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Ion - RNA interactions: thermodynamic analysis of the effects of mono- and divalent ions on RNA conformational equilibria

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Abstract

RNA secondary and tertiary structures are strongly stabilized by added salts, and a quantitative thermodynamic analysis of the relevant ion-RNA interactions is an important aspect of the RNA folding problem. Because of long-range electrostatic forces, an RNA perturbs the distribution of both cations and anions throughout a large volume. Binding formalisms that require a distinction between ‘bound’ and ‘free’ ions become problematic in such situations. A more fundamental thermodynamic framework is developed here, based on preferential interaction coefficients; linkage equations derived from this framework provide a model-free description of the ‘uptake’ or ‘release’ of cations and anions that accompany an RNA conformational transition. Formulas appropriate for analyzing the dependence of RNA stability on either mono- or divalent salt concentration are presented and their application to experimental data is illustrated. Two example data sets are analyzed with respect to the monovalent salt dependence of tertiary structure formation in different RNAs, and three different experimental methods for quantitating the ‘uptake’ of Mg²⁺ ions are applied to the folding of a riboswitch RNA. Advantages and limitations of each method are discussed.

I. Introduction

It is well known that the conformations and stabilities of nucleic acids are sensitive to the concentrations and identities of salts that are present. With regard to RNAs, the strongly stabilizing effect of Mg²⁺ ion on tertiary structures has been of particular interest (Stein and Crothers, 1976), but virtually any RNA conformational equilibrium may shift in response to changes in either monovalent or divalent ion concentrations.

In describing the effects of ions on RNA folding equilibria, a widely-used formalism assumes that ions are simple ligands that bind to RNA sites according to stoichiometric mass action equations. For instance, the effect of Mg²⁺ on the equilibrium between a folded (F) and unfolded (U) RNA has been written as

$$U + n\text{Mg}^{2+} \rightleftharpoons F \bullet (\text{Mg}^{2+})_n \quad K = \frac{[F \bullet (\text{Mg}^{2+})_n]}{[U][\text{Mg}^{2+}]^n} \quad (1)$$

(Fang, *et al.*, 1999, Latham and Cech, 1989, Schimmel and Redfield, 1980). This particular equilibrium and its corresponding equilibrium constant have been the starting point for

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deriving equations used to calculate the n ions ‘taken up’ in a conformational transition or to extrapolate the free energy of RNA folding to different Mg^{2+} concentrations.

Reaction equilibria such as Eq. (1) apply to neutral ligands that bind stoichiometrically to defined sites on a macromolecule, but their interpretation becomes problematic when used to describe ions and charged macromolecules. Mass action schemes overlook the essential electrostatic character of these interactions in two ways. First, the long-range character of electrostatic interactions is neglected. Ions a considerable distance from an RNA surface interact significantly with the RNA (García-García and Draper, 2003), and the total free energy of such long-range interactions may constitute the major source of ion-induced stabilization for many RNAs (Soto, *et al.*, 2007). Second, ions can be added to a solution only as electroneutral salts. When cations are titrated into an RNA solution, the unfavorable electrostatic interactions between the accompanying anions and the RNA are part of the overall energetic picture and generally must be taken into account. As developed in the next sections, only under special circumstances can the effects of the added anion be ignored.

To circumvent the above problems with mass action schemes, it is necessary to use a more general thermodynamic formalism based on parameters known as interaction coefficients, also called Donnan coefficients in some contexts (Record, *et al.*, 1998). This approach is completely general; it requires no assumptions about the types of interactions the ions may make with the RNA or the kinds of environments the ions may occupy. Although interaction parameters are a fundamental concept in thermodynamics and have been widely applied to biophysical problems, the literature on this topic can be difficult to access for anyone not already familiar with the formalism, and the application of interaction coefficients to the mixed monovalent-divalent cation solutions commonly used for RNA studies has received only limited attention (Grilley, *et al.*, 2006, Misra and Draper, 1999). For these reasons, the following theory section sets out the main concepts of the preferential interaction formalism in some detail, and outlines derivations of formulas relevant to monovalent ion - RNA interactions. A third section presents example analyses of experimental data, and extends the preferential interaction formalism to solutions of mixed salts (i.e., KCl and MgCl_2). The section includes discussions of potential sources of error and practical considerations in data analysis for experiments with both monovalent and divalent ions.

II. Thermodynamic descriptions of ion-RNA interactions

1. “Excess” ions in an equilibrium dialysis experiment

The key concept of the analysis developed here is the interaction coefficient, which we will use to assess the net interactions (favorable or unfavorable) taking place between ions and an RNA. We first introduce interaction coefficients by describing the way they might be measured in an equilibrium dialysis experiment, and give an overview of their significance. These parameters are defined in more formal thermodynamic terms in the next section (II.2) and are subsequently used to derive formulas useful in the interpretation of experimental data.

Consider two chambers separated by a dialysis membrane permeable to ions and water. After the chambers are filled with a KCl solution and allowed to come to equilibrium, the concentration of K^+ and Cl^- ions will be the same on each side of the membrane. An RNA with Z negative charges is then added to the left chamber (Figure 1A). Because the RNA must be added as a neutral salt, Z cations are also added. In the example diagrammed in Figure 1A, $Z = 16$ and the K^+ salt of the RNA, K_ZRNA , is illustrated. The two dialysis chambers are no longer in equilibrium after the addition, because of the excess of 16 K^+ on the left side of the membrane as compared to the right side. The RNA itself is confined to the left side, but ions may migrate across the membrane to achieve equilibrium. For

simplicity, suppose that the volume of the right side is so large that the migration of ions in or out of it does not appreciably change its KCl concentration; that is, the illustrated volume is only a small fraction of the total. (As a further simplification, suppose that the RNA molecule occupies a negligible fraction of the left side volume.) The excess K^+ ions in the left chamber will tend to flow down the concentration gradient into the right chamber. To maintain a net zero charge on each side of the membrane, an equivalent number of negative charges must accompany the flow of cations. In the Figure 1A example, equilibrium is achieved after two $K^+ - Cl^-$ ion pairs migrate from the left to the right. There are now two ion concentration gradients: the initial excess of K^+ ions accompanying the RNA persists, though reduced in magnitude, and a deficiency of Cl^- anions has been created in the RNA solution. (Figure 1A shows a reduction in the number of the dark blue K^+ ions that accompanied the RNA, but obviously there is no way to distinguish dissolved K^+ ions on the basis of their original ionic partners.)

The two ion gradients that develop in the dialysis experiment are conveniently quantitated in terms of interaction coefficients. A histogram (Figure 1B) diagrams the ion concentrations in a similar experiment as cartooned in Figure 1A. The total number of KCl ion pairs that migrates across the membrane as equilibrium is established, when normalized by the number of RNA molecules present, becomes the interaction coefficient Γ_{KCl} . Another way to find the same number is to count the total number of KCl ion pairs on each side of the membrane at equilibrium,

$$\Gamma_{KCl} \cong \frac{[KCl]_L - [KCl]_R}{[RNA]} \quad (2)$$

where the subscripts on the salt concentrations indicate the side of the dialysis membrane in Figure 1A, and refer to concentrations at dialysis equilibrium. (Note that on the left hand side, each K^+ ion can be paired with either a Cl^- or RNA phosphate anion for the purposes of determining the concentrations of the two electroneutral salts, KCl and K_ZRNA . On either side of the membrane in the Figure 1A example, $[KCl] = [Cl^-]$.)

Another way of representing the ion concentration differences at equilibrium is by way of single ion interaction coefficients, which count the cations and anions separately:

$$\Gamma_+ \cong \frac{[K^+]_L - [K^+]_R}{[RNA]} \quad \Gamma_- \cong \frac{[Cl^-]_L - [Cl^-]_R}{[RNA]} \quad (3)$$

These three different Γ parameters are related to each other and to the total number of RNA charges, Z (see “ Γ relationships” in Figure 1B). First, the number of KCl pairs lost from the left hand side during the approach to equilibrium is the same as the equilibrium difference in the number of Cl^- ions between the two chambers, $\Gamma_{KCl} = \Gamma_-$. Electroneutrality also requires that $\Gamma_+ = \Gamma_- + Z$, *i.e.* the left side excess of cations (relative to the right side solution) must be balanced by an equivalent number of negative charges (note that $\Gamma_- < 0$).

Figure 1 has been drawn to suggest that $\Gamma_+ > |\Gamma_-|$. This inequality is generally true for nucleic acids in low to moderate salt, a phenomenon sometimes called the ‘polyelectrolyte effect’ (Draper, 2008, Record and Richey, 1988). Any RNA conformational change that increases the density of phosphate charges will also increase Γ_+ at the expense of $|\Gamma_-|$ (Record, *et al.*, 1998). However, Γ_+ may be similar to $|\Gamma_-|$ at high salt concentrations: for

instance, $\Gamma_+ = 0.46$ and $\Gamma_- = -0.54$ ions/nucleotide for DNA in 0.98 M NaBr (Strauss, *et al.*, 1967).

