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The influence of monovalent cation size on the stability of RNA tertiary structures

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Abstract

Many RNA tertiary structures are stable in the presence of monovalent ions alone. To evaluate the degree to which ions at or near the surfaces of such RNAs contribute to stability, the salt-dependent stabilities of a variety of RNA structures were measured with each of the five group I cations. The stabilities of hairpin secondary structures and a pseudoknot tertiary structure are insensitive to the ion identity, but the tertiary structures of two other RNAs, an adenine riboswitch and a kissing loop complex, become more stable by 2–3 kcal/mol as ion size decreases. This “default” trend is attributed to the ability of smaller ions to approach the RNA surface more closely. The degree of cation accumulation around the kissing loop complex was also inversely proportional to ion radius, perhaps because of the presence of sterically restricted pockets that can be accessed only by smaller ions. An RNA containing the tetraloop-receptor motif shows a strong (up to ~3 kcal/mol) preference for Na⁺ or K⁺ over other group I ions, consistent with the chelation of K⁺ by this motif in some crystal structures. This RNA reverts to the “default” dependence on ion size when a base forming part of the chelation site is mutated. Lastly, an RNA aptamer for cobinamide, which was originally selected in the presence of high LiCl concentrations, binds ligand more strongly in the presence of Li⁺ than other monovalent ions.

Based on these trends in RNA stability with group I ion size, it is argued that two features of RNA tertiary structures may promote strong interactions with ions at or near the RNA surface: negative charge densities that are higher than found in secondary structures, and the occasional presence of chelation sites, electronegative pockets that selectively bind ions of an optimum size.

Keywords

pseudoknot; riboswitch; tetraloop receptor; kissing loop; salt dependence; group I ions

Introduction

It is well-known that the stability of an RNA structure is sensitive to the concentrations and types of ions that are present, as first noted by studies in the 1970s of the sensitivity of tRNA tertiary structures to both Na⁺ and Mg²⁺-ions.^{1; 2} Although Mg²⁺ tends to be much more effective than monovalent ions at stabilizing RNA tertiary structures, it is not always essential; many RNAs form all or part of their tertiary contacts in the presence of monovalent ions alone.

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3; 4; 5 This class of RNAs is convenient for experimental and computational studies of ion - RNA interactions, since the complexities associated with competition between mono- and divalent ions can be bypassed. In the present work, we use the variation in size among group I ions (0.6 - 1.69 Å ionic radius for Li⁺ through Cs⁺)⁶ as a probe for evaluating the importance of ions at or near the RNA surface for the stabilization of RNA tertiary structures. These studies were undertaken because of their potential for yielding two kinds of insights into ion - RNA interactions. First, several RNA crystal structures show Na⁺ or K⁺ ions making at least three direct contacts with the RNA surface, in effect forming ion - RNA chelates.^{7; 8; 9; 10} Because K⁺ is the dominant physiological monovalent cation,¹¹ it might be expected that RNA structures have been optimized by natural selection for specific interactions with K⁺, and in fact functional specificity for K⁺ has been observed in several RNAs.^{7; 12; 13; 14} The specificity of a chelating RNA structure for a particular size ion can help gauge how strongly the RNA depends on the occupancy of the chelation site for stability.

The second motivation for these studies is the possibility that the stabilities of some RNA tertiary structures could be sensitive to monovalent ion size, even in the absence of specific ion binding sites. All nucleic acids, by virtue of their high charge densities and negative electrostatic potentials, accumulate concentrated cation “atmospheres” which extend many Ångstroms from the nucleic acid surfaces. When the distance between a cation and RNA is large, the interaction energy depends only on the charge of the ion and the RNA electrostatic potential it feels; the size of the ion is unimportant. But as an ion approaches an RNA surface, there are at least two ways that ion diameter could become a consideration. First, the minimum distance from the surface to the center of the ion is shorter for smaller ions; narrow grooves or pockets on the RNA surface might also be sterically accessible only to smaller ions. Second, the hydration energy of an ion is inversely proportional to its radius; thus any perturbation of ion hydration that may occur near an RNA surface will be more costly for smaller ions.¹⁵ One study found an inverse correlation between the stability of tRNA tertiary structure and size of the group I ion present, at least at very high salt concentrations (1 M),¹⁶ but whether steric and hydration factors generally affect the overall energetics of ion interactions with RNA tertiary structures is an open question.

Five RNAs were chosen for this study. Two show a chelated ion (Na⁺ or K⁺) in x-ray crystal structures, a third is an aptamer with higher ligand affinity in the presence of Li⁺, and the potential for ion specificity of the other two was unknown. In examining the stability of these RNAs with the five group I cations, we find that two RNAs show a general trend towards greater tertiary structure stability in the presence of smaller ions, and suggest that this is a “default” effect of ion size on compact RNA tertiary structures. One exception to this trend is an RNA which shows selectivity for Na⁺ and K⁺, but reverts to the “default” trend when an RNA base contributing to a crystallographic K⁺ chelation site is mutated. The other exception is the RNA aptamer, which was originally selected in the presence of 1 M LiCl. It has a strong preference for Li⁺ over all other ions, suggesting that RNA is versatile enough to develop specific interactions with a variety of ions, depending on the ionic environment in which it is selected to function.

Results

BWYV pseudoknot

A pseudoknot structure in the Beet Western Yellow Virus (BWYV) RNA induces translational frameshifting.¹⁷ Within the pseudoknot, a number of tertiary hydrogen bonds form where loop segments cross the major or minor grooves of two helices (Figure 1A). In a high resolution (1.2 Å) crystal structure of this RNA, two Na⁺ ions were identified bridging between loop 2 and helix 1: each ion directly contacts one or the other of the A21 non-bridging phosphate oxygens and two additional N or O ligands each from C5, G6, and G16 (Figure 1A).¹⁰ As

K⁺ and Mg²⁺ ions were also present in the crystallization buffer, these two sites must have some selectivity for Na⁺.

