Defining the Catalytic Metal Ion Interactions in the *Tetrahymena* Ribozyme Reaction†

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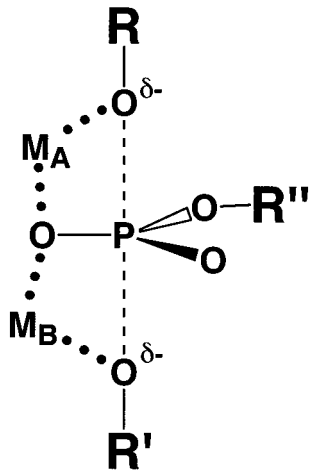
Received December 19, 2000; Revised Manuscript Received February 23, 2001

ABSTRACT: Divalent metal ions play a crucial role in catalysis by many RNA and protein enzymes that carry out phosphoryl transfer reactions, and defining their interactions with substrates is critical for understanding the mechanism of biological phosphoryl transfer. Although a vast amount of structural work has identified metal ions bound at the active site of many phosphoryl transfer enzymes, the number of functional metal ions and the full complement of their catalytic interactions remain to be defined for any RNA or protein enzyme. Previously, thiophilic metal ion rescue and quantitative functional analyses identified the interactions of three active site metal ions with the 3′- and 2′-substrate atoms of the *Tetrahymena* group I ribozyme. We have now extended these approaches to probe the metal ion interactions with the nonbridging *pro-S*$_P$ oxygen of the reactive phosphoryl group. The results of this study combined with previous mechanistic work provide evidence for a novel assembly of catalytic interactions involving three active site metal ions. One metal ion coordinates the 3′-departing oxygen of the oligonucleotide substrate and the *pro-S*$_P$ oxygen of the reactive phosphoryl group; another metal ion coordinates the attacking 3′-oxygen of the guanosine nucleophile; a third metal ion bridges the 2′-hydroxyl of guanosine and the *pro-S*$_P$ oxygen of the reactive phosphoryl group. These results for the first time define a complete set of catalytic metal ion/substrate interactions for an RNA or protein enzyme catalyzing phosphoryl transfer.

Many RNA and protein enzymes that carry out phosphoryl transfer reactions use active site metal ions for catalysis (e.g., refs *1*−*10*). The number of active site metal ions and their modes of interaction are subjects of much investigation and discussion, and catalytic models have been proposed that involve one, two, and more active site metal ions (*4*, *6*, *10*−*22*). The most widespread and commonly accepted proposal is a "two-metal-ion" mechanism. This general model is supported by structural observations with alkaline phosphatase, polymerases, and restriction endonucleases (*4*, *6*, *12*, *19*, *20*, *22*) and has been proposed for numerous phosphoryl transfer enzymes (*4*, *6*, *8*, *12*, *17*−*19*, *22*). In this model, two active site metal ions coordinate a single nonbridging oxygen of the reactive phosphoryl group, with one also coordinating the bridging oxygen of the leaving group and the other the attacking nucleophile (Scheme 1; *12*, *19*).

The structural work alone, however, while powerful in identifying the presence of metal ions bound at or near an enzymatic active site, cannot unambiguously define catalytic interactions. Indeed, different numbers of metal ions have been observed in active sites of the same enzymes (*23*, *24*), and different catalytic interactions have been proposed for enzymes containing two closely spaced active site metal ions (e.g., refs *10*, *20*, *22*, *25*, *26*). To define the number of metal ions that contribute to catalysis and their interactions with substrate groups, functional studies are required (*1*, *2*). Nevertheless, despite decades of enzymology, functional evidence that fully defines the catalytic metal ion interactions for any enzyme, protein or RNA, has yet to be obtained.

Scheme 1



Several active site metal ion interactions have been demonstrated in the *Tetrahymena* ribozyme (E). This ribo-

† This work was supported by NIH Grant GM49243 to D.H. and a grant from the Howard Hughes Medical Institute to J.A.P.

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10.1021/bi002887h CCC: $20.00
Published on Web 04/06/2001

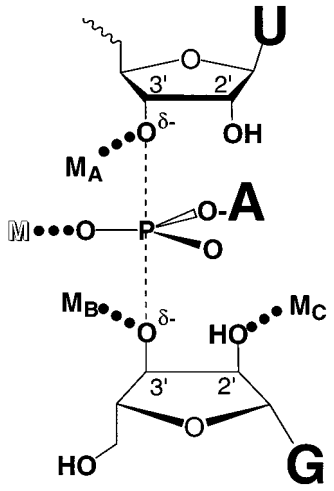

FIGURE 1: Metal ion interactions previously identified at the *Tetrahymena* ribozyme active site. A model for the reaction's transition state is shown. The dashed lines (- - -) depict the partial bonds between the reactive phosphorus and the leaving group and the incoming nucleophile, and $\delta-$ depicts the partial negative charges on the leaving group and the nucleophile. M_A is the metal ion coordinating the 3′-bridging oxygen of S (*3*), M_B is the metal ion coordinating the nucleophilic 3′-oxygen of G (*8*), M_C is the metal ion coordinating the 2′-hydroxyl of G (*27*, *28*), and M (open letter) refers to the metal ion coordinating the *pro*-S_P oxygen of the reactive phosphoryl group (*30*) that is the focus of this investigation. This metal ion was identified in a study using a dithioate susbtrate containing sulfur substitutions for the *pro*-S_P oxygen of the reactive phosphoryl group as well as the 3′-bridging oxygen (*30*). M_A, M_B, and M_C are three distinct metal ions (*10*). This work addresses whether M is the same as M_A, M_B, or M_C or a fourth active site metal ion; this metal ion is therefore depicted by an open letter. The results herein strongly suggest that the metal ion interaction with the transferred phosphoryl group is mediated by both M_A and M_C but not by M_B or a fourth active site metal ion.

