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The hammerhead cleavage reaction in monovalent cations

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ABSTRACT

Recently, Murray et al. (*Chem Biol*, 1998, *5*:587–595) found that the hammerhead ribozyme does not require divalent metal ions for activity if incubated in high (≥1 M) concentrations of monovalent ions. We further characterized the hammerhead cleavage reaction in the absence of divalent metal. The hammerhead is active in a wide range of monovalent ions, and the rate enhancement in 4 M Li^+ is only 20-fold less than that in 10 mM Mg^{2+}. Among the Group I monovalent metals, rate correlates in a log-linear manner with ionic radius. The pH dependence of the reaction is similar in 10 mM Mg^{2+}, 4 M Li^+, and 4 M Na^+. The exchange-inert metal complex $Co(NH_3)_6^{3+}$ also supports substantial hammerhead activity. These results suggest that a metal ion does not act as a base in the reaction, and that the effects of different metal ions on hammerhead cleavage rates primarily reflect structural contributions to catalysis.

Keywords: cobalt hexammine; divalent ions; hammerhead ribozyme; monovalent ions

INTRODUCTION

Originally identified in the genomes of certain plant viroids and virusoids, the hammerhead ribozyme is a small catalytic RNA that cleaves itself at a specific phosphodiester linkage to generate 5′ hydroxyl and 2′,3′-cyclic phosphate termini (Hutchins et al., 1986; Forster & Symons, 1987; Fig. 1). It has been extensively studied in an attempt to better understand RNA catalysis (McKay, 1996; Thomson et al., 1996; Stage-Zimmermann & Uhlenbeck, 1998), but despite its small size, the mechanism by which the hammerhead accelerates the cleavage of RNA has proven difficult to elucidate.

Until recently, it was thought that the hammerhead ribozyme required divalent cations for activity, and based on a correlation between hammerhead cleavage rate and metal pK_a, it was suggested that either a solvated metal hydroxide (Dahm et al., 1993) or metal ion directly coordinated to the 2′-OH at the site of cleavage (Sawata et al., 1995) acts as a base in the reaction. However, Murray et al. (1998) found that high (≥1 M) concentrations of monovalent ions can substitute for divalent ions in the hammerhead cleavage mechanism. Because monovalent ions have little effect on the acidity of water molecules to which they are bound, and because the hammerhead cleavage rate in 4 M Li^+ has been reported to be only 30-fold slower than that in 10 mM Mg^{2+} (Murray et al., 1998), this is not consistent with the hypothesis that a metal ion acts as a base in the reaction.

To better understand the reaction in the absence of divalent metal, we determined rate enhancements and pH dependence of the cleavage rate in the presence of various monovalent ions, and also examined hammerhead activity in the exchange-inert metal complex $Co(NH_3)_6^{3+}$. Our results do not support the idea that a metal ion acts as a base in the reaction, and suggest that the primary role of metal ions in the reaction may be structural rather than catalytic.

RESULTS AND DISCUSSION

The rate enhancement in Li^+ approaches that in Mg^{2+}

In comparing the catalytic proficiency of the hammerhead in different metal ions, it is informative to compare not only the ribozyme-catalyzed rates, but also the intrinsic abilities of different metals to cleave RNA. The stability of RNA depends on both the identity and concentration of metal ions present, and rates of background RNA cleavage can differ by more than 1000-fold in different ions (Kazakov, 1996; Li & Breaker, 1999). If the nonenzymatic reaction was much faster in 4 M

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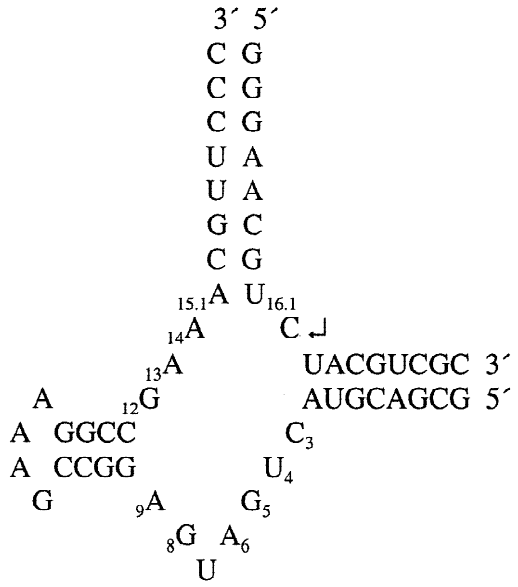

FIGURE 1. Secondary structure of hammerhead HH16.1. Conserved nucleotides are numbered, and the cleavage site is indicated by an arrow.

