Divalent ion competition reveals reorganization of an RNA ion atmosphere upon folding

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ABSTRACT

Although RNA interactions with K⁺ and Mg²⁺ have been studied extensively, much less is known about the third most abundant cation in bacterial cells, putrescine²⁺, and how RNA folding might be influenced by the three ions in combination. In a new approach, we have observed the competition between Mg²⁺ and putrescine²⁺ (in a background of K⁺) with native, partially unfolded and highly extended conformations of an adenine riboswitch aptamer. With the native state, putrescine²⁺ is a weak competitor when the ratio of the excess Mg²⁺ (which neutralizes phosphate charge) to RNA is very low, but becomes much more effective at replacing Mg²⁺ as the excess Mg²⁺ in the RNA ion atmosphere increases. Putrescine²⁺ is even more effective in competing Mg²⁺ from the extended conformation, independent of the Mg²⁺ excess. To account for these and other results, we propose that both ions closely approach the surface of RNA secondary structure, but the completely folded RNA tertiary structure develops small pockets of very negative electrostatic potential that are more accessible to the compact charge of Mg²⁺. The sensitivity of RNA folding to the combination of Mg²⁺ and putrescine²⁺ found in vivo depends on the architectures of both the unfolded and native conformations.

INTRODUCTION

It is well-known that most of the negative charge of an RNA in solution is compensated by the accumulation of excess cations in its vicinity, with only a small fraction of the phosphate charges balanced by the nearby depletion of mobile anions (1,2). The identity of the cation may strongly influence the folding equilibrium of an RNA: moderate concentrations of K⁺ stabilize many RNAs in their native structure (3), but it has been known for 40 years that Mg²⁺ promotes RNA folding far more effectively, even in the presence of the large excess of monovalent ions present in vivo (4). One reason Mg²⁺ is so effective is the entropic advantage of its divalent charge, which allows the same amount of positive charge to be positioned near an RNA with half the number of particles needed with monovalent ions; the energetic advantage is larger for RNAs with more compact structures that tend to concentrate cations closer to their surfaces (5). Another consideration is that Mg²⁺ is generally more effective at stabilizing RNA tertiary structure than larger group II cations, in part because its small size allows it to accumulate closer to the convoluted surfaces of RNAs tertiary structures (6–8).

Among divalent cations that stabilize RNA tertiary structures, of particular relevance is putrescine²⁺, which is abundant in bacteria. The total cellular putrescine²⁺ concentration varies as much as 8-fold in response to changes in growth conditions and may approach the total Mg²⁺ concentration in Escherichia coli (9,10); the ion is probably essential for optimum translation rates and cell growth (11–13). We previously found that some RNA tertiary structures are stabilized by putrescine²⁺, though not as effectively as by Mg²⁺; it is possible that putrescine²⁺ contributes a small additional stability to these RNAs at concentrations of Mg²⁺ and putrescine²⁺ found in vivo (14). Putrescine²⁺ acts very differently with a class of RNA structures that are known to chelate Mg²⁺; these RNAs are slightly destabilized by putrescine²⁺, again at Mg²⁺ and putrescine²⁺ concentrations comparable to those found in vivo (14). These observations underscore the importance of extending RNA folding studies to include putrescine²⁺ as well as Mg²⁺ and K⁺.

A simple thermodynamic cycle illustrates how the stability of an RNA structure is affected by the strength of ion interactions with both the folded and unfolded forms of the RNA (Figure 1A). Thus, to understand the differing responses of RNA tertiary structures to Mg²⁺ and putrescine²⁺, it is necessary to know how effectively the two ions compete with each other for interactions with both extended and compact conformations of an RNA. In this work, we examine Mg²⁺–putrescine²⁺ competition
RNA is largely secondary structure, becomes less effective as the RNA becomes more compact, and is an extremely weak competitor when Mg$^{2+}$ interacts primarily with the one or two regions where RNA phosphates are brought closest together by the RNA tertiary structure.

The idea that Mg$^{2+}$ plays a special role in RNA folding because of its access to small pockets of negative charge created by tertiary structures was proposed in early studies with tRNA (4,16) and supported by later electrostatic computations (17). Subsequent quantitative measurements of Mg$^{2+}$–RNA interaction free energies have recently been useful benchmarks for more sophisticated computational studies of the distribution of Mg$^{2+}$ near an RNA tertiary structure (18,19). The present data add to this developing picture by providing quantitative free energy measurements of two divalent ions competing for interactions with different RNA conformations, and are relevant to the more complex in vivo environment where RNA functions in the presence of multiple kinds of cations.

**MATERIALS AND METHODS**

**Preparation of solutions and RNA**

All solutions were prepared using water at 18.3 MΩ resistivity. All buffers and salts were ≥99.5% purity. MOPS buffer was obtained from Sigma, and brought to pH 6.8 with KOH (K•MOPS). The standard buffer was 40 mM K•MOPS (with 13 mM K$^+$), 10 μM ethylenediaminetetraacetic acid (EDTA) (Sigma) and 37 mM KCl (Fluka) to give a total K$^+$ concentration of 50 mM. MgCl$_2$ and/or diamine were also added as indicated. The first acid dissociation constant of putrescine is pK$_a$ = 9.36 (14); thus, at pH 6.8 more than 99% of the putrescine is the di-protonated species. Solutions of MgCl$_2$ (Fluka) were standardized by titration into an EDTA solution (pH 8.0) of known concentration, while monitoring absorbance at 230 nm (20). 8-hydroxyquinoline sulfonic acid (HQS, Sigma Chemicals) was purified by recrystallization as described (20).

