The ionic environment determines ribozyme cleavage rate by modulation of nucleobase pK_a

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ABSTRACT

Several small ribozymes employ general acid–base catalysis as a mechanism to enhance site-specific RNA cleavage, even though the functional groups on the ribonucleoside building blocks of RNA have pK_a values far removed from physiological pH. The rate of the cleavage reaction is strongly affected by the identity of the metal cation present in the reaction solution; however, the mechanism(s) by which different cations contribute to rate enhancement has not been determined. Using the *Neurospora* VS ribozyme, we provide evidence that different cations confer particular shifts in the apparent pK_a values of the catalytic nucleobases, which in turn determines the fraction of RNA in the protonation state competent for general acid–base catalysis at a given pH, which determines the observed rate of the cleavage reaction. Despite large differences in observed rates of cleavage in different cations, mathematical models of general acid–base catalysis indicate that k_1 , the intrinsic rate of the bond-breaking step, is essentially constant irrespective of the identity of the cation(s) in the reaction solution. Thus, in contrast to models that invoke unique roles for metal ions in ribozyme chemical mechanisms, we find that most, and possibly all, of the ion-specific rate enhancement in the VS ribozyme can be explained solely by the effect of the ions on nucleobase pK_a . The inference that k_1 is essentially constant suggests a resolution of the problem of kinetic ambiguity in favor of a model in which the lower pK_a is that of the general acid and the higher pK_a is that of the general base.

Keywords: ribozyme mechanism; metal ions; general acid–base catalysis; pK_a ; kinetic ambiguity

INTRODUCTION

The small nucleolytic ribozymes (hammerhead, hairpin, HDV, VS, and GlmS) all perform site-specific RNA self-cleavage, yielding products with 2',3' cyclic phosphate and 5' hydroxyl termini. These products imply a common reaction pathway in which the 2' hydroxyl attacks the adjacent phosphorous and the downstream 5' oxygen departs, thereby breaking the backbone; however, the mechanism(s) by which these ribozyme catalyze cleavage is still incompletely understood (Lilley and Eckstein 2008).

The observed rate of RNA cleavage by a given ribozyme can differ by several orders of magnitude depending on which cation is present in the reaction solution (Murray et al. 1998; Curtis and Bartel 2001; O'Rear et al. 2001; Perrotta and Been 2006; Poon et al. 2006; Roychowdhury-Saha and Burke 2006). Typically, the observed rate of a

cleavage reaction is much faster in divalent than in monovalent ions. Hypothetically, this might be due to a direct increase in the rate of the bond-breaking step if, for example, the divalent ion were specifically positioned to neutralize the developing negative charge on the phosphate during the transition state or interact with the 2' or 5' oxygens, as has been found for other ribozymes, such as Group I and II introns (Shan et al. 1999; Zhang and Doudna 2002; Stahley and Strobel 2005; Gordon et al. 2007). Alternatively, the divalent ion may play an indirect role, such as in positioning of the catalytic functional groups, in a variety of electrostatic effects in the transition state and/or ground state, or in modulating the pK_a of a functional group involved in general acid or general base catalysis (Fedor 2002; Emilsson et al. 2003; Kraut et al. 2003; Lonnberg and Lonnberg 2005; Hoogstraten and Sumita 2007; Scott 2007; Sigel and Pyle 2007). The mechanism(s) by which cations contribute to rate enhancement continues to be a matter of considerable discussion (Fedor 2002; Perrotta and Been 2006; Sigel and Pyle 2007).

