A two-metal ion mechanism operates in the hammerhead ribozyme-mediated cleavage of an RNA substrate

(ribozyme function/metal ion activation/RNA cleavage/magnesium ion/lanthanum ion)

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ABSTRACT   **Evidence for a two-metal ion mechanism for cleavage of the HH16 hammerhead ribozyme is provided by monitoring the rate of cleavage of the RNA substrate as a function of La^{3+} concentration in the presence of a constant concentration of Mg^{2+}. We show that a bell-shaped curve of cleavage activation is obtained as La^{3+} is added in micromolar concentrations in the presence of 8 mM Mg^{2+}, with a maximal rate of cleavage being attained in the presence of 3 μM La^{3+}. These results show that two-metal ion binding sites on the ribozyme regulate the rate of the cleavage reaction and, on the basis of earlier estimates of the K_d values for Mg^{2+} of 3.5 mM and >50 mM, that these sites bind La^{3+} with estimated K_d values of 0.9 and >37.5 μM, respectively. Furthermore, given the very different effects of these metal ions at the two binding sites, with displacement of Mg^{2+} by La^{3+} at the stronger (relative to Mg^{2+}) binding site activating catalysis and displacement of Mg^{2+} by La^{3+} at the weaker (relative to Mg^{2+}) (relative to Mg^{2+}) binding site inhibiting catalysis, we show that the metal ions at these two sites play very different roles. We argue that the metal ion at binding site 1 coordinates the attacking 2′-oxygen species in the reaction and lowers the pK_a of the attached proton, thereby increasing the concentration of the attacking alkoxide nucleophile in an equilibrium process. In contrast, the role of the metal ion at binding site 2 is to catalyze the reaction by absorbing the negative charge that accumulates at the leaving 5′-oxygen in the transition state. We suggest structural reasons why the Mg^{2+}–La^{3+} ion combination is particularly suited to demonstrating these different roles of the two-metal ions in the ribozyme cleavage reaction.**

Ribozymes are rightly classified as metalloenzymes because metal ions in the reaction mixture are obligatory for catalytic activity (1–5). However, the precise role of metal ion cofactors in these systems is not fully understood. The currently debated models describing the involvement of metal ions in ribozyme-mediated cleavage are generally divided into one-metal ion and two-metal ion classes. In a popular single metal ion model a solvated metal hydroxide coordinates to the pro-R oxygen of the phosphate at the cleavage site (6–8). This metal hydroxide is favorably positioned to act as a base to abstract the proton from the 2′-OH in the first step of the cleavage reaction. The resulting 2′-alkoxide then acts as a nucleophile, attacking the electropositive phosphorus by an intramolecular reaction and displacing the 5′-oxygen of the leaving nucleotide. The general two-metal ion model (3) is functionally much the same as the single metal ion model, except that a second metal ion is invoked that coordinates to the 5′-oxygen of the leaving nucleotide to dissipate the accumulating negative charge on

this atom in the transition state of the rate-determining step. Similar two-metal ion models have been proposed for a variety of other enzymatic phosphoryl transfer reactions (3, 9–11). In a previous publication, we critically examined the literature on this subject and concluded that all of the available data were at least consistent with a two-metal model (12). Other relevant reviews of metalloenzymes and ribozymes are also available (5, 13–18).

An experimental design that can definitively distinguish between these two general classes of models has been elusive for a variety of reasons. By using a DNA–RNA hybrid hammerhead ribozyme, Hendry and McCall (19) observed a biphasic relationship between the observed rate constant for RNA cleavage and the concentration of Mg^{2+} ion, which they interpreted as evidence for a strong metal ion binding site and a weak metal ion binding site in a two-metal ion model. We also observed this biphasic behavior as a function of Mg^{2+} concentration for the fully RNA HH16 hammerhead ribozyme shown in Fig. 1 (12) and estimated apparent dissociation constants for the binding of Mg^{2+} to these sites at 3.5 mM and >50 mM, respectively (B.W.P., unpublished data). Measuring the binding constant of Mg^{2+} to the putative weaker site in such experiments, in particular, requires studies at very high divalent salt concentrations. As a consequence it is difficult to differentiate between general polyelectrolyte effects on the structure of the RNA complex and specific binding at the defined site(s) as the Mg^{2+} concentration is increased in this range.

