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Analysis on a Cooperative Pathway Involving Multiple Cations in Hammerhead Reactions

Yasuomi Takagi,[†,‡] Atsushi Inoue,[†,§] and Kazunari Taira*[,†,§]

Contribution from the Gene Function Research Center, National Institute of Advanced Industrial Science and Technology (AIST), Central 4, 1-1-1 Higashi, Tsukuba Science City, 305-8562, Japan, iGENE Therapeutics, Inc., c/o AIST, Central 4, 1-1-1 Higashi, Tsukuba Science City, 305-8562, Japan, and Department of Chemistry and Biotechnology, School of Engineering, The University of Tokyo, Hongo, Tokyo 113-8656, Japan

Received December 29, 2003; E-mail: taira@chembio.t.u-tokyo.ac.jp

Abstract: The hammerhead ribozyme reaction is more complex than might have been expected, perhaps because of the flexibility of RNA, which would have enhanced the potential of RNA during evolution of and in the RNA world. Divalent Mg^{2+} ions can increase the rate of the ribozyme-catalyzed reaction by approximately 10^9-fold as compared to the background rate under standard conditions. However, the role of Mg^{2+} ions is controversial since the reaction can proceed in the presence of high concentrations of monovalent ions, such as Li^+, Na^+, and NH_4^+ ions, in the absence of divalent ions. We thus carried out ribozyme reactions under various conditions, and we obtained parameters that explain the experimental data. On the basis of the analysis, we propose a new pathway in the hammerhead ribozyme reaction in which divalent metal ions and monovalent ions act cooperatively.

Introduction

Catalytic RNAs that are found in nature include hammerhead, hairpin, hepatitis delta virus (HDV), and Varkud satellite (VS) ribozymes; Group I and II introns; and the RNA subunit of RNase P.[1−13] Furthermore, structural and chemical analyses strongly suggest that ribosomal RNA is a ribozyme,[14−18] and the possibility exists that the RNA component of the spliceosome might also be a ribozyme.[19] Early research on ribozymes suggested that all ribozymes might be metalloenzymes that require divalent metal ions, in particular Mg^{2+} ions, for catalysis, and that all might operate by a basically similar mechanism. However, extensive subsequent studies revealed that the catalytic activity of hairpin ribozymes is independent of divalent metal ions.[20−26] Although Group I and II introns and the RNA subunit of RNase P apparently exploit several divalent metal ions as catalysts,[27−34] the HDV ribozyme uses a combination of a divalent metal ion and a nucleobase,[35] while hairpin ribozymes seem to use a nucleobase only.[26] It has been suggested that ribosomal RNA also makes use of a nucleobase in the peptidyl transferase reaction rather than a divalent metal ion.[15−17] Thus, the various types of ribozyme appear to exploit different cleavage mechanisms, which depend, in turn, upon the architecture of each individual ribozyme.[36] Even hammerhead

[†] Gene Function Research Center, AIST.
[‡] iGENE Therapeutics, Inc.
[§] The University of Tokyo.

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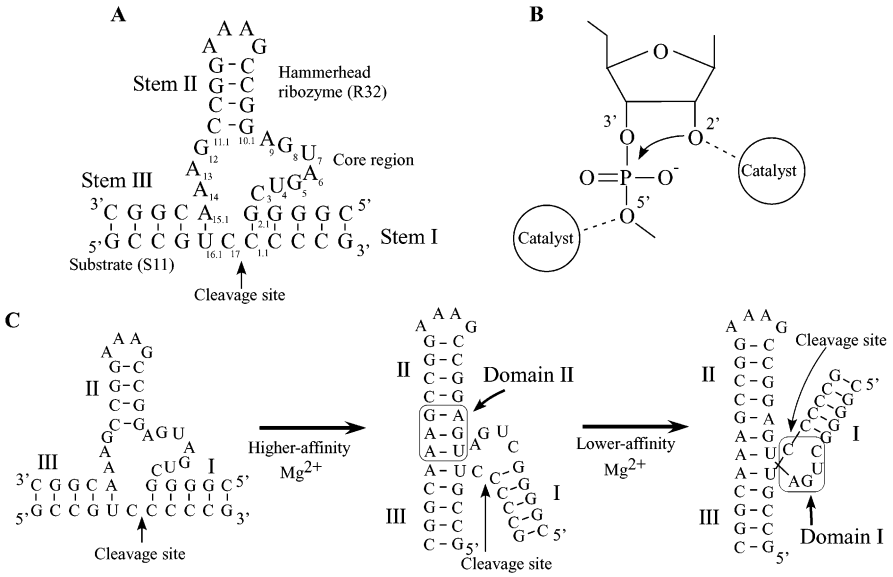

Figure 1. (A) Sequence and secondary structure of the hammerhead ribozyme (R32) and the substrate (S11) used in this study. (B) Schematic representation of the proposed mechanism of the hammerhead ribozyme reaction. The 2′-hydroxyl moiety is activated by the catalyst and then launches a nucleophilic attack on the adjacent phosphate, with subsequent cleavage of the bond at the 5′-oxygen. The developing negative charge on the leaving 5′-oxygen is stabilized by another catalyst. (C) Proposed two-stage scheme for folding of the ribozyme−substrate complex. The higher-affinity Mg^{2+} ion(s) drives the formation of domain II, which includes non-Watson−Crick base pairs, and the lower-affinity Mg^{2+} ion(s) rotates around helix I, forming the catalytic core.

ribozymes, which have generally been characterized as typical metalloenzymes, can no longer be categorized unambiguously.[26,36−38]

