Effects of Divalent Metal Ions on Individual Steps of the *Tetrahymena* Ribozyme Reaction†

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Received January 13, 1997; Revised Manuscript Received May 8, 1997⊗

ABSTRACT: The *Tetrahymena thermophila* L-21 *Sca*I ribozyme utilizes Mg^{2+} to catalyze a site-specific endonuclease reaction analogous to the first step of self-splicing. To better understand the contribution of Mg^{2+} to ribozyme activity, the Mg^{2+} concentration dependence of individual rate constants was examined at concentrations greater than those required for ribozyme folding (>2 mM; at 50 °C and pH 6.7). Analysis of metal ion inhibition of the chemical step of the reaction indicated that two Ca^{2+} ions compete with two Mg^{2+} ions involved in active site chemistry. These Mg^{2+} ions are bound tightly to the E·S complex (K_d < 2 mM). The rate constant for association of the oligoribonucleotide substrate (S) increased 12-fold from 2 to 100 mM Mg^{2+} and exhibited saturation behavior, consistent with a single Mg^{2+} ion involved in S association that binds to the free ribozyme with a K_d for Mg^{2+} of 15 mM. The preference for the divalent metal ion ($Mg^{2+} \cong Ca^{2+} > Ba^{2+} \gg Sr^{2+}$) suggested that enhancing the rate constant of S association is not simply a function of ionic strength, but is due to a distinct metal ion binding site. Even though Ca^{2+} does not support reaction, the RNA substrate S was able to bind in the presence of Ca^{2+}. Upon addition of Mg^{2+}, S was cleaved without first dissociating. A model is proposed in which the inactive Ca^{2+} form of E·S is structurally equivalent to the open complex along the reaction pathway, which has the RNA substrate bound but not docked into the active site. Weaker binding of S in Ca^{2+} was shown to result from an increase in the rate constant of S dissociation, leading to the proposal that a tight Mg^{2+} binding site or sites in the E·S complex contribute to the strong binding of S. In summary, the data provide evidence for four functions for bound Mg^{2+} ions in the catalytic cycle: one increases the rate of RNA substrate binding, one or more decrease the rate of dissociation of S, and two are involved in the chemical step.

Catalytic RNAs are considered to be metalloenzymes (Guerrier-Takada et al., 1986; Cech et al., 1992; Pan et al., 1993; Pyle, 1993; Smith & Pace, 1993; Steitz & Steitz, 1993; Yarus, 1993). However, because RNA has so many divalent cation binding sites, it is difficult to separate the roles of individual ions. For group I ribozymes, Mg^{2+} has been shown to participate in RNA folding, substrate binding, and catalysis of transphosphoesterification (Grosshans & Cech, 1989; Pyle et al., 1990; Celander & Cech, 1991; Piccirilli et al., 1993). By manipulating the [Mg^{2+}] and by adding other competing divalent cations, we now demonstrate the involvement of Mg^{2+} in individual steps of the established reaction pathway and provide some characteristics of the sites holding these functionally important metals.

The L-21 *Sca*I ribozyme, a truncated form of the group I intron from *Tetrahymena thermophila*, catalyzes an endonuclease reaction (eq 1) (Zaug et al., 1988).[1,2]

$$\underset{\text{S}}{\overset{-3\ \ -1}{\text{GGCCCUCUpA(AAAA)}}} + \text{G} \rightleftharpoons \underset{\text{P}}{\text{GGCCCUCU}} + \text{GpA(AAAA)} \qquad (1)$$

The forward reaction is an intermolecular version of the first step of RNA self-splicing, the cleavage of the 5′-splice site by an intron-bound guanosine nucleophile, G (Zaug et al.,

† This work was supported by National Institutes of Health Grant GM28039 to T.R.C. D.H. is a Lucille P. Markey Scholar in Biomedical Science. T.R.C. is an investigator of the Howard Hughes Medical Institute and an American Cancer Society Professor. We thank the W. M. Keck Foundation for support of RNA science on the Boulder Campus.

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⊗ Abstract published in *Advance ACS Abstracts,* June 15, 1997.

[1] Abbreviations: E, *Tetrahymena* L-21 *Sca*I ribozyme; G, guanosine; pG, guanosine 5′-monophosphate; GpA, guanyl-3′−5′-adenosine; Mes, 2-(*N*-morpholino)ethanesulfonic acid; S and P, oligonucleotide substrate and product, respectively, whose identity depends on the experiment; IGS, internal guide sequence, which is the six nucleotides at the 5′ end of the ribozyme that base pair with S and P; E, enzymatic RNA or ribozyme; $(E\cdot S)_o$, open complex in which the substrate is bound only by base pairing to the IGS; $(E\cdot S)_c$, closed complex in which the substrate is bound and docked by tertiary interactions into the ribozyme active site; k_{on}, rate constant for association of an oligonucleotide substrate or product to E; k_{off}, rate constant for dissociation of an oligonucleotide substrate or product from E; $(k_{cat}/K_m)^S$, second-order rate constant for the reaction of E·G and S; $(k_{cat}/K_m)^G$, second-order rate constant for the reaction of E·S and G; $k_c(-G)$, observed rate of reaction of E·S in the absence of added G; $K_d(Mg^{2+})$, binding constant for Mg^{2+}; $K_d^{app}(Mg^{2+})$, apparent binding constant for Mg^{2+}.

[2] The four adenosines in parentheses were present in oligonucleotides used in some but not all of the experiments. For example, the reverse reaction described herein utilized GpA, not GpAAAAA. In several studies, the number of 3′ adenosines has been shown to have no significant effect on substrate binding or reactivity (McConnell et al., 1993; Narlikar et al., 1995, 1997).

S0006-2960(97)00067-6 CCC: $14.00

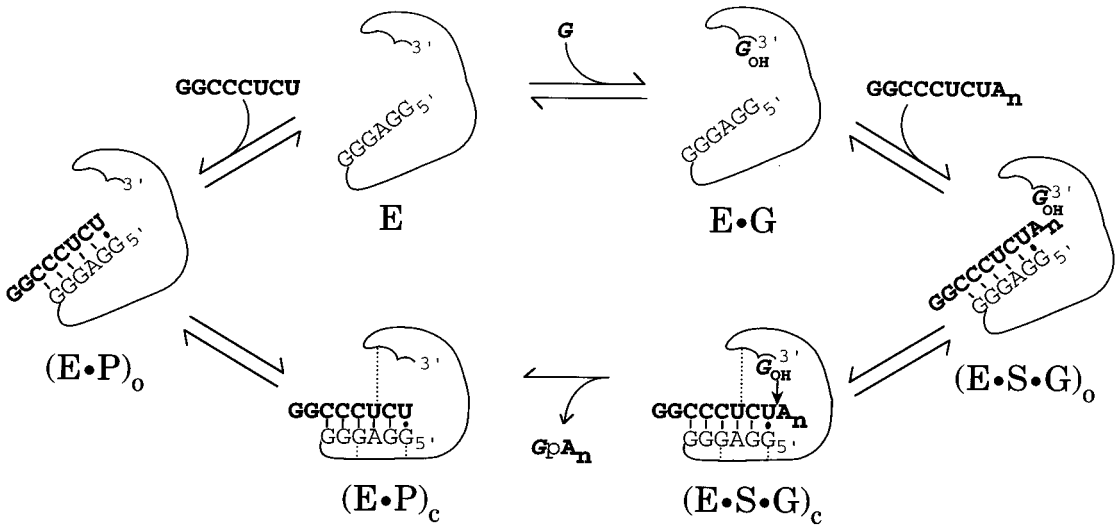


FIGURE 1: Endonuclease reaction catalyzed by the L-21 *Sca*I ribozyme, a shortened form of the *Tetrahymena* intron missing 21 nucleotides from its 5′ end and 5 nucleotides from its 3′ end (Zaug et al., 1988; Herschlag & Cech, 1990a; Herschlag, 1992). The ribozyme (E) binds guanosine (G) and an oligonucleotide substrate (S) in random order (not shown). The substrate shown here is GGCCCUCUA$_n$, where *n* indicates that different substrates contain different numbers of 3′ A residues. Binding of the substrate occurs in two steps. The first step forms the "open" complex [(E·S·G)$_o$] in which the substrate is bound primarily by base pairing with the internal guide sequence (5′-GGAGGG), forming helix P1. In the second step, the open complex docks into the core of the ribozyme via tertiary interactions (dotted lines) to form the "closed" complex [(E·S·G)$_c$]. Chemistry occurs in the closed complex, producing, in this case, GpA$_n$ and the oligonucleotide product P (GGCCCUCU). As with the binding of S, the dissociation of P occurs in two steps.

1986, 1988). Prior to this chemical step, the oligonucleotide substrate is bound in two steps: base pairing to the ribozyme's internal guide sequence (IGS), followed by docking of the resulting substrate helix into the active site (Bevilacqua et al., 1992; Herschlag, 1992; Figure 1). Docking is stabilized by tertiary interactions with several 2′-hydroxyl groups (U-3 of the substrate and G22 and G25 of the internal guide sequence) and with the exocyclic amine of the G22·U-1 wobble pair at the cleavage site (Pyle et al., 1992, 1994; Strobel & Cech, 1993, 1995; Knitt et al., 1994).