At these high Mg^{2+} concentrations, the reaction also proceeds so fast that it is difficult to follow manually under single-turnover reaction conditions. Single-turnover reaction conditions are preferred because (e.g.) a rate-limiting product dissociation reaction could obscure the metal ion concentration dependence of the cleavage reaction in multiturnover measurements. An additional implication is that the extremely weak binding site might only exist in a high-energy conformation near the transition state. Such a metal ion binding site would only be transiently present along the reaction pathway, and nonkinetic detection methods, including x-ray crystallography, would fail to observe its presence.

In this study, we present the results of an experiment that effectively circumvents these problems. Cleavage of the HH16 hammerhead ribozyme (Fig. 1) was carried out in the presence of a constant concentration of Mg^{2+} ion, with La^{3+} as a competing metal ion species. k_{obs}, the observed rate constant of the cleavage reaction, was measured as a function of La^{3+} concentration. In this fashion, bound Mg^{2+} ions were effectively titrated (displaced) by La^{3+} ions as the concentration of the latter cation was increased. Because a kinetic parameter was used to monitor the cleavage reaction, the resulting activity titration as a function of total La^{3+} ion concentration

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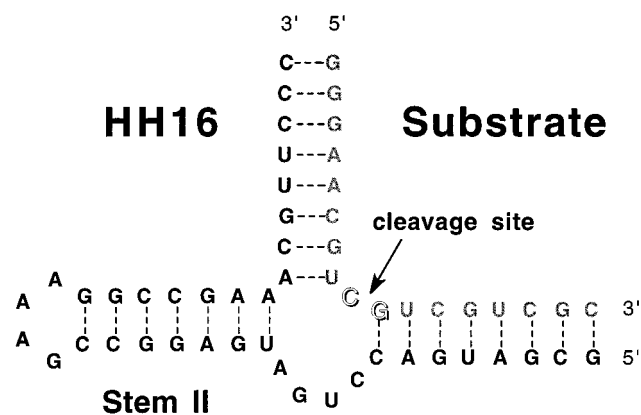
FIG. 1. HH16 hammerhead ribozyme–substrate complex. The base pairing within the ribozyme complex is shown as observed in the crystal structure of the hammerhead ribozyme (7, 8, 20–22).

defines the relative concentrations of the metal ion species in the transition state of the rate-determining step.

MATERIALS AND METHODS

The RNA substrate oligomer for the hammerhead ribozyme cleavage reaction was purchased from Midland Certified Reagent (Midland, TX) and purified electrophoretically on a 20% polyacrylamide gel. The hammerhead ribozyme itself was transcribed from a synthetic DNA template by using a published procedure (23). All metal chlorides were obtained as reagent grade salts of >99.9% purity from Aldrich and were not further purified. The final HH16 hammerhead ribozyme–substrate complex used in this study is shown in Fig. 1.

Cleavage reactions were carried out at 37°C in 20 mM Hepes (pH 7.0) under single-turnover conditions, in 50-μl reaction volumes containing a 40 nM concentration of radioactively 5′-end-labeled RNA substrate and an 80 nM concentration of unlabeled ribozyme. The reaction solutions contained 200 mM NaCl to provide an essentially constant ionic strength for the reaction and to neutralize nonspecific electrostatic effects. The ribozyme and substrate RNA oligomers were allowed to hybridize in the reaction solution for 5 min at 37°C, and then cleavage was initiated by adding stock solution mixtures of di- and trivalent metal ions to obtain the required final Mg^{2+} and La^{3+} concentrations. Each solution was sampled at designated time intervals by removing 5 μl of the reaction mixture and quenching with 10 μl of a 90% formamide solution containing 50 mM EDTA. The cleaved (and end-labeled) reaction products were separated from the remaining unreacted (and labeled) ribozyme complex on a 20% polyacrylamide denaturing gel and quantitated with a Molecular Dynamics Storm model
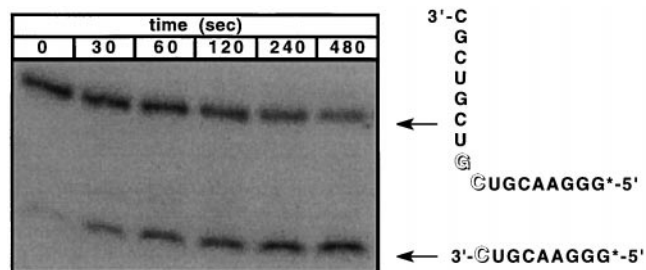