Hammerhead ribozymes, which act *in cis* during viral replication by the rolling circle mechanism, were identified originally in certain RNA viruses.[39] In the laboratory, ribozymes have been engineered such that they act on other RNA molecules *in trans* and catalyze the cleavage of phosphodiester bonds at specific sites to generate specific products, each of which has a 2′,3′-cyclic phosphate and a 5′-hydroxyl group (Figure 1A).[40−43] The transesterification reaction includes deprotonation of the 2′-hydroxyl moiety of a ribose group, nucleophilic attack of the 2′-oxygen on the adjacent phosphorus atom, and stabilization (neutralization) of the 5′-oxyanion leaving group (Figure 1B).[44] A large body of evidence also indicates that the $P9/G_{10.1}$ site binds a metal ion with high affinity, with other metal ion-binding sites being located around the G_5 nucleobase and the A_{13} phosphate near the site of cleavage.[45−48] Thus, the idea that ribozymes are metalloenzymes has become generally accepted. However, it was reported recently that ribozymes are active in the presence of very high concentrations of monovalent cations, such as Li^+ or NH_4^+ ions, or at moderate (below 100 mM) concentrations of the exchange-inert complex ion $Co(NH_3)_6^{3+}$, in the absence of divalent metal ions.[26,37] These findings raise the possibility that it might be inappropriate to classify hammerhead ribozymes as metalloenzymes. By contrast, we observed a difference in numbers of protons transferred in the transition state between ribozyme-catalyzed reactions that were allowed to proceed in the presence of Mg^{2+}, Li^+, and NH_4^+ ions, and we proposed that the catalyst in the reaction might depend on the conditions of the reaction.[36,49−51]

Our previous kinetic analysis supported the "two-phase folding model" that was originally proposed by Lilley and coworkers (Figure 1C).[50,52−56] In this study, we examined the validity of the two-phase folding model and the reaction pathway by performing stoichiometric analyses of our hammerhead ribozyme's activity as a function of the concentration of different monovalent ions in the presence and in the absence of Mg^{2+} ions. Our results strongly support the existence of the novel cooperative pathway in the hammerhead ribozyme reaction. We also found that our hammerhead ribozyme has a very unique characteristic with respect to the dependence of its reaction on Mg^{2+} ions, even in the presence of approximately 1 M Mg^{2+} ions. Such dependence suggests that the ribozyme's activity

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reaches more than 100 min^{-1} at pH 8 and 25 °C in the presence of more than 800 mM Mg^{2+} ions. Our ribozyme might, thus, be very suitable for studies of the mechanism of catalysis as is the very fast so-called "kissing ribozyme" reaction described by Khvorova et al.[57]

Experimental Section

Preparation of the Hammerhead Ribozyme and Substrate. The ribozyme (R32) and its substrate (S11) were synthesized chemically on a DNA/RNA synthesizer (model 394; PE Applied Biosystems, Foster City, CA) using phosphoramidic chemistry with 2′-*tert*-butyldimethylsilyl (TBDMS) protection, as described elsewhere.[47] The chemically synthesized oligonucleotides R32 and S11 were deprotected by incubation in a mixture of 28% ammonia and ethanol (3:1, v/v) at 55 °C for 8 h. Each mixture was evaporated to dryness, and the residue was allowed to dissolve in 1 mL of 1 M tetrabutylammonium fluoride (TBAF; Sigma-Aldrich Japan K. K., Tokyo, Japan) at room temperature for 12 h. After the addition of 1 mL of water, the mixture was desalted on a gel-filtration column (Bio-Gel P-4; Bio-Rad Laboratories, Hercules, CA). Fully deprotected oligonucleotides were purified by gel electrophoresis on a 20% polyacrylamide gel that contained 7 M urea, the respective bands were excised from the gel, and oligonucleotides were extracted in water. The oligonucleotides were recovered by ethanol precipitation, and then solutions were desalted on a gel-filtration column (TSK-GEL G3000PW; TOSOH, Tokyo, Japan) with ultrapure water. The RNA oligomers were quantitated in terms of absorbance at 260 nm.

We radiolabeled the substrate S11 using [γ-^{32}P]-ATP and T4 polynucleotide kinase (TaKaRa Bio Inc., Shiga, Japan) and purified the radiolabeled S11 on a 20% polyacrylamide gel that contained 7 M urea. After purification by the standard procedure as described above, the preparation of S11 was desalted on a gel-filtration column (NAP-10 column; Amersham Biosciences K. K., Tokyo, Japan).

Quantification of the Ribozyme Reaction. All ribozyme reactions were performed under ribozyme-saturated single-turnover conditions to ensure that conversion of the ribozyme−substrate complex to the ribozyme−product complex could be monitored kinetically without complications due to complex formation and slow release of product in particular at a high concentration of metal ions. The solution for the ribozyme reaction contained a trace amount of 5′-^{32}P-labeled S11 in 25 mM Bis-Tris buffer at pH 6.0 or pH 7.5 and 25 °C. The pH values of all 1.25× stock Bis-Tris buffers that contained appropriate metal ions (metal-ion buffers) were adjusted appropriately with HCl, and we confirmed that each buffer had the appropriate pH under the chosen reaction conditions. The error in the reading of pH meter under very high concentrations of metal ions is expected to be negligible.[58] Each reaction was initiated by addition of the substrate to a mixture of metal-ion buffer and ribozyme, and aliquots were removed from the reaction mixture at appropriate intervals. Each aliquot was mixed with more than three volumes of a stop solution that contained 100 mM MES (pH 6), 100 mM EDTA, 7 M urea, xylene cyanol (0.1%), and bromophenol blue (0.1%), and then it was stored at −80 °C prior to analysis. Since EDTA is known not to chelate Mg^{2+} ions efficiently at lower pH values and does not effectively chelate monovalent cations, we confirmed that reactions did not continue in the stop solution and that effective quenching was achieved as a result of the high concentration of urea in this solution. Uncleaved substrate and 5′-cleaved products were separated on a 20% polyacrylamide gel that contained 7 M urea. The extent of each cleavage reaction was quantitated with an image analyzer (Storm 830; Molecular Dynamics, Sunnyvale, CA). For each reaction, an observed rate constant was determined by nonlinear least-squares fitting of the time course of the reaction, using the following pseudo-first-order equation:

$$P_t = P_e - (P_e - P_0)\exp(-k_{obs}t)$$

where P_t is the amount of a product at reaction time t (min), P_e is the amount of a product at the endpoint, P_0 is the amount of a product at the start of the reaction, and k_{obs} is the observed rate constant (min^{-1}).

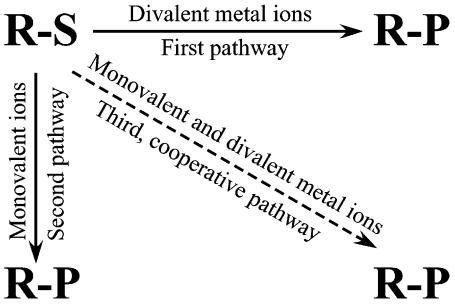

Figure 2. Possible pathways in the hammerhead ribozyme reaction. R−S and R−P stand for the ribozyme−substrate complex and the ribozyme−product complex, respectively. The first pathway requires only divalent metal ions, such as Mg^{2+} ions; the second pathway requires monovalent ions, such as Li^+ ions; and the third pathway involves both divalent and monovalent cations.

