute. In the solution conditions of this study, all melts were essentially 100% reversible up to 80 °C; above this temperature, RNA hydrolysis becomes significant. The measured thermodynamics parameters are apparently not significantly affected by hydrolysis, given that DSC scans performed at rates of 0.5, 1.0 or 1.5 K/min were not significantly different (see Supplemental Figure S4). DSC experiments were performed on a VP-DSC (Microcal, Inc), and UV melts were carried out at 260 nm using a Cary 100Bio UV-Vis Spectrometer with a 1 cm pathlength cuvette.

Data for the TR construct were fit using a 5–state unfolding model:

This process is characterized by four equilibria, the first of which describes dissociation of the bivalent bimolecular complex:

$$K_d = \frac{[N]^2}{[C]} \quad \text{Equation (10)}$$

K_d is the dissociation constant (the inverse of K_a , the association constant calculated by ITC), $[N]$ is the concentration of native (binding-competent) monomers, and $[C]$ is the concentration of the complex. Subsequent unfolding of the monomer, or the entirety of the T_{GUGA}R construct unfolding process, is relatively well-described by a 4–state mechanism:

This process is described by three equilibria:

$$K_{I1} = \frac{[I_1]}{[N]} \quad K_{I2} = \frac{[I_2]}{[I_1]} \quad K_U = \frac{[U]}{[I_2]}$$

Equation (12a-c)

K_{I1} is the equilibrium constant for conversion from the native monomer, N, to the unfolding intermediate, I_1 . K_{I2} is the equilibrium constant for conversion from I_1 to the unfolding intermediate, I_2 . K_U is the equilibrium constant for denaturation of I_2 to the fully unfolded species, U. Using this set of equations, f_N , the fraction of RNA in the native monomer state can be determined according to:

$$f_N = \frac{K_d}{4[R]_{\text{tot}}} \left(- \left(1 + K_{I1} (1 + K_{I2} (1 + K_U)) \right) + \sqrt{\left(1 + K_{I1} (1 + K_{I2} (1 + K_U)) \right)^2 + \frac{8[R]_{\text{tot}}}{K_d}} \right)$$

Equation (13)

where $[R]_{\text{tot}}$ is the total concentration of RNA molecules, and $f_N = [N]/[R]_{\text{tot}}$. The fractions of the remaining species (f_C , f_{I1} , f_{I2} , f_U) could then be determined using (equation 10) and (equation 12).

Global fitting was performed using a reference temperature equation set that assumed temperature-independent $\Delta C_{p,\text{obs}}^\circ$ values (equation (3)). In order to obtain a unique fit, $\Delta C_{p,\text{obs}}^\circ$ for each transition was fixed to reasonable values (Table 2), and the equilibrium constant for the first isomerization step (K_{I1}) was held to some value. Supplemental Table 3 shows the effect of various K_{I1} values on the minimized set of remaining parameters.

DSC Data Analysis—Raw excess heat capacity (C_p^{ex}) data were generated by subtracting a reference (no RNA) thermogram from that of the RNA sample (Figure 7a, inset), then

normalizing by the experimental molar [RNA] (Figure 7a). The partial molar $C_{p,\text{obs}}^\circ$ ($\bar{C}_{p,\text{obs}}^\circ$) of the RNA was determined as approximately 7.5 kcal·mol⁻¹·K⁻¹ at 25 °C, using a partial molar volume of 11,000 mL/mol (71). The appropriate constant offset was applied to each dataset to generate the plots in Figures 7b, 7c and Figure 9.