A variety of structural probes have shown that Mg^{2+} promotes tertiary structure formation of group I ribozymes (Latham & Cech, 1989; Downs & Cech, 1990; Heuer et al., 1991; Nakamura et al., 1995). Minimum estimates of the number of Mg^{2+} ions that bind during the folding process range from 3 to 8 (Celander & Cech, 1991; Wang & Cech, 1994). Tight binding of the RNA substrate in the closed complex requires Mg^{2+} (Pyle et al., 1990). Furthermore, a Mg^{2+} ion interacts with the 3′-oxygen at the cleavage site (U-1) to give specific transition state stabilization (Piccirilli et al., 1993). Phosphorothioate interference studies have identified phosphates likely to be involved in binding Mg^{2+} ions that promote the function of the intron in self-splicing (Christian & Yarus, 1992, 1993). Some of these sites may be important for folding the RNA into the active structure, while others may be directly involved in binding substrates or in catalysis.

The metal ion specificity of the ribozyme has also been studied. Group I RNA reactions require Mg^{2+} or Mn^{2+} (Cech & Bass, 1986). Ca^{2+} and Sr^{2+} can reduce the Mg^{2+}/Mn^{2+} requirement under steady state reaction conditions but cannot fully substitute (Grosshans & Cech, 1989). Fe(II)−EDTA structure mapping has shown that the Ca^{2+} and Sr^{2+} forms of the ribozyme fold in a manner similar to that of the Mg^{2+} form (Celander & Cech, 1991). By native gel binding studies, the Ca^{2+} form has been shown to bind oligonucleotides, although more weakly than the Mg^{2+} form of the ribozyme (Pyle et al., 1990). Yet, no reaction is observed in Ca^{2+} alone [$(k_{cat}/K_m)^S$ is reduced by more than a factor of 1700 relative to the value measured in Mg^{2+}; Celander & Cech, 1991]. A potential explanation for this large decrease came from studies utilizing a cross-linking probe on the IGS (which base pairs with S or P to form the substrate helix); the location of this helix relative to the rest of the ribozyme was different in Ca^{2+} versus Mg^{2+} (Wang et al., 1993). Interestingly, *in vitro* selection has identified sequence variants of the *Tetrahymena* ribozyme that can promote endonucleolytic cleavage of an RNA substrate in 10 mM Ca^{2+} without Mg^{2+} (Lehman & Joyce, 1993).

A better understanding of how Mg^{2+} and Ca^{2+} affect individual steps of the ribozyme reaction should help elucidate the reason Mg^{2+} is special for catalysis. In the current work, we identify specific contributions of Mg^{2+} ions in promoting the binding of the RNA substrate. Furthermore, we provide experimental evidence for a model with two metal ions in the active site of a group I ribozyme.

MATERIALS AND METHODS

L-21 ScaI Ribozyme. Ribozyme (E) was prepared by transcription of the *Sca*I-cut pT7L-21 DNA template using T7 RNA polymerase and purification by polyacrylamide gel electrophoresis and size exclusion chromatography (Zaug et al., 1988). The ribozyme concentration was determined spectrophotometrically (Zaug et al., 1988). The concentration was also confirmed by an active site titration experiment, as follows. The second-order rate constant, $(k_{cat}/K_m)^S$, for reaction of the weak-binding substrate ^{32}pGGCCCGCUAAAAA (<1 nM) was measured using subsaturating E (50 nM). Increasing concentrations of the matched product, P (GGCCCUCU, 10−200 nM), were added to reaction mixtures until all ribozyme was bound with P and only a slow reaction rate was observed. After subtraction of this slow rate, the rate was plotted against [P]; extrapolation gave an *x*-intercept which equals the [P] required for complete inhibition. This method provides an accurate kinetic measurement of the fraction of the ribozyme (95 ± 10%) that can bind P. This measurement agreed with the spectropho-

tometric calculation of [E]. Treatment of ribozyme with calf intestine phosphatase or use of a ribozyme constructed without phosphates at the 5′ end (Strobel & Cech, 1993) did not affect the values of $(k_{cat}/K_m)^S$, $(k_{cat}/K_m)^G$, or $k_c(-G)$ at several Mg^{2+} concentrations.

Oligonucleotide Substrates and Products. All RNA oligonucleotides that start with two guanosine residues (GGCCCUCU, GGCCCUCUAAAAA, and GGCCCUCUAGU) were transcribed from synthetic DNA templates by T7 RNA polymerase (Lowary et al., 1986; Milligan et al., 1987; Zaug et al., 1988) and ethanol precipitated. All other oligonucleotides were synthesized chemically on an Applied Biosystems (ABI) model 380B DNA synthesizer or model 394 DNA/RNA synthesizer (Wu et al., 1989; Scaringe et al., 1990) using phosphoramidites (ABI, American Bionetics, Milligen Biosystems, or Glen Research). All oligonucleotides were purified by electrophoresis on 20% polyacrylamide/7 M urea gels. After cutting out the UV-visualized gel bands, elution, and removal of the gel matter by centrifugation and filtration, the RNA was precipitated in ethanol and resuspended in 10 mM Tris·HCl and 1 mM EDTA at pH 7.5. Concentrations were measured spectrophotometrically at 260 nm, using an extinction coefficient that is the sum of those of the individual nucleotides (P-L Biochemicals Circular OR-10). For ^{32}P labeling, the 5′-phosphates of transcribed oligonucleotides were removed by treatment with calf intestine phosphatase. All oligonucleotides were then 5′ end-labeled by treatment with T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]-ATP (New England Nuclear) and purified by electrophoresis on a 20% polyacrylamide/7 M urea gel, essentially as described by Zaug et al. (1988). Concentrations of labeled oligonucleotides were estimated on the basis of specific activity.

Kinetics. All reactions were carried out at 50 °C in 50 mM Mes sodium salt, which was measured to be pH 7.0 at 25 °C and calculated to be pH 6.7 at 50 °C [$\Delta pK_a = 0.011$ °C^{-1} for Mes (Good et al., 1966)] as previously described (Herschlag & Cech, 1990a). The ionic strength was not kept constant by addition of monovalent ions, because such ions behave as competitive inhibitors (Knitt & Herschlag, 1996). For each reaction, samples were preincubated in the presence of buffer and divalent metal ion with or without G for 20 min to allow formation of a single active species (≥90%) of ribozyme, as previously described (Herschlag & Cech, 1990a; McConnell et al., 1993). As long as the concentration of divalent ion was above that required for folding, preincubation at various divalent concentrations or preincubation at 10 mM Mg^{2+} and then dilution into various divalent concentrations in the reaction gave similar ribozyme activity [differences in $(k_{cat}/K_m)^S$, $(k_{cat}/K_m)^G$, and $k_c(-G)$ were always ≤50% and typically ≤20%]. It is therefore unlikely that the ribozyme undergoes slow conformational changes subsequent to initial metal addition when metal ion conditions are altered within the concentration range studied. Reaction was initiated by either addition of oligonucleotide or addition of ribozyme preincubated in Mg^{2+}-containing buffer to the rest of the reaction components. Typically, six 1.5−2 μL aliquots were removed from a 20 μL reaction mixture and quenched with 3 volumes of stop buffer containing 80% formamide, 50 mM EDTA, 0.01% bromophenol blue, 0.01% xylene cyanole, and 2 mM Tris·boric acid at pH 7.5. When reactions were followed for more than 1 h, the solutions were kept submerged and/or centrifuged periodically to prevent condensation of the sample by evaporation. Reaction products were separated on a 20% polyacrylamide/7 M urea gel. The fraction of the substrate remaining relative to the total substrate and product(s) at each time point was quantitated using an AMBIS radioanalytic scanner or using a PhosphorImager (Molecular Dynamics).

Single-turnover kinetics were performed with excess (10-fold or greater) ribozyme and only trace amounts (<0.5 nM) of the labeled substrate. First-order kinetics could be observed for about three half-lives. In multiple-turnover kinetic experiments, initial rates corresponding to the first 20% of reaction were used to obtain rate constants. Labeled oligonucleotides had a single 5′-phosphate, and those added unlabeled had 5′-triphosphate ends.

Estimation of error limits is essentially as previously discussed (Herschlag & Cech, 1990a). The deviation of rates of the identical experiment on the same day can be as great as 20% but is typically less than 10%. Ribozyme activity can vary as much as 2-fold; however, the overall effects from varying [Mg^{2+}] were within 20% from day to day.

Divalent Cations. Trace amounts of EDTA from RNA purification were calculated never to exceed 0.1 mM and for inhibition experiments involving divalent metal ions never exceeded 0.01 mM. Several stocks of $MgCl_2$, as well as $MgSO_4$ and $Mg(C_2H_3O_3)_2$, showed identical kinetic behavior in side-by-side reactions. Accurate concentrations of $CaCl_2$, $BaCl_2$, $SrCl_2$, and $MgCl_2$ were determined from the density of the stock solutions (*CRC Handbook of Chemistry and Physics*, 66 ed., 1985). In several experiments, both pure (Mallinckrodt) and ultrapure (Aldrich) $CaCl_2$ and $MgCl_2$ gave no difference in the measured rate constants.

Pulse-Chase Experiments. "Pulse-chase" experiments were performed essentially as described previously (Herschlag & Cech, 1990a). To measure the rate constant of oligonucleotide association, 5−50 nM ribozyme preincubated (20 min) with the desired divalent metal ion concentration and Mes buffer at pH 6.7 was mixed with labeled oligonucleotide (≤1 nM). At different times, aliquots were diluted 10-fold into a chase solution of excess unlabeled CCCUCU (1 μM, ≥20[E]), 1 mM G, 10 mM Mg^{2+}, and 50 mM Mes at pH 6.7. After 2 min to allow all the bound substrate to react, aliquots were quenched into ∼3 volumes of stop buffer.