zyme, derived from a self-splicing group I intron, catalyzes the transesterification reaction shown in eq 1, in which an

$$\underset{\text{(S)}}{\text{CCCUCU}_{\text{P}}\text{A}} + \text{G}_{\text{OH}} \rightarrow \underset{\text{(P)}}{\text{CCCUCU}_{\text{OH}}} + \text{G}_{\text{P}}\text{A} \qquad (1)$$

exogenous guanosine nucleophile (G) cleaves a specific phosphodiester bond of an oligonucleotide substrate (S). Three distinct metal ions at this RNA active site interact with the 3′-bridging oxygen of S and the 3′- and 2′-oxygens of G (Figure 1, M_A, M_B, and M_C, respectively; *3*, *8*, *10*, *27*, *28*). There was also evidence for active site interaction with the *pro*-S_P nonbridging oxygen of the reactive phosphoryl group (*29*), but this interaction was not defined.

Thio substitution of the *pro*-S_P oxygen of the reactive phosphoryl group reduces the reactivity of the bound substrate by 10^4-fold (*30*, *31*), suggesting catalytic interactions with the *pro*-S_P oxygen. Recently, soft metal ions such as Cd^{2+} and Zn^{2+} were found to rescue this deleterious thio effect, providing evidence for direct metal ion interactions with the *pro*-S_P oxygen [Figure 1, M (*30*)]. However, a critical question remained: What is the identity of the metal ion or ions interacting with the *pro*-S_P oxygen? It is possible that this interaction is mediated by M_A, M_B, or both metal ions, as proposed in the two-metal-ion mechanism (*12*, *19*). Alternatively, the *pro*-S_P oxygen may interact with M_C, the metal ion coordinating the 2′-OH of G (*27*, *28*), with a previously identified metal ion at or near the active site (*32*) or with an as yet unidentified active site metal ion.

Table 1: List of Oligonucleotide Substrates[a]

abbreviation	oligonucleotide substrate										
		−5		−3		−1	+1		+3		+5
$S_{3'S,P-S}$[b]	mC	mC	mC	rU	rC	dU	$_{3'S,P-S}$rA				
dS	dC	dC	dC	dU	dC	dT	$_{3'O,P-O}$dA	dA	dA	dA	dA
−1r,dS	dC	dC	dC	dU	dC	rU	$_{3'O,P-O}$dA	dA	dA	dA	dA
rS	rC	rC	rC	rU	rC	rU	$_{3'O,P-O}$rA	rA	rA	rA	rA
S_{P-S}[b]	mC	mC	mC	rU	rC	dU	$_{3'O,P-S}$rA				
$S_{3'S}$[b]	mC	mC	mC	rU	rC	dU	$_{3'S,P-O}$rA				

[a] r = 2′-OH; d = 2′-H; m = 2′-OCH_3; 3′S and 3′O refer to the presence of a sulfur and oxygen as the 3′-bridging atom of S, respectively; P-S and P-O refer to the presence of a sulfur and oxygen as the S_P or *pro*-S_P atom of the reactive phosphoryl group, respectively. [b] 2′-OCH_3 groups are introduced into the (−4) to (−6) residues of $S_{3'S,P-S}$, S_{P-S}, and $S_{3'S}$; the sole effect of this modification is to prevent miscleavage of the thio-substituted substrates, allowing more accurate determination of rate constants for cleavage at the correct position (*37*, *39*, *45*).

In this work, we have quantitatively characterized the effect of soft metal ions on reactions of substrates bearing various combinations of site-specific modifications. The binding properties of the metal ion sites that rescue the deleterious effect of thio substitution at the *pro*-S_P oxygen have allowed determination of the identity of these metal ions. The results reveal a novel set of metal ion interactions at this RNA active site and provide a functionally defined model for the participation of metal ions in the catalysis of phosphoryl transfer.

MATERIALS AND METHODS

Materials. Ribozyme and oligonucleotides (Table 1) were prepared and 5′-end-labeled using standard methods (*10*). Oligonucleotides with thio substitutions were prepared by published procedures (*33*).

General Kinetic Methods. All reactions were single turnover, with E in excess of labeled S (S*) and were carried out at 30 °C in 50 mM buffer. Reactions were followed and analyzed as previously described (*28*, *34*).

All the rate constants herein were first-order rate constants for the reaction $E{\cdot}S{\cdot}G_X \rightarrow$ products (k_c; G_X = G or G_N) and were determined with E saturating with respect to S* (S* < 0.1 nM; 0.1−1 μM E; $K_d^S = 0.5-1$ nM) and with saturating G_X [1.8−2 mM; $K_d^G = 360$ μM and $K_d^{G_N} \leq 280$ μM (*28*, *35*)].