The dialysis experiment is a convenient way to conceptualize the meaning of the interaction coefficient, but the formal definition of a Γ does not depend on the presence of a membrane (see section II.2 below). Γ essentially measures the tendency of an RNA molecule to create ion concentration gradients in solution, manifested as the accumulation of cations and depletion of anions in a volume surrounding the RNA, relative to the ‘bulk’ concentration of ions a large distance away from the RNA surface. These gradients are related to the sum of all the attractive and repulsive forces experienced by the ions, and therefore to the overall energetics of ion - RNA interactions. The advantages of using this interaction formalism when considering ions are evident in a comparison with the interpretation of an equilibrium dialysis experiment by a binding formalism. v , the ‘binding density’ of a ligand with a macromolecule, would be calculated from a dialysis experiment in exactly the same way as Γ_+ (equation 3), but interpreted as the number of ligands ‘bound’ to the macromolecule. However, as pointed out in the Introduction, v fails to capture two aspects of ion - RNA interactions that are represented by Γ . First, Γ_{KCl} (or Γ_-) properly represents the depletion of anions near the RNA. The concept of a ‘binding density’ obviously breaks down when applied to Cl^- ; a negative binding density cannot be meaningfully discussed in terms of binding sites. Second, manipulations of v (i.e., in the calculation of equilibrium constants and interaction free energies) presume that v ligands are bound per macromolecule and the remaining ‘free’ ligands have no interactions at all. This distinction between free and bound ligands is valid only if all ligand-macromolecule interactions are short range. Because of long-range interactions, *all* the ions in a solution interact with an RNA, and it is not possible to parse the ions into distinct bound and free fractions. The interaction coefficients, by contrast, are completely model-independent, in that they reflect the influences of all the energetic costs that are present: long-range electrostatic attraction and repulsion as described by Coulomb’s law, as well as hydration changes and all short-range factors.

2. Thermodynamic definition of interaction coefficients

The preceding sections have used standard molar concentration units for RNA and ions, indicated by brackets or the abbreviation M. Thermodynamic definitions of interaction coefficients are made in terms of molal units, abbreviated m , the moles of solute per kilogram of solvent water. Molal units have the convenient properties that the concentration of water is a constant 55.5 m regardless of the amount of solute(s) present, and the molality of one solute is unaffected by addition of a second solute. For dilute solutions, M and m units are interchangeable. We use molal units for the thermodynamic derivations in this section, and indicate later (section III.1) the salt concentrations where a correction for molar - molal conversion is required.

Interaction coefficients are formally defined as partial derivatives, *e.g.*:

$$\Gamma_+ = \left(\frac{\partial m_+}{\partial m_{RNA}} \right)_{\mu_+} \quad \Gamma_- = \left(\frac{\partial m_-}{\partial m_{RNA}} \right)_{\mu_-} \quad (4a)$$

$$\Gamma_{KCl} = \left(\frac{\partial m_{KCl}}{\partial m_{K^+RNA}} \right)_{\mu_{KCl}} \quad (4b)$$

where concentrations of ions (m_+ , m_-), RNA (m_{RNA}), and neutral salts (m_{KCl} and m_{KzRNA}) are in molal units. The derivatives are the limits of Eqs. (2) and (3) as the added concentration of RNA becomes very small (see equation 1 in (Record, *et al.*, 1998). In the dialysis examples (Figure 1), the chemical potentials μ_{KCl} or μ_+ and μ_- are set by the concentration of KCl in the right chamber, and therefore are held constant as RNA is added. Hence Γ_+ reports on the number of cations that should accompany the introduction of a single RNA molecule, in order to keep a constant cation chemical potential in the solution, and Γ_- reports the number of anions that must be simultaneously removed to maintain constant μ_- . Note that the neutral salt coefficient is defined in terms of the addition of the neutral RNA salt, as illustrated in Figure 1, while the single ion coefficients consider the hypothetical addition of a charged RNA.

In the definitions of Γ , two variables in addition to the ion chemical potential must also be specified as constant. In an equilibrium dialysis experiment, these are temperature and the chemical potential of water. This partial derivative is known as the Donnan coefficient. (Note that the hydrostatic pressure is higher in the RNA-containing solution.) In making connections between Γ and the Gibbs free energy, it is more convenient if temperature and pressure are held constant instead. At the concentrations of RNA and ions typically used in experiments, the quantitative difference between these two kinds of interaction coefficients is inconsequential (Anderson, *et al.*, 2002). For all partial derivatives in the following sections, the conditions of constant temperature and pressure apply but are not explicitly written.

3. Implications of Γ_- and Γ_+ for ion activities in the presence of RNA

The *effective* concentration of a ion, better known as its *activity*, must be the same on each side of a dialysis membrane at equilibrium; in other words, there is no driving force for net ion migration from one side of the membrane to the other. The *actual* ion concentrations, used to calculate Γ_- and Γ_+ , are different on either side of the membrane because of RNA - ion interactions. The ratio of ion activity to concentration in the presence of an RNA will provide a starting point for derivations that link interaction coefficients to the effects of ions on RNA folding transitions. As background for these derivations, the relationships between interaction coefficients, ion concentrations, and ion activities are outlined here.

In Figure 1, the condition for thermodynamic equilibrium is that the chemical potential of the membrane-permeable ions is identical between the left and right side solutions. The chemical potential μ can be defined either for the 1:1 salt or for the individual ions,

$$\mu_{\text{KCl}} = \mu_{\text{KCl}}^0 + RT \ln a_{\text{KCl}} \quad (5a)$$

$$\mu_{\text{K}^+} = \mu_{\text{K}^+}^0 + RT \ln a_{\text{K}^+} \quad \mu_{\text{Cl}^-} = \mu_{\text{Cl}^-}^0 + RT \ln a_{\text{Cl}^-} \quad (5b)$$

where μ^0 is a standard state defined for a particular temperature and pressure, and a is the activity of the salt or ion. Activities of cations and anions usually cannot be measured separately; instead, a mean ionic activity, $a_{\pm}$, is used:

$$a_{\text{KCl}} = (a_{\text{K}^+})(a_{\text{Cl}^-}) = a_{\pm}^2 \quad (6)$$

Activities are related to concentrations by the activity coefficient γ ,

$$a_{KCl} = \gamma_{K^+} m_{K^+} \gamma_{Cl^-} m_{Cl^-} = \gamma_{\pm}^2 m_{K^+} m_{Cl^-} \quad (7)$$

where $\gamma_{\pm}$ is the mean ionic activity coefficient. Because ion chemical potentials (and thus the ion activities) must be the same on each side of a dialysis membrane at equilibrium, the ion concentration differences in the Figure 1A example imply that the ion activity coefficients are different in the presence and absence of RNA, and the following sets of inequalities apply:

$$(m_{K^+})_L > (m_{K^+})_R \quad \Gamma_+ > 0 \quad \gamma_{+L}/\gamma_{+R} < 1 \quad (8a)$$

$$(m_{Cl^-})_L < (m_{Cl^-})_R \quad \Gamma_- < 0 \quad \gamma_{-L}/\gamma_{-R} > 1 \quad (8b)$$

These inequalities are a consequence of the net attractive (8a) or repulsive (8b) interactions between RNA and cations or anions, respectively. The reciprocal effect of the ions on the RNA activity coefficient is taken up in the next section.

4. Application of interaction coefficients to RNA conformational changes

The interconversion between any two conformations of an RNA, here named folded (F) and unfolded (U) for simplicity, is associated with a free energy change which is expressed in terms of the chemical potentials of the two conformations,

$$\Delta G = \mu_F - \mu_U = \mu_F^0 - \mu_U^0 + RT \ln \left(\frac{a_F}{a_U} \right) = \Delta G^0 + RT \ln \left(\frac{a_F}{a_U} \right) \quad (9)$$

where μ is the chemical potential, μ^0 is the standard state chemical potential, and a is the activity; subscripts specify the RNA conformation. Note the distinction between the actual free energy change, ΔG , and the free energy difference between U and F in their standard states, ΔG^0 (more about the definition of standard states below). The condition for equilibrium between U and F is $\Delta G = 0$; in this circumstance, the thermodynamic equilibrium constant is defined as

$$K_{eq} = \frac{a_F}{a_U} = \frac{\gamma_F m_F}{\gamma_U m_U} \quad (10)$$

and the standard relation $\Delta G^0 = -RT \ln K_{eq}$ applies. The definition of the RNA standard state (μ^0) in Eq. (9) is critical. Thermodynamic standard states refer to hypothetical ideal states in which the molecular interactions of interest (those that cause non-ideal behavior in the real world) are turned off. If it were possible to carry out a dialysis experiment with such an 'ideal' RNA, one that had no net interactions with the ions of a salt, then the salt activity coefficients would be the same on each side of the dialysis membrane,

