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An unconventional origin of metal-ion rescue and inhibition in the *Tetrahymena* group I ribozyme reaction

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ABSTRACT

The presence of catalytic metal ions in RNA active sites has often been inferred from metal-ion rescue of modified substrates and sometimes from inhibitory effects of alternative metal ions. Herein we report that, in the *Tetrahymena* group I ribozyme reaction, the deleterious effect of a thio substitution at the *pro-*S_P position of the reactive phosphoryl group is rescued by Mn^{2+}. However, analysis of the reaction of this thio substrate and of substrates with other modifications strongly suggest that this rescue does not stem from a direct Mn^{2+} interaction with the S_P sulfur. Instead, the apparent rescue arises from a Mn^{2+} ion interacting with the residue immediately 3′ of the cleavage site, A(+1), that stabilizes the tertiary interactions between the oligonucleotide substrate (S) and the active site. This metal site is referred to as site D herein. We also present evidence that a previously observed Ca^{2+} ion that inhibits the chemical step binds to metal site D. These and other observations suggest that, whereas the interactions of Mn^{2+} at site D are favorable for the chemical reaction, the Ca^{2+} at site D exerts its inhibitory effect by disrupting the alignment of the substrates within the active site. These results emphasize the vigilance necessary in the design and interpretation of metal-ion rescue and inhibition experiments. Conversely, in-depth mechanistic analysis of the effects of site-specific substrate modifications can allow the effects of specific metal ion–RNA interactions to be revealed and the properties of individual metal-ion sites to be probed, even within the sea of metal ions bound to RNA.

Keywords: chemical modification; enzymatic catalysis; metal ion; ribozyme

INTRODUCTION

It has long been recognized that metal ions play critical roles in catalysis by RNA enzymes. For most RNA enzymes, however, little is known about the number of active-site metal ions and their mechanistic roles. Our ability to study individual metal-ion sites on RNA has mainly been impeded by the sea of metal ions that coat the charged RNA backbone and facilitate RNA folding (see, e.g., Quigley et al., 1978; Bujalowski et al., 1986; Celander & Cech, 1991; Pan et al., 1993; Pan, 1995; Bassi et al., 1996; Beebe et al., 1996; Cate & Doudna, 1996; Draper, 1996; Cate et al., 1997; Correll et al., 1997; Gluick et al., 1997; Walter et al., 1998, and references therein).

Metal-ion sites on RNA have been probed by monitoring ribozyme structure or activity as a function of metal-ion concentration and identity. In favorable cases, this has allowed metal ions to be separated into different classes. For example, global folding of the *Tetrahymena* group I ribozyme can be supported by a variety of metal ions including Mg^{2+}, Mn^{2+}, and Ca^{2+}; however, only Mg^{2+} and Mn^{2+} support ribozyme activity, whereas Ca^{2+} inhibits the chemical step (Grosshans & Cech, 1989; Latham & Cech, 1989; Celander & Cech, 1991; Downs & Cech, 1996; McConnell et al., 1997). These observations led to the postulation of two types of metal-ion sites in this ribozyme: those involved in RNA folding, a role which can be fulfilled by Mg^{2+}, Mn^{2+}, and Ca^{2+}, and those involved in the chemical

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Abbreviations: E: the *Tetrahymena* L-21 *Sca*I ribozyme; G: guanosine; S_A: generically, the oligonucleotide substrates with the sequence CCCUCUA or $CCCUCUA_5$; S_{Me}: oligonucleotide substrates with A(+1) replaced by a methoxy group, without specification of the sugar identity or other modifications; S: S_A or S_{Me} (the individual oligonucleotides used in this work are defined in Table 1). The presence of additional A residues at the (+2) to (+5) position of some oligonucleotide substrates (see Table 1) does not affect the binding and the interaction of metal ions investigated herein (data not shown). The asterisk after an oligonucleotide substrate denotes that the substrate is ^{32}P-labeled at its 5′ end. IGS: the internal guide sequence of the ribozyme, which has the sequence GGAGGG; P1: the duplex formed between the oligonucleotide substrate and the IGS strand; $(E•S)_o$ and $(E•S)_c$: the open and closed ribozyme•oligonucleotide substrate complexes, respectively, described in Scheme 1 in the text. MES: [4-morpholine]ethanesulfonic acid; MOPS: [4-morpholine]propanesulfonic acid; HEPES: [4-(2-hydroxyethyl)-1-piperazine]ethanesulfonic acid; EPPS: [4-(2-hydroxyethyl)-1-piperazine]propanesulfonic acid.

reaction, a role which can only be met by Mg^{2+} and Mn^{2+}.

For several well-studied RNA enzymes such as the *Tetrahymena* ribozyme, the RNA component of RNase P, and the hammerhead ribozyme, the chemical step has been investigated as a function of the concentration of Mg^{2+}, Ca^{2+}, or other metal ions (see, e.g., Smith & Pace, 1993; Beebe et al., 1996; McConnell et al., 1997; Lott et al., 1998; Warnecke et al., 1999). The observed concentration dependences have led to proposals of catalytic mechanisms involving one, two, or three metal ions (see, e.g., Smith & Pace, 1993; McConnell et al., 1997; Lott et al., 1998; Warnecke et al., 1999). Analogous conclusions have been drawn for protein enzymes (Dismukes, 1996; Wilcox, 1996; Cowan, 1997; Groll et al., 1997; Black & Cowan, 1998; Horton et al., 1998; Keck et al., 1998). However, the results of these experiments do not distinguish between effects of active-site metal ions that directly participate in the chemical reaction and indirect effects of metal ions located away from the active site, nor do they shed light on the roles of individual metal ions. Further, as numerous metal ions are required to fold an RNA, the effects of high affinity active-site metal ions are likely to be masked, because they are already bound at the Mg^{2+} concentration required for RNA folding.

These problems have been addressed by the application of metal-ion specificity switch experiments to studies of nucleic acids. Specific metal-ion ligands on the substrates or ribozyme can, in principle, be identified by changing specific oxygen ligands to sulfur or nitrogen and exploiting the preference of these modified ligands to interact with metal ions softer than Mg^{2+}, such as Mn^{2+}, Zn^{2+}, or Cd^{2+} (Jaffe & Cohn, 1978; Burgers & Eckstein, 1979, 1980; Connolly & Eckstein, 1981; Christian & Yarus, 1993). These experiments have provided strong evidence for active-site metal-ion interactions in several RNA enzymes (Piccirilli et al., 1993; Warnecke et al., 1996; Chen et al., 1997; Peracchi et al., 1997; Sjögren et al., 1997; Sontheimer et al., 1997; Weinstein et al., 1997; Scott & Uhlenbeck, 1999; Shan & Herschlag, 1999; Yoshida et al., 1999; see Fig. 1 for the *Tetrahymena* ribozyme). However, even with these modified substrates, the effects of metal ions require cautious interpretation, as the metal ions being probed represent a minuscule fraction of the metal ions that coat an RNA. Soft metal ions bound to metal-ion sites other than the site of interest can indirectly affect the ribozyme's structure and reactivity (see, e.g., Basu & Strobel, 1999). Conversely, the absence of metal-ion rescue does not rule out a metal-ion interaction, as thio substitution can disrupt a metal-ion binding site surrounding the normal ligand (see, e.g., Brautigam & Steitz, 1998).

In this work, the apparent Mn^{2+} rescue of a phosphorothioate substitution has been investigated in the well-characterized *Tetrahymena* group I ribozyme reaction (Cech & Herschlag, 1996, and references therein).

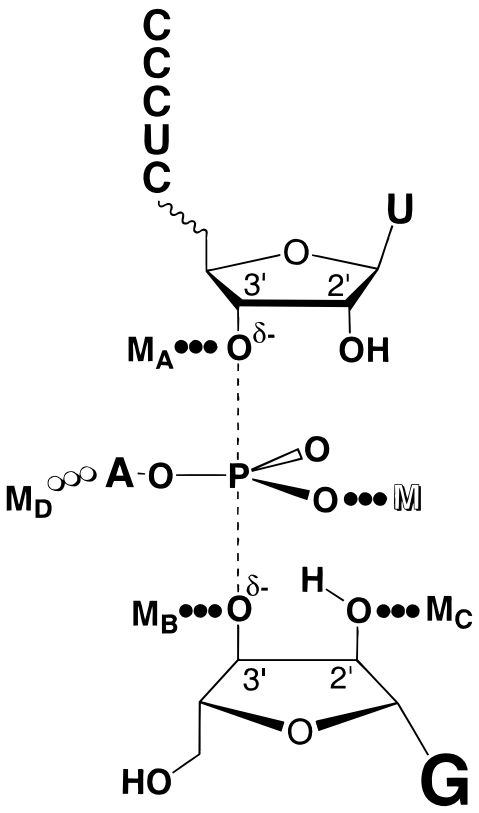


FIGURE 1. Metal-ion interactions in the transition state of the *Tetrahymena* ribozyme reaction. The dashed lines (---)depict the partial bonds between the reactive phosphorus and the 3′-oxygen of S and the 3′-oxygen of G, and "δ-" depicts the partial negative charges on the 3′-oxygens of S and G. M_A is the metal ion that interacts with the 3′-oxygen of U(−1) of S (Piccirilli et al., 1993); M_B is the metal ion interacting with the 3′-moiety of G (Weinstein et al., 1997); M_C is the metal ion interacting with the 2′-moiety of G (Sjögren et al., 1997; Shan & Herschlag, 1999); and M_D is the metal-ion site investigated in this work. The solid circles (●●●) depict direct metal-ion interactions, and the open circles (○○○) indicate that the interaction of M_D with the A(+1) residue may be direct or indirect. Recent results suggest that M_A, M_B, and M_C are three distinct metal ions (Shan et al., 1999). There is evidence for an additional active site metal-ion interaction with the *pro-*S_P oxygen of the reactive phosphoryl group (Yoshida et al., 1999); this metal ion is depicted by the open Ⓜ, as it is not known whether this metal ion is the same as M_A, M_B, M_C, or a fourth metal ion within this ribozyme active site. Results described herein suggest that M_D is distinct from M_A, M_B, and M_C.

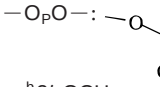
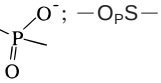
This ribozyme (E) catalyzes a transesterification reaction shown in Equation (1),

$$\underset{(S)}{CCCUCU_pA} + G_{OH} \rightarrow \underset{(P)}{CCCUCU_{OH}} + G_pA \qquad (1)$$

with an exogenous guanosine nucleophile (G) cleaving a specific phosphodiester bond of an oligonucleotide substrate (S). Thio substitution of the *pro-*S_P oxygen of the reactive phosphoryl group reduces the rate of the chemical step 10^4-fold (Rajagopal et al., 1989; Yoshida et al., 2000), suggesting the presence of interactions with the *pro-*S_P oxygen that could involve active-site metal ions (Yoshida et al., 1999). In this study, we found

TABLE 1. List of oligonucleotide substrates.[a]

Abbreviation	Oligonucleotide substrate or product											
		−5		−3		−1		+1		+3		+5
rS_A	rC	rC	rC	rU	rC	rU	—OpO—	rA	rA	rA	rA	rA
—1d,rS_A	rC	rC	rC	rU	rC	dT	—OpO—	rA	rA	rA	rA	rA
—1d,$rS_{A,P\text{-}S}$[b]	mC	mC	mC	rU	rC	dU	—OpS—	rA				
mS_A	rC	rC	rC	mU	rC	rU	—OpO—	dA				
—1d,mS_A	rC	rC	rC	mU	rC	dU	—OpO—	dA				
rS_{Me}	rC	rC	rC	rU	rC	rU	—OpO—	Me				
mS_{Me}	rC	rC	rC	mU	rC	rU	—OpO—	Me				
—1d,$rS_{Me,P\text{-}S}$[b]	mC	mC	mC	rU	rC	dU	—OpS—	Me				
—1r,dS_A	dC	dC	dC	dU	dC	rU	—OpO—	dA	dA	dA	dA	dA

[a] r: 2′-OH; d: 2′-H; m: 2′-OCH_3; Me: OCH_3;
—O_PO—:  —O_PS—: 

[b] 2′-OCH_3 groups are introduced into the (−4) to (−6) residues of —1d,$rS_{A,P\text{-}S}$ and —1d,$rS_{Me,P\text{-}S}$; the sole effect of this modification is to prevent miscleavage of these oligonucleotides, allowing more accurate determination of rate constants for cleavage at the correct position (Herschlag, 1992; Knitt et al., 1994; Narlikar et al., 1997).

that Mn^{2+} stimulates the reaction of a modified oligonucleotide substrate with a thio substitution at the *pro-*S_P oxygen relative to reaction of the unsubstituted substrate. However, further analysis revealed that this rescue is not due to a Mn^{2+} that directly interacts with the S_P sulfur. Rather, the apparent rescue arises from the indirect effect of a metal-ion site interacting with the A(+1) residue, referred to as metal site D herein (Fig. 1, M_D).

The inhibitory effect of Ca^{2+} on the *Tetrahymena* ribozyme reaction has also been reinvestigated. Previously, the ability of Ca^{2+} to inhibit the chemical step of this reaction and the observation that the inhibitory Ca^{2+} is competitive with Mg^{2+} led to the suggestion that the inhibitory Ca^{2+} ions displace catalytic Mg^{2+} ions at the ribozyme active site, such as the Mg^{2+} ions interacting with the 3′-bridging oxygen of S, the 3′-OH of G, and/or the nonbridging reactive phosphoryl oxy-

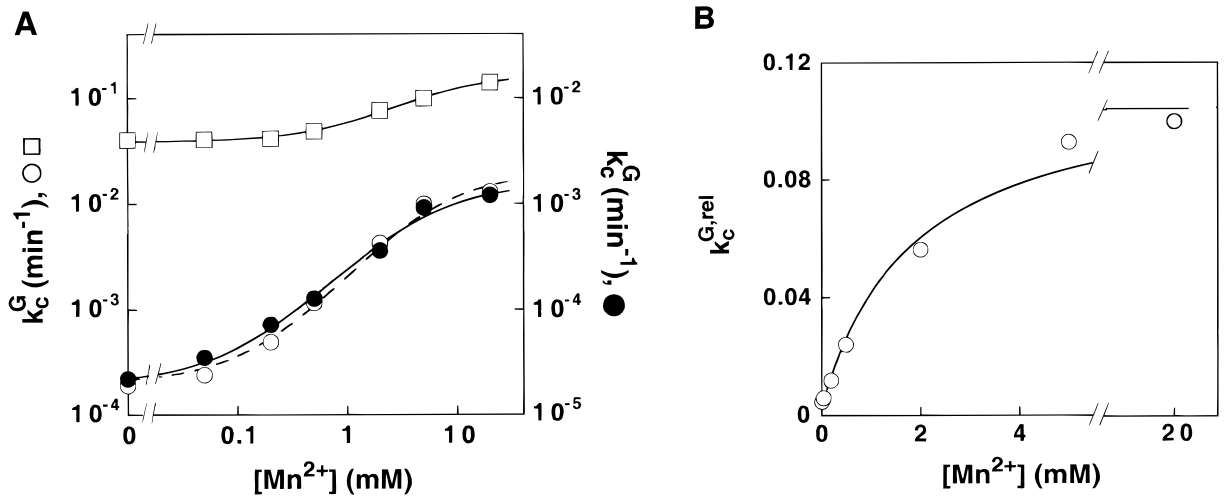
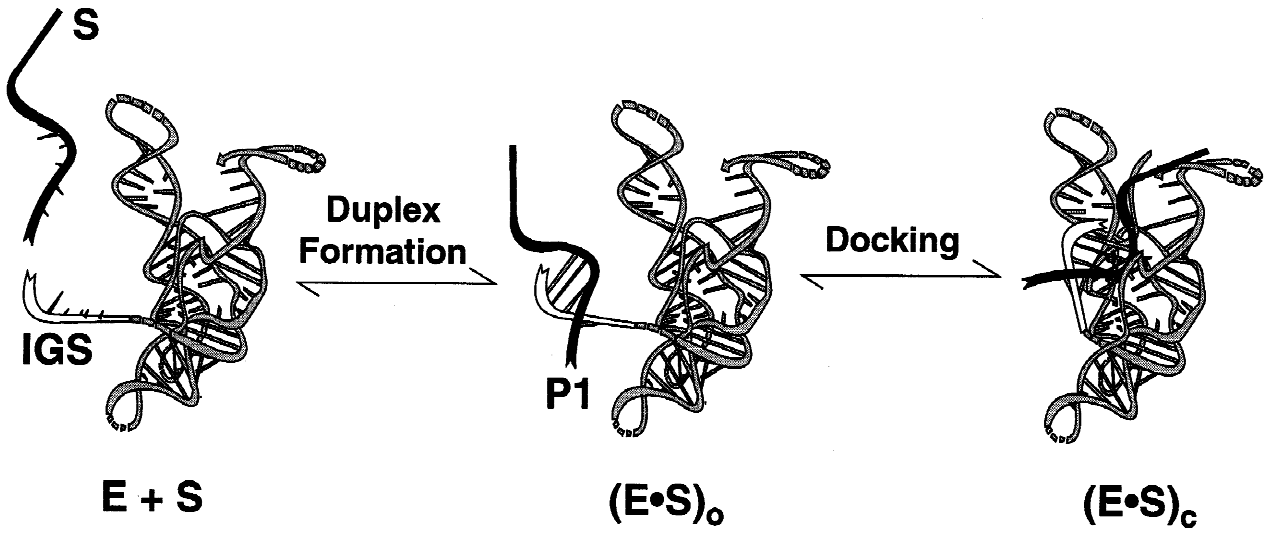


