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Effects of Preferential Counterion Interactions on the Specificity of RNA Folding

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Abstract

The real-time search for native RNA structure is essential for the operation of regulatory RNAs. We previously reported that a fraction of the *Azoarcus* ribozyme achieves a compact structure in less than a millisecond. To scrutinize the forces that drive initial folding steps, we used time-resolved SAXS to compare the folding dynamics of this ribozyme in thermodynamically isostable concentrations of different counterions. The results show that the size of the fast-folding population increases with the number of available counterions and correlates with the flexibility of initial RNA structures. Within 1 ms of folding, Mg^{2+} exhibits a smaller preferential interaction coefficient per charge, Γ_+/Z , than Na^+ or $[\text{Co}(\text{NH}_3)_6]^{3+}$. The lower Γ_+/Z corresponds to a smaller yield of folded RNA, although Mg^{2+} stabilizes native RNA more efficiently than other ions at equilibrium. These results suggest that strong Mg^{2+} -RNA interactions impede the search for globally native structure during early folding stages.

Graphical Abstract

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ASSOCIATED CONTENT

Supporting Information.

The Supporting Information is available free of charge on the ACS Publications website at DOI: sample preparation methods; X-ray scattering data; data analysis methods (PDF)

RNA molecules must fold into well-defined three-dimensional structures to activate many RNA-dependent biological events.^{1–3} The self-assembly of RNA into compact tertiary structures is opposed by electrostatic repulsion of the phosphates, the reduced configurational entropy of the RNA itself, and the concomitant ordering of ions and water molecules around the folded RNA.^{4–8} These unfavorable energetic changes are compensated by favorable Coulombic interactions with counterions which allows for chain flexibility, as well as by base stacking and hydrogen bonding.^{9–10} Understanding how dynamics of folding intermediates is coupled with the interactions of counterions with the RNA is central to unraveling the mechanism of RNA folding.^{2, 11–13}

We have used the stable, ~200 nt *Azoarcus* group I ribozyme as a model system for the folding of RNA structures comprising multiple helical domains. For the wild type ribozyme, rapid helix assembly (1–50 ms) produces compact, native-like structures (I_C) that can be monitored by changes in the solution scattering of the RNA particles.^{14–16} Further reorganization of the I_C intermediate in 2 mM MgCl_2 is required to stabilize all of the native tertiary interactions and for ribozyme activity.^{17–19}

A number of studies have shown how counterions influence the folding pathways of large ribozymes (eg, ^{20–28}) such as the *Azoarcus* group I ribozyme^{12, 29}. Metal ions such as Mg^{2+} interact more strongly with the folded RNA than with the unfolded RNA.^{30–35} Although some metal ions bind specific sites in the folded RNA,^{27–28} dehydration of counterions is energetically costly,^{12, 36–37} and most ions interact dynamically and non-specifically with the RNA's electrostatic field.^{30, 35, 38}

As predicted by counterion condensation theory,⁴ lower concentrations of multivalent ions than monovalent ions are needed to stabilize the folded RNA^{2, 12, 31–35, 39}. In addition, owing to interactions between cations that directly or indirectly interact with the negatively charged RNA, folding becomes more cooperative with respect to ion concentration when the ionic radius of the hydrated cation is small.^{29, 40–41} Therefore, the valence and size of the hydrated cations both affect the free energy gap between the unfolded and folded RNA.^{40, 42}

The strength of cation-anion pairs in the solvent has also been reported to contribute the preferential interaction of different cations with the RNA.⁴³

Understanding how different counterions influence the earliest stages of RNA folding intermediates remains a daunting challenge. RNA folding kinetics is often heterogeneous, with the RNA population partitioning into native-like and non-specific intermediates, followed by a slower rearrangement of misfolded states.^{30, 44–48} Using stopped-flow SAXS, we and others previously observed that the initial collapse transition, which we attributed to specific nucleation of native-like structures in the *Azoarcus* ribozyme, occurs within 1–10 ms.^{49–53} Importantly, the amplitudes of initial and subsequent folding phases depended on the Mg^{2+} concentration, demonstrating that interactions between the cations and RNA affect the early partitioning of the RNA population into different folding pathways. How different counterions dictate this partitioning mechanism is not known.