Because of the complexity of the fitting models, baseline $\bar{C}_{p,\text{obs}}^\circ$ values were applied empirically in a manner that most greatly simplified fitting. Each dataset consisted of low (0 – 50 °C), intermediate (50 – 90 °C) and high temperature (90 – 110 °C) baseline regions. For the low temperature region, where DSC plots are linear, the baseline was calculated from the best fit line to each dataset below 45 °C. For TR, the best fit line equation was used directly, and for T_{GUGAR}, the fitted slope was subtracted by 8 cal mol⁻¹ K⁻² to model the native baseline. For the high temperature region, where DSC plots are constant, the baseline $\bar{C}_{p,\text{obs}}^\circ$ value was set to the average experimental $\bar{C}_{p,\text{obs}}^\circ$ above 104 °C for each dataset. A linear $\bar{C}_{p,\text{obs}}^\circ$ baseline joining the low and high temperature baselines was used in the intermediate region. The employed baselines are displayed with the fitted curves as the lowermost gray curve in each panel (Figure 9). The observed signal at each temperature was determined by summing the baseline ($\bar{C}_{p,\text{obs}}^\circ$) and the transition enthalpy contribution to the excess heat capacity (ΦC_p):

$$\Phi C_p = \Delta H_{I_2 \rightarrow U} \left(\frac{\partial f_U}{\partial T} \right) + \Delta H_{I_1 \rightarrow I_2} \left(\frac{\partial f_{I_2}}{\partial T} + \frac{\partial f_U}{\partial T} \right) + \Delta H_{F \rightarrow I_1} \left(\frac{\partial f_{I_1}}{\partial T} + \frac{\partial f_{I_2}}{\partial T} + \frac{\partial f_U}{\partial T} \right) + \Delta H_{C \rightarrow 2F} \left(\frac{\partial f_F}{\partial T} + \frac{\partial f_{I_1}}{\partial T} + \frac{\partial f_{I_2}}{\partial T} + \frac{\partial f_U}{\partial T} \right)$$

Equation (14)

In global fitting, the population of each species at each temperature was calculated using (equation 3), (equation 10), (equation 12), and (equation 13), and the temperature derivatives were then taken to calculate ΦC_p at each temperature. The $\Delta C_{p,\text{obs}}^\circ$ values for each individual

step cannot be resolved through fitting; instead, the sum of the fixed $\Delta C_{p,obs}^\circ$ values was set to agree with the DSC-observed cumulative $\Delta C_{p,obs}^\circ$. Also, to account for the slight difference in ΔH_{obs}° for the 12 bp hairpin unfolding in the two constructs (Supplemental Figure S4), an empirical correction of five kcal/mol ($< 5\%$ of the ΔH_{obs}° for the transition) was applied to the fitted enthalpy change for the final unfolding step for T_{GUGAR}

(for $\Delta H_{I_2 \rightarrow U}^{TGIGAR} = \Delta H_{I_2 \rightarrow U}^{TR} + 5 \text{ kcal/mol}$). This small difference in the ΔH_{obs}° for the final unfolding steps of the two RNAs may derive from a difference in extinction coefficients.

UV Spectroscopy Data Analysis—UV hyperchromicity plots (Figure 6 and Figure 9) display the fractional increase in absorbance at 260 nm, relative to the absorbance at 20 °C. In data fitting, the lower baseline for either T_{GUGAR} or TR (the modeled hyperchromicity of either N or C, respectively) was set to a constant value of zero hyperchromicity. These modeled baselines are consistent with the lack of any temperature-dependence in the experimental absorbance data for either T_{GUGAR} or TR below 45 °C. The high temperature baseline (the modeled hyperchromicity of U) was a linear function determined by fitting to the experimental hyperchromicity data for each dataset from 93 – 97 °C.

For fitting simplicity, the observed signal was calculated according to the fractional signal progress (P_X) toward the upper baseline, logical because the magnitude of the temperature dependence of the absorbance for each species should scale with the number of unpaired bases. The $N \rightarrow I_1$ transition was assumed to occur with no hyperchromic effect, so the modeled signal for I_1 was identical to that for N. This is consistent with the small structural change proposed for this transition and the absence of any hyperchromic effect for T_{GUGAR} below 50 °C. Therefore, for T_{GUGAR}, the fractional hyperchromicity (f.h.) data were fit to the following equation at each temperature

$$f.h._{T_{GUGAR}} = U_{baseline} P_{I_2} (f_{I_2}) + U_{baseline} (f_U) \quad \text{Equation (15)}$$