All RNAs were prepared by transcription of linearized plasmid DNA with a hexa-histidine-tagged bacteriophage T7 RNA polymerase; the plasmids have been described previously (21,22). Transcription products were purified by preparative electrophoresis on denaturing, 12% polyacrylamide gels. The desired product band was excised from the gel, from which the RNA was electroeluted in an Elutrap Electrophoresis Chamber (Schleicher & Schuell). Centricron filter units (Millipore) with a 3K molecular weight cutoff were used to equilibrate RNA to the desired buffer. An excess of a high-affinity ligand for the A-riboswitch, 2,6-diaminopurine (DAP), was used to maintain the RNA in its native conformation (22).

**Measurement of excess Mg$^{2+}$**

The excess Mg$^{2+}$ that neutralizes RNA charge, $\Gamma_{Mg^{2+}}$, is defined below (Background). It was measured by titration of MgCl$_2$ into RNA solutions that contained HQS as a fluorescent reporter of bulk Mg$^{2+}$ concentration. RNA samples for HQS titrations were dialyzed against a given concentration of putrescine$^{2+}$, which is therefore the bulk con-
centration of putrescine$^{2+}$ as reported in the legend to Figure 3. (The total putrescine$^{2+}$ concentration will be higher than the bulk, because of putrescine$^{2+}$ interactions with the RNA.) The titrat of these experiments contained the standard buffer described above (40 mM K•MOPS pH 6.8, 37 mM KCl, 10 $\mu$M EDTA, for a total of 50 mM K$^+$) with 20 mM MgCl$_2$ and the given putrescine$^{2+}$ concentration.

Two cuvettes with either dialyzed RNA sample or the dialysis buffer alone were titrated in an Aviv ATF 105 fluorometer outfitted with Hamilton automatic titrators. Both cuvettes contained 20 $\mu$L M HQS; RNA samples were 2–4 mM in phosphate concentration. Reported titration curves are the averages of three to five experiments. The larger error bars for the ensemble RNA titrations are due to the smaller number of titrations (three) and larger variability between runs compared to the others, possibly related to the RNA conformation changes taking place during the titration. Data collection and analysis have been described in detail (20).

X-ray scattering

RNA samples (1–2 mg/ml) were exchanged extensively into standard buffer (described above) with 0–50 mM putrescines•(HCl)$_2$ as indicated. The samples were heated to 65°C for 5 min and incubated at room temperature for at least 30 min. Samples were then passed through a 0.1 $\mu$m filter (Millipore) prior to beam exposure. SAXS measurements were performed at beamline 12-ID at the Advanced Photon Source, Argonne National Laboratory. The beam energy was set to 12 keV with an exposure time of ∼0.5 s. Samples were moved through an X-ray flow cell to minimize radiation damage. The ambient temperature was ∼25°C. Thirty shots were collected for each sample condition in order to obtain good statistics. Radii of gyration ($R_g$) were determined from the Guinier fit to averaged data; P(r) plots were generated using GNOM after finding approximate $D_M$ values in AUTOGNOM (23). Envelopes were generated for the N and Ex-state using DAMMIF. The results of the simulated annealing procedure were uninterpretable for the Ex-state mutant in normal P1 mode, and the simulation was therefore rerun with P2 symmetry imposed.

UV titrations

UV absorbance titrations were carried out in an Aviv 14-DS spectrophotometer. Titrations were carried out either with a Hamilton automatic titrator or by manual titrations while observing the 260 and 280 nm wavelengths. The results of both methods were identical. The initial absorbance of each sample was ∼0.6 OD at 260 nm. The data were normalized to 1 by dividing the absorbance at a given concentration of divalent ion by the initial absorbance in the absence of divalent ion. Resulting titrations were fit to the Hill equation with the Hill coefficient and fractional change in OD as variables.

BACKGROUND

The method we use here to measure the excess Mg$^{2+}$ accumulated by an RNA is based on the same principle as an equilibrium dialysis experiment, in which an RNA solution is kept separate from buffer solution by a membrane permeable only to ions and water. Comparison of the concentrations of a particular cation or anion in the RNA solution (‘in’) and buffer only solution (‘out’) approximates the preferential interaction coefficient for that ion,

$$\Gamma_{\text{ion}} = \frac{C_{\text{ion}}^{\text{In}} - C_{\text{ion}}^{\text{Out}}}{C_{\text{RNA}}^{\text{In}}} \approx \left( \frac{\partial C_{\text{ion}}}{\partial C_{\text{RNA}}} \right)_{\mu_{\text{ion}}} \mu_{\text{ion}}$$

(1)

where the chemical potential of the ion, $\mu_{\text{ion}}$, must be identical on both sides of the membrane at thermodynamic equilibrium. Positive $\Gamma_{\text{ion}}$ values are called the excess of a particular cation; negative values are characteristic of an anion deficiency. The value of $\Gamma_{\text{ion}}$ measured by equilibrium dialysis or equivalent means (see Materials and Methods) can be an excellent approximation of the Equation (1) partial derivative that defines $\Gamma_{\text{ion}}$ (20). A more detailed yet accessible discussion of the meaning of $\Gamma_{\text{ion}}$ is in reference (24).