$$(\gamma_{KCl})_L = (\gamma_{KCl})_R \quad (11)$$

(cf. Eqs. 8a and 8b). (This relation does not imply that electrostatic interactions are absent, only that the *net* interactions of the RNA with all ions in solution sums to zero. Under this condition, added salt cannot affect any RNA conformational transition; K_{eq} is therefore independent of salt concentration.) In this hypothetical ideal state, the RNA activity is the same as its concentration ($a_{\text{RNA}} = m_{\text{RNA}}$ and $\gamma_{\text{RNA}} = 1$). Deviation from ideal behavior in the form of net favorable interactions between the salt ions and RNA will cause γ_{RNA} to take on values less than 1. In experiments, only the concentrations of various RNA conformers can be measured directly, not their activities. An “observed” equilibrium constant is expressed in terms of the actual concentrations,

$$K_{obs} = \frac{m_F}{m_U} \quad (12)$$

By omitting activity coefficients, this formula implies that the corresponding free energy change ($\Delta G^\circ_{obs} = -RT \ln K_{obs}$) does not refer to the ideal standard states described above, but to RNAs interacting with salt under the specific set of solution conditions used for evaluating K_{obs} . If the salt concentration changes, both the standard states and K_{obs} change. Substituting Eq. (12) into (10) gives the relation between thermodynamic and observed equilibrium constants as

$$K_{eq} = \frac{\gamma_F}{\gamma_U} K_{obs} \quad (13)$$

This equation will give us a way to use changes in an experimental observable, K_{obs} , to access changes in RNA activity coefficients.

At this point, we have defined an ideal reference state for the RNA in which there are no net interactions with ions, and introduced the RNA activity coefficient as a factor that assesses the deviation of the RNA from ideal behavior due to its interactions with all the ions in solution. No assumptions have been made about the nature of the ion interactions: anions and cations, long and short-range interactions all contribute. The ion interaction coefficients (Eqs. 4a and 4b) also reflect the ion - RNA interactions that create concentration differences in a dialysis experiment, and there is an intimate relationship between activity coefficients (γ) and interaction coefficients (Γ), as developed below. This relationship will be extremely useful: γ comes from the chemical potential and gives access to free energies and other thermodynamic functions, while Γ is directly accessible by both experiment and computation (see Pappu et al., this volume, III.20).

To show how γ , Γ , and free energies are linked, we first find an expression for Γ_{KCl} in terms of γ_{RNA} , and then connect Γ_{KCl} to Eq. (13). We begin by expanding the definition of Γ_{KCl} using a standard property of partial derivatives, the Euler chain rule,

$$\Gamma_{\text{KCl}} = \left(\frac{\partial m_{\text{KCl}}}{\partial m_{\text{RNA}}} \right)_{\ln a_{\text{KCl}}} = - \left(\frac{\partial \ln a_{\text{KCl}}}{\partial m_{\text{RNA}}} \right)_{m_{\text{KCl}}} \left(\frac{\partial m_{\text{KCl}}}{\partial \ln a_{\text{KCl}}} \right)_{m_{\text{RNA}}} \quad (14)$$

(It is equivalent whether μ_{KCl} or $\ln a_{\text{KCl}}$ is held constant.) Because interactions are always mutual (if A attracts B, B must attract A), any ion - RNA interaction must cause both the ion and RNA activity coefficients to change. This principle is embodied in Euler reciprocity

relations (discussed in standard physical chemistry textbooks, e.g. p 116 of Levine, 2002); in this case the relation takes on the form

$$\left(\frac{\partial \ln a_{KCl}}{\partial m_{RNA}}\right)_{m_{KCl}} = \left(\frac{\partial \ln a_{RNA}}{\partial m_{KCl}}\right)_{m_{RNA}} \quad (15)$$

Substituting Eq. (15) into (14) and simplifying by application of the standard chain rule, we obtain

$$\Gamma_{KCl} = -\left(\frac{\partial \ln a_{RNA}}{\partial \ln a_{KCl}}\right)_{m_{RNA}} = -\left(\frac{\partial \ln \gamma_{RNA}}{\partial \ln a_{KCl}}\right)_{m_{RNA}} \quad (16)$$

(The second equality follows because m_{RNA} is being held constant.) If this equation is now applied to a difference between Γ_{KCl} for the U and F states of an RNA,

$$\Delta \Gamma_{KCl} = \Gamma_{KCl}^F - \Gamma_{KCl}^U = -\left(\frac{\partial \ln(\gamma_F/\gamma_U)}{\partial \ln a_{KCl}}\right)_{m_{RNA}} \quad (17)$$

Differentiation of Eq. (13) with respect to $\ln a_{KCl}$ while holding m_{RNA} constant gives

$$\left(\frac{\partial \ln(\gamma_F/\gamma_U)}{\partial \ln a_{KCl}}\right)_{m_{RNA}} = -\left(\frac{\partial \ln K_{obs}}{\partial \ln a_{KCl}}\right)_{m_{RNA}} \quad (18)$$

(Recall that K_{eq} is independent of salt activity, and so does not appear in the derivative.) Combining Eqs. (17) and (18) and making use of the standard relation between ΔG° and an equilibrium constant, the final equation relating a change in the RNA-salt interaction coefficient to a change in the observed RNA folding free energy is

$$\Delta \Gamma_{KCl} = \left(\frac{\partial \ln K_{obs}}{\partial \ln a_{KCl}}\right) = -\left(\frac{1}{RT}\right) \left(\frac{\partial \Delta G_{obs}^0}{\partial \ln a_{KCl}}\right) \quad (19)$$

(The specification of constant m_{RNA} has been dropped because we are assuming that these equations are being applied to dilute enough ranges of RNA concentrations that Γ_{KCl} can be considered a constant with respect to m_{RNA} . Constant temperature and pressure still apply.)

A last point to consider is the relation of $\Delta \Gamma_{KCl}$ to single-ion coefficients. If a_{KCl} is put into terms of the mean ionic activity $a_{\pm}$ (see Eq. 6, II.3 above), Eq. (19) becomes

$$\left(\frac{\partial \ln K_{obs}}{\partial \ln a_{\pm}}\right) = 2\Delta \Gamma_{KCl} = \Delta \Gamma_+ + \Delta \Gamma_- = 2\Delta \Gamma_{\pm} \quad (20)$$

The way the second equality is written is justified by two relationships: Γ_{KCl} is numerically equivalent to Γ_- (cf. Figure 1A and Eqs. 2 and 3), and electroneutrality (Figure 1B, $\Gamma_+ = \Gamma_- + Z$) requires that $\Delta\Gamma_+ = \Delta\Gamma_-$. Suppose an RNA conformational change causes Γ_+ to decrease, in effect a ‘release’ of cations that (in terms of the Figure 1A diagram) flow out of the left dialysis chamber. The same number of anions must flow across the membrane in order to maintain charge neutrality, which causes Γ_- to become more negative. (Mass action equations which suppose that an RNA ‘takes up’ or ‘releases’ only cations, as Eq. 1, violate the principle of electroneutrality by ignoring the corresponding changes in anions.) We call the final result $2\Delta\Gamma_{\pm}$ to emphasize that *both* cations and anions are affected to the same extent when an RNA changes conformation.

Eqs. (19–20) are connections between the sensitivity of an RNA folding equilibrium to added salt, and the ‘uptake’ or ‘release’ of cations and anions as measured by the preferential interaction parameter. A similar kind of thermodynamic linkage between an added solute and a macromolecular conformational change was derived by Wyman for the case of O₂ binding to specific sites on haemoglobin. A binding formalism (as in Eq. 1) was originally used (Wyman, 1948), but later generalized in a way which bypasses specific binding models, as described above (Wyman, 1964). The modifications of linkage equations necessary when equilibria involve ions and charged polymers has been extensively considered by Record and colleagues (equation 47 of ref. (Record, *et al.*, 1998) is identical to Eq. 21 above).

III. Analysis of experimental salt dependence data

1. 1:1 salt dependence of RNA folding reactions

RNA secondary structures and many tertiary structures are stable in the absence of any divalent cation, and may require only moderate concentrations of a monovalent salt such as KCl or NaCl. In this section we give two examples of the calculation of $\Delta\Gamma_{\pm}$ for such RNAs.

Example 1: A-riboswitch RNA—The adenine riboswitch RNA (A-riboswitch) adopts a folded tertiary structure upon binding a purine ligand; in the absence of the ligand, only secondary structure remains (Serganov, *et al.*, 2004). In melting experiments with this RNA, inclusion of a purine ligand results in the appearance of a new unfolding transition that is generally well-resolved from the unfolding of secondary structure (Lambert and Draper, 2007, Leipply and Draper, 2009). From the melting temperature (T_m) and ΔH° of the folding transition, K_{obs} is readily calculated at a desired temperature (see legend to Figure 2; analysis of melting curves has been reviewed by (Draper, *et al.*, 2000). Values of K_{obs} extrapolated to 20 °C for the A-riboswitch in various KCl concentrations are shown in Figure 2A. The solutions were originally made volumetrically using molar concentration units, as shown; two manipulations were needed to convert molarity to activity on the molal scale, as needed for application of the linkage Eq. (20):

- i. Molar concentration units were converted into molal units using the partial molar volume of the salt. (We assume that RNA and other buffer components are present in such low concentrations that the density of the solution is not significantly different than a solution of water and salt alone.) The formula is

$$m_{\text{MCl}} = \frac{[\text{MCl}]}{(1/\rho_1)(1 + [\text{MCl}]\bar{V}_{\text{MCl}}/1000)} \quad (21)$$

where

$\bar{V}_{MCl}$ is the partial molar volume of the salt MCl, in units of mL/mol;

ρ_1 is the density of water, 0.998 g/mL at 20 °C;

the brackets indicate molar concentrations, and m is the molal concentration.

