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Importance in catalysis of a magnesium ion with very low affinity for a hammerhead ribozyme

Atsushi Inoue[1,3], Yasuomi Takagi[1,2] and Kazunari Taira[1,2,3,*]

[1]Gene Function Research Center and [2]iGENE Therapeutics, Inc., National Institute of Advanced Industrial Science and Technology (AIST), Central 4, 1-1-1 Higashi, Tsukuba Science City 305-8562, Japan and [3]Department of Chemistry and Biotechnology, School of Engineering, The University of Tokyo, Hongo, Tokyo 113-8656, Japan

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ABSTRACT

Available evidence suggests that Mg^{2+} ions are involved in reactions catalyzed by hammerhead ribozymes. However, the activity in the presence of exclusively monovalent ions led us to question whether divalent metal ions really function as catalysts when they are present. We investigated ribozyme activity in the presence of high levels of Mg^{2+} ions and the effects of Li^{+} ions in promoting ribozyme activity. We found that catalytic activity increased linearly with increasing concentrations of Mg^{2+} ions and did not reach a plateau value even at 1 M Mg^{2+} ions. Furthermore, this dependence on Mg^{2+} ions was observed in the presence of a high concentration of Li^{+} ions. These results indicate that the Mg^{2+} ion is a very effective cofactor but that the affinity of the ribozyme for a specific Mg^{2+} ion is very low. Moreover, cleavage by the ribozyme in the presence of both Li^{+} and Mg^{2+} ions was more effective than expected, suggesting the existence of a new reaction pathway—a cooperative pathway—in the presence of these multiple ions, and the possibility that a Mg^{2+} ion with weak affinity for the ribozyme is likely to function in structural support and/or act as a catalyst.

INTRODUCTION

Naturally existing catalytic RNAs include hammerhead, hairpin, hepatitis delta virus (HDV) and Varkud Satellite (VS) ribozymes, group I and II introns, and the RNA subunit of RNase P (1–7). In addition, recent structural and chemical analyses strongly suggest that ribosomal RNA might also be a ribozyme (8–11). In addition, the possibility exists that the RNA component of the spliceosome might be a ribozyme too (12). The earliest 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, subsequent, extensive studies revealed that the catalytic activity of hairpin ribozymes is independent of divalent metal ions (7,13–18). Thus, the various types of ribozymes appear to exploit different cleavage mechanisms, which depend upon the architecture of the individual ribozyme. Even hammerhead ribozymes, which have generally been characterized as typical metalloenzymes, can no longer be categorized unambiguously.

Naturally existing hammerhead ribozymes were originally identified in some RNA viruses, and it was demonstrated that they act *in cis* during viral replication by the rolling circle mechanism (3). In the laboratory, ribozymes have been engineered such that they act *in trans* against other RNA molecules 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 (19–22). The transesterification mechanism includes deprotonation of the 2′-hydroxyl moiety of a ribose group, nucleophilic attack of the 2′-oxygen on the adjacent phosphorus atom, and protonation of the 5′-oxyanion leaving group (Figure 1A). 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 A_{13} phosphate near the site of cleavage (23–36). Thus, the idea that ribozymes are metalloenzymes has been generally accepted. However, it was reported recently that hammerhead ribozymes are active in the presence of very high concentrations of monovalent cations, such as Li^{+} or NH_4^{+} ions, in the absence of divalent metal ions (37). This finding raises the possibility that hammerhead ribozymes should not be classified as metalloenzymes. Therefore, we decided to investigate ribozyme activity in the presence of Mg^{2+} ions and the effects of Mg^{2+} ions in the presence of Li^{+} ions on ribozyme activity, using a well-studied model hammerhead ribozyme (R32) and its substrate (S11), both of which are shown in Figure 1B (5–7,25,28,32–34,38–41).