For an RNA with $Z_{\text{phosphate}}$ negative charges in a solution with the chloride salts of K$^+$, Mg$^{2+}$ and putrescine$^{2+}$, electroneutrality of the system requires the following relation between the interaction coefficients of the ions:

$$Z = 2\Gamma_{\text{Mg}^{2+}} + 2\Gamma_{\text{Put}^{2+}} + \Gamma_{\text{K}^+} + \Gamma_{\text{Cl}^-}$$

(2)

If the chemical potential of one of the ionic species in solution is changed, the system will equilibrate accordingly. For instance if the chemical potential of putrescine$^{2+}$ is increased, $\Gamma_{\text{Put}^{2+}}$ will increase while $\Gamma_{\text{Mg}^{2+}}$, $\Gamma_{\text{K}^+}$ and $\Gamma_{\text{Cl}^-}$ change in a compensatory fashion. A change in RNA conformation may also induce a set of compensatory changes among the $\Gamma_{\text{ion}}$ terms.

In this paper, we use the term ‘ion exchange’ to refer to the complete set of compensatory changes among the four $\Gamma_{\text{ion}}$ terms of Equation (2) in response to the change in chemical potential of one ionic species. Alternatively, we will refer to a reduction in $\Gamma_{\text{Mg}^{2+}}$ caused by an increase in the concentration of putrescine$^{2+}$ as a ‘competition’ between the two divalent ions. Neither ‘exchange’ or ‘competition’ is meant to imply that putrescine$^{2+}$ and Mg$^{2+}$ are vying for a fixed number of specific ‘sites’, or that competition occurs only if one ion replaces another at a specific location. In fact, the long-range electrostatic interactions that define ion interactions with RNA couple ion interactions in such a way that an ion chelated at a specific site may be displaced by mobile ions within the ion atmosphere (25).

The partial derivative defining $\Gamma_{\text{ion}}$ (Equation (1)) can be transformed into an integral that gives the free energy of ion − RNA interactions. For Mg$^{2+}$, this free energy is (15)

$$\Delta G_{\text{RNA}−\text{Mg}^{2+}} \approx −RT \int_0^{C_{\text{Mg}^{2+}}} \Gamma_{\text{Mg}^{2+}} d\ln C_{\text{Mg}^{2+}}.$$ (3)

When MgCl$_2$ is titrated into RNA in the presence of a large enough excess of KCl, the total Cl$^−$ concentration does not change significantly; thus $\Delta G_{\text{RNA}−\text{Mg}^{2+}}$ can be obtained independently of the free energy of RNA − anion interactions.
RESULTS
Characterization of folded and extended adenine riboswitch conformations

The RNA used in these studies is a modified form of the aptamer domain of the add adenine riboswitch (A-riboswitch), an exceptional system for probing the details of RNA folding (26) (Figure 1A). (In conjunction with its expression platform, the aptamer domain regulates translation of a gene product responsible for adenine metabolism (27).) In the presence of adenine or select purine derivatives, the RNA folds to a compact tertiary structure in which the ligand is completely surrounded by the binding pocket and the two hairpin loops are docked in a kissing interaction (28). In the absence of both ligand and Mg²⁺, the two hairpin loops tend to undock and the RNA samples extended conformations (29). Both ligand and Mg²⁺ favors docking of the hairpin loops, and together act synergistically to stabilize the native state (22,29,30). The strength of the kissing loop interaction varies with the exact sequence of the riboswitch (31,32); in the extreme case of the disruptive mutation C60G (29), the RNA folding equilibrium heavily favors an extended, T-shaped conformation even at moderate Mg²⁺ concentrations ((22) and Figure 2B, C). Over the range 0–0.1 mM Mg²⁺, SAXS experiments found that the native (N state) structure is adopted by the A-riboswitch sequence when high ligand concentration is present, and an extended conformation (Ex state) by the C60G variant in the absence of ligand (22).

We first used SAXS and UV hypochromicity experiments to examine the effect of putrescine²⁺ on the A-riboswitch and its C60G variant (Figure 3). The radius of gyration (Rg) as measured by SAXS is primarily sensitive to the overall dimensions of a macromolecule; there is an easily detectable decrease (~15%) in A-riboswitch Rg in going from the Ex to N states. Previous experiments showed that the A-riboswitch approaches the same Rg with or without ligand present, as Mg²⁺ is added to 1 mM (22). Putrescine²⁺ is apparently less effective, as the reduction in Rg appears to plateau at ~80% of the compaction seen in the presence of ligand (Figure 3A). (The P(r) distribution of the A-riboswitch with putrescine²⁺ is also subtly different from that found with Mg²⁺, Supplementary Figure S1A.) A potential caveat is that concentrations of putrescine²⁺ > ~10 mM significantly increase the concentration of Cl⁻ beyond the 50 mM already present with KCl, which could change the docking equilibrium. However, UV absorption experiments (described below) suggest the plateau is not an artifact.

Two additional results from the SAXS experiments are important for interpretation of the Mg²⁺ measurements that follow. First, with A-riboswitch in the presence of ligand, putrescine²⁺ does not alter either the native structure Rg (Figure 3A) or distance distribution profile (P(r), Supplementary Figure S1C) from that observed with Mg²⁺. Second, C60G RNA remains in an extended conformation at high putrescine²⁺ concentrations (Figure 3A, Supplementary Figure S1B), as previously observed with Mg²⁺ (22). Thus, at the level of resolution available from SAXS experiments, putrescine²⁺ and Mg²⁺ do not alter the N or Ex state structures within the ion concentration ranges used in our studies. It is not critical for this study whether C60G RNA is rigidly fixed in an extended conformation; some flexibility is suggested by the fact that high divalent ion concentrations drive it into a somewhat more compact conformation. The important conclusion for our competition experiments is that, within the ionic concentration range used here, C60G RNA maintains a constant Rg approximately that of unfolded A-riboswitch RNA in the absence of both divalent ions and ligand.