Li^+ than in 10 mM Mg^{2+}, then the idea that divalent metals are crucial for a significant proportion of hammerhead catalysis might be retained. On the other hand, an identical rate enhancement in 4 M Li^+ and in 10 mM Mg^{2+} would support the idea that Li^+ can fully replace Mg^{2+} in hammerhead catalysis and would not be consistent with metal acting as a base in the reaction. With these considerations in mind, we examined the ability of different ions to cleave RNA.

Background rates of RNA cleavage were determined in 4 M Li^+, Na^+, K^+, Rb^+, Cs^+, and NH_4^+, as well as in 10 mM Mg^{2+}. At the hammerhead cleavage site, nonenzymatic rates varied by as much as 20-fold in different monovalent ions (Table 1). In 4 M K^+, rates were comparable to those predicted by Li and Breaker (1999), and rates in 10 mM Mg^{2+} were similar to those observed previously for unconstrained RNA linkages (Soukup & Breaker, 1999). Rates in K^+, Rb^+, and Cs^+ approached the rate in buffer alone (Table 1). This result is consistent with metal hydroxide being one of the active species in the nonenzymatic cleavage reaction, since the pK_as of these ions (Burgess, 1988) appear to differ little from the pK_a of water (15.74; Jencks, 1969). The background rate in Li^+ is threefold faster than in Mg^{2+}, further supporting this idea. Although the pK_a of Mg^{2+} (11.4) is lower than that of Li^+ (13.8), the concentration of metal hydroxide in 4 M Li^+ is about twofold greater than in 10 mM Mg^{2+} (calculated using the Henderson–Hesselbach equation). This idea is also consistent with the observation that RNA is unstable in the presence of Pb^{2+} (pK_a = 7.2), Eu^{3+} (pK_a = 8.5), and Zn^{2+} (pK_a = 9.6), all of which have low pK_as (Ciesiolka et al., 1998). However, other factors must also be important, because the background rate of RNA cleavage is 40-fold faster in 4 M NH_4^+ than in buffer alone (Table 1). Furthermore, Mg^{2+} has a greater affinity for RNA than does Li^+ (Kazakov, 1996), which would be expected to increase the effective concentration of the Mg^{2+} metal hydroxide, yet the rates correlate with bulk metal hydroxide concentration.

The hammerhead was active in all monovalent ions tested, and rate enhancements ranged from 50,000-fold in Li^+ to 130-fold in Cs^+ (Table 1). The rate enhancement in 4 M Li^+ is only 20-fold less than that in 10 mM Mg^{2+} (Table 1). This comparable rate enhancement demonstrates that the hammerhead is catalytically proficient in monovalent ions alone. The reason for the 20-fold greater rate enhancement in Mg^{2+} is unclear. If the 20-fold difference is due to the pK_a difference between Mg^{2+} and Li^+, it suggests that the hammerhead is much less sensitive to metal ion pK_a than was previously thought. Hydration number could also be important, as Li^+ ions tend to coordinate fewer water molecules than Mg^{2+} ions do (Feig & Uhlenbeck, 1999). Another possibility is that the hammerhead structure is more efficiently stabilized by the higher charge density of Mg^{2+}.

TABLE 1. Cleavage rates in monovalent and divalent ions at pH 8.0.