See section 2.1 of (Eisenberg, 1976) for a full discussion of the relation between molal and molar units. Table I lists partial molar volumes of different salts. The difference between molar and molal units is about 1% for a 0.3 molar KCl solution, and rises to only 3% for 1 M KCl. These percentages are smaller than the error in most measurements of K_{obs} .

- ii. Molal salt concentrations must be translated into mean ionic activity (Eq. 7). The mean ionic activity coefficients of many salts are compiled in the literature as a function of salt concentration (see appendix 8.10 to (Robinson and Stokes, 2002). Table I provides polynomial coefficients needed to calculate $\ln(\gamma_{\pm})$ at a given $\ln(m_{\pm})$ for common 1:1 chloride salts. A plot of $\ln(\gamma_{\pm})$ as a function of $\ln(m_{\pm})$ generally turns concave upwards as the salt concentration approaches 1 m ; this curvature is particularly severe for LiCl, which has a minimum in $\gamma_{\pm}$ at about 0.5 m . Therefore two sets of coefficients are given in Table I, one adequate for salt concentrations up to 0.5 m , and a higher order polynomial adequate up to ~0.8 m .

Figure 2A contains a second set of data points for which the molar units used in carrying out the experiments have been re-cast as activities on the molal scale. Both sets of data are linear within experimental error, but the slopes differ by about 10%:

$$\left(\frac{\partial \ln K_{obs}}{\partial \ln m_{KCl}}\right) = 3.33 \pm 0.09 \quad \left(\frac{\partial \ln K_{obs}}{\partial \ln a_{\pm}}\right) = 2\Delta\Gamma_{\pm} = 3.67 \pm 0.10$$

A second way to find $2\Delta\Gamma_{\pm}$ from salt dependence data takes advantage of the following application of the standard chain rule to Eq. (22):

$$2\Delta\Gamma_{\pm} = \left(\frac{\partial \ln K_{obs}}{\partial \ln a_{\pm}}\right) = \left(\frac{\partial \ln K_{obs}}{\partial \ln m_{KCl}}\right) \left(\frac{\partial \ln m_{KCl}}{\partial \ln a_{\pm}}\right) \quad (22)$$

The first term on the right is the salt dependence of $\ln K_{obs}$, expressed in molal concentration units. The second term can be calculated from the dependence of the salt activity coefficient on salt concentration,

$$\left(\frac{\partial \ln a_{\pm}}{\partial \ln m_{KCl}}\right) = 1 + \left(\frac{\partial \ln \gamma_{\pm}}{\partial \ln m_{KCl}}\right) = 1 + \varepsilon \quad (23)$$

For many salts, ε is nearly constant over a wide concentration range; $(1+\varepsilon)$ is then a simple correction factor that can be applied to the slope of the salt dependence. This approximation is valid for KCl in the 0–0.5 m range; from the constant a_2 in Table I, $\varepsilon = -0.1059$. Applying this factor to the Figure 2A data, we obtain

$$\left(\frac{\partial \ln K_{obs}}{\partial \ln m_{KCl}}\right) \left(\frac{1}{1+\varepsilon}\right) = (3.33)(1 - 0.1059)^{-1} = 3.72$$

which is within experimental error of the result obtained above from the plot of $\ln(K_{\text{obs}})$ as a function of $\ln(a_{\pm})$.

Example 2: tar-tar*—A so-called ‘kissing loop’ complex forms by Watson-Crick base pairing between the loops of two hairpins. Melting experiments with one such complex, between hairpins named tar and tar*, easily resolve disruption of the bimolecular complex from denaturation of the individual hairpins at higher temperatures (Chang and Tinoco, 1994, Lambert and Draper, 2007). The dependence of $\log(K_{\text{obs}})$ on $\log(a_{\pm})$ for the tar-tar* complex is plotted in Figure 2B. In contrast to the A-riboswitch data in Figure 2A, these data are best fit by a second order polynomial. Although the dependences of RNA folding transitions on salt tend to be linear, there is no necessity that they be so. Whether a polynomial should be used to describe the salt dependence depends on the size of the errors in $\ln(K_{\text{obs}})$ and the range of salt concentrations over which data have been collected. In this case, there are systematic deviations of the data from a linear fit that are larger than the error in the data, and the polynomial is a better description of the data.

The fitted polynomial for the tar-tar* data is

$$\log(K_{\text{obs}}) = 0.9084 + 0.6983 \log(a_{\pm}) - 1.238 (\log(a_{\pm}))^2$$

therefore

$$2\Delta\Gamma_{\pm} = 0.6983 - 2.476 \log(a_{\pm})$$

The slope evaluated at the middle of the data range ($a_{\pm} = 0.268$) is $2\Delta\Gamma_{\pm} = 2.11$. It would of course be unwise to extrapolate values of $2\Delta\Gamma_{\pm}$ to activities outside of the salt concentration range over which data were collected.

In both of the above examples we used an anionic buffer (MOPS or cacodylate). The buffer anions have only repulsive interactions with RNA and can be grouped with chloride ions when calculating mean ion activities. Thus we apply mean ionic activity coefficients measured with KCl solutions to solutions in which K^+ ions are contributed both by KCl and K-buffer salts. We strongly advise against the use of cationic buffers such as Tris, because of its idiosyncratic interactions with nucleic acids as compared to group I ions, and particularly against mixing KCl with Tris buffer, which creates a cationic mixture of unknown activity.

2. MgCl_2 dependence of folding reactions

Interaction coefficients in mixed divalent - monovalent cation solutions—

Suppose the K^+ salt of an RNA is added to a dialysis chamber that has been equilibrated with a mixture of MgCl_2 and KCl (Figure 3A). As in the monovalent salt example (Figure 1A), KCl ion pairs will tend to diffuse into the right chamber, leaving an excess of K^+ and creating a deficiency of Cl^- in the left chamber. However, there will also be a tendency for the RNA to accumulate Mg^{2+} in preference to K^+ ; the resulting net diffusion of Mg^{2+} ions into the left chamber must be accompanied by enough Cl^- ions to neutralize the Mg^{2+} charge. There are now two neutral salt interaction coefficients; Γ_{MgCl_2} is positive and Γ_{KCl} is negative. The ion gradients can also be represented by three single ion coefficients; Γ_+ and Γ_- are defined as in Eq. (4a), and Γ_{2+} is

$$\Gamma_{2+} \cong \frac{[Mg^{2+}]_L - [Mg^{2+}]_R}{[RNA]} \quad (24)$$

This coefficient is always positive. The relations between the neutral salt coefficients, the single ion coefficients, and the number of RNA negative charges are illustrated by the histograms and equations in Figure 3B. The neutral salt coefficient Γ_{MgCl_2} is identical to Γ_{2+} , which becomes important in the interpretation of linkage expressions (section III.2, below). Another important relation is the condition for electroneutrality, which is now

$$2\Gamma_{2+} + \Gamma_+ - \Gamma_- = Z \quad (25)$$

Any RNA conformational change that alters one of the three Γ values must also alter one or both of the other two. This is another aspect of RNA – ion equilibria that is generally not included in mass action equilibrium expressions: any change in the interactions between one kind of ion and an RNA is always accompanied by reciprocal changes in the interactions of the other ion species.

The linkage relation in titrations with $MgCl_2$ —The linkage equation that quantifies the dependence of an RNA folding equilibrium on added divalent cation is

$$\Delta\Gamma_{MgCl_2} = \left(\frac{\partial \ln K_{obs}}{\partial \ln a_{MgCl_2}} \right)_{m_{KCl}} \quad (26)$$

where the concentration of monovalent salt must be held constant. The derivation of this relation proceeds as outlined in Eqs. (14)-(19), except that Eq. (13) is differentiated with respect to $\ln a_{MgCl_2}$ with the additional stipulation that m_{KCl} is held constant.