Data Analysis. (A) Use of the Relative Rate Constant, k^{rel}, To Isolate the Effect of Rescuing Cd^{2+} and Zn^{2+}. To control for effects of Cd^{2+} and Zn^{2+} not specific to the thio substitutions, the rate constant for the $S_{3'S,P-S}$ reaction *relative to* dS or −1r,dS, k^{rel}, was analyzed. This approach has been described in detail and has proven powerful in isolating the effects of specific metal ions on the reaction (*10*, *28*, *32*). Evidence that supports the validity of the use of k^{rel} in the analysis herein is described in detail in Supporting Information.

(B) Analysis of Metal Ion Concentration Dependences. The Cd^{2+} and Zn^{2+} concentration dependences for rescue were fit to standard models and equations that involve one, two, or three rescuing metal ions. These models and equations are described in Supporting Information.

Determination of the Effects of Competing Mg^{2+} and Mn^{2+} on Cd^{2+} and Zn^{2+} Rescue. As described in the text, the competing effect of Mg^{2+} and Mn^{2+} was determined from the shift in the Cd^{2+} (or Zn^{2+}) concentration dependences in the presence of changing Mg^{2+} or added Mn^{2+}. The analysis was simplified because Mn^{2+} and Mg^{2+} do not have any effect on the S_{P-S} and $S_{3'S,P-S}$ reactions (*30*, *31*, *32*); i.e., the sole effect of adding Mn^{2+} or increasing Mg^{2+} is to weaken the apparent Cd^{2+} (or Zn^{2+}) affinity of the rescuing metal sites [the Cd^{2+} and Zn^{2+} affinities described in this work are apparent affinities because Cd^{2+} and Zn^{2+} compete with Mg^{2+} ions bound at the rescuing metal sites (see Results)], thereby shifting the metal ion concentration dependence to higher concentrations without changing the shape of the concentration dependence. The relationship between the amount of shift in the $[Cd^{2+}]$ dependence and the weakening of the apparent Cd^{2+} affinities by competing Mg^{2+} (or Mn^{2+}) is quantitatively described by eq 2. In eq 2,

$$\text{fold competition} \equiv \frac{[\text{Cd}]'}{[\text{Cd}]} = \sqrt{mn} \qquad (2)$$

[Cd] and [Cd]′ are the Cd^{2+} concentrations required to achieve the same amount of rescue in the absence and presence of competing Mg^{2+} (or Mn^{2+}), respectively, and *m* and *n* are the fold of increase in the apparent Cd^{2+} dissociation constant for each of the two rescuing sites caused by increasing Mg^{2+} or adding Mn^{2+} (for derivation of eq 2, see Supporting Information).

Equation 2 also allows prediction of the competing effects of Mn^{2+} and Mg^{2+} at sites A, B, and C from the Mn^{2+} and Mg^{2+} affinities of these sites determined previously (*10*). For competition with Mg^{2+}, the weakening of apparent Cd^{2+} affinity at a particular metal site, *m* or *n*, caused by increasing Mg^{2+} concentration from [Mg] to [Mg]′ is described by eq 3, in which K^{Mg} is the Mg^{2+} dissociation constant of this

$$m \text{ (or } n) = \frac{K^{\text{Mg}} + [\text{Mg}]'}{K^{\text{Mg}} + [\text{Mg}]} \qquad (3)$$

site (see Supporting Information for derivation of eq 3). The effect of increasing Mg^{2+} on the Cd^{2+} concentration dependence is then described by eq 4, derived by combining eqs 2 and 3, in which K^{Mg1} and K^{Mg2} are the Mg^{2+} affinities of the two rescuing metal sites.

$$(\text{fold competition})_{\text{Mg}} = \sqrt{mn} = \sqrt{\left(\frac{K^{\text{Mg1}} + [\text{Mg}]'}{K^{\text{Mg1}} + [\text{Mg}]}\right)\left(\frac{K^{\text{Mg2}} + [\text{Mg}]'}{K^{\text{Mg2}} + [\text{Mg}]}\right)} \qquad (4)$$

The application of eq 4 for calculation of the competing effect from Mg^{2+}_A and Mg^{2+}_B or Mg^{2+}_A and Mg^{2+}_C, used in Figure 3, is described in Supporting Information.