The Basic Strategy for the Establishment of the Pathways of Reactions Catalyzed by the Hammerhead Ribozyme. The basic strategy for establishment of the pathways of the reaction is shown in Figure 2. We suggested previously that a third pathway might be operative in the ribozyme reaction in addition to the two that have already been well characterized.[50] The first and second pathways involve only Mg^{2+} ions and only Li^+ ions, respectively. The third pathway involves the cooperative effects of two kinds of metal ion. We first investigated the dependence of the reaction on either Mg^{2+} or Li^+ ions independently and fitted the results to each scheme that has been postulated on the basis of our earlier data to choose values for basic parameters by nonlinear least-squares method. Finally, we established the third pathway on the basis of the reaction that occurred in the presence of the two metal ions, proceeding step by step and satisfying the parameters established in our analyses of the first and second pathways.

Results and Discussion

Li^+ Ions Have an Inhibitory Effect but Are Also a Better Accelerator Than Other Monovalent Ions of the Ribozyme Reaction in the Presence of Mg^{2+} Ions. We examined the activity of the hammerhead ribozyme as a function of the concentration of various monovalent metal ions, namely, Li^+, Na^+, K^+, Cs^+, and NH_4^+ in the presence of 10 mM Mg^{2+} ions. The reactions were performed at pH 6 and 25 °C, at concentrations on monovalent cations from 0 to 3 M. As shown in Figure 3, all monovalent ions, the Group I metal ions and NH_4^+ ions, had an inhibitory effect at a few hundred mM in the presence of 10 mM Mg^{2+} ions. This inhibitory effect can be explained by the simple hypothesis that monovalent ions prevent necessary binding of Mg^{2+} ions to the ribozyme−substrate complex, as validated by DeRose and colleagues by EPR.[53]

In the case of Li^+, Na^+, and NH_4^+ ions, the ribozyme activity was elevated at higher concentrations of each monovalent ion individually. This observation is consistent with our previous observations of reactions in the presence of either Mn^{2+} plus Na^+ ions or Mg^{2+} plus Na^+ ions.[50] The inhibitory effect of Na^+ ions has also been observed in kinetic studies by other

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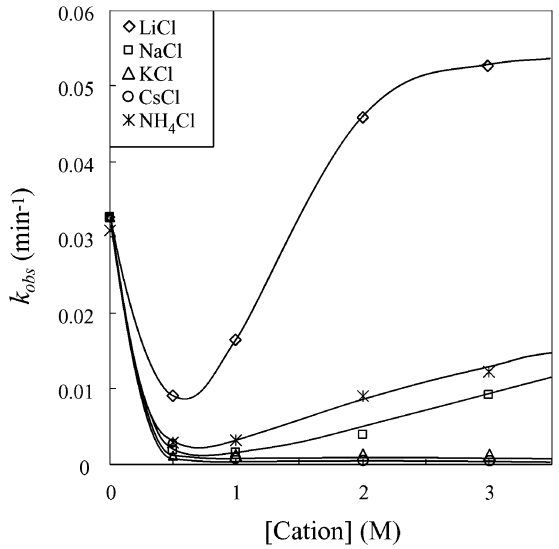

Figure 3. Dependence on various monovalent ions of the activity of the hammerhead ribozyme (R32) in the presence of Mg^{2+} ions. Values of k_{obs} are plotted as a function of concentration of Li^+, Na^+, K^+, Cs^+, and NH_4^+ ions, respectively. All reactions were performed in the presence of 10 mM Mg^{2+} ions under ribozyme-saturated single-turnover conditions at pH 6 and 25 °C. All monovalent ions had an inhibitory effect at 500 mM, and Li^+ ions accelerated the reaction at higher concentrations.

researchers at 100 mM Na^+ ions in the presence of Mg^{2+} ions.[59] In addition to their inhibitory effect, Li^+ ions at higher concentrations were the best accelerator of the ribozyme reaction in the presence of Mg^{2+} ions.

In the case of K^+ and Cs^+ ions, respectively, there was no acceleration of the reaction and both ions had the same effect (Figure 3). The rank order, in terms of the acceleration of ribozyme activity of the various monovalent ions in the presence of Mg^{2+} ions, was rather similar to that of these monovalent ions in the absence of divalent metal ions (data not shown). As noted by Curtis and Bartel,[37] such dependence is correlated with the radius of each anhydrous ion and appears to be manifested even on a background of Mg^{2+} ions (Figure 3).

It is particularly noteworthy that the observed accelerated activities were higher than the calculated sums of the activities in the presence of Li^+ ions and in the presence of Mg^{2+} ions, suggesting that the divalent and monovalent ions act cooperatively in the reaction catalyzed by the hammerhead ribozyme (Figure 3).[50] We chose the combination of Mg^{2+} and Li^+ ions for further stoichiometric analysis since it yielded the most pronounced profile in terms of inhibition and acceleration and the highest activities of all the combinations that we tested (Figure 3).

The Mg^{2+}-Mediated Ribozyme Reaction and Elucidation of the First Pathway. We examined the dependence on Mg^{2+} ions of the activity of the hammerhead ribozyme up to a concentration of Mg^{2+} ions close to 1 M at pH 6 and 25 °C under single-turnover conditions (ribozyme-saturating with respect to the substrate) to ensure that the kinetics of the cleavage reaction could be monitored without complications due to formation of the ribozyme−substrate complex and the slow release of products. As reported previously, the reaction in the presence of Mg^{2+} ions is accelerated by increases in pH with a slope of unity.[36,44,60−62] Thus, we adjusted the pH of the reactions in this study to 6.0 to slow the reaction. Under these conditions, we were able to measure the rate constant of the rapid reaction precisely.