	Background (min^{-1})	Catalyzed (min^{-1})	Rate enhancement
10 mM $MgCl_2$	1.9×10^{-6}	2.2	1.2×10^{6}
4 M LiCl	6×10^{-6}	2.9×10^{-1}	5×10^{4}
4 M NaCl	8×10^{-7}	7.5×10^{-3}	9×10^{3}
4 M KCl	2.7×10^{-7}	4.7×10^{-4}	1.7×10^{3}
4 M RbCl	7×10^{-7}	1.8×10^{-4}	2.6×10^{2}
4 M CsCl	2.8×10^{-7}	3.7×10^{-5}	1.3×10^{2}
4 M NH_4Cl	5×10^{-6}	1.4×10^{-2}	2.8×10^{3}
50 mM Tris	1.3×10^{-7}	not determined	not determined

Correlation between ionic radius and cleavage rate

Among the Group I metals, we noted a log-linear relationship between ionic radius and cleavage rate (Fig. 2A). We suggest that this relationship reflects the stabilizing effects of different ions on the catalytically active conformation of the hammerhead. The idea that cations could stabilize the catalytically active conformation of the hammerhead relative to that of the ground state is consistent with both crystallographic (Murray et al., 2000) and biochemical data (Peracchi et al., 1997; Wang et al., 1999), which suggest that the catalytically active and ground-state structures are significantly different. Smaller monovalent ions are more efficient than larger ones at stabilizing tRNA, RNA pseudoknots, and RNA dimers, and in some cases the relationship between ionic radius and thermal stability is linear (Ur-

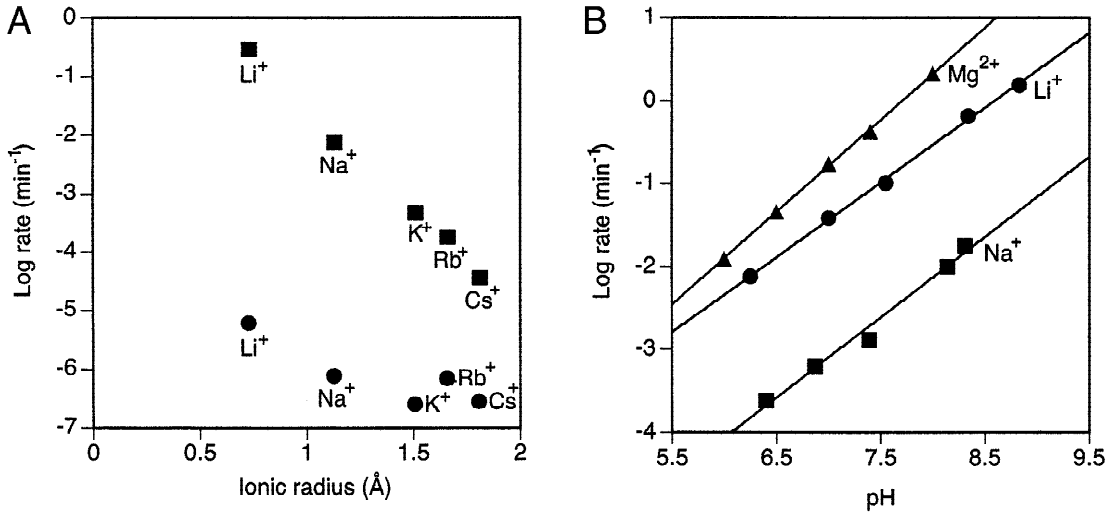

FIGURE 2. RNA cleavage as a function of metal ionic radius and pH. **A:** Relationship between the ionic radius of Group I monovalent metals and hammerhead-catalyzed (squares) and background (circles) RNA cleavage rates. Ionic radii are from Cotton et al. (1999). All rates were measured at pH 8.0. **B:** The pH dependence of the hammerhead cleavage rate in 10 mM Mg^{2+} (triangles), 4 M Li^+ (circles), and 4 M Na^+ (squares). Slopes of the lines are 1.1 in Mg^{2+}, 0.90 in Li^+, and 0.97 in Na^+.

banke et al., 1975; Labuda & Augustyniak, 1977; Heerschap et al., 1985; Torrent et al., 1994; Gluick et al., 1997). In the case of yeast tRNA-Phe, the first unfolding transition occurs 25 °C lower in Cs^+ than in Li^+ (Urbanke et al., 1975; Heerschap et al., 1985), indicating that the magnitude of this effect can be significant. Also, NH_4^+ stabilizes tRNA and RNA pseudoknots more efficiently than would be expected based on its size (Heerschap et al., 1985; Gluick et al., 1997), and we find that the hammerhead cleavage rate in NH_4^+ (ionic radius = 1.61 Å) is 80-fold faster than that in Rb^+ (ionic radius = 1.66 Å). Furthermore, differences in the stability of various RNA structures in different monovalent ions are typically much greater than differences in different divalent ions (Gluick et al., 1997). This could explain why hammerhead cleavage rates vary 8000-fold among the Group I monovalent metals (Table 1), but only 130-fold among the Group II divalent metals (Dahm & Uhlenbeck, 1991).