UV absorption is sensitive to base stacking in RNA, and in the A-riboswitch hypochromicity associated with folding likely originates from structures formed within both the docked loops and the binding pocket. In the presence of ligand, putrescine²⁺ and Mg²⁺ titrate A260 to the same endpoints (Figure 3B). A smaller hyperchromic change is seen in the absence of ligand when either divalent ion is present, possibly because the binding pocket does not become fully structured. Putrescine²⁺ induces marginally less...
hyperchromicity than does Mg$^{2+}$, but the titration curve notably approaches a plateau at less than 10 mM putrescine$^{2+}$. This endpoint suggests the apparent plateau of the SAXS titration curve is not an artifact of increasing Cl$^{-}$ concentration, and that putrescine$^{2+}$ in the absence of ligand stabilizes an RNA conformation in which the hairpin loops occupy an intermediate position between that of the docked native structure and the fully extended loop–loop configuration.

We conclude from these experiments that $\Gamma_{\text{Mg}^{2+}}$ can be calculated by Equation (3) for the A-riboswitch RNA–ligand complex and for the variant C60G RNA when ions are varied from 0 to $\sim$10 mM putrescine$^{2+}$ and 0 to $\sim$0.1 mM Mg$^{2+}$. In the absence of ligand, the A-riboswitch RNA undergoes a significant shift in dimensions over the same ranges of concentrations, and Equation (3) is not applicable.

Measurement of excess Mg$^{2+}$ upon addition of putrescine$^{2+}$

To study the effect of putrescine$^{2+}$ on Mg$^{2+}$–A-riboswitch interactions, we quantified excess Mg$^{2+}$ ions accumulated by the RNA during titrations in the presence of a fluorescent Mg$^{2+}$ chelator, HQS (see Materials and Methods). Titrations were carried out in the presence of fixed concentrations of Mg$^{2+}$, putrescine$^{2+}$ and a wild-type A-riboswitch in the presence of ligand (Native state, Figure 2B) or the C60G variant without ligand (Extended state, Figure 2C). A third set of titrations was made with A-riboswitch RNA without ligand, which permits a partial folding transition as divalent ions are titrated (compare SAXS and UV titrations in the absence of ligand, Figure 3). We refer to RNA under these conditions as ‘ensemble’ RNA, because it may adopt multiple conformations.

The titration curves show that increasing putrescine$^{2+}$ concentration for any of the three conditions results in a net decrease in $\Gamma_{\text{Mg}^{2+}}$ over the entire curve, which implies that excess putrescine$^{2+}$ accumulates at the expense of excess Mg$^{2+}$ (Equation (2)). As observed previously (22), $\Gamma_{\text{Mg}^{2+}}$ depends on the dimensions of the RNA with the more compact form (N state) possessing a larger $\Gamma_{\text{Mg}^{2+}}$ than the less compact Ex state (Figure 4A and B); this difference holds true when putrescine$^{2+}$ is present. However, the relative decrease in $\Gamma_{\text{Mg}^{2+}}$ caused by putrescine$^{2+}$ tends to be much greater for the Ex state. For instance 10 mM putrescine$^{2+}$ reduces $\Gamma_{\text{Mg}^{2+}}$, at 0.1 mM Mg$^{2+}$ by $\sim$55% (to $\sim$3.6 ions/RNA) for the N state, but by over 80% for the Ex state (to $\sim$1 ion/RNA). $\Gamma_{\text{Mg}^{2+}}$ for the ensemble RNA generally lies between values found for the N and Ex states as illustrated for the titrations with 5 mM putrescine$^{2+}$ (Figure 4D): the ensemble curve is similar to that of the Ex state at low C$_{\text{Mg}^{2+}}$, but rises toward the N state curve at higher C$_{\text{Mg}^{2+}}$.

Free energies of RNA–Mg$^{2+}$ interaction

By integrating the $\Gamma_{\text{Mg}^{2+}}$ curves in Figure 4A and B, RNA–Mg$^{2+}$ interaction free energy values ($\Delta\mu_{\text{RNA-Mg}^{2+}}$, as defined in Figure 1A) are obtained per Equation (3) (Figure 5). (Only the curves for the N and Ex state titrations provide reliable $\Delta\mu_{\text{RNA-Mg}^{2+}}$ values, since these structures are unchanged during the titration.) We see approximately linear decreases in $\Delta\mu_{\text{N-Mg}^{2+}}$ and $\Delta\mu_{\text{Ex-Mg}^{2+}}$, with the log of the putrescine$^{2+}$ concentration, though with different slopes: $\Delta\mu_{\text{Ex-Mg}^{2+}}$ is more sensitive to the addition of putrescine$^{2+}$. The difference between these free energies, $\Delta\mu = \Delta\mu_{\text{N-Mg}^{2+}} - \Delta\mu_{\text{Ex-Mg}^{2+}}$, would be the contribution of Mg$^{2+}$ to the stability of an Ex $\rightarrow$ N folding transition, and should increase in magnitude as the putrescine$^{2+}$ concentration increases. (See the thermodynamic cycle, Figure 1A.) Because $\Delta\mu_{\text{RNA-Mg}^{2+}}$ is directly related to $\Gamma_{\text{Mg}^{2+}}$, another way to visualize this observation is that the number of Mg$^{2+}$ ions taken up in an Ex $\rightarrow$ N transition, $\Delta\Gamma_{\text{Mg}^{2+}}$, should increase upon putrescine$^{2+}$ addition despite the fact that putrescine$^{2+}$ decreases $\Gamma_{\text{Mg}^{2+}}$ for both states of the RNA. (Note that an Ex $\rightarrow$ N transition is hypothetical: in the absence of the C60G mutation to enforce an extended conformation, A-riboswitch RNA tends to adopt more compact conformations when titrated with Mg$^{2+}$, and thus reduce $\Delta\Gamma_{\text{Mg}^{2+}}$.)