We investigated the dependence on the concentration of Mg^{2+} and Li^{+} ions of ribozyme activity over a range from 5 mM to ~1 M Mg^{2+} ions and 1 to 5 M Li^{+} ions, respectively. Although several research groups have reported similar analyses (42–50), the concentrations of Mg^{2+} ions used in most of these studies were <100 mM, and little information is available about activity at a higher concentration, such as 1 M. The activity at high concentrations of Mg^{2+} ions, as compared

*To whom correspondence should be addressed at Department of Chemistry and Biotechnology, School of Engineering, The University of Tokyo, Hongo, Tokyo 113-8656, Japan. Tel: +81 3 5841 8828 or +81 29 861 3015; Fax: +81 29 861 3019; Email: taira@chembio.t.u-tokyo.ac.jp

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

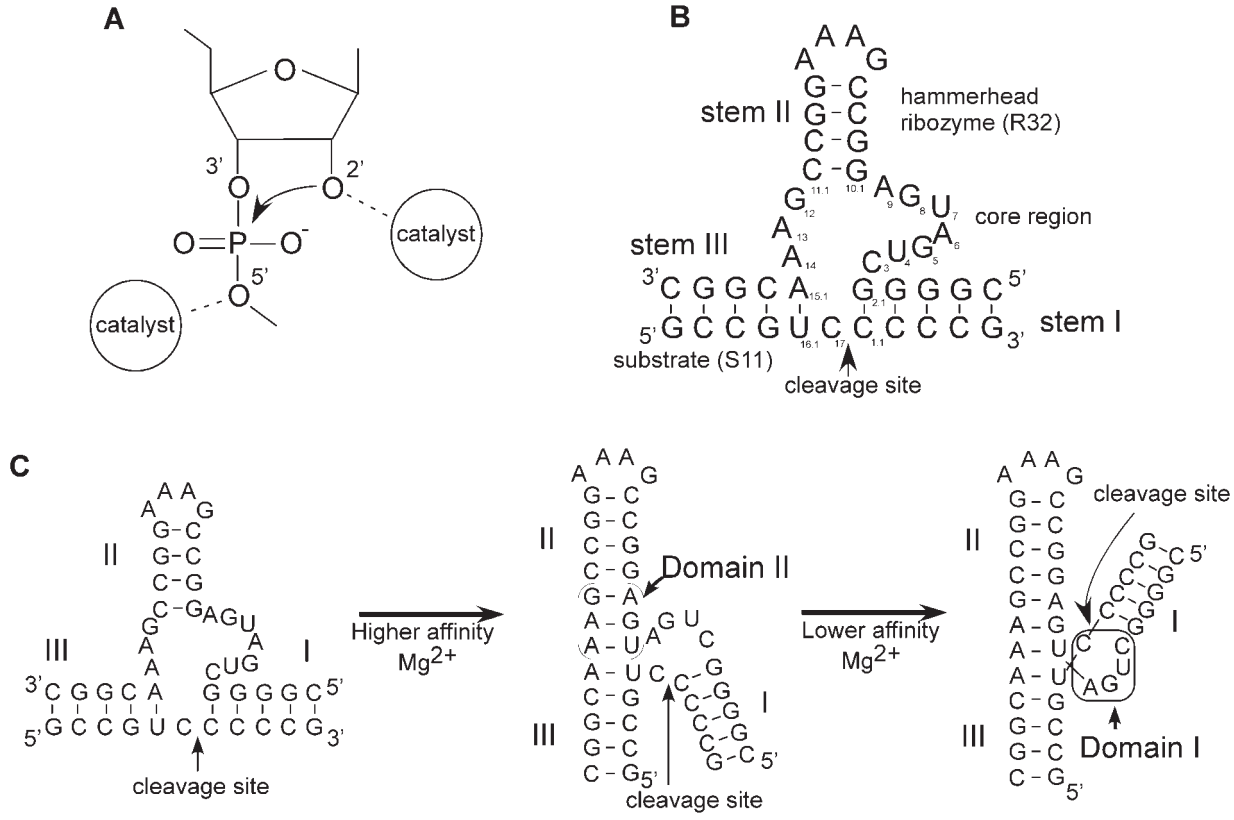

Figure 1. (**A**) Schematic representation of the proposed mechanism of the hammerhead ribozyme reaction. The 2′-hydroxyl moiety is activated by the catalyst and attacks the adjacent phosphate nucleophilically, with subsequent cleavage of the bond at the 5′-oxygen. The developing negative charge on the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

to physiological concentrations, is useful for studies of the chemistry of the hammerhead ribozyme and it allowed us to estimate the intrinsic cleavage rate constant and to compare an enzymatic reaction to a non-enzymatic reaction. We were also able to calculate acceleration energy more precisely and to investigate the dependence on Mg^{2+} ions in the presence of high concentrations of Li^{+} ions. Considering the results of such analyses, we propose the existence of a new cooperative pathway that involves divalent metal ions, such as Mg^{2+}, and monovalent ions, such as Li^{+}, in the reaction catalyzed by a hammerhead ribozyme. We also discuss the nature of the catalysts.