In RNA cleavage via general acid–base catalysis, an unprotonated functional group, the general base, removes

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a proton from the 2'-OH and a protonated functional group, the general acid, donates a proton to the 5' oxygen leaving group. In protein ribonucleases, such as RNaseA, histidine residues function well in general acid–base catalysis because the imidazole side chain of histidine has a pK_a near neutral pH that can readily accept or donate a proton under physiological conditions (Raines 1998). In contrast, the ribonucleoside building blocks of RNA have pK_a values around pH 4 for adenosine and cytosine and above pH 9 for guanosine and uridine (Clauwaert and Stockx 1968), superficially making it unlikely that ribozymes could use general acid–base catalysis effectively in the biological environment. Nonetheless, experimental evidence supports a role for general acid–base catalysis in all of the small ribozymes (Nakano et al. 2000; Pinard et al. 2001; Shih and Been 2001; Das and Piccirilli 2005; Han and Burke 2005; McCarthy et al. 2005; Wilson et al. 2006, 2007; Cochrane et al. 2007; Klein et al. 2007; Smith and Collins 2007; Bevilacqua 2008). In some cases, the apparent pK_a values of unpaired or non-Watson–Crick paired bases are shifted substantially toward neutrality by the local environment, for example, by base stacking, hydrogen bonding, and proximity to the strong negative charge of backbone phosphates or the strong positive charge of bound divalent cations (Luptak et al. 2001; Fedor 2002; Sigel 2004; Gong et al. 2007).

In this paper, we present evidence that most, if not all, of the ion-dependent rate enhancement observed in cleavage by the VS ribozyme can be accounted for by the effect of the ions on nucleobase pK_a and not to direct participation of the metal ion in the chemical step of the reaction. The relationships among the ionic environment, catalytic pK_a values, and the observed reaction rate may also apply to other catalysts that employ similar mechanisms, including other nucleolytic ribozymes and possibly protein enzymes.

RESULTS AND DISCUSSION

In the simplest model of general acid–base catalysis, the experimentally observed rate of cleavage, k_{obs} , is related to the intrinsic rate of the bond-breaking step, k_1 , by

$$k_{\text{obs}} = F \cdot (k_1),$$

where F represents the fraction of RNA in the catalytically competent state. k_1 is a function of the Brønsted α and β values of the general acid and base, respectively, and is not affected by pH. F is the proportion of the RNA capable of participating in catalysis: in the simplest model, this is the proportion of RNA in which the general acid and general base are in their protonated and deprotonated states, f_{HA^+} , and f_{B^-} , respectively. f_{HA^+} and f_{B^-} are functions of pH and depend on the pK_a values of the general acid and

base and the pH at which the reaction is performed (Bevilacqua 2003; Jaikaran et al. 2008). We have recently incorporated an additional term into F that describes the cooperative loss of activity at very low pH; this term is a function of pH and the number of sites that contribute to the inactivation of the ribozyme when protonated (Bevilacqua 2003; Jaikaran et al. 2008; see Materials and Methods).

We have previously found that the fast *cis*-cleaving RG version of the VS ribozyme exhibits a bell-shaped k_{obs} versus pH curve with apparent pK_a values of 5.8 and 8.3 in saturating concentrations of Mg^{2+} (Fig. 1A; Table 1; Smith and Collins 2007). Similar observations have been made with a *trans*-cleaving version of the ribozyme (Wilson et al. 2007). A kinetic solvent isotope effect has been observed for the RG ribozyme, indicative of a rate-limiting proton transfer step in a general acid–base catalyzed reaction (Smith and Collins 2007).

In the VS ribozyme, nucleobases A756 and G638 have been implicated as the most likely candidates for the catalytic nucleobases (Andersen and Collins 2000; Hiley et al. 2002; Jones and Strobel 2003; Smith and Collins 2007; Wilson et al. 2007; Jaikaran et al. 2008). Determining which nucleobase functions as the general acid and which as the general base is challenging for any enzyme because a k_{obs} versus pH curve obtained under a single set of experimental conditions can be equally explained by either of two kinetic models (Fig. 1; for the principle of kinetic ambiguity, see Jencks 1969; see Materials and Methods). In Model 1, the lower pK_a represents the general base and the higher pK_a represents the general acid; in Model 2, these assignments are reversed. Importantly for the analysis below, the models also differ substantially in their estimate of k_1 (Fig. 1). Analyzing k_{obs} versus pH curves for the RG ribozyme in different ionic environments provided evidence that Model 2 provides the more parsimonious explanation of the overall data (see below), implying that the nucleobase with the lower pK_a , possibly A756, is the general acid and the nucleobase with the higher pK_a , possibly G638, is the general base.