FIGURE 2. Mn_D^{2+} stimulates reaction from the open complex relative to reaction from the closed complex. **A**: Effect of Mn^{2+} on the reaction E•S•G → products (k_c^G) for —1d,$rS_{A,P\text{-}S}$ (●), —1d,mS_A (○), and —1d,rS_A (□). Values of k_c^G for —1d,mS_A and —1d,rS_A were determined at pH 7.0, and those for —1d,$rS_{A,P\text{-}S}$ were determined at pH 7.9 with 10 mM Mg^{2+}, as described in Materials and methods. The data for —1d,rS_A were fit to a model in which a single Mn^{2+} gives the observed small stimulation. The data for —1d,$rS_{A,P\text{-}S}$ and —1d,mS_A are fit to a model in which this Mn^{2+} has the same effect on reaction of these substrates, and an additional Mn^{2+}, Mn_D^{2+}, has an additional, independent stimulatory effect (see footnote 2). **B**: Effect of Mn^{2+} on the reaction rate of —1d,mS_A relative to —1d,rS_A: $k_c^{G,rel} = k_{c,rS}^G/k_{c,mS}^G$. The data are fit to Equation (3), derived from Scheme 2, and give $K_{(E\bullet S\bullet G)_o}^{Mn,app} = 1.8$ mM and $k_{c,Mn}^{G,rel} = 0.10$.



SCHEME 1. Two-step binding of the oligonucleotide substrate to the *Tetrahymena* ribozyme.

gen (McConnell et al., 1997; Fig. 1). Herein we present data that suggest that one of the inhibitory Ca^{2+} ions binds to metal site D. Analysis of the Ca^{2+} effect on individual reaction steps suggests that this Ca^{2+} exerts its inhibitory effect by mispositioning the substrates within the active site.

RESULTS

Rescue of a phosphorothioate substitution arises from an indirect Mn^{2+} effect

In this section, we first present evidence that the deleterious effect of a phosphorothioate substitution at the *pro-*S_P oxygen of the reactive phosphoryl group can be rescued by a Mn^{2+} ion. We then show that the apparent rescue arises from an indirect effect of Mn^{2+}, resulting because different reaction steps are monitored for the thio and the wild-type substrates. We then describe results that suggest that this Mn^{2+} ion, referred to as Mn^{2+}_D, interacts with A(+1), the residue immediately 3′ of the cleavage site (Table 1). Evidence that Mn^{2+}_D competes with a bound Mg^{2+} is presented at the end of this section.

Apparent Mn^{2+} rescue from an indirect effect

Thio substitution of the *pro-*S_P oxygen of the reactive phosphoryl group slows the chemical step ~10^4-fold (Rajagopal et al., 1989; Yoshida et al., 2000). To test for a potential metal-ion interaction with the *pro-*S_P oxygen, we determined the effect of Mn^{2+} on the chemical step for −1d,r$S_{A,P\text{-}S}$ (Table 1), in which this oxygen is replaced by sulfur. Addition of Mn^{2+} increases the rate of the chemical step for −1d,r$S_{A,P\text{-}S}$ ~60-fold (10 mM Mg^{2+}; Fig. 2A, closed circles). In contrast, Mn^{2+} has only a fourfold effect on the chemical step for −1d,rS_A (Table 1), which lacks the thio substitution (Fig. 2A, open squares).

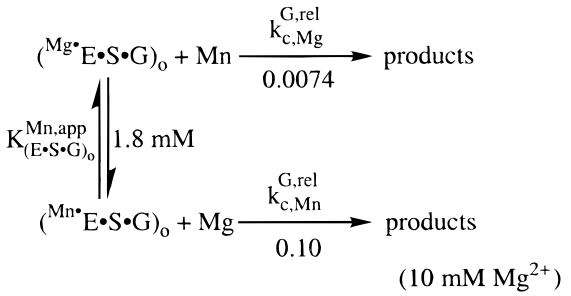
The simplest explanation for this Mn^{2+} stimulation is that a Mn^{2+} coordinates the S_P sulfur, thereby rescuing the deleterious effect from the thio substitution. However, stimulatory effects of Mn^{2+} can also arise indirectly, especially if different reaction steps are monitored for the thio and the wild-type substrate. Previous work showed that binding of the oligonucleotide substrate occurs in two distinct steps (Scheme 1): first, the open complex, $(E•S)_o$, is formed, in which S is held solely by base-pairing interactions with the internal guide sequence (IGS) of E to form the P1 duplex; second, the closed complex, $(E•S)_c$, is formed, in which the P1 duplex docks into the catalytic core of the ribozyme via tertiary interactions (Bevilacqua et al., 1992; Herschlag, 1992; Narlikar & Herschlag, 1996; Narlikar et al., 1997; Szewczak et al., 1998, and references therein). The oligonucleotide substrate −1d,rS_A binds the ribozyme in the closed complex (Bevilacqua et al., 1992; Herschlag, 1992; Narlikar & Herschlag, 1997; Narlikar et al., 1996, and see below). In contrast, −1d,r$S_{A,P\text{-}S}$ binds the ribozyme predominantly in the $(E•S)_o$ complex.[1] This

[1]The following observations strongly suggest that −1d,r$S_{A,P\text{-}S}$ binds the ribozyme to form an $(E•S)_o$ complex (Yoshida et al., 2000, and our unpub. results): (1) −1d,r$S_{A,P\text{-}S}$ binds the ribozyme with an affinity comparable to that expected for the open complex, even in the presence of added Mn^{2+} [dissociation constants for −1d,r$S_{A,P\text{-}S}$ of 1.2 and ≥0.7 nM were observed with 10 mM Mg^{2+} and 10 mM Mn^{2+}/10 mM Mg^{2+}, respectively (data not shown); the dissociation constant of S from the open complex is expected to be ~0.5–1 nM (Narlikar et al., 1997)]; (2) the guanosine nucleophile binds to the E•S complex formed by −1d,r$S_{A,P\text{-}S}$ with an affinity of K_a^G = 3.1 mM^{-1} (see Results), the same as the G affinity expected for the open complex (McConnell et al., 1993; Shan & Herschlag, 1999). In contrast, the affinity of G for the closed complex is expected to be approximately fivefold higher, with K_a^G = 16 mM^{-1} (McConnell et al., 1993; Shan & Herschlag, 1999).

means that different reaction steps are monitored for the two substrates under comparison: for $-1d,rS_A$, the reaction $(E•S•G)_c \rightarrow$ products was followed, whereas for $-1d,rS_{A,P\text{-}S}$, the reaction $(E•S•G)_o \rightleftharpoons (E•S•G)_c \rightarrow$ products was followed. Thus, any Mn^{2+} ion that increases docking of S could stimulate the reaction of $-1d,rS_{A,P\text{-}S}$ without affecting the reaction of $-1d,rS_A$, providing an apparent rescue unrelated to the phosphorothioate substitution.

To test the alternative model that a Mn^{2+} ion affects S docking, we determined the effect of Mn^{2+} on reaction of $-1d,mS_A$ (Table 1). In this substrate, the 2′-OH of the U(−3) residue is replaced by a methoxy group, disrupting docking of S and resulting in a stable open complex (Pyle & Cech, 1991; Herschlag, 1992; Herschlag et al., 1993a; Knitt et al., 1994; Narlikar et al., 1997; Narlikar & Herschlag, 1996). Thus, the reaction $(E•S•G)_o \rightleftharpoons (E•S•G)_c \rightarrow$ products can be monitored with $-1d,mS_A$, as with $-1d,rS_{A,P\text{-}S}$. The reaction of $-1d,mS_A$ is stimulated 60-fold by addition of Mn^{2+}, just as reaction of $-1d,rS_{A,P\text{-}S}$, and the Mn^{2+} concentration dependences are the same (Fig. 2A, closed vs. open circles). These results strongly suggest that the Mn^{2+} stimulation of the $-1d,rS_{A,P\text{-}S}$ reaction arises from a Mn^{2+} ion that stabilizes S docking, rather than a direct Mn^{2+} interaction with the S_P-sulfur.

This Mn^{2+} effect was isolated by plotting the reaction rate for $-1d,mS_A$ relative to $-1d,rS_A$, as the reaction of $-1d,rS_A$ does not include the docking step (Fig. 2B, $k_c^{G,rel}$).[2] The Mn^{2+} concentration dependence suggests that a single Mn^{2+}, referred to as Mn_D^{2+}, is responsible for the stimulation (Scheme 2). This Mn^{2+} concentration dependence further suggests that Mn_D^{2+} binds to the $(E•S•G)_o$ complex with an apparent dissociation constant[3] of $K^{Mn,app}_{(E•S•G)_o} = 1.8$ mM and provides



SCHEME 2. Mg^{2+} binding and its effect on reactivity of the E•S•G open complex.

a 13-fold stimulation of $k_c^{G,rel}$ relative to Mg^{2+} ($k_{c,Mn}^{G,rel}/k_{c,Mg}^{G,rel} = 0.10/0.0074 = 13$).

To provide an independent test for a Mn^{2+} that increases S docking, we directly determined the effect of Mn^{2+} on the binding affinity of $-1d,rS_A$. This oligonucleotide substrate binds the ribozyme predominantly in the closed complex (Narlikar & Herschlag, 1996), so that a Mn^{2+} that increases S docking would be expected to increase the affinity of $-1d,rS_A$. As expected, addition of Mn^{2+} decreased the equilibrium dissociation constant of $-1d,rS_A$ 10-fold (Fig. 3, K_d^S and $K_d^{S\prime}$ for the $E•S_A$ and $E•S_A•G$ complexes, respectively). In contrast, Mn^{2+} has no significant effect on the equilibrium for formation of the open complex, with dissociation constants of 0.70 and 0.82 nM from $(E•S)_o$ in the presence or absence of 5 mM Mn^{2+} ($-1d,mS_A$, 10 mM Mg^{2+} background; data not shown). Thus, the observed 10-fold Mn^{2+} effect in Figure 3 represents an effect on the equilibrium for S docking.

The Mn^{2+} concentration dependences in Figure 3 suggest that a single Mn^{2+} is responsible for stabilizing docking of $-1d,rS_A$, as depicted in Scheme 3. The

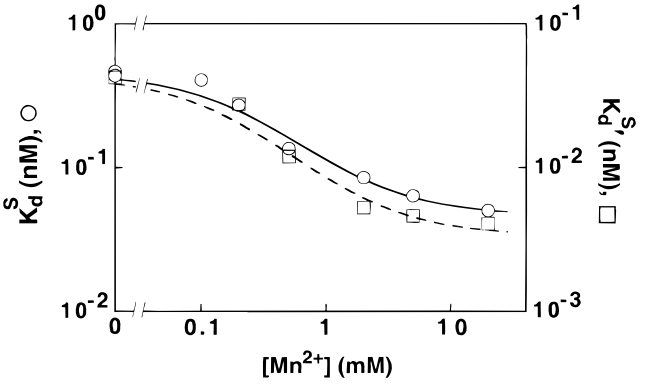
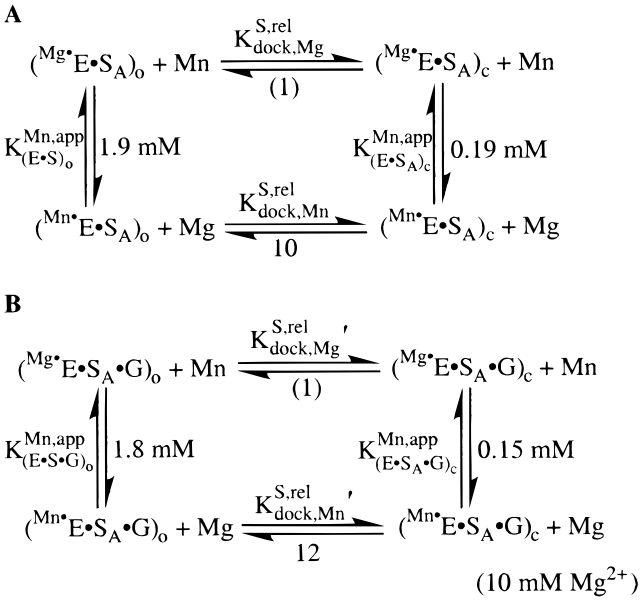


FIGURE 3. Mn^{2+} increases the affinity of $-1d,rS_A$ for E (○, K_d^S) and E•G complex (□, $K_d^{S\prime}$). Values of K_d^S and $K_d^{S\prime}$ were obtained from $K_d^S = k_{off}^S/k_{on}^S$, as described in Materials and methods, and were determined at pH 6.8–7.9 and pH 5.0–5.8, respectively. The data are fit to Equation (4), derived from Scheme 3, and give $K^{Mn,app}_{(E•S)_c} = 0.19$ mM and $K^{Mn,app}_{(E•S•G)_c} = 0.15$ mM (solid and dashed lines, respectively).