As shown in Figure 4A, we found an approximately first-order dependence on the concentration of Mg^{2+} ions, and the rate constant did not reach a plateau value under our conditions, even at more than 800 mM Mg^{2+} ions. The continuous increase in the rate constant with the addition of more and more Mg^{2+} ions indicates the involvement of a Mg^{2+} ion that has very low affinity for the hammerhead ribozyme−substrate complex. At 800 mM Mg^{2+} ions, the rate constant approached 1 min^{-1} at pH 6 and 25 °C, which is the limit of detection of a rapid cleavage reaction under standard laboratory conditions. The dependence of the activities of the hammerhead ribozymes on Mg^{2+} ions has been studied by many researchers, generally of concentrations of Mg^{2+} ions below 200 mM.[59−61,63,64] In most cases, the rate constant reached or approached a plateau value.[59,60,63] In this respect, our ribozyme exhibits unusual dependence on Mg^{2+} ions.

Earlier analyses of the structure of hammerhead ribozymes and of the conformational changes caused by interactions with Mg^{2+} ions suggested that major conformational changes occur in two stages, with the formation of domain II and then domain I, as indicated in Figure 1C.[52,54−56] The formation of domain II occurs first and results in the coaxial stacking of helices II and III. This conformational change is induced by the binding of a higher-affinity Mg^{2+} ion(s) to the ribozyme−substrate complex. A divalent metal ion bound to the $A9/G_{10.1}$ site is a strong candidate for the ion that is involved in this first transition.[47d,65,66]

The second conformational change is the formation of the catalytic domain of the ribozyme, with movement of stem I toward stem II, and this change is induced by the binding of a lower-affinity Mg^{2+} ion(s). The K_d (equilibrium dissociation constant) of each transition has been investigated by various methods and appears to be several hundred micromolar and several millimolar for the first and the second transition, respectively.[52−56] Therefore, we chose 100 μM and 1 mM as K_{d1} and K_{d2}, the fixed dissociation constants of the first and the second Mg^{2+} ion(s), respectively, for the fitting in the first pathway (Figures 1C, 2, and 4B). The ribozyme reaction in the presence of high concentrations of Mg^{2+} ions did not reach saturation. Therefore, we included additional Mg^{2+} ions (indicated as x Mg^{2+}) in the pathway, as shown in Figure 4B, and we fitted the reaction profiles to the following equation:

$$k_{obs} = \frac{\dfrac{[Mg^{2+}]^{(2+x)}}{K_{d1}K_{d2}K_{d6}}}{1 + \dfrac{[Mg^{2+}]}{K_{d1}} + \dfrac{[Mg^{2+}]^2}{K_{d1}K_{d2}} + \dfrac{[Mg^{2+}]^{(2+x)}}{K_{d1}K_{d2}K_{d6}}} k_1$$

where K_{d6} is the dissociation constant of the additional Mg^{2+}

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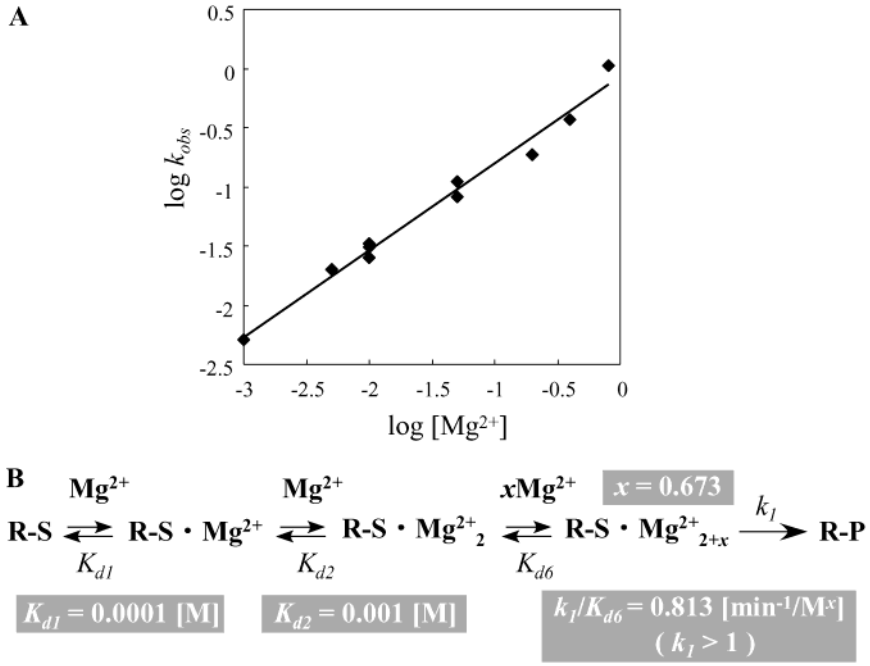

Figure 4. (A) Dependence on Mg^{2+} ions of the activity of the hammerhead ribozyme (R32). All reactions were performed at pH 6 and 25 °C under ribozyme-saturated single-turnover condition. The concentration of Mg^{2+} ions ranged from 1 mM to 800 mM. The theoretical curve (−−−) as drawn after parameters had been fitted to the equation given in the text. (B) The first pathway, involving Mg^{2+} ions, of the ribozyme reaction. R−S stands for the ribozyme−substrate complex. All parameters are explained in the text.

ion(s), k_1 is the rate constant for cleavage at a saturating concentration of Mg^{2+} ions, and x is the additional number of Mg^{2+} ions.

Since the reaction did not reach saturation even at 800 mM Mg^{2+} ions, we could not define k_1 and K_{d6}, but we were able to determine the values of k_1/K_{d6} and x after fitting the results to the equation. The calculated values of k_1/K_{d6} and x were 0.813 [min^{-1}/M^x] and 0.673, respectively. However, k_1 must be greater than 1 because k_{obs} is close to 1 min^{-1} at 800 mM Mg^{2+} (k_{obs} should be nearly equal to k_1 at saturating concentration of Mg^{2+} ions). The theoretical curve, obtained after putting these results in the equation, is shown in Figure 4A.

