Characterization of the kinetic and thermodynamic landscape of RNA folding using a novel application of isothermal titration calorimetry

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ABSTRACT

A novel isothermal titration calorimetry (ITC) method was applied to investigate RNA helical packing driven by the GAAA tetraloop–receptor interaction in magnesium and potassium solutions. Both the kinetics and thermodynamics were obtained in individual ITC experiments, and analysis of the kinetic data over a range of temperatures provided Arrhenius activation energies ($\Delta H^\ddagger$) and Eyring transition state entropies ($\Delta S^\ddagger$). The resulting rich dataset reveals strongly contrasting kinetic and thermodynamic profiles for this RNA folding system when stabilized by potassium versus magnesium. In potassium, association is highly exothermic ($\Delta H_{25\, ^\circ C} = -41.6 \pm 1.2 \text{ kcal/mol}$ in 150 mM KCl) and the transition state is enthalpically barrierless ($\Delta H^\ddagger = -0.6 \pm 0.5$). These parameters are significantly positively shifted in magnesium ($\Delta H_{25\, ^\circ C} = -20.5 \pm 2.1 \text{ kcal/mol}$, $\Delta H^\ddagger = 7.3 \pm 2.2 \text{ kcal/mol}$ in 0.5 mM MgCl$_2$). Mixed salt solutions approximating physiological conditions exhibit an intermediate thermodynamic character. The cation-dependent thermodynamic landscape may reflect either a salt-dependent unbound receptor conformation, or alternatively and more generally, it may reflect a small per-cation enthalpic penalty associated with folding-coupled magnesium uptake.

INTRODUCTION

Proper tertiary folding of RNA is crucial to its array of enzymatic and regulatory functions (1). Due to the significant increase in anionic charge density and structure-dependent apposition of phosphate oxygens that accompany RNA tertiary folding, metal ions play a significant and nuanced role in the folding equilibrium. Beginning with the seminal series of tRNA folding studies (2–8), the highly stabilizing role of the divalent cation magnesium (Mg$^{2+}$) has been an experimental focal point. Misra and Draper (9) demonstrated that Mg$^{2+}$ can confer this stability to folded tRNA entirely through diffuse (hydrated) ion accumulation, consistent with the fact that tRNA can fold in magnesium salt or monovalent salt alone (2). For some other RNA structures, magnesium is directly coordinated and essential for folding (10,11); in principle, intermediate hydration states are also possible although the prevalence and thermodynamic stability afforded by these types of ions is not well understood. Such molecular considerations may underlie the systematic underestimation of magnesium accumulation by Poisson–Boltzmann theory (12). As theoretical work continues in this area (13–15), additional complementary experiments studying this crucial component of RNA stability are also necessary.

In addition to the nature of association (11) and the change in charge density accompanying RNA folding (16), the quantitative role of magnesium depends strongly on the concentration and identity of other metal ions (17). This is an important consideration given in vivo free potassium (K$^+$) concentrations near 150 mM. The Tetrahymena thermophila Group I self-splicing intron provides an excellent case study. For the P4–P6 domain of this ribozyme, the GAAA tetraloop–receptor tertiary interaction can form in solutions containing either MgCl$_2$ or monovalent salt (NaCl) alone (18–20), but formation of the A-rich bulge tertiary contact requires magnesium (20). While the uptake of Mg$^{2+}$ and Na$^+$ is tied to tertiary folding, and addition of either improves folding stability via a reduced entropic penalty, in some concentration regimes when both ions are present they act competitively (21). Interestingly, salt-dependent differences in RNA folding kinetics have also been observed. For example, at salt concentrations that elicit similar overall folding stabilities, intron folding is faster when driven by monovalent cations than when driven by a multivalent cation such as magnesium (22–24). The underlying
thermodynamic composition of these findings is not yet understood.

The ubiquitous GAAA tetraloop receptor mediates helical packing (25) in Groups I and II self-splicing introns (26–28) and RNase P (29,30). In this work we examine a bimolecular helical packing event driven by this interaction (Figure 1) (31), in solutions containing either one of the physiologically dominant cations, K⁺ or Mg²⁺. This domain separation strategy has proven successful in determining RNA folding principles (32–35) and allows access to the rich thermodynamic information provided by calorimetry. The approach involves two helical RNA molecules, one containing two tetraloop receptor motifs phased by one helical turn (termed RR) and another containing two cognate GAAA tetraloops (TT). The helices therefore assemble in parallel into a well-defined structure mediated solely by the tetraloop–receptor interaction (Figure 1), as demonstrated by our previous thermodynamic investigation (32) and excellent agreement with single molecule fluorescence resonance energy transfer (FRET) studies of tethered tetraloop receptors (36). We have also solved the nuclear magnetic resonance structure of this interaction, which within experimental error is superimposable with previous crystal structures (18,28,37).