[2] The use of relative rate constant in data analysis is based on the assumption that the Mn^{2+} increasing S docking is distinct from the Mn^{2+} that increases the $-1d,rS_A$ reaction from the closed complex fourfold (see Materials and methods). The following observations strongly support this assumption: (1) There are different Mg^{2+} effects on the Mn^{2+} that increases S docking and the Mn^{2+} that affects the $-1d,rS_A$ reaction: the Mn^{2+} stabilization of S docking is the same from 2 to 50 mM Mg^{2+} (see text), whereas the Mn^{2+} enhancement of the $-1d,rS_A$ reaction from the closed complex diminishes with increasing Mg^{2+} (not shown); (2) if the same Mn^{2+} ion affected the $-1d,mS_A$ and $-1d,rS_A$ reactions from the open and closed complex, respectively, the ~10-fold larger Mn^{2+} effect on reaction of $-1d,mS_A$ than $-1d,rS_A$ predicts that this Mn^{2+} would bind ~10-fold weaker to the open than the closed complex, with a dissociation constant of ~50 mM. This is obtained from the dissociation constant of 5.5 mM for the Mn^{2+} that enhances the $-1d,rS_A$ reaction, the 10-fold greater effect of Mn^{2+} on reaction of $-1d,mS_A$ than $-1d,rS_A$, and a thermodynamic cycle analogous to that in Scheme 3. However, the Mn^{2+} effect on the $-1d,mS_A$ reaction saturates above 5 mM Mn^{2+}, indicating that the effect of Mn^{2+} on reaction from the closed complex arise from a distinct Mn^{2+} ion(s) (Fig. 2A).

[3] The Mn^{2+} and Ca^{2+} dissociation constants are apparent because Mn_D^{2+} (or Ca_D^{2+}) competes with a Mg^{2+} ion under the experimental conditions (see Results). This is denoted by the superscript "app" accompanying the K^{Mn} (or K^{Ca}) values.



SCHEME 3. Thermodynamic analysis of Mg^{2+} binding and its effect on docking.

equilibrium constants for docking of S in E•S and E•S•G, K^{S}_{dock} and $K^{S'}_{dock}$, are expressed *relative to* the docking equilibrium in 10 mM Mg^{2+} ($K^{S,rel}_{dock,Mg}$ and $K^{S,rel'}_{dock,Mg}$ in Scheme 3A,B), such that the values of $K^{S,rel}_{dock,Mg}$ and $K^{S,rel'}_{dock,Mg}$ are defined as equal to 1. The Mn^{2+} concentration dependences of K^{S}_{d} and $K^{S'}_{d}$ are similar, suggesting that the binding and effect of this Mn^{2+} is independent of bound G. Fitting these Mn^{2+} concentration dependences to Scheme 3 gives apparent Mn^{2+} dissociation constants (see footnote 3) of $K^{Mn,app}_{(E\bullet S_A)_c}$ = 0.19 mM and $K^{Mn,app}_{(E\bullet S_A)_c}$ = 0.15 mM for the $(E\bullet S_A)_c$ and $(E\bullet S_A\bullet G)_c$ complexes, respectively, which are the same within error. These Mn^{2+} affinities and the 10- and 12-fold increase in K^{S}_{dock} and $K^{S'}_{dock}$ then give the affinity of the Mn^{2+} for the $(E\bullet S)_o$ and $(E\bullet S\bullet G)_o$ complexes, according to the thermodynamic cycles in Scheme 3:

$$K^{Mn,app}_{(E\bullet S)_o} = K^{Mn,app}_{(E\bullet S_A)_c} \times \frac{K^{S,rel}_{dock,Mn}}{K^{S,rel}_{dock,Mg}} = 0.19\ mM \times 10 = 1.9\ mM$$

and

$$K^{Mn,app}_{(E\bullet S\bullet G)_o} = K^{Mn,app}_{(E\bullet S_A\bullet G)_c} \times \frac{K^{S,rel'}_{dock,Mn}}{K^{S,rel'}_{dock,Mg}} = 0.15\ mM \times 12 = 1.8\ mM.$$

The Mn^{2+} affinity obtained for $(E\bullet S\bullet G)_o$ from the Mn^{2+} effect on the affinity of $-1d,rS_A$ is the same as that determined independently from the Mn^{2+} stimulation of the $-1d,mS_A$ and $-1d,rS_{A,P\text{-}S}$ reaction (Fig. 2). The 10–12-fold stabilization of $-1d,rS_A$ binding by Mn^{2+} (Scheme 3) is also the same, within error, as the 13-fold Mn^{2+} stimulation of reactions of $-1d,mS_A$ and $-1d,rS_{A,P\text{-}S}$ (Fig. 2). The quantitative agreement of the Mn^{2+} affinity and the magnitude of its effect strongly suggest that the same Mn^{2+} ion, Mn^{2+}_D, is responsible for increasing docking of S and for stimulating the reaction of $-1d,mS_A$ and $-1d,rS_{A,P\text{-}S}$.

Mn^{2+}_D interacts with A(+1)

Replacing A(+1), the residue 3′ of the reactive phosphoryl group, with a methoxy group (rS_{Me}; Table 1) has less than a twofold effect on the binding affinity of S or its reaction rate, and the affinity of rS_{Me} is stronger than that of an open complex, suggesting that rS_{Me} binds the ribozyme predominantly in the closed complex (Narlikar et al., 1995). However, this modification abolishes the effect of Mn^{2+}_D on S docking: addition of up to 10 mM Mn^{2+} has less than a 2-fold effect on the affinity of rS_{Me}, in contrast to the 10-fold increase in the affinity of $-1d,rS_A$ (Fig. 4A).[4] This suggests that Mn^{2+}_D interacts with A(+1), either directly or indirectly.

To further test this model, we asked whether the Mn^{2+} stimulation of reaction from the open complex is also lost when A(+1) is replaced by $-OCH_3$. The $(E\bullet S_{Me})_o$ complex was obtained by introducing a 2′-methoxy substitution for the 2′-OH of U(−3) to give mS_{Me} (Table 1). As predicted, the mS_{Me} reaction was not stimulated by addition of up to 20 mM Mn^{2+}, in marked contrast to the stimulation of the reaction of $-1d,mS_A$ (Fig. 4B). Further, the apparent Mn^{2+} rescue of the thio effect at the *pro-*S_P oxygen is abolished when the A(+1) residue is replaced by a methoxy group ($-1d,rS_{Me,P\text{-}S}$, Table 1; data not shown). These results strongly suggest that the Mn^{2+} stimulation of the $-1d,rS_{A,P\text{-}S}$ reaction arises indirectly.

Mn^{2+}_D competes with a bound Mg^{2+}.

To determine whether metal site D is normally occupied by a Mg^{2+}, the effect of Mg^{2+} on the affinity of Mn^{2+}_D was investigated. If site D is unoccupied, then the observed affinity of Mn^{2+}_D would not be altered by changes in Mg^{2+} concentration; on the other hand, if a Mg^{2+} is bound at site D, then the observed affinity of Mn^{2+}_D would be weakened proportionately to the increase in Mg^{2+} concentration (see Equation (6) in Materials and methods).

The affinity of Mn^{2+}_D for the open complex was determined at a series of Mg^{2+} concentrations from the Mn^{2+} concentration dependence of the reactivity of $-1d,mS_A$ relative to $-1d,rS_A$, as described above

[4] Oligonucleotide substrates with both 2′-OH and 2′-H at U(−1) have been used in this study for the practical reasons outlined in Materials and methods. The following observations strongly suggest that the affinity and effect of metal ions at site D are independent of the 2′-OH or 2′-H at U(−1): (1) The Mn^{2+} effect on reaction of mS_A and $-1d,mS_A$ are the same, within error (not shown); (2) Mn^{2+} has the same effect on the affinity of rS_A and $-1d,rS_A$ within experimental error (not shown); (3) The Ca^{2+} effects are the same, within error, for reaction of rS_A and $-1d,rS_A$ (Fig. 5).

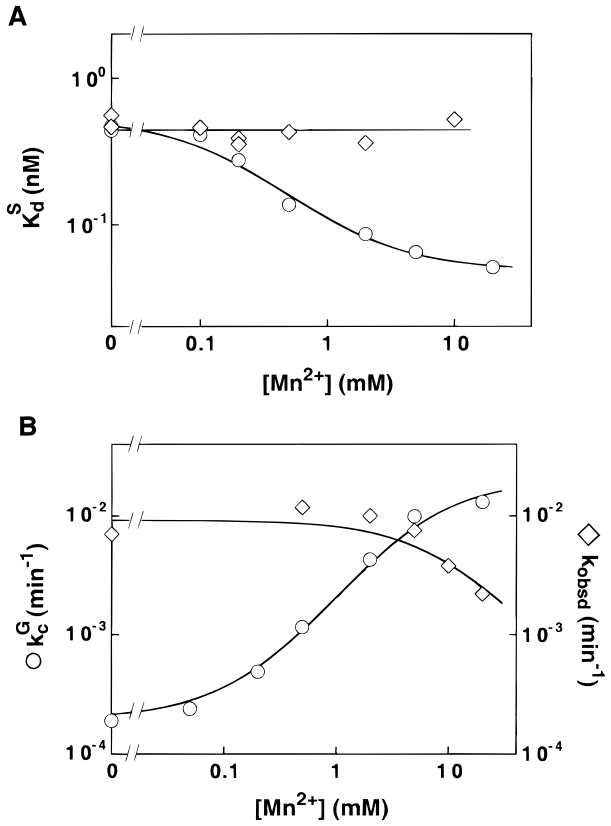


FIGURE 4. Replacement of A(+1) residue by $-OCH_3$ removes the Mn_D^{2+} effect on S docking. **A**: Effect of Mn^{2+} on the equilibrium dissociation constant of rS_{Me} (◇) and $-1d,rS_A$ (○) from E•S. Values of K_d^S for rS_{Me} were determined from $K_d^S = k_{off}^S/k_{on}^S$ as described in Materials and methods (10 mM Mg^{2+}; pH 5.4–6.4). The data for $-1d,rS_A$ are from Figure 3 and are shown for comparison. **B**: Effect of Mn^{2+} on the reaction $(E•S)_o + G \rightarrow$ products for mS_{Me} (◇; k_{obsd}), determined at pH 7.5 with 10 mM Mg^{2+}, as described in Materials and methods. The data for $-1d,mS_A$ (○) are from Figure 2A and are shown for comparison. The inhibitory effect of Mn^{2+} on the mS_{Me} reaction above 5 mM Mn^{2+} may arise from an unfavorable interaction with Mn_D^{2+} or with a distinct Mn^{2+}.

(Fig. 2 and data not shown). Increasing the Mg^{2+} concentration from 2 to 100 mM increases the observed dissociation constant of Mn_D^{2+} from $(E•S)_o$ by 50-fold, from 0.42 to 21 mM (Table 2, $K_{(E•S)_o}^{Mn,app}$), consistent with competitive binding of Mn_D^{2+} with a Mg^{2+} in the open complex.

The apparent affinity of Mn_D^{2+} for the $(E•S_A)_c$ complex can be obtained from the Mn_D^{2+} affinity for $(E•S)_o$ and the rate enhancement for $-1d,mS_A$ relative to $-1d,rS_A$ provided by Mn_D^{2+} at each Mg^{2+} concentration (Table 2, $K_{(E•S_A)_c}^{Mn,app}$). As with the $(E•S)_o$ complex, the affinity of Mn_D^{2+} for the $(E•S_A)_c$ complex is weakened with increasing Mg^{2+} concentration, suggesting that Mn_D^{2+} also competes with a bound Mg^{2+} in the closed complex.

The competitive binding of Mn_D^{2+} with Mg^{2+} and the apparent Mn_D^{2+} affinity at each Mg^{2+} concentration give the specificity of site D for Mn^{2+} relative to Mg^{2+}. The results suggest that in the open complex, Mn_D^{2+} binds approximately fivefold more strongly than Mg^{2+} (Table 2, $K_{(E•S)_o}^{Mn,rel}$). In contrast, Mn_D^{2+} binds ~60-fold more strongly than Mg^{2+} to the $(E•S_A)_c$ complex (Table 2, $K_{(E•S_A)_c}^{Mn,rel}$).

An inhibitory Ca^{2+} binds to metal site D

In previous studies, Ca^{2+} was found to inhibit the chemical step for the *Tetrahymena* ribozyme reaction, and the Ca^{2+} concentration dependence suggests the presence of two inhibitory Ca^{2+} ions. It was proposed that the inhibitory Ca^{2+} bind to catalytic metal-ion sites, such as sites A and B in Figure 1 (McConnell et al., 1997). To test these proposals and understand how Ca^{2+} inhibits the chemical step, the inhibitory effect of Ca^{2+} has been further investigated in the course of this study. In this section, we first describe characterization of the affinity and specificity of an inhibitory Ca^{2+} site, and then present evidence that the inhibitory Ca^{2+} binds to metal site D. Results that suggest that the inhibitory Ca^{2+} disrupts the positioning of S are described at the end of this section.

A Ca^{2+} ion inhibits the chemical step

Addition of Ca^{2+} decreases the rate of the reaction $E•S + G \rightarrow$ products ($S = rS_A$ or $-1d,rS_A$; Fig. 5A, open symbols). In contrast to previous observation of two inhibitory Ca^{2+} ions at 50 °C, the Ca^{2+} concentration dependences at 30 °C are consistent with an effect from a single Ca^{2+} (Scheme 4A). This difference presumably arises from different temperature effects on the interactions of the inhibitory Ca^{2+} ions, so that one of the Ca^{2+} ions binds more weakly or lacks an inhibitory effect at 30 °C. The Ca^{2+} concentration dependences for rS_A and $-1d,rS_A$ are the same, within error, suggesting that the inhibitory effect of Ca^{2+} is independent of the 2′-substituent of U(−1) (Fig. 5A, open circles and squares). The Ca^{2+} concentration dependence further suggests that the inhibitory Ca^{2+} binds to the $(E•S_A)_c$ complex formed by rS_A or $-1d,rS_A$ with an apparent dissociation constant (see footnote 3) of $K_{(E•S_A)_c}^{Ca,app} = 0.055$ mM in the presence of 2 mM Mg^{2+} (Scheme 4A). A similar inhibitory effect of Ca^{2+} was observed at 10 mM Mg^{2+}, but with an approximately fivefold weaker apparent Ca^{2+} affinity ($K_{(E•S_A)_c}^{Ca,app} = 0.28$ mM; Table 2 and Fig. 6). The weakening of Ca^{2+} affinity with increasing Mg^{2+} concentration suggests that the inhibitory Ca^{2+} competes with a bound Mg^{2+}. The competitive binding and the high apparent Ca^{2+} affinity at each Mg^{2+} concentration suggest that the inhibitory Ca^{2+} binds ~40-fold more strongly than Mg^{2+} to the $(E•S_A)_c$ complex (Table 2, $K_{(E•S_A)_c}^{Ca,rel}$).

In initial experiments, we noticed that a higher Ca^{2+} concentration was required to inhibit the reaction when

TABLE 2. Effects of Mg^{2+} on binding of Mn_D^{2+} and Ca_D^{2+}.

$[Mg^{2+}]$ (mM)	$K^{Mn,app\ a}_{(E•S)_o}$ (mM)	$K^{Mn,rel\ b}_{(E•S)_o}$	$K^{Mn,app}_{(E•S_A)_c}$ (mM)	$K^{Mn,rel\ c}_{(E•S_A)_c}$	$K^{Ca,app\ d}_{(E•S_A)_c}$ (mM)	$K^{Ca,rel\ e}_{(E•S_A)_c}$
2	0.42	4.8	0.037[f]	54	0.055	37
10	1.8	5.6	0.15[f](0.19[g])	53	0.28	36
100	21	4.8	1.6[f]	62	—	—

[a] $K^{Mn,app}_{(E•S)_o}$ is the observed Mn_D^{2+} dissociation constant from $(E•S)_o$ at each Mg^{2+} concentration. These values are equal to $K^{Mn,app}_{(E•S•G)_o}$, the Mn_D^{2+} affinity for the $(E•S•G)_o$ complex, as there is no Mn^{2+} effect on G binding (Shan & Herschlag, 1999). $K^{Mn,app}_{(E•S•G)_o}$ values were determined from the data in Figure 2 and analogous experiments (not shown).