At several hundred millimolar Mg^{2+} ions, the formation of both domains II and I would be complete because of the low values of K_{d1} and K_{d2}. If we take the global changes induced by the two types of Mg^{2+} ion into consideration, it seems reasonable to conclude that the Mg^{2+} ion with very low affinity that we detected might be involved in some step other than the formation of domains II and I. The step might be a further conformational change or the binding of a catalytic species to the ribozyme−substrate complex. Rueda et al. reported that a third and previously undetected metal ion at a rather high concentration might play a role in the induction of a minor conformational adjustment that leads to formation of the active state, after the formation of domains II and I.[59]

Although we are unable to calculate a Hill coefficient for Mg^{2+} ions and cannot estimate the number of Mg^{2+}-binding sites from our current data, our results and those of others strongly support the possible existence of a very-low-affinity metal-binding site(s). Misra and Draper propose a model for the stabilization of RNA by Mg^{2+} ions that involves two distinct binding modes, "diffuse binding" and "site binding".[67] Diffusely bound Mg^{2+} ions are described as fully solvated Mg^{2+} ions that interact with RNA through long-range electrostatic interactions exclusively. Site-bound Mg^{2+} ions are described as partially desolvated ions that are attracted to electronegative pockets. In general, the affinity of diffusely bound Mg^{2+} ions seems to be lower than that of site-bound Mg^{2+} ions. Thus, it is possible that the very-low-affinity Mg^{2+} ions that we detected might be involved in diffuse binding. Diffuse binding of metal ions appears, sometimes, to play a dominant role in the stabilization of the tertiary structures of small RNAs,[67] but it might also participate in the ribozyme reaction at some specific site(s) in the ribozyme−substrate complex.

The activity of the hammerhead ribozyme in 800 mM Mg^{2+} ions is unusual because the observed rate constant is estimated to be about 100 min^{-1} at pH 8 and 25 °C from the dependence on pH, which has a slope of unity as noted above. The rate constant at concentrations of Mg^{2+} ions above 800 mM should be even higher because it is clear that 800 mM Mg^{2+} is not a saturating concentration for the cleavage reaction. The rate constant approaches the estimated observed rate constant for the so-called "kissing ribozyme" at optimum conditions with respect to a concentration of Mg^{2+} ions and pH.[57] The Mg^{2+} ion has very low affinity for the ribozyme−substrate complex, and the number of truly active ribozyme species, at concentrations of Mg^{2+} ions of several millimolar, is less than 1% of the number of ribozyme−substrate complexes (compare 1 min^{-1} in 10 mM $MgCl_2$ at pH 8 and 25 °C[68] with 100 min^{-1} in 800 mM $MgCl_2$ at the same pH and the same temperature).

The Li^+-Mediated Ribozyme Reaction and Elucidation of the Second Pathway. We examined the activity of the hammerhead ribozyme as a function of the concentration of Li^+

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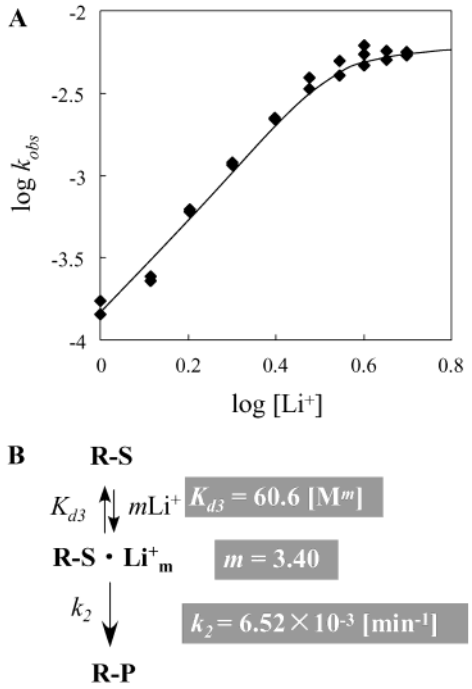

Figure 5. (A) Dependence on Li^+ ions of the activity of the hammerhead ribozyme (R32) at pH 6. All reactions were performed at pH 7.5 and 25 °C under the ribozyme-saturated single-turn over conditions and the concentration of Li^+ ions ranged from 1 to 5 M. Data at pH 6 were extrapolated from the experimental data obtained at pH 7.5 as described in the text. The theoretical curve (−−) was drawn after parameters had been fitted to the equation given in the text. (B) The second pathway, involving Li^+ ions, of the hammerhead ribozyme reaction. R−S stands for the ribozyme−substrate complex. All parameters are explained in the text.

ions, in the absence of divalent metal ions, under ribozyme-saturating single-turnover conditions at pH 7.5 and 25 °C. The experimental data obtained at pH 7.5 were extrapolated to give results at pH 6 on the basis of the fact that the dependence of activity on pH has a slope of unity.[37,50b] The results are shown by diamonds in Figure 5A.

It is clear that the slope of the linear part of the curve in Figure 5A is steeper than that in Figure 4A (in the presence of Mg^{2+} ions) and the activity reaches a plateau at high concentrations of Li^+ ions. Using the simple pathway shown in Figure 5B, we fitted the data to the following equation:

$$k_{obs} = \frac{\frac{[Li^+]^m}{K_{d3}}}{1 + \frac{[Li^+]^m}{K_{d3}}} k_2$$

where m is the number of Li^+ ions, K_{d3} is the dissociation constant of Li^+ ions, and k_2 is the rate constant for cleavage under Li^+-saturating conditions. The data fit well when m = 3.40, K_{d3} = 60.6 M^m, and $k_2 = 6.52 \times 10^{-3}$ min^{-1} at pH 6. The theoretical curve obtained after fitting these values is shown in Figure 5A.

O'Rear et al. reported that the hammerhead reaction has second-order dependence on Li^+ ions, without a plateau.[38] By contrast, our ribozyme exhibited more than third-order dependence, and a plateau was observed (Figure 5A). Although there are some differences between our results and those of O'Rear et al., in both cases the order of dependence on Li^+ is greater than that on Mg^{2+} ions, suggesting that Li^+ ions bind cooperatively to the ribozyme−substrate complex, while Mg^{2+} ions bind sequentially.

From the value of k_2 at pH 7.5 and 25 °C, we recalculated the value at pH 6 and 25 °C (again, the ribozyme reaction is dependent, with a slope of a unity, on pH at high concentrations of Li^+ ions).[50b] We estimated k_2 to be 6.52×10^{-3} min^{-1} at pH 6 and 25 °C and used this value for stoichiometric analysis of the third pathway (see below).