FIG. 2.   Gel autoradiogram of the time course of a typical HH16-mediated cleavage reaction with a 5′ end-labeled RNA substrate. The gel bands corresponding to the complex and to the cleaved RNA are indicated at increasing reaction times. These experiments typically resulted in 80–90% cleavage of the substrate over the measured time course of the reaction.

860 PhosphoImager. A typical reaction gel is shown in Fig. 2. The reaction rate data obtained were fit to a first-order reaction by using the KALEIDOGRAPH graphing software program to obtain apparent rate constants for the ribozyme cleavage reaction as a function of the concentration of metal ion cofactor.

RESULTS AND DISCUSSION

If a general two-metal model is assumed for the ribozyme-catalyzed cleavage process (see Fig. 5), a close examination of the likely mechanism suggests that the two-metal ions involved must play very different roles in the reaction (12). The metal ion that binds at or near the 2′-OH (metal binding site 1 in Fig. 5) should function primarily to change the pK_a for the deprotonation of this hydroxyl group. Whether this results from the coordination of the metal ion to the 2′-oxygen itself, leading to a lower pK_a for this entity (24), or from the binding of a metal hydroxide species coordinated to the pro-R phosphoryl oxygen, functioning as a specific base to deprotonate the 2′-OH (6), is unclear. Both mechanisms would produce the observed correlation between the pK_a values of the aqueous metal ions and the measured cleavage rate constants (12).

Metal ion binding at site 1 modulates the distribution between the protonated and deprotonated forms of the 2′-OH in a thermodynamic process that is fast relative to the rate-determining cleavage event, with binding of the metal ion at this position increasing the rate of cleavage by increasing the equilibrium population of the active (deprotonated) species. The putative second metal cation (which binds at site 2 in Fig. 5) in the two-metal ion model plays a true kinetic role, binding coordinately to the 5′-oxygen of the leaving nucleotide to absorb the negative charge that accumulates on this atom in the transition state of the cleavage reaction. The activation energy barrier for the cleavage reaction is thus lowered by this binding and the reaction is accelerated.

When the diverse roles that must be played by the metal cations in a two-metal ion reaction model are considered, it is highly unlikely that the same cationic species would function optimally at both of the postulated metal ion binding sites. Thus, some selectivity between the two sites would be expected for a reaction system in which two or more different metal ion species are available. We have exploited this putative selectivity difference by establishing a competition for the two putative metal ion binding sites between divalent and trivalent metal cations with very different physical properties.

Choice of Metal Ions. Mg^{2+} is likely to be the cation used in ribozyme cleavage reactions *in vivo* and thus represents a natural choice as the representative divalent ion for this study. A fixed 8 mM concentration of $MgCl_2$ was chosen for our reactions because this concentration falls well above the dissociation constant for Mg^{2+} for the tighter metal ion binding site and also provides a convenient range of reaction times for following cleavage under single-turnover conditions.

We chose La^{3+} as the competing cation for a number of reasons. First, the ionic radius of La^{3+} is roughly the same as that of Ca^{2+}, which has been well characterized in the HH16 system (6, 25). In addition, the La^{3+} ion is well known as an inhibitor of biochemical processes requiring Ca^{2+}, Mg^{2+}, or other divalent metal ions (26). Finally, in contradistinction to other transition state metal ions that have been commonly used in biochemical reactions, La^{3+} has a roughly spherical orbital symmetry due to its completely filled outer d orbital, which obviates the need to consider issues related to orbital shape and directionality (27).