Equation (26) generally cannot be applied directly to experimental data, because of the problem of determining the activity of the added $MgCl_2$ in the presence of a monovalent salt. (Note that it is not feasible to carry out a titration with Mg^{2+} as the only cation, because buffer always contributes a monovalent cation, usually at comparable or larger concentrations than the added Mg^{2+} .) The Cl^- that is introduced with the Mg^{2+} affects the activities of both K^+ and Mg^{2+} ions. Although it is possible to estimate mean ion activities in solutions of mixed electrolytes (Harned and Robinson, 1968), it is easier in this case to design the experiment in a way that minimizes changes in the $MgCl_2$ activity coefficient during the course of a titration. The strategy is to insure that the solution always has a large excess of KCl over the added $MgCl_2$, such that the K^+ and Mg^{2+} ions see a relatively constant concentration of anions during the course of the titration. Because salt activity coefficients at low to moderate salt concentrations are primarily determined by cation - anion interactions, the activity coefficients change very little despite the increasing Mg^{2+} concentration.

The approximation that the $MgCl_2$ mean ionic activity coefficient remains constant is introduced into the linkage relation by first expanding Eq. (26) by the standard chain rule:

$$\left(\frac{\partial \ln K_{obs}}{\partial \ln a_{MgCl_2}}\right)_{m_{KCl}} = \left(\frac{\partial \ln K_{obs}}{\partial \ln m_{MgCl_2}}\right)_{m_{KCl}} \left(\frac{\partial \ln m_{MgCl_2}}{\partial \ln a_{MgCl_2}}\right)_{m_{KCl}} \quad (27)$$

We next examine the last term, the dependence of m_{MgCl_2} on a_{MgCl_2} . The activity of $MgCl_2$ in a mixed solution with KCl is defined as

$$a_{MgCl_2} = (a_{Mg^{2+}})(a_{Cl^-})^2 = \gamma_{MgCl_2} m_{MgCl_2} (2m_{MgCl_2} + m_{KCl})^2 \quad (28)$$

which, upon differentiation with respect to $\ln m_{MgCl_2}$ while holding m_{KCl} constant, yields

$$\left(\frac{\partial \ln a_{MgCl_2}}{\partial \ln m_{MgCl_2}}\right)_{m_{KCl}} = \left(\frac{\partial \ln \gamma_{MgCl_2}}{\partial \ln m_{MgCl_2}}\right)_{m_{KCl}} + 1 + \frac{4m_{MgCl_2}}{2m_{MgCl_2} + m_{KCl}} \quad (29)$$

The last term is simply computed from the concentrations of the two salts. The partial derivative of $\ln(\gamma_{MgCl_2})$ can be estimated from a theory of mixed electrolytes (Harned and Robinson, 1968), and is small and of opposite sign compared to the last term. We have previously estimated from Eq. (29) that $(\partial \ln m_{MgCl_2} / \partial \ln a_{MgCl_2}) \approx 1$ as long as the ratio $[KCl]/[MgCl_2]$ remains larger than ~ 30 (Grilley, *et al.*, 2006). In an experimental test, the titration of a fluorescent chelator dye with $MgCl_2$ gave an anomalous binding curve unless excess KCl was included in the titration buffer. A 30:1 $K^+ : Mg^{2+}$ ratio suppressed most of the anomaly (Leipply and Draper, 2009).

Upon substitution of Eq. (27) into the linkage Eq. (26), and including the above approximation that $(\partial \ln m_{MgCl_2} / \partial \ln a_{MgCl_2}) \approx 1$ as well as the additional approximation that molarity and molality units are interchangeable, the linkage relation for $MgCl_2$ becomes

$$\left(\frac{\partial \ln K_{obs}}{\partial \ln [Mg^{2+}]}\right)_{[MCl]} \approx \Delta \Gamma_{MgCl_2} \equiv \Delta \Gamma_{2+} \quad (30)$$

The last equivalence comes from the definitions of neutral salt and single ion interaction coefficients (Figure 3). Because ions must always be added to a solution as neutral salts, it is usually impossible to distinguish the effects of the cation from those of the anion. Equation (30) is therefore an unusual result, in that a term containing the single ion interaction coefficient for Mg^{2+} may be extracted from the titration of an RNA with $MgCl_2$. This outcome depends entirely on the fact that the effects of $MgCl_2$ on RNA folding can usually be observed in the presence of a large excess of monovalent ion, which allows the approximation that Eq. (29) takes on the value of one. It has not generally been appreciated that the way to isolate the effect of Mg^{2+} on an RNA from other factors is not to minimize the monovalent cations in the experiment, but to make sure that they are in large excess.

The first two of the following examples show how $\Delta \Gamma_{2+}$ may be determined by application of Eq. (30), using two different methods to find K_{obs} . In a third example, these $\Delta \Gamma_{2+}$ values are then compared with those obtained by a direct measurement which bypasses linkage relations and determination of K_{obs} .

Example 1: $\Delta\Gamma_{2+}$ from isothermal titration with MgCl_2 —In a titration of RNA with MgCl_2 , the extent of folding can be monitored in several ways, for instance by chemical probing experiments (Koculi, *et al.*, 2007, Ralston, *et al.*, 2000) or by following the change in a spectroscopic signal such as UV absorbance or ellipticity (CD) (Pan and Sosnick, 1997). UV absorption data for a titration of the A-riboswitch RNA with MgCl_2 are shown in Figure 4A. K_{obs} can be calculated from the signal and used in Eq. (30) to extract $\Delta\Gamma_{2+}$. With spectroscopic data, calculation of K_{obs} frequently requires correction for ‘baselines’ at high and low Mg^{2+} concentrations. The most common origins of baselines are

- i. Mg^{2+} - dependent extinction coefficients for either form of the RNA;
- ii. instrument drift during the time of the titration.

The two possibilities are easily distinguished by altering the concentration and timing of the titrant schedule, for instance by adding fewer aliquots of larger volume (or higher concentration) over a shorter time span. If instrument drift is a factor, an increase in the elapsed time of the titration should increase the slopes of the baselines. In Figure 4A, the slight positive slope of the 260 nm data set at high adenine concentrations is attributable to instrument drift, but the larger slope of the 295 nm data is mostly due to the effect of Mg^{2+} on the RNA extinction at this wavelength.

To correct for a time-dependent baseline, the data points are simply plotted as a function of the time of addition and a linear fit to the longest time points is subtracted from the data set. After normalization, the baseline-subtracted data set is then fit to Eq. (34), derived below. As a decision must be made as to which points to include in the linear fit, data must be collected over a long enough time span to identify unambiguously a linear portion of the curve. For many data sets, including the 260 nm data in Figure 4A, the correction for instrument drift is small enough that subtraction of either a time-dependent baseline (as just described) or a concentration-dependent baseline (described below, Eq. 31) give the same set of RNA folding parameters, within the reproducibility of the experiment.

When the effect of Mg^{2+} on the RNA extinction must be factored out of the data set, a single equation that incorporates both baseline(s) and the folding transition can be used. The approach is similar to the methods used to fit baselines and thermodynamic parameters to melting data (Albergo, *et al.*, 1981). The equation assumes a linear dependence of the folded and unfolded RNA extinction coefficients on Mg^{2+} concentration:

$$Abs = (a_U + m_U [\text{Mg}^{2+}]) (1 - \theta) + (a_F + m_F [\text{Mg}^{2+}]) (\theta) \quad (31)$$

where a_U , m_U , a_F , and m_F are the intercepts and slopes of the unfolded and folded RNA baselines, respectively, and θ is the fraction of the RNA in the folded form. To obtain an expression for the dependence of θ on the Mg^{2+} concentration, we start with the integrated form of Eq. (30),

$$\ln K_{\text{obs}} = \Delta\Gamma_{2+} \ln [\text{Mg}^{2+}] + C \quad (32)$$

where C is an integration constant. Note that the integration assumes $\Delta\Gamma_{2+}$ is a constant which may be factored out of the integral; whether this assumption is warranted will be discussed at the end of this section and in example 3. When $K_{\text{obs}} = 1$, *i.e.* at the midpoint of the folding transition, C takes on the value $(\Delta\Gamma_{2+}) \ln [\text{Mg}^{2+}]_0$. The desired expression for K_{obs} is then

$$\ln K_{obs} = \Delta\Gamma_{2+} \ln \left(\frac{[Mg^{2+}]}{[Mg^{2+}]_0} \right) \quad (33)$$

θ is related to K_{obs} as $K_{obs} = \theta/(1-\theta)$. Making this substitution and solving for θ gives

$$\theta = \frac{([Mg^{2+}]/[Mg^{2+}]_0)^{\Delta\Gamma_{2+}}}{1 + ([Mg^{2+}]/[Mg^{2+}]_0)^{\Delta\Gamma_{2+}}} \quad (34)$$

Both 295 and 260 nm data sets in Figure 4A have been fit to Eqs. (31) and (34). A problem with these data sets is that the folding transition takes place at low Mg^{2+} concentrations, leaving insufficient data to determine a lower baseline. Fitting six variables (four variables in Eq. 31 and two variables in Eq. 34) gave unrealistic values for m_U that were an order of magnitude larger than m_F . However, essentially identical values of $[Mg^{2+}]_0$ and $\Delta\Gamma_{2+}$ are obtained whether m_U is fixed as 0 or made to take on the same value as m_F .