The Hammerhead Ribozyme Reaction in the Presence of Mg^{2+} and Li^+ Ions and Characterization of the Third, Cooperative Pathway. To characterize the third pathway, we examined the rate constant of the ribozyme reaction as a function of the concentration of Mg^{2+} ions on a background of 2 M Li^+ ions and as a function of the concentration of Li^+ ions on a background of 10 mM Mg^{2+} ions. All reactions were performed under ribozyme-saturating single-turnover conditions at pH 6 and 25 °C. The results are shown in Figure 6A,B. Parts A and B of the figure show apparent saturation at a high concentration of either Mg^{2+} or Li^+ ions. We tested many possible third pathways for a good fit to these data, using the parameters of the first and second pathways that we had already fixed. We were able to identify a third, cooperative pathway that satisfied all the experimental data. The entire scheme, including the first, second, and third pathways, is shown in Figure 6C, and the corresponding equation is as follows:

$$k_{obs} = f_1 k_1 + f_2 k_2 + f_3 k_3$$

where

$$f_1 = \frac{1}{g} \cdot \frac{[Mg^{2+}]^{(2+x)}}{K_{d1} K_{d2} K_{d6}}$$

$$f_2 = \frac{1}{g} \cdot \frac{[Li^+]^m}{K_{d3}}$$

$$f_3 = \frac{1}{g} \cdot \frac{[Mg^{2+}]^{(1+y)}[Li^+]^{(n+n')}}{K_{d1} K_{d4} K_{d5} K_{d7}}$$

$$g = 1 + \frac{[Mg^{2+}]}{K_{d1}} + \frac{[Mg^{2+}]^2}{K_{d1}K_{d2}} + \frac{[Li^+]^m}{K_{d3}} + \frac{[Mg^{2+}][Li^+]^n}{K_{d1}K_{d4}} + \frac{[Mg^{2+}][Li^+]^{(n+n')}}{K_{d1}K_{d4}K_{d5}} + \frac{[Mg^{2+}]^{(2+x)}}{K_{d1}K_{d2}K_{d6}} + \frac{[Mg^{2+}]^{(1+y)}[Li^+]^{(n+n')}}{K_{d1}K_{d4}K_{d5}K_{d7}}$$

and where n is the number of Li^+ ions as indicated in Figure 6C, K_{d4} is the dissociation constant of these n Li^+ ions, n' is the number of further additional Li^+ ions as indicated in Figure 6C, K_{d5} is the dissociation constant of these n' Li^+ ions, y is the number of Mg^{2+} ions indicated in Figure 6C, K_{d7} is the dissociation constant of these y Mg^{2+} ions, and k_3 is the rate constant for cleavage in the third pathway.

The third pathway involves interactions of Mg^{2+} ions and Li^+ ions with the ribozyme−substrate complex. In the third pathway, n Li^+ ions compete with a Mg^{2+} ion (in the first pathway) to form a R−S·Mg^{2+}·Li^+_n complex. This competition is the main cause of the observed inhibitory effect (Figure 3; see also below for details). Upon addition of more Li^+ ions, the relative activity of the first Mg^{2+}-only pathway becomes

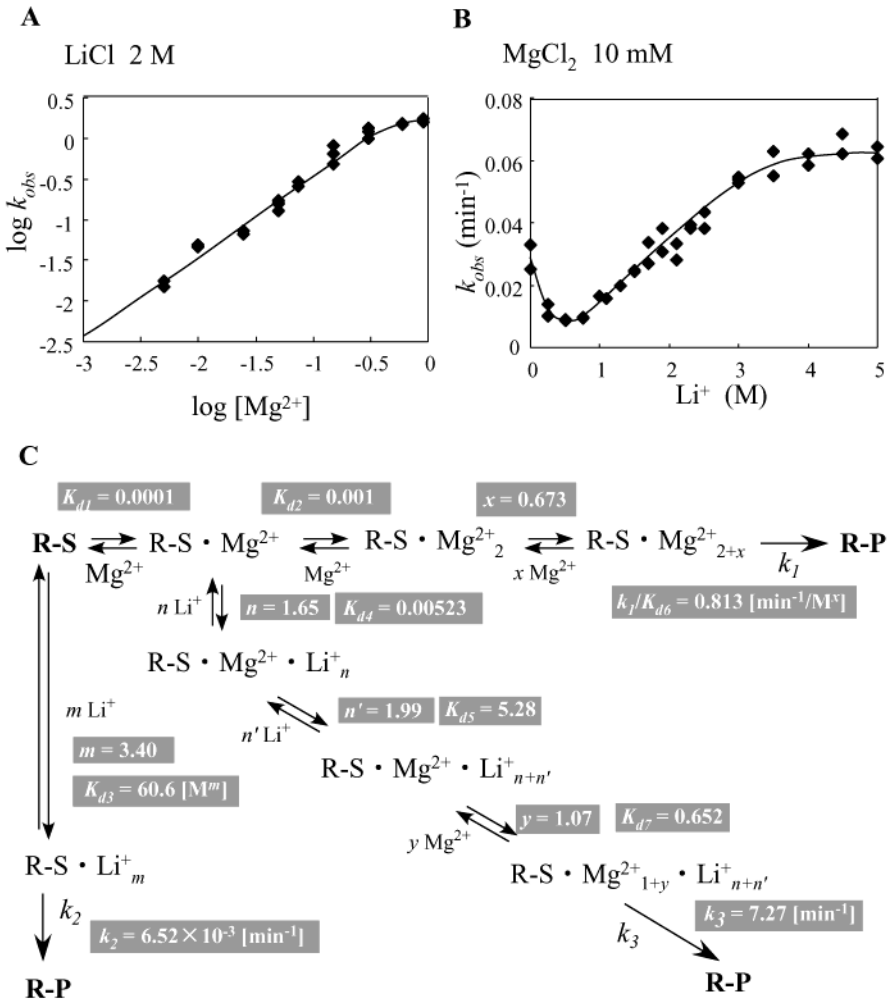

Figure 6. (A) Dependence on Mg^{2+} ions of the activity of the hammerhead ribozyme (R32) in the presence of 2 M Li^+ ions. All reactions were performed at pH 6 and 25 °C under the ribozyme-saturated single-turnover conditions and the concentration of Mg^{2+} ions ranged from 5 mM to 800 mM. The theoretical curve (−−−) was drawn after parameters had been fitted to the equation given in the text. (B) Dependence on Li^+ ions of the activity of the hammerhead ribozyme (R32) on a background of 10 mM Mg^{2+} ions. All reactions were performed at pH 6 and 25 °C under the ribozyme-saturated single-turnover conditions at concentration of Li^+ ions that ranged from 0 to 5 M. The theoretical curve (−−−) was drawn after parameters had been fitted to the equation given in the text. (C) The third pathway, involving both Li^+ and Mg^{2+} ions, for the ribozyme reaction at pH 6 and 25 °C. R−S and R−P stand for complexes of the ribozyme−substrate and the ribozyme−product, respectively. All parameters are explained in the text.

smaller. We might imagine that the second Li^+-only pathway would become the major pathway upon addition of more Li^+ ions. However, the activity, indicated by k_2, of the second pathway is much lower than the Mg^{2+}-based rate, and the affinity of Li^+ ions for the ribozyme−substrate complex in the second pathway is very low.