Figure 1. Structure of the TT–RR system. Red/salmon: tetraloop; green/mint: receptor. (A) Secondary structure of dual receptor (RR) and dual tetraloop (TT) constructs employed in this study. (B) Cartoon model of TT–RR complex. (C) Close-up view of tetraloop–receptor interaction, structurally stabilized by stacking of the tetraloop 5' adenine on the 3' adenine of the receptor AA platform, in addition to 10 intermolecular hydrogen bonds (PDB code 1HR2).

Mg²⁺ accumulation in the tetraloop–receptor contact is entirely or largely diffuse (10,11) in character (18,28). Thus a direct comparison of folding in MgCl₂ and KCl can potentially uncover core energetic differences in Mg²⁺ and K⁺ accumulation. Here, by application of a novel titration calorimetry approach, we characterize the thermodynamic and kinetic landscape of tetraloop–receptor mediated helical packing interaction when stabilized by either K⁺ or Mg²⁺. We find that the thermodynamic and kinetic profiles for tetraloop–receptor association are indeed significantly dependent on the identity of the cation, such that the transition state enthalpy barrier is on average 9 kcal/mol larger and the overall binding enthalpy is 19 kcal/mol less exothermic in MgCl₂ relative to the values in KCl.

MATERIALS AND METHODS

Preparative methods

TT and RR RNA were prepared by in vitro transcription and quantitatively as described previously (32,37). All samples were prepared for calorimetry by extensive dialysis. Magnesium solutions contained 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and MgCl₂ at the stated concentration; potassium solutions contained 20mM HEPES, 1 mM ethylenediaminetetraacetic acid, and the stated KCl concentration. Both solutions were titrated to pH 7.0 at room temperature before dialysis.

ITC thermodynamics analysis

Binding data were analyzed as described previously. A small fraction of TT misfolds during preparation (32). This is accounted for using a TT ‘activity’ term in the binding data and quantitated as described previously (32,37). All samples were prepared for calorimetry by extensive dialysis. Magnesium solutions contained 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and MgCl₂ at the stated concentration; potassium solutions contained 20 mM HEPES, 1 mM ethylenediaminetetraacetic acid, and the stated KCl concentration. Both solutions were titrated to pH 7.0 at room temperature before dialysis.

ITC kinetics analysis

Following baseline subtraction and deconvolution ([38], Supplementary Data), an injection power trace reflects the real-time derivative of the negative of heat evolution within the calorimeter cell, and subsequent integration results in the negative of the corresponding heat evolution as a function of time (Qₑᵥ). Qₑᵥ for each injection is approximately described as the time-dependent
concentration of new complex formed \([C^*(t)]\) scaled by the binding enthalpy and the sample cell volume \((V_0 = 1.42 \text{ ml})\).

\[ Q_{ev}(t) = -\Delta H V_0 C^*(t) \]  

(1)

The equilibrium involves three species, so knowledge of the total TT and RR concentrations reduces the description of all solution concentrations to a single degree of freedom, which can be defined in terms of the displacement from equilibrium, \( \lambda \). Here we define \( \lambda \) as the difference between the equilibrium concentration \([C]_\text{eq}\) and the current concentration \([C]\). The reaction rate can then be written

\[ \frac{d[C]}{dt} = -\frac{\partial \lambda}{\partial t} = k_{\text{on}}([\text{TT}]_\text{eq} + \lambda)([\text{RR}]_\text{eq} + \lambda) - k_{\text{off}}([C]_\text{eq} - \lambda) \]  

(2)

Assuming only that \( K_d = k_{\text{off}}/k_{\text{on}} \), Equation (3) is the resulting most general expression for \( \lambda \), which cannot be expressed analytically.

\[ \ln \left( \frac{(\lambda t=0)}{\lambda} \cdot \frac{[\text{TT}]_\text{eq} + [\text{RR}]_\text{eq} + K_d t + \lambda}{[\text{TT}]_\text{eq} + [\text{RR}]_\text{eq} + K_d t + \lambda t=0} \right) \]

\[ = -([\text{TT}]_\text{eq} + [\text{RR}]_\text{eq} + K_d)k_{\text{on}}t \]  

(3)