The pH–rate profile for RG cleavage in saturating concentrations of Li^+ is bell-shaped between pH 4.5 and 9 (Fig. 1C). The plateau of the curve is flatter and broader in Li^+ than in Mg^{2+} , with apparent pK_a values of 4.9 and 8.8, and the maximum k_{obs} is about 50-fold slower than in Mg^{2+} (Table 1). Repeating the Li^+ pH–rate experiments in D_2O instead of H_2O showed a kinetic solvent isotope effect, indicating that the reaction in Li^+ is rate-limited by proton transfer, consistent with general acid–base catalysis, as in Mg^{2+} (Fig. 2A; Smith and Collins 2007).

It would be tempting to conclude that self-cleavage is faster in Mg^{2+} than in Li^+ because Mg^{2+} performs an additional role in catalysis that contributes a 50-fold enhancement to k_1 , the intrinsic rate of chemistry. For example, it is easy to imagine that the stronger electronegativity or the

FIGURE 1. Cleavage rate versus pH curves for the VS ribozyme in different ionic environments. The apparent first-order cleavage rate constant, k_{obs} , of the RG version of the VS ribozyme was determined in the presence of the indicated cations over the pH range indicated (open symbols): (A) 200 mM MgCl_2 ; (B) 2.5 M LiCl + 20 mM MgCl_2 ; (C) 2.5 M LiCl ; (D) 2.5 M NaCl . Control experiments showed that the concentrations of cations used were saturating across the pH range examined (see Materials and Methods). Data were fit to either of two kinetically ambiguous models of general acid–base catalysis that also included a term describing the cooperative loss of activity at low pH (Jaikaran et al. 2008) (solid lines; see Materials and Methods). In Model 1, the lower pK_a is assigned to the general base and the higher pK_a to the general acid; these assignments are reversed in Model 2. Dotted and dashed lines describe the titration of the general acid ($f_{\text{HA}^+} \cdot k_1$), and the general base plus the contribution from cooperative loss of activity below pH 4 ($f_{\text{B}^-} \cdot f_{\text{C}} \cdot k_1$), respectively, having the pK_a values inferred from the data. The intrinsic rate of the bond-breaking step, k_1 , is indicated by the arrows. Parameter values are presented in Table 1.

particular coordination geometry of Mg^{2+} might allow it to contribute more effectively to nucleobase positioning, or electrostatic catalysis than Li^+ , thereby making catalysis intrinsically faster in Mg^{2+} . Fitting the k_{obs} versus pH data to Model 1 could accommodate this interpretation because k_1 effectively becomes a variable that is free to acquire different values in different reaction conditions, as needed to fit the observed data (Table 1). However, when we fit the data using Model 2, we noticed that the differences in pK_a values in Mg^{2+} versus Li^+ produced estimates of k_1 that were within twofold of each other ($9 \times 10^4 \text{ min}^{-1}$ versus $5 \times 10^4 \text{ min}^{-1}$) despite the 50-fold difference in k_{obs} (Table 1). The slightly higher estimate of k_1 in Mg^{2+} may represent a small Mg^{2+} -specific rate enhancement, or it may be the result of cumulative errors of the parameters used to make the estimate. Putting this another way, if we assume that k_1 is truly constant for a given ribozyme, the differences in apparent pK_a values of the ribozyme in Mg^{2+} versus Li^+ are enough to explain almost all of the 50-fold difference between the observed cleavage rates.