MATERIALS AND METHODS

Preparation of the hammerhead ribozyme and its 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 previously (25). Chemically synthesized oligonucleotides were deprotected in a mixture of 28% ammonia and ethanol (3:1) at 55°C for 8 h. The 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) by high-performance liquid chromatography (HPLC) with ultrapure water. All of the RNA oligomers were quantitated in terms of absorbance at 260 nm.

The substrate S11 was labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase (Takara Bio, Inc., Shiga, Japan). Radiolabeled S11 was purified on a 20% polyacrylamide gel that contained 7 M urea and then purified by the standard procedure, as described above, with desalting on a gel-filtration column (NAP^{TM}-10 column; Amersham Biosciences, K.K., Tokyo, Japan).

Quantification of the ribozyme reaction

All ribozyme reactions were performed under 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 products. The solution for the ribozyme reaction contained a trace amount of 5′-^{32}P-labeled S11 and 25 mM Bis-Tris buffer at pH 6.0 and 25°C. The pH values of all 1.25× pre-stock Bis-Tris buffers that contained appropriate metal ions (metal-ion–buffer) were adjusted appropriately with HCl and we confirmed that each buffer had the appropriate pH under the chosen reaction conditions. 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 does not chelate Mg^{2+} and Li^+ ions efficiently at lower pH values, we confirmed that reactions did not continue in the stop solution and that quenching was effective due to 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 imageanalyzer (Storm 830; Molecular Dynamics, Sunnyvale, CA). For each reaction, an observed rate constant was determined by non-linear least-squares fitting of the time course of reaction using a pseudo-first-order equation.

RESULTS AND DISCUSSION

The dependence of the activity of the hammerhead ribozyme on the concentration of Li^+ ions

We examined the dependence of the activity of the ribozyme on the concentration of Li^+ ions to confirm that Li^+ ions affect the ribozyme's activity, as reported previously by others (51). We performed reactions under single-turnover conditions at pH 7.5 and 25°C. The results are shown in Figure 2A. Although the activity reached a plateau at ∼3 M Li^+ ions, the dependence on the concentration of Li^+ ions was observed (with a slope of three) below the plateau. In the study by O'Rear *et al*. (51), the hammerhead ribozyme reaction exhibited second-order dependence on Li^+ ions up to 4 M at pH 7.5. Although our curve is steeper than the curve that they obtained under the same conditions, with the exception of the concentrations of Li^+ ions, the slopes of both profiles obtained with Li^+ ions are clearly steeper than those of profiles obtained with Mg^{2+} ions. Our profile is unique insofar as we observed saturation of the ribozyme reaction in the range of 3–5 M (Figure 2A). This plateau suggests that the ribozyme reaction might involve the binding of Li^+ ions to the ribozyme–substrate complex.

We also confirmed the dependence on pH, with a slope of unity, of the activity in the presence of a high concentration of Li^+ ions, as shown in Figure 2B. This result supports the hypothesis that the cleavage step is the rate-limiting step even at such a high ionic strength. The pH profile is also consistent with the previous report by Curtis and Bartel (52). As shown in Figure 2A, the observed rate constant at a saturating concentration of Li^+ ions (3–5 M) at pH 7.5 was ∼0.17 min^{-1}. The estimated observed rate constant in the presence of 800 mM Mg^{2+} ions was 32 min^{-1} at pH 7.5, as determined from the value of 1 min^{-1} at pH 6 at the same temperature as that at which the Li^+ experiment was performed (see later). The difference was, thus, approximately 200-fold under similar conditions. The true difference might be even greater because, in contrast to the results with Li^+, the rate at 800 mM Mg^{2+} does not reach a plateau value (see later). These data indicate clearly that Mg^{2+} ions are more suitable for an effective hammerhead ribozyme reaction than any other monovalent ions (Li^+ ions have the highest activity of all monovalent ions in the ribozyme reaction).