Here \( \lambda t=0 \) is the displacement from equilibrium following addition of RNA. More precisely in the case of an ITC experiment, it is the displacement at a theoretical time point following the injection period but preceding formation of any new complexes. \( \lambda t=0 \) also reflects concentration changes caused by the addition of titrant and by the dilution of all species due to the injection volume \( \lambda t=0 = [C]_\text{eq} - [C]_t=0, \) where \([C]_t=0\) is the starting \([C]\) after accounting for the injection-caused dilution. Equation (3) shows that in the most general case, determination of \( \lambda (t) \) requires prior knowledge of \( k_{\text{on}}, \) \( K_a \) (i.e. \( k_{\text{off}} \)), and the total starting concentrations. In our application, the latter two terms are known: the total RNA concentrations are defined for each injection by the experimental setup, and \( K_a \) is obtained directly in the same experiment or through an extrapolation from higher temperature according to the van’t Hoff equation.

Because \( \lambda \) cannot be determined analytically, its numerical solution from Equation (3) is wrapped within a least squares procedure for determining \( k_{\text{on}} \). The predicted heat evolution function is given by

\[ Q_{ev} = -\Delta H V_0 (\lambda t=0 - \lambda) \]  

(4)

where \( \lambda t=0 \) is a known quantity defined by the \( K_a \) and the RNA concentrations and \( \lambda \) is a function of \( k_{\text{on}} \). Data were fit to a time regime starting at the minimum in the raw power trace (to exclude the injection period from analysis), and the final fitted time point never exceeded 10 times a prior-estimated half-time (to avoid over-weighting the baseline). Only injections from the first half of a titration were analyzed because signal-to-noise decreases significantly for subsequent injections.

Kinetic data fitting may be performed by integrating the post-deconvolution power trace to obtain \( Q_{ev} \) for least squares minimization with target Equation (4).

Alternatively, Equations (3) and (4) can be differentiated for comparison with the deconvolved power trace \( (E_{dc}) \). We implemented the latter approach here (Figure 2).

All experiments were performed on a GE Healthcare Microcal VP-ITC, using a feedback setting of ‘high’, a stirring rate of 307 rpm, and an injection rate of 0.5 \( \mu \text{L/sec} \). The feedback setting was chosen to maximize the instrument response rate constant; the latter two settings have little effect on the instrument response time. The slow kinetics are also not complicated by any RNA dilution effect (Supplementary Figure S1).

Error analysis

A weighted average \( k_{\text{on}} \) was determined for each titration experiment from the population of individual injection
measurements. The underlying error in these individual determinations is a combination of random noise, baseline drift and uncertainty in the instrumental rate constant ($k_{ITC}$). The true uncertainty also contains contributions from uncertainty in $k_a$ and in the RNA concentrations. All of these error sources were incorporated into both $k_{on}$ and $k_{off}$ measurements through error propagation as described in Supplementary Data. Higher order analysis of data, such as measurement of slope values to determine $\Delta H^\circ$, used a ‘nested’ bootstrap approach to properly estimate error (Supplementary Data). All data processing and analysis routines were implemented and performed using the high-level programming language in Igor Pro 6.

RESULTS

Extraction of thermodynamic and kinetic parameters from a single ITC experiment

Figure 2 shows a typical ITC experiment in which both thermodynamic and kinetic measurements are obtained. The experiment depicted is a titration in which TT RNA is serially injected into a cell initially containing 10 µM RR. The data demonstrate exothermic binding as a series of TT injection-induced negative deflections in the compensatory power trace followed by a slow decay back to the relative baseline value of 0.0 µW (Figure 2A). Integration of each peak yields the heat evolved by binding, which is plotted against the corresponding molar ratio ([TT]/[RR]) for each injection to generate an ITC binding isotherm (Figure 2B). The binding enthalpy ($\Delta H$) and binding constant ($K_a$) are determined through least squares fits to these binding data (‘Materials and Methods’ section).

A typical titration microcalorimeter power compensation trace can be viewed as the convolution of a binding-generated ‘impulse’ heat evolution function with the calorimeter ‘response’ function. Using a Laplace Transform approach with an instrumental time constant of 12.5 s, the heat evolution function is recovered (deconvolved) for each injection ([38], Supplementary Data). A representative deconvolved peak and the associated least squares-minimized kinetics curve are displayed in Figure 2C. This fitting procedure in general requires knowledge of the equilibrium RNA concentrations (‘Materials and Methods’ section); in Figure 2, these values were obtained from the total RNA concentrations and the measured binding constant (Figure 2B). Below, the thermodynamic results from this study are analyzed first, then the corresponding kinetic data are considered.