For competition with Mn^{2+}, the change in apparent Cd^{2+} affinity in the presence of added Mn^{2+}, represented by *m* and *n* for the two rescuing metal sites, is described by eq 5,

$$m \text{ (or } n) = 1 + \frac{[\text{Mn}]}{K^{\text{Mn,app}}} \qquad (5)$$

in which $K^{Mn,app}$ is the apparent Mn^{2+} dissociation constant of the metal site (for derivation of eq 5, see Supporting Information). The effect of competing Mn^{2+} on the Cd^{2+} rescue can therefore be predicted by combining eqs 2 and 5 to give eq 6. For values at 10 mM Mg^{2+}, the following Mn^{2+}

$$(\text{fold competition})_{\text{Mn}} = \sqrt{mn} = \sqrt{\left(1 + \frac{[\text{Mn}]}{K^{\text{Mn1,app}}}\right)\left(1 + \frac{[\text{Mn}]}{K^{\text{Mn2,app}}}\right)} \qquad (6)$$

dissociation constants were used: $K^{MnA,app} = 0.8$ mM, $K^{MnB,app} = 7$ mM, and $K^{MnC,app} = 0.28$ mM. For values at 50 mM Mg^{2+}, the following Mn^{2+} dissociation constants were used: $K^{MnA,app} = 3.2$ mM, $K^{MnB,app} = 13$ mM, and $K^{MnC,app} = 1.3$ mM (*10*).

RESULTS

A metal ion interaction with the *pro*-S_P oxygen of the oligonucleotide substrate in the *Tetrahymena* ribozyme reaction was previously identified on the basis of rescue of the deleterious effect of thio substitution at this oxygen upon addition of soft metal ions such as Cd^{2+} and Zn^{2+} in the context of dithioate substrate [Figure 1, M (*30*)]. Herein we use various combinations of site-specific substrate modifications combined with quantitative characterization of the affinities of different metal ion sites to determine the identity of the metal ions responsible for this rescue. The results of this study, combined with previous work, provide strong evidence for a novel set of catalytic metal ion interactions in this RNA active site.

The metal ion interaction with the *pro*-S_P oxygen eluded detection for a long time because a single thio substitution at this oxygen was not rescued by soft metal ions such as Mn^{2+} or Zn^{2+} (*30*, *32*, and data not shown) and, as shown herein, is only inefficiently rescued by Cd^{2+} (see below). However, with a phosphorodithioate substrate in which both the 3′-bridging oxygen and the *pro*-S_P oxygen are replaced by sulfur ($S_{3'S,P-S}$; Table 1), the thio effect at the *pro*-S_P oxygen can be efficiently rescued by Cd^{2+} or Zn^{2+} (*30*). This substrate was therefore used primarily in the characterization of metal ion interactions with the *pro*-S_P oxygen in this work. The results herein also explain the inefficient rescue of the single thio substitution at the *pro*-S_P oxygen.

We first show that two Cd^{2+} or two Zn^{2+} ions are required to rescue the $S_{3'S,P-S}$ reaction, indicating that a metal ion in addition to M_A, the metal ion coordinating the 3′-bridging oxygen of S, is required to rescue the thio effect at the *pro*-S_P oxygen. We then describe experiments in which Mg^{2+} or Mn^{2+} is allowed to compete with the rescuing Cd^{2+} and Zn^{2+}; these experiments, combined with the known affinities of Mg^{2+} and Mn^{2+} for metal sites A, B, and C, strongly argue against the involvement of M_B, the metal ion coordinating the 3′-OH of G, in the rescue. Instead, the results are quantitatively consistent with binding of one of the rescuing metal ions to metal site C, the site that coordinates the 2′-OH of G. We then describe an independent test for the model that M_C is responsible for rescuing the thio effect at the *pro*-S_P oxygen. Finally, we describe results that provide evidence for an additional interaction of the *pro*-S_P oxygen with M_A, the metal ion that coordinates the 3′-bridging oxygen of S.