Where $U_{baseline}$ is the upper baseline value at each temperature and P_{I_2} is the fractional progress toward the upper baseline in the second intermediate I_2 (0.36 in Table 2). The fractional hyperchromicity of the final step is obtained as the remainder: $(1 - P_{I_2})$. Table 2 reports the fractional hyperchromicity (f.h.) contributed by each step, where $f.h. = P_X \times (0.286)$. To account for $C \rightarrow 2N$, the TR data contain an additional term:

$$f.h._{TR} = U_{baseline} P_{I_2} (f_{I_2}) + U_{baseline} (f_U) + (1 - f_C) (d.h.) \quad \text{Equation (16)}$$

d.h. (dissociative hyperchromicity) is the temperature-independent hyperchromic effect observed upon unfolding, and the expression $(1 - f_C)(d.h.)$ offsets the baseline (by approximately 0.03, Table 2) above the dissociation temperature.

The T_m 's for both UV-monitored helical denaturation steps occur approximately 2 °C below the value determined by DSC, suggesting that the hyperchromicity-measured value prematurely reports unfolding because of increasing nucleotide dynamics. Therefore, a factor of three (in free energy terms, $\Delta G_{ref}^{UV} \approx \Delta G_{ref}^{DSC} + 0.75$) was applied to the equilibrium constants for these steps (K_{I_2} and K_U) in global fits.

NMR Spectroscopy—NMR experiments were performed at 80 μM in 2 mM MgCl_2 , 20 mM KCl, 20 mM HEPES pH 7.0 at room temperature as previously described (17,18).

RNA Preparation

RNA was prepared by *in vitro* transcription from a chemically synthesized DNA oligonucleotide template (Integrated DNA Technologies) using His₆-tagged T7 polymerase, as previously described (18). RNA concentrations were determined by UV absorbance spectroscopy following hydrolysis in 0.3 M NaOH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

ITC, isothermal titration calorimetry; DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance; nt, nucleotide; bp, base-pair.

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Figure 1.

RNA constructs in this study. (A) “RR-TT” heterodimer used in ITC experiments. The double GAAA tetraloop construct (TT) binds in a bivalent fashion to the double 11 nt receptor construct (RR). The tetraloop is indicated with a red box, and the receptor is indicated with a green box. (B) Homodimerizing (“TR”) construct used in DSC and optical and NMR spectroscopy. (C) Monomeric construct (“T_{GUGA}R”), in which the tetraloop sequence has been altered to GUGA to eliminate bimolecular association. (D) Wide-eyed stereo-view of the tetraloop – receptor interaction (PDB code 1HR2). Hydrogen bonds are indicated with dashed lines.

Figure 2.

Representative forward ITC titration at 30 °C. The calorimeter cell containing 6.0 μM RR was titrated with 70 μM TT. The upper panel displays the raw electrical power trace of the binding titration (baseline set at 0 $\mu\text{cal/sec}$), as well as that of a control injection series (Y-offset of +0.2 $\mu\text{cal/sec}$), in which 70 μM TT was serially injected into a cell containing only the buffer solution. The lower panel plots the integrated and normalized heat signal for each injection in the binding titration against the stoichiometry. The experiment employed an injection volume of 10 μL and 3 – 5 minute spacing between injections to allow chemical and instrumental equilibration.

Figure 3.

Representative ITC forward titration data over a range of temperatures. All experiments were carried out in a buffer solution of 2 mM MgCl_2 , 20 mM KCl, 20 mM HEPES near pH 7.0. The starting cell concentration of RR was 5 – 10 μM , and the injecting syringe contained 70 – 100 μM TT.

Figure 4.

Temperature-dependence of the ITC-determined thermodynamics of the TT-RR interaction.