To examine how the valence, size, and number of condensed counterions affect the early RNA folding kinetics, we compared the folding rates of the *Azoarcus* ribozyme in seven different salts by time-resolved stopped-flow SAXS. The *Azoarcus* ribozyme is advantageous for these studies because its structure is known, the wild type RNA folds cooperatively in solution at equilibrium, and its size and mass yield a robust change in the small angle X-ray scattering function as it folds. We find that different counterions alter the average compactness of the folding intermediates, which we interpret as reflecting different partitioning of the RNA ensemble into fast and slow folding pathways. We analyze this partitioning in terms of the preferential interaction coefficients of the counterion charges, and use the solution scattering curves to obtain information about the flexibility of the RNA chain during the early stages folding.

Our previous SAXS observation that the early folding intermediate ensemble of the *Azoarcus* ribozyme population varies with the Mg^{2+} concentration implied that kinetic partitioning between folding paths depends on the intrinsic stability of the tertiary interactions, or the degree of charge neutralization, or both.⁴⁹ To systematically compare the influence of different cations on the folding kinetics of the ribozyme, we established conditions in which the folding equilibria are the same by titrating the unfolded ribozyme with Na^+ , K^+ , Mg^{2+} , Ca^{2+} , Ba^{2+} , $[\text{Co}(\text{NH}_3)_6]^{3+}$, or spermidine (spd^{3+}) (Figures S1 and S2). The resulting solution scattering curves were consistent with our previous data on this RNA²⁹, and $P(r)$ or Guinier analysis of the scattering intensity showed that the R_g decreased to 31–34 Å when ribozyme was refolded in any of these counterions (Figure S2a and Table S1). Because the main folding transition at equilibrium is dominated by two states, as judged by a previous SVD analysis of the scattering functions²⁹, we used R_g as an order parameter of the system to calculate the average fraction of unfolded RNA, Φ_U (eq. S1 and Figure S2b). The change in R_g and Φ_U with counterion concentration C was well fit by a two-state folding equilibrium (eq. S4 and S5) as described in MATERIALS and METHODS. As previously observed,^{29, 54} much higher concentrations (0.1 to 0.2 M) of monovalent Na^+ and K^+ are needed to reach the midpoint of the folding transition, compared to 0.4 to 1.2 mM for divalent and trivalent ions, in the order $[\text{Co}(\text{NH}_3)_6]^{3+} \sim \text{spd}^{3+} < \text{Ca}^{2+} < \text{Ba}^{2+} < \text{Mg}^{2+} \ll \text{Na}^+ \sim \text{K}^+$ (Figure S2 and Table S1).

Real-time folding of the *Azoarcus* ribozyme (1 mg/mL) was measured by stopped-flow SAXS with a mixing dead time of 0.6 ms as previously described.⁴⁹ To understand how different counterions affect the RNA folding pathways, we compared the folding kinetics in two isostable conditions, at which the RNA was 90% or 95% folded, respectively. These conditions provide the best resolution of the kinetic phases. We refer to the concentrations of each counterion at these isostable states as C_{090} and C_{095} (Table S1). Just 1 ms after mixing with any of the counterions at C_{090} , dimensionless Kratky plots of the scattering intensity⁵⁵ showed that the ribozyme RNA was already substantially more compact than the unfolded state (Figure 1a). The pair probability density distribution $P(r)$ at C_{090} showed that the maximum distance, $R_{\max}$, decreased by 20 ~ 50 % after 1 ms, compared to $R_{\max} \sim 200$ Å for the unfolded RNA in 20 mM Tris-HCl (Figure 1b). This change in scattering function correlates with a decrease in the average R_g from 60 Å to ~ 40 Å.