Although the free energy of Mg$^{2+}$ interaction with ensemble RNA cannot be calculated, the effects of putrescine$^{2+}$ addition on $\Gamma_{\text{Mg}^{2+}}$ of N, Ex and ensemble RNAs can be compared: Supplementary Figure S2 shows how rapidly $\Gamma_{\text{Mg}^{2+}}$ decreases with increasing putrescine$^{2+}$ concentration while C$_{\text{Mg}^{2+}}$ is held constant. The ensemble RNA resembles Ex RNA in its sensitivity to putrescine$^{2+}$ at a lower C$_{\text{Mg}^{2+}}$ (Supplementary Figure S2B) and is midway between N and Ex RNAs at a higher C$_{\text{Mg}^{2+}}$ that induces a small degree of compaction in A-riboswitch RNA in the absence of ligand (Supplementary Figure S2A and (22)).

Mg$^{2+}$–putrescine$^{2+}$ exchange at constant $\Gamma_{\text{Mg}^{2+}}$

An alternative perspective on the competition between Mg$^{2+}$ and putrescine$^{2+}$ is suggested by the horizontal line in Figure 4 panels A and B, drawn for $\Gamma_{\text{Mg}^{2+}} = 1.0$. The intersections of this line with the successive titration curves at increasing concentrations of putrescine$^{2+}$ ask: if a given amount of putrescine$^{2+}$ is added to a system with an initial $\Gamma_{\text{Mg}^{2+}}$, how much Mg$^{2+}$ must also be added in order to return to the same value of $\Gamma_{\text{Mg}^{2+}}$? It is helpful to view the ion concentrations in terms of chemical potentials, where for two solutions with different ion concentrations

$$\Delta\mu_{\text{Mg}^{2+}} = \mu_{\text{Mg}^{2+},2} - \mu_{\text{Mg}^{2+},1} \approx -RT \ln(C_{\text{Mg}^{2+},2}/C_{\text{Mg}^{2+},1}) \tag{4}$$

and a similar relation holds for $\Delta\mu_{\text{Put}^{2+}}$. (The activity coefficients of divalent ions in a solution of excess KCl are relatively insensitive to the overall MgCl$_2$ or putrescine • Cl$_2$ concentration; hence the activity coefficients should approximately cancel and have been omitted in Equation (4).) The plots in Figure 6A shows that there is an approximately linear relation between the two chemical potential changes needed to keep $\Gamma_{\text{Mg}^{2+}}$ constant, i.e.

$$\Delta\mu_{\text{Mg}^{2+}} = m\Delta\mu_{\text{Put}^{2+}} \tag{5}$$

where $m$ is the slope of the plots. For the Ex state, $m \approx 1$: a given increase in $\mu_{\text{Mg}^{2+}}$ is countered by an equivalent in-
crease in $\mu_{\text{Put}^{2+}}$. However, putrescine$^{2+}$ is much less effective at displacing Mg$^{2+}$ from the N state excess ions: $m \approx 0.2$, and a given increase in $\mu_{\text{Mg}^{2+}}$ requires a five fold larger increase in $\mu_{\text{Put}^{2+}}$ to maintain constant $\Gamma_{\text{Mg}^{2+}}$. With ensemble RNA, $m \approx 0.65$ is intermediate between N and Ex state RNAs. Not only do putrescine$^{2+}$ and Mg$^{2+}$ compete against each other as $\mu_{\text{Mg}^{2+}}$ and $\mu_{\text{Put}^{2+}}$ increase, but the increase in total divalent ion concentration presumably decreases $\Gamma_{K^+}$ and possibly also $\Gamma_{\text{Cl}^-}$, to establish a final set of $\Gamma_{\text{Ion}}$ that satisfy Equation (2). Although a complete determination of all four $\Gamma_{\text{Ion}}$ from the Figure 5 data are not possible, the Figure 6A plot provides a simple quantitation of the efficiency with which putrescine$^{2+}$ ions 'exchange' with other excess ions of an RNA. Qualitatively, the plot suggests that ion exchange is much more efficient with more extended RNA conformations. (Note that $m$ should be taken only as a relative measure of ion exchange efficiency; without knowing all four $\Gamma_{\text{Ion}}$ values, we cannot interpret the absolute value of $m$. In particular, $m$ is not a simple stoichiometric ratio: e.g., $m = 1$ does not imply that one putrescine$^{2+}$ replaces one Mg$^{2+}$.)