[b] $K^{Mn,rel}_{(E•S)_o}$ is the specificity of site D for binding Mn^{2+} relative to Mg^{2+} in the $(E•S)_o$ complex, calculated from $K^{Mn,rel}_{(E•S)_o} = [Mg^{2+}]/K^{Mn,app}_{(E•S)_o}$ (see Materials and methods).

[c] $K^{Mn,rel}_{(E•S_A)_c}$ is the specificity of site D for binding Mn^{2+} relative to Mg^{2+} in the $(E•S_A)_c$ complex, obtained from $K^{Mn,rel}_{(E•S_A)_c} = [Mg^{2+}]/K^{Mn,app}_{(E•S_A)_c}$ (Equation (6)).

[d] $K^{Ca,app}_{(E•S_A)_c}$ is the observed Ca^{2+} dissociation constant from the $(E•S_A)_c$ complex at each Mg^{2+} concentration, determined from the data in Figures 5 and 6.

[e] $K^{Ca,rel}_{(E•S_A)_c}$ is the specificity of metal site D for binding Ca^{2+} relative to Mg^{2+} in the $(E•S_A)_c$ complex, obtained from $K^{Ca,rel}_{(E•S_A)_c} = [Mg^{2+}]/K^{Ca,app}_{(E•S_A)_c}$ (Equation (7)).

[f] $K^{Mn,app}_{(E•S_A)_c}$ is the observed Mn_D^{2+} dissociation constant from the $(E•S_A)_c$ complex at each Mg^{2+} concentration. The values are calculated from the $K^{Mn,app}_{(E•S)_o}$ values in this table and the maximal Mn^{2+} stimulation of the reaction of $-1d,mS_A$ relative to $-1d,rS_A$ of 11-, 13-, and 13-fold Mn^{2+} observed at 2, 10, and 100 mM Mg^{2+}, respectively

$$K^{Mn,app}_{(E•S_A)_c} = K^{Mn,app}_{(E•S)_o} \Big/ \left(\frac{k^S_{dock,Mn}}{k^S_{dock,Mg}}\right) = K^{Mn,app}_{(E•S)_o} \Big/ \left(\frac{k^{G,rel}_{c,Mn}}{k^{G,rel}_{c,Mg}}\right);$$

these expressions are equal because the Mn^{2+} stimulation of reaction from the open complex relative to the closed complex ($k^{G,rel}_{c,Mn}/k^{G,rel}_{c,Mg}$) arises solely from stabilization of the docked complex by Mn^{2+} ($k^S_{dock,Mn}/k^S_{dock,Mg}$) (see footnote 2); cf. Schemes 2 and 3].

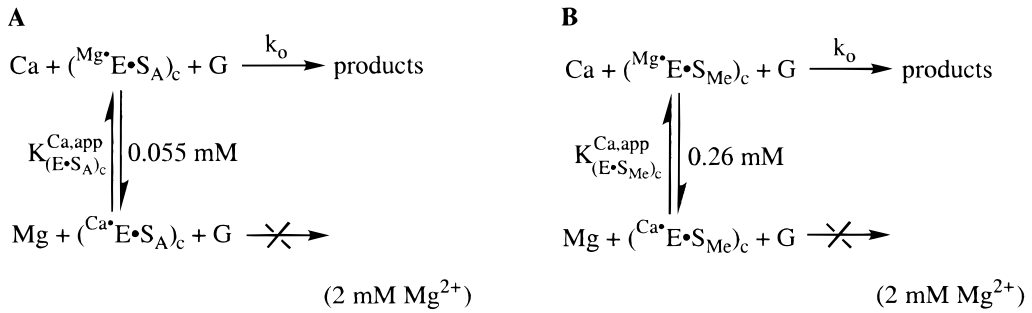
[g] The value in parenthesis was independently determined from the binding data in Figure 3.

the A(+1) residue is replaced by a methoxy group (Fig. 5A), with an apparent Ca^{2+} affinity of $K^{Ca,app}_{(E•S_{Me})_c}$ = 0.26 mM (Scheme 4B). The ~5-fold weaker Ca^{2+} affinity for the $(E•S_{Me})_c$ than the $(E•S_A)_c$ complex is analogous to the ~10-fold weaker affinity of Mn_D^{2+} for $(E•S_{Me})_c$ than $(E•S_A)_c$. This raised the possibility that the inhibitory Ca^{2+} also binds to metal site D.

The inhibitory Ca^{2+} competes with Mn_D^{2+}

To test whether the inhibitory Ca^{2+} site coincides with metal site D, we determined the affinity of Ca^{2+} under conditions such that site D is occupied by Mn^{2+}. If the inhibitory Ca^{2+} were to bind to metal site D, it would need to compete with Mn^{2+}, which binds strongly to site D. That is, added Mn^{2+} sufficient to occupy metal site D would weaken the apparent affinity of Ca^{2+}, and the apparent Ca^{2+} affinity in the presence of Mn^{2+}, $K^{Ca,app\prime}$, could be predicted from the apparent affinities of Mn_D^{2+} and Ca^{2+} determined above according to the relationship of Equation (9) in Materials and methods.

Figure 5B shows the Ca^{2+} inhibition of the reaction $E•rS_A + G \rightarrow$ products in the presence of varying concentrations of added Mn^{2+} with a background of 2 mM Mg^{2+}. Apparent Ca^{2+} affinities obtained from these concentration dependences are in reasonable agreement



SCHEME 4. Ca^{2+} binding and inhibition.

with the values predicted for Ca^{2+} binding to metal site D (Table 3). Analogous results were obtained when the A(+1) residue is replaced by a methoxy group (Fig. 5C and Table 3). These results provide strong evidence that the inhibitory Ca^{2+} competes with the Mn^{2+} at site D.

The simplest interpretation for the competitive binding of Ca^{2+} with Mn_D^{2+} is that the inhibitory Ca^{2+} occupies metal site D. This Ca^{2+} ion is therefore referred to as Ca_D^{2+} below. It is possible, nevertheless, that some of the ligands differ for Ca^{2+} or Mn^{2+}, or even that these metal ions bind at different sites that are mutually exclusive, either because of electrostatic repulsion or conformational rearrangements. Some differences in the ligands or coordination geometry for Mn^{2+} and Ca^{2+} is implied by the distinct effects of Ca^{2+} and Mn^{2+} on the chemical step and on guanosine binding described below.

TABLE 3. The inhibitory Ca^{2+} competes with binding of Mn_D^{2+}.

	$K^{Ca,app\prime}$, mM[a]			
	$(E•S_A)_c$		$(E•S_{Me})_c$	
$[Mn^{2+}]$, mM	observed	predicted	observed	predicted
0	0.055	N.A.[b]	0.26	N.A.[b]
0.2	0.26	0.35	—[c]	—[c]
0.5	0.63	0.80	—[c]	—[c]
1	1.9	1.6	0.64	0.88
5	—[c]	—[c]	3.3	3.4

[a] $K^{Ca,app\prime}$ is the apparent affinity of the inhibitory Ca^{2+} at each Mn^{2+} concentration (2 mM Mg^{2+} background). Observed $K^{Ca,app\prime}$ values were determined in Figure 5. Predicted $K^{Ca,app\prime}$ values were calculated from Equation (9).
[b] N.A.: not applicable.
[c] —: not determined.

The inhibitory Ca^{2+} increases docking of S

In an effort to understand how Ca_D^{2+} inhibits the chemical step, we determined the effect of Ca^{2+} on the binding affinity of —1d,rS_A. Addition of Ca^{2+} increased the affinity of —1d,rS_A at least 10-fold (Fig. 6A; 10 mM Mg^{2+}). This affinity increase follows the Ca^{2+} concentration dependence predicted for an effect from Ca_D^{2+} (Fig. 6A, dashed line), suggesting that the inhibitory Ca^{2+} causes the *increase* in the affinity of —1d,rS_A.

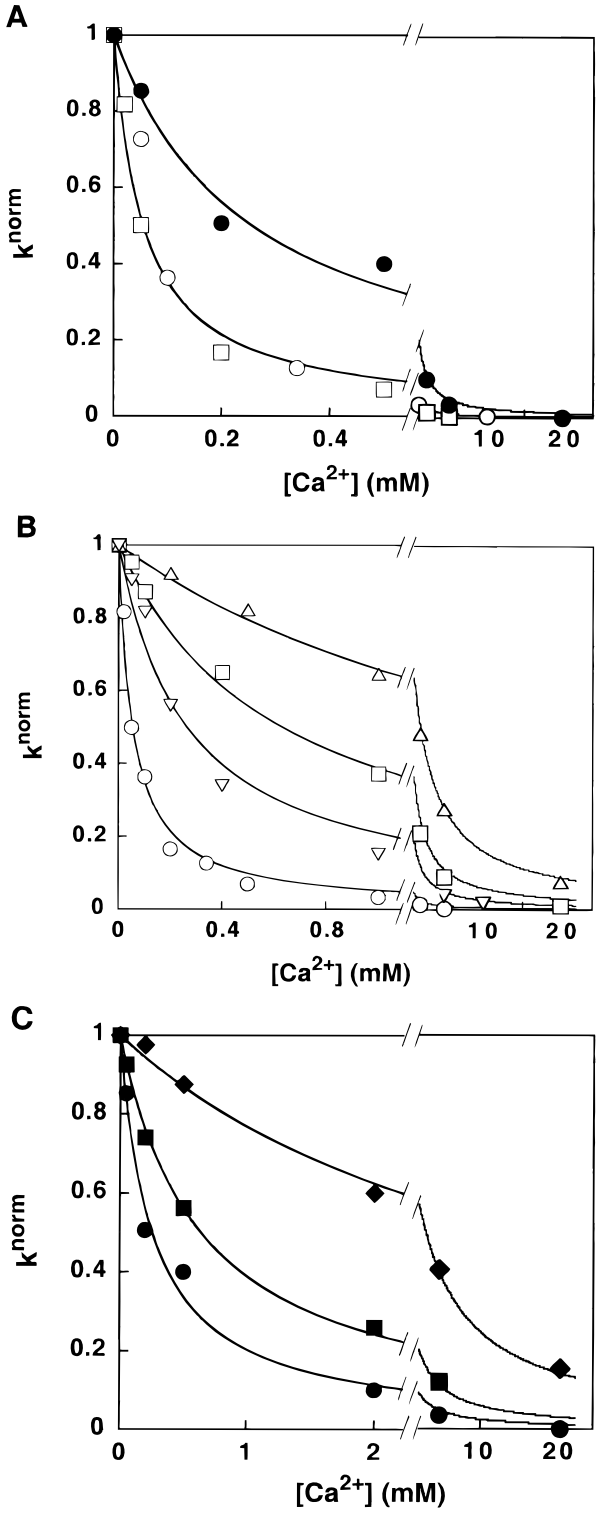


FIGURE 5. Ca^{2+} inhibition of the ribozyme reaction. **A**: Ca^{2+} inhibition of the reaction E•S + G → products for rS_A (○), —1d,rS_A (□), and rS_{Me} (●). Reactions were carried out with subsaturating G (1 μM for rS_A and rS_{Me}, pH 6.4; 10 μM for —1d,rS_A, pH 7.2) at 2 mM Mg^{2+} as described in Materials and methods. Rate constants were normalized such that $k^{norm} = 1$ in the absence of added Ca^{2+}. The data are fit to the inhibition model of Scheme 4 according to Equation (5), and give $K^{Ca,app}_{(E•S_A)_c}$ = 0.055 mM and $K^{Ca,app}_{(E•S_{Me})_c}$ = 0.26 mM. **B**: Ca^{2+} inhibition of the reaction E•S + G → products with —1d,rS_A at pH 7.2 in the presence of 2 mM Mg^{2+} with 0 (○; from part A), 0.2 (▽), 0.5 (□), or 1 mM (△) added Mn^{2+}. The rate constants were normalized as described in **A**. The lines are fits of the data as in **A**, and give $K^{Ca,app\prime}_{(E•S_A)_c}$ = 0.26, 0.63, and 1.9 mM in the presence of 0.2, 0.5, and 1 mM Mn^{2+}, respectively. **C**: Ca^{2+} inhibition of the reaction: E•rS_{Me} + G → products in the presence of 0 (●; from **A**), 1 (■), and 5 mM (♦) Mn^{2+} (2 mM Mg^{2+}; pH 6.4). The rate constants were determined and normalized as in **A**. The lines are fits of the data as in **A**, derived from Scheme 4, and give $K^{Ca,app\prime}_{(E•S_{Me})_c}$ = 0.64 and 3.3 mM in the presence of 1 and 5 mM Mn^{2+}, respectively.

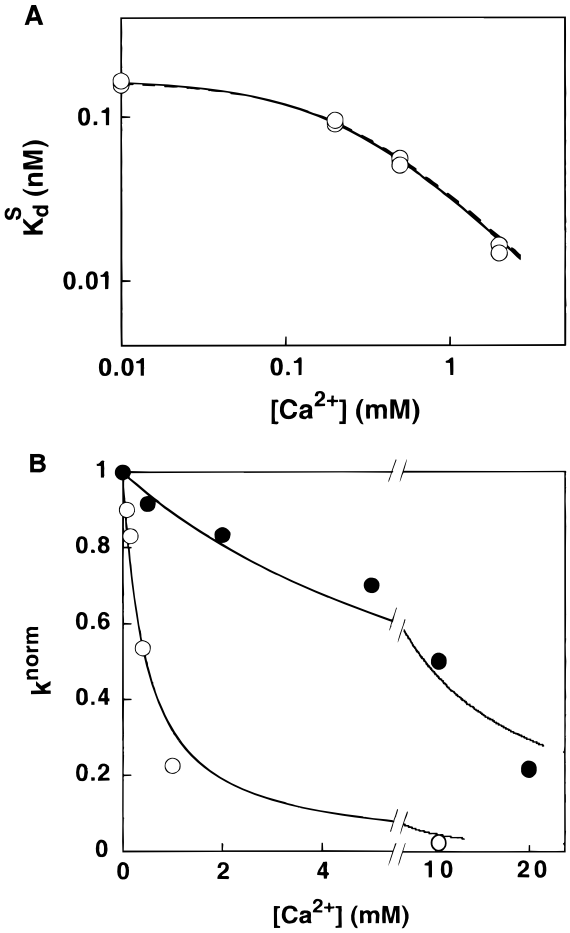
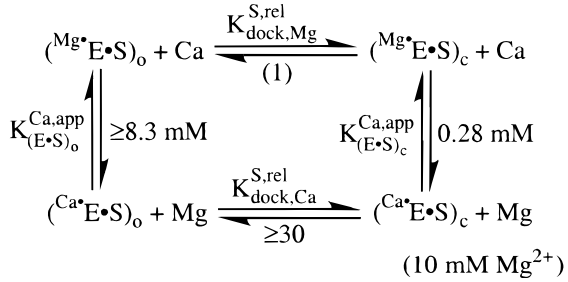


FIGURE 6. The inhibitory Ca^{2+} increases docking of S. **A**: Ca^{2+} decreases the equilibrium dissociation constant of $-1d,rS_A$ from the E•S complex. K_d^S was determined from $K_d^S = k_{off}^S/k_{on}^S$ as described in Materials and methods (10 mM Mg^{2+}). The solid line is the best fit of the data to a model in which a single Ca^{2+} decreases K_d^S, and the dashed line (barely visible) is a predicted Ca^{2+} concentration dependence if the inhibitory Ca^{2+}, with $K_{(E•S_A)_c}^{Ca,app} = 0.28$ mM (Table 2), is responsible for the observed effect. **B**: Ca^{2+} inhibition of the reaction E•S + G → products for rS_A (○) and $-1r,dS_A$ (●). Reactions were carried out with a subsaturating G concentration (1 μM) at 10 mM Mg^{2+}, pH 6.4, as described in Materials and methods and were normalized as in Figure 5. The data are fit to Equation (5), derived from Scheme 4, and give $K_{(E•S)_c}^{Ca,app} = 0.28$ mM (○) and $K_{(E•S)_o}^{Ca,app} = 8.3$ mM (●).