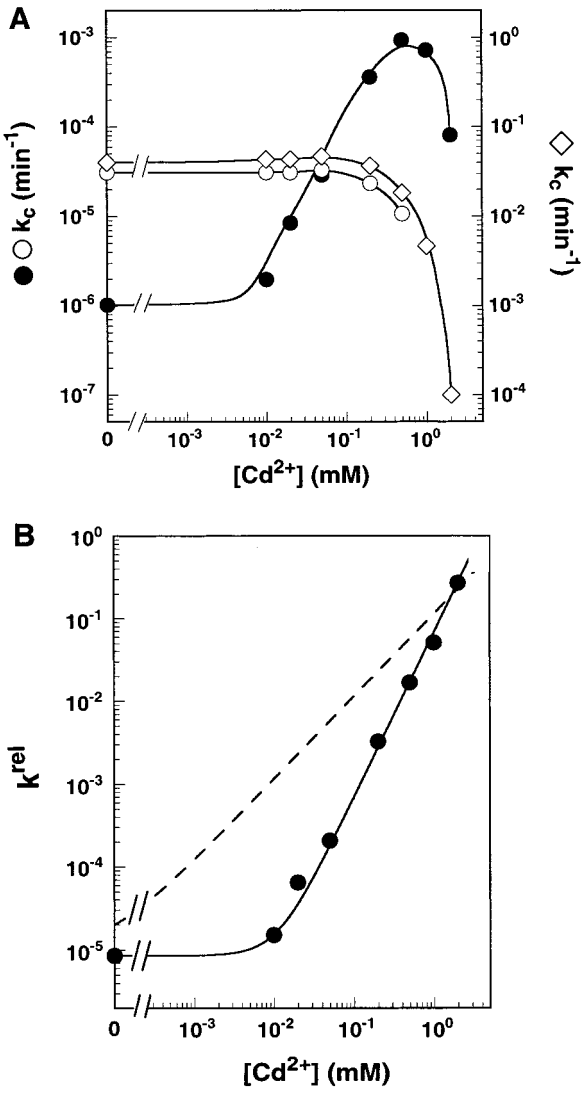

FIGURE 2: Two Cd^{2+} ions are required to rescue the $S_{3'S,P-S}$ reaction. (A) [Cd^{2+}] dependences of the rate of reaction E·S·G → products (k_c) for $S_{3'S,P-S}$ (●), −1r,dS (◇), and dS (○), determined as described in Materials and Methods (pH 6.5, 10 mM Mg^{2+}). (B) [Cd^{2+}] dependence of the rate constant of the $S_{3'S,P-S}$ reaction relative to −1r,dS (k^{rel}; data from part A). The solid line is a fit of the data to a model in which two Cd^{2+} ions are required for rescue, and the dashed line is the fit to a model in which a single Cd^{2+} rescues the reaction (see Materials and Methods).

A Metal Ion Distinct from M_A Is Required To Rescue the $S_{3'S,P-S}$ Reaction. To address whether the metal ion interacting with the 3′-bridging oxygen (Figure 1, M_A) is solely responsible for rescue of the $S_{3'S,P-S}$ reaction, we determined the number of metal ions required to rescue this reaction.

The reaction, E·S·G → products (k_c) for $S_{3'S,P-S}$, was followed as a function of Cd^{2+} concentration (Figure 2A, ●). To isolate the effect of Cd^{2+} specific to the phosphorodithioate substitutions, the reaction was followed in parallel for dS and −1r,dS (Table 1), in which the 3′-bridging oxygen and the *pro-*S_P oxygen are unmodified (Figure 2A, ○ and ◇). It was necessary to use dS and −1r,dS to control for the nonspecific Cd^{2+} effects, because previous work showed that the ribozyme binds S in two steps: first, an open complex is formed, in which S is held solely via base-pairing interactions with the internal guide sequence of E to form the P1 duplex; second, a closed complex is formed, in which the P1 duplex docks into tertiary interactions with the ribozyme active site (*36*−*40*). The wild-type oligonucleotide substrate rS (Table 1) binds E to form a closed complex (*36*−*39*), whereas modified substrates such as dS and −1r,dS and those with thio substitutions bind E predominantly in the open complex, as determined by dissociation rate and equilibrium constants for these S analogues and the absence of coupled binding with G (*32* and data not shown). Formation of the open complex by both −1r, dS (ordS) and $S_{3'S,P-S}$ allows appropriate control for the effects of metal ions on all of the microscopic reaction steps followed with $S_{3'S,P-S}$ (*32*).

Addition of Cd^{2+}, up to 0.5 mM, stimulates the $S_{3'S,P-S}$ reaction 10^3-fold, whereas this Cd^{2+} concentration has no effect on the dS and −1r,dS reactions. Above 0.5 mM Cd^{2+}, an inhibitory effect was observed for the $S_{3'S,P-S}$ reaction; this inhibitory effect was also present in the dS and −1r,dS reactions, suggesting that it is not a specific property of the $S_{3'S,P-S}$ reaction. To control for this inhibitory Cd^{2+} effect, the relative rate constant for reaction of $S_{3'S,P-S}$ relative to −1r,dS was plotted (Figure 2B, k^{rel}; see Materials and Methods). The relative reactivity of $S_{3'S,P-S}$ has a steep dependence on Cd^{2+} concentration, consistent with rescue by two Cd^{2+} ions (Figure 2B, solid line). In contrast, rescue by a single Cd^{2+} is not predicted to give such a steep concentration dependence (Figure 2B, dashed line). k^{rel} continues to increase log-linearly with a slope of 2 at the highest Cd^{2+} concentration, suggesting that Cd^{2+} does not saturate either metal site over the entire concentration range. Thus, the increase in k^{rel} observed at the highest Cd^{2+} concentration, 3×10^4-fold, represents a lower limit for the amount of rescue provided by the two Cd^{2+} ions [rescue is quantitatively defined as the increase in k^{rel} for the thio-substituted substrates with soft metal ions (Mn^{2+}, Cd^{2+}, or Zn^{2+}) bound at the rescuing metal sites relative to the k^{rel} value with Mg^{2+} bound].

Analogous results were observed with Zn^{2+}-mediated rescue of the $S_{3'S,P-S}$ reaction. The rate constant of the $S_{3'S,P-S}$ reaction relative to dS has a Zn^{2+} concentration dependence consistent with rescue of the $S_{3'S,P-S}$ reaction by two Zn^{2+} ions but inconsistent with rescue by a single Zn^{2+} ion [in all cases, the Zn^{2+} concentration dependences of k^{rel} are analogous to those observed with Cd^{2+} (Figure s1 in Supporting Information)].

These results suggest that two metal ions are required to rescue the phosphorodithioate substitutions at the 3′-bridging oxygen of S and the *pro-*S_P oxygen. Presumably, the rescue is mediated by M_A, the metal ion previously shown to coordinate the 3′-bridging atom of S (*3*), and by a distinct metal ion that coordinates the *pro-*S_P atom of the reactive phosphoryl group.