A further point, revealed by Figure 6B, is that $m$ for the N state increases substantially as the constant value of $\Gamma_{\text{Mg}^{2+}}$ is raised, reaching $\sim 0.6$ when $\Gamma_{\text{Mg}^{2+}} = 3.6$ ions/RNA. Thus, putrescine$^{2+}$ becomes a progressively more effective replacement for Mg$^{2+}$ as the number of excess Mg$^{2+}$ increases. Although $m$ values for Ex and ensemble state RNAs are available over only a limited range of $\Gamma_{\text{Mg}^{2+}}$ values, it appears...
that $m$ is approximately constant with these more extended RNA conformations (Figure 6C). (Note that the maximum values of $\Gamma_{Mg^{2+}}$ in Figure 6C are low, with $<10\%$ of the A-riboswitch phosphates neutralized by excess Mg$^{2+}$. The trends observed in these plots may well diverge at much higher $\Gamma_{Mg^{2+}}$.) These results are interpreted in the Discussion in terms of the interaction free energies available to putrescine$^{2+}$ and Mg$^{2+}$ near different types of RNA surfaces.

**DISCUSSION**

**Divalent ion–RNA interactions**

One of the main driving forces of RNA tertiary folding is the uptake of cations that accompanies the transition from an extended, partially structured conformation to the compact native structure. It is well established that divalent ions are taken up in strong preference to monovalent ions (4,33,34), and Mg$^{2+}$ is generally a more potent stabilizer of ions are taken up in strong preference to monovalent ions. A first consideration is the entropic advantage that accompanies the transition positive charge in similar locations at the RNA surface. Two putrescine$^{2+}$ interactions are found in the major groove of A-form RNA segments, each with one amino group directly hydrogen bonded to a phosphate and the other 4-4.5 Å from a second phosphate (Supplementary Figure S3). In RNA crystal structures, Mg$^{2+}$ is frequently observed within A-helix major grooves with its first hydration layer hydrogen bonded to bases or phosphates (45,46). The estimated electrostatic potential within the groove is very negative (17,47), and a study that combined MD simulations with X-ray scattering data suggested that a large fraction of excess Mg$^{2+}$ accumulates deep in the RNA major groove (48). Thus, in the A-helix major groove, Mg$^{2+}$ and putrescine$^{2+}$ can position positive charge in close proximity to several phosphates. A recent crystal structure of the ribosome (44) suggests that putrescine$^{2+}$, despite its size, may readily occupy locations at the RNA surface. Two putrescine$^{2+}$ ions are found in the major groove of A-form RNA segments, each with one amino group directly hydrogen bonded to a phosphate and the other 4-4.5 Å from a second phosphate (Supplementary Figure S3). In RNA crystal structures, Mg$^{2+}$ is frequently observed within A-helix major grooves with its first hydration layer hydrogen bonded to bases or phosphates (45,46). The estimated electrostatic potential within the groove is very negative (17,47), and a study that combined MD simulations with X-ray scattering data suggested that a large fraction of excess Mg$^{2+}$ accumulates deep in the RNA major groove (48). Thus, in the A-helix major groove, Mg$^{2+}$ and putrescine$^{2+}$ can position positive charge in close proximity to several phosphates. Putrescine$^{2+}$ may also occupy pockets of negative charge associated with tertiary folding, as suggested by putrescine$^{2+}$ hydrogen-bonded to three phosphates brought into close proximity by the 23S rRNA tertiary structure (44).

**Competition between Mg$^{2+}$ and putrescine$^{2+}$**

Our previous study showed that about an order of magnitude higher concentration of putrescine$^{2+}$ than Mg$^{2+}$ is needed to induce folding of the A-riboswitch tertiary structure (14). The contribution of a divalent ion to the folding free energy is the difference in the ion interaction free energies between folded and unfolded RNA: $\Delta G = AG_{N,A}^{\text{Mg}^{2+}} - AG_{N,A}^{\text{Put}^{2+}}$. Figure 1A implies that the weaker efficiency of putrescine$^{2+}$ could arise from many combinations of increases or decreases in $\Delta G_{Ex-Put}^{\text{Mg}^{2+}}$ and $\Delta G_{N-Put}^{\text{Mg}^{2+}}$ relative to a small volume surrounding the RNA (17). This entropic principle applies to putrescine$^{2+}$ as well as Mg$^{2+}$.

A second factor to consider is the overall free energy of interaction between the divalent cations and the RNA ($\Delta G_{RNA-Mg^{2+}}$ or $\Delta G_{RNA-Put^{2+}}$), which is strongly electrostatic in origin: the most favorable free energies are developed when ions occupy regions of negative electrostatic potential created by several nearby phosphates. At one extreme, so-called ‘diffuse’ ions remain fully hydrated near or some distance from the RNA surface (43). At the other extreme, partially dehydrated Mg$^{2+}$ may make two or more direct contacts with the RNA in small pockets of concentrated negative charge, sometimes entirely buried within the solvent-accessible surface of the RNA (39–41). These ‘chelation’ sites cannot accommodate the elongated putrescine$^{2+}$ ion, and in fact RNAs with well-defined Mg$^{2+}$ chelation sites cannot be driven to adopt their native structure when putrescine$^{2+}$ is the sole divalent ion (14). Because putrescine$^{2+}$ does substitute for Mg$^{2+}$ in stabilizing the A-riboswitch native structure (14), and crystal structures of the A-riboswitch with resolved Mg$^{2+}$ have not revealed any chelated Mg$^{2+}$ (28,42), this special type of Mg$^{2+}$–RNA interaction is unlikely to occur in the A-riboswitch.