The thermal unfolding pathway of the BWYV pseudoknot has been thoroughly analyzed.⁴ The tertiary structure unfolds in two steps as the temperature is raised; disruption of loop 2 - helix 1 hydrogen bonding (with consequent disordering of the Na⁺ sites) comes first, followed by denaturation of helix 2 (Figure 1A). The remaining hairpin formed by helix 1 unfolds in a third and last step. The pseudoknot tertiary structure is stable with monovalent ions concentrations as low as ~20 mM.¹⁸ In the present study, a series of melting profiles, each obtained in buffer containing 124 mM of a different group I ion, were analyzed as three unfolding transitions. An overall free energy of tertiary structure formation, summed from the free energies of the first two transitions, was independent of the type of ion present; neither did the helix 1 hairpin stability depend on ion type, within experimental error (Figure 1B). There is no indication from these data that Na⁺ confers any special stability on the pseudoknot tertiary structure.

Adenine Riboswitch

The adenine riboswitch RNA (A-riboswitch) folds into a compact tertiary structure upon binding adenine or other purine derivatives (Figure 2A).^{19; 20} A crystal structure of this RNA has resolved penta- and hexahydrate Mg²⁺ in grooves (PDB file 1Y26) but has not revealed any monovalent ion associated with the structure.²⁰ A stable A-riboswitch - ligand complex is observed in the absence of Mg²⁺ if moderate concentrations of monovalent ions are present.²¹ In thermal denaturation experiments, disruption of the tertiary structure and release of ligand occurs in a first unfolding transition, usually well-resolved from subsequent unfolding of the secondary structure. In a series of melting experiments carried out with different group I ions, the stability of the tertiary structure was strongly influenced by the identity of the ion (Figure 2B): the RNA became progressively more stable as ion radius decreased, with Li⁺ more effective than Cs⁺ by nearly 3 kcal/mol.

Tar-tar* RNA kissing loops

The formation of a complex between two RNA hairpins with complementary loop sequences, tar and tar* (Figure 3A), has been studied as a model of “kissing loop” interactions that are key to a number of antisense regulatory pathways^{22; 23}. The tar-tar* complex is stable in moderate monovalent salt concentrations in the absence of Mg²⁺. In this system, it has been possible to measure the free energy of tar and tar* association by two methods, isothermal titration and UV melting experiments. If tar and tar* are present together in solution, the UV absorbance is less than expected from the individual hairpins (inset, Figure 3B). This hypochromic change is convenient for monitoring interaction of tar and tar* and was used in isothermal titration experiments to characterize the reaction (Figure 3B). Although use of isothermal titration is somewhat limited by the strong UV absorbance of the RNA and the relatively small absorbance change that occurs when tar and tar* interact, it has the great advantage of directly giving both ΔG° and the stoichiometry of the reaction. Thermal melting can be used over a wider range of conditions than titration, but the calculation of ΔG is less direct and assumes a reaction stoichiometry. In the present case, analysis was made possible by the fact that melting experiments easily resolve the dissociation of the bimolecular tar-tar* complex from the melting of the more stable monomeric tar and tar* hairpins²².

Where it was possible to compare titration and melting experiments under identical solution conditions, similar free energies were obtained. For example, at 20 °C (the temperature of the titration experiments), the ΔG° 's of tar-tar* association by titration and melting are, respectively, -6.78 ± 0.04 and -7.04 ± 0.13 kcal/mol in 0.1 M LiCl, and -8.83 ± 0.17 kcal/mol and -9.04 ± 0.03 kcal/mol in 0.4 M LiCl. In all titration experiments, independent of the

particular group I cation or its concentration, the expected 1:1 stoichiometry of association of tar and tar* was observed. There was no indication of self-association of either tar or tar*.

The stability of the tar-tar* complex is much more dependent on the identity of the group I ion than are the individual hairpins (Figure 3C): Li⁺ is 1.9 - 2.3 kcal more stabilizing than Cs⁺ (depending on the salt concentration), while the tar* hairpin stability varies by only ~0.4 kcal/mol with the same ions. (Similar data with the tar hairpin show an even smaller range, ~0.2 kcal/mol.) The smooth increase in tertiary structure stability with decreasing ion size is similar to the trend seen with the A-riboswitch RNA (Figures 2B).

Dependence of $\Delta\Gamma_{\pm}$ on ion type

The primary parameters describing ion - nucleic acid interactions in 1:1 salts are the so-called single ion interaction coefficients, Γ_{+} (the excess number of cations per RNA phosphate) and Γ_{-} (the deficiency of anions per phosphate, a negative number). (See the Discussion and ref. ²⁴ for further definition.) Γ_{+} and Γ_{-} are individually accessible from equilibrium dialysis experiments,²⁵ in principle, but are difficult to measure and to our knowledge have not been systematically studied for any RNA tertiary structure. However, changes in Γ_{+} or Γ_{-} that are caused by RNA folding can be derived from a linkage relation,

$$\left(\frac{\partial \ln K_{obs}}{\partial \ln a_{\pm}} \right)_{T,P} = \Delta\Gamma_{+} + \Delta\Gamma_{-} = 2\Delta\Gamma_{\pm} \quad (1)$$

where K_{obs} is the observed two-state equilibrium constant for folding RNA and $a_{\pm}$ is the mean ionic activity of the monovalent ions (cf. equation 49 in ref. ²⁶). Electroneutrality requires that $\Delta\Gamma_{+}$ and $\Delta\Gamma_{-}$ be equal, and for convenience we refer to either as $\Delta\Gamma_{\pm}$. For RNA folding, $\Delta\Gamma_{\pm}$ is positive, i.e., the reaction entails a net increase of excess cations and a similar decrease in the number of excluded anions. It is likely that cation interactions with RNA secondary structure are relatively insensitive to the identity of the monovalent ion (see Discussion). To the extent that this is true, differences in $\Delta\Gamma_{+}$ between group I ions imply that ion interactions with the native form of the RNA are size dependent.

Log-log plots of the A-riboswitch folding equilibrium constant as a function of mean ion activity are linear (Figure 4A), yielding values of $\Delta\Gamma_{\pm}$ that are similar for each of the salts tested. Within the error of the experiment, we conclude that there is no trend in $\Delta\Gamma_{\pm}$ with ion size ($2\Delta\Gamma_{\pm} \approx 3.6$). Similar measurements with the tar-tar* RNA gave slightly curved salt dependences (Figure 4B). Choosing a salt activity near the middle of the measured range, $a_{\pm} = 0.30$ (molal activity scale); the values of $2\Delta\Gamma_{\pm}$ show about a 30% increase as ion size decreases from that of Cs⁺ to Li⁺ (Figure 4C). The range of $\Delta\Gamma_{\pm}$ is slightly larger at lower salt activities, but the same trend of increasing $\Delta\Gamma_{\pm}$ with smaller ion size holds over the entire range of salt concentrations used in these experiments.