Mg^{2+} and Mn^{2+} Competition To Probe the Identity of the Rescuing Metal Ion. To determine whether site B, C, or an unidentified metal site is responsible for rescuing the $S_{3'S,P-S}$ reaction, we determined the Mg^{2+} and Mn^{2+} binding properties of the rescuing metal sites by measuring the competing effect of Mg^{2+} and Mn^{2+} on the affinity of the rescuing Cd^{2+} and Zn^{2+}. These properties were then compared to those of the known metal sites determined previously (*10*). As $S_{3'S,P-S}$ binds in the open complex (*32* and see above), the affinity of the rescuing metal ions is unaffected by the bound thio substrates, as is critical for this approach (*10*); this allows

direct comparison of the metal ion affinities of the rescuing metal sites with those of the previously identified active site metal ions.

As Cd^{2+} and Zn^{2+} do not saturate the rescuing metal sites, the affinity of the rescuing Cd^{2+} and Zn^{2+} and the changes in the metal ion affinities by competing Mg^{2+} or Mn^{2+} could not be determined directly. As an alternative way to determine the effect of Mg^{2+} and Mn^{2+} on Cd^{2+} (or Zn^{2+}) affinity, we took advantage of the fact that the amount of rescue is proportional to the fraction of ribozyme that has Cd^{2+} (or Zn^{2+}) bound at both rescuing site(s). Binding of Mg^{2+} (or Mn^{2+}) will decrease the fraction of E with Cd^{2+} (or Zn^{2+}) bound at a subsaturating Cd^{2+} (or Zn^{2+}) concentration. Therefore, to achieve the same amount of rescue with a higher Mg^{2+} concentration (or with added Mn^{2+}), a higher Cd^{2+} (or Zn^{2+}) concentration will be required. This results in a shift in the Cd^{2+} (or Zn^{2+}) concentration dependences to higher concentrations, providing a quantitative measure of the competition between Mg^{2+} (or Mn^{2+}) and the rescuing Cd^{2+} (or Zn^{2+}; eq 2 in Materials and Methods).

Competition of the Rescuing Metal Ions with Mg^{2+}. To rule out the involvement of site B or site C in the rescue, we determined the effect of Mg^{2+} on the Cd^{2+} and Zn^{2+} ions that rescue the $S_{3'S,P-S}$ reaction. Previous work suggested that site B binds Mg^{2+} weakly, with a dissociation constant of $K^{Mg} \approx 20$ mM (*10*), so that changing Mg^{2+} concentration would not be expected to affect the binding of the rescuing Cd^{2+} or Zn^{2+} to site B below 20 mM Mg^{2+}. In contrast, sites A and C are occupied by Mg^{2+} above 2 mM Mg^{2+}, so that increasing Mg^{2+} would weaken the binding of Cd^{2+} and Zn^{2+} to these metal sites proportionately (*10*). Thus, if sites A and C are responsible for the rescue, the rescuing Cd^{2+} and Zn^{2+} ions would need to compete with two Mg^{2+} ions over the entire Mg^{2+} concentration range, whereas if sites A and B were responsible, the observed competition from Mg^{2+} would be much smaller, as site B is predominantly occupied only above 20 mM Mg^{2+}.

The Cd^{2+} concentration dependences for the $S_{3'S,P-S}$ reaction relative to −1r,dS were determined at a series of Mg^{2+} concentrations from 2 to 100 mM (Figure 3A). The competing effect of Mg^{2+} was quantitated from the change in the amount of Cd^{2+} required to give the same extent of rescue as with 10 mM Mg^{2+}. In all cases, the effect of Mg^{2+} is larger than that predicted from the model in which the rescuing Cd^{2+} ions compete with Mg_A^{2+} and Mg_B^{2+} (Figure 3B, dashed line). In contrast, the effects are quantitatively consistent with competition from Mg_A^{2+} and Mg_C^{2+} (solid line).

The same result was obtained with the Zn^{2+}-mediated rescue of the $S_{3'S,P-S}$ reaction: increasing Mg^{2+} shifts the Zn^{2+} concentration dependences with an effect consistent with competition from Mg_A^{2+} and Mg_C^{2+} but larger than that expected for competition from Mg_A^{2+} and Mg_B^{2+} (Figure 3B). These results strongly suggest that M_B, the metal ion that coordinates the 3′-OH of G (Figure 1), is not responsible for rescuing the $S_{3'S,P-S}$ reaction. In contrast, the rescuing metal site (Figure 1, M) is already occupied by a Mg^{2+} ion above 2 mM Mg^{2+}, which could be site C or a distinct active site metal ion. To distinguish between these alternative models and to further rule out the involvement of site B in the rescue, we determined the effect of Mn^{2+} on the rescuing Cd^{2+} ions as described in the next section.