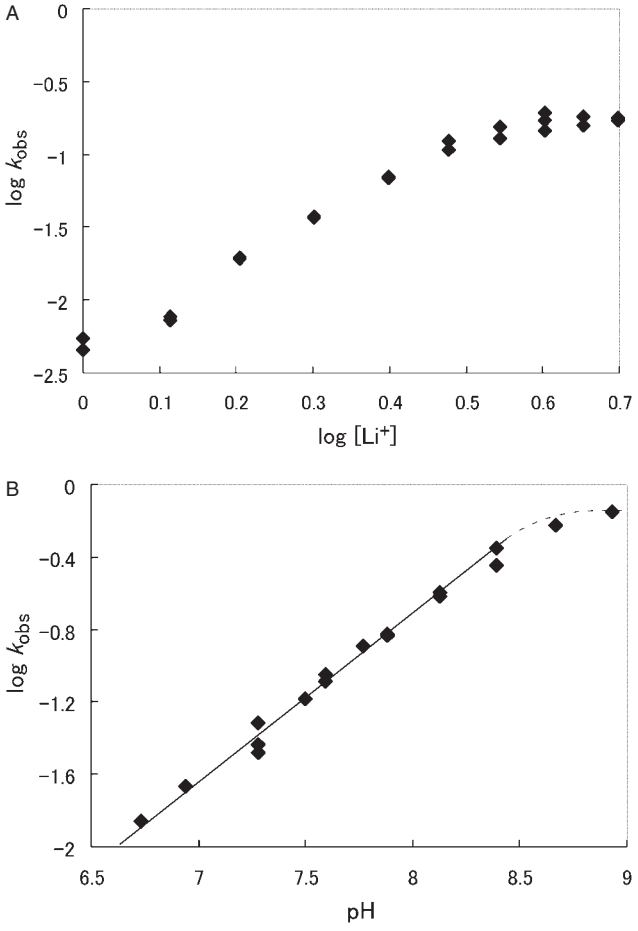

Figure 2. (**A**) Dependence of cleavage activity on the concentration of Li^+ ions. The activity increased linearly with the concentration of Li^+ ions until ∼3 M. Reaction conditions: 25 mM Bis-Tris, pH 7.5, and varying concentrations of Li^+ ions as indicated. (**B**) Dependence on pH of the activity in 2 M Li^+ ions. The Li^+-containing buffer used in this experiment included Bis-Tris propane and Tris. The cleavage rate increased linearly with pH, yielding a slope of 0.9.

The dependence of the activity of the hammerhead ribozyme on the concentration of Mg^{2+} ions

We attempted first to determine how many Mg^{2+} ions might be involved in our model ribozyme reaction and the saturating concentration of Mg^{2+} ions in the reaction, beyond which the cleavage rate constant no longer increases. We examined the dependence on the concentration of Mg^{2+} ions of the activity