A possible clue to the mechanism of structural stabilization comes from analysis of a defined metal-binding site in the hammerhead. The affinity of metal ion binding to the G_5 site in the hammerhead correlates in a log-linear manner with the ionic potential [(charge)^2/ionic radius] of different monovalent and divalent ions (Feig et al., 1999). We observe a similar correlation between catalytic rate and ionic potential in monovalent ions, suggesting a link between a metal ion's affinity to RNA and its ability to support the transition-state conformation of the hammerhead. Such a correlation between affinity and activity has also been suggested for the reaction in divalent ions (Hunsicker & DeRose, 2000). For the G_5 site, the slope of the line relating affinity and ionic potential is 0.6 (Feig et al., 1999). For cleavage in Group I monovalent ions, the slope of an analogous graph (not shown) is seven times steeper, suggesting involvement of numerous additional metal–RNA interactions in the transition-state conformation compared to those of the ground state. The structural basis for these additional interactions could be a conformational change similar to that proposed by Peracchi et al. (1997).

Hammerhead activity in $Co(NH_3)_6^{3+}$

To further explore the role played by metal ions in the hammerhead cleavage reaction, we tested ribozyme activity in the presence of $Co(NH_3)_6^{3+}$. The amine ligands in this complex are exchange-inert over the time scale of the reaction, so that rates measured under these conditions reflect catalysis in the absence of inner-sphere coordination between metal ions and ribozyme (Hampel & Cowan, 1997; Nesbitt et al., 1997; Young et al., 1997; Suga et al., 1998). It was previously reported that in 500 μM Mn^{2+} the hammerhead is inhibited by $Co(NH_3)_6^{3+}$ (Horton & DeRose, 2000) and that it is inactive in $Co(NH_3)_6^{3+}$ alone (Nesbitt et al., 1997). We also found that the hammerhead is inactive in low concentrations of $Co(NH_3)_6^{3+}$ ($\leq$10 mM), but between 10 mM and 100 mM the cleavage rate increases dramatically. In 100 mM $Co(NH_3)_6^{3+}$, the hammerhead cleavage rate is 0.0071 min^{-1}, and the rate enhancement is 7000-fold. The hammerhead does not appear to be saturated under these conditions, but the solubility of $Co(NH_3)_6^{3+}$ prevented us from using higher concentrations. Rates did not change in the presence of

25 mM EDTA, indicating that activity is not due to divalent metal contamination. These results show that millimolar concentrations of cations can support significant levels of hammerhead activity in the absence of inner-sphere coordination.

Hammerhead activity in $Co(NH_3)_6^{3+}$ is intriguing, because two important metal-binding ligands believed to involve inner-sphere coordination have been identified by phosphorothioate experiments (Dahm & Uhlenbeck, 1991; Peracchi et al., 1997; Scott & Uhlenbeck, 1999; Wang et al., 1999; Derrick et al., 2000). One possible explanation for this activity is that divalent metal ions and $Co(NH_3)_6^{3+}$ make outer-sphere rather than inner-sphere contacts at these sites. Thio effects and apparent thiophilic metal rescue of these effects have been reported at sites of outer-sphere contacts (Basu & Strobel, 1999). Another possibility is that divalent metal ions bind at these sites by inner-sphere coordination, but $Co(NH_3)_6^{3+}$ makes outer-sphere contacts instead. For example, although Tb^{3+} appears to make three inner-sphere contacts at the G_5 binding site in the hammerhead, it can be displaced by $Co(NH_3)_6^{3+}$ (Feig et al., 1999). A third possibility, supported by recent phosphorothioate experiments (O'Rear et al., 2001), is that the 310-fold difference in rate and the 170-fold difference in rate enhancement between the reactions in Mg^{2+} and $Co(NH_3)_6^{3+}$ are due to the inability of $Co(NH_3)_6^{3+}$ to productively bind at these sites. If this is true, then there must also be a compensatory effect of high concentrations of $Co(NH_3)_6^{3+}$, because thio effects at several positions in the hammerhead are much greater than 310-fold. For example, the thio effect at the pro-R_p oxygen at the scissile phosphate is 2000- to 80,000-fold (Scott & Uhlenbeck, 1999; but see also Derrick et al., 2000).