Tetraloop receptor motif

A tertiary structure motif that docks a GAAA tetraloop into the non-canonical minor groove of a receptor helix was first detected by sequence analysis and mutagenesis of group I and group II introns.²⁷ The structure was subsequently resolved by crystallography,^{28; 29} and shown to incorporate a K⁺ ion within the receptor sequence. The ion makes a total of five direct contacts to base and backbone atoms (Figure 5B). Under certain conditions the self-splicing activity of the *Azoarcus* group I intron is stimulated by K⁺ relative to other group I ions, an effect potentially related to occupancy of the receptor ion site.⁷

To ask whether the tetraloop-receptor tertiary interaction is preferentially stabilized by K^+ , we used an RNA designed to dimerize *via* two such motifs (Figure 5A).³⁰ The dimer complex is quite stable and its structure has been solved by NMR.^{31; 32} We found that disruption of the dimer could be observed in melting experiments carried out in monovalent ion concentrations as low as ~200 mM in the absence of Mg^{2+} ion, consistent with recent studies of the same RNA³³ and of an intramolecular tetraloop - receptor complex.³⁴ Melting of the dimer is detected as an unusual transition with a small hyperchromicity at 260 nm and small hypochromicity at 280 nm (see Supplementary Information), which together imply that only minor changes in the net extent of base stacking accompany disruption of the complex. This conclusion is consistent with what is known about the tetraloop-receptor motif: the tetraloop is a stable, well-stacked structure on its own,³⁵ and receptor bases are extensively stacked in the absence of the tetraloop.³⁶

Melting experiments carried out in different group I ions (400 mM MCl) established that the tetraloop - receptor interaction has about the same stability in either Na^+ or K^+ salts, but smaller or larger ions are much less effective (Figure 6A). The stability difference between Cs^+ and Na^+ is about 2.9 kcal/mol under these conditions. A study of a similar intramolecular complex also found no stability difference between Na^+ and K^+ , but did not test other monovalent ions.³⁴ Mg^{2+} stabilized the structure substantially, but was somewhat more effective in the presence of larger monovalent ions; thus K^+ became the most stabilizing of the group I ions in the presence of Mg^{2+} (Figure 6A).

To draw a more direct connection between selective stabilization of the RNA by K^+/Na^+ and the crystallographically-observed ion site, we mutated G39 to A (Figure 5). This variant replaces the electronegative O6 carbonyl of G, one of the ligands chelating K^+ , with the exocyclic amino group of adenine. The amine should not be an effective ligand for ions. Because the equivalent of an A39 substitution is commonly observed among the sequences of group I intron tetraloop-receptor complexes (making either a A39-U5 base pair or an A39•C5 noncanonical pair),³⁷ the G39A mutation presumably allows formation of a functional complex.

The G39A mutation substantially destabilized the dimer complex; in 400 mM salt, the structure was observed only with LiCl. 5 mM $MgCl_2$ was therefore included to bring the complex stability into a measurable range (Figure 6A). Under these conditions, the G39A mutation has virtually no effect on the RNA stability when LiCl is present, but is substantially destabilizing in the presence of all other group I ions. The net result is that the RNA no longer shows any preference for K^+ . Instead, there is a weak trend similar to what has been seen with other RNAs in this study, *viz.* decreasing stability with increasing ion size. It is not possible to compare the magnitude of this trend with ion size-dependence of the other RNAs reported here, as Mg^{2+} may accumulate close to the RNA surface in preference to monovalent ions and potentially suppress stabilization differences between the group I ions.

We also asked whether the selective stabilization of the tetraloop-receptor by K^+ would be reflected in values of $\Delta\Gamma_{\pm}$. To calculate K_{obs} from T_m differences, it is necessary to know ΔH° of the folding transition. We were not confident of the ΔH° values obtained from analysis of the UV melting profiles, for two reasons: the amplitudes of the signal changes are extremely small, leading to large errors, and the presence of hysteresis below ~20 °C suggested that some of the melting transitions could have been artifactually sharpened. Scanning calorimetry was therefore carried out with the tetraloop receptor RNA in each of the group I ions (see Materials & Methods and Supplementary Information). No significant trend in ΔH° (range 28.7 - 33.1 kcal/mol) was observed with ion size. The calculated values of $\Delta\Gamma_{\pm}$ (Figures 6B and C) show a substantial and nearly monotonic decrease with ion size, similar in overall magnitude to the

trend seen with the kissing loop complex (Figure 4C). A possible reason why this trend does not track with stability (Figure 6A) is suggested in the Discussion.

Vitamin B₁₂ aptamer RNA

An aptamer that specifically binds vitamin B₁₂ was isolated from RNA sequence pools by its retention on vitamin B₁₂-agarose; the elution buffer contained 1 M LiCl and 25 mM MgCl₂ to suppress non-specific binding.³⁸ The selected sequence with the highest affinity for vitamin B₁₂ in that study (Figure 7A) was found to bind the ligand tightly only in the presence of high LiCl concentrations; NaCl or KCl could not be substituted. For comparison with other RNAs presented in this study which are also stabilized by LiCl, we measure the stability of the B₁₂-aptamer complex with group I ions.

Melting experiments were used to explore the solution conditions over which this aptamer is able to bind the vitamin B₁₂ derivative dicyanocobinamide, which we here call cobinamide. Cobinamide is missing the purine ribonucleotide moiety of B₁₂, but this moiety is oriented away from the aptamer surface in crystal structures.^{39; 40} The lack of purine - RNA contacts is consistent with vitamin B₁₂ and cobinamide having essentially indistinguishable aptamer binding properties in our hands (data not shown). As reported elsewhere, methanol stabilizes many RNA tertiary structures and enables folding studies at a lower range of salt concentrations.^{12; 21} A combination of 16% methanol and a low concentration of MgCl₂ permitted detection of a cobinamide-aptamer complex in relatively low monovalent salt concentrations. An example set of melting profiles is shown in Figure 7B. In the absence of ligand, the RNA unfolded in two main transitions with similar stabilities in either 185 mM LiCl or KCl (2 mM MgCl₂ and 16% methanol are also present). Inclusion of cobinamide greatly increased the unfolding hyperchromicity when LiCl is present but had no effect with KCl as the monovalent salt. The T_m of the first unfolding transition increased with increasing concentration of cobinamide (data not shown). These experiments qualitatively reproduce the ion selectivity observed in the original study of this aptamer;³⁸ we conclude that the different buffer conditions we use have not altered its fundamental ligand binding properties.