All seven counterions tested, including monovalent ions and the polyamine spermidine, trigger the collapse of the *Azoarcus* ribozyme into more compact structures within a millisecond. However, each counterion produced a different scattering profile within this window (Figures 1 and S3), although the thermodynamic stability of the folded RNA at the equilibrated conditions was the same. Surprisingly, the RNA was less compact after 1 ms of folding in Mg^{2+} ($R_{\max} = 135$ Å) and Ca^{2+} ($R_{\max} = 160$ Å), although these divalent ions strongly stabilize the native ribozyme at equilibrium (Table S1). By contrast, the ensemble of intermediate structures after 1 ms was most compact in $[Co(NH_3)_6]^{3+}$ and Na^+ at both C_{090} and C_{095} . Interestingly, the $P(r)$ curves for RNA folded in Mg^{2+} and Ca^{2+} display a shoulder at ~ 25 Å (Figure 1b). These differences demonstrate that the early folding intermediates depend on the types of counterions that are available to interact with the RNA, and suggest that the counterions directly affect how the RNA partitions among different folding pathways.

Our previous time-resolved SAXS studies showed that the *Azoarcus* ribozyme folds in at least three kinetic phases: a fast transition $\tau_{\text{fast}} \sim 1$ ms that occurs within the mixing time of the experiment described above, a second phase $\tau_{\text{med}} \sim 10 - 40$ ms, and a very slow phase $\tau_{\text{slow}} \sim 740$ ms. Because the amplitude of the fast phase increases when the initial RNA ensemble is partially denatured with urea, and tertiary interactions detected by footprinting appear throughout the ribozyme within 50 ms,¹⁴ we inferred that the fast process ($\tau_{\text{fast}} \sim 1$ ms) likely represents a specific collapse to native-like structures.^{49, 56} The observed R_g at this early stage was smaller than that of an equilibrium folding intermediate.⁵⁷ This observation and the early appearance of tertiary interactions throughout the ribozyme are inconsistent with a purely sequential folding mechanism, and better explained by a parallel folding process that yields a mixture of native-like and non-native structures. Because the amplitude and time constant of the second phase decrease in urea, we postulated that the second phase with $\tau_{\text{med}} \sim 10$ to 40 ms corresponds to a conformational search for the correct topology after non-specific collapse^{30, 49}. The difference between the extent of folding after 200 ms and at equilibrium correlates with very slow ($\tau_{\text{slow}} \sim 100$ s) reorganization of RNAs which are kinetically trapped in misfolded intermediates.^{50, 58-59}

To examine the partitioning of the folding reaction between different kinetic phases as shown in the plot of time dependent R_g values (Figure 2), we fit a double exponential decay

function to the time dependence of the fractional weight (Φ_U , see MATERIALS and METHODS for its relationship with R_g) over the first 200 ms after mixing, which corresponds to the first two kinetic phases (Figure S4),

$$\Phi_U(t) = 1 - P_{fast} \left(1 - e^{-t/\tau_{fast}} \right) - P_{med} \left(1 - e^{-t/\tau_{med}} \right) \quad (1)$$

In this bi-exponential expression of a parallel folding mechanism, the observed amplitudes of each kinetic phase are taken to be roughly equal to the populations P of each path, which is a valid approximation when the time constants for each phase differ by more than tenfold. The amplitude of the slowest phase is taken from the difference between the extents of folding at 200 ms and 10 min. The parameter Φ_U describes the fractional saturation of tertiary interactions overall and is thus independent of which model one uses to interpret the kinetics data.

Stopped-flow SAXS experiments conducted in varied concentrations of Na^+ , Mg^{2+} , spd^{3+} and $[\text{Co}(\text{NH}_3)_6]^{3+}$ showed that the kinetics evolves as the amount of counterion is raised (Figures 2 and S4), with the greatest heterogeneity at C_{090} or C_{095} as observed previously in Mg^{2+} .⁵⁸ While the magnitudes of the fast and medium kinetic phases differed among the various counterions, the time constant of the medium phase changed little within the error of the measurement. These observations can be explained if the counterions alter partitioning of the RNA population among different folding pathways.