Between the two extremes of chelated and diffuse ion interactions, ions may occupy regions within the first few hydration layers of the RNA where the energetics of the interaction are influenced by the ability of the ion to hydrogen bond to the RNA surface, directly or via the ion’s own hydration layer, and where the ion may be in close proximity to several phosphates. A recent crystal structure of the ribosome (44) suggests that putrescine$^{2+}$, despite its size, may readily occupy locations at the RNA surface. Two putrescine$^{2+}$ ions are found in the major groove of A-form RNA segments, each with one amino group directly hydrogen bonded to a phosphate and the other 4-4.5 Å from a second phosphate (Supplementary Figure S3). In RNA crystal structures, Mg$^{2+}$ is frequently observed within A-helix major grooves with its first hydration layer hydrogen bonded to bases or phosphates (45,46). The estimated electrostatic potential within the groove is very negative (17,47), and a study that combined MD simulations with X-ray scattering data suggested that a large fraction of excess Mg$^{2+}$ accumulates deep in the RNA major groove (48). Thus, in the A-helix major groove, Mg$^{2+}$ and putrescine$^{2+}$ can position positive charge in close proximity to several phosphates. Putrescine$^{2+}$ may also occupy pockets of negative charge associated with tertiary folding, as suggested by putrescine$^{2+}$ hydrogen-bonded to three phosphates brought into close proximity by the 23S rRNA tertiary structure (44).

**Figure 5.** The RNA-Mg$^{2+}$ interaction free energy calculated at 0.1 mM Mg$^{2+}$ as a function of bulk putrescine$^{2+}$ concentration for the N and Ex states. Points on the y-axis are $\Delta G_{RNA-Mg^{2+}}$ values in the absence of putrescine$^{2+}$. **Figure 6.** Putrescine$^{2+}$ ions are found in the major groove of A-form RNA segments, each with one amino group directly hydrogen bonded to a phosphate and the other 4-4.5 Å from a second phosphate (Supplementary Figure S3). In RNA crystal structures, Mg$^{2+}$ is frequently observed within A-helix major grooves with its first hydration layer hydrogen bonded to bases or phosphates (45,46). The estimated electrostatic potential within the groove is very negative (17,47), and a study that combined MD simulations with X-ray scattering data suggested that a large fraction of excess Mg$^{2+}$ accumulates deep in the RNA major groove (48). Thus, in the A-helix major groove, Mg$^{2+}$ and putrescine$^{2+}$ can position positive charge in close proximity to several phosphates. Putrescine$^{2+}$ may also occupy pockets of negative charge associated with tertiary folding, as suggested by putrescine$^{2+}$ hydrogen-bonded to three phosphates brought into close proximity by the 23S rRNA tertiary structure (44).
that a higher concentration of putrescine$^{2+}$ is needed to halve the corresponding Mg$^{2+}$–RNA interaction strengths. The ion titration data presented in this work (Figure 4, as interpreted in Figures 5, 6 and Supplementary Figure S2) reveal these differences in detail.

We first infer that Mg$^{2+}$ develops a more favorable interaction free energy with the Ex state conformation than does putrescine$^{2+}$, i.e. $\Delta G_{\text{Ex-Mg}^{2+}} > \Delta G_{\text{Ex-Put}^{2+}}$. For instance, a 20- to 30-fold excess of putrescine$^{2+}$ over Mg$^{2+}$ is needed to halve $\Delta G_{\text{Ex-Mg}^{2+}}$ (Figure 5). The surface of Ex state RNA must be largely A-form helices; as discussed above both divalent ions may lie within the major groove, where electrostatic interactions are optimal. But $\Delta G_{\text{Ex-Put}^{2+}}$ includes not only a favorable electrostatic term, but also an entropic penalty for restricted motion of the putrescine$^{2+}$ butyl chain. The penalty could be more than 1 kcal/mol (14) and largely account for the weaker putrescine$^{2+}$ interactions. Indirect measurements of Mg$^{2+}$ and putrescine$^{2+}$ interactions with helical DNA found that Mg$^{2+}$ is favored by $\sim-0.7$ kcal/mol (49), qualitatively consistent with the Ex state RNA results.

Turning to the N state RNA conformation, we first note that an even higher concentration of putrescine$^{2+}$ is needed to displace Mg$^{2+}$ with this RNA than with the Ex state RNA, evidenced by both a lesser rate of decrease in $\Delta G_{\text{N-Mg}^{2+}}$ with increasing putrescine$^{2+}$ concentration (Figure 5), and a weaker effect on $\Gamma_{\text{Mg}^{2+}}$ (Supplementary Figure S2). The reduced effectiveness of putrescine$^{2+}$ with the N state is doubtlessly linked to the formation of tertiary structures; both the ligand binding pocket and kissing loop (Figure 2A) contain closely spaced phosphates, which may create pockets of negative electrostatic potential that are more accessible to Mg$^{2+}$ than putrescine$^{2+}$. Evidence that putrescine$^{2+}$ might not be able to access some regions of the native tertiary structure as effectively as Mg$^{2+}$ comes from the SAXS and UV hypochromicity observations that putrescine$^{2+}$, without the help of ligand, does not fold the RNA into a structure that is as compact as the one stabilized by Mg$^{2+}$ (Figure 3).