The pH dependence of the cleavage rate in monovalent ions

The finding that the rate enhancement in Li^+ approaches that in Mg^{2+}, and that the hammerhead retains significant activity in $Co(NH_3)_6^{3+}$, weakens the case for a metal ion acting as a base in the reaction. Furthermore, the relationship between ionic radius and cleavage rate suggests that the differences among ions reflect their differential abilities in stabilizing the catalytically active conformation of the hammerhead. This contrasts to the picture emerging for the hepatitis delta virus (HDV) ribozyme, a different ribozyme that also carries out site-specific cleavage leaving 5′ hydroxyl and 2′,3′-cyclic phosphate termini (Been & Wickham, 1997). For the HDV ribozyme, a divalent metal ion is thought to act as a base in the cleavage reaction: removal of the divalent metal reduces the rate significantly (5,000-fold at pH 7), and exposes the underlying acid catalysis, as indicated by an inversion of the pH dependence (Nakano et al., 2000). With this in mind, we investigated the pH dependence of HH16.1 in monovalent and divalent metals.

In 10 mM Mg^{2+}, the hammerhead cleavage rate increases approximately 10-fold with each one unit increase in pH (Dahm et al., 1993; Fig. 2B). We observed a similar pH dependence in 4 M Li^+ and in 4 M Na^+ (Fig. 2B). For both monovalent and divalent cations, it appears there is a single deprotonation prior to the rate-limiting step, most likely deprotonation of the 2′-OH at the site of cleavage (Dahm et al., 1993; Kuimelis & McLaughlin, 1995; but also see Sawata et al., 1995). Throughout this pH range, deprotonation of the 2′-OH might be only about sevenfold greater in Mg^{2+} than in Li^+.

The pH-rate profiles can be added to the list of similarities between the reaction in monovalent ions compared with that in divalent ions, which includes: requirement for a 2′-OH at the site of cleavage (Murray et al., 1998), formation of a 2′,3′-cyclic phosphate product (Murray et al., 1998), requirement for G_5 in the conserved core of the ribozyme (Murray et al., 1998), requirement for numerous other residues and functional groups in the hammerhead core (O'Rear et al., 2001), activity in a wide range of ions (Table 1), and similar rate enhancements (Table 1). These similarities suggest that monovalent and divalent ions play essentially the same roles in hammerhead catalysis. Regarding the nature of the base in the hammerhead reaction, this suggests two possibilities: that both monovalent and divalent ions can act as bases in the reaction, or that neither can act as bases in the reaction. The latter possibility appears more likely for several reasons. First, the pK_a of Li^+ (13.8) is considerably higher than that of Mg^{2+} (11.4), and is also higher than the 2′-OH of ribose (13.1; Li & Breaker, 1999), yet the rates and rate enhancements are similar in Li^+ and Mg^{2+}. The hammerhead also retains significant activity in $Co(NH_3)_6^{3+}$, arguing against the role of a solvated metal hydroxide, or a metal directly coordinated to the 2′-OH at the site of cleavage, as a base in the reaction. Finally, the relationship between ionic radius and cleavage rate suggests that a primary role played by metal ions in the hammerhead cleavage reaction might be structural.

What is the base in the hammerhead cleavage reaction?

If a metal ion does not act as a base in the hammerhead cleavage reaction, what does? One possibility is that a nucleotide base in the ribozyme core functions in this manner. Work by Ferre-D'Amare et al. (1998), Perrotta et al. (1999), and Nakano et al. (2000) suggests that such a role is possible for bases in the HDV ribozymes. Furthermore, recent experiments indicate that a conserved adenosine in domain V of 23S rRNA might act as a general acid–base catalyst in the peptidyl transferase reaction of the ribosome (Ban et al., 2000; Muth