To measure the rate constant of oligonucleotide dissociation, saturating ribozyme (100−1000 nM) preincubated for 20 min with the desired divalent metal ion and Mes buffer at pH 6.7 was allowed 15 s to 2 min to fully bind the labeled oligonucleotide (≤1 nM). The solution was then diluted 5- or 10-fold into excess GGCCCUCU (1−2 μM, >20[E]) in the presence or absence of 1 μM G with the desired divalent metal ion and Mes buffer. At varying times, aliquots were quenched into ∼3 volumes of stop buffer. In the case where k_{off}^S was measured in the presence of inhibitory Ca^{2+}, at varying times, samples from the first chase reaction were diluted 2−10-fold into a second chase containing excess (100 mM) Mg^{2+} and 1 mM G to promote reaction of all bound S. After the second chase had proceeded for 2−3 min, aliquots were quenched into ∼3 volumes of stop buffer.

To measure an equilibrium dissociation constant for the E·S complex in 10 mM Ca^{2+}, varying concentrations of E (2−2000 nM) preincubated as above were mixed with labeled substrate and allowed time to reach equilibrium (2−20 min). Then, aliquots were diluted into excess GGCCCUCU (1−2 μM, ≥10[E]), 100 mM Mg^{2+}, and 1 mM G to promote the reaction of bound S and prevent the reaction

of unbound S. After 2−5 min, aliquots were quenched as above. In each of the pulse-chase experiments, reversing the order of addition of the chase solution and the labeled oligonucleotide resulted in negligible reaction, indicating that the chase was effective. Also, the concentration of unlabeled GGCCCUCU was varied to ensure all free ribozyme was bound during the chase.

RESULTS

Mg^{2+} Enhances the Rate Constant of Oligonucleotide Association. Kinetic measurements were performed at a Mg^{2+} of ≥2 mM, because at these concentrations the ribozyme appears to be fully folded as judged by a photocross-link that monitors tertiary structure and by Fe(II)−EDTA free radical probing at 45 °C (Downs & Cech, 1990; Celander & Cech, 1991). At each [Mg^{2+}], the second-order rate constant, $(k_{cat}/K_m)^S$, for a single turnover was determined. A 12-fold increase in $(k_{cat}/K_m)^S$ was observed from 2 to 100 mM Mg^{2+} (Figure 2A). Previously, it was shown that at 10 mM Mg^{2+} this second-order rate constant is equal to the rate constant of S association, k_{on}^S (Herschlag & Cech, 1990a). To test whether $(k_{cat}/K_m)^S$ is equal to k_{on}^S throughout the Mg^{2+} concentration range, pulse-chase experiments were used to measure k_{on}^S directly. These values of k_{on}^S were the same as those measured for $(k_{cat}/K_m)^S$ [$(2.0 \pm 0.5) \times 10^7$ and $(1.8 \pm 0.5) \times 10^7$ M^{-1} min^{-1}, respectively, at 2 mM Mg^{2+} and $(2.5 \pm 0.5) \times 10^8$ and $(2.6 \pm 0.5) \times 10^8$ M^{-1} min^{-1}, respectively, at 100 mM Mg^{2+}]. Thus, the rate of association of the RNA substrate is enhanced by increasing [Mg^{2+}]. While the change in ionic strength upon increasing [Mg^{2+}] may contribute to this enhancement, the effects of changing the ion identity provide evidence for ion binding rather than simple change screening (see below).

The [Mg^{2+}] effect on $(k_{cat}/K_m)^S$ with a variety of substrates (S = GGCCCUCUpAAAAA, CCCUCUpA, or GGCCCUCUpAGU, where the phosphate at the cleavage site is shown) fit a simple binding curve with a $K_d^{app}(Mg^{2+})$ of 15 ± 4 mM (Figure 2A and data not shown). A larger rate effect (~60-fold) was seen with a substrate that forms a single G·A mismatch with the ribozyme at position −3 from the cleavage site (GGCCC**G**CUpAAAAA); in this case, half-maximal velocity occurred at ~20 mM Mg^{2+} (data not shown). This curve was distinctly sigmoidal, indicating that more than one Mg^{2+} was involved in increasing the reaction rate. For this mismatched substrate, the binding and chemical steps both contribute to $(k_{cat}/K_m)^S$ (Herschlag & Cech, 1990b).

The rate of association of oligonucleotide product P increased 4-fold from 2 to 100 mM Mg^{2+} as measured by pulse-chase experiments (Figure 2A). The enhancement, which is smaller for binding of P than for binding of S, arises because product association is significantly faster than that of substrate at low [Mg^{2+}]. The apparent binding of Mg^{2+} [$K_d^{app}(Mg^{2+}) = 15 \pm 6$ mM; Figure 2A] is similar in the two cases. Because the product of the forward reaction (P) is the substrate for the reverse reaction, Mg^{2+} facilitates oligonucleotide substrate association in both the forward and reverse reactions. In pre-steady state experiments, the value of the apparent second-order rate constant, $(k_{cat}/K_m)^{P,app}$, for the reverse reaction of E, GpA, and P was shown to increase 20-fold from 2 to 100 mM Mg^{2+} [at 1 mM GpA, $(k_{cat}/K_m)^{P,app} = (7 \pm 2) \times 10^6$ and $(1.4 \pm 0.2) \times 10^8$ M^{-1} min^{-1} at 2 and 100 mM Mg^{2+}, respectively], consistent with a Mg^{2+} effect on k_{on}^P. The effect, which is larger on $(k_{cat}/K_m)^{P,app}$ than on k_{on}^P, is consistent with the observation that, at low [Mg^{2+}], $(k_{cat}/K_m)^{P,app}$ may be at least partially limited by the chemical step of the reverse reaction, a step which is itself Mg^{2+}-dependent (McConnell, 1995).

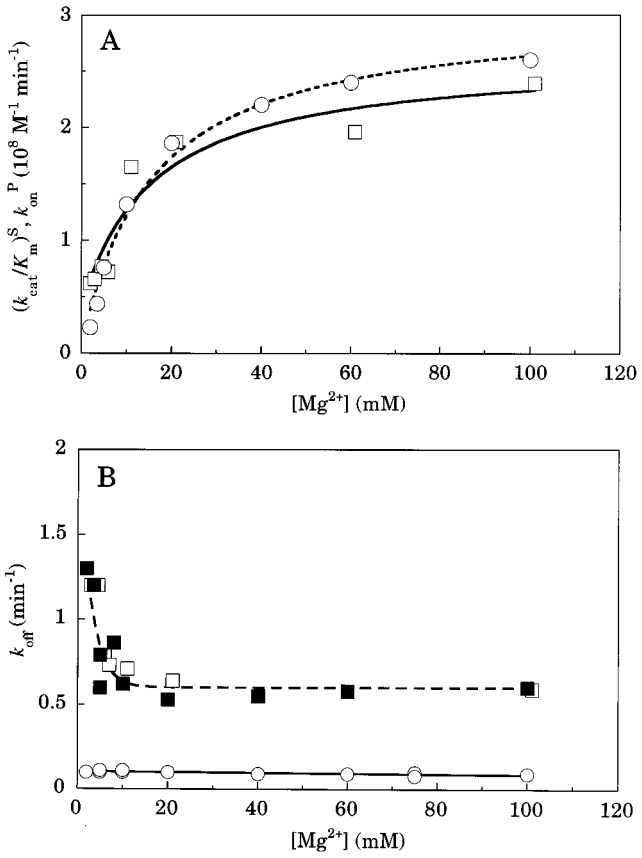


FIGURE 2: Mg^{2+} effect on binding of the oligonucleotide substrate and product. (A) The second-order rate constant $(k_{cat}/K_m)^S$ was measured as a function of [Mg^{2+}]. Single-turnover reactions with 3−5 nM ribozyme, trace amounts of [5′-^{32}P]GGCCCUCUAAAAA (○), and high G concentrations (1 mM) to ensure rapid cleavage of any bound substrate are shown. The second-order rate constant for the association of product, k_{on}^P, was measured by pulse-chase experiments; 5−10 nM ribozyme and trace ^{32}pGGCCCUCU (□) were allowed to bind for varying times and then chased into 2 mM GpA and 100 nM GGCCCUCU to ensure rapid ligation of any bound product. $K_d^{app}(Mg^{2+})$ values of 15 ± 4 and 15 ± 6 mM for E (unsaturated with G) reacting with substrate and product, respectively, were determined using least-squares fits to the equation $k_{obs} = k_o + (k_{cat}/K_m)^{S,max}[Mg^{2+}]/(K_d^{app}(Mg^{2+}) + [Mg^{2+}])$. The $(k_{cat}/K_m)^{S,max}$ value of $(3.0 \pm 0.4) \times 10^8$ M^{-1} min^{-1} [or $(k_{cat}/K_m)^{P,max} = (2.2 \pm 0.4) \times 10^8$ M^{-1} min^{-1}] is the second-order rate constant for reaction of free enzyme and oligonucleotide at saturating Mg^{2+}. The k_o values, which are the rates in the absence of Mg^{2+}, were determined by extrapolation of the data at low Mg^{2+} concentrations, since the ribozyme is denatured when [Mg^{2+}] = 0. The resulting uncertainty in k_o does not significantly affect the determination of $K_d^{app}(Mg^{2+})$. (B) Values of k_{off} for GGCCCUCU (○) and for GGCCCUCUA (□) as a function of [Mg^{2+}] were taken to be equal to k_{cat} for multiple-turnover reactions. Reaction mixtures (○) contained 0.1 μM E, 1 μM [5′-^{32}P]GGCCCUCUAAAAA, and 1 mM G, while reaction mixtures (□) contained 0.1 μM E, 1 μM [5′-^{32}P]GGCCCUCU, and 1 mM GpA. The k_{off} value for GGCCCUCUAAAAA (■) was directly measured in pulse-chase experiments (see Materials and Methods).