Figure 3 displays the sizes of the fast, medium and slow folding populations, P_{fast} , P_{med} and P_{slow} , obtained from the fit of $\Phi_U(t)$ (Figure S5) under the isostable C_{090} and C_{095} conditions (Figures 3a and 3b). Among the cations tested, the apparent population of the fast phase (P_{fast}) was smallest in Mg^{2+} and Ca^{2+} and largest in Na^+ and $[\text{Co}(\text{NH}_3)_6]^{3+}$. Other counterions presented intermediate effects, with K^+ most similar to Na^+ , and Ba^{2+} between K^+ and Mg^{2+} . Thus, while monovalent counterions produce the most gradual equilibrium folding transitions, they direct a larger fraction of the RNA ensemble into the compact intermediate state at short times. By contrast, Mg^{2+} is less able to promote very rapid collapse, although it stabilizes the folded RNA more efficiently than Na^+ , K^+ or Ba^{2+} . $[\text{Co}(\text{NH}_3)_6]^{3+}$ effectively induced compact structures in 1 ms (large P_{fast}). However, no further reduction in R_g occurred over the next 200 ms, suggesting that reorganization of the remaining unfolded population was too slow to observe in this time window (the largest gray bars, Figures 3). $[\text{Co}(\text{NH}_3)_6]^{3+}$ has been previously found to increase the kinetic barriers to RNA refolding²⁶. At longer times, footprinting showed that the RNA forms similar tertiary interactions in $[\text{Co}(\text{NH}_3)_6]^{3+}$ and Mg^{2+} , except around the active site⁶⁰.

Further analysis of the scattering curves indicated that kinetic partitioning of the RNA population into specific (fast) versus non-specific (medium) folding pathways is directly related to the structural flexibility of the RNA at 1 ms. To compare the structures of the folding intermediates produced by different counterions, we evaluated the Porod region of the scattering curves ($0.5 \text{ \AA}^{-1} \leq Q \leq 0.15 \text{ \AA}^{-1}$) after 1 ms folding (see Figure S6 for the details of the fits). The exponent ν of fits to $I(Q) \sim Q^{-\nu}$ over this region reflects the average

compactness and local flexibility of the chain, with $\nu = 2$ corresponding to a random coil and $\nu = 4$ corresponding to a sphere. Values of ν less than 2 indicate greater local stiffness, compared to an ideal Gaussian chain. In general, the flexibility of a polyelectrolyte chain depends on the degree of electrostatic neutralization, which approaches a random coil when neutralization is complete.⁶¹ Remarkably, larger P_{fast} correlates with an increase in ν (Figure 4), implying that counterions that favor partitioning of the RNA into the fast collapse pathway also increase the local flexibility of the RNA backbone at early times in the folding process.

The value of ν increases more between 1 ms and 170 ms when the RNA is folded in Mg^{2+} and Ca^{2+} more than when it is folded in other counterions (Table S3 and Figure S7), indicating that Mg^{2+} and Ca^{2+} delay the appearance of compact or relaxed structures. Likewise, the kinetic partitioning of the RNA ensemble into non-specific collapse pathways, $P_{\text{med}} + P_{\text{slow}}$, is more accentuated in Mg^{2+} and Ca^{2+} than in Na^+ , K^+ , and $[\text{Co}(\text{NH}_3)_6]^{3+}$ (Figure S9). These results prove that counterion charge density is not directly linked to the partitioning of the RNA between native-like and non-specific folding.

The excess counterions associated with each RNA molecule at a given bulk concentration of counterions can be described by a preferential interaction coefficient, Γ_+ ($= C/C_{\text{RNA}}$)¹¹ in which $C = C_{\text{total}} - C_{\text{free}}$, and C_{free} denotes the molar concentration of unbound counterions and C_{RNA} is the concentration of RNA. Γ_+ is related to condensation of counterions around the RNA. According to Wyman linkage analysis, and the thermodynamic cycle for ion interactions with the unfolded and folded RNA (Scheme S1),¹² the free energy of an RNA conformational transition is related to the change of the excess counterions for the two different conformers, Γ_+ , which is akin to the steepness of the transition with respect to total counterion activity $a \approx C$,^{12, 62–64} yielding

$$\Delta \Gamma_+ = -\frac{1}{RT} \frac{d \Delta G_{\text{obs}}}{d \ln a} \approx -\frac{1}{RT} \frac{d \Delta G_{\text{obs}}}{d \ln C}. \quad (2)$$

The error introduced into our analysis by replacing ion activities with concentrations in eq. 2 is likely small for our assays that meet the conditions of (1) a large excess of counterions (cations) and co-ions (anions) over RNA concentration (16 μM in our RNA samples), and (2) stable RNA secondary structure buffered by monovalent cations (20 mM Tris-HCl at pH 7.5 in our samples).^{11, 62, 65}