Additional experiments indicated that addition of up to 5 mM Ca^{2+} does not affect the equilibrium for formation of the $(E•S)_o$ complex ($-1d,mS_A$; data not shown). Thus, the observed Ca^{2+} effect on K_d^S represents an effect on the equilibrium for docking of S, K_{dock}^S (Scheme 5). As in Scheme 3, K_{dock}^S is expressed relative to the value at 10 mM Mg^{2+}, $K_{dock,Mg}^S$, such that $K_{dock,Mg}^{S,rel}$ is defined as equal to 1. As K_d^S continues to decrease at the highest Ca^{2+} concentrations, the observed 10-fold increase in K_{dock}^S represents a lower limit for the effect of Ca_D^{2+}.

To further test the model that Ca_D^{2+} stabilizes S docking, we measured the affinity of the inhibitory Ca^{2+} for the $(E•S)_o$ complex. The stronger docking of S with



SCHEME 5. Ca^{2+} binding and effect on docking.

Ca^{2+} bound at site D relative to Mg^{2+} predicts that Ca^{2+} would bind to site D with a stronger affinity for the $(E•S)_c$ than the $(E•S)_o$ complex, according to the thermodynamic cycle of Scheme 5

$$\left[\frac{K_{(E•S)_o}^{Ca,app}}{K_{(E•S)_c}^{Ca,app}} = \frac{K_{dock,Ca}^{S,rel}}{K_{dock,Mg}^{S,rel}}\right].$$

Binding of Ca^{2+} to the open complex was followed from the Ca^{2+} concentration dependence of the reaction $E•-1r,dS_A + G \rightarrow$ products (Table 1). Removal of the 2′-OH groups at positions (−2) to (−6) disrupts docking of S, so that $-1r,dS_A$ binds the ribozyme predominantly in the $(E•S)_o$ complex (Narlikar & Herschlag, 1996). Indeed, a substantially higher concentration of Ca^{2+} is required to inhibit the reaction for $-1r,dS_A$ than for rS_A (Fig. 6B). The Ca^{2+} concentration dependence of the $-1r,dS_A$ reaction gives an observed Ca^{2+} dissociation constant of 8.3 mM; this represents a lower limit for the dissociation constant of Ca^{2+} from the $(E•S)_o$ complex [Scheme 5, $K_{(E•S)_o}^{Ca,app} \geq 8.3$ mM], because the inhibitory effect above 8 mM Ca^{2+} could arise from one or more Ca^{2+} ions distinct from Ca_D^{2+}. Following the analysis of Scheme 5, Ca_D^{2+} binds at least 30-fold more strongly to the $(E•S)_c$ than the $(E•S)_o$ complex. This provides independent evidence that the inhibitory Ca^{2+} increases S docking

$$\left[\frac{K_{dock,Ca}^{S,rel}}{K_{dock,Mg}^{S,rel}} = \frac{K_{(E•S)_o}^{Ca,app}}{K_{(E•S)_c}^{Ca,app}} \geq 30\right].$$

The inhibitory Ca^{2+} disrupts coupled binding between G and S

The results described above indicate that Ca_D^{2+} increases docking of S but inhibits reaction from the closed complex, suggesting that the oligonucleotide substrate might dock nonproductively in the presence of Ca_D^{2+}. Previous results indicated that binding of S and G strengthen one another when S is docked within the ribozyme active site (McConnell et al., 1993). Additional observations further suggested that this cou-

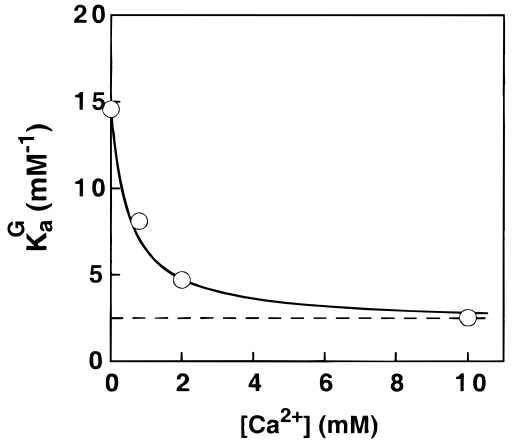
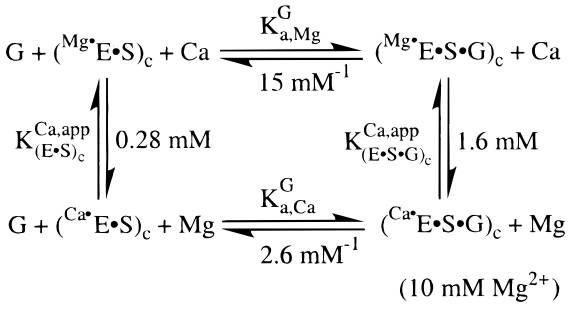


FIGURE 7. Ca^{2+} weakens binding of G. Equilibrium association constants of G for the E•S complex, K_a^G, were determined with −1d,rS_A at pH 7.1, 10 mM Mg^{2+}, as described in Materials and methods. The solid line is the Ca^{2+} concentration dependence *predicted* from the model of Scheme 6. The dashed line represents the affinity of G for the ribozyme without bound S in the absence of Ca^{2+}.

pled binding reflects positioning of the substrates within the ribozyme active site (McConnell & Cech, 1995; Shan & Herschlag, 1999). Thus, if the inhibitory Ca^{2+} were to disrupt positioning of the oligonucleotide substrate, coupled binding between S and G might be lost.

The equilibrium association constant of G for the $(E•S)_c$ complex (S = −1d,rS_A) was determined at a series of Ca^{2+} concentrations (K_a^G; 10 mM Mg^{2+}). In the absence of added Ca^{2+}, G binds to the $(E•S)_c$ complex with an affinity of $K_{a,Mg}^G = 15\ mM^{-1}$, approximately sixfold higher than the affinity for free E of 2.8 ± 0.4 mM^{-1}, consistent with previous determinations (Fig. 7A and data not shown; McConnell et al., 1993; McConnell & Cech, 1995). Addition of Ca^{2+} weakens the affinity of G for $(E•S)_c$ (Fig. 7). The Ca^{2+} effect on K_a^G follows the concentration dependence *predicted* from the model of Scheme 6, in which a Ca^{2+} ion binds to the $(E•S)_c$ complex with an apparent affinity of $K_{(E•S)_c}^{Ca,app} = 0.28$ mM and weakens the binding of G to $K_{a,Ca}^G = 2.8\ mM^{-1}$ (Fig. 7, solid line). The Ca^{2+} affinity is that of the inhibitory Ca^{2+} determined from independent experiments (Fig. 5), and the value of $K_{a,Ca}^G$ is the G affinity for free E, also determined independently (Fig. 7, dashed line; McConnell & Cech, 1995; Shan & Herschlag, 1999). These data provide strong evidence that the inhibitory Ca^{2+} disrupts coupling between S and G.



SCHEME 6. Thermodynamic analysis of Ca^{2+} and G binding.

DISCUSSION

Indirect metal-ion rescue and inhibition in RNA catalysis

The presence of catalytic metal ions in RNA catalysis has been inferred from the rescuing effect of soft metal ions on reactions of modified substrates (Christian & Yarus, 1993; Piccirilli et al., 1993; Warnecke et al., 1996; Chen et al., 1997; Peracchi et al., 1997; Sjögren et al., 1997; Sontheimer et al., 1997; Weinstein et al., 1997; Scott & Uhlenbeck, 1999; Shan & Herschlag, 1999; Yoshida et al., 1999). On face value, the Mn^{2+} stimulation of reaction of the substrate with a thio substitution at the *pro-*S_P oxygen relative to the unmodified substrate observed in this work might have been taken as evidence for a Mn^{2+} interaction with the S_P sulfur in the *Tetrahymena* group I ribozyme reaction. However, analysis of the effect of Mn^{2+} on individual reaction steps revealed an alternative origin for the Mn^{2+} stimulation. The apparent rescue arises because different reaction steps are followed with the thio and the unmodified substrate, and Mn^{2+} affects a step other than chemical cleavage.

The origin of this effect was traced to a Mn^{2+} ion, referred to as Mn_D^{2+}, that stabilizes docking of S into its tertiary interactions with the ribozyme core (Scheme 1), and this effect is observed for both the thio and the unmodified oligonucleotide substrates. Because the thio substrate reacts from the undocked or open complex whereas the unmodified substrate reacts from the closed complex, an additional docking step was monitored for reaction of the thio substrate. Mn_D^{2+} thus rescues reaction of the thio substrate, despite the absence of a direct Mn^{2+} coordination to the S_P sulfur. Thio substitution of another ligand, the 3′-bridging oxygen of S, also causes the modified substrate to bind predominantly in the open complex, and the effect of Mn_D^{2+} has also been observed in the Mn^{2+} concentration dependence for its reaction (Shan et al., 1999). These observations underscore the importance of ensuring that the same microscopic reaction steps are followed for the modified and unmodified substrates in metal-ion specificity switch experiments.

As Mn_D^{2+} does not specifically rescue the deleterious effect of phosphorothioate substitution at the *pro-*S_P reactive phosphoryl oxygen (see above), the simplest interpretation of the results is that Mn_D^{2+} binds to a site distinct from the metal site interacting with the *pro-*S_P

oxygen (Fig. 1, M_D; Yoshida et al., 1999). Additional unpublished experiments strongly suggest that M_D does not interact with the *pro-*S_P oxygen.

Several observations strongly suggest that metal site D is distinct from the previously identified active-site metal ions that interact with the 3′-bridging oxygen of S and with the 3′ and 2′ moieties of G (Fig. 1, M_A, M_B, and M_C; Piccirilli et al., 1993; Sjögren et al., 1997; Weinstein et al., 1997; Shan & Herschlag, 1999). The Mn^{2+} affinity and Mn^{2+}/Mg^{2+} specificity of site D for the free ribozyme and ribozyme•substrate complexes are distinct from these other metal-ion sites. In addition, the Mn^{2+} concentration dependences for rescue of modifications at each of these other positions suggest stimulation by two distinct Mn^{2+} ions, the Mn^{2+} specific to the modified substrate and Mn_D^{2+} (Shan & Herschlag, 1999; Shan et al., 1999). Finally, the binding and stimulatory effect of the Mn^{2+} interacting with the 2′ moiety of G (M_C) is unaffected when A(+1), the residue 3′ to the cleavage site, is replaced by a methoxy group, whereas this modification eliminates the effect of Mn_D^{2+}. Site D thus represents a novel metal-ion site in the vicinity of the active site of this ribozyme.

Previously, the inhibitory effect of Ca^{2+} on the *Tetrahymena* ribozyme reaction was proposed to arise from two Ca^{2+} ions that displace catalytic Mg^{2+} ions (McConnell et al., 1997). Similar observations and interpretations have been made with other RNA enzymes (see, e.g., Lott et al., 1998; Warnecke et al., 1999). However, the results of this work suggest that at least one of the inhibitory Ca^{2+} sites is distinct from the metal-ion sites directly involved in the chemical reaction. Rather, the inhibitory Ca^{2+} binds to metal site D, a site that interacts with the A(+1) residue.

The results herein exemplify the complexity of metal-ion effects possible in RNA. Another example is the indirect rescue by soft metal ions observed for folding of the P4–P6 domain of the *Tetrahymena* ribozyme (Basu & Strobel, 1999). In this previous work, Mn^{2+} restored folding of several mutants with thio substitutions at phosphoryl oxygens or deoxy substitutions at 2′-hydroxyl groups, even though these groups are not directly involved in metal-ion coordination in the crystal structure. On the other hand, Mn^{2+} did not restore folding of several mutants with thio substitution at phosphoryl oxygens that provide ligands for Mg^{2+} ions (Cate & Doudna, 1996; Cate et al., 1996). These observations are most simply accounted for by an indirect Mn^{2+} effect on ribozyme folding analogous to the Mn^{2+} effect observed herein. That is, if replacing Mg^{2+} with Mn^{2+} globally stabilizes the folded structure, a destabilizing effect from *any* mutation can be overcome by Mn^{2+}, provided that the destabilization is not too large. The observed inability of Mn^{2+} to rescue thio substitution of atoms that directly coordinate metal ions may arise because these mutations have larger destabilizing effects due to steric and geometrical constraints at metal-ion sites situated within networks of interactions.

The results herein demonstrate that metal-ion rescue of substrate or ribozyme modifications can arise from indirect metal-ion effects that are unrelated to the particular modification. Instead of a direct metal-ion interaction, a rescuing effect of soft metal ions can also arise if the modified group recruits additional metal ions that are not present in the wild-type substrate or ribozyme, or if the reaction follows a different pathway or mechanism. Conversely, the absence of metal-ion rescue does not rule out a metal-ion interaction with the modified atom. Introduction of a bulky sulfur atom could potentially disrupt the surrounding active-site groups and metal-ion ligands, preventing the binding or interaction of metal ions (see, e.g., Herschlag et al., 1991; Brautigam & Steitz, 1998). Analogously, metal-ion inhibition can arise from indirect effects, from metal ions not interacting with substrate groups undergoing chemical transformation.

Given the numerous metal ions that are typically bound to an RNA molecule, the results of metal-ion rescue and inhibition experiments should be interpreted with caution. Nevertheless, quantitative mechanistic analysis of metal-ion effects on modified substrates has been successful, in several previous studies and in the current work, in distinguishing different metal-ion sites (Shan et al., 1999) and in probing the properties and roles of the individual sites (Shan & Herschlag, 1999, and below).

Probing specific metal ion-RNA interactions

Previous work has provided strong evidence for at least three distinct metal ions at the active site of the *Tetrahymena* ribozyme (Shan et al., 1999). In the current study, a metal-ion site, referred to as site D, has also been uncovered. Further, kinetic and thermodynamic analyses of the metal-ion effects have provided a quantitative description of the metal-ion specificity of this site along the reaction pathway and, correspondingly, the effects of different metal ions bound at this site on individual reaction steps. These results are summarized in Figure 8 and discussed below.