et al., 2000; Nissen et al., 2000). We tested the hypothesis that G_5 acts as a base in the hammerhead cleavage reaction but our results were inconclusive.[1] Another possibility is that the pK_a of the 2′-OH at the site of cleavage is perturbed by its environment, defined by the three-dimensional structure of the hammerhead. Several examples of perturbed pK_a values in RNA structures have been reported (Connell & Yarus, 1994; Legault & Pardi, 1994, 1997; Perrotta et al., 1999; Muth et al., 2000; but also see Narlikar & Herschlag, 1997). A third possibility is that the hammerhead does not accelerate deprotonation of the 2′-OH at all, and instead relies on solution levels of OH^-. But regardless, because the rate of a well-behaved hammerhead (10/min at pH 8.5) is at least 450-fold faster than the rate of uncatalyzed RNA cleavage when the attacking 2′-hydroxyl is fully deprotonated (0.022/min), the hammerhead must employ additional catalytic strategies (Li & Breaker, 1999).

In summary, our results suggest that monovalent and divalent ions play essentially the same roles in the hammerhead cleavage reaction. Inner-sphere coordination is not required for a substantial fraction of the hammerhead rate enhancement. Furthermore, a solvated metal hydroxide or a metal ion directly coordinated to the 2′-OH at the site of cleavage does not appear to enhance deprotonation at the site of hammerhead cleavage. This does not rule out a catalytic role for a metal ion in the reaction. For example, a fully hydrated metal ion could still provide electrostatic neutralization of developing negative charge in the transition state (Cowan, 1998). An interesting alternative to a metal ion acting as a base in the reaction is that a core nucleotide plays a direct role in the cleavage chemistry, but further experiments will be needed to explore this hypothesis.

MATERIALS AND METHODS

The initial report describing hammerhead activity in high monovalent salt used the hammerhead HH16.1 construct (Murray et al., 1998). Because we wished to build on these findings, HH16.1 (Fig. 1; Clouet-d'Orval & Uhlenbeck, 1997) was used for all experiments. RNA was transcribed from gel-purified DNA templates using T7 RNA polymerase. Following alkaline phosphatase treatment, substrate RNA was 5′-radiolabeled using T4 polynucleotide kinase and γ-^{32}P ATP. RNA was purified on denaturing polyacrylamide gels. RNA concentrations were determined by optical density (Dawson et al., 1986).

Because 4 M approaches the solubility limit of some of the salts used in this study, reactions were initiated using a modified protocol. Enzyme and substrate strand were combined in 4 μL, incubated at 95 °C for 2 min, and cooled to 25 °C over 5 min. Then, samples were dried using a SpeedVac Concentrator and reactions initiated with a solution containing 4 M monovalent salt, 50 mM buffer, and 25 mM EDTA. Rates in divalent metal were measured in 10 mM Mg^{2+} and 50 mM buffer. Final concentrations of enzyme and substrate strands were 0.65–7.3 μM and $<$0.088 μM, respectively. Rates did not change over the 10-fold range in ribozyme concentration used, confirming that ribozyme was saturating under these conditions. For each time point, a 1 μL aliquot was removed and quenched in 20 μL of a stop solution containing 8 M urea and 25 mM EDTA, and placed on dry ice. Product and substrate were separated on 20% denaturing polyacrylamide gels, and quantitated on a Fujix Phosphorimager using MacBAS and Image Reader software. To calculate rates, fraction reacted was plotted against time, and, depending on the extent of the reaction, was fitted to either a line or to equation (1),

$$\text{Fraction reacted} = F(1 - e^{-kt}), \quad (1)$$

where F is the maximum fraction reacted (typically 0.9), k is the observed rate constant, and t is time. All rates were measured at least twice, and independent determinations differed by less than 15% for catalyzed rates, and less than twofold for nonenzymatic cleavage rates.