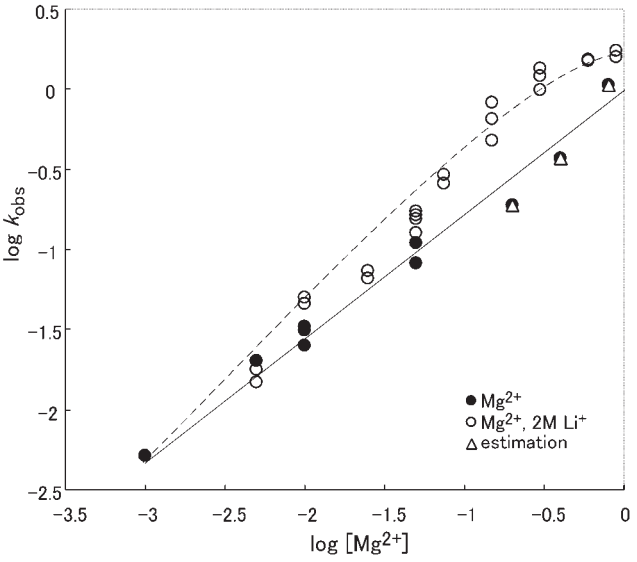

Figure 3. The Mg^{2+}-titration curve for cleavage activity in the presence (open circles) and absence (filled circles) of Li^+ ions. In the absence of Li^+ ions, the line drawn with the linear best fit had a slope of 0.7. The activity increased with increasing concentrations of Mg^{2+} ions and did not reach a plateau value even at 800 mM Mg^{2+} ions. In the presence of 2 M Li^+ ions, the activity increased with increases in the concentration of Mg^{2+} ions up to ~600 mM. Triangles are the calculated rates in the presence of Mg^{2+} ions plus 2 M Li^+ ions, which were determined simply by adding the respective reaction rates together. Since the observed rate in the presence of 2 M Li^+ ions only at pH 6 was <0.01 min^{-1}, triangles are at almost the same positions as the filled circles. The rates observed in the presence of Mg^{2+} ions and Li^+ ions together (open circles) were clearly higher than the calculated values (triangles).

of the R32 hammerhead ribozyme up to ~1 M Mg^{2+} ions. We performed reactions with R32-S11 (Figure 1B) under single-turnover conditions with a saturating amount of ribozyme with respect to the amount of S11 for the same reasons as noted above. The reaction in the presence of Mg^{2+} ions is accelerated with increases in pH, with a slope of unity (7,40,43,44,47,53). We adjusted the pH of reactions to 6.0 to slow down the reaction so that we could determine the rate constants of rapid reactions precisely. As shown by closed circles in Figure 3, the dependence on Mg^{2+} ions was approximately first-order. However, no plateau was reached under our conditions, even above 800 mM Mg^{2+} ions. The continuous increase in rate constant upon the addition of more and more Mg^{2+} ions indicates the involvement of one Mg^{2+} ion that has low affinity for the hammerhead ribozyme–substrate complex. At 800 mM Mg^{2+} ions, the rate constant approached 1.1 min^{-1} at pH 6.0, and this is the limit of detection of a rapid cleavage reaction under standard laboratory conditions.

Analyses of the structure of hammerhead ribozymes and of the conformational changes caused by interactions with Mg^{2+} ions have indicated that two major conformational changes occur: the formation of domain II, which is followed by the formation of domain I, as shown in Figure 1C (38,45, 48,50,54). The formation of domain II results in coaxial stacking of helices II and III, induced by the binding of a higher-affinity Mg^{2+} ion(s) to P9 phosphate and N7 of $G_{10.1}$ (P9/G10.1) of the ribozyme–substrate complex (38,45,48,50,54–58). The second transition is the formation of the catalytic domain with movement of stem I toward stem II, which is induced by the binding of a lower-affinity Mg^{2+} ion(s). The K_d values of the two domains have been determined by various methods to be several hundred micromolar and several millimolar, respectively (45,46,48,50,54). Thus, at several hundred millimolar Mg^{2+} ions, the formation of domains II and I should be complete.

Taking this structural information into consideration, we can reasonably conclude that the very-low-affinity Mg^{2+} ion that we detected in the present study might be involved in some step other than the formation of domains I and II. This step might be a conformational change or the binding of a catalytic species to the ribozyme–substrate complex. Walter and coworkers recently reported that a third and previously undetected metal ion at rather high concentrations might play a role in the induction of a minor conformational adjustment that leads to the formation of the active state after the formation of domains I and II (59). The Mg^{2+} ion that we detected had very low affinity, and the relative level of truly active ribozyme species at a concentration of several millimolar Mg^{2+} ions corresponded to <1% of all the ribozyme–substrate complexes in the reaction mixture [compare 1 min^{-1} in 10 mM $MgCl_2$ at pH 8 and 25°C (60) with 100 min^{-1} in 800 mM $MgCl_2$ at the same pH and the same temperature (see later)].