Evaluating the kinetics based on the approximation above yields an estimate for the stability of the intermediate population relative to the unfolded ensemble,

$$\int_{C_m}^C \Delta \Gamma_+ d \ln C = -\frac{1}{RT} \int_{C_m}^C d \Delta G_{\text{obs}} \quad (3)$$

$$\frac{\Delta \Delta G_{obs}}{RT} = - \Delta \Gamma_+ \ln\left(\frac{C}{C_m}\right). \quad (4)$$

The term C/C_m in eq. 4 represents the concentration C of cations needed to reach a given folding ensemble from the reference condition, C_m . In general, C/C_m is smallest for multivalent cations including Ca^{2+} , Mg^{2+} , Ba^{2+} , spd^{3+} and $[\text{Co}(\text{NH}_3)_6]^{3+}$ in which the collapse transition is steep ($n > 3$; Table S1), and largest for monovalent ions in which the transition is shallow ($n \approx 1.5$).

The size of the fast-folding population, P_{fast} , increases as the number of ions exceeds the midpoint of the folding equilibrium (Figure 3). However, P_{fast} increased more strongly with the concentration of multivalent counterions than with the concentration of Na^+ (Figure S10). We calculated an effective $G_{\text{fast}} = -RT \ln[P_{\text{fast}}/(1-P_{\text{fast}})]$ that represents the stability of folding intermediates leading to collapse within 1 ms, at a given counterion concentration C . Figure 5 shows that G_{fast}/RT calculated over C to C_m varies linearly with $\ln(C/C_m)$ for each set of mono-, di- and trivalent cations (Na^+ ; Mg^{2+} ; $[\text{Co}(\text{NH}_3)_6]^{3+}$). The resulting slope corresponds to Γ_+ (eq. 4) which ranges from 0.63 to 1.89 in the order of $\text{Na}^+ < \text{Mg}^{2+} < [\text{Co}(\text{NH}_3)_6]^{3+}$ (Table 1). Thus, the ability of different counterions to trigger rapid folding of the RNA correlates with their preferential interaction coefficients.

At the isostable counterion concentrations C_{090} or C_{095} , the statistical weights $-G_{\text{fast}}/RT$ are greater for Na^+ and $[\text{Co}(\text{NH}_3)_6]^{3+}$ than for Mg^{2+} (circled data points in Figure 5). This correlates with a smaller preferential interaction coefficient per charge, Γ_+/Z , for Mg^{2+} , followed by Na^+ and $[\text{Co}(\text{NH}_3)_6]^{3+}$ (Table 1). These differences may be linked to the physical reason why the fast-folding population P_{fast} is smallest in Mg^{2+} ions at the isostable condition, compared to Na^+ or $[\text{Co}(\text{NH}_3)_6]^{3+}$.

Condensed counterions stabilize RNA tertiary structures, and a number of studies have explored how counterions of different charge and size affect the equilibrium stability of large ribozymes.^{9, 12, 29–30, 39, 48} In general, multivalent ions stabilize the folded RNA more efficiently than monovalent ions, and singular value decomposition analyses of SAXS signals of the *Azoarcus* and *Tetrahymena* group I ribozymes indicated that the cooperativity of the equilibrium transition increases with the charge density of counterions.^{40, 66} Our current SAXS measurements of the equilibrium folding transition in different counterions follow this same trend (Figure S2). This dependence of the folding equilibrium on counterion charge density arises in part because smaller, more highly charged ions more effectively neutralize the negative charges of the phosphate groups.^{29, 40}

The forces that drive the early stages of folding are more difficult to understand because counterion interactions are thermodynamically linked to the formation of the RNA tertiary structure, and because the folding pathways are heterogeneous.^{26, 67–69} On the one hand, strong ion-RNA interactions are expected to efficiently seed native tertiary structure.^{68–72} On the other hand, ions that strongly stabilize the RNA can impede relaxation of the

molecular interactions during the folding reaction, as seen in studies of protein folding and the relaxation of small RNA hairpins^{73–74}.