Equation (34) can only be used to fit data for which K_{obs} is small, less than ≈ 0.02 , under the initial conditions of the experiment ($[Mg^{2+}] = 0$). We use a melting experiment under the buffer conditions of the isothermal titration to establish a value for K_{obs} at the titration temperature. If a significant fraction of the RNA is folded in the absence of Mg^{2+} , baseline(s) are subtracted by an appropriate method and the data are normalized to the appropriate range of θ , $\theta = K_{obs}/(1 + K_{obs})$ for the initial zero Mg^{2+} concentration point and 1.0 for the maximum value of θ . The following equation works well for fitting these curves:

$$\theta = \frac{([Mg^{2+}] + C_0)/K)^n}{1 + ([Mg^{2+}] + C_0)/K)^n} \quad (35)$$

where the exponent n , the offset C_0 , and K are empirical parameters without specific physical significance. When $K_{obs} > 1$, the midpoint of the titration curve ($\theta = 0.5$) is given by $[Mg^{2+}]_0 = K - C_0$. The value of $\Delta\Gamma_{2+}$ at a particular value of $[Mg^{2+}]$ is extracted from the fitted parameters by taking the derivative specified by Eq. (30):

$$K_{obs} = \frac{\theta}{1-\theta} = (1/K)^n ([Mg^{2+}] + C_0)^n$$

$$\Delta\Gamma_{2+} = -\frac{\partial \ln K_{obs}}{\partial \ln [Mg^{2+}]} = n \left(\frac{[Mg^{2+}]}{[Mg^{2+}] + C_0} \right) \quad (36)$$

In contrast to Eq. (34), which assumes that $\Delta\Gamma_{2+}$ is a constant over the entire range of fitted Mg^{2+} concentrations, Eq. 36 more realistically allows $\Delta\Gamma_{2+}$ to be a function of $[Mg^{2+}]$. The hyperbolic dependence of $\Delta\Gamma_{2+}$ on $[Mg^{2+}]$ that is assumed by the equation is a good approximation of the experimentally observed dependence, as measured in Example 3 below. The application of Eq. (36) to simulated data sets is shown in Figure 5 as part of a discussion of the errors associated with different methods for measuring $\Delta\Gamma_{2+}$.

By using different purine derivatives as ligands or different concentrations of ligand, the midpoint of the riboswitch folding transition, $[Mg^{2+}]_0$, was varied by more than an order of magnitude (Figure 4D) (Leipply and Draper, 2009). $\Delta\Gamma_{2+}$ is clearly dependent on $[Mg^{2+}]_0$, in contrast to the assumption made in the derivation of Eqs. (33) and (34) that $\Delta\Gamma_{2+}$ is

constant. More complete information about the variability of $\Delta\Gamma_{2+}$ is presented in Example 3 of this section, and the question of the errors incurred when $\Delta\Gamma_{2+}$ is obtained by application of Eq. (34) is taken up in the subsequent comparison of the different methods for finding $\Delta\Gamma_{2+}$.

Equation (34) has the same mathematical form as the Hill equation, which is commonly used to analyze RNA folding data (Fang, *et al.*, 1999, Latham and Cech, 1989, Schimmel and Redfield, 1980). It is frequently supposed that the Hill exponent n corresponds to a stoichiometric uptake of ions to defined sites on the RNA, as written in Eq. (1). But the derivation of Eq. (34) shows that such an interpretation of the Hill exponent is unwarranted. First, the Hill exponent becomes equivalent to $\Delta\Gamma_{2+}$ and takes on a molecular interpretation only when there is an excess of monovalent ion over Mg^{2+} (Eq. 29), and when $\Delta\Gamma_{2+}$ can be considered a constant (Eq. 33). Second, $\Delta\Gamma_{2+}$ represents a change in the excess number of Mg^{2+} ions associated with the RNA. When long-range electrostatic interactions are present, it is not possible to identify ions in any particular environment as the ‘excess’ ions; all the ions present in the solution interact with the RNA to some degree. Only at extremely high monovalent salt concentrations or at very low Mg^{2+} concentrations may long-range Mg^{2+} -RNA interactions be reduced to the degree that $\Delta\Gamma_{2+}$ approaches the number of site-bound ions (Bukhman and Draper, 1997, Das, *et al.*, 2005).

Example 2: $\Delta\Gamma_{2+}$ from melting data—Melting curves for the A-riboswitch RNA were obtained at Mg^{2+} concentrations up to 10 mM (Figure 4B), under the same buffer conditions as used for the isothermal titration in the previous example. The tertiary unfolding transition was well-resolved from the unfolding of secondary structure up to ~ 1 mM Mg^{2+} ; global fitting of transitions to melting curves observed at 280 and 295 nm allowed deconvolution of the unfolding transitions at the higher Mg^{2+} concentrations (Draper, *et al.*, 2000). K_{obs} was calculated from the T_{m} s of the melting transitions (Figure 4B) in the same way as done for the data in Figure 2A. According to Eq. (30), the slope of the curve in Figure 4B is $\Delta\Gamma_{2+}$. The middle part of the plot, between Mg^{2+} concentrations of ~ 50 – 500 μM , is approximately linear; the slope over this part of the graph gives $\Delta\Gamma_{2+} = 2.8 \pm 0.1$. However, the slope clearly approaches zero at both low and high Mg^{2+} concentrations. This behavior is expected: at extremely low Mg^{2+} neither the folded nor the unfolded form interacts significantly with Mg^{2+} , and at high enough concentrations Mg^{2+} almost entirely displaces K^+ as the excess cation with both forms of the RNA. The melting experiments are able to access a much larger range of K_{obs} values than the isothermal titration (Figure 4A), such that the dependence of $\Delta\Gamma_{2+}$ on Mg^{2+} concentration becomes evident. For this particular RNA, we are able to observe T_{m} over more than 40 degrees, corresponding to a 5 order of magnitude range in the calculated K_{obs} . Isothermal titration experiments cannot accurately calculate K_{obs} over more than a 100 fold range (θ varying between 0.1 and 0.9 in Eq. 34), which is usually not enough to detect curvature in the dependence of $\ln K_{\text{obs}}$ on $\ln[\text{Mg}^{2+}]$.

To work out the dependence of $\Delta\Gamma_{2+}$ on $[\text{Mg}^{2+}]$, we first fit the following curve to the Figure 4B data:

$$\ln(K_{\text{obs}}) = \ln(K_0) + \ln\left(\frac{K_{\text{max}}}{K_0}\right) \left[\frac{([\text{Mg}^{2+}]/[\text{Mg}^{2+}]_0)^n}{1 + ([\text{Mg}^{2+}]/[\text{Mg}^{2+}]_0)^n} \right] \quad (37)$$

This is simply an empirical equation for fitting a smooth curve between the data points, where K_0 and K_{max} are the values of K_{obs} in the absence of Mg^{2+} and the presence of saturating Mg^{2+} , respectively, $[\text{Mg}^{2+}]_0$ is the midpoint of the curve, and n controls the

steepness of the curve at the midpoint. Differentiation of Eq. (35) with respect to $\ln[Mg^{2+}]$ yields an expression for $\Delta\Gamma_{2+}$,

$$\Delta\Gamma_{2+} \approx \left(\frac{\partial \ln K_{obs}}{\partial \ln [Mg^{2+}]} \right)_{[MCl]} = n \ln \left(\frac{K_{max}}{K_0} \right) \left[\frac{([Mg^{2+}]/[Mg^{2+}]_0)^n}{(1 + ([Mg^{2+}]/[Mg^{2+}]_0)^n)^2} \right] \quad (38)$$

This function is plotted in Figure 4D, using parameters derived from the curve that was fit to the A-riboswitch data in Figure 4B. Comments on the approximations and potential errors of this approach are deferred until after the next example.

Example 3: direct measurement of Γ_{2+} —It is possible to measure the different single ion interaction coefficients directly by equilibrium dialysis (Bai, *et al.*, 2007, Strauss, *et al.*, 1967). An alternative method for measuring Γ_{2+} takes advantage of a fluorescent dye that chelates Mg^{2+} (Grilley, *et al.*, 2006). In essence, the method uses the dye to sense differences in the Mg^{2+} activity in the presence or absence of an RNA. Detailed theoretical justification, titration protocols, and data analysis for the method have been presented elsewhere (Grilley, *et al.*, 2009). For the A-riboswitch, titrations carried out in the presence or absence of ligand measure Γ_{2+} for the folded or unfolded state of the RNA, respectively (Figure 4C). As expected, Γ_{2+} is always larger for the folded RNA. The difference between these two curves is $\Delta\Gamma_{2+}$ (Figure 4D), the identical thermodynamic quantity as obtained in the previous two examples but measured without recourse to a linkage relation (Eq. 30).