In the free ribozyme, site D has low specificity for Mn^{2+} and Ca^{2+} relative to Mg^{2+}, with Mn^{2+} binding fivefold more strongly, and Ca^{2+} binding with equal or lower affinity than Mg^{2+} (Fig. 8). The Mn^{2+} and Ca^{2+} specificities of site D remain the same in the $(E•S)_o$ complex. This is expected because S lacks tertiary interactions with active site groups and bound metal ions in the open complex (Bevilacqua et al., 1992; Herschlag, 1992; Narlikar et al., 1997; Narlikar & Herschlag, 1996). As oxygen ligands typically have low specificity for Mn^{2+} relative to Mg^{2+} (Martell & Smith, 1976; see also Table 2 in Shan & Herschlag, 1999), the simplest interpretation of the low Mn^{2+} specificity is that in the free ribozyme and the open com-

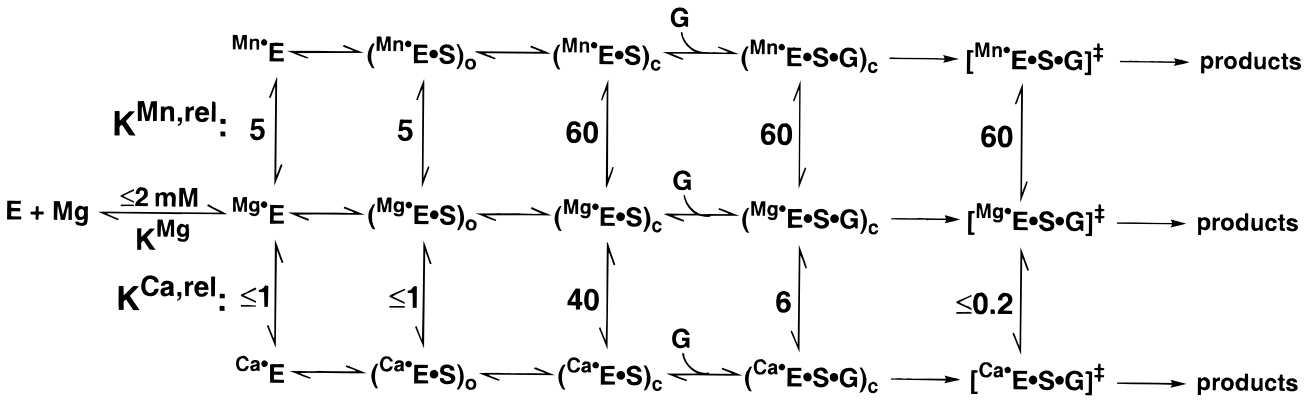


FIGURE 8. Summary of metal-ion specificities of site D in the ribozyme and ribozyme•substrate complexes. The competitive binding studies herein indicate that Mg^{2+} saturates at site D at the lowest Mg^{2+} concentration used, so that the Mg^{2+} dissociation constant of site D, K^{Mg}, is below 2 mM. $K^{Mn,rel}$ and $K^{Ca,rel}$ are the specificities of site D for Mn^{2+} and Ca^{2+}, respectively, relative to Mg^{2+}. The values are for oligonucleotide substrates with an A(+1) residue. For simplicity metal-ion interactions are discussed for free E and the $(E•S)_o$ complex in the absence of bound G. Binding of G to free E and the open complex does not affect Mn_D^{2+} and Ca_D^{2+} affinity (see Results; data not shown). $K^{Mn,rel}$ and $K^{Ca,rel}$ values were obtained as described in Materials and methods. The metal-ion specificities for free E are equal to those determined for $(E•S)_o$, as Mn^{2+} and Ca^{2+} do not affect formation of the open complex (see Results). For $(E•S)_o$, the value of $K^{Mn,rel}_{(E•S)_o}$ is from Table 2, and the value of $K^{Ca,rel}_{(E•S)_o}$ is from $K^{Ca,rel}_{(E•S)_o} = [Mg^{2+}]/K^{Ca,app}_{(E•S)_o} \leq 10$ mM/8.3 mM ≈ 1 (see Materials and methods and Fig. 6). The $K^{Mn,rel}$ and $K^{Ca,rel}$ values for the $(E•S)_c$ complex are from Table 2. The value of $K^{Mn,rel}$ for the $(E•S•G)_c$ complex and the transition state, $[E•S•G]^‡$, is the same as for $(E•S)_c$, because Mn^{2+} has no effect on binding of G or on reaction from the $(E•S)_c$ complex. The value of $K^{Ca,rel}$ for $(E•S•G)_c$ was obtained from $K^{Ca,rel}_{(E•S•G)_c} = [Mg^{2+}]/K^{Ca,app}_{(E•S•G)_c} = 10$ mM/1.6 mM ≈ 6 (see Materials and methods and Scheme 6). The value of $K^{Ca,rel}$ for $[E•S•G]^‡$ was obtained from the $\geq$200-fold slower chemical step with Ca^{2+} than with Mg^{2+} bound (Fig. 5), the value of $K^{Ca,rel}_{(E•S)_c} = 40$ (Table 2), and a thermodynamic cycle analogous to Scheme 6, which gives $K^{Ca,rel}_{[E•S•G]^‡} \leq K^{Ca,rel}_{(E•S)_c}/200 = 40/200 = 0.2$.

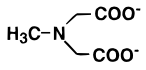
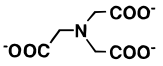
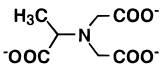
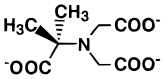
plex, site D may consist primarily of oxygen ligands, such as water, phosphoryl oxygens, and 2′-hydroxyl groups.

The specificity of site D for Mn^{2+} and Ca^{2+} relative to Mg^{2+} increases in going from the open to the closed complex [Fig. 8, $(E•S)_o$ vs. $(E•S)_c$], with 60- and 40-fold higher affinity than Mg^{2+}, respectively. This is in contrast to the low Mn^{2+} and Ca^{2+} specificities in free E and in the open complex (Fig. 8). The increase in metal-ion specificity means that binding of Mn^{2+} and Ca^{2+} to site D stabilizes docking of S into the closed complex. The increased metal-ion specificity could arise because of additional ligands in the $(E•S)_c$ complex, or because of more stringent steric and geometric constraints on ligands already present at site D in the open complex.

The stabilizing effect of Mn_D^{2+} on docking of oligonucleotide substrates is observed only in the presence of the A(+1) residue, indicating that the increased Mn^{2+} specificity of site D in the closed complex arises from an interaction of Mn_D^{2+} with A(+1). The presence of the A(+1) residue also increases the Ca^{2+} specificity of site D approximately fivefold, reflecting an interaction of Ca^{2+} with A(+1). Unlike Mn^{2+}, however, the Ca^{2+} specificity for site D remains at least 10-fold higher in the $(E•S)_c$ than the $(E•S)_o$ complex even in the absence of A(+1), suggesting that Ca_D^{2+} makes additional active site interactions besides A(+1). We do not know whether the contribution of A(+1) to the increased Mn^{2+} and Ca^{2+} specificity arises from a direct interaction of these metal ions with groups on the A(+1) residue, or from indirect effects such as rearrangement of the active site upon removal of A(+1). In contrast to the effects observed with Mn^{2+} and Ca^{2+}, replacing A(+1) with a methoxy group has less than a twofold effect on binding of the oligonucleotide substrate or on the chemical step in Mg^{2+}. This suggests that the Mg^{2+} ion that normally occupies metal site D does not exhibit preferential interactions with the A(+1) residue. It thus appears that different metal ions bound at site D interact differently with the oligonucleotide substrate (see also, e.g., Pan et al., 1993; Feig et al., 1998; Jose et al., 1999; and references therein).

In general, Ca^{2+} has weaker affinity for compounds containing a single oxygen or nitrogen ligand than Mg^{2+} (Table 4, compounds **I–III**). How, then, does the ribozyme bind Ca^{2+} more strongly than Mg^{2+} in the closed complex? The larger size and the higher coordination number of Ca^{2+} than Mg^{2+} could allow Ca^{2+} to more favorably make multiple inner-sphere interactions with ribozyme functional groups, and docking of S within the ribozyme core may impose more stringent steric or geometrical constraints for these ligands than those in the open complex, thereby giving rise to the higher Ca^{2+} specificity of site D in the $(E•S)_c$ complex (Amburgey et al., 1994; Linse & Forsen, 1995; Katz et al., 1996; and references therein). The higher affinities of Ca^{2+} than Mg^{2+} observed for compounds containing multiple, sterically constrained ligands (Table 4, cf. **VI–VII** vs. **XIII–X**; see also Amburgey et al., 1994; Linse &

TABLE 4. Comparison of equilibrium constants for association of Mg^{2+} and Ca^{2+} with model compounds.

Compound		log K^{M} [a]			
		Mg^{2+}	Ca^{2+}	$K^{Ca,rel}$ [b]	ΔΔG (kcal/mol) [c]
(I)	OH^-	2.58	1.30	0.052	1.8
(II)	NH_3	0.23	−0.20	0.37	0.6
(III)	PO_4^{3-}	2.81	2.66	0.71	0.2
(IV)	ATP^{4-}	4.06	3.77	0.59	0.3
(V)	UTP^{4-}	4.01	3.69	0.48	0.4
(VI)	$H_2N\text{-}CH_2\text{-}COO^-$	2.22	1.40	0.14	1.2
(VII)		3.5	3.8	2	−0.4
(VIII)		5.5	6.4	8	−1.3
(IX)		5.84	7.0	15	−1.7
(X)		6.30	8.32	110	−2.8

[a] K^{M} is the equilibrium association constant for the reaction $M + L \rightleftharpoons M{\bullet}L$, in which M is Mg^{2+} or Ca^{2+} and L is the compound that provides ligand(s) for M. Values of K^{M} are in units of M^{-1} and were determined at 25 °C at ionic strength 0.1. From Martell & Smith (1976).

[b] $K^{Ca,rel} = K^{Ca}/K^{Mg}$, and gives the specificity for association with Ca^{2+} relative to Mg^{2+}.

[c] $\Delta\Delta G = -RT \ln K^{Ca,rel}$, and gives the difference in binding free energy for Ca^{2+} and Mg^{2+}. Negative values of ΔΔG denote a preference for Ca^{2+}.

Forsen, 1995) provide examples of geometrical effects that could give rise to high specificity for Ca^{2+}.

Mn^{2+}_D has no effect on binding of the guanosine nucleophile or the chemical step from the $(E{\bullet}S{\bullet}G)_c$ complex. The Mn^{2+} specificities of site D in $(E{\bullet}S{\bullet}G)_c$ and in the transition state, $[E{\bullet}S{\bullet}G]^{\ddagger}$, are therefore the same as in the $(E{\bullet}S)_c$ complex (Fig. 8). These results suggest that the interactions of Mn^{2+}_D are maintained in the $(E{\bullet}S{\bullet}G)_c$ complex and in the transition state. Thus, these interactions are conducive to the chemical reaction.

In contrast, Ca^{2+}_D weakens the binding of G to the $(E{\bullet}S)_c$ complex and inhibits reaction once S is docked within the active site. In reactions with Mg^{2+}, binding of G is strengthened by the presence of bound S at the active site (McConnell et al., 1993; Shan & Herschlag, 1999). This coupled binding appears to reflect alignment of the substrates within the active site for their chemical reaction (McConnell et al., 1993; McConnell & Cech, 1995; Shan & Herschlag, 1999; and references therein). This coupled binding is lost when Ca^{2+} is bound at site D, such that the affinity of G for $(E{\bullet}S)_c$ is the same as that for free E. This is equivalent to a decrease in the Ca^{2+} specificity upon binding of G to $(E{\bullet}S)_c$ (Fig. 8). We suggest that this disruption of coupling reflects a disruption in the positioning of S and G with respect to one another induced by the interactions of Ca^{2+}_D with the oligonucleotide substrate and/or the active site. This could also account for the Ca^{2+}_D inhibition of the chemical step by more than 200-fold, which is reflected in a Ca^{2+}/Mg^{2+} specificity of site D that is even lower in the chemical transition state than in the $(E{\bullet}S{\bullet}G)_c$ ground state, with $K^{Ca,rel}_{[E{\bullet}S{\bullet}G]^{\ddagger}} \leq 0.2$. Instead of displacing catalytic metal ions, the Ca^{2+} at site D appears to exert its inhibitory effect by mispositioning the substrates within the ribozyme active site. Analogous effects have been observed for binding of Tb^{3+} to the hammerhead ribozyme (Feig et al., 1998).

Variants of the *Tetrahymena* ribozyme have been selected that retain catalytic activity in the presence of Ca^{2+} (Lehman & Joyce, 1993). Most of the mutations were found to occur at or near the conserved regions P7, J8/7, and J4/5 (Lehman & Joyce, 1993), which form the catalytic core of this ribozyme that binds the guanosine nucleophile and contacts the oligonucleotide substrate (Michel & Westhof, 1990; Golden et al., 1998; Strobel et al., 1998; and references therein). Replacing a Mg^{2+} ion with Ca^{2+} could perturb the alignment of this closely-packed active site, consistent with the inhibitory effects of Ca^{2+} observed in this work.

CONCLUSIONS

In this work, quantitative mechanistic analysis with modified substrates has revealed an unconventional origin for the stimulatory and inhibitory effects of metal ions in the *Tetrahymena* group I ribozyme. A novel metal-ion site, referred to as site D, has been uncovered. These analyses have also allowed characterization of the properties of metal site D and the functional consequences of different metal ions bound at this site. The structural or catalytic role, if any, of the Mg^{2+} bound at site D, the ligands and coordination geometry of the Mg^{2+}, Mn^{2+}, and Ca^{2+} bound at this site, and the mechanism by which Ca^{2+}_D disrupts the alignment of the substrates remain to be addressed.

MATERIALS AND METHODS

Materials

Ribozyme was prepared by in vitro transcription with T7 RNA polymerase as described previously (Zaug et al., 1988). Oligonucleotides (Table 1) were made by solid phase synthesis and supplied by the Protein and Nucleic Acid Facility at Stanford University or were gifts from L. Beigelman (Ribozyme Pharmaceuticals Inc.). Oligonucleotide substrates were 5′-end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase and purified by electrophoresis on 24% nondenaturing polyacrylamide gels as described (Zaug et al., 1988; Herschlag et al., 1993a).

General kinetic methods

All reactions were single-turnover, with ribozyme in excess of labeled oligonucleotide substrate (S*) and were carried out at 30 °C in 50 mM buffer. The buffers used were: NaMES, pH 5.4–7.0; NaMOPS, pH 6.4–7.1; NaHEPES, 6.8–7.5; NaEPPS, pH 7.1–8.5. Reactions were followed and analyzed essentially as previously described (Herschlag & Cech, 1990; McConnell et al., 1993). Briefly, ribozyme was preincubated in 10 mM $MgCl_2$ and 50 mM buffer at 50 °C, cooled to 30 °C, and Mg^{2+}, Mn^{2+}, and/or Ca^{2+} was added to obtain the desired metal-ion concentrations prior to initiating the reaction by addition of S* (<0.1 nM). Six aliquots of 2-μL reaction mixture were removed from 20-μL reactions at specified times, and further reaction was quenched by addition of 4 μL of stop solution [90% formamide with EDTA in ≥2-fold excess of total divalent metal ion (20–200 mM), 0.005% xylene cyanole, 0.01% bromophenol blue, and 1 mM Tris, pH 7.5]. Oligonucleotide substrates and products were separated by electrophoresis on 20% polyacrylamide/7 M urea gels, and their ratio at each time point was quantitated with a Molecular Dynamics PhosphorImager.

Reactions were followed for $\geq 3t_{1/2}$ except for very slow reactions. Good first-order fits to the data, with endpoints of ≥90%, were obtained (KaleidaGraph). The slow reactions were typically linear for up to 20 h, and an endpoint of 95% was assumed in obtaining observed rate constants from these initial rates.

Kinetic constants

k_c^G, the first-order rate constant for the reaction E•S•G → products, was determined with ribozyme saturating with respect to S (50–1,000 nM E; $K_d^S \leq 1$ nM) and with saturating G (2 mM; $K_d^G \leq 500$ μM). $(k_c/K_m)^G$, the second-order rate constant for the reaction E•S + G → products, was determined with E saturating with respect to S as above, but with G subsaturating (≥3 concentrations, each more than fivefold below K_d^G).