The fully hydrated La^{3+} ion has a pK_a of 9.06, which is more than two pK_a units lower than that of either Mg^{2+} (pK_a = 11.41) or Ca^{2+} (pK_a = 12.67) (28). The observed correlation between the pK_a of the various divalent metal ions tested with the k_{obs} of HH16 cleavage would predict a larger cleavage rate

in the presence of La^{3+} ion than is observed with either of these divalent cations. However, if a metal ion were indeed directly coordinated to the 2′-oxygen, a La^{3+} in this position might be expected to result in a lower k_{obs} for the reaction, compared with that seen with a divalent metal ion in this position, because the nucleophilicity of the 2′-alkoxide bound to a trivalent metal ion would be greatly reduced relative to that of the same alkoxide bound to a divalent cation.

In support of the first expectation listed above, La^{3+} has been shown to increase the rate of phosphodiester cleavage by 10^6 compared with Mg^{2+} ion in a nonenzymatic phosphodiester cleavage reaction (29). In that work a complexing moiety was engineered into the molecule to increase the coordination of metal ions at the leaving oxygen. These results suggested that a La^{3+} cation coordinated to the 5′-oxygen of the leaving nucleotide would likely increase k_{obs} for ribozyme cleavage well above the rate seen with the divalent Mg^{2+} cation. We therefore chose La^{3+} as a "foil" for Mg^{2+} in this study because we expected that profound kinetic changes would result when this cation was substituted for Mg^{2+} in the cleavage reaction and that these effects would be quite different depending on which of the two putative metal ion binding sites was involved.

Titration by Increasing Concentrations of La^{3+} in the Presence of a Constant Concentration of Mg^{2+} Is Consistent with a Two-Metal Ion Model. Fig. 3 shows the effect of increasing (micromolar) concentrations of LaCl$_3$ in the presence of 8 mM MgCl$_2$ on the apparent rate constant for the ribozyme cleavage reaction. The most striking feature of this titration curve is its bell-shaped character, with the midpoints of the two sides of the curve centered at total La^{3+} concentrations of ≈2 μM and ≈6 μM, respectively. The demonstration of this bell-shaped curve, and of the two titrations that it represents, provides in itself clear and convincing qualitative evidence for the two-metal ion model of ribozyme catalysis. Thus these observations alone show that two-metal ions are involved in controlled the apparent rate of the cleavage reaction and that these two ions must play very different roles in the reaction mechanism.

Because the biphasic titration of Fig. 3 must represent the replacement of one Mg^{2+} and then the other by La^{3+} (Fig. 3, see structures *a*–*c*), it is clear that the replacement of the former cation by the latter in the two binding sites produces opposite effects on k_{obs} for the ribozyme-mediated cleavage reaction. With 3 μM LaCl$_3$ and 8 mM MgCl$_2$ present together in the solution (the conditions that apply at the peak of the titration curve), we see that the measured value of k_{obs} is greater than that measured in the presence of either 3 μM LaCl$_3$ or 8 mM MgCl$_2$ alone. Thus in the absence of La^{3+} ions and at 8 mM MgCl$_2$, Mg^{2+} ions are expected to fully occupy the tighter metal ion binding site and to partially occupy the weaker binding site (see estimates of Mg^{+2} dissociation constants given above). A measured value of k_{obs} = 4.5 × 10^{-3}·sec^{-1} corresponds to the cleavage rate constant for this concentration of MgCl$_2$ alone.

As LaCl$_3$ is added to a total concentration of about 3 μM, the first of the two-metal ion binding sites is saturated with La^{3+} and the measured value of k_{obs} increases to a maximum of ≈11 × 10^{-3}·sec^{-1}. We speculate that the Mg^{2+} ion bound to the 5′-oxygen of the leaving nucleotide (see Fig. 5, binding site 2) is the one that is displaced in this initial titration. The metal ion in this position absorbs the developing charge in the transition state of the rate-determining step of this reaction, and the increased charge of a trivalent metal cation at this binding site would be expected to produce greater transition state stabilization than would a divalent cation.