To further test the correlation between k_{obs} and pK_a , we measured the cleavage rate versus pH for the same ribozyme in the presence of other cations. The observed rate in Na^+ is more than 3000-fold slower than in Mg^{2+} , and we find that the apparent pK_a values are even further apart (Fig. 1D, <4.5 and >9), in fact, sufficiently far from neutral pH that additional effects observed at extreme pH prevent their accurate measurement (Jaikaran et al. 2008; see Materials and Methods). Kinetic solvent isotope experiments confirmed that the reaction in Na^+ was limited by proton transfer, as in Mg^{2+} and Li^+ (Fig. 2B). If we assume that the lower apparent pK_a in Na^+ has a value typical of an adenosine in a single-stranded RNA ($\text{pK}_a \approx 3.7$) (Moody et al. 2005) and the upper pK_a has a value typical of guanosine ($\text{pK}_a \approx 9.3$) (Da Costa and Sigel 2003), then the estimate from Model 2 is $k_1 \approx 4 \times 10^4 \text{ min}^{-1}$, almost the same as in Li^+ and about twofold lower than in Mg^{2+} (Table 1). Considering the 3000-fold range in k_{obs} among reactions in Mg^{2+} , Li^+ , and Na^+ , the small differences in the estimates of k_1 among these ionic conditions are

TABLE 1. Estimation of cleavage rate constants and pK_a values in different ionic environments^a

Ion	k_{obs}^{max} (min^{-1})	pK_aA	pK_aB	ΔpK_a	Estimated k_1 (min^{-1})		pK_aC	n
					Model 1	Model 2		
Mg^{2+}	280	5.8	8.3	2.5	292	9×10^4	4.1	5
$Mg^{2+}+Li^+$	26	5.2	8.5	3.3	26	5×10^4	4.1	7
Li^+	6	4.9	8.8	3.9	6.4	5×10^4	4.1	7
Na^+	0.1	3.7	9.3	5.6	0.1	4×10^4	4.6	4
NH_4^+	0.1	3.7	—	—	—	—	4.5	6
Mn^{2+}	94	5.4	—	—	—	—	ND	ND

ND, not determined; dashes in cells indicate ionic conditions where pK_aB could not be estimated (see Materials and Methods) and, therefore, where other dependent parameters could not be calculated.

^aSee Materials and Methods for definitions of parameters and estimates of errors.

consistent with the idea that the value of k_1 is essentially the same in each ionic condition.

Cleavage in the presence of only K^+ is rate limited by a step that does not involve proton transfer and is thus not relevant to the current analysis (Fig. 2C). The concentration of Ca^{2+} required for saturation varied substantially with pH (see Materials and Methods), complicating analysis of pH–rate data. We were able to measure single pK_a values in NH_4^+ or Mn^{2+} (Fig. 3; Table 1); however, chemical properties of the individual cations prevented detection of putative pK_a values above pH 8 (see Materials and Methods). Therefore, to further test the correlation between ribozyme pK_a and k_{obs} , we measured pK_a values in a combination of cations that gave maximal k_{obs} values between those of the individual ions (Fig. 1B). The maximal k_{obs} in 2.5 M Li^+ plus 20 mM Mg^{2+} is approximately fivefold faster than in Li^+ alone, and the apparent pK_a values are shifted a combined ~ 0.5 pH unit closer together, leading to an estimate of $k_1 = 5 \times 10^4 min^{-1}$, similar to the estimates in all of the other ionic conditions examined (Table 1).

The theoretical relationship between k_{obs} of a ribozyme reaction employing general acid–base catalysis and the magnitude of the difference between the pK_a of the general acid and base ($\Delta pK_a = |pK_aA - pK_aB|$) has been described previously (Fig. 4A, solid line; Bevilacqua 2003): For every pH unit that these pK_a values move farther apart, k_{obs} decreases by a factor of 10, thus a plot of $\log_{10} k_{obs}$ versus ΔpK_a would have a slope of -1 if k_{obs} were completely determined by general acid–base catalysis. The observed data fit this relationship quite well (Fig. 4A, open symbols). Because k_1 is a true constant in Model 2 but a variable in Model 1, Model 2 provides a more parsimonious explanation of the relationship between k_{obs} and ΔpK_a . Alternative roles of individual types of ions are not consistent with this relationship. For example, if each type of ion made unique electrostatic or other contributions to the observed rate of cleavage without affecting nucleobase pK_a , the data points

in Figure 4A would fall on a vertical line with an x-axis value equal to the intrinsic ΔpK_a of the catalytic nucleobases; if each type of ion indeed altered the pK_a of the catalytic nucleobases, but such alterations did not cause the observed change in the rate of cleavage, the k_{obs} versus ΔpK_a data points would be scattered on this graph. The observed relationship suggests that the rate of cleavage of the RG ribozyme in a given ionic environment is largely determined by the difference between the apparent pK_a values of the general acid and general base.