To confirm that accurate rates could be determined using our modified protocol, some rates were also determined using a standard annealing protocol (Stage-Zimmermann & Uhlenbeck, 1998). The two protocols yielded indistinguishable values, and these values were similar to those previously reported (Clouet-d'Orval & Uhlenbeck, 1997; Murray et al., 1998). One difference between our modified protocol and the standard protocol is that, when using the modified protocol, a small amount of cleavage (about 5%) is typically observed during the evaporation. Such cleavage has been observed by others (Seyhan & Burke, 2000), and, as mentioned above, we confirmed that it had no effect on observed rates. We also note that reactions in 4 M salt were not effectively terminated when 10 vol of stop solution were used to quench the reaction. A primary role of the stop solution was to reduce the concentration of monovalent salt by dilution, because urea does not effectively denature the hammerhead in the presence of high concentrations of monovalent salt. For example, in 4 M Li^+ the reaction rate is reduced only about 40-fold in 8 M urea, whereas in 10 mM Mg^{2+} the reaction rate is reduced about 200-fold in 2 M urea, and about 60,000-fold in 8 M urea (Fig. 3). Consequently, we stopped reactions by diluting in 20 vol of stop solution and freezing in dry ice.

Background rates of RNA cleavage were measured in the same way as ribozyme-catalyzed rates, but in the absence of

[1]The hypothesis that the N^1 nitrogen of G_5 acts as a base in the reaction is consistent with a recent crystal structure, in which the keto oxygen of G_5 is positioned 3 Å away from the 2′-OH at the cleavage site, suggesting that it, or a nearby functional group, could abstract a proton from this -OH (Murray et al., 2000). Furthermore, replacement of G_5 with 1-methylguanosine reduces the hammerhead cleavage rate to background levels (Limauro et al., 1994), and binding of Tb^{3+} to the Watson–Crick face of G_5 similarly inhibits hammerhead activity (Feig et al., 1998). To test this idea, we examined the activity of a hammerhead in which the guanosine at G_5 (pK_a = 9.4) was replaced by 7-methylguanosine (pK_a = 6.7; Hendler et al., 1970). If the N^1 nitrogen of G_5 acts as a base in the reaction, its lowered pK_a with the 7-methyl substitution might lead to a faster rate. However, at pH 6.0, substitution at G_5 was inhibitory, although at G_8 this substitution increased the hammerhead cleavage rate fivefold (data not shown). Inhibition at G_5 is likely due to either the positive charge introduced at N^7 or to the methyl group at N^7, so this result is inconclusive. But because N^7 appears to be one of the few positions of G_5 that can be modified without loss of function (Fu et al., 1993; McKay 1996), perhaps other substitutions at this position could better address our hypothesis.

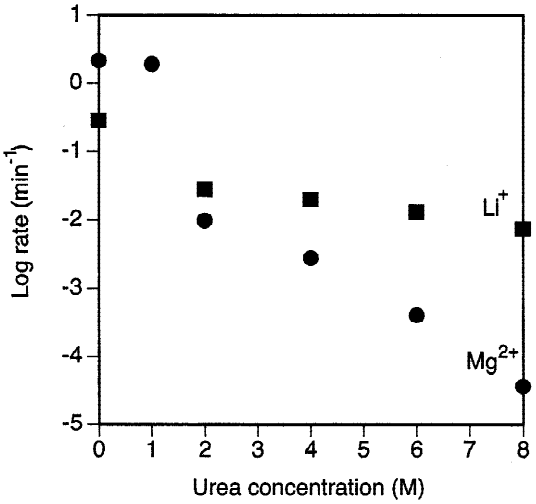

FIGURE 3. Effect of urea on the hammerhead cleavage rate in 10 mM Mg^{2+} (circles) and in 4 M Li^+ (squares). All rates were measured at pH 8.0.

enzyme-strand RNA. A ladder of cleavage products was observed when time points were run on denaturing polyacrylamide gels, and rate constants were calculated for the hammerhead cleavage site as well as eight neighboring phosphodiester linkages. For each metal, cleavage rates at different linkages systematically varied up to 12-fold, but usually no more than 5-fold. The background rates at the hammerhead cleavage site were representative of rates at neighboring linkages.

The buffers MES (pH 6–6.5), BES (pH 6.3–7.5), MOPS (pH 7), and Tris (pH 7.5–8.8) were used to determine rates at different pHs. Experiments using different buffers at the same pH indicated that changing buffers did not affect rate. Because high ionic strength can affect buffers, pHs were adjusted using HCl or the appropriate metal hydroxide after adding monovalent salt. Values were determined on a Beckman ϕ200 pH Meter with a Futura Refillable Micro Calomel Combination pH Electrode, and were consistent with those determined by indicator dyes on pH paper.

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