One might agree that Figure 3 does not support the existence of a very-low-affinity binding site for an Mg^{2+} ion on the ribozyme–substrate complex since the binding curve does not reach a plateau. If the dependence that we observed here were due only to ionic strength, no plateau would be observed. However, we did observe a plateau in the case of Li^+ ions (Figure 2A). Because reactions were so rapid, even at low pH, we could not monitor the kinetics at even higher concentrations of Mg^{2+} ions, such as 3 M. However, it is likely that, in ribozyme reactions, ionic strength is less important than the ion radius and/or the electron density of metal ions since (i) a correlation between the ion radius of Group I monovalent metal ions and ribozyme activity has been observed (52,57) and (ii) an apparent plateau was reached in the presence of high concentrations of Mg^{2+} ions plus 2 M Li^+ ions (Figure 3). Although we cannot calculate a Hill constant for Mg^{2+} ions and estimate the number of binding sites for Mg^{2+} ions from our current data, all the available data strongly support the possibility of the existence of a very-low-affinity metal-binding site(s).

Misra and Draper (61) proposed a model for the stabilization of RNA by Mg^{2+} ions that arises from two distinct binding modes, diffuse binding and site binding. Diffusely bound Mg^{2+} ions are defined as fully solvated ions that interact with RNA only through long-range electrostatic interactions. Site-bound Mg^{2+} ions are defined as partially desolvated ions that are attracted to electronegative pockets. In general, the affinity of diffusely bound Mg^{2+} ions appears 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 here might be involved in diffuse binding. Although diffuse binding can sometimes play a dominant role in stabilizing the tertiary structures of small RNAs (61), we cannot exclude the possibility that such diffuse binding Mg^{2+} ion might play a role in catalysis in the ribozyme reaction at a specific site in the ribozyme–substrate complex.

In 800 mM Mg^{2+} ions, the observed rate constant of the reaction catalyzed by the hammerhead ribozyme can be estimated to be ~100 min^{-1} at pH 8 and 25°C, from the

dependence on pH with a slope of unity. The estimated rate constant at concentrations of Mg^{2+} ions >800 mM should be much higher than that at 800 mM since the cleavage reaction did not reach saturation at 800 mM Mg^{2+} and approached the estimated observed rate constant of a 'kissing ribozyme', under saturating conditions with respect to Mg^{2+} ions and pH (62).

The dependence of the activity of the hammerhead ribozyme on Mg^{2+} ions in the presence of a high concentration of Li^+ ions

In order to investigate the properties of Mg^{2+} ions in the ribozyme reaction in further detail, we examined the dependence on Mg^{2+} ions in the presence of a high concentration (2 M) of Li^+ ions. We chose the Li^+ ion as the monovalent cation because this ion is the most active of a large variety of monovalent cations (57). At 2 M Li^+ ions at pH 6.0, the hammerhead ribozyme had detectable activity in the absence of Mg^{2+} ions. We varied the concentration of Mg^{2+} ions from 5 to 900 mM and measured the rate constant. As shown by open circles in Figure 3, we observed approximately first-order dependence on the concentration of Mg^{2+} ions up to 600 mM, and then the rate constant started to reach a plateau from 600 to 900 mM Mg^{2+} ions. These data indicate that, even in the presence of a very high concentration of Li^+ ions, an Mg^{2+} ion with very low affinity plays an important role in the hammerhead ribozyme reaction. The plateau that seemed to appear in the presence of high concentrations of Li^+ ions (Figure 3, open circles) was not observed in the absence of Li^+ ions (closed circles). This apparent plateau was not due to misfolding of RNA at such a high ionic concentration because the extent of cleavage at the end of the reaction was always >90% under these conditions. The plateau might indicate that Li^+ ions help the ribozyme–substrate complex to fold into a more active form, contributing to the enhanced binding of the very-low-affinity Mg^{2+} ion(s) to the complex.