A second titration is observed as the concentration of LaCl$_3$ increases from 3 μM to 12 μM. The apparent value of k_{obs} diminishes in this region of the La^{3+} titration curve, approaching a value of ≈2.2 × 10^{-3}·sec^{-1} at LaCl$_3$ concentrations above 12 μM. This value of k_{obs} is also observed for the reaction

performed in 3 μM LaCl$_3$ alone (Fig. 3, ◇), confirming that both metal ion binding sites are fully occupied by La^{3+} ions under these conditions. The observation that La^{3+} occupation of the binding site near the 2′-oxygen lowers k_{obs} is consistent with direct coordination of a metal ion to the 2′-oxygen, whereas the deprotonation of the 2′-OH by a metal hydroxide species would be expected to produce the opposite effect.

These Results Are Not Compatible with a Solvated Metal Hydroxide Cleavage Mechanism. With a pK$_a$ value of 9.06 for a bound water in the fully hydrated La^{3+} ion, the low k_{obs} for the cleavage reaction involving the La^{3+} ion in both positions is not compatible with the observed correlation between the pK$_a$ of a water bound to a metal ion and the k_{obs} produced by different divalent metal ions. That correlation has been interpreted in the metal hydroxide model (Fig. 4) as an effect on the concentration of the aqueous metal hydroxide, which then serves as a Brønsted base in the abstraction of the proton from the 2′-oxygen. We have argued (12) that this logic is flawed, because the metal hydroxide complexes formed with metal ions with lower pK$_a$ values are weaker bases and, therefore, should be less able to abstract the 2′-OH proton, despite their greater concentration. This conclusion is supported by the data presented in Fig. 3 because the pK$_a$ of the 2′-OH is two or more pK$_a$ units higher than those of any of the aqueous metal ions studied, making the metal hydroxide poorly suited to the task of deprotonating the 2′-OH. It has been convincingly shown that proton transfer does not occur in the rate-determining step of the ribozyme cleavage reaction (30). The observed pH dependence and the correlation between the pK$_a$ values of the aqueous metal ions and k_{obs} must, therefore, reflect the effects
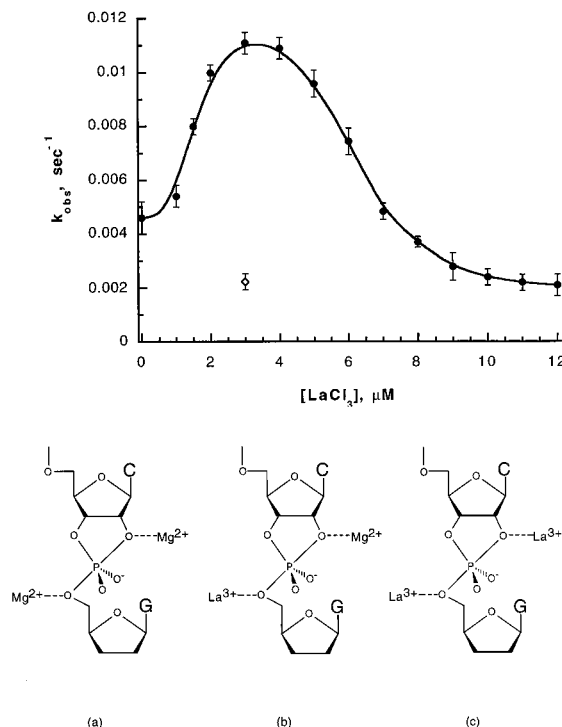