Comparison of the thermodynamics of tetraloop–receptor association in KCl and MgCl$_2$

Analysis of TT–RR binding data reveals strikingly different thermodynamic profiles for this helical packing event in MgCl$_2$ and KCl solutions (Figure 3). Representative experiments performed at 20°C in either 0.5 mM MgCl$_2$ or 150 mM KCl demonstrate that, while binding is enthalpy-driven in both salts, in KCl it is significantly more exothermic (for these conditions, on average $\Delta \Delta H = -21.7 \pm 3.7$ kcal/mol) and less favorable entropically ($\Delta (\Delta T \Delta S) = +22.4 \pm 3.8$ kcal/mol) (Figure 3A). The differences in these terms represent contributions to $\Delta G$ that are nearly three times greater than the overall stabilities.

To more fully describe the folding landscapes in MgCl$_2$ and KCl, we performed calorimetry experiments in a range of salt concentrations and temperatures (Tables 1 and 2). In aggregate the thermodynamic data are highly self-consistent. $K_a$ increases as a function of added MgCl$_2$ or KCl, and it decreases with increasing temperature.

Figure 3B plots the thermodynamics for TT–RR binding across the full set of examined salt conditions at a common temperature (25°C); the values are also listed in Table 3. The significant discrepancy in MgCl$_2$ and KCl thermodynamic profiles persists across the examined salt concentrations. $\Delta H_{25^\circ C}$ is bounded by $-24.6$ and $-20.5$ kcal/mol in the ensemble of MgCl$_2$ solutions; in KCl the minimum and maximum values are $-41.6$ and $-34.0$, respectively. Contrastingly, $T \Delta S_{25^\circ C}$ ranges from $-16.5$ to $-10.7$ kcal/mol in MgCl$_2$ while it is much more unfavorable in KCl solutions, ranging from $-27.4$ to $-33.8$ kcal/mol.

These 25°C thermodynamic quantities are maximum likelihood (i.e. ‘optimized’) values from the full set of measurements across a range of experimental temperatures. $\Delta H_{25^\circ C}$ values were calculated for each salt condition from linear fits of individual $\Delta H$ measurements plotted against the experimental temperature. The average slopes from these measurements are also given in Table 3 as the heat capacity change ($\Delta C_p = \partial \Delta H / \partial T$). For data collected in 1.0 mM MgCl$_2$, the fit excluded data...
above 30°C, where the binding enthalpy displays a statistically significant change in slope (Supplementary Figure S2). This observation corroborates our previous hypothesis that the free receptor exists in a temperature-dependent conformation (32). In other words, the enthalpy becomes increasingly temperature dependent at temperatures where the melting intermediate becomes significantly populated. While the shift in ΔC_p may begin between 20 and 30°C (Tables 1 and 2, Supplementary Figure S2), overall the ΔC_p is small and negative for the full set of experiments conducted below 30°C. The average ΔC_p below 30°C are similar in MgCl₂ and KCl solutions: bootstrap analysis yields −0.14 (±0.02) kcal/mol/K in MgCl₂ versus −0.04 (±0.08) kcal/mol/K in KCl. The weighted average ΔC_p for a single tetraloop–receptor interaction is therefore −0.06 (±0.02) kcal/mol/K. This value is not far from that predicted by a coarse-grained surface area burial calculation (−0.002 kcal/mol/K) (39).

The free energy at 25°C (ΔG_{25°C}) in Table 3 is also a maximum likelihood value (interpolated in 5 of the 7 salt conditions) determined from K_a (ΔG = −RTlnK_a) according to its temperature dependence using the van’t Hoff equation.

\[
\frac{\partial \ln K_a}{\partial 1/T} = -\frac{\Delta H_{\text{VH}}}{R}
\]