By resolving fast and slow phases of the folding process for the *Azoarcus* ribozyme, our stopped-flow SAXS results show that the ability of different counterions to promote rapid folding does not correlate with counterion charge density and the equilibrium stability of the folded RNA. Instead, we find that the magnitude of the fast folding phase (P_{fast} , $\tau < 1$ ms), which we associate with specific, native-like collapse,^{50, 56} is smallest in Mg^{2+} and Ca^{2+} , and larger in monovalent ions and trivalent amines (Figure 3). These trends can be qualitatively rationalized by the predicted strength of direct metal ion-RNA interactions, and the flexibility of the early RNA folding intermediates. In our results, the RNA after 1 ms is more rigid (or extended) on the length scale of a nucleotide to a helix in Mg^{2+} and Ca^{2+} than in Na^+ or K^+ (Figure 4). One possibility is that Mg^{2+} ions efficiently nucleate folding by binding different regions of the *Azoarcus* ribozyme. However, impaired diffusion of the intrachain interactions reduces the probability of reaching a globally native-like structure, reducing the yield of folded RNA at 1 ms. Conversely, monovalent ions stabilize the final native state less efficiently than divalent ions, yet have greater preferential charge interactions during initial folding, produce more relaxed structures that can sample different intrachain interactions, and result in a larger fast folding population.

These observations are consistent with previous findings that Mg^{2+} ions stabilize and rigidify helix junctions in DNA and RNA, resulting in faster folding toward the native configuration of the hairpin ribozyme and a rRNA three-helix junction, yet slower exchange between alternative conformations.^{68–70} They are also consistent with slower refolding kinetics and increased partitioning through non-native folding pathways of the *Tetrahymena* ribozyme^{26, 67, 75} and the RNase P catalytic domain,⁷⁶ in Mg^{2+} compared to Na^+ and K^+ .

Although cobalt hexammine interacts strongly with the RNA owing to its high charge density, its preferential interaction coefficient per unit charge is similar to that of Na^+ , and more favorable than Mg^{2+} with respect to the fast-folding population (Figure 5). Unlike Mg^{2+} and Ca^{2+} , $[\text{Co}(\text{NH}_3)_6]^{3+}$ cannot directly coordinate the RNA phosphates (inner sphere) because its amine ligands resist exchange. Instead, $[\text{Co}(\text{NH}_3)_6]^{3+}$ ions interact with the hydrated RNA via hydrogen bonding with the amines. In this sense, $[\text{Co}(\text{NH}_3)_6]^{3+}$ is more similar to Na^+ , for which dehydration is also energetically unfavorable. Accordingly, the larger fast folding population and more favorable Γ_+/Z for Na^+ and $[\text{Co}(\text{NH}_3)_6]^{3+}$ than Mg^{2+} could reflect the smaller energetic cost of shedding the outer hydrating waters, if Mg^{2+} ions form inner sphere bonds that exchange more slowly.

Our finding that fast native-like folding population is linked to preferential charge interactions rather than to charge density suggests that the initial tertiary structures form from specific ion-RNA interactions. This concept is in agreement with recent simulations of the folding pathway of the *Azoarcus* ribozyme, which showed that Mg^{2+} ions nucleate assembly of the tertiary structure by stabilizing local interaction motifs.⁷¹ Nevertheless, the observation that Mg^{2+} and Ca^{2+} ions decrease the size of the fast folding population suggests that some reorganization of the RNA interactions is still needed to search out native-like structures, which may be impeded by strong association of Mg^{2+} and Ca^{2+} ions

with the RNA. It will be of interest to know how competition between different ion-RNA interactions alters the efficiency of RNA folding in the cell.

Supplementary Material

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Figure 1. Structures of early folding intermediates.

(a) Dimensionless Kratky plots $(QR_g)^2 I(Q)/I(0)$ vs QR_g ,⁵⁵ and (b) pair probability density distribution function, $P(r)$, as a function of real length scale measured for folding of RNA in different counterions (Na^+ , blue; K^+ , red; Mg^{2+} , green; Ca^{2+} , pink; Ba^{2+} , black; $[\text{Co}(\text{NH}_3)_6]^{3+}$, orange; spd^{3+} , purple) at 1 ms under C_{090} (Table S1). Folded (long dash) and unfolded (short dash) states were measured at equilibrium. The $P(r)$ was obtained from indirect inverse Fourier transform of $I(Q)$ (eq. S2). Dimensionless Kratky plots reflect the shape of the particle normalized for its molecular mass.