Following the chemical step of the reaction

Previous results showed that the rate-limiting step for $(k_c/K_m)^G$ for reaction of rS_A (Table 1), the wild-type oligonucleotide substrate, is the chemical step below pH 7, but changes to a binding or conformational step above pH 7 (Herschlag & Khosla, 1994; see also Yoshida et al., 2000). To ensure that the chemical step is rate limiting under all the pH and metal-ion concentrations investigated, oligonucleotide substrates with 2′-H substitutions at U(−1) were used in several experiments (Table 1). The sole effect of this modification is to slow down the chemical step 10^3-fold, without affecting other reaction steps (Herschlag et al., 1993b; Yoshida et al., 2000). Use of substrates with 2′-H at U(−1) has also allowed rate constants with saturating G to be measured manually. For substrates containing a 2′-OH at U(−1), the reactions were followed only up to pH 6.4 to ensure that the chemical step is rate limiting (Herschlag & Khosla, 1994). The following strongly suggest that the chemical step is rate limiting for reaction of substrates with 2′-OH under the experimental conditions used herein: (1) below pH 6.4, the observed $(k_c/K_m)^G$ for these substrates has a log-linear dependence on pH (Yoshida et al., 2000; data not shown); (2) the observed $(k_c/K_m)^G$ is always below 1×10^5 $M^{-1}min^{-1}$, lower than the maximal value of $(k_c/K_m)^G = 5 \times 10^5$ $M^{-1}min^{-1}$ for rS_A (Herschlag & Khosla, 1994; Yoshida et al., 2000).

Determination of the affinity of oligonucleotide substrates

The equilibrium dissociation constant of S (−1d,rS_A or rS_{Me}; Table 1) from the E•S complex, K_d^S, was measured with subsaturating G (≤10 μM) from the rate constants for binding and dissociation of S (k_{on}^S and k_{off}^S, respectively) according to the relationship $K_d^S = k_{off}^S/k_{on}^S$. Values of k_{off}^S were determined by pulse-chase measurements as described previously (Rose et al., 1974; Herschlag & Cech, 1990). The k_{on}^S value for −1d,rS_A was assumed to be the same as that for rS_A in the experiments herein with Mn^{2+} or Ca^{2+} present, as substitution of the 2′-OH of U(−1) with 2′-H has no effect on k_{on}^S with 10 mM Mg^{2+} (Herschlag et al., 1993b; Narlikar et al., 1999). The values of k_{on}^S for rS_A in the presence of Mn^{2+} or Ca^{2+} were determined by measuring $(k_{cat}/K_m)^{S,app}$, the apparent second-order rate constant for the reaction E + S* → products. With a subsaturating concentration of G that is sufficient to ensure that cleavage of rS_A* is faster than its dissociation, the observed $(k_{cat}/K_m)^{S,app}$ values for rS_A is equal to k_{on}^S (Herschlag & Cech, 1990; McConnell et al., 1997). As the affinity and reactivity of rS_{Me} is within twofold of that of rS_A, the k_{on}^S value for rS_{Me} can also be obtained from its observed $(k_{cat}/K_m)^{S,app}$ at a subsaturating G concentration sufficient to ensure that cleavage of rS_{Me}* is faster than its dissociation.

The equilibrium dissociation constant of −1d,rS_A from the E•S•G ternary complexes, $K_d^{S\prime}$, was analogously obtained from k_{on}^S and k_{off}^S, determined by experiments analogous to those described above except that a saturating concentration of G (2 mM) was present.

Determination of the affinity of G for E•S

The equilibrium dissociation constant of G from the E•S•G ternary complex, K_d^G, was determined with −1d,rS_A (Table 1) with ribozyme saturating with respect to S (50–200 nM E, $K_d^S \leq 0.5$ nM). K_d^G values were obtained by following the reaction rate at varying G concentrations (1–2,000 μM). Under the conditions of these reactions, the concentration of G that provides half of the maximal rate, $K_{1/2}^G$, equals K_d^G, as described previously (McConnell et al., 1993). The data were fit to Equation (2), in which k_{obsd} is the observed rate constant at a particular G concentration and k_{max} is the rate constant with saturating G.

$$k_{obsd} = k_{max} \times \frac{[G]}{[G] + K_{1/2}^G} \quad (2)$$

Determination of Mn^{2+} and Ca^{2+} affinities for the ribozyme•substrate complexes

As described in the Results, Mn^{2+} and Ca^{2+} alter the binding affinity of S and the rate of the reaction. The Mn^{2+} and Ca^{2+} concentration dependences are consistent with the effect ex-

pected for a single Mn^{2+} or Ca^{2+}, referred to as Mn_D^{2+} and Ca_D^{2+}, respectively. The following strongly suggest that the concentration of Mn^{2+} (or Ca^{2+}) required to reach half-saturation (or half-inhibition), $K_{1/2}^{Mn}$ (or $K_{1/2}^{Ca}$), equals $K^{Mn,app}$ (or $K^{Ca,app}$), the apparent equilibrium dissociation constant (see footnote 3) of Mn_D^{2+} (or Ca_D^{2+}): (1) the reaction rate is not affected by the time of incubation with Mn^{2+} or Ca^{2+} before initiation of the reaction or by the order of addition of metal ions and other reaction components after preincubation of the ribozyme; (2) reactions follow good first-order kinetics without a lag or burst phase; (3) the $K_{1/2}^{Mn}$ (or $K_{1/2}^{Ca}$) value is not affected by changing pH or by using substrates with 2′-OH or 2′-H at U(−1), changes that alter the observed reaction rate. Further, previous studies showed that when E•S formed in Ca^{2+} is diluted into Mg^{2+}, S reacts without a lag phase as if it is originally bound in Mg^{2+} (McConnell et al., 1997). These and other observations suggest that Mn^{2+} and Ca^{2+} achieve equilibrium binding prior to the chemical step (McConnell et al., 1997; Shan & Herschlag, 1999).

Binding of Mn_D^{2+} to the $(E•S•G)_o$ complex, $K_{(E•S•G)_o}^{Mn,app}$

Mn^{2+} stimulates the reaction for −1d,mS_A (Table 1), which reacts from the open complex, relative to that for −1d,rS_A, which reacts from the closed complex (Scheme 1). This provides a signal for binding of Mn_D^{2+} to the open complex. The apparent dissociation constant of Mn_D^{2+} from the $(E•S•G)_o$ complex, $K_{(E•S•G)_o}^{Mn,app}$, was obtained from the Mn^{2+} concentration dependence of $k_c^{G,rel}$, the rate constant for reaction of the E•S•G complex for −1d,mS_A relative to −1d,rS_A. The data were fit to Equation (3), which was derived from the model of Scheme 2. $k_{c,obsd}^{G,rel}$ is the observed reactivity of −1d,mS_A relative to −1d,rS_A at a particular Mn^{2+} concentration, and $k_{c,Mg}^{G,rel}$ and $k_{c,Mn}^{G,rel}$ are the relative reactivities of −1d,mS_A with zero and saturating Mn^{2+}, respectively.

$$k_{c,obsd}^{G,rel} = k_{c,Mg}^{G,rel} \times \frac{K_{(E•S•G)_o}^{Mn,app}}{[Mn^{2+}] + K_{(E•S•G)_o}^{Mn,app}} + k_{c,Mn}^{G,rel} \times \frac{[Mn^{2+}]}{[Mn^{2+}] + K_{(E•S•G)_o}^{Mn,app}} \quad (3)$$

The use of relative rate constant in the analyses of Equation (3) assumes that replacing Mg^{2+} with Mn^{2+} at site D has no effect on the reaction of −1d,rS_A and that any additional Mn^{2+} ions that affect the reaction of −1d,rS_A (see footnote 2) have the same effect on the reaction of −1d,mS_A. The following observations strongly support these assumptions: (1) over the range of Mn^{2+} concentration that gives stimulation of the reaction of −1d,mS_A, there is no significant change in the reaction rate of −1d,rS_A; (2) the data are fit well by Equation (3), which was derived with these assumptions; (3) the Mn_D^{2+} affinity for the $(E•S•G)_o$ complex determined using this approach is the same, within error, as that obtained independently from the Mn_D^{2+} effect on binding of S (see Results).

Binding of Mn_D^{2+} to the $(E•S_A)_c$ and $(E•S_A•G)_c$ complexes, $K_{(E•S_A)_c}^{Mn,app}$ and $K_{(E•S_A•G)_c}^{Mn,app}$

Mn^{2+} decreases the equilibrium dissociation constant of −1d,rS_A from the $(E•S_A)_c$ and $(E•S_A•G)_c$ complexes (K_d^S and $K_d^{S\prime}$, respectively). The Mn^{2+} concentration dependences of K_d^S and $K_d^{S\prime}$ are consistent with an effect from a single Mn^{2+}, Mn_D^{2+}. Values of $K_{(E•S_A)_c}^{Mn,app}$, the equilibrium dissociation constant of Mn_D^{2+} from $(E•S_A)_c$, was determined from nonlinear least-squares fit of the Mn^{2+} concentration dependence of K_d^S to Equation (4a), derived from the model of Scheme 3A. In this equation, $K_{d,obsd}^S$ is the observed dissociation constant of −1d,rS_A from $(E•S_A)_c$ at each Mn^{2+} concentration, and $K_{d,Mg}^S$ and $K_{d,Mn}^S$ are the dissociation constants of −1d,rS_A with zero and saturating Mn^{2+}, respectively. Analogously, the equilibrium dissociation constant of Mn_D^{2+} from $(E•S_A•G)_c$, $K_{(E•S_A•G)_c}^{Mn,app}$, was determined by fitting the Mn^{2+} concentration dependence of $K_d^{S\prime}$ to Equation(4b), derived from Scheme 3B. In Equation(4b), $K_{d,obsd}^{S\prime}$ is the observed dissociation constant of S from $(E•S_A•G)_c$ at each Mn^{2+} concentration, and $K_{d,Mg}^{S\prime}$ and $K_{d,Mn}^{S\prime}$ are the dissociation constants of S from $(E•S_A•G)_c$ with zero and saturating Mn^{2+}, respectively.

$$K_{d,obsd}^S = K_{d,Mg}^S \times \frac{K_{(E•S_A)_c}^{Mn,app}}{[Mn^{2+}] + K_{(E•S_A)_c}^{Mn,app}} + K_{d,Mn}^S \times \frac{[Mn^{2+}]}{[Mn^{2+}] + K_{(E•S_A)_c}^{Mn,app}} \quad (4a)$$

$$K_{d,obsd}^{S\prime} = K_{d,Mg}^{S\prime} \times \frac{K_{(E•S_A•G)_c}^{Mn,app}}{[Mn^{2+}] + K_{(E•S_A•G)_c}^{Mn,app}} + K_{d,Mn}^{S\prime} \times \frac{[Mn^{2+}]}{[Mn^{2+}] + K_{(E•S_A•G)_c}^{Mn,app}} \quad (4b)$$

Binding of Ca_D^{2+} to $(E•S_A)_c$ and $(E•S_{Me})_c$, $K_{(E•S_A)_c}^{Ca,app}$ and $K_{(E•S_{Me})_c}^{Ca,app}$

The apparent dissociation constant of Ca_D^{2+} from the $(E•S_A)_c$ and $(E•S_{Me})_c$ complexes, $K_{(E•S_A)_c}^{Ca,app}$ and $K_{(E•S_{Me})_c}^{Ca,app}$, were determined from the Ca^{2+} concentration dependence of the reaction E•S + G → products with −1d,rS_A and rS_{Me}, respectively. The data were fit to Equations (5a) and (5b), respectively, derived from Schemes 6A and 6B; k_{obsd} is the observed rate constant of the reaction at a particular Ca^{2+} concentration, and k_0 is the rate constant of the reaction in the absence of added Ca^{2+}.

$$k_{obsd} = k_0 \times \frac{K_{(E•S_A)_c}^{Ca,app}}{[Ca^{2+}] + K_{(E•S_A)_c}^{Ca,app}} \quad (5a)$$

$$k_{obsd} = k_0 \times \frac{K_{(E•S_{Me})_c}^{Ca,app}}{[Ca^{2+}] + K_{(E•S_{Me})_c}^{Ca,app}} \quad (5b)$$

Determination of the Mn^{2+} and Ca^{2+} specificity of site D: $K^{Mn,rel}$ and $K^{Ca,rel}$

As described previously (Shan & Herschlag, 1999), the apparent affinity of a metal site for Mn^{2+} at a particular Mg^{2+} concentration, $K^{Mn,app}$, is defined by Equation (6a) if the metal site is predominantly occupied by either Mg^{2+} or Mn^{2+}, that is, $[E] \ll [E^{Mg}] + [E^{Mn}]$ (see Results); E denotes the ribozyme form with the metal site unoccupied, E^{Mg} and E^{Mn} denote ribozyme forms with the metal site occupied by Mg^{2+} and Mn^{2+}, respectively, and $[E_t]$ denotes the total concentration of all ribozyme forms. Under these conditions, the specificity

of a metal site for Mn^{2+} relative to Mg^{2+}, $K^{Mn,rel}$, can be calculated from Equation (6b) (Shan & Herschlag, 1999).

$$K^{Mn,app} = \frac{([E_t] - [E^{Mn}]) \times [Mn^{2+}]}{[E^{Mn}]} \approx \frac{[E^{Mg}][Mn^{2+}]}{[E^{Mn}]}; \quad (6a)$$

$$K^{Mn,rel} \approx [Mg^{2+}]/K^{Mn,app} \quad (6b)$$

Analogously, the apparent Ca^{2+} affinity of a metal site at a particular Mg^{2+} concentration is defined by Equation (7a) if the metal site is predominantly occupied by either Mg^{2+} or Ca^{2+}, that is, $[E] \ll [E^{Mg}] + [E^{Ca}]$ (E^{Ca} denotes the ribozyme form with Ca^{2+} bound). The Ca^{2+} specificity of the metal site relative to Mg^{2+}, $K^{Ca,rel}$, can be obtained from Equation (7b) under these conditions.

$$K^{Ca,app} = \frac{([E_t] - [E^{Ca}]) \times [Ca^{2+}]}{[E^{Ca}]} \approx \frac{[E^{Mg}][Ca^{2+}]}{[E^{Ca}]} \quad (7a)$$

$$K^{Ca,rel} \approx [Mg^{2+}]/K^{Ca,app} \quad (7b)$$

Prediction of the apparent affinity of Ca^{2+} with Mn^{2+} bound at site E, $K^{Ca,app\prime}$

The apparent Ca^{2+} affinity in the presence of both Mg^{2+} and Mn^{2+}, $K^{Ca,app\prime}$, is defined by Equation (8), under conditions such that $[E] \ll [E^{Ca}] + [E^{Mn}] + [E^{Mg}]$.

$$K^{Ca,app\prime} = \frac{([E_t] - [E^{Ca}]) \times [Ca^{2+}]}{[E^{Ca}]}$$

$$\approx \frac{([E^{Mg}] + [E^{Mn}]) \times [Ca^{2+}]}{[E^{Ca}]} \quad (8)$$

Combining Equation (8) with Equations (6) and (7) gives Equation (9), which was used to calculate the predicted apparent Ca^{2+} affinities in Table 3.

$$K^{Ca,app\prime} \approx K^{Ca,app} \times \left(1 + \frac{[Mn^{2+}]}{K^{Mn,app}}\right) \quad (9)$$

For $(E{\bullet}S_A)_c$, values of $K^{Ca,app} = 0.055$ mM and $K^{Mn,app} = 0.037$ mM from Table 2 were used to calculate $K^{Ca,app\prime}$. For $(E{\bullet}S_{Me})_c$, values of $K^{Ca,app} = 0.26$ mM from Figure 5A and $K^{Mn,app} = 0.42$ mM were used. The $K^{Mn,app}$ value of 0.42 mM is the apparent Mn_D^{2+} affinity for the $(E{\bullet}S)_o$ complex (Table 2); this would be expected to be equal to the Mn_D^{2+} affinity for the $(E{\bullet}S_{Me})_c$ complex because Mn^{2+} has no effect on docking of substrates with A(+1) replaced by $-OCH_3$.