(5)
Table 3. Average Thermodynamic Parameters

<table>
<thead>
<tr>
<th>Salt</th>
<th>Temp (°C)</th>
<th>ΔG_{25C} (kcal/mol)</th>
<th>ΔH_{25C} (kcal/mol)</th>
<th>ΔTΔS_{25C} (kcal/mol)</th>
<th>ΔC_p (kcal/mol/K)</th>
<th>ΔH_VH (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3 mM MgCl₂</td>
<td>10–30</td>
<td>-8.1 (±0.8)</td>
<td>-24.6 (±4.3)</td>
<td>-16.5 (±4.4)</td>
<td>-0.21 (±0.15)</td>
<td>-24.2 (±0.6)</td>
</tr>
<tr>
<td>0.5 mM MgCl₂</td>
<td>2–30</td>
<td>-9.0 (±0.8)</td>
<td>-20.5 (±2.1)</td>
<td>-11.6 (±2.1)</td>
<td>-0.12 (±0.10)</td>
<td>-17.4 (±2.9)</td>
</tr>
<tr>
<td>0.7 mM MgCl₂</td>
<td>10–30</td>
<td>-9.5 (±0.8)</td>
<td>-23.9 (±2.0)</td>
<td>-14.4 (±2.0)</td>
<td>-0.17 (±0.06)</td>
<td>-17.8 (±1.0)</td>
</tr>
<tr>
<td>1.0 mM MgCl₂</td>
<td>5–45</td>
<td>-10.2 (±0.8)</td>
<td>-20.9 (±2.1)</td>
<td>-10.7 (±2.3)</td>
<td>-0.11 (±0.05)</td>
<td>-31.0 (±6.7)</td>
</tr>
<tr>
<td>100 mM KCl</td>
<td>5–15</td>
<td>-6.6 (±0.3)</td>
<td>-34.0 (±4.1)</td>
<td>-27.4 (±4.5)</td>
<td>0.11 (±0.31)</td>
<td>-36.4 (±3.8)</td>
</tr>
<tr>
<td>150 mM KCl</td>
<td>5–25</td>
<td>-7.8 (±0.1)</td>
<td>-41.6 (±1.3)</td>
<td>-33.8 (±1.4)</td>
<td>-0.13 (±0.07)</td>
<td>-42.4 (±2.5)</td>
</tr>
<tr>
<td>200 mM KCl</td>
<td>5–30</td>
<td>-8.8 (±0.1)</td>
<td>-40.4 (±3.3)</td>
<td>-31.7 (±3.3)</td>
<td>-0.16 (±0.10)</td>
<td>-37.6 (±3.5)</td>
</tr>
</tbody>
</table>

* Determined from 30–45°C; all other values determined using temperatures ≤30°C.

The van’t Hoff enthalpies (ΔH_VH) determined from Equation (5) agree well with the ΔH_{25C} values (Table 3). Only in 1.0 mM MgCl₂ is there a noticeable discrepancy between ΔH_{25C} and ΔH_VH, because K₉ measurements in 1.0 mM MgCl₂ were performed across the temperature range 30–45°C, where ΔH is significantly more exothermic than it is from 10 to 30°C. The overall strong agreement shown in Table 3 corroborates homogeneous, one-to-one binding and underscores the robustness of van’t Hoff equation-based extrapolations of K₉ for kinetics analysis (below).

The 25°C entropy contribution (TΔS_{25C}) in Table 3 was calculated according to the Gibbs equation using ΔG_{25C} and ΔH_{25C}. TΔS_{25C} is large and negative in all cases, indicating an unfavorable contribution. Parameter uncertainty and the relatively small number of salt concentrations examined in this study do not allow an unambiguous determination of whether the [salt]-dependence of the stability is largely entropically or enthalpically based, but additional data show it to be largely entropic as expected (16,40,41) (Supplementary Figure S3).

Comparison of the kinetics of tetraloop–receptor association in KCl and MgCl₂

Representative kinetic analyses of the titrations in Figures 2 and 3 are displayed in Figure 4. Figure 4A plots the deconvolved power trace and respective fitted curves for injections 2, 7, and 12 from the representative titration collected in 0.5 mM MgCl₂, 20°C, and a similar sampling of peaks and fits from the 150 mM KCl, 20°C dataset is displayed in Panel B. The resulting forward rate constants, k_on, are plotted for each injection from the first half of each titration (all injections such that [TT]_{total} > [RR]_{total}) in Figure 4C. As is visible in the raw data (Figure 2A), the observed rate of association decreases appreciably with each subsequent injection over the course of the first half of the titration, reflecting the decreasing concentration of unbound RR. Because k_on is a microscopic rate constant, it is independent of the RNA concentration (Figure 4C); the weighted-average k_on for these experiments are 860 (± 50) M⁻¹ s⁻¹ in MgCl₂ and 2180 (± 240) M⁻¹ s⁻¹ in KCl. The faster association of TT–RR in KCl versus MgCl₂ solution at ambient temperatures and respective salt concentrations eliciting similar stabilities is consistent with extant RNA folding literature (22–24).