Three situations could produce the results described in Figure 4A: the pK_a of the general base could be unchanged in the different ionic conditions, and the pK_a of the general acid could be altered, or vice versa, or the pK_a values of

FIGURE 2. Cleavage of RG in Li^+ or Na^+ but not K^+ exhibits a kinetic solvent isotope consistent with rate-limiting proton transfer in a general acid–base catalyzed reaction. Cleavage reactions were performed and analyzed as described in Materials and Methods using (A) 2.5 M $LiCl$, (B) 2.5 M $NaCl$, or (C) 2.5 M KCl . “pL” refers to pH (for reactions in H_2O ; closed symbols) or pD (for reactions in D_2O ; open symbols).

FIGURE 3. Rate versus pH curves for RG in NH_4^+ or Mn^{2+} . The apparent first-order cleavage rate constant, k_{obs} , of the RG version of the VS ribozyme was determined in the presence of (A) 3.75 M NH_4Cl and (B) 25 mM MnCl_2 . Data in NH_4Cl were fit to Equation 3 and data in MnCl_2 were fit to Equation 4 (solid lines), as described in Materials and Methods. Parameter values are presented in Table 1.

both could change. Plotting the maximal observed cleavage rate in each ionic condition as a function of the individual apparent pK_a values of the general acid or general base in each ionic condition produced striking log-linear relationships in both cases (Fig. 4B). Cleavage rates in Mn^{2+} or NH_4^+ , for which only the lower pK_a could be estimated (Fig. 3), also fell on the same line (Fig. 4B). Thus, the observed differences in ΔpK_a in different ionic environments are the result of both pK_a values being shifted toward neutrality by specific amounts characteristic of each type of cation.

There are a number of mechanisms by which metal ions could function as modulators of the pK_a of a nucleobase (for a recent review, see Sigel and Pyle 2007). The most direct would be that the metal ion binds to the nucleobase and stabilizes or destabilizes a particular protonation state. Indirect effects, in which the metal ion binds via a water molecule or even to another nearby nucleobase and alters the electrostatic environment of the catalytic nucleobase, could also account for our observations. The relationships

described in Figure 4 among cation identity, pK_a of the catalytic nucleobases, and observed catalytic rate might apply to other ribozymes and maybe even to proteins or other catalysts that utilize general acid–base catalysis.

FIGURE 4. Ionic environment determines the apparent pK_a of the general acid and general base and the observed rate of cleavage. (A) The logarithm of the maximal observed rate of cleavage in a given ionic condition, $\log_{10} k_{\text{obs}}^{\text{max}}$, is plotted as a function of the absolute value of the difference between pK_aA and pK_aB ($|\Delta\text{pK}_a|$). The solid line describes the theoretical relationship between ΔpK_a and $\log_{10} k_{\text{obs}}^{\text{max}}$ for a reaction catalyzed solely by general acid–base catalysis with $k_1 = 5.8 \times 10^4 \text{ min}^{-1}$ (the mean of the estimates of k_1 in the four ionic conditions examined in Fig. 1; see Table 1). Dotted gridlines are included to emphasize the linear relationship between the y - and x -axis variables over most of the pH range. (B) $\log_{10} k_{\text{obs}}^{\text{max}}$ is plotted as a function of the values of pK_aA (open symbols) or pK_aB (filled symbols) in Mg^{2+} ($\circ$); Mn^{2+} (∇); $\text{Mg}^{2+} + \text{Li}^+$ ($\triangle$); Li^+ ($\square$); NH_4^+ ($*$); and Na^+ ($\diamond$). Symbols for NH_4^+ and Na^+ lie on top of each other in B. Error bars indicate the upper and lower range of pK_a estimates including uncertainties from all sources (see Materials and Methods); error bars in A are the sum of the error bars in B. pK_aB could not be experimentally determined for some cations due to oxidation of the metal ion, RNA degradation, or buffering by the metal ion at high pH (see Materials and Methods).