Thus, the third cooperative pathway, instead of the second pathway, becomes the major pathway upon addition of more Li^+ ions. The R−S·Mg^{2+}·Li^+_n complex is converted to a R−S·Mg^{2+}·$Li^+_{(n+n')}$ (Figure 6C) upon binding of n' Li^+ ions. We then added y Mg^{2+} ions to the R−S·Mg^{2+}·$Li^+_{(n+n')}$ complex because we failed to obtain a good fit without the addition of such Mg^{2+} ions. Thus, in the third pathway, two different metal ions, namely $(1+y)$ Mg^{2+} ions and $(n+n')$ Li^+ ions, are involved in formation of the final complex. Using this model, we fit all the data to the equation and obtained by nonlinear least-squares method the following values: $n = 1.65$, $K_{d4} = 0.00523$, $n' = 1.99$, $K_{d5} = 5.28$, $y = 1.07$, $K_{d7} = 0.652$, and $k_3 = 7.27$ at pH 6 and 25 °C (Figure 6C).

The theoretical curves, obtained after inserting these parameters in the equation, are shown in Figure 6A,B. If we assume that k_1 is 1 min^{-1} (this value is an underestimate because the reaction did not reach saturation even at 800 mM Mg^{2+} ions; see above), the relative extent of each pathway on a background of Mg^{2+} ions is as follows: 100% via the first pathway at 0 M Li^+ ions; 92% via the first pathway and 8% via the third pathway at 500 mM Li^+ ions; 48% via the first pathway and 52% via the third pathway at 1 M Li^+ ions; and 100% via the third pathway at 5 M Li^+ ions (Figure 7).

These parameters imply that (i) the pathway involving Li^+ ions alone (the second pathway) is negligible when both Li^+ and Mg^{2+} ions are available (Figure 7A,B), (ii) an increase in the concentration of Li^+ ions on a background of Mg^{2+} ions induces cooperation between Li^+ and Mg^{2+} ions (the third pathway; Figure 7A), and (iii) an increase in the concentration of Mg^{2+} ions on a background of Li^+ ions induces cooperation between Li^+ and Mg^{2+} ions (the third pathway), although higher concentrations of Mg^{2+} ions induce the first pathway and dramatically reduce the activity of the third pathway (Figure 7B). Simulations made on the assumption that k_1 is 5 or 10 min^{-1} (rather than 1 min^{-1}) did not affect these conclusions. It should also be emphasized that, in the measurements of reaction

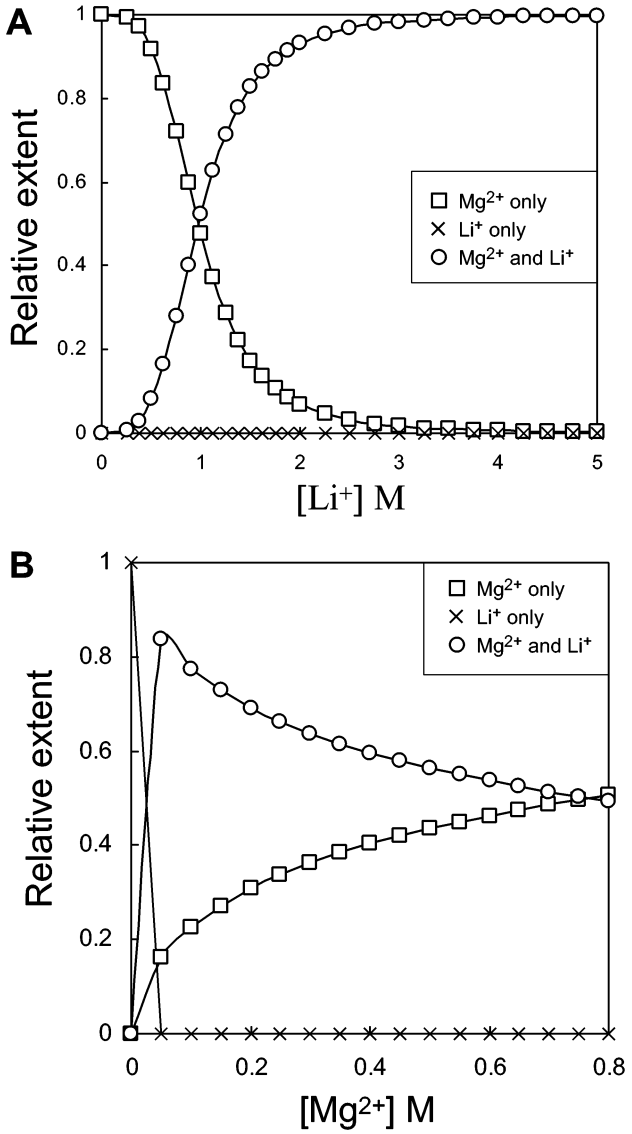

Figure 7. Simulations showing the relative extent of involvement of each of the three pathways in the proposed scheme for hammerhead ribozyme reactions. The first pathway involved only Mg^{2+} ions (□), the second pathway involved only Li^+ ions (×), and the third pathway involved both Li^+ and Mg^{2+} ions (○). In this scheme, k_1 was taken as 1 min^{-1}. The relative values were obtained from calculations of f_1 (Mg^{2+} alone), f_2 (Li^+ alone), and f_3 (Mg^{2+} and Li^+; see the equations in the text) after fitting of the various parameters. (A) Simulation, in the presence of 10 mM Mg^{2+} ions, of the relative extent of involvement of each pathway as a function of the concentration of Li^+ ions. (B) Similar simulations performed as a function of the concentration of Mg^{2+} ions in the presence of 2 M Li^+ ions.

rates summarized in Figure 6C, we were unable to adjust the ionic strength at each concentration of metal ions because different cations we tested had differently affected the ribozyme reaction, as shown in Figure 3. Although we cannot exclude the possibility that the obtained parameters might have been influenced by the ionic strength, the major finding that the ribozyme reaction can proceed by a new cooperative pathway involving both monovalent and divalent metal ions remains valid.