FIG. 3.   Kinetic titration curve of k_{obs} for the cleavage of the HH16 ribozyme with increasing concentrations of LaCl$_3$ in a solution containing 8 mM MgCl$_2$ (●). At low concentrations of LaCl$_3$ both metal ion binding sites are occupied with Mg^{2+}, as illustrated in structure *a*. At intermediate concentrations (1–8 μM LaCl$_3$), the two sites are heterogeneously complexed with one La^{3+} ion and one Mg^{2+} ion (structure *b*). At high concentrations of LaCl$_3$ both sites are occupied by La^{3+} (structure *c*). The combination of 3 μM LaCl$_3$ and 8 mM MgCl$_2$ in the reaction solution yields a higher value of k_{obs} than is observed in the presence of either 3 μM LaCl$_3$ alone (◇) or 8 mM MgCl$_2$ alone (● at [LaCl$_3$] = 0 μM).
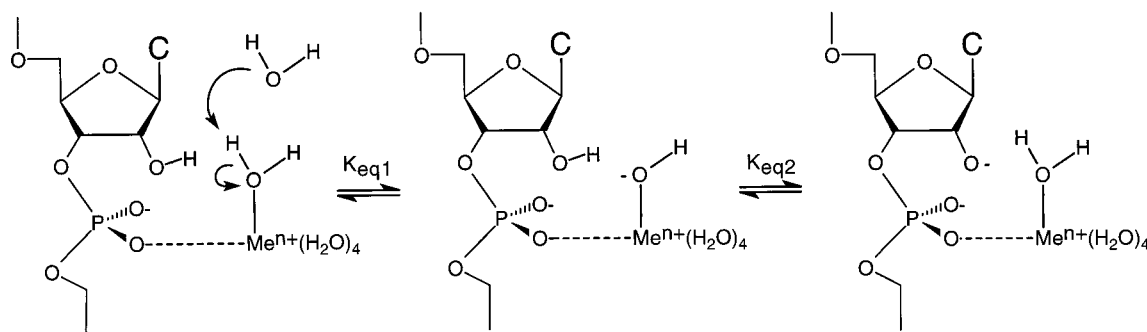
FIG. 4.   Solvated metal hydroxide model. The concentration of metal hydroxide coordinated to the pro-R oxygen depends upon the pK_a of a water molecule coordinated to the metal ion. Use of hydrated metal ions with lower pK_a values would produce a larger $K_{eq,1}$ but would also result in a smaller $K_{eq,2}$. Because the active nucleophile in the rate limiting step is the 2′-alkoxide and not the metal hydroxide, the observed pK_a correlation is not supported by a solvated metal hydroxide model.

of the metal ions on the equilibrium concentration of the active species in solution, i.e., the 2′-alkoxide.

Roles of the Two Metal Cations. We have proposed that the metal ion in binding site 1 is directly coordinated to the 2′-oxygen. The metal ion would then directly effect the pK_a of the 2′-OH and would result in the correlation that is experimentally observed. Although the equilibrium concentration of the resulting 2′-alkoxide would increase, this metal ion binding would also decrease the nucleophilicity of the 2′-alkoxide toward the electropositive phosphorus. However, because the nucleophilic attack does indeed take place in the rate-determining step, the sensitivity of the reaction rate to the strength of the nucleophile will be strongly dependent upon the position of the transition state.

Thus, if the transition state for the cleavage reaction occurs relatively early in the reaction process, k_{obs} would not be overly sensitive to the reactivity of the nucleophile, requiring instead that the 2′-OH simply be deprotonated. This would need to be true at least throughout the series of divalent metal ions previously studied (6). Coordination of a trivalent metal at this position reduces the nucleophilicity of the resulting alkoxide much more dramatically than the differences that would be expected within a series of divalent ions. When the La^{3+} ion is used in place of Mg^{2+}, a greater equilibrium concentration of the 2′-alkoxide is expected, whether or not direct coordination to the 2′-oxygen is assumed. However, direct coordination of the La^{3+} ion to the 2′-oxygen would result in the observed reduced rate of cleavage. This result cannot be reconciled with a metal hydroxide abstraction of the 2′-OH proton.
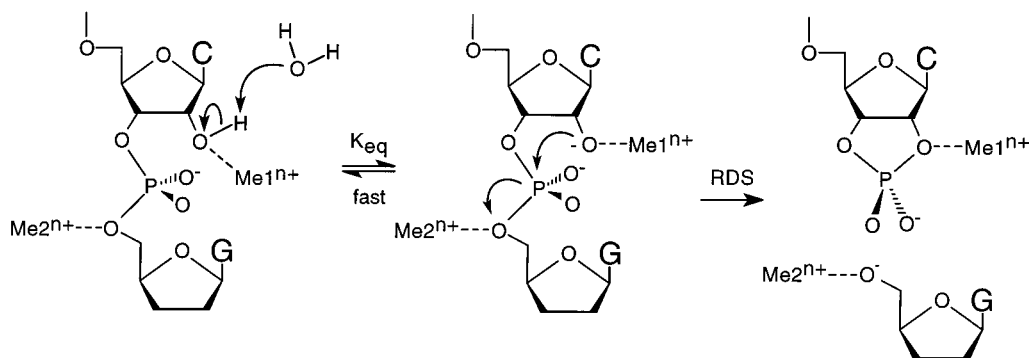
Estimation of the Binding Constants of La^{3+} to the Metal Ion Binding Sites of the Ribozyme. This displacement titration method allows indirect estimation of dissociation constants for metal ions that are difficult to measure directly. As discussed earlier, the K_d for the weaker binding Mg^{2+} ion is experimentally difficult to observe, and we have only been successful at estimating it as >50 mM. The problem arises largely from the unusually high concentrations of $MgCl_2$ that must be added to measure such a high dissociation constant. Because the concentration of La^{3+} bound to a metal ion binding site is equal to the concentration of Mg^{2+} bound to this same site at the half-equivalence point, it can be shown mathematically from the individual K_d expressions for these two ions that:

$$K_{d,Mg^{2+}} = K_{d,La^{3+}}([Mg^{2+}]/[La^{3+}]), \qquad [1]$$

where $[Mg^{2+}]$ is the constant concentration of $MgCl_2$ used in the experiment (8 mM) and $[La^{3+}]$ is the value of the $LaCl_3$ concentration read from the abscissa on the graph of Fig. 3 at the inflection point for each of the two titrations. Eq. **1** is only exact, of course, when (*i*) the total concentrations of Mg^{2+} and La^{3+} in solution are much greater than the concentrations of these ions bound at the metal ion binding site and (*ii*) the two-metal ion binding sites titrate independently of one another. Assumption *i* is obviously approximately true in our experiments because the concentration of ribozyme complexes (and thus of metal ion binding sites) used is 40 nM, whereas the total concentration of Mg^{2+} is 8 mM and the lowest concentration of La^{3+} used is 1 μM. The overall shape of the bell-shaped titration curve of Fig. 3 suggests that assumption

FIG. 5.   Modified two-metal ion model for ribozyme cleavage. The metal ion in binding site 1 (Me_1^{n+}) coordinates directly to the 2′ oxygen, lowering the pK_a of the 2′-OH proton. The 2′-OH proton is lost to the solvent in an equilibrium that is fast relative to the rate-determining cleavage step. The resulting 2′-alkoxide serves as the attacking nucleophile, displacing the 5′-oxygen of the leaving nucleotide (see arrows). The metal ion in binding site 2 (Me_2^{n+}) absorbs the developing charge on the leaving 5′-oxygen in the transition state of the rate-determining step. The nature of the metal ion coordination to the pro-R oxygen is not explicit in this model, although there is evidence for such an interaction. RDS, rate-determining step.

ii is also likely to be correct and that the titration of the two-metal ion binding sites is likely to be noncooperative.

In principle, if the K_d values for one-metal ion are known and the assumptions *i* and *ii* listed above apply, then the K_d values for the other metal ion can be calculated from Eq. **1**. By using the values of ≈ 3.5 mM and > 50 mM previously estimated for the K_d values for the binding of Mg^{2+} to the two sites in the absence of La^{3+}, we can use this relation to estimate the K_d values for La^{3+} at the two binding sites as ≈ 0.9 μM and > 37.5 μM, respectively. (We note that these values apply to a ribozyme titration carried out against a constant background of 200 mM NaCl.) Because these values are relatively small, it would be easier to measure these K_d values directly for La^{3+} than for Mg^{2+}. Then, with an experimentally measured binding constant for La^{3+} to the weaker binding site of the HH16 hammerhead ribozyme, an accurate value of the K_d for Mg^{2+} at that site can also be calculated. Such measurements are currently in progress.