Isothermal titration of the aptamer RNA with cobinamide caused changes in the UV absorption spectrum of both the ligand and the RNA, including a strong hypochromic signal centered between 255 and about 260 nm, depending on solution conditions. The presence of isosbestic wavelengths in the difference spectra suggest that binding is two-state (data not shown). The results of all titrations could be fit with high precision by an equation that assumed 1:1 binding stoichiometry (Figure 8A). Titration at 12°C showed weak ligand - aptamer binding with cations other than Li⁺ (Fig. 8B). (There is a moderate temperature dependence of cobinamide binding B₁₂ aptamer RNA, with an apparent $\Delta H^\circ = -17.1 \pm 1.5$ kcal/mole in buffer with 10 mM LiCl, 0.3 mM MgCl₂, and 16% MeOH (data not shown).) The ligand-RNA complex is ~20 fold more stable in the presence of LiCl than any other ion (Figure 8B), but in contrast to most of the other RNAs examined in this study, there is no gradual trend with increasing ion size: all other monovalent cations (including NH₄⁺) stabilize the complex to about the same degree.

Because cobinamide is not ionic, the intrinsic affinity of the aptamer for cobinamide is not expected to change with ionic strength, though both hydrophobic cobinamide-RNA interactions and RNA conformational changes accompanying ligand binding could render the observed binding affinity salt dependent. In the event, cobinamide binding was enhanced by increasing salt concentrations. Essentially the same effect was seen whether LiCl is the only added monovalent salt, or whether a constant total monovalent ion concentration was maintained by a combination of LiCl and KCl (Figure 8C). This observation implies that increased ligand affinity is not a general effect of increasing ionic strength, but is specific for Li⁺. The logarithmic dependence of the ligand affinity on LiCl concentration, and the relatively low salt concentrations at which the dependence is observed, are both characteristic of salt

effects on nucleic acid stability.²⁶ In contrast, hydrophobic interactions are usually only weakly affected by salts in molar concentration ranges.⁴¹

When the LiCl concentration is varied, a value of $2\Delta\Gamma_{\pm} \approx -0.80 \pm 0.06$ is found (Figure 8C). (This value probably underestimates the magnitude of $\Delta\Gamma_{\pm}$ slightly, because the low concentration of Mg^{2+} present in these experiments competes with monovalent ions for interactions with the RNA.⁴²) In this context, $\Delta\Gamma_{\pm}$ (equation 1) is associated with the presumed RNA conformational change coupled to dissociation of the cobinamide ligand. The sign of $\Delta\Gamma_{\pm}$ indicates that cations are released when cobinamide dissociates, suggesting that the RNA adopts a more compact conformation when bound to ligand. In experiments that varied the Li^+ concentration while keeping a constant total concentration of Li^+ and K^+ (Figure 8C), the slope of the Li^+ -concentration dependence indicates the degree to which Li^+ and K^+ compete in stabilizing the cobinamide - aptamer complex. When the concentration of the two cations are equal, the slopes of the Figure 8C plots measure the quantity $(\Delta\Gamma_{Li^+} - \Delta\Gamma_{K^+})$, where the two terms refer to the change of the Li^+ and K^+ ion interaction coefficients, respectively, upon dissociation of the cobinamide ligand. (The negative sign appears because any increase in Li^+ concentration is matched by a decrease in K^+ concentration.) At two different total salt concentrations, the slopes are slightly more negative (~ -0.92) than the value of $2\Delta\Gamma_{\pm}$ obtained when the LiCl concentration was varied in the absence of K^+ . This can be the case only if $\Delta\Gamma_{Li^+} \approx -\Delta\Gamma_{K^+} \approx \Delta\Gamma_{\pm}$, that is, any uptake of Li^+ ion (upon ligand binding) is accompanied by a compensating release of K^+ ion. We conclude that the RNA conformational change accompanying cobinamide binding is strongly coupled to an uptake of Li^+ , and that K^+ competition for this uptake is undetectable, within the sensitivity of this experiment. Weak competition between the ions does take place, as noted by experiments in which addition of either KCl or $MgCl_2$ to a constant concentration of LiCl weakens the cobinamide-RNA complex (data not shown).

Discussion

Factors that influence the free energies of cation - RNA interactions

A simple view of ions and RNA distinguishes two major ways cations may interact with an RNA.²⁴ “Diffuse” ions remain fully hydrated, are affected only by the electrostatic potential of the RNA, and interact with the RNA without making direct physical contact. “Chelated” ions, in contrast, make a set of specific, direct contacts with the RNA; the water molecules that would normally hydrate the ion must be partially or entirely displaced. This distinction between diffuse and chelated ions has been a useful framework for electrostatic calculations with both mono- and divalent cations,^{9; 43} but is clearly a simplification of the varieties of interactions that may be taking place between ions and an RNA. Ions located less the diameter of a water molecule from the RNA surface (here called “surface” ions for convenience) are distinct from chelated ions but may be subject to different energetic considerations than diffuse ions. For instance, surface ions may penetrate layers of water that hydrate the RNA and are differently structured from bulk water, a potentially similar situation to the group I ions that have been observed substituting for ordered water molecules near some sequences in DNA minor grooves.^{44; 45} Although calculations based on diffuse ions and a continuum water model fully account for the observed stabilization of DNA duplex and RNA triplex structures by monovalent ions,⁴⁶ it is possible that similar calculations will not be as successful with RNAs having higher charge densities and irregular structures. A goal of the present study was to use ion size as a probe of the relative importance of chelated, diffuse, and surface ions for the stabilities of different RNA structures.