Comparison of three different $\Delta\Gamma_{2+}$ measurement methods—Three methods for measuring $\Delta\Gamma_{2+}$ have been described in the preceding examples. Two of the methods depend on a linkage relation (Eq. 30) but find K_{obs} in different ways; the third method is based on the direct measurement of Γ_{2+} for the two RNA conformations under consideration. The error bars in the Figure 4D comparison of the sets of $\Delta\Gamma_{2+}$ values illustrate the difficulty in quantitating $\Delta\Gamma_{2+}$ very accurately by any method, but there are some systematic differences between the measurements (particularly at higher concentrations of Mg^{2+}) which most likely reflect the different assumptions made by each method. Here we first summarize the assumptions that go into the formulas and analyses, and then comment on the merits and drawbacks of each approach.

Assumptions underlying measurements of $\Delta\Gamma_{2+}$: Derivation of the linkage Eq. (30) and its integrated form Eq. (34) requires three assumptions:

- that the folding transition being observed is two-state (Eq. 19);
- that $\Delta\Gamma_{2+}$ is constant over the range of Mg^{2+} concentrations being analyzed;
- that sufficient excess monovalent salt is present to suppress changes in the Mg^{2+} ion activity coefficient as $MgCl_2$ is titrated (Eq. 29). This condition is an issue only at the highest concentration of $MgCl_2$ reached in Figure 4C.

The isothermal UV titrations depend further on the assumption

- that titration baselines have a linear dependence on $[Mg^{2+}]$.

The derivation of $\ln K_{obs}$ from UV melting profiles depends on the extrapolation of $\ln K_{obs}$ to a temperature different than the T_m using a value of ΔH°

- that is derived from the melting profile using the ‘two-state’ assumption (*i.e.*, only the folded (N) and unfolded (U) forms are ever present in significant concentrations);
- that is constant with temperature (*i.e.*, $\Delta C_p \approx 0$).

In addition, comparison of data obtained by isothermal titration and melting profile methods must assume

- that the structure of the unfolded state does not change with temperature.

Derivation of $\Delta\Gamma_{2+}$ from direct measurements of Γ_{2+} (Example 3) only requires the same assumption of excess monovalent salt as needed for the other two methods. The calculation does *not* invoke the two-state assumption, as measurements on folded and unfolded RNAs are made separately.

Advantages and disadvantages of the different measurement methods: In the application of linkage equations to folding equilibria, it can be difficult to know how much error is introduced by the two-state assumption. For the isothermal titrations, the difference in transition midpoints obtained when folding is monitored at 260 or 295 nm (Figure 3A) suggests that the A-riboswitch may deviate from a strict two-state model. The presence of a significant concentration of intermediate RNA form(s) during the titration will generally broaden the curve and thereby reduce the apparent value of $\Delta\Gamma_{2+}$. The tendency of the titration $\Delta\Gamma_{2+}$ values to land below those obtained by direct measurement (Figure 4D) is possibly another indication that folding intermediates are present in the isothermal titrations.

A second uncertainty associated with linkage equations is the degree to which the Mg^{2+} concentration dependence of $\Delta\Gamma_{2+}$ might affect the derived value of $\Delta\Gamma_{2+}$. To see how large these potential errors might be, we simulated Mg^{2+} titration curves (θ , the fraction of folded RNA, vs. $[Mg^{2+}]$) using an expression derived by integration of Eq. (30) with a polynomial function for $\Delta\Gamma_{2+}$ that approximated the $[Mg^{2+}]$ -dependence of $\Delta\Gamma_{2+}$ displayed in Figure 4D (solid gray data points). When the midpoint (where $K_{obs} = 1$) of a simulated titration was set to 30 or 100 μM Mg^{2+} , a fit of Eq. (34) to the data points gave values of $\Delta\Gamma_{2+}$ that were very close to the actual values reached at the titration midpoints (see legend to Figure 5). The Eq. (34) parameters are very sensitive to the data in the vicinity of $\theta = 0.5$ and tend to report the slope at this point correctly, even when the fitted curve shows systematic deviations from the data (see plots of residuals in the lower panels of Figure 5). This bias of the equation makes it surprisingly reliable for extracting $\Delta\Gamma_{2+}$ from titration data, though it should be emphasized that the fitted value of $\Delta\Gamma_{2+}$ applies only to the Mg^{2+} concentration at the midpoint of the titration curve.

There is a caveat in using Eq. (34), illustrated by the simulated titration curve at 10 μM Mg^{2+} (Figure 5). This titration curve shows a significant fraction of folded RNA in the absence of Mg^{2+} , where $K_{obs} \approx 0.15$. Spectroscopic titrations and most other methods used to assess K_{obs} assume a baseline value of $\theta = 0$ when $[Mg^{2+}] = 0$ and normalize the titration curve accordingly. Some of the calculated curves in Figure 5 have been fit to a modified Hill equation that incorporates this assumption. The apparent value of $\Delta\Gamma_{2+}$ at the midpoint of the curve (1.99) is larger than the value used to calculate the titration curve (1.45; see legend to Figure 5).

Because of the potential source of error just noted, it is advisable to have independent confirmation of K_{obs} in the absence of Mg^{2+} . For the A-riboswitch RNA, moderate concentrations of the most tightly-binding ligand (2,6-diaminopurine) do in fact stabilize the native structure to some degree at 20 °C; from the melting experiments used to generate Figure 4B, $K_{obs} = 1.3$ when Mg^{2+} was omitted from the buffer. Equation (35) allows $\theta > 0$

when $[Mg^{2+}] = 0$; when this equation was used to fit the simulated data in Figure 5, much better fits to the data were obtained than with Eq. (34) (see residuals plotted in the lower panels of Figure 5), and the $\Delta\Gamma_{2+}$ value calculated from the fitted parameters reproduced the values used to generate the curves.

Direct measurements of Γ_{2+} are, in principle, the most reliable and informative way to obtain $\Delta\Gamma_{2+}$; the two-state assumption is not required and the $[Mg^{2+}]$ -dependence of $\Delta\Gamma_{2+}$ is observed. However, a drawback of the direct measurements is that the errors tend to be large: because $\Delta\Gamma_{2+}$ is a small difference between two large numbers, errors in the measurement of the individual Γ_{2+} are amplified. A second limitation is obtaining the RNA in both conformations of interest. Some RNAs do not adopt the folded state in the absence of Mg^{2+} , which limits the concentration range over which $\Delta\Gamma_{2+}$ can be obtained (Grilley, *et al.*, 2006). The A-riboswitch is particularly convenient in that the unfolded state can be obtained simply by omitting the purine ligand. For other RNAs it is necessary to use a mutant that is incapable of folding as a model for the unfolded state (Grilley, *et al.*, 2007, Soto, *et al.*, 2007); the mutations may perturb the ensemble of unfolded states and bias the measurements.

Melting experiments have an advantage over isothermal titrations in that they are able to provide an absolute measurement of K_{obs} (there is no assumption that $\theta = 0$ when $[Mg^{2+}] = 0$) and extend the measurement of K_{obs} over several orders of magnitude, enough to detect the Mg^{2+} concentration dependence of $\Delta\Gamma_{2+}$. However, uncertainties may arise in calculating K_{obs} from T_m and ΔH° values. The partially unfolded state of the RNA is likely to be increasingly unstacked as the T_m of the RNA increases, and result in a temperature-dependent ΔH° . This phenomenon has been observed as an apparent heat capacity change in reactions involving single-stranded polynucleotides (Ferrari and Lohman, 1994), but has not been thoroughly explored for RNA tertiary folding reactions. In the case of the melting data presented in Figure 4B, there was no significant correlation of ΔH° with T_m .

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Figure 1.

Ion - RNA interaction coefficients defined with respect to equilibrium dialysis. **A**, Cartoon of a dialysis experiment. An RNA with 16 negative charges (red), together with 16 K⁺ ions, is added (gray box) to a solution of KCl previously in equilibrium across a dialysis membrane (blue, K⁺; yellow, Cl⁻; different shades of blue are used to distinguish K⁺ contributed as a counterion to RNA (dark blue) or KCl (light blue).) In the approach to equilibrium (arrow), two K⁺ and two Cl⁻ ions migrate from left to right across the dialysis membrane. (The right side dialysis chamber is presumed to be very large compared to the left side, such that the ion migration does not appreciably change the right side salt concentration.) Calculations of the single ion interaction coefficients (Γ_{+} , Γ_{-}) are shown as the differences between the left and right side ion concentrations (Eq. 3). **B**, Histogram illustrating charge balance in the equilibrium dialysis experiment cartooned in panel A. The vertical heights of the colored bars represent the total concentration of the various ions. Color coding of mobile ions and RNA phosphate is as in panel A. The left-most pair of bars (L_{init}) represents the addition of the potassium salt of an RNA with Z negative charges ($K_Z\text{-RNA}$) to the left dialysis chamber. The middle pair of bars (marked L_{eq}) represents the ion concentrations in the left chamber after dialysis equilibrium has been achieved with the concentrations of salts indicated in the right dialysis chamber (marked R_{eq}). The relations between the single ion coefficients (Γ_{+} , Γ_{-}) and histogram heights are marked, and the relationships between the interaction coefficients and the number of RNA charges (Z) are listed.