The bimolecular kinetics equation applied to obtain k_on requires knowledge of K₉ (Materials and Methods’ section). For many conditions in this work, the binding strength is in an optimal window, so K₉ can be obtained with high precision and the measured value can be directly inserted into the kinetic analysis. However, in many of the low temperature experiments, the binding constant is greater than 2 × 10⁷ M⁻¹ (i.e. c > 200), so in these cases K₉ was determined by van’t Hoff extrapolation. Note that for these roughly stoichiometric binding scenarios, error in K₉ only weakly influences k_on (Materials and Methods’ section). k_off was subsequently calculated according to the assumption of a single step mechanism, i.e. k_off = k_on / K₉. k_on and k_off measurements for all conditions are listed alongside the aforementioned thermodynamic results in Tables 1 and 2. The rate constants observed in this work are consistent with those obtained by Downey et al. (19), using a tethered tetraloop–receptor construct (Supplementary Data).

Analysis of the temperature dependence of k_on and k_off using the Eyring equation allows a thermodynamic characterization of the transition state.

\[
\ln(k) = \ln(\alpha) + \frac{\Delta S^T}{R} - \frac{\Delta H^T}{RT}
\]

The slope in a plot of ln(k) against the inverse temperature reveals the transition state enthalpy (ΔH^T) in the slope and a term containing the transition state entropy (ΔS^T) in the y-intercept. The so-called pre-exponential term (α) (42) is necessary to recover ΔS^T and has been discussed in the context of RNA folding elsewhere (43,44); here we simply assume a value within the range of possible values for macromolecular folding (43–46), as our intention is merely to provide ΔS^T values for comparison among salt conditions. Because turnover probability is a fundamental property of the transition state, it is almost certainly not influenced by salt type or concentration, thus application of a single α-value to all conditions is the only requirement for rigorous comparative analysis. Additionally, the logarithmic relationship between ΔS^T and α means the likely maximum error in TΔS^T is no more than a few kcal/mol.

Figure 5 plots the logarithms of k_on and k_off against inverse temperature for all of the experiments in this work. In MgCl₂ (Figure 5A), the slopes (−ΔH^T/R) for each salt concentration and both reaction directions are...
negative, indicating that $\Delta H^\ddagger$ is large and positive for both association and dissociation. The full set of transition state theory thermodynamic quantities are listed in Table 4. $\Delta H^\ddagger_{on}$ is on average 8.1 (±0.5) kcal/mol in MgCl₂ solutions. This is consistent with a previous measurement, which used an oligonucleotide linker to monitor intraconstruct tetraloop–receptor association via FRET, and determined an upper limit $\Delta H^\ddagger_{on}$ of 12.7 kcal/mol in 10 mM MgCl₂ (19). Using the aforementioned $\alpha$-value, the average entropy contribution to forming the transition state ($\Delta S^\ddagger_{on}$) from the unbound starting state is small and slightly favorable: 2.4 (±0.5) kcal/mol. For dissociation ($k_{off}$), the slopes are significantly steeper than the association plots, indicating even larger values for $\Delta H^\ddagger_{off}$.

The difference in association and dissociation transition state enthalpies is consistent with the binding exothermicity (Table 3). Also notable in Figure 5A is the difference between the [MgCl₂]-dependence of the association and dissociation profiles. In plots of $k_{on}$ there exists a significant and systematic trend in the rate constants measured at a common temperature, manifested as a horizontal offset. However, for $k_{off}$ the temperature-dependent plots are nearly collinear. This behavior reflects a much more significant [MgCl₂]-dependence in $k_{on}$ than in $k_{off}$.

Eyring plots obtained in KCl are displayed in Figure 5B. The data bear a strong resemblance to MgCl₂ data with one particularly striking difference: the temperature-dependence of $k_{on}$ is almost entirely absent and is in fact slightly in the opposite direction as the MgCl₂ data for all KCl solutions, indicating that $\Delta H^\ddagger_{on}$ is slightly negative. Thus, the cation identity-based difference in $\Delta H^\ddagger_{on}$ parallels the observed difference in $\Delta H$ for the equilibrium binding process (Figure 3 and Table 3). The average $\Delta H^\ddagger_{on}$ value in KCl is −0.5 (±0.3) kcal/mol in KCl, a slightly favorable contribution that is much less than the unfavorable average value of 8.1 (±0.5) in MgCl₂. $\Delta S^\ddagger_{on}$ is similarly reduced in KCl to an unfavorable average value of −5.9 (±0.3) kcal/mol, contrasting with the transition state entropy term in MgCl₂.