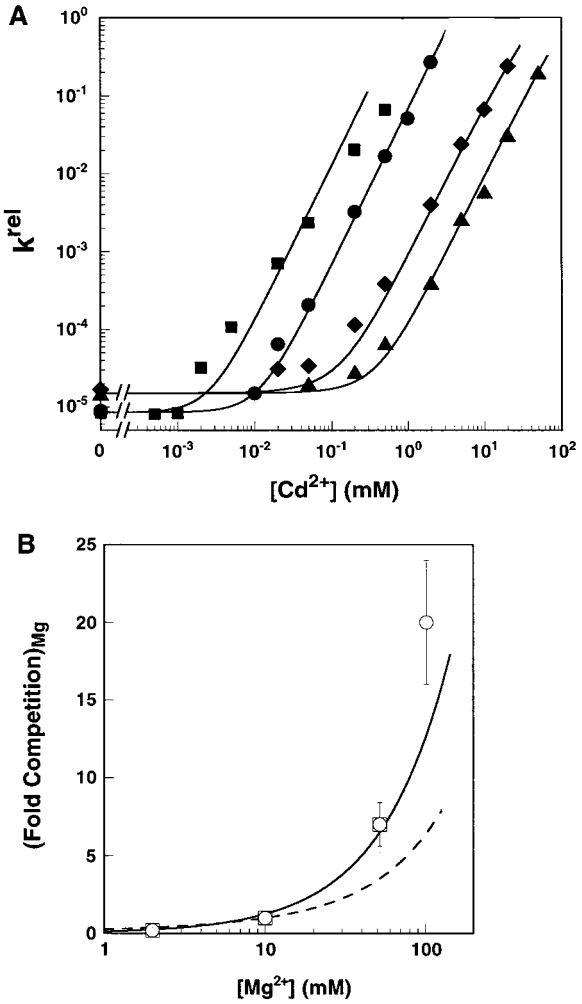

FIGURE 3: The effect of Mg^{2+} on the rescue of the $S_{3'S,P-S}$ reaction by Cd^{2+} and Zn^{2+}. (A) [Cd^{2+}] dependences of the rate constant for the $S_{3'S,P-S}$ reaction relative to −1r,dS (k^{rel}) in the presence of 2 (■), 10 (●), 50 (◆), and 100 (▲) mM Mg^{2+}, determined and analyzed as in Figure 2. The data with 10 mM Mg^{2+} are from Figure 2B. The data at 2 mM Mg^{2+} have more scatter compared to those at higher [Mg^{2+}], probably because there is larger error in determining the slow rate constants at low [Mg^{2+}]. (B) Mg^{2+} competition for rescue by Cd^{2+} (□) and Zn^{2+} (○). The amount of Mg^{2+} competition was quantitated as described in the text. The solid line is the *predicted* effect from a model in which the rescuing Cd^{2+} and Zn^{2+} compete with two Mg^{2+} ions that are bound at and above 2 mM Mg^{2+} such as Mg_A^{2+} and Mg_C^{2+}; the dashed line is the *predicted* effect from the model in which the rescuing metal ions compete with the Mg^{2+} ions at sites A and B (see Materials and Methods).

Competition of the Rescuing Metal Ions with Mn^{2+}. Mn^{2+} does not rescue the thio effect at the *pro-*S_P oxygen with either a single thio substitution at this oxygen or the phosphorodithioate substitution in $S_{3'S,P-S}$ (*30*, *32*; and data not shown). Thus, the requirement for the rescuing Cd^{2+} remains in the presence of added Mn^{2+}. However, Mn^{2+} ions bound at the site of rescue are expected to compete with the binding of rescuing Cd^{2+}. The Mn^{2+} affinity of sites A, B, and C are known from previous determinations (*10*), allowing quantitative prediction of the amount of competition from the Mn^{2+} ions bound at these sites (eq 6 in Materials and Methods).

The Cd^{2+} concentration dependences of the $S_{3'S,P-S}$ reaction relative to −1r,dS were determined in the presence of