Figure 2.

Monovalent salt dependences for the formation of RNA tertiary structures. For both RNAs shown, $\log(K_{\text{obs}})$ was calculated at 20 °C from the melting temperature (T_m) and enthalpy (ΔH°) of the tertiary folding transition observed in melting curves by the formula $\ln(K_{\text{obs}}) = -(\Delta H^\circ/R)(1/T_m - 1/T_0)$, where R is the gas constant and T_0 is 293 K.

A, $\log(K_{\text{obs}})$ for folding of the A-riboswitch (buffer: 4 μM 2,6-diaminopurine, 20 mM MOPS adjusted to pH 6.8 with KOH, 0.1 mM EDTA, and various KCl concentrations). Data are taken from (Lambert, *et al.*, 2009). Each point is the average of three experiments; error bars are smaller than the data points. The salt molality and mean ionic activity are calculated from the total K^+ concentration contributed by both KCl and MOPS buffer.

B, $\log(K_{\text{obs}})$ for formation of the complex between tar and tar* hairpins (buffer: 5 mM cacodylic acid adjusted to pH 6.4 with KOH, 0.1 mM EDTA, and various KCl concentrations). Data are taken from (Lambert, *et al.*, 2009). Error bars are standard deviations from the average of three experiments.

Figure 3.

Definition of ion - RNA interaction coefficients in mixed monovalent-divalent salt solutions. K^+ and Cl^- ions are identified by the same colors as in Figure 1; green circles represent Mg^{2+} .

A, migration of ions taking place after addition of an RNA and its neutralizing K^+ ions (in gray rectangle) to a mixture of KCl and MgCl_2 previously in equilibrium across a dialysis membrane. As equilibrium is re-established (arrow), there is a net migration of K^+ into the right chamber and a net migration of Mg^{2+} into the left chamber. The movement of each cation type is accompanied by a neutralizing number of anions; in this example, there is a *net* flow of two Cl^- out of the left chamber. (As in Figure 1, the right side dialysis chamber is presumed to be very large compared to the left side, such that the ion migration does not appreciably change the right side salt concentration.) Calculations of all three single ion interaction coefficients (Γ_+ , Γ_- , Γ_{2+}) are shown as the differences between the left and right side ion concentrations (Eq. 3 and 24).

B, histogram illustrating charge balance in the equilibrium dialysis experiment cartooned in panel **A**. The vertical heights of the colored bars represent the total concentration of *charge* contributed by various ions, with the same color coding as in panel **A**. (The Mg^{2+} ion concentration is half the charge concentration.) The pair of bars labeled L_{init} represents the combination of salts added to the “left” chamber of a dialysis apparatus: the potassium salt of an RNA with Z negative charges ($K_Z\text{-RNA}$), KCl , and MgCl_2 . The next pair of bars (marked L_{eq}) represents the ion charge concentrations in the left chamber after dialysis equilibrium has been achieved with the concentrations of salts indicated in the right dialysis chamber (marked R_{eq}). The L_{eq} and R_{eq} concentrations of the three ions are individually

compared on the right side of the diagram, and relationships between the single ion coefficients, neutral salt coefficients, and the number of RNA charges are listed.

Figure 4.

Three different measurements of $\Delta\Gamma_{2+}$ for the A-riboswitch RNA, all reported under the same temperature and ionic conditions (50 mM KCl, 20 mM K-MOPS pH 6.8, 20 °C). **A**, isothermal titration of A-riboswitch RNA (3 μ M) with buffer containing MgCl_2 . 11 μ M adenine was also present. Absorbances at 260 and 295 nm were collected after each addition. After correction of the absorbance for dilution, the data were normalized to the initial reading before MgCl_2 addition. The curves are least squares fits of Eqs. (31) and (34), with the assumption that $m_U = m_F$.

B, $\ln(K_{\text{obs}})$ was calculated from the T_{ms} and ΔH° (average value, 56.3 ± 2.4 kcal/mol) obtained from melting experiments with A-riboswitch RNA in buffer containing 11 μ M 2,6-diaminopurine and various concentrations of MgCl_2 . The curve is a least squares fit of Eq. (37). Errors in $\ln(K_{\text{obs}})$ are principally the uncertainty in ΔH° .

C, direct measurement of Γ_{2+} by titration with MgCl_2 in the presence of a fluorescent dye that senses Mg^{2+} activity. The '+DAP' titration contained 250 μ M 2,6-diaminopurine. The points and error bars are the averages and standard deviations of five different titrations for each data set.

D, comparison of $\Delta\Gamma_{2+}$ obtained by three different methods. Data points with black or gray centers are from isothermal titrations observed at 260 and 295 nm, respectively, in which different ligands (purine, adenine, 2-aminopurine, or 2,6-diaminopurine) were used to vary the midpoint of the folding transition. The fitted midpoint of the folding transition, $[\text{Mg}^{2+}]_0$ in Eq. (34), is plotted on the x-axis. Errors on both axes are standard deviations from three independent experiments. The solid curve is the calculated slope of the fitted line in panel B; the single error bar is the uncertainty in the slope at the inflection point. Solid gray data points are the difference between the two curves in panel C, with the corresponding cumulative error. Data in panels A, C, and D were taken from (Leipply and Draper, 2009).

Figure 5.

Potential errors introduced by the assumption that $\Delta\Gamma_{2+}$ is constant in the derivation of the linkage Eq. (30). The dependence of $\Delta\Gamma_{2+}$ on $[Mg^{2+}]$ was modeled by a polynomial fit to the solid gray data points in Figure 4D. The polynomial was used in the integration of Eq. (30) to give expressions for $\ln K_{obs}$ and θ with $[Mg^{2+}]$ -dependent $\Delta\Gamma_{2+}$ (in contrast to Eqs. 33 and 34, which assume $\Delta\Gamma_{2+}$ is constant). θ is plotted for the calculated titration curve when the midpoint of the titration, $[Mg^{2+}]_0$, is 10 μ M (circles), 30 μ M (squares), or 100 μ M (diamonds). $\Delta\Gamma_{2+}$ (as used to calculate the displayed curves) at the titration midpoints ($\theta = 0.5$) is 1.45, 2.30, and 2.73, respectively. The simulated data points have been fit to either a modified version of Eq. (34) that assumes the y-intercept of the curve has the value $\theta = 0$,

$$\theta = \theta_0 + (1 - \theta_0) \left(\frac{[Mg^{2+}]}{[Mg^{2+}]_0} \right)^n / \left[1 + \left(\frac{[Mg^{2+}]}{[Mg^{2+}]_0} \right)^n \right],$$

, or to an equation that allows a non-zero y-intercept, Eq. (35). The residuals of the fits are shown in the lower three panels; closed symbols correspond to Eq. (34) and open symbols to Eq. (35). For the curve with a midpoint of $[Mg^{2+}]_0 = 100 \mu M$, Eq. (35) could not be fit to the data because the value of C_0 became vanishingly small. The values of $\Delta\Gamma_{2+}$ at the titration curve midpoints obtained from the modified Eq. (34) are 1.99, 2.39, and 2.73, in order of increasing $[Mg^{2+}]_0$. $\Delta\Gamma_{2+}$ obtained by fitting of Eq. (35) and application of Eq. (36) are 1.43 and 2.29 (10 and 30 μM transition midpoints, respectively).

Table I

Solution properties of some commonly used monovalent salts

salt	V_{MC} , cm ³ /mol ^a	dependence of $\ln(\gamma_{\pm})$ on $\ln(m_{MCI})^b$			
		a_1	a_2	a_3	a_4
LiCl	16.0	-0.2609	0.1098	8.2973e-2	1.3270e-2
		-0.2946	0.0283	0.02344	
NaCl	12.9	-0.4215	-3.3369e-2	3.6126e-2	8.0234e-3
		-0.4423	-8.3134e-2		
KCl	23.6	-0.5081	-0.1139	-2.8418e-3	
		-0.5052	-0.1059		
RbCl	28.76	-0.5429	-0.1306	-4.9786e-3	
		-0.5339	-0.1159		
CsCl	36.13	-0.6140	-0.1739	-1.2411e-2	
		-0.1739	-0.1369		
NH ₄ Cl	33.92	-0.5099	-0.1147	-2.8509e-3	
		-0.5048	-0.1064		

^a Partial molar volumes are taken from (Millero, 1971). See also (Marcus, 1994) for an extensive compilation and evaluation of V values for salts.

^b The listed coefficients allow calculation of $\ln(\gamma_{\pm})$ from a polynomial,

$$\ln(\gamma_{\pm}) = a_1 + a_2 [\ln(m_{MCI})] + a_3 [\ln(m_{MCI})]^2 + a_4 [\ln(m_{MCI})]^3$$

For each salt, the first line is appropriate for the range 0–0.8*m*, and the second line for the range 0–0.5 *m*. The coefficients were obtained by least squares fitting of polynomials to the activity coefficient data compiled in (Robinson and Stokes, 2002).