Trends in the salt concentration dependence of binding and rate constants are listed in Table 5, according to the log-log slope, using the molal salt activity ($a_{salt}$) as the dependent variable. These dependences report the change in the salt preferential interaction coefficient ($\Delta T_{salt}$) for a process (16,34), which conveys the net cation uptake into the RNA coulombic atmosphere. The salt activity dependence for the equilibrium binding event, $\ln k_{on}/\ln(a_{salt})$ is 6.2 (±0.5) in KCl and 2.8 (±0.1) in MgCl₂. This approximately 2-fold difference is consistent with the cation valences.

In MgCl₂, the [salt]-dependence of the binding constant is almost entirely explained through the on-rate. The dependence of $k_{on}$ ($\ln k_{on}/\ln(a_{MgCl₂})$) accounts for most of the dependence of $K_a$ on $a_{MgCl₂}$, whereas the off-rate exhibits a negligible $a_{MgCl₂}$-dependence (Table 5). In KCl the situation is slightly more balanced: the $k_{on}$ $a_{KCl}$-dependence is 61% as large as $\ln K_a/\ln(a_{KCl})$, while the relative magnitude of the reverse rate constant quantity is approximately half as large.

**DISCUSSION**

**TT–RR association in the context of RNA folding**

As generally observed for RNA folding, tetraloop–receptor driven helical packing exhibits highly salt-dependent behavior (20,47,48) and is enthalpy-driven (2,4,49–55). The results here demonstrate that the exothermicity can be extremely solution dependent, with the association enthalpy varying by as much as 20 kcal/mol. Additional data indicate that it is the balance of Mg²⁺ and K⁺ concentrations that is most relevant to the thermodynamic structure of the folding landscape (17) (Supplementary Figures S4 and S5). Mikulecky et al. (54) also observed a significantly less
negative enthalpy for folding the hammerhead ribozyme in a solution containing added MgCl2, with a $\Delta H$ of 24 kcal/mol in solutions containing 100 mM NaCl and either 0 or 10 mM MgCl2. Kinetic studies of RNA folding have generally observed a (large) positive activation enthalpy (3–5,44,47,56–63), and these studies were almost all conducted in solutions containing millimolar concentrations of MgCl2.

Interestingly though, Cole and Crothers (3) identified a tRNA tertiary structure transition with $\Delta H = 0$ kcal/mol for formation in a solution lacking MgCl2 (4). Here we observe a large positive $\Delta H$ in MgCl2 solutions but a lower, near-zero enthalpic barrier in KCl solutions. The tRNA result and the [MgCl2]-dependent hammerhead ribozyme $\Delta H$ suggests potential generality to these findings.

Figure 6 summarizes the thermodynamic reaction coordinate profiles for TT–RR binding in solutions containing either MgCl2 or KCl, using representative conditions (0.5 mM MgCl2 or 150 mM KCl). A sizeable enthalpic
barrier to association in MgCl₂ of 7.3 kcal/mol contrasts with the slightly negative $\Delta H^{\text{on}}$ of $-0.6$ kcal/mol in KCl (Figure 6A). The overall binding process is 21.7 kcal/mol more exothermic in KCl than in MgCl₂ (by 7.9 and 13.8 kcal/mol, respectively). The entropic contribution to binding ($T\Delta S$) is essentially the reverse energetically of the enthalpic diagram (Figure 6B). In other words, formation of the transition state and subsequent conversion to the bound state are each more entropically opposed in KCl than in MgCl₂. The reason for the stepwise disparity cannot be resolved from our data; it may relate to the bivalent construct design if the folding pathway traverses two similar activation barriers on the way to the final bound state.

The data suggest that the transition state is relatively compact; in other words, the transition state is at least a partially associated TT–RR complex. In both MgCl₂ and KCl, the forward rate constant [salt]-dependence, $\partial \ln k_{\text{on}}/\partial \ln(a_{\text{salt}})$ constitutes most of the $a_{\text{salt}}$-dependence of the overall association process, $\partial \ln K_{\text{on}}/\partial \ln(a_{\text{salt}})$ (Table 5). This result is in line with the analogous process of double helix formation (64), and suggests the charge density of the rate-limiting transition state is most similar to that of the final bound state. The [salt]-dependence breakdown for TT–RR is also similar to that previously identified by Nesbitt and co-workers using a tethered tetraloop–receptor design. They found $\partial \ln k_{\text{on}}/\partial \ln[MgCl₂] = 0.49$ to be approximately 2/3 of the overall $\partial \ln K_{\text{on}}/\partial \ln[MgCl₂] = 0.74$ (65). This agreement suggests that a compact transition state is not specific to the bimolecular design in our study.