Increasing the [Mg^{2+}] Has Only a Modest Effect on Oligonucleotide Dissociation. The Mg^{2+} dependencies of the dissociation rates were measured by two methods: (1) multiple-turnover experiments, in which k_{cat} for the forward and the reverse reactions is rate-limited by the dissociation of the reaction product (GGCCCUCU and GGCCCUCUA, respectively) (data not shown; Herschlag & Cech, 1990a),

Table 1: Effect of Mg^{2+} on the Binding of Oligonucleotides to the Ribozyme[a]

	2 mM Mg^{2+}	100 mM Mg^{2+}
k_{on}^S (M^{-1} min^{-1})	2.0×10^7	2.5×10^8
k_{on}^P (M^{-1} min^{-1})	6.0×10^7	2.2×10^8
k_{off}^S (min^{-1})	1.2	0.6
k_{off}^P (min^{-1})	0.1	0.1
K_d^S (nM)	60	2
K_d^P (nM)	2	0.5

[a] Values reflect binding to the free ribozyme, although in the case of k_{on}^S similar values were obtained for the E·G complex by measuring $(k_{cat}/K_m)^S$. k_{off}^P values measured by multiple-turnover experiments are in the presence of subsaturating G (1 mM G, $K_m^G = 2$ mM for E·P; McConnell et al., 1993). K_d is calculated as k_{off}/k_{on}.

and (2) pulse-chase experiments to measure k_{off} of GGCCCUCUAAAAA. As shown in Figure 2B, both methods give rate constants of substrate dissociation (k_{off}^S) and product dissociation (k_{off}^P) that have $[Mg^{2+}]$ dependencies different from that seen for k_{on}^S. There is only an ~2-fold decrease in k_{off}^S and essentially no change in k_{off}^P from 2 to 100 mM Mg^{2+}.

The increased difference between dissociation of S (□ and ■) and P (○) at low $[Mg^{2+}]$ may be another manifestation of the ground state destabilization of S bound in the active site. The destabilization has been localized to interactions at the reaction site phosphate, a moiety which is not present in the oligonucleotide product P, and has been suggested to arise from an interaction of a Mg^{2+} ion with the 3′-oxygen of this phosphate (Piccirilli et al., 1993; Narlikar et al., 1995). Even though an observed rate constant is unaffected by $[Mg^{2+}]$ in the range of 2−100 mM, a tightly bound Mg^{2+} ($K_d \leq 2$ mM) may be involved in the strong binding of the oligonucleotide in the closed complexes. The overall effects of $[Mg^{2+}]$ on oligonucleotide association and dissociation are compared in Table 1.

Specificity for Mg^{2+} in Enhancing $(k_{cat}/K_m)^S$. It seemed possible that the mechanism by which Mg^{2+} increases the rate of RNA substrate binding could involve an electrostatic effect in which Mg^{2+} ions in solution facilitate the association of the two polyanionic RNA molecules. The model of cation condensation is well documented for nucleic acid duplex formation; the effect does not involve cation binding, and it is a function of the ionic strength and ion charge (De Marky & Manning, 1976; Manning, 1978; Record et al., 1978; Record & Lohman, 1978; Williams et al., 1989).

To determine if the observed rate enhancements of $(k_{cat}/K_m)^S$ by Mg^{2+} were the result of nonspecific cation condensation effects, monovalent cations (Na^+, Li^+, K^+, Cs^+, and NH_4^+) and divalent cations (Ba^{2+}, Ca^{2+}, Sr^{2+}, Mn^{2+}, Cu^{2+}, Ni^{2+}, and Cd^{2+}) were added to reaction mixtures containing 2 and 10 mM Mg^{2+}. Monovalent ions added at 10 or 100 mM had no effect on the rate, even at 2 mM Mg^{2+}. (Note that ~50 mM Na^+ ion is already present in the reaction as a counterion for the Mes buffer.) Cu^{2+}, Ni^{2+}, and Cd^{2+} were inhibitory without visible precipitate. Mn^{2+} at 2 mM did not affect the rate, but precipitate was observed at higher concentrations. Ba^{2+}, Ca^{2+}, and Sr^{2+} showed both stimulatory and inhibitory effects on the rate (see below), without showing any evidence of precipitates. These alkaline earth metal ions do not by themselves promote catalysis for this ribozyme (Celander & Cech, 1991). Also, structural studies have shown that these cations promote the same general ribozyme structure as Mg^{2+} [data not shown and Celander and Cech (1991)], although the local structure may vary (Wang et al., 1994). The effects of these ions were examined more closely.

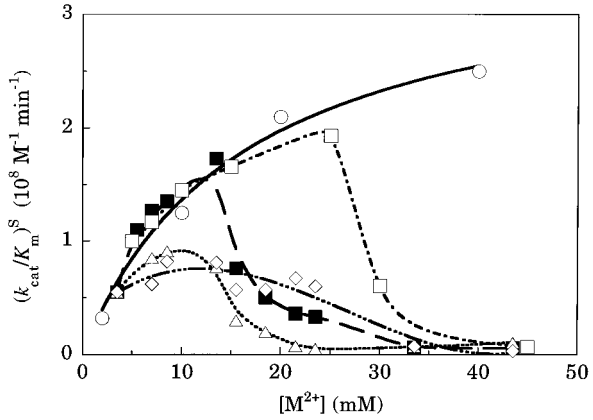


FIGURE 3: Rate enhancement and inhibition by Ca^{2+}, Ba^{2+}, and Sr^{2+}. Values of $(k_{cat}/K_m)^S$ were determined under single-turnover conditions (3−5 nM E, trace concentrations of ^{32}pGGCCCUCUAAAAA, and 1 mM G) with increasing $[Mg^{2+}]$ (○, solid line), 3.5 mM Mg^{2+} and increasing $[Ca^{2+}]$ (■, dashed line), 5 mM Mg^{2+} and increasing $[Ca^{2+}]$ (□, dashed, one-dot line), 3.5 mM Mg^{2+} and increasing $[Ba^{2+}]$ (△, dotted line), and 3.5 mM Mg^{2+} and increasing $[Sr^{2+}]$ (◇, dashed three-dot line). $[M^{2+}]$ represents the total divalent metal ion concentration. Except for the data for Mg^{2+} alone, the curves are presented only to follow the trends and do not represent theoretical fits to the data.

The value of $(k_{cat}/K_m)^S$ was measured at low concentrations of Mg^{2+} with increasing concentrations of Ca^{2+}, Ba^{2+} and Sr^{2+} (Figure 3). As $[Ca^{2+}]$ was increased with 3.5 (■) or 5 mM Mg^{2+} (□), the observed rate constant first increased and then decreased. Addition of Ca^{2+} up to ~10 mM gave an observed rate constant that mimicked the curve with Mg^{2+} only, suggesting that Ca^{2+} can substitute for Mg^{2+} in stimulation of $(k_{cat}/K_m)^S$. The inhibitory effect of Ca^{2+} occurred at a higher $[Ca^{2+}]$ with 5 mM Mg^{2+} than with 3.5 mM Mg^{2+}, suggesting that Ca^{2+} is competing with Mg^{2+}. Since there are two effects, one in which Ca^{2+} duplicates the function of Mg^{2+} and another in which it inhibits the function of Mg^{2+}, at least two classes of metal binding events are being observed.

Compared to Ca^{2+}, Ba^{2+} gave only a slight rate enhancement when added to 3.5 mM Mg^{2+} (△) and produced inhibition at lower concentrations. However, Sr^{2+} added to 3.5 mM Mg^{2+} (◇) showed almost no rate enhancement and even weaker inhibition than Ca^{2+} (compare to ■). Although the Ba^{2+} data are consistent with a counterion condensation model, the Sr^{2+} data are not easily reconciled. Having the same charge as Mg^{2+} but being the weakest inhibitor, Sr^{2+} should have produced the largest rate enhancement before inhibition was observed, if the counterion condensation model correctly described the Mg^{2+} effect on k_{on}^S (Figure 2A). Instead, Sr^{2+} appears to fit poorly into a binding site that has some specificity for Mg^{2+} (ionic radii are 0.65 Å for Mg^{2+}, 0.99 Å for Ca^{2+}, and 1.13 Å for Sr^{2+}; Huheey, 1983). Moreover, the inhibition does not appear to be an aggregation effect since no precipitate was seen and the inhibition was relieved by the addition of more Mg^{2+} (data not shown). The strong inhibition seen at higher $[Ca^{2+}]$ and $[Ba^{2+}]$ occurs over a concentration range too narrow to be explained by a model of one inhibition site (curve fits not shown). As described below, the decrease in $(k_{cat}/K_m)^S$ at higher competitor cation concentrations can be explained by a change in the rate-limiting step from binding to the

Scheme 1

(A) $E + Ca^{2+} \xrightarrow{(1)} E^{Ca} \xrightarrow{(2)} E^{Ca} \cdot S^* \xrightarrow[P,\ Mg^{2+},\ G]{(3)} E^{Mg} + P^*$

(B) $E + Mg^{2+} \xrightarrow{(1)} E^{Mg} \xrightarrow{(2)} E^{Mg} \cdot S^* \xrightarrow[P,\ Ca^{2+},\ G]{(3)} E^{Ca} + S^*$

chemical step. The inhibition over a narrow Ca^{2+} concentration range presumably arises because there are two Ca^{2+} ions that inhibit the chemical step and two or more Ca^{2+} ions that weaken substrate binding (see below).