Among the five RNAs used in this study, size-dependent differences among group I cations of as much as 3 kcal/mol in stability were observed. Two distinctive trends in RNA stability vs. ion size were observed. The first trend, which we refer to as the “default”, is a monotonic

tendency for smaller ions to be more effective stabilizers than larger ions; we attribute this trend to the steric advantage of smaller ions in more closely approaching the RNA and accessing narrow grooves or pockets. In the second trend, K^+ is a more effective stabilizer than smaller or larger ions, which we ascribe to chelation of K^+ by a specific set of RNA contacts. These two aspects of ion-RNA interactions are developed in more detail in the following two sections.

RNAs for which smaller ions are more stabilizing

Formation of simple hairpin secondary structures and BWYV tertiary structure are both insensitive to ion size (<0.5 kcal/mol range; Figures 1B and 3C). Similar results have been obtained for the MMLV pseudoknot, which does not show any difference in either stability or $\Delta\Gamma$ with group I ions or NH_4^+ .⁴⁷ However, both the A-riboswitch and tar-tar* kissing loop RNAs show a monotonic correlation between ion size and RNA folding free energy, with a difference in stability of nearly 3 kcal/mol between Li^+ and Cs^+ (Figures 2B and 3C). We argue that a stronger “default” dependence of stability on ion size should appear in RNAs with higher charge density. Clearly, ion size factors into energetic considerations only for “surface” ions: smaller ions have a shorter distance of closest approach to the RNA surface, which gives them access to higher (more negative) electrostatic potential and to sterically constricted grooves or pockets, which tend to have very negative potentials.⁴⁸ Any RNA transition to a more compact, higher charge density structure is expected to increase Γ_+ and to draw surrounding cations closer to the surface of the RNA; both factors increase the number of ions that are at the surface of the RNA at any one time, and thus the relative importance of size-dependent energetic factors. We accordingly expect that both secondary structures and the BWYV pseudoknot are of low enough charge density that most of their salt-dependent stabilization originates from diffuse ions at some distance from the RNA surface; by the same argument, the A-riboswitch and tar-tar* tertiary structures must have higher charge densities than BWYV RNA. High charge density also favors the accumulation of Mg^{2+} over monovalent ions in the ion atmosphere; Mg^{2+} interactions with BWYV RNA are indeed weaker than with A-riboswitch RNA under similar conditions, consistent with this interpretation (ref. 18, and D. Leipply, unpublished data).

A steric rationalization for the advantage of small ions has to be tempered by the fact that the strength of water - ion interactions is inversely proportional to ion radius.^{15; 49} Any perturbations of the waters hydrating an ion near the RNA surface are therefore more costly for smaller ions, potentially offsetting the steric advantage of a small radius for these ions. It follows that, in the cases of the A-riboswitch and tar-tar* RNAs, the gain in electrostatic energy that smaller ions obtain close to the RNA surface is large enough to outweigh any cost that might have been incurred in altering ion - water interactions. The size-selectivity of ion electrodes and ion channels have been rationalized by similar considerations of the ways steric access and hydration energies vary with ion size.⁵⁰

The structure of the tar-tar* RNA suggested to us an explanation for the strong ion-size dependence of its stability. Formation of the tar-tar* complex creates a short helix of five Watson-Crick base pairs; the major groove of this helical segment is nearly bridged by the two hairpin loop phosphates at which the backbone makes a sharp turn (Figure 9). The result is a “tunnel” of such restricted size that group I ions would be unable to penetrate into this region without some perturbation of the ion hydration or accommodation by the RNA structure (the diameter of the Cs^+ ion is ~ 3.4 Å). The highest negative potential of an RNA A-form helix is within its major groove;^{48; 51} the non-bridging phosphates of tar C6 and tar* U6 point into the major groove and can only make its potential even more negative. In the crystal structure of a related kissing loop complex, the tunnel is occupied by $Mg(H_2O)_6^{2+}$,⁵² presumably because of the electrostatic potential. To ask whether monovalent ions are also able to access this region and to explore the hydration of ions at different distances from the RNA, Chen *et*

al have carried out molecular dynamics simulations on the tar-tar* complex in the presence of NaCl, KCl, or CsCl (see accompanying paper). These computations suggest that the tunnel is maximally occupied by any of the three cations, and therefore not the main source of the ion discrimination. However, there are distinct differences in ion distribution near the closely juxtaposed tar C6 and tar* U6 phosphates (Figure 9) and further out from the RNA surface. The thermodynamic quantities measured in experiments (differences in stabilization free energy and ion uptake among group I ions) reflect the aggregate behavior of many ions, and at the molecular level apparently result from small differences widely distributed around the kissing loops.

Cation selectivity of the tetraloop-receptor complex

An early observation of K^+ selectivity by a nucleic acid structure was made in studies of the G-quadruplex,⁵³ in which the arrangement of guanine carbonyls in the center of a fourstranded helical structure has the potential for 8-fold coordination of ions. Solution and crystal studies of these structures suggest that ions occupying these sites are completely dehydrated, and that the differential dehydration penalty is largely responsible for the ion selectivity.^{54; 55; 56; 57} Another instance of K^+ selectivity was seen with a 58mer rRNA fragment, which is as much as 3 kcal/mol more stable with K^+ than other group I ions;¹² a crystal structure shows a K^+ ion completely buried within the RNA solvent-accessible surface.⁹ In contrast to these two K^+ - selective nucleic acids, the five RNA ligands contacting the K^+ binding site in the tetraloop-receptor RNA are distributed over one hemisphere of the ion, leaving the other side exposed to solvent (Figure 5B). K^+ and Na^+ stabilize this RNA to about the same extent, but both ions are nearly 3 kcal/mol more effective than Li^+ or Cs^+ .

The reversion of the G39A tetraloop receptor mutant to the default trend in ion selectivity is good evidence that its preference for K^+ indeed originates from the crystal chelation site, but it is unlikely that the destabilization brought about by the mutation is solely due to weakened ion binding at this site. A crystal structure of the *Azoarcus* group I intron contains two tetraloop-receptor complexes, both similar in sequence to the structure used here²⁹, but K^+ was found at only one of the sites. At the second site, a hydrogen-bond network has changed in a way that rotates the equivalent of G39 out of position for K^+ coordination by either N7 or O6. Apparently there are alternative conformations of the tetraloop-receptor complex, some of which are not associated with bound K^+ . (It is interesting to note that the unusual conformation of the A37-A38-G39 sequence is reproduced in a different structural context in the 23S rRNA, where the equivalent of the G39 base and A37 2'OH, as well as two other ligands, directly contact a Na^+ ion.⁵⁸) The tetraloop-receptor complex may well adopt slightly different structures depending on the particular sequence variant, the ionic conditions, and the larger structural context.