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REFERENCES

Amburgey JC, Huh N-M, Pedersen LG, Hiskey RG. 1994. Small molecule analogs of phospholipid-metal ion binding sites: Potentiometric and spectroscopic studies of Mg(II) and Ca(II) complexes of cyclohexane-1,2,4,-triol trisphosphates. *Bioorg Chem 22*:198–215.

Bassi GS, Murchie AIH, Lilley DMJ. 1996. The ion-induced folding of the hammerhead ribozyme: Core sequence changes that perturb folding into the active conformation. *RNA 2*:756–768.

Basu S, Strobel SA. 1999. Thiophilic metal ion rescue of phosphorothioate interference within the *Tetrahymena* ribozyme P4–P6 domain. *RNA 5*:1399–1407.

Beebe JA, Kurz JC, Fierke CA. 1996. Magnesium ions are required by *Bacillus subtilis* Ribonuclease P RNA for both binding and cleaving precursor $tRNA^{Asp}$. *Biochemistry 35*:10493–10505.

Bevilacqua PC, Kierzek R, Johnson KA, Turner DH. 1992. Dynamics of ribozyme binding of substrate revealed by fluorescence-detected stopped-flow methods. *Science 258*:1355–1358.

Black CB, Cowan JA. 1998. A critical-evaluation of metal-promoted Klenow $3' \rightarrow 5'$ exonuclease activity: Calorimetric and kinetic analyses support a one-metal-ion mechanism. *J Biol Inorg Chem 2*:292–299.

Brautigam CA, Steitz TA. 1998. Structural principles for the inhibition of the $3' \rightarrow 5'$ exonuclease activity of *Escherichia coli* DNA polymerase I by phosphorothioates. *J Mol Biol 277*:363–377.

Bujalowski W, Graester E, McLaughlin LW, Porschke D. 1986. Anticodon loop of $tRNA^{Phe}$: Structure, dynamics, and Mg^{2+} binding. *Biochemistry 25*:6365–6371.

Burgers PMJ, Eckstein F. 1979. A study of the mechanism of DNA polymerase I from *E. coli* with diastereomeric phosphorothioate analogs of deoxyadenosine triphosphate. *J Biol Chem 254*:6889–6893.

Burgers PMJ, Eckstein F. 1980. Structure of the metal-nucleotide complex in the creatine kinase reaction. *J Biol Chem 255*:8229–8233.

Cate JH, Doudna JA. 1996. Metal-binding sites in the major groove of a large ribozyme domain. *Structure 4*:1221–1229.

Cate JH, Gooding AR, Podell E, Zhou K, Golden BL, Kundrot CE, Cech TR, Doudna JA. 1996. Crystal structure of a group I ribozyme domain reveals principles of higher order RNA folding. *Science 273*:1678–1685.

Cate JH, Hanna RL, Doudna JA. 1997. A magnesium ion core at the heart of a ribozyme domain. *Nat Struct Biol 7*:553–558.

Cech TR, Herschlag D. 1996. Group I ribozymes: Substrate recognition, catalytic strategies, and comparative mechanistic analysis. *Nucleic Acids Mol Biol 10*:1–17.

Celander DW, Cech TR. 1991. Visualizing the higher order folding of a catalytic RNA molecule. *Science 251*:401–407.

Chen Y, Li X, Gegenheimer P. 1997. Ribonuclease P catalysis requires Mg^{2+} coordinated to the *pro-R*p oxygen of the scissle bond. *Biochemistry 36*:2425–2438.

Christian EL, Yarus M. 1993. Metal coordination sites that contribute to structure and catalysis in the group I intron from *Tetrahymena*. *Biochemistry 32*:4475–4480.

Connolly BA, Eckstein F. 1981. Structures of the monovalent and divalent metal nucleotide complexes in the myosin ATPase. *J Biol Chem 256*:9450–9456.

Correll CC, Freeborn B, Moore PB, Steitz TA. 1997. Metals, motifs, and recognition in the crystal structure of a 5S rRNA domain. *Cell 91*:705–712.

Cowan JA. 1997. Metal-mediated hydrolysis of biological phosphate-esters: A critical analysis of the essential metal-ion stoichiometry for magnesium-dependent nuclease activation. *J Biol Inorg Chem 2*:168–176.

Dismukes GC. 1996. Manganese enzymes with binuclear active sites. *Chem Rev 96*:2909–2926.

Downs WD, Cech TR. 1996. Kinetic pathway for folding of the *Tetrahymena* ribozyme revealed by three ultraviolet-inducible cross-links. *RNA 2*:718–732.

Draper DE. 1996. Strategies for RNA folding. *Trends Biochem Sci 21*:145–149.

Feig AL, Scott WG, Uhlenbeck, OC. 1998. Inhibition of the hammerhead ribozyme cleavage reaction by site-specific binding of Tb. *Science 279*:81–84.

Gluick TC, Wills NM, Gesteland RF, Draper DE. 1997. Folding of an

mRNA pseudoknot required for stop codon readthrough: Effects of mono- and divalent ions on stability. *Biochemistry 36*:16173–16186.
Golden BL, Gooding AR, Podell ER, Cech TR. 1998. A preorganized active site in the crystal structure of the *Tetrahymena* ribozyme. *Science 282*:259–264.
Groll DH, Jeltch A, Selent U, Pingoud A. 1997. Does the restriction endonuclease *Eco*RV employ a two-metal-ion mechanism for DNA cleavage? *Biochemistry 36*:11389–11401.
Grosshans CA, Cech TR. 1989. Metal ion requirements for sequence-specific endonuclease activity of the *Tetrahymena* ribozyme. *Biochemistry 28*:6888–6894.
Herschlag D. 1992. Evidence for processivity and two-step binding of the RNA substrate from studies of J1/2 mutants of the *Tetrahymena* ribozyme. *Biochemistry 31*:1386–1398.
Herschlag D, Cech TR. 1990. Catalysis of RNA cleavage by the *Tetrahymena thermophila* ribozyme. 1. Kinetic description of the reaction of an RNA substrate complementary to the active site. *Biochemistry 29*:10159–10171.
Herschlag D, Eckstein F, Cech TR. 1993a. Contribution of 2′-hydroxyl groups of the RNA substrate to binding and catalysis by the *Tetrahymena* ribozyme. An energetic picture of an active site composed of RNA. *Biochemistry 32*:8299–8311.
Herschlag D, Eckstein F, Cech TR. 1993b. The importance of being ribose at the cleavage site in the *Tetrahymena* ribozyme reaction. *Biochemistry 32*:8312–8321.
Herschlag D, Khosla M. 1994. Comparison of pH dependencies of the *Tetrahymena* ribozyme reactions with RNA 2′-substituted and phosphorothioate substrates reveals a rate-limiting conformational step. *Biochemistry 33*:5291–5297.
Herschlag D, Piccirilli JA, Cech TR. 1991. Ribozyme-catalyzed and nonenzymatic reactions of phosphate diesters: Rate effects upon substitution of sulfur for a nonbridging phosphoryl oxygen atom. *Biochemistry 30*:4844–4854.
Horton NC, Newberry KJ, Perona JJ. 1998. Metal ion-mediated substrate-assisted catalysis in type II restriction endonucleases. *Proc Natl Acad Sci USA 95*:13489–13494.
Jaffe EK, Cohn M. 1978. Divalent cation-dependent stereospecificity of adenosine 5′-*O*-(2-thiotriphosphate) in the hexokinase and pyruvate kinase reactions. *J Biol Chem 253*:4823–4825.
Jose TJ, Conlan LH, Dupureur CM. 1999. Quantitative evaluation of metal ion binding to PvuII restriction endonuclease. *J Biol Inorg Chem 4*:814–823.
Katz AK, Glusker JP, Beebe SA, Bock CW. 1996. Calcium ion coordination: A comparison with that of beryllium, magnesium, and zinc. *J Am Chem Soc 118*:5752–5763.
Keck JL, Goedken ER, Marqusee S. 1998. Activation/attenuation model for RNase H. *J Biol Chem 273*:34128–34133.
Knitt DS, Narlikar GJ, Herschlag D. 1994. Dissection of the role of the conserved G•U pair in group I RNA self-splicing. *Biochemistry 33*:13864–13879.
Latham JA, Cech TR. 1989. Defining the inside and outside of a catalytic RNA molecule. *Science 245*:276–282.
Lehman N, Joyce GF. 1993. Evolution *in vitro* of an RNA enzyme with altered metal dependence. *Nature 361*:182–185.
Linse S, Forsen S. 1995. Determinants that govern high-affinity calcium binding. In: Means AR, ed. *Advances in second messenger and phosphoprotein research*. New York: Raven Press. pp 89–151.
Lott WB, Pontius BW, von Hippel PH. 1998. A two-metal ion mechanism operates in the hammerhead ribozyme-mediated cleavage of an RNA substrate. *Proc Natl Acad Sci USA 95*:542–547.
Martell AE, Smith RM. 1976. *Critical stability constants*. New York: Plenum.
McConnell TS, Cech TR. 1995. A positive entropy change for guanosine binding and for the chemical step in the *Tetrahymena* ribozyme reaction. *Biochemistry 34*:4056–4067.
McConnell TS, Cech TR, Herschlag DH. 1993. Guanosine binding to the *Tetrahymena* ribozyme: Thermodynamic coupling with oligonucleotide binding. *Proc Natl Acad Sci USA 90*:8362–8366.
McConnell TS, Herschlag D, Cech TR. 1997. Effects of divalent metal ions on individual steps of the *Tetrahymena* ribozyme reaction. *Biochemistry 36*:8293–8303.
Michel F, Westhof E. 1990. Modeling of the three-dimensional architecture of group I catalytic introns based on comparative sequence analysis. *J Mol Biol 216*:585–610.
Narlikar GJ, Bartley LE, Khosla M, Herschlag D. 1999. Characterization of a local folding event of the *Tetrahymena* group I ribozyme: Effects of oligonucleotide substrate length, pH, and temperature on the two substrate binding steps. *Biochemistry 38*:14192–14204.
Narlikar GJ, Gopalakrishnan V, McConnell TS, Usman N, Herschlag D. 1995. Use of binding energy by an RNA enzyme for catalysis by positioning and substrate destabilization. *Proc Natl Acad Sci USA 92*:3668–3672.
Narlikar GJ, Herschlag D. 1996. Isolation of a local tertiary folding transition in the context of a globally folded RNA. *Nat Struct Biol 3*:701–710.
Narlikar GJ, Khosla M, Usman N, Herschlag D. 1997. Quantitating tertiary binding energies of 2′-OH groups on the P1 duplex of the *Tetrahymena* ribozyme: Intrinsic binding energy in an RNA enzyme. *Biochemistry 36*:2465–2477.
Pan T. 1995. Higher order folding and domain analysis of the ribozyme from *Bacillus subtilis* ribonuclease P. *Biochemistry 34*:902–909.
Pan T, Long DM, Uhlenbeck OC. 1993. Divalent metal ions in RNA folding and catalysis. In: Gesteland RF, Atkins JF, eds. *The RNA world*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 271–302.
Peracchi A, Beigelman L, Scott EC, Uhlenbeck OC, Herschlag DH. 1997. Involvement of a specific metal ion in the transition of the hammerhead ribozyme to its catalytic conformation. *J Biol Chem 272*:26822–26826.
Piccirilli JA, Vyle JS, Caruthers MH, Cech TR. 1993. Metal ion catalysis in the *Tetrahymena* ribozyme reaction. *Nature 361*:85–88.
Pyle AM, Cech TR. 1991. Ribozyme recognition of RNA by tertiary interactions with specific ribose 2′-OH groups. *Nature 350*:628–631.
Quigley GJ, Teeter MM, Rich A. 1978. Structural analysis of spermine and magnesium ion binding to yeast phenylalanine transfer RNA. *Proc Natl Acad Sci USA 75*:64–68.
Rajagopal J, Doudna JA, Szostak JW. 1989. Stereochemical course of catalysis by the *Tetrahymena* ribozyme. *Science 244*:692–694.
Rose IA, O'Connell EL, Litwin S, Bar Tana J. 1974. Determination of the rate of hexokinase-glucose dissociation by the isotope-trapping method. *J Biol Chem 249*:5163–5168.
Scott EC, Uhlenbeck OC. 1999. A re-investigation of the thio effect at the hammerhead cleavage site. *Nucleic Acids Res 27*:479–484.
Shan S, Herschlag D. 1999. Probing the role of metal ions in RNA catalysis: Kinetic and thermodynamic characterization of a metal ion interaction with the 2′-moiety of the guanosine nucleophile in the *Tetrahymena* ribozyme reaction. *Biochemistry 38*:10958–10975.
Shan S, Yoshida A, Sun S, Piccirilli J, Herschlag D. 1999. Three distinct metal ions at the active site of the *Tetrahymena* group I ribozyme. *Proc Natl Acad Sci USA 96*:12299–12304.
Sjögren A-S, Pettersson E, Sjöberg B-M, Strömberg R. 1997. Metal ion interaction with cosubstrate in self-splicing of group I introns. *Nucleic Acids Res 25*:648–653.
Smith D, Pace NR. 1993. Multiple magnesium ions in the ribonuclease P reaction mechanism. *Biochemistry 32*:5273–5281.
Sontheimer EJ, Sun S, Piccirilli J. 1997. Metal ion catalysis during splicing of pre-messenger RNA. *Nature 388*:801–805.
Strobel SA, Ortoleva-Donnelly L, Ryder SP, Cate JH, Moncoeur E. 1998. Complementary sets of noncanonical base pairs mediate RNA helix packing in the group I intron active site. *Nat Struct Biol 5*:60–66.
Szewczak AA, Ortoleva-Donnelly L, Ryder SP, Moncoeur E, Strobel SA. 1998. A minor groove RNA triple helix within the catalytic core of a group I intron. *Nat Struct Biol 5*:1037–1042.
Walter F, Murchie AIH, Thomson JB, Lilley DMJ. 1998. Structure and activity of the hairpin ribozyme in its natural junction conformation: Effect of metal ions. *Biochemistry 37*:14195–14203.
Warnecke JM, Furste JP, Hardt W-D, Erdmann VA, Hartmann RK. 1996. Ribonuclease P (RNase P) RNA is converted to a Cd^{2+}-ribozyme by a single *R*p-phosphorothioate modification in the precursor tRNA at the RNase P cleavage site. *Proc Natl Acad Sci USA 93*:8924–8928.
Warnecke JM, Held R, Busch S, Hartmann RK. 1999. Role of metal ions in the hydrolysis reaction catalyzed by RNase P RNA from *Bacillus subtilis*. *J Mol Biol 290*:433–445.

Weinstein LB, Jones BC, Cosstick R, Cech TR. 1997. A second catalytic metal ion in a group I ribozyme. *Nature 388*:805–808.

Wilcox DE. 1996. Binuclear metallohydrolases. *Chem Rev 96*:2435–2458.

Yoshida A, Shan S, Herschlag D, Piccirilli JA. 2000. The role of the cleavage site 2′-OH in the *Tetrahymena* ribozyme reaction: Evidence for catalysis by hydrogen bond donation to the 3′-leaving group oxygen. *Chem Biol 7*:85–96.

Yoshida A, Sun S, Piccirilli JA. 1999. A new metal ion interaction in the *Tetrahymena* ribozyme reaction revealed by double sulfur substitution. *Nat Struct Biol 6*:318–321.

Zaug AJ, Grosshans CA, Cech TR. 1988. Sequence-specific endoribonuclease activity of the *Tetrahymena* ribozyme: Enhanced cleavage of certain oligonucleotide substrates that form mismatched ribozyme-substrate complexes. *Biochemistry 27*:8924–8931.