Catalysis after Formation of the E·S Complex in Ca^{2+}. It has been shown that the ribozyme can fold in Ca^{2+} in a manner similar to that in Mg^{2+} and is also competent to bind S (Pyle et al., 1990; Celander & Cech, 1991). If the Ca^{2+} form of the ribozyme resembles the active ribozyme structure, it seemed possible that substrate bound to the Ca^{2+}-folded ribozyme might be able to react upon addition of Mg^{2+} without first dissociating and rebinding. This possibility was tested using a "mixed-metal" pulse-chase experiment. As shown in Scheme 1, ribozyme (1 μM) was incubated in the presence of 10 mM Ca^{2+} (A, step 1) or 10 mM Mg^{2+} (B, step 1) for 20 min. Then, ^{32}pGGCCCUCUAAAAA (S*) was allowed to bind to each form of the ribozyme (step 2, 0.25 min) followed by a 10-fold dilution into a solution of 2 μM GGCCCUCU, 1 μM or 1 mM G, and 10 mM Mg^{2+} (A, step 3) or 10 mM Ca^{2+} (B, step 3). Time points were then taken to follow the fate of S*.

When the substrate was bound to the Ca^{2+} form of the ribozyme and then diluted into Mg^{2+}, it reacted completely with a single-exponential decay (or prior to the first time point when 1 mM G was used in the chase) as if substrate were originally bound in Mg^{2+} (data not shown). However, the Mg^{2+} form of the E·S* complex did not react when diluted into 10 mM Ca^{2+} in the presence of 1 mM G and excess GGCCCUCU. (A small fraction of S reacted during the binding step as determined by stopping the reaction after binding in step 2.) Two conclusions can be discerned from these experiments. (1) Exchange of the metal ions important for catalysis is fast relative to the binding and reaction of G, and (2) no slow conformational changes are required to change from the Ca^{2+} form to the Mg^{2+} form of the ribozyme [i.e., none with a $t_{1/2}$ of $\geq$10 s, based on the fact that reaction was complete at the first time point taken (15 s) and based on the estimate of a $t_{1/2}$ of $\simeq$10 s for dissociation of S from the open complex]. Because docking of the substrate helix is estimated to occur with a $t_{1/2}$ of $\simeq$1 ms at this temperature (50 °C) and [Mg^{2+}] (G. J. Narlikar, M. Khosla, and D. Herschlag, unpublished results), there is plenty of time for P1 to dock after addition of Mg^{2+} and before the first time point. These results are consistent with the Ca^{2+} form of the ribozyme having a structure close to that which is catalytic, if only Mg^{2+} ions could replace Ca^{2+} ions in the metal sites essential for proper substrate orientation and catalysis.

Using the mixed-metal pulse-chase method, the equilibrium binding constant, K_d(S), of substrate and ribozyme in 10 mM Ca^{2+} was determined (Figure 4A). The value of K_d(S) is 80 $\pm$ 20 nM, representing a binding of S 40-fold weaker in 10 mM Ca^{2+} than in 10 mM Mg^{2+}. The dissociation constant in Ca^{2+} is similar to values of $\sim$100 nM obtained in 10 mM Mg^{2+} for simple oligonucleotide duplexes and for E·S complexes trapped in the open state (Narlikar & Herschlag, 1996; Narlikar et al., 1997). This

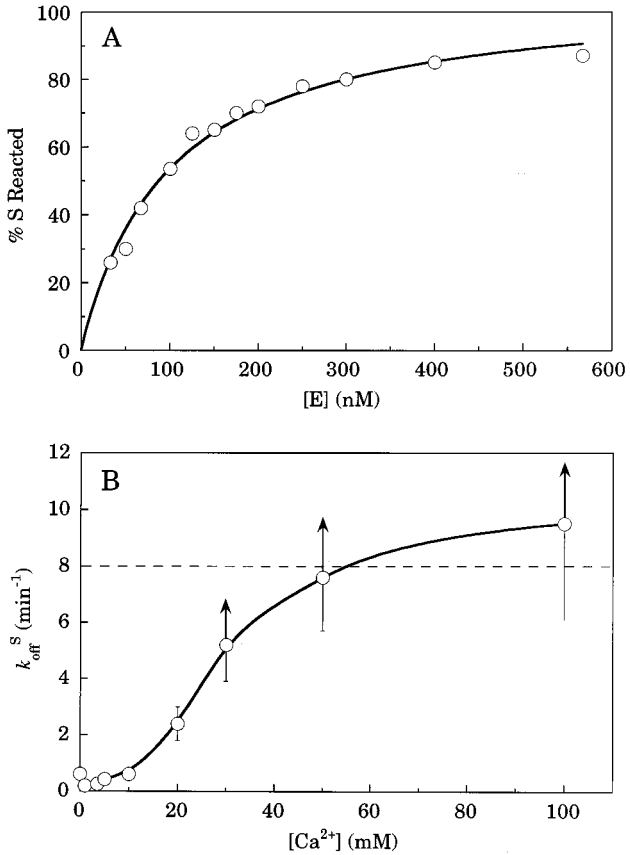


FIGURE 4: RNA substrate bound in Ca^{2+} is rapidly cleaved upon addition of Mg^{2+}. (A) In mixed metal pulse-chase experiments, ^{32}P-labeled substrate was bound in 10 mM Ca^{2+} at various E concentrations, allowed to reach equilibrium over 5 min, and then diluted 10-fold into 1 mM G, 10 mM Mg^{2+}, and 2 μM GGCCCUCU to allow reaction of bound S. The K_d of 80 $\pm$ 20 nM for GGCCCUCUAAAAA in 10 mM Ca^{2+} was obtained from the fit to a single-site binding equation (solid line), assuming a maximum of 100% binding. Variation in the time of incubation or a larger dilution in the chase did not affect the results. (B) The rate constant of the dissociation of GGCCCUCUAAAAA (k_{off}^S) was measured as a function of [Ca^{2+}] at 3.5 mM Mg^{2+} in mixed-metal pulse-chase experiments. At high Ca^{2+} concentrations, the rate of S dissociation becomes too fast to measure accurately, and the arrows indicate that the measured values should be considered lower limits. The dashed line (8 min^{-1}) represents the estimated rate of substrate dissociation in 10 mM Ca^{2+} alone, which was determined using the K_d^S value of 80 nM (part A) and an estimated k_{on}^S value of 10^8 M^{-1} min^{-1}. This estimate is based on the observation that binding appears to represent simple duplex formation, which should be similar in Ca^{2+} and Mg^{2+}, and the estimate is consistent with the limit of $k_{on}^S \geq 4 \times 10^7$ M^{-1} min^{-1} in Ca^{2+} (see the text).

supports the suggestion that in Ca^{2+} the substrate binds the ribozyme in an open complex. Binding of GGCCCUCU being considerably weaker in 10 mM Ca^{2+} than in 10 mM Mg^{2+} had been seen previously using native gel methods at 25 °C (Pyle et al., 1990). Further, in similar mixed-metal pulse-chase experiments, lower limits could be set for a k_{on}^S of $>4 \times 10^7$ M^{-1} min^{-1} and a k_{off}^S of >4 min^{-1} at 50 °C and 10 mM Ca^{2+} (data not shown). This limit for k_{on}^S is close to the observed value of $\sim 10^8$ M^{-1} min^{-1} for binding in 10 mM Mg^{2+}, but k_{off}^S is >20 times larger than that in 10 mM Mg^{2+}. These values confirm the conclusion that Mg^{2+} plays a specific role in binding the oligonucleotide (Pyle et al., 1990) and are consistent with one or more Mg^{2+} ions decreasing k_{off}^S.

In a similar mixed-metal pulse-chase experiment, the effect of Ca^{2+} on k_{off}^S at 3.5 mM Mg^{2+} was demonstrated (Figure 4B). The amount of Ca^{2+} required to increase k_{off}^S to $^1/_2$

Table 2: Divalent Cation Inhibition of the Chemical Step [$(k_{cat}/K_m)^G$] of the Ribozyme Reaction[a]

inhibitor	$[Mg^{2+}]$ (mM)	IC_{50} (mM)[b]	$[I]_{90}/[I]_{10}$[c]	$K_i^{app,1}$ (mM)[d]	$K_i^{app,2}$ (mM)[d]	Hill plot m_1[e]	Hill plot m_3[e]
Ca^{2+}	2	0.3	20	0.4	1.0	1	2
	3.5	0.5	25	0.6	1.5	1.4	2.3
	5	1.6	19	1.5	3.5	0.9	2.1
	10	2	23	4	7	1.1	2.5
Sr^{2+}	3.5	3.7	20	4	12	1.0	2.0

[a] Values are accurate to within ±25%, on the basis of the goodness of fit of theoretical curves spanning a range of K_i values. [b] Concentration giving 50% inhibition. [c] Ratio of inhibitor concentration that gave 90% inhibition of the reaction rate to the concentration that gave 10% inhibition. [d] Determined for the best fit of the data with two inhibition sites according to Scheme 2 using KINSIM (Barshop et al., 1983). Values are "apparent" because they are sensitive to the $[Mg^{2+}]$ present in the reaction. [e] Initial and final slopes of the data plotted on inhibition Hill plots (see Figure 5B). The final slope represents the number of metal binding sites being competed for.

the predicted value in Ca^{2+} alone was between 20 and 30 mM, indicating a 6−9-fold preference for Mg^{2+} in the sites responsible for the slow dissociation of S. While the points at higher Ca^{2+} have greater uncertainty, it is clear that the curve is sigmoidal. This suggests that more than one Ca^{2+} is involved.