The ~3 kcal/mol preference of the tetraloop receptor RNA for K^+ is comparable to the 2-3 kcal/mol range of the A-riboswitch and tar-tar* stabilities between Li^+ and Cs^+ . Apparently, chelation and “surface effects” can be equally important aspects of the overall stabilization of an RNA by monovalent ions. To put the magnitude of the 2-3 kcal/mol range of stabilizations in perspective, a 2-8 fold change in salt concentration would be needed to obtain the same range of stabilities for the RNAs studied here.

Trends in $\Delta\Gamma_{\pm}$

The interaction coefficients Γ_+ and Γ_- describe the way the negative charge of an RNA is neutralized by ions. If an RNA solution is in dialysis equilibrium with a solution of monovalent salt, Γ_+ is the excess number of cations present in the RNA solution, and Γ_- is the deficiency of anions, relative to the salt solution. To preserve electroneutrality, Γ_+ and $|\Gamma_-|$ must add to the total number of RNA charges. The important principle needed to interpret the experiments

presented here is that higher nucleic acid charge density is accompanied by an increase in both Γ_+ and Γ_- (Γ_- becomes less negative)²⁴. Thus, added salt stabilizes more compact (higher charge density) conformations of an RNA, and $\Delta\Gamma_{\pm}$ is positive for RNA folding reactions.

We were able to measure $\Delta\Gamma_{\pm}$ as a function of ion size for three RNAs. Of the two RNAs that show the default dependence of stability on ion size, only with the tar-tar* complex does $\Delta\Gamma_{\pm}$ vary with the group I ion, smoothly increasing by about 30% in the series from Cs^+ to Li^+ . This increase corresponds to only ~0.3 more excess Li^+ ions than Cs^+ ions associated with the kissing loop complex (assuming that interactions with the hairpins are the same for all ions). For comparison, the total number of excess cations (Γ_+) for a six base pair segment of helical RNA (the approximate size of the structure formed between the two hairpin loops) is ~11⁴⁶. As a qualitative way to account for this difference between the two RNAs, we suggest that smaller ions access the volume of solvent and electrostatic potential in the kissing loop “tunnel” (Figure 9) more readily than larger ions; consequently large ions “see” a tar-tar* complex of effectively larger volume and lower charge density. The A-riboswitch RNA, which does not show any trend in $\Delta\Gamma_{\pm}$ with ion size, does not have any similar pockets or surfaces that might be inaccessible to the largest ions.

The tetraloop-receptor shows a similar inverse relation between $\Delta\Gamma_{\pm}$ and ion size as does the tar-tar* complex, even though the tetraloop-receptor complex is optimally stable with intermediate-sized ions (cf. Figures 6A and 6C). The dimerized form of the RNA used in this study brings two helices into close juxtaposition (Figure 5A). Although the gap between the helices is not as constricted as the tar-tar* “tunnel”, it still has the potential to reduce the access of hydrated ions to the RNA surface. This large area of constricted access may be a bigger contributor to $\Delta\Gamma_{\pm}$ than the relatively small pocket that chelates K^+ .

Evolution and RNA - ion interactions

Cellular RNAs have evolved in the presence of K^+ as the most abundant cation, and so it is not surprising that some RNAs, such as the tetraloop-receptor motif, take specific advantage of this ion to achieve specific structures or needed stability. Chelated K^+ has also been observed near the active site of the *Azoarcus* group I intron,²⁹ in a protein-RNA complex derived from the signal recognition particle,¹³ in some riboswitches,^{14; 59} and in a number of locations within ribosomal RNA.^{9; 58} The presence of sites selective for Na^+ over other ions in the BWYV pseudoknot structure is harder to rationalize in evolutionary terms, but our failure to find any thermodynamic preference for Na^+ in forming the structure suggests that the crystallographic sites might be adventitious, and not the consequence of selective pressure on RNA stability. Lastly, the unusual preference of the B_{12} aptamer for Li^+ over all other ions suggests that RNAs are versatile enough to take specific advantage of ions with properties much different from K^+ . Had life evolved with a different set of cations than Mg^{2+} and K^+ , RNA structures might have explored different regions of conformational space.

Materials and Methods

Chemical, RNAs, and solutions

All solutions were prepared using distilled deionized water at 18.3M Ω resistivity. Chloride salts of group I ions were obtained from Fluka, and were >99.5% purity. Buffers (MOPS, HEPES, or cacodylate) were purchased in the acid form and titrated to the desired pH with the hydroxide of the appropriate monovalent cation; they were used at the concentrations indicated in the figure legends. Buffers also standardly included 0.1 mM EDTA. To measure the stabilities of the different secondary and tertiary structures under consideration, different ranges of salt concentrations had to be used for the different RNAs. Tar and tar* hairpin RNA sequences were purchased from Dharmacon. All other RNAs were transcribed by T7 RNA

polymerase from DNA templates using described methods: the BWYV pseudoknot and B12 aptamer RNAs from DNA oligomers synthesized by the Core Facility at the M.S. Hershey Medical Center, Hershey, PA,¹⁸ the A-riboswitch and tetraloop-receptor RNAs from plasmid DNA cleaved with Sma I restriction nuclease. The necessary plasmid sequences were obtained by cloning synthetic DNA into pLL2, which contains a T7 promoter immediately followed by a Stu I cleavage site.⁶⁰ Transcription products were purified by preparative electrophoresis through 20% denaturing acrylamide gels, followed by electroelution in an Elutrap Electrophoresis Chamber (Schleicher & Schuell). Centricon filter units (Millipore, Billerica, MA) were used to equilibrate RNAs in the desired buffers for experiments.

UV melting and calorimetry

Thermal denaturation experiments were carried out in a Cary 400 spectrophotometer equipped with a six position thermostatted cuvette holder. To insure that equilibrium unfolding of RNAs was being observed, the temperature was ramped in three stages: heating from room temperature to 60-65 °C, cooling to 2-5 °C, and then heating to 95°C. The temperature was ramped at $\pm 0.5 - 0.8$ deg/min in the second and third stages, which were compared to check for hysteresis. Data were generally collected at both 260 and 280 nm. Differential scanning calorimetry was carried out in a Microcal VP instrument using a similar heating/cooling protocol, with temperature changing at 0.45 deg/min.