Shifted pK_a values are observed in the active sites of several protein enzymes (for review, see Harris et al. 2002). Examples in which metal ion identity affects the apparent pK_a of protein-catalyzed reactions include the nucleotidyl transfer by a viral RNA polymerase and GTP hydrolysis by p21^{ras} (Schweins et al. 1997; Bowers et al. 2007; Castro et al. 2007). Further testing of these relationships should be possible using enzymes in which (1) the cation does not play an obligatory role in the catalytic mechanism; (2) general acid–base catalysis is the rate-limiting step across the pH range examined; and (3) both pK_a values can be experimentally measured.

In summary, we provide evidence that the intrinsic rate of the bond-breaking step, k_1 , is constant irrespective of the identity of the cation(s) in the reaction solution for a ribozyme that employs general acid–base catalysis. We propose that the differences in experimentally observed cleavage rates in different ions are the result of the ionic environment endowing the nucleobases that participate in general acid–base catalysis with particular pK_a values. This in turn determines the fraction of the RNA in the catalytically competent protonation state at a given pH, which determines the experimentally observed cleavage rate.

MATERIALS AND METHODS

RNA synthesis and cleavage reactions

RNAs were synthesized by in vitro transcription and purified as described (Hiley and Collins 2001). Cleavage reactions were initiated by mixing 1 volume of 1× reaction mix (50 mM buffer, 2 mM spermidine, 50 mM KCl) containing 2× RNA with 1 volume of 1× reaction mix containing 2× cation to obtain final concentrations of RNA=5–10 nM and cation as indicated in Figures 1–3 and 5. Reactions with rate constants $>6 \text{ min}^{-1}$ were performed using a Kintek RQF-3 instrument (Kintek Corp.). Buffers, pH measurements, and data quantification were done as previously described (Jaikaran et al. 2008). Estimates of k_{obs} in a given reaction condition typically varied by less than $\pm 20\%$ in replicate experiments.

As will be reported in detail elsewhere (M.D. Smith and R.A. Collins, unpubl.) we observed effects of high salt concentrations on the measured pH of the reaction solutions. Others have also

FIGURE 5. Determining the concentration of cation required to obtain maximal cleavage rate over the pH range examined. Cleavage rate constants were determined in 1× reaction solutions at the indicated pH containing the indicated concentrations of (A) MgCl_2 , (B) LiCl , (C) NaCl , (D) NH_4Cl , (E) CaCl_2 , and (F) MnCl_2 . Data are plotted as a fraction of maximal k_{obs} at the indicated pH. Data in B were fit to the Hill equation (solid lines); other lines simply connect the data points. The dashed line in B illustrates inhibition, by an unknown mechanism, at LiCl concentrations above 2.5 M at pH 9.1. High concentrations of Ca^{2+} at high pH also inhibit ribozyme cleavage by an unknown mechanism.

reported effects of high ionic strength on the real solution pH and/or the pH electrode used for measurement (Perrotta and Been 2006; Salis et al. 2006). Extensive control experiments provided evidence that the pH values that we report in Figures 1–3 and 5 are not confounded by errors due to ionic effects on pH measurement but are indeed the pH values (± 0.1 pH unit) of the 1× reaction solutions at 37°C. pH–rate profiles in Figures 1–3 were collected using a concentration of metal ion that was saturating for ribozyme activity across the entire pH range (as determined in Fig. 5). Because metal ions altered the pH of the 1× reaction solutions, the pH of the 1× reaction solutions containing different concentrations of cations were adjusted to be within <0.1 pH unit of the pH indicated in Figure 5. This ensured that changes in cleavage rate were due solely to differences in metal ion concentrations and not to an effect of metal ions on the pH of the