Determining the Number of Mg^{2+} Sites Involved in the Chemical Step. Since Ca^{2+}, Ba^{2+}, and Sr^{2+} alone do not allow ribozyme catalysis, it is possible that their inhibition of $(k_{cat}/K_m)^S$ is due to a change in the rate-limiting step from the association of GGCCCUCUAAAAA to the chemical step. This would occur as increasing concentrations of these divalent cations competed for metal binding sites in the active site. Inhibition of the chemical step was tested by measuring $(k_{cat}/K_m)^G$ for the transesterification reaction. [Chemistry is rate-limiting for $(k_{cat}/K_m)^G$ in 10 mM Mg^{2+} below pH 7 (Herschlag & Cech, 1990a; Herschlag et al., 1991; McConnell et al., 1993; Herschlag & Khosla, 1994).] All three metal ions inhibited the chemical step; inhibition by Sr^{2+} and Ca^{2+} is summarized in Table 2. Strikingly, these divalent cations inhibited at very low concentrations, far lower than those required for the inhibition of $(k_{cat}/K_m)^S$ or for the increase in k_{off}^S caused by Ca^{2+}.

The rate constants for the G-dependent reaction [$(k_{cat}/K_m)^G$] and for the reaction in the absence of added G [$k_c(-G)$] were essentially independent of $[Mg^{2+}]$ in the range of 2−100 mM (the data are not shown because they can be described by simple lines with a slope of 0). One control involved preincubation of the ribozyme at 10 mM $MgCl_2$ prior to the addition or dilution of $MgCl_2$ to the final concentration; the data matched those obtained by preincubation of the ribozyme at the final $[Mg^{2+}]$. These results support a model in which metal ions required for catalysis are tightly bound in the E·S complex (K_d < 2 mM). Thus, the proposed active site Mg^{2+} ions essential for ribozyme catalysis (Cech et al., 1992; Piccirilli et al., 1993; Steitz & Steitz, 1993) would have dissociation constants lower than those required for folding.

For the RNA substrate−ribozyme complex, the binding of pG was only affected 2−3-fold by $[Mg^{2+}]$ in the range of 10−100 mM [data not shown and McConnell and Cech (1995)]. Furthermore, there is evidence against the possibility that replacement of a tightly bound Mg^{2+} by Ca^{2+} significantly weakens G binding. $K_d(G) \cong 0.4$ mM in the presence of 3.5 mM Mg^{2+} and 10 mM Ca^{2+} (data not shown), which is only ∼2-fold weaker binding of G than in the presence of 10 mM Mg^{2+} alone (McConnell et al., 1993). Under these conditions, the value of $(k_{cat}/K_m)^G$ was 240 times slower than that at 10 mM Mg^{2+}, and the observed rate constant k_{obs} of 1.2 min^{-1} at near-saturating G (2 mM) was ∼200 times slower than the estimated rate of chemistry in 10 mM Mg^{2+} (Herschlag & Cech, 1990a; Herschlag et al., 1993). These results are consistent with the inhibition by Ca^{2+} affecting the chemical step with minimal impact on the binding of G.

Is it possible that the apparent inhibition of the chemical step resulted from weaker binding of the oligonucleotide substrate? Recall that the binding constant of the oligonucleotide substrate is 80 nM in 10 mM Ca^{2+} (Figure 4A). In the experiments testing inhibition of the chemical step, the ribozyme concentrations were 200−500 nM, high enough to keep S bound despite its weaker affinity in Ca^{2+} alone. However, if S is bound in the open complex rather than in the closed complex, an additional docking step is required before the E·S·G complex can undergo its chemical transformation. This would decrease the observed rate of reaction of the E·S·G ternary complex even if the rate of reaction from the closed complex were unaffected (Narlikar et al., 1995, 1997; Narlikar & Herschlag, 1996). However, the concentration of Ca^{2+} required to inhibit the chemical step is well below the $[Ca^{2+}]$ that weakens substrate binding (compare Figures 4B and 5A).

A more detailed examination revealed that Ca^{2+} inhibition is not a simple competition of one Ca^{2+} for one Mg^{2+} (Figure 5A). The ratio of $[Ca^{2+}]$ required for 90% inhibition over that required for 10% inhibition is ∼20 (Table 2). For single-site inhibition, this ratio is calculated to be 81 (Segel, 1975),[3] significantly greater than the value observed. For two independent inhibitor sites, the ratio is calculated to be 18. A cooperative model, in which the first Ca^{2+} increases the binding of the second by 10-fold, gives a smaller ratio of 11.6; thus, the data do not warrant this sort of a more elaborate model. The simplest model that fits the data well involves two metal sites, with two Ca^{2+} ions competing independently for two Mg^{2+} ions essential for the catalytic activity of the ribozyme. The fits are consistently best with the apparent inhibition constants differing by a factor ∼3 (Table 2 and Figure 5A). The inhibition of $(k_{cat}/K_m)^G$ by Ca^{2+} occurs at Ca^{2+} concentrations lower than are required for inhibition of $(k_{cat}/K_m)^S$ (Table 2 and Figure 3); this observation is consistent with the model in which the decrease in $(k_{cat}/K_m)^S$ is due to a change in the rate-limiting step from binding substrate to chemistry. The apparent inhibition constants (Table 2 and Figure 5A), determined by a fit to the data with the mechanism shown in Scheme 2, are weaker with increasing $[Mg^{2+}]$, suggesting direct competition of Ca^{2+} for Mg^{2+} at sites important for catalysis. Sr^{2+} is a weaker inhibitor than Ca^{2+} (Table 2), showing that

[3] The ratios $[I]_{90}/[I]_{10}$ are calculated using the equation $k_{obs} = k_c[Mg^{2+}]/\{K_d(Mg^{2+})(1 + [I]/K_i)^n + [Mg^{2+}]\}$ [where n is equal to the number of metal binding sites, k_c is the maximal rate with the metal sites completely occupied with Mg^{2+}, $K_d(Mg^{2+})$ is the binding constant for Mg^{2+}, and K_i is the dissociation constant for the inhibitory metal, I; it is assumed that each Mg^{2+} has a dissociation constant $K_d(Mg^{2+})$ and each inhibitory metal ion has a dissociation constant K_i.] The equation was solved for [I] with values for k_{obs} at 10 and 90% of the rate observed without inhibitor present assuming $[Mg^{2+}]$ is at or near saturation. Ratios in the text were obtained using $[Mg^{2+}] = 10 \times K_d(Mg^{2+})$ and are insensitive to changes in $[Mg^{2+}]$ provided that it remains well above $K_d(Mg^{2+})$.

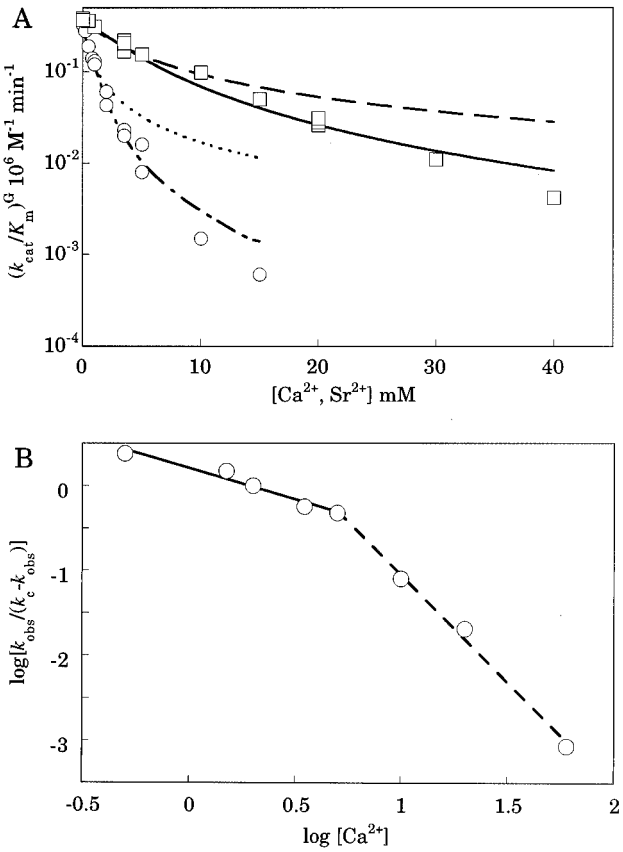
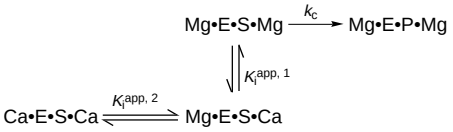


FIGURE 5: Ca^{2+} inhibition of the reaction indicates that two Ca^{2+} ions compete for two Mg^{2+} ions that are important for the chemical step. (A) The second-order rate constant, $(k_{cat}/K_m)^G$, for a reaction in 3.5 mM Mg^{2+} as a function of [Ca^{2+}] (○) and [Sr^{2+}] (□) at pH 6.7 (note that the *y*-axis is logarithmic). The dotted and dashed lines are the best fits to a model of a single inhibition site for the Ca^{2+} and Sr^{2+} data, respectively. The best fits to a model of two inhibition sites (Scheme 2) are shown for Ca^{2+} (dashed, one-dot line) with a $K_i^{app,1}$ of 0.7 mM and a $K_i^{app,2}$ of 1.5 mM and for Sr^{2+} (solid line) with a $K_i^{app,1}$ of 4 mM and a $K_i^{app,2}$ of 12 mM. (B) Hill plot for competitive inhibition of the ribozyme reaction. The logarithm of $k_{obs}/(k_c - k_{obs})$ is plotted as a function of the logarithm of the Ca^{2+} concentration in millimolar, where k_{obs} is the observed second-order rate constant $(k_{cat}/K_m)^G$ at a given [Ca^{2+}] and 10 mM Mg^{2+} and k_c is $(k_{cat}/K_m)^G$ at 10 mM Mg^{2+}. The solid line represents a slope of −1 and the dashed line a slope of −2.