(A) Enthalpy of binding ($\Delta H_{\text{obs}}^{\circ}$) versus temperature. Each datapoint represents an individual experiment. Open squares, forward titrations; closed circles, reverse titrations. Black symbols are $\Delta H_{\text{obs}}^{\circ}$ values that were calculated by simultaneously fitting a forward and reverse titration carried out on the same sample preparation, which allowed for determination of the activity of the TT construct (i.e., datasets were fit as ‘partner titrations’ as described in Materials and Methods and Supplemental Material). Symbols shown in gray are $\Delta H_{\text{obs}}^{\circ}$ determinations from experiments that did not employ partner titrations, and the error bars illustrate the uncertainty

introduced by an imprecisely-determined TT activity. The trendline shows the best linear fit to the data, with slope $-0.24 (\pm 0.04) \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$. (B) Plot of the logarithm of the binding constant ($\log K_{\text{obs}}$) versus temperature. Symbols are as in (A), except that all symbols are in black because the presence or absence of partner titrations had a negligible effect on K_{obs} (Supplemental Table 1). The dotted curve, which demonstrates consistency between enthalpy and binding stability measurements, plots the predicted temperature dependence of $\log K_{\text{obs}}$ using a 40 °C reference state with $\log K_{40\text{C}} = 7.1$, $\Delta H_{40\text{C}}^{\circ} = -33.1 \text{ kcal/mol}$, and $\Delta C_{p,\text{obs}}^{\circ} = -0.24 \text{ kcal} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$.

Figure 5. Temperature-dependence of the tetraloop – receptor complex investigated by NMR spectroscopy. 1D ^1H NMR spectra of 80 μM T_{GUGAR} and TR constructs at 30 °C, in 2 mM MgCl_2 , 20 mM KCl, 20 mM HEPES, pH 7.0. Identities of imino-protons are labeled above each peak. The chemical shift of G9 in the bound state is labeled 9, and the free (monomeric) state is labeled 9*.

Figure 6.

Ultraviolet wavelength absorbance-monitored denaturation of TR (black) and T_{GUGAR} (red) constructs, performed in 2 mM MgCl₂, 20 mM KCl, 20 mM HEPES (pH 7.60 at room temperature) at 1.5 μM. Three sets of symbols (open squares, open circles, and closed circles) represent three separate experiments performed with each construct. The data are plotted as fractional hyperchromicity relative to the absorbance at 20 °C at 260 nm. Inset to the figure is a first derivative plot for each construct ($\partial \text{Fractional Hyperchromicity} / \partial T$). The dots display the averaged derivative plot using the three raw datasets, and the line shows the same result using sliding average-smoothed datasets.

Figure 7.

Differential Scanning Calorimetry raw experimental data with TR and T_{GUGAR} constructs. (A) Sample dataset performed with 430 μM TR. The figure plots the raw, molar concentration-normalized excess heat capacity (C_p^{ex}) contributed by the RNA relative to a buffer-only solution. The negative offset is a trivial consequence of the displacement of water volume. The dotted and dashed-dotted lines illustrate empirically-determined low and high temperature baselines used in data fitting. Inset to (A), the direct experimental data illustrating the differential power (C_p/K) required to maintain a temperature increase equivalent to that of the reference cell for either the solution alone (dashed line) or solution containing RNA (solid line). (B) Excess heat

capacity plots for the TR construct performed over a > 10-fold range of [RNA]. The figure plots the excess heat capacity, which includes the RNA partial molar heat capacity ($\bar{C}_{p,obs}$) and the contribution from the heat input required to drive conformational transitions (ϕC_p). The total concentration of TR for each experiment is noted in the legend. (C) Excess heat capacity plots for the T_{GUGA}R construct at 60, 120, and 430 μ M, which demonstrate no [RNA]-dependence.

Figure 8.

Thermodynamic and proposed structural mechanisms for the unfolding process, whereby dissociation is followed by 3 isomerization steps. TR undergoes the full 5-state mechanism, and T_{GUGAR}, which does not dimerize under the solution conditions of this study, follows the unfolding mechanism represented by the final 4-states only. The first isomerization step is hypothesized to occur within the receptor, and is indicated with a color change from green to yellow. The tetraloop is shown in red. C, complex; N, native monomer; I₁, intermediate 1, I₂, intermediate 2; U, unfolded.

Figure 9.