As developed in Results, Equations (4) and (5), $m$ is a measure of how hard the putrescine$^{2+}$ chemical potential has to ‘push’ against an increase in the Mg$^{2+}$ chemical potential to keep $\Gamma_{\text{Mg}^{2+}}$ constant. To interpret the way $m$ increases with $\Gamma_{\text{Mg}^{2+}}$ with the folded N state RNA (Figure 6C), we suppose that volumes near the surface of the native RNA exhibit a wide range of electrostatic potential, from smaller pockets of dense negative charge formed by the tertiary structure to larger volumes in the helix grooves with more widely spaced phosphates and less favorable interactions with cations. We further suppose that putrescine$^{2+}$ is unable to place positive charge within the smaller volumes with the most negative potential as effectively as Mg$^{2+}$, but that the two ions comes closer to parity within the more weakly interacting regions associated with secondary structure. Since Mg$^{2+}$ will tend to first populate the small regions of most negative potential at low $\Gamma_{\text{Mg}^{2+}}$ and appear more frequently around the secondary structure as $\Gamma_{\text{Mg}^{2+}}$ increases, this simple model predicts that progressively smaller increases in $\Delta \mu_{\text{Mg}^{2+}}$ will be needed to counter increases in $\Delta \mu_{\text{Put}^{2+}}$ as $\Gamma_{\text{Mg}^{2+}}$ increases, as observed. The constant value of $m$ for the Ex state (Figure 6C) suggests that this RNA conformation gives about the same energetic advantage to Mg$^{2+}$ relative to putrescine$^{2+}$ over its entire surface.

The ‘ensemble RNA’ used in these experiments is a collection of conformations intermediate between the native and extended states. At the Mg$^{2+}$ and putrescine$^{2+}$ concentrations used to maintain $\Gamma_{\text{Mg}^{2+}}$ values between 0.5 and 1.5 ions/RNA in Figure 6A and C, the average $R_g$ is much less than that of the extended form (cf. Figure 3A, 1–10 mM putrescine$^{2+}$), which implies that the helical segments of the riboswitch are on average closer to each other than in the extended variant. (This is consistent with the known sta-
The stabilizing influence of Mg\(^{2+}\) on the kissing loop contacts of purine riboswitches (29,32). The intermediate \(m\) value for the ensemble RNA is also consistent with reduced accessibility of RNA surfaces to putrescine\(^{2+}\), compared to the Ex state, but it is interesting to note that the partially compacted conformations of the ensemble show no hint of developing the regions with very high discriminating in favor of Mg\(^{2+}\) seen in the native structure. The very low \(m\) values seen for the N state at low \(\Gamma_{\text{Mg}}\) probably correspond to Mg\(^{2+}\)-accessible volumes that only appear when the RNA tertiary structure is fully in place. If the A-riboswitch native structure could accommodate several Mg\(^{2+}\) ions in volumes that were highly inaccessible to putrescine\(^{2+}\), one might expect \(m\) to remain very small as \(\Gamma_{\text{Mg}}\) increases. Instead, Figure 6C shows a constantly increasing value of \(m\) from the first value at \(\Gamma_{\text{Mg}} = 0.5\) ions per RNA; we conclude that putrescine\(^{2+}\) can access all but a small fraction of the RNA surface.

In summary, the model we present here is that the RNA folds from an extended form in which putrescine\(^{2+}\) is equally disadvantaged in competing against Mg\(^{2+}\) throughout the RNA, to a tertiary structure with a more highly differentiated surface that strongly discriminates against putrescine\(^{2+}\) in limited regions. As sketched in Figure 1B, putrescine\(^{2+}\) interacts less strongly than Mg\(^{2+}\) with both Ex and N conformations of the riboswitch, but the difference is more pronounced with the native state. Although putrescine\(^{2+}\) is nevertheless able to stabilize the A-riboswitch, presumably RNAs tertiary structures with more convoluted surfaces would interact even less strongly with putrescine\(^{2+}\); likewise, partially unfolded RNAs with more structure than the highly extended state of the C60G RNA may be less favorable to putrescine\(^{2+}\). An extreme case is the very compact M box RNA, which takes up Mg\(^{2+}\) into several pockets that would be completely inaccessible to putrescine\(^{2+}\) (41). This RNA readily folds when Mg\(^{2+}\) is present but is actually destabilized when putrescine\(^{2+}\) is in excess over Mg\(^{2+}\) (14); putrescine\(^{2+}\) therefore must interact more strongly with partially unfolded RNA than with the native fold. An implication of the present and previous (14) work is that the architectures of both folded and unfolded forms of an RNA determine how its stability responds to competing Mg\(^{2+}\) and putrescine\(^{2+}\) ions.

Our model of Mg\(^{2+}\) versus putrescine\(^{2+}\) competition is consistent with ideas put forward by others, who have argued from experiments and model calculations on the folding of large RNAs that the ability of a divalent ion to stabilize large RNA tertiary structures decreases in proportion to the ion’s size (6). The competition experiments and free energy measurements presented here give a more quantitative picture of how the folding of an RNA structure influences the competition between the ions, and are unique in measuring free energies of ion–RNA interactions for specific RNA conformations (Figure 1), rather than just the net effect of ions on \(\Delta G_{\text{Fold}}\). As such, the data provide benchmarks for computational studies that examine the relation between an RNA structure and the stabilizing ion atmosphere that surrounds it.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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