Scheme 2



these effects are not a simple consequence of changing ionic strength.

Analysis of the mixed-metal inhibition data by a Dixon plot (not shown) further clarified the participation of more than one inhibitory Ca^{2+} ion. At four concentrations of Mg^{2+}, Ca^{2+} inhibition of $(k_{cat}/K_m)^G$ showed biphasic behavior, indicating inhibition by two Ca^{2+} ions. These plots of the effect of Ca^{2+} ions at different Mg^{2+} concentrations also confirmed that the inhibition was competitive. For example, when the [Mg^{2+}] was doubled, it took approximately twice as much Ca^{2+} to inhibit the rate by an equivalent magnitude in both transitions of the inhibition curve.

Hill (1913) derived an equation to determine graphically the number of sites involved in competition for analysis of the number of CO_2 molecules competing for O_2 binding sites in hemoglobin. This method is also applicable to kinetic measurements (Segel, 1975). Unlike the more commonly used Hill plot, the final slope does not indicate the cooperativity, but the number of sites involved. As shown in Figure 5B, the Hill plot for inhibition has an initial slope of −1 and a final slope of −2, again indicating that a two-metal inhibition model best fits the data. The $K_d(Ca^{2+})$ values are ≤0.4 and ≤1.0 mM for the two sites (Table 2). Similar inhibition behavior was measured for $k_c(-G)$ (data not shown). In summary, these data suggest a model in which two Mg^{2+} ions important for the chemical step are bound in sites that can be competed for by two Ca^{2+} ions in a noncooperative manner.

DISCUSSION

It is now widely accepted that group I ribozymes, and perhaps all naturally occurring ribozymes, are metalloenzymes, utilizing divalent metal ions for both structure formation and active site chemistry. Metal ions involved in the formation of the catalytic core of the *Tetrahymena* group I ribozyme have been studied thermodynamically (Celander & Cech, 1991) and to some extent located (Waring, 1989; Christian & Yarus, 1992, 1993; Wang & Cech, 1994). The current work extends our knowledge of metal ions more directly involved in the catalytic cycle, addressing the question of how Mg^{2+} ions contribute to individual steps of the ribozyme reaction. The overall conclusions are summarized in Figure 6 and Table 3. Two Mg^{2+} binding sites (sites 1 and 2) in the E·S complex contribute to the chemical step. A divalent metal ion (Mg^{2+} or Ca^{2+}) in a site (site 3) in the free ribozyme increases the rate constants for the association of the oligonucleotide substrate and product. Finally, a Mg^{2+} site or sites [site(s) 4] reduce the rate constants of oligonucleotide substrate and product dissociation.

It is important to realize that these key functional sites represent a small fraction of those required to properly fold the ribozyme. In the crystal structure of the 160-nucleotide P4−P6 domain of the ribozyme, 28 metal ions per RNA dimer in the asymmetric unit have been modeled at 2.8 Å resolution (Cate et al., 1996). Furthermore, based on tRNA which has 4 "strong" binding sites for Mg^{2+} [$K_d(Mg^{2+})$ in the 10−100 μM range] and 20−50 "weak" binding sites [$K_d(Mg^{2+})$ in the millimolar range] (Rialdi et al., 1972; Römer & Hach, 1975; Bina-Stein & Stein, 1976; Stein & Crothers, 1976; Reid & Cowan, 1990), the 389-nucleotide ribozyme may well have 20 tight binding sites and 200 weak binding sites.

Despite the multitude of Mg^{2+} sites, it is likely that few specifically require Mg^{2+}. This would explain the ability of the Ca^{2+} ribozyme to convert rapidly to the Mg^{2+} form and catalyze substrate cleavage before the substrate can dissociate (Figure 4A). Furthermore, mutation of only five or six positions allows the Ca^{2+} form of the ribozyme to catalyze the cleavage reaction (Lehman & Joyce, 1993). Here, we have provided evidence for one weak-binding Mg^{2+} (∼15 mM) in the free E and three stronger-binding Mg^{2+} ions (<2 mM) in the E·S complex that are important for ribozyme function. It is possible that the site responsible for the increase in k_{on} is the same as one of the other three sites, but the results suggest that the three functional sites in the E·S complex are distinct.

Two Magnesium Ions in the Active Site. The data presented here are consistent with two distinct metal ions

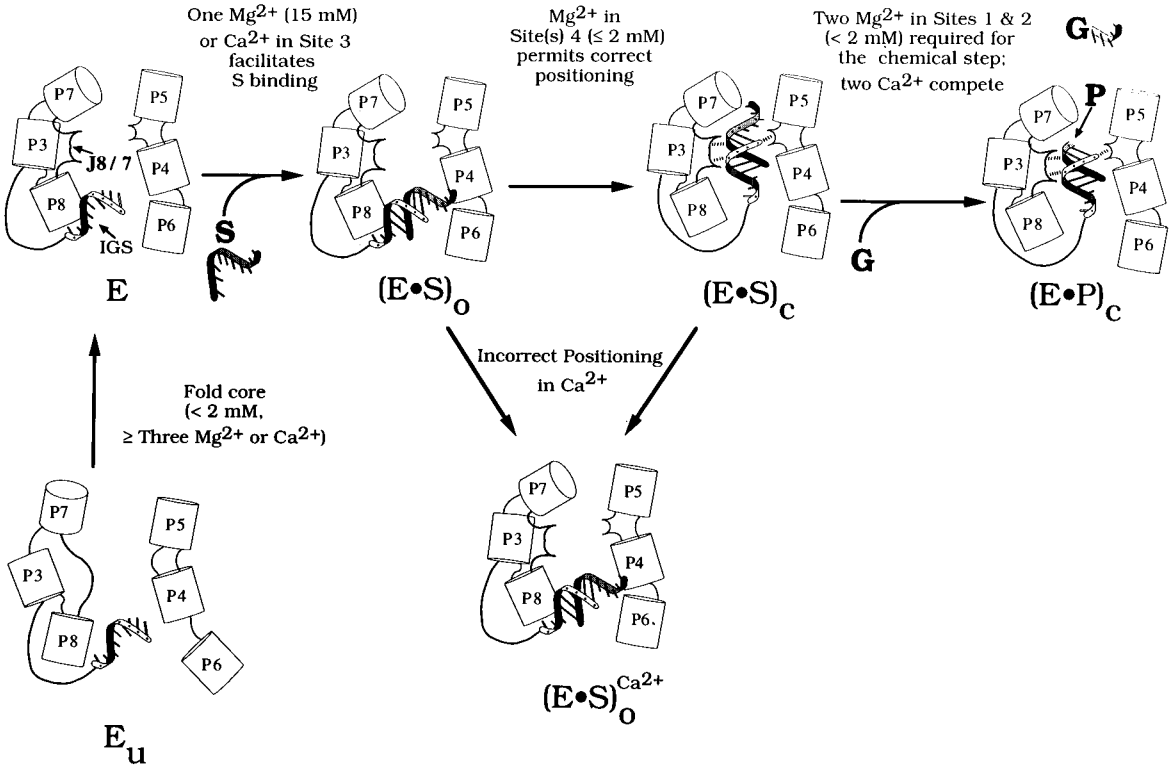


FIGURE 6: Metal requirements for individual steps of the ribozyme reaction. A cartoon of the core structure of the ribozyme is used to demonstrate the effect of metal ions on major steps of the ribozyme reaction based on these and previous results. The unfolded ribozyme (E_u) requires binding of at least three Mg^{2+} to fold into the active structure (E). One additional Mg^{2+} (or Ca^{2+}) binds to the free ribozyme to facilitate substrate association. One or more Mg^{2+} ions bind to the closed complex $(E{\cdot}S)_c$ tightly ($K_d \leq 2$ mM), promoting tertiary interactions between the ribozyme and the IGS and S. If these Mg^{2+} ions are replaced with Ca^{2+}, the IGS·S duplex is not docked correctly $[(E{\cdot}S)_o^{Ca^{2+}}]$ and resembles the open complex, $(E{\cdot}S)_o$. Two Mg^{2+} ions are required for the chemical step; Ca^{2+} acts as a competitive inhibitor for these sites. [The affinity for Mg^{2+} in site 3 (15 mM) represents binding to free E, while the affinities for sites 4, 1, and 2 (≤2 mM) represent binding to $(E{\cdot}S)_c$.]