Comparison of Our Data with Previous Data. In a previous report, we suggested the possible existence of various catalytic channels for the hammerhead ribozyme, depending on the reaction conditions. For example, a Mg^{2+} ion functions as a catalyst in the transition state in Mg^{2+}-containing solutions while an NH_4^+ ion functions as a catalyst in NH_4^+-containing solutions.[51] Our data support an anhydrate direct interaction between these ions and the 5′-leaving oxygen atom at the cleavage site.[51]

However, since $Co(NH_3)_6^{3+}$ ions enhance the ribozyme's activity, we cannot exclude the possibility that, for example, a hydrated cation also functions as a catalyst in the transition state.[66] A multiple-channel model has also been proposed for reactions catalyzed by the HDV genomic ribozyme in which a base catalyst changes according to the environment in which the HDV ribozyme reaction occurs.[35] We propose similarly that several pathways are possible in the reaction catalyzed by hammerhead ribozymes. One of the pathways, the cooperative pathway, involves several different cations simultaneously. In the case of the reaction catalyzed by the RNA subunit of RNase P, similar cooperativity by Mg^{2+} and Ca^{2+} ions has been reported.[69]

Our data also suggest the existence of a very-low-affinity Mg^{2+} ion (corresponding to K_{d6} in Figures 4B and 6C), which was not included in the "two-phase folding model" proposed by Lilley and co-workers.[52,54−56] In a previous report, we postulated that this very-low-affinity Mg^{2+} ion might play a catalytic or a structural role.[50] Rueda et al. reported recently that a third Mg^{2+} ion might play a role, at a rather high concentration, in a minor conformational adjustment that results in formation of the active state after the formation of domains II and I (Figure 1C).[59] The Mg^{2+} ion that we detected in the present study might be the same as the Mg^{2+} ion that they discussed.

Our ribozyme did not yield a plateau even at 800 mM Mg^{2+} ions (Figure 4A), while HHα (another hammerhead ribozyme that has been used in studies of reaction mechanisms by other researchers) yields a plateau at 100 mM Mg^{2+} ions.[59] Breaker and co-workers proposed recently that a speed limit might exist for RNA-cleaving ribozymes that adopt $\alpha\gamma$ catalytic strategies, when the α, β, γ, and δ steps in catalysis are equivalent, respectively, to in-line nucleophilic attack, neutralization of negative charge on a nonbridging oxygen atom, deprotonation of the 2′-hydroxyl group, and neutralization of negative charge on the 5′-oxygen atom.[70,71] The reaction catalyzed by our hammerhead ribozyme cannot be categorized in terms of these steps because the k_{max} of the reaction under optimal conditions can be extrapolated to exceed the speed limit of $\alpha\gamma$ catalysis at neutral pH and 25 °C (see above). This observation suggests the existence of some other catalytic strategy, for example, a δ-metalation strategy or a β-metalation strategy. We noted earlier that no direct interaction occurs between a nonbridging oxygen atom and Mg^{2+} ions at the cleavage site during cleavage by the hammerhead ribozyme,[47] while the direct coordination of a Mg^{2+} ion to the 5′-leaving oxygen atom in the transition state was realized.[44,49,51] Thus, it appears that the hammerhead ribozyme exploits an $\alpha\gamma\delta_{metalation}$ strategy, rather than an $\alpha\beta_{metalation}\gamma$ strategy in Mg^{2+}-containing solutions. While this scenario might be valid in the Mg^{2+}-mediated ribozyme reaction, we cannot ignore the possibility that the same hammerhead

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(70) Emilsson, G. M.; Nakamura, S.; Roth, A.; Breaker, R. R. *RNA* **2003**, *9*, 907−918.
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ribozyme might adopt a different strategy in a different environment.[51]

Rueda et al. reported that Na^+ ions had an inhibitory effect on the HHα ribozyme at 100 mM Na^+ ions on a background of Mg^{2+} ions.[59] Hendry and McCall also reported the inhibitory effect on their hammerhead ribozyme of Na^+ ions on a background of Mg^{2+} ions.[61] Such effects are consistent with our previous and current observations, namely, that inhibitory effects are greater at 500 mM Na^+ ions in the presence of a low concentration of Mg^{2+} or Mn^{2+} ions. However, the activity of our ribozyme can be restored by the addition of high concentrations of K^+ ions, Na^+ ions, or Li^+ ions (Figures 3 and 6B).[50] The activity of our ribozyme depended on the concentration of Mg^{2+} ions even at around 1 M Mg^{2+} ions. The observed rate constant of $\sim$1 min^{-1} at pH 6 and 25 °C in 800 mM Mg^{2+} ions under single-turnover conditions allowed us to estimate that the ribozyme activity would be 100 min^{-1} at pH 8. Since we observed no sign of saturation by Mg^{2+} ions (Figure 4A), concentrations of Mg^{2+} ions above 800 mM should lead to a rate constant greater than 100 min^{-1} at pH 8. This rate constant is similar to that of the so-called "kissing ribozyme with the optimum activity" under optimum conditions with respect to a concentration of Mg^{2+} ions and pH.[57]

Conclusions

We have established a novel third pathway, namely, a cooperative pathway that involves monovalent and divalent metal ions, for the hammerhead ribozyme reaction. Moreover, complete kinetic parameters were obtained that explain the ribozyme activities under different conditions. The ribozyme reaction is more complex than might have been expected, perhaps because of the flexibility of RNA, which would have enhanced the potential of RNA during evolution of and in the RNA world. Our ribozyme, used as a model, might mimic the chemical cleavage of the so-called "kissing ribozyme with the optimum activity" because there was no upper limit to the rate constant of cleavage even at high concentrations of Mg^{2+} ions. Further studies with our ribozyme might help to elucidate the catalytic mechanism of the "kissing ribozyme". Our analysis further indicates that the high concentrations of monovalent ions are inhibitory for the ribozyme in cells that is the disadvantage to the ribozyme compared to other catalysts such as siRNA whose activity can be enhanced by intracellular factors.

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