UV melting data were plotted as the first derivative of absorbance with respect to temperature, and the T_m and ΔH° for each unfolding transition were extracted by simultaneous fitting of 260 and 280 nm data using the program Global Melt Fit.⁶¹ The same software was used to fit unfolding transitions to heat capacity data. Uncertainties in the fitted parameters were estimated either by a bootstrap routine included in the program or from 3-5 data sets obtained under identical conditions. Typical UV melting profiles, fitted curves, and transition enthalpies for tar-tar*, BWYV pseudoknot, and A-riboswitch are in the supplementary information of reference.²¹ UV and heat capacity profiles for the tetraloop-receptor complex are in Supplementary Data.

Folding free energies at temperature T_0 were calculated from T_m s by the formula, $\Delta G^\circ(T_0) = \Delta H^\circ(T_0)(1/T_m - 1/T_0)$. For calculations of $\Delta \Gamma_\pm$, molar concentrations of ions were first converted to the molal scale using partial molar volumes tabulated in ref.⁶², and then to mean ionic activities by use of the activity coefficients compiled in ref.⁶³. The total monovalent ion concentration (from added buffer and chloride salt) was used in calculating the mean ionic activity. $\Delta \Gamma_\pm$ values and the ΔH° values used in their calculation Supplementary Information.

Isothermal titrations

To quantitate stock solutions of tar and tar*, aliquots were hydrolysed in 1M KOH at 37° for 24 hours, the hydrolysates diluted into 1M KOH at room temperature, and absorbance at 260nm recorded. Concentrations were found by comparing with the calculated molar absorbance of a solution of the appropriate mix of nucleotides under comparable conditions. To obtain the tar-tar* difference spectrum, the spectra of equimolar solutions of tar and tar* in 0.4 M LiCl (5 mM Li-cacodylate, pH 6.4, 20°C) were recorded, then equal weights (approximately 0.500 g each measured to ± 0.2 mg) of these solutions were mixed, equilibrated at 20°C for 20 min, and the spectrum of the mixture recorded. The tar-tar* difference spectrum = spectrum of the mixture minus 1/2(spectrum tar solution + spectrum tar* solution).

Isothermal titrations were at 20°C. At the start, tar (or tar*) was in the sample cuvette. The reference cuvette contained all components except the RNA. Equal amounts of tar* (or tar) were then added to the sample and reference cuvettes. After time to reach equilibrium (~5 min), the absorbance spectrum from 230 to 340 nm was recorded for each solution. This process

was repeated until the designated titration endpoint was reached. Since addition of titrant changes the solution volume, absorbance spectra were corrected to the starting (original) sample volume. The original sample RNA spectrum was then subtracted from each volume-corrected spectrum, giving a set of “difference spectra”. These spectra are not exactly correct. Small differences (a few percent) in the titrant volumes pipetted into the sample and reference cuvettes are unavoidable, as a result these “difference spectra” are contaminated to an unknown extent with highly absorbing, uncomplexed titrant RNA.

To avoid problems caused by these pipetting errors, a Δ Absorbance (Δ Abs) due to tar-tar* complex formation was measured between wavelengths at which titrant RNA absorbance does not change. The appropriate wavelengths (266.75 and 251.25 nm in Fig.3B) were chosen from a spectrum of titrant RNA obtained under the same conditions of buffer and temperature used in the titration. This Δ Abs is proportional to the amount of tar-tar* complex in solution and is the number plotted on the graph and used in curve fitting. Appropriate dilution factors were included in the fitting equation, since tar-tar* complex formation described by the successive Δ Abs's took place in larger volumes, and hence at lower concentrations, than indicated by the volume corrected absorbances. The fit of the titration curve yields an equilibrium constant for the interaction of tat and tar* from which the ΔG° was calculated.

Titration of the B12 aptamer with dicyanocobinamide was done in a similar manner to the titration of tar by tar* given above. Prior to a titration experiment, the aptamer was renatured by heating in the complete experiment buffer to 65°C for 20 min, then allowed to equilibrate at 25°C for an additional 25 min. The buffer in all experiments was 2.5 mM HEPES, pH 7.6, neutralized with the monovalent cation used in that experiment. The cation concentration of the buffer was taken into account in determining the cation concentration of the experiment. Titrations in the presence of LiCl were carried at 25 or 12 °C; for monovalent cations other than Li⁺, the titration temperature was always 12°C. The aptamer was in the sample cuvette and the reference cuvette contained all components except the aptamer. In the titration equal aliquots of cobinamide were added to the sample and reference cuvettes, the solutions mixed and after 3-5 minutes a spectrum was obtained of the region from 240 to 400 nm. Cobinamide has significant absorption in the 390-400 nm region that is not affected by binding to the B12 aptamer. Therefore, for each spectrum, we used the averaged absorption from 390 to 400 nm to correct for any differences in addition of cobinamide to the sample and reference cuvettes. As with tar/tar* titrations, the spectra were then corrected to the original sample volume and the spectrum of the B12 aptamer subtracted to yield difference spectra due to addition of cobinamide to B12 aptamer. These difference spectra have an absorption minimum at about 257 nm. The titration curve of Figure 8A is a plot of Δ Absorbance of this minimum vs vs cobinamide concentration.

As with tar and tar*, appropriate dilution factors were included in the fitting equation to give the correct concentrations under which the reactions took place. The fit of the titration curve yields an equilibrium constant for the interaction of the Vitamin B12 aptamer and dicyanocobinamide.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Effects of group I ions on the stability of the BWYV pseudoknot RNA. **A**, structure of the BWYV pseudoknot RNA. Black bars indicate Watson-Crick base pairs; gray bars and dot indicate tertiary interactions between the loops (L1 and L2) and helices (H1 and H2). Lines with arrows point in the 5' to 3' direction of the backbone. The phosphate and three nucleosides providing ligands for chelation of two Na⁺ ions (PDB file 1L2X¹⁰) are drawn in outline. **B**, ΔG° of folding vs. ion size as derived from melting experiments: open circles, secondary structure (formation of H1 from fully unfolded RNA); filled circles, tertiary folding (formation of native structure from the H1 hairpin). All free energies are calculated at 40 °C. Buffers (10 mM MOPS, pH 7.0) contained 124 mM of (left to right) Li⁺, Na⁺, K⁺, Rb⁺, and Cs⁺. Ionic

radii are those originally determined by Pauling.⁶ Confidence intervals were estimated from bootstrap analysis of the melting profiles; linear least squares best fits to the data (dashed lines) suggest that there are no significant trends in stability with ion type. See Materials and Methods for further experimental details and calculations.