Table 3: Characteristics of Mg^{2+} Binding Sites

site	proposed contribution	$K_d^{app}(Mg^{2+})$[a]	metal ion specificity[a,b]
1	catalysis; substrate destabilization and charge stabilization of 3′-O(U-1)	<2 mM (E·S)	only Mg^{2+} (and Mn^{2+}) function; Ca^{2+} (inhibitor) ≥ Mg^{2+} (E·S)
2[c]	catalysis; Lewis acid to deprotonate the 3′-OH of the G nucleophile	<2 mM (E·S)	Only Mg^{2+} (and Mn^{2+}) function; Ca^{2+} (inhibitor) ≥ Mg^{2+} (E·S)
3[d]	facilitates oligonucleotide association	15 mM (E)	$Mg^{2+} = Ca^{2+} > Ba^{2+} > Sr^{2+}$ (E)
4[e]	stabilizes E·S and E·P (located near J8/7)	≤2 mM (E·S)	$Mg^{2+} > Ca^{2+}$ (inhibitor) (E·S)

[a] (E·S) and (E) indicate that metal ion binding was assessed with the E·S complex or free E, respectively. [b] The order of affinity of different ions for the site is indcated by >, ≥, or =. (inhibitor) means that filling the site with the indicated ion inhibits function. [c] Unique from site 1 on the basis of evidence presented for two metal ions in the active site. [d] Cannot be uniquely distinguished from the other sites because the metal ion in this site is observed to affect free E; the information for all other metal ion sites pertains to E·S. [e] The preference for Mg^{2+} over Ca^{2+} differentiates this site from sites 1 and 2.

involved in reaction of the E·S complex. Both metal sites appear to require magnesium ions, which are bound tightly (<2 mM). We did not test Mn^{2+} but already have evidence that it would substitute (Grosshans & Cech, 1989; Piccirilli et al., 1993). Even though Ca^{2+} can bind at least as strongly to these sites,[4] Ca^{2+} cannot catalyze the reaction. Ca^{2+} may not be able to perform the functions of the metal ions in these sites because of its larger size or because its preferred coordination geometry does not allow it to bind the ligands properly. The inhibition by Sr^{2+}, which is weaker than that by Ca^{2+}, supports the model of specific sites, rather than nonspecific ionic strength effects.

There is a good basis for concluding that these two ions are involved in the chemical step. The kinetic parameter $(k_{cat}/K_m)^G$ is rate-limited by chemistry below pH 7 in 10 mM $MgCl_2$, as indicated by several criteria, including the rate decrease upon phosphorothioate substitution and upon reduction of the stability of the leaving group (Herschlag et al., 1991, 1993; Herschlag & Khosla, 1994). Could the rate-limiting step change upon addition of Ca^{2+}? There is good evidence that the modest concentrations of Ca^{2+} used in these experiments do not cause the undocking of the substrate from the active site; the $[Ca^{2+}]$ required to weaken the binding of S is 6−9 times greater than $[Mg^{2+}]$, whereas the strong inhibition of chemistry occurs when $[Ca^{2+}] \leq [Mg^{2+}]$ (Figure 5). Furthermore, the binding constant for the guanosine nucleophile is hardly affected (see Results). However, we cannot dismiss the possibility that some rapid conformational change, such as the one responsible for the leveling off of the reaction rate above pH 7 (Herschlag & Khosla, 1994), becomes slow upon addition of Ca^{2+} and limits the rate.

[4] The inhibitory metal ions may block Mg^{2+} binding by binding in the same site as Mg^{2+}, by binding in an overlapping site, or by binding in a distinct site that causes a conformational change in the Mg^{2+} binding site.

We now describe our current model of a two-metal ion active site incorporating available data. The simplest expectation is that one of the two Mg^{2+} ions we have identified as being involved in the chemical step is the Mg^{2+} that interacts with the 3′-oxygen of U-1 (Piccirilli et al., 1993); it is defined as site 1 in our model (Table 3). The metal ion in site 1 is proposed to stabilize the developing negative charge on the 3′-oxygen of the leaving group in the transition state. There is evidence that it also catalyzes the reaction by electrostatic destabilization of bound S (Narlikar et al., 1995). It remains to be determined whether this site has an affinity for Ca^{2+} stronger or similar to that for Mg^{2+}.

The other of the two metal sites (site 2) inferred from the competition studies on $(k_{cat}/K_m)^G$ and $k_c(-G)$ could also contain a Mg^{2+} ion directly involved in catalysis. It has been speculated that the Mg^{2+} in site 2 interacts with the *pro-*S_p oxygen of the reactive phosphate and the 3′-hydroxyl group of guanosine (Steitz & Steitz, 1993). A metal ion interaction with the *pro-*S_p oxygen is not supported by direct evidence such as the restoration by thiophilic metals of the activity of a substrate containing an S_p phosphorothioate at the cleavage site. However, geometric or size constraints in the active site may prevent such a straightforward metal ion specificity switch. On the other hand, evidence has been recently found for a metal ion interaction with the 2′-hydroxyl of G (Sjögren et al., 1997) and with the 3′-hydroxyl of G (L. B. Weinstein, B. C. N. M. Jones, R. Cosstick, and T. R. Cech, submitted for publication). Considering that our Ca^{2+} inhibition experiments provide a lower limit for the number of metal sites that affect the chemical step, the metal ion interactions with the hydroxyl groups of G may involve one or two different ions, and additional active site metal ions may also be present.

Mg^{2+} Ions in Site(s) 4 Promote the Docking of the Oligonucleotide. Substrate dissociation occurs in two steps: undocking of the substrate helix followed by melting of base pairs with the IGS (Bevilacqua et al., 1992; Herschlag, 1992). Thus, the metal ion sites that stabilize the bound substrate, called here site(s) 4, are inferred to be involved in tertiary interactions in the closed E·S complex (Figure 6). They have a 6−9-fold preference for Mg^{2+} over Ca^{2+} when substrate is bound. Once bound, Mg^{2+} does a much better job of stabilizing the docked substrate helix than Ca^{2+}; addition of Ca^{2+} weakens the equilibrium binding of S (Figure 4A) and increases k_{off}^S (Figure 4B). This is in agreement with photo-cross-linking results, which have provided physical evidence for undocking of the substrate helix upon addition of Ca^{2+} [Wang & Cech, 1994; see also Narlikar and Herschlag (1996)]. The abrupt change in k_{off}^S with increasing $[Ca^{2+}]$ provides evidence for the involvement of more than one Mg^{2+} and Ca^{2+}.

Previous work has implicated the J8/7 element of the ribozyme in binding the oligonucleotide substrate. We suggest that site(s) 4 may reside at least partially within J8/7. Nucleotide A302 in J8/7 is involved in an interaction with the 2′-hydroxyl of U-3 of the oligonucleotide substrate (Pyle et al., 1992). Phosphorothioate substitution of U306 and A307 in J8/7 interferes with the first step of self-splicing; splicing is restored by the more thiophilic Mn^{2+}, suggesting that these phosphates coordinate metal ions required for folding or reactivity (Christian & Yarus, 1993). Two studies have used metal−hydroxide cleavage (Brown et al., 1985) to identify regions of the ribozyme in three-dimensional proximity to bound metal ions. McConnell (1995) found that Ca^{2+} cleavage of J8/7 was inhibited by increased Mg^{2+} or by bound oligonucleotide and concluded that metal ions bound to J8/7 are involved in the docking of the substrate helix into the ribozyme core. Streicher et al. (1996) ascribed similar metal-catalyzed cleavage within the J8/7 region in the bacteriophage T4 *td* intron to active site metal ions, although other possibilities were not ruled out. For the present, the involvement of a Mg^{2+}−J8/7 complex in formation of tertiary interactions with the docked P1 duplex remains an intriguing hypothesis.

Binding of a Mg^{2+} Ion to the Ribozyme Facilitates the Initial Base Pairing of the Substrate. The conclusion that a site-bound Mg^{2+} or Ca^{2+} ion increases the rate of association of RNA oligonucleotides to the ribozyme was surprising. This step involves formation of complementary base pairs (RNA hybridization) as shown in Figure 1. Thus, an ionic strength effect would seem reasonable, but an ion-selective, saturable site is unexpected for facilitation of open complex formation.

One possible explanation is that a divalent cation in site 3 on the free ribozyme causes a local conformational rearrangement, making the internal guide sequence more accessible for hybridization. Because the rate constant for association of the substrate (k_{on}^S) is 3−10-fold below measured values for simple duplex formation (Bevilacqua et al., 1992; Narlikar et al., 1997), there is room for improvement in the hybridization rate. Furthermore, cross-linking experiments with a photoactivatable group on the 5′ end of the IGS suggest that it has a preferred resting state on the ribozyme surface rather than being disordered (Wang et al., 1993; Wang & Cech, 1994). By making the IGS more available for interaction, a specific ion-binding site could enhance the rate of substrate−IGS base pairing.

Site 3 is representative of the class of Mg^{2+}-binding sites described by Laing et al. (1994), sites which show moderate specificity for Mg^{2+} and likely occur in regions of high negative charge density on the RNA. Such Mg^{2+} sites may be a common feature of catalytic RNAs that have to negotiate structural switching of their polyanionic components to promote function [e.g., Dahm and Uhlenbeck (1991), Franzen et al. (1994), Weeks and Cech (1995), and Beebe et al. (1996)]. It remains to be determined whether site 3 is physically distinct from the other three sites. Because $(k_{cat}/K_m)^S$ measures properties of the free ribozyme, we do not know the affinity of metal ions for this binding site in the E·S complex.

Conclusions. In the catalytic cycle of the ribozyme, Mg^{2+} ions bind in specific sites on the ribozyme promoting distinct functions: binding of substrate, docking of the substrate helix into the core, and enhancing the chemical step directly (Figure 6). We have provided evidence for two Mg^{2+} binding sites involved in the chemical step of the ribozyme reaction, on the basis of their different inhibitions by Ca^{2+}. We propose that there are at least two additional sites involved in oligonucleotide binding. One of these holds a Mg^{2+} that enhances the rate constant of oligonucleotide association to the ribozyme. At least one additional Mg^{2+} site stabilizes the ribozyme−oligonucleotide complex by decreasing the rate constant of substrate and product dissociation. Thus, the cation Mg^{2+} plays many roles in the ribozyme catalysis: as a counterion capable of assisting the folding of a polyanion into a specific three-dimensional structure, as a means of facilitating formation of the substrate helix and recognition of that helix by the rest of the ribozyme,

and as a metal cofactor in a metalloenzyme catalyzing the cleavage of a specific phosphodiester linkage.

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BI9700678