Other Studies of Ribozyme Cleavage Rates with Two Different Metal Ions. Recently a conceptually similar competition experiment involving two different metal ions was reported for a "leadzyme" ribozyme (31). In that experiment, concentrations of Pb^{2+} and Nd^{3+} in the same reaction mixture were varied while keeping the total concentrations of these metal ions constant. An experiment of this sort requires that the binding affinities of the ribozyme for the two catalytically active metal ions be comparable. If these binding constants differ by more than an order of magnitude, the effects of the two different ions on the cleavage reaction will be obscured. In our titration experiment with La^{3+} against a constant concentration of Mg^{2+}, the binding affinities of the two ionic species for the putative binding sites are obviously very different, and thus such a varying mole fraction strategy could not be employed. In fact, as Fig. 3 shows, the titration of the first putative metal ion binding site begins at concentrations of La^{3+} as low as 2 μM in the presence of an 8 mM concentration of Mg^{2+}. Thus, for the HH16 hammerhead ribozyme, the apparent binding affinities of La^{3+} for both binding sites is approximately three orders of magnitude stronger than the binding affinities of Mg^{2+} for these sites. Because the dissociation constants for the La^{3+}–Mg^{2+} cation pair are so vastly different, a standard "varying mole fraction" type of experiment would have failed to demonstrate the different binding (and catalytic) effects of these two ions.

Structural Roles of Metal Ions? We must also consider the structural role(s) that divalent metal ions may play in maintaining a particular functional nucleic acid conformation in aqueous solution. Thus we were concerned that at the low concentrations of La^{3+} required for catalysis in the absence of Mg^{2+}, the ribozyme might lack sufficient metal ions to support the required conformation of the ribozyme complex during catalysis. In the titration experiment described herein, sufficient Mg^{2+} ions were present to satisfy such structural requirements, even at low concentrations of La^{3+}. It is interesting to note, however, that as the second metal ion binding site is titrated with La^{3+}, the value of k_{obs} approaches $\approx 2.2 \times 10^{-3}$ sec^{-1}, which is the same value obtained in solutions containing 3 μM $LaCl_3$ in the absence of $MgCl_2$ (see Fig. 3). This suggests that structural metal ion requirements, if any, are fully satisfied by La^{3+} alone, even at micromolar concentrations of this ion.

This conclusion may not be surprising, because the hammerhead is among the smallest ribozymes known and may well have simpler structural requirements than do the larger ribozyme systems. On the other hand, we find that no cleavage of the RNA substrate is observed at millimolar concentrations of $LaCl_3$ (data not shown), either in the presence or in the absence of 8 mM $MgCl_2$. This may mean that at millimolar concentrations the La^{3+} cation does bind to structural binding sites on the hammerhead ribozyme complex to produce a conformation that is unfavorable to cleavage. (We note that

this experiment also serves as a control to show that even relatively elevated concentrations of La^{3+} ion do not catalyze nonspecific degradation of the ribozyme under our reaction conditions.) By operating at micromolar concentrations of La^{3+} and (when present) at Mg^{2+} concentrations that are comparable to the dissociation constant for that ion from such putative structural binding sites, we have been able to avoid such potentially unfavorable conformational effects.

Conclusions. On the basis of the results presented herein, it is clear that a two-metal ion model for the mechanism of ribozyme action best explains the available data for the cleavage of the hammerhead HH16 ribozyme. Our data support the two-metal ion model illustrated in Fig. 5, requiring that metal ions directly coordinate to the attacking 2′-oxygen and to the 5′-oxygen of the leaving nucleotide. The metal ion coordinated at the 2′-oxygen lowers the pK_a of the attached proton, thereby increasing the equilibrium concentration of the attacking nucleophilic alkoxide species. The role of the putative second metal ion positioned at the leaving 5′-oxygen is to absorb the negative charge that accumulates at that position in the transition state.

Since our earlier examination of the mechanistic hammerhead ribozyme literature (12), several papers have been published that support the present version of the general two-metal ion model (11, 30–33). In addition to the leadzyme experiments (32) mentioned above, a very recent paper supporting a two-metal ion model presents data from competition experiments between Mg^{2+} and Ca^{2+} on the rate enhancement of the cleavage of the *Tetrahymena thermophila* L-21 *Sca*I ribozyme (11). Thus, including the work with the hammerhead ribozyme presented herein, three distinct ribozyme systems have now been shown to exhibit similar two-metal ion dependencies. It is increasingly clear that a general two-metal ion model is operating in these systems and that this model is likely to be common to most, if not all, ribozyme-catalyzed phosphodiester cleavage reactions. In addition, as predicted by Steitz *et al.* (3), this mechanism may also apply to many of the phosphoryl transfer reactions catalyzed by polymerases and related enzymes in the processes of gene expression and their regulation.

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