We examined reactions in the presence of Mg^{2+} ions only (Figure 3, closed circles) and in the presence of Li^+ ions only (57). We then calculated rate constants (Figure 3, triangles) on the assumption that Mg^{2+} and Li^+ ions function independently. It should be noted that the observed rate constant in the presence of both Mg^{2+} ions and 2 M Li^+ ions together (Figure 3, open circles) was definitely higher than the calculated rates (Figure 3, triangles) at pH 6 (compare open circles with triangles in Figure 3). One might expect that, in such an experiment (i) the activities in the presence of both metal ions together would be lower than the estimated activities (triangles) because of the lower ability of Mg^{2+} ions to bind to RNA in the presence of high ionic strength due to 2 M Li^+ ions and (ii) the stimulation by Mg^{2+} ions in the presence of such a high concentration of Li^+ ions would be smaller than in the absence of Li^+ ions. It has been reported that stimulation by Cd^{2+} ions is reduced in a ribozyme reaction in the presence of 4 M Li^+ ions (51), while stimulation by Cd^{2+} ions is observed in reaction mixtures that contain 10 mM Ca^{2+} ions instead of 2 M Li^+ ions (23). However, the results in the present study were quite opposite: (i) the activities in the presence of Mg^{2+} ions and 2 M Li^+ ions together (open circles in Figure 3) were higher than the calculated activities (triangles in Figure 3), and (ii) the stimulation by Mg^{2+} ions in the presence of 2 M Li^+ ions (open circles in Figure 3) was even greater than that by Mg^{2+} ions in the absence of Li^+ ions (closed circles in Figure 3).

Our observations suggest the existence of a new cooperative pathway that involves Li^+ and Mg^{2+} ions, in which both metal ions function cooperatively in structural support and/or as the catalyst(s) to increase the rate constant of cleavage (57). In the case of cleavage of RNA by another type of ribozyme, the RNA subunit of RNase P, a similar cooperativity between the metal ions, e.g. Mg^{2+} and Ca^{2+}, has been reported (63).

What is the catalyst in the hammerhead reaction?

To the best of our knowledge, this report is the first to describe the dependence of ribozyme activity on a high concentration of Mg^{2+} ions under single-turnover conditions (Figure 3). The reaction did not reach a plateau even at 800 mM Mg^{2+} ions. Under these conditions at pH 6, the observed rate constant was 1.1 min^{-1}. The activity of the ribozyme is known to depend on pH, with a slope of unity. Thus, we can estimate an observed rate constant for the ribozyme reaction of 110 min^{-1} at pH 8. It is very unlikely that this rate constant of cleavage can occur without an effective catalyst(s) (64). So, what is the catalyst(s) of the reaction?

In studies of solvent isotope effects on the ribozyme reaction in the presence of deuterium and Li^+, Mg^{2+} or NH_4^+ ions, we failed previously to observe any proton transfers in the transition state during the ribozyme reaction in the presence of either Li^+ or Mg^{2+} ions but we did observe proton transfer in the presence of NH_4^+ ions (53,65). We interpreted such results by suggesting that metal ions, such as Mg^{2+} and Li^+, function as a Lewis acid catalyst while NH_4^+ ions function as a general acid catalyst during the ribozyme reaction. Thus, the catalyst can change according to the conditions around the ribozyme. On the basis of this hypothesis and the involvement of two other kinds of Mg^{2+} ions in the formation of domains I and II, it is possible that the newly identified Mg^{2+} ion with very low affinity that we observed in this study might be the true catalyst. Our novel cooperative pathway might involve an Mg^{2+} ion in a catalytic role after monovalent cations and other Mg^{2+} ions have acted to generate the pre-active conformation just before chemical cleavage by the hammerhead ribozyme. In the presence of monovalent ions exclusively, monovalent ions can also function as the catalyst but they are not as effective. Mg^{2+} ions have a higher charge density than Li^+ ions and would function better as catalyst in the reaction. Thus, when both Mg^{2+} and Li^+ ions are present in the reaction mixture, the high activity of the ribozyme reaction is likely due to an Mg^{2+} ion catalyst. The Li^+ ions act to support the formation of the domains I and II in cooperation with Mg^{2+} ions, as discussed above.

We are at present constructing a reaction scheme for ribozyme reactions in the presence of both Mg^{2+} and Li^+ ions, namely, a 'cooperative pathway', by determining the details of the stoichiometric relationships among these metal ions (57). Examination of ribozyme-catalyzed reactions in the presence of various metal ions in combination should clarify the roles of metal ions in catalysis.

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