FIGURE 6. Simulations showing the effect of pH on the proportion of RNA in the state capable of participating in catalysis for Models 1 and 2 (see Materials and Methods). The figure shows individual plots of $\log_{10}$ of the fraction of RNA (F) in which the general acid (f_{HA^+} , short dashed line) or general base (f_{B^-} , long dashed line) are in the appropriate protonation state to participate in catalysis as a function of pH; in Model 1, the general base $\text{pK}_a\text{B} = 6$ and the general acid $\text{pK}_a\text{A} = 8$; in Model 2, the general base $\text{pK}_a\text{B} = 8$ and the general acid $\text{pK}_a\text{A} = 6$. A third plot (solid black line) describes the fraction of RNA that retains critical tertiary interactions (f_{C}) that are disrupted below an apparent $\text{pK}_a\text{C} = 4$ and $n = 5$ (see Materials and Methods). Multiplying the three proportions ($f_{\text{HA}^+} \cdot f_{\text{B}^-} \cdot f_{\text{C}}$) describes F , the overall fraction of RNA in the catalytically competent state at a given pH (O).

solution. Data were not collected above pH 7.5 in NH_4Cl because high concentrations of NH_4^+ strongly buffer above pH 7.5. Data in MnCl_2 could not be collected above the pH 8.0 due to oxidation of Mn^{2+} .

Data analysis

Rate constants and pK_a values were determined from the following relationships, as described elsewhere (Bevilacqua 2003; Jaikaran et al. 2008):

$$k_{\text{obs}} = F \cdot k_1, \quad (1)$$

where k_{obs} is the experimentally estimated apparent rate constant, F is the fraction of RNA in the catalytically competent state at a given pH, and k_1 is the intrinsic rate constant of the bond-breaking step, which is unaffected by pH.

F is the product of several proportions, including: (1) f_{HA^+} and f_{B^-} , which describe the proportion of the RNA in which the general acid and general base (having pK_aA and pK_aB) are in their protonated and deprotonated states, respectively; and (2) f_{C} , which represents the fraction of RNA retaining critical tertiary interactions, described by an additional pK_a (pK_aC) and a cooperativity coefficient (n). Thus, F can be described by

$$F = \left[\frac{1}{1 + 10^{(\text{pK}_a\text{B} - \text{pH})} + 10^{(\text{pK}_a\text{B} - \text{pK}_a\text{A})} + 10^{(\text{pH} - \text{pK}_a\text{A})}} \right] \times \left[\frac{1}{1 + 10^{(\text{pK}_a\text{C} - \text{pH})}} \right]^n. \quad (2)$$

Simulations showing the effect of pH on F (Equation 2) for Models 1 and 2 are presented in Figure 6. By substituting

Equation 2 into Equation 1, experimental k_{obs} versus pH data were fit using nonlinear least-squares minimization to allow simultaneous estimation of optimal values for k_1 , pK_aA , pK_aB , pK_aC , and n in each ionic environment examined (Jaikaran et al. 2008). Due to the inability to collect data at high pH, data collected in NH_4Cl or MnCl_2 were fit with Equation 3 or 4, respectively:

$$F = \left[\frac{1}{1 + 10^{(\text{pK}_a\text{B} - \text{pH})}} \right] \times \left[\frac{1}{1 + 10^{(\text{pK}_a\text{C} - \text{pH})}} \right]^n, \quad (3)$$

$$F = \left[\frac{1}{1 + 10^{(\text{pK}_a\text{B} - \text{pH})}} \right]. \quad (4)$$

Multiple cycles of enforcing different values on pK_a estimates and re-estimating the remaining parameters suggested that our estimates for pK_a values of bell-shaped curves are within ± 0.1 pH unit. As discussed by Jaikaran et al. (2008), pK_a values that are low enough to be in the region of the curve affected by the cooperative loss of activity at low pH (described by pK_aC and n) cannot be estimated accurately. This is the situation for reactions in NaCl or NH_4Cl . In these cases, we have estimated pK_aA as that of an unshifted adenosine in a single-stranded RNA (Moody et al. 2005). The error bars for these ions in Fig. 4B represent the range of values that pK_aA may have, which would otherwise be obscured by the cooperative loss of activity.

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