Figure 2. Time-dependent ribozyme folding in various counterions.

Radius of gyration, R_g values of *Azoarcus* ribozyme obtained from stopped-flow SAXS experiments for (a) Na^+ , (b) K^+ , (c) Mg^{2+} , (d) Ca^{2+} , (e) Ba^{2+} , (f) spd^{3+} , and (g) $[\text{Co}(\text{NH}_3)_6]^{3+}$. Counterion concentrations after mixing are indicated in the key for each panel. The isostable conditions for the different ions were, for C_{090} : 0.55 M NaCl, 0.8 M KCl, 1.5 mM MgCl_2 , 0.9 mM CaCl_2 , 1.3 mM BaCl_2 , 0.6 mM $[\text{Co}(\text{NH}_3)_6]\text{Cl}_3$ and 0.6 mM spermidine-HCl; and for C_{095} : 0.9 M NaCl, 1.1 M KCl, 2.0 mM MgCl_2 , 1.2 mM CaCl_2 , 1.6 mM BaCl_2 , 0.78 mM $[\text{Co}(\text{NH}_3)_6]\text{Cl}_3$ and 0.8 mM spermidine-HCl. Solid line is the best fit to a double exponential decay function. The dotted lines indicate the R_g values of the RNAs at 10 min. equilibrium states. Error bars represent the uncertainty in R_g from the data inversion.

Figure 3. Partitioning of the RNA ensemble into populations with different folding times. Color bars indicate the size of each population: fast (1 ms), medium (20 – 40 ms) and slow (> 150 s). The amplitude of the slow phase (gray bars) was calculated from the difference between the extent of folding after 200 ms ($P_{\text{fast}} + P_{\text{med}}$) and at equilibrium. Errors were calculated from the deviation of the least squares fits to the exponential decay function (eq. 1). Counterion concentrations were: (a) C_{090} and (b) C_{095} .

Figure 4. Structural flexibility and kinetic partitioning.

The exponent, ν , of Q -dependent scattering intensity, $I(Q)$, versus P_{fast} at C_{090} and C_{095} , respectively. The ν values were determined from $I(Q, 1 \text{ ms}) \sim Q^{-\nu}$ for $0.05 \leq Q \leq 0.15 \text{ \AA}^{-1}$ (see Figure S6). Error bars represent the standard deviation of the fit. The ν values reflect average local flexibility of the RNA: $\nu = 1$ represents a rod-like chain, 2 a random coil, and 4 an ideal sphere. Insets: schematic illustration of the representative ensemble conformations that are more compact (flexible) or less compact than a random coil.

Figure 5. Probability of rapid folding correlates with the excess counterions.

$\Delta G_{\text{fast}}/RT$ corresponding to the probability of collapse within 1 ms for counterion concentrations C above the folding midpoint C_m (eq. 4). The uncertainty in $\Delta G_{\text{fast}}/RT$ is $\pm 5\%$. The dashed straight lines represent a linear regression fit with $R^2 \sim 0.94$. The estimated slopes that correspond to $-\Gamma_+$ for Na^+ , Mg^{2+} , and $[\text{Co}(\text{NH}_3)_6]^{3+}$ are -0.63 ± 0.08 , -1.07 ± 0.12 , and -1.89 ± 0.29 , respectively. Straight and dashed circles indicate isostable states of C_{090} and C_{095} , respectively. The inset is a schematic view of ΔG_{fast} between C_m and each of the isostable states, C_{090} and C_{095} .

Table 1.Preferential interaction coefficients estimated from P_{fast}

Cation	Γ_+	Γ_+/Z
Na ⁺	0.63 ± 0.08	0.63
Mg ²⁺	1.07 ± 0.12	0.53
[Co(NH ₃) ₆] ³⁺	1.89 ± 0.29	0.63

 Γ : preferential interaction coefficient

Z: charge valence of cations.