While the evidence points to a compact transition state, it may not involve tertiary contacts. Previous investigations of RNA folding identified an ‘early’ transition state; i.e. a state lacking specific stabilizing tertiary contacts (43,44,66). Bokinsky et al. (66) went on to also characterize the [salt]-dependence of hairpin ribozyme folding. As is true here for tetraloop–receptor mediated helical packing, the [salt]-dependence of hairpin ribozyme folding is almost entirely in the forward direction in MgCl₂. Moreover, in the monovalent salt NaCl, the hairpin ribozyme [salt]-dependence is more balanced between the forward and reverse rate constants, further paralleling our TT–RR association data. Bokinsky et al. concluded that the transition state is compact yet early, and further hypothesized that in monovalent salt, the transition state may be slightly less compact than in MgCl₂. This interpretation is consistent with the data herein for tetraloop–receptor mediated helical packing.

**Potential explanations for cation-dependent $\Delta H$ and $\Delta H^\ddagger$**

We consider two conceptual explanations for the cation-dependent TT–RR binding profile highlighted by Figure 6. Either there is a cation-dependent difference in the RNA structure that influences the thermodynamics of folding, or the explanation lies in the difference in the thermodynamics of potassium and magnesium ion uptake that occurs concomitant with tetraloop–receptor association. The difference is not directly related to the potassium ‘binding’ site (28,34,67) (Supplementary Table S2 and Figure S6). In the discussion below we focus on the cation-dependence in enthalpy terms because entropy terms are strongly [salt]-dependent, whereas $\Delta H$ is only weakly [salt]-dependent in these physiological salt concentration regimes (16,40) (Supplementary Figure S3).

In terms of structural explanations, we think the most likely interpretation invokes a salt-dependent unbound receptor conformation. For example, if a free receptor conformation such as that previously observed (68) were stabilized by magnesium, the unbound state would be enthalpically stabilized and a corresponding penalty for obligatory disruption of this structure would be observed in forming the transition state and final bound state in MgCl₂, but not in KCl. There is some precedence for this functional behavior as kinetic traps in large RNA folding are more prevalent in solutions containing Mg$^{2+}$ and little or no monovalent salt (22,48,57). The effect here does not appear to be related to a structural effect induced by some level of ‘ionic strength’, however (Supplementary Figure S4).

Alternatively and potentially of more generality, cation hydration may explain the divergent thermodynamic
profiles in KCl and MgCl₂. Formation of the transition or bound state from the unbound state requires an uptake of cations due to the increased charge density (Table 5). Thus it may be that the uptake of diffuse monovalent and divalent cations by the tetraloop–receptor contact in particular, or in folded RNA structures in general, occurs with different thermodynamic signatures. Most simply, partial dehydration of one or more Mg²⁺ accumulated in the local domain of the RNA might incur an additional enthalpic penalty concomitant with formation of both the transition state and final bound state. Given a Mg²⁺ dehydration enthalpy of ~450 kcal/mol, even a slight disturbance in an outer hydration shell could explain this difference (69).

We favor this ion hydration hypothesis because it is consistent with an early but compact transition state. There is evidence that Mg²⁺ accumulation by nucleic acids is accompanied by partial dehydration and is partially non-coulombic in nature (70,71), and replacement of sodium with magnesium in the local domain of polyU or polyA-polyU may be accompanied by a slightly positive ΔH (72). On the other hand, low-resolution data argue against a difference in ΔH for magnesium and monovalent cation accumulation near RNA (2,6).

The magnitude and generality of the phenomenon observed here may of course depend on RNA structural details. A slight variation on (or addition to) the ion hydration hypothesis focuses on differences in how cations affect RNA hydration. Magnesium, more than a monovalent cation, preferentially accumulates in the most electro-negative regions of nucleic acids (9,73). Thus relative to K⁺, complexation in the presence of Mg²⁺ is accompanied by a disproportionate release of RNA hydrating waters from these regions; if these water molecules possess a significantly favorable solvating enthalpy term, then their displacement will be observed as a positive ΔH contribution. Accumulation of Mg²⁺ may therefore displace a larger quantity of such highly enthalpically stabilized hydrating waters from the RNA than does K⁺. As above this would yield the observed behavior that magnesium-driven RNA folding is associated with a larger enthalpic penalty.

Overall the data provide new insight into RNA folding in a physiological, mixed salt solution. Preliminary data suggest tertiary folding in physiological conditions may exhibit a balance of K⁺ and Mg²⁺ stabilization (Supplemental Figure S5). One consequence of this finding would be that RNA folding in vivo does not exhibit the extreme temperature dependence in the rate of folding predicted by most in vitro studies, which have been conducted in solutions dominated by MgCl₂.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR online.

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