Experimental and simulated unfolding curves using a semi-quantitatively optimized 5-state TR unfolding parameter set. (A) Experimental (colored circles, using the same scheme as Figure 7B) and simulated (black curves) excess heat capacity plots for the TR construct at all concentrations examined. The $C_{p,obs}$ baselines used in fitting and the predicted additional heat capacity contributions from each transition (ϕC_p) are displayed in light gray lines. The total TR concentration is listed at the top of each panel. (B) Representative excess heat capacity data for the T_{GUGAR} construct at 430 μM (circles) plotted with the simulated curve (black). (C) Representative UV fractional hyperchromicity melting data and best-fit curves for TR (top panel, black) and T_{GUGAR} (lower panel, red) at 1.5 μM . The experimentally-determined

fractional hyperchromicity data are plotted as closed circles and best-fit simulations are plotted as smooth curves.

Figure 10.

Comparison of experimental $K_{d,app}$ values and the simulated $K_{d,app}$ function from the optimized 5-state TR unfolding model. $K_{d,app}$ determinations from UV, NMR and DSC thermal denaturation experiments with the homodimeric construct are plotted as open symbols, and ITC data using the heterodimeric construct are plotted as closed circles (the ITC error bars are within the scale of the data points). The solid curve shows the 5-state simulated homodimer $K_{d,app}$ over the range of temperatures.

Table 1
Averaged thermodynamics of the TT-RR interaction determined by ITC

Temp (°C)	Buffer	$K_{obs}(M^{-1}) \times 10^6$	ΔG_{obs}° (kcal/mol)	ΔH_{obs}° (kcal/mol)	$T \Delta S_{obs}^{\circ}$ (kcal/mol)
10	HEPES	nd	nd	-26.0 ($\pm$ 1.8)	nd
20	HEPES	nd	nd	-29.9 ($\pm$ 2.0)	nd
30	HEPES	nd	nd	-30.1 ($\pm$ 1.5)	nd
40	HEPES	11 ($\pm$ 0.3)	-10.04 ($\pm$ 0.32)	-27.6 ($\pm$ 1.7)	-17.6 ($\pm$ 2.0)
42.5	HEPES	8.7 ($\pm$ 2.8)	-9.99 ($\pm$ 0.32)	-34.5 ($\pm$ 2.2)	-24.5 ($\pm$ 2.5)
45	HEPES	5.5 ($\pm$ 1.6)	-9.77 ($\pm$ 0.29)	-33.8 ($\pm$ 2.0)	-24.0 ($\pm$ 2.3)
47.5	HEPES	4.0 ($\pm$ 0.9)	-9.65 ($\pm$ 0.23)	-37.2 ($\pm$ 1.8)	-27.6 ($\pm$ 2.0)
20	Tris	nd	nd	-28.8 ($\pm$ 1.4)	nd
30	Tris	nd	nd	-27.1 ($\pm$ 1.4)	nd

nd= not determined ($K_{obs} > 2 \times 10^7 M^{-1}$)

Table 2

Optimal Simulated Unfolding Parameters

Equilibrium	T _m	ΔH_{Tm}° (kcal/mol)	$\Delta C_{p,obs}^{\circ}$ kcal·mol ⁻¹ ·K ⁻¹	f.h.	ΔH_{nn} (kcal/mol)
C↔2N	[f(RNA)] [*]	[10.7]	[0]	0.03 (± 0.01)	---
N↔I ₁	[50 °C]	[22.5]	[0.12]	[0]	---
I ₁ ↔I ₂	75.6 (± 1.7) °C	46.6 (± 6.3)	[0.28]	0.10 (± 0.01)	39.3
I ₂ ↔U	86.1 (± 0.1) °C	116.6 (± 1.0)	[1.00]	0.18 (± 0.01)	119.4

T_m, melting temperature

$\Delta H_{Tm}^{\circ}, \Delta H_{obs}^{\circ}$ at the T_m

^{*} the T_m for dissociation is a function of [RNA] (here, K_d at 65 °C = 2.8 × 10⁻⁶ M)

[], parameter constrained

f.h., fractional hyperchromic effect (relative to absorbance at 20 °C)

ΔH_{nn} , nearest-neighbor model-predicted ΔH_{obs}° for 5 bp lower helix (I₁ ↔ I₂) or 12 bp hairpin (I₂ ↔ U) unfolding