Figure 3.

Ion-dependent stability of the tar-tar* complex. **A**, secondary structure of the kissing loop complex. Gray bars represent tertiary base pairing between the two hairpins. **B**, K_{obs} determined from changes in absorbance upon titration of $2.75 \mu\text{M}$ tar with tar* (20°C in buffer containing 0.4 M LiCl). The line is a least squares fit of a binding isotherm with dissociation constant $0.198 \mu\text{M}$. Inset: UV difference spectrum of the tar-tar* complex (see Materials & Methods). **C**, $\Delta G^\circ(25^\circ\text{C})$ for formation of the tar - tar* complex (square symbols, solid lines) or the tar hairpin (circles, dashed lines) at either 0.2 M monovalent ion (closed symbols) or 0.4 M cation (open symbols). 5 mM cacodylate, $\text{pH } 6.4$, was included in all buffers.

Figure 4.

Effects of group I ion activity on RNA folding equilibria. **A**, plot of $\log(K_{\text{obs}})$ vs. $\log(a_{\pm})$ for formation of the A-riboswitch tertiary structure in salts of each of the five group I ions. Each data point is the average of three or four determinations; in most cases, the range of averaged values is smaller than the size of the symbol. Data have been fit to lines with the slopes and errors listed in the figure. **B**, plot of $\log(K_{\text{obs}})$ vs. $\log(a_{\pm})$ for formation of the tar-tar* RNA complex. Each data point is the average of three determinations; data sets are color-coded by ion in the same way as in panel A. Error bars are shown but most are smaller than the size of the symbols. Data have been fit to second order polynomials. **C**, values of $2\Delta\Gamma_{\pm}$ for each group I ion with the tar-tar* complex were calculated as the slopes of the fitted polynomials shown in panel B, evaluated at a salt activity of 0.30 ($\log(a_{\pm}) = 0.523$). The error bars are the uncertainties in the slopes of the fitted curves in panel B, based on bootstrap analysis.

Figure 5. Tetraloop - receptor complex. **A**, sequence designed by Jaeger *et al* (molecule 1 in reference 30) to dimerize *via* two tetraloop — receptor interactions (boxed sequences). Arrow indicates the G39A mutation made in the putative K⁺ chelation site. **B**, K⁺ chelation by a tetraloop receptor in the *Azoarcus* self-splicing intron (PDB file 1T42²⁹). Intron nucleotides homologous to U5 and G39-A41 of the structure in panel A are labeled.

Figure 6.

Monovalent ion interactions with the tetraloop receptor motif. **A**, tetraloop-receptor stability as a function of group I ion size. Melting experiments were carried out in buffer with 400 mM MCl (black) or 400 mM MCl and 5 mM MgCl_2 (blue, sequence in Figure 5A; red, G39A variant). Points are averages from three melting curves; error bars are smaller than the symbols. **B**, salt dependences of the tetraloop receptor in different ions. Error bars (based on three measurements) are shown, but are smaller than most of the symbols. The lines are least squares fits. **C**, dependence of $2\Delta\Gamma_{\pm}$ on group I ion size. Error bars are from the uncertainties in the slopes in panel B. 10 mM MOPS, pH 7.0, was included in all buffers.

Figure 7.

Vitamin B₁₂ aptamer RNA. **A**, aptamer RNA secondary structure. Thin red and black lines represent backbone, arrows indicate 5' – 3' directions. Canonical (thick black bars) and non-canonical (•) base pairings are indicated, based on an x-ray crystal structure.^{39; 40} The planes of the blue and green sets of bases are oriented at roughly 90° in the crystal, and form the B₁₂ binding site. **B**, 260 nm melting profiles of the aptamer. Dark blue, aptamer in 16% methanol buffer containing 185 mM LiCl and 2 mM MgCl₂. Cyan, the same LiCl buffer plus 30 μM cobinamide. Red and orange curves were obtained under the same buffer conditions only substituting KCl for LiCl; 30 μM cobinamide is present in the orange data set. 2.5 mM HEPES, pH 7.6, was included in all buffers.

Figure 8.

Binding of cobinamide to the B12-aptamer RNA. **A**, change in UV difference spectrum at 257 nm upon titration of 4.4 μM B₁₂ aptamer RNA with cobinamide at 25 °C (16 % methanol, 0.3 mM MgCl₂, 10 mM KCl, 10 mM LiCl). Data are fit to a single site binding isotherm with $K_d = 7.9 \mu\text{M}$; based on a bootstrap method, the uncertainty in the fit is estimated as $\pm 0.6 \mu\text{M}$. **B**, dependence of K_d (12 °C) on ion size (16% methanol, 0.3 mM MgCl₂, 10 mM MCl). Uncertainties in K_d based on the fit of the data to the titration curve are shown as error bars for all points. In this set of experiments, NH_4^+ (open circles) was included in place of Rb^+ . **C**, log-log plot of K_d vs. Li^+ molarity at 25 °C (16% methanol, 0.3 mM MgCl₂). Closed circles, LiCl concentration varied (slope = -0.80); open circles, concentrations of Li^+ and K^+ totaled 10 mM (slope = -0.93); open squares, concentrations of Li^+ and K^+ totaled 30 mM (slope = -0.91). All titrations included 2.5 mM HEPES, pH 7.6, neutralized with the hydroxide of the monovalent cation used in that experiment.

Figure 9.

Tar-tar* kissing loop complex. (PDB file 1KIS²³). Only the two hairpin loop sequences are shown (tar C5-A11 and tar* U5-G11). The black bar is $\sim 3\text{\AA}$. The backbones of the two loops make sharp bends at C6 (tar) and U6 (tar*), placing their 5' non-bridging oxygens (identified by black dots) nearly in Van der Waals contact ($2.49\text{\AA}$ distant) as a “bridge” over the major groove of the kissing helix.. (Figure prepared with PyMol.)