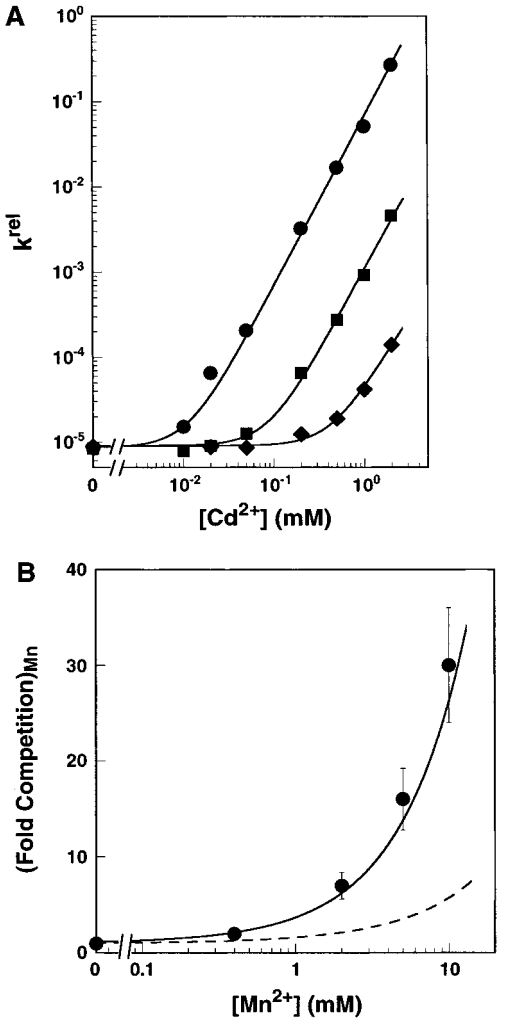

FIGURE 4: Effect of Mn^{2+} on the Cd^{2+} ions that rescue the $S_{3'S,P-S}$ reaction. (A) $[Cd^{2+}]$ dependences of the rate constant for the $S_{3'S,P-S}$ reaction relative to −1r,dS (k^{rel}) in the presence of 0 (●), 2 (■), and 10 (◆) mM Mn^{2+}, determined and analyzed as in Figure 2 (10 mM Mg^{2+}). The data with 0 Mn^{2+} are from Figure 2B. (B) Mn^{2+} competition for rescue by Cd^{2+} (10 mM Mg^{2+}). The amount of Mn^{2+} competition was quantitated as described in the text. The solid line is the *predicted* effect from a model in which the rescuing Cd^{2+} compete with Mn_A^{2+} and Mn_C^{2+}, and the dashed line is the *predicted* effect from the model in which the rescuing Cd^{2+} compete with Mn_A^{2+} and Mn_B^{2+} (see Materials and Methods).

various amounts of Mn^{2+} in a background of 10 mM Mg^{2+}. Representative concentration dependences at 0, 2, and 10 mM Mn^{2+} are shown in Figure 4A. As with Mg^{2+}, the competing effect of Mn^{2+} was quantitated from the increase in Cd^{2+} concentration required to achieve the same amount of rescue in the presence of added Mn^{2+} relative to that in the absence of Mn^{2+} (Figure 4B). The Mn^{2+} competition experiments were also performed in a background of 50 mM Mg^{2+}. (Competition data at 50 mM Mg^{2+} are available as Supporting Information.) Increasing Mg^{2+} from 10 to 50 mM weakens the Mn^{2+} affinities for sites A and C by 5-fold but has less than a 2-fold effect on the Mn^{2+} affinity for site B (*10*). This differential weakening of the Mn^{2+} affinity leads to different predicted changes in the Mn^{2+} competition effects from models involving sites B and C, providing an additional quantitative test to differentiate between these models.

In all cases, the effects of Mn^{2+} are substantially larger than the effects expected from the model in which the rescuing Cd^{2+} ions compete with the Mn^{2+} ions at sites A and B (Figure 4B, dashed lines, and Supporting Information, Figure S3), providing further evidence against the involvement of metal site B in the rescue. In contrast, the observed Mn^{2+} effects are quantitatively consistent with predictions from the model in which the rescuing Cd^{2+} ions compete with the Mn^{2+} ions bound at sites A and C (Figure 4B, solid lines, and Supporting Information, Figure S3), suggesting that site C is responsible for rescuing the thio effect at the *pro-*S_P oxygen. Alternatively, rescue could arise from a distinct metal site that has Mg^{2+} and Mn^{2+} affinities similar to those of site C, with Mg^{2+} already bound at the site above 2 mM Mg^{2+} and with Mn^{2+} binding ~50-fold stronger than Mg^{2+} to this site. We therefore carried out an independent experiment to distinguish between these possibilities, as described in the next section.

Independent Evidence for Rescue of the $S_{3'S,P-S}$ Reaction by M_C. The interaction of M_C, the metal ion coordinating the 2′-OH of G (Figure 1), can be probed using the modified guanosine analogue G_N, in which the 2′-OH of G is replaced by a 2′-NH_2 group. The G_N reaction is slower than the G reaction in the presence of Mg^{2+} but can be rescued by soft metal ions such as Mn^{2+}, Zn^{2+}, and Cd^{2+} (*27*, *28*, and see below). To address whether M_C is responsible for rescuing the thio effect at the *pro-*S_P oxygen, we determined the number of Cd^{2+} ions required to rescue the $S_{3'S,P-S}$ reaction in the presence of G_N. If site C were responsible for the rescue, then the reaction of $S_{3'S,P-S}$ with G_N would be rescued with a dependence on two Cd^{2+} (or Zn^{2+}) ions, as for the reaction of $S_{3'S,P-S}$ with G. In contrast, if a metal site distinct from M_C were responsible, then the reaction of $S_{3'S,P-S}$ with G_N would exhibit a steeper Cd^{2+} (or Zn^{2+}) concentration dependence than the reaction of $S_{3'S,P-S}$ with G, as three Cd^{2+} (or Zn^{2+}) ions would be required for rescue.

The reaction $E{\cdot}S_{3'S,P-S}{\cdot}G_N \rightarrow$ products was followed as a function of Cd^{2+} concentration. To control for Cd^{2+} effects that are not specific to the three substrate modifications, the rate constant for this reaction relative to the reaction of −1r,dS with G (k^{rel}) was plotted (Figure 5A). In the absence of added Cd^{2+}, the reaction of $S_{3'S,P-S}$ with G_N was ~20-fold slower than the $S_{3'S,P-S}$ reaction with G, consistent with previous observations with unmodified oligonucleotide substrates (*28*). The $S_{3'S,P-S}$ reaction with G_N starts to be rescued at a lower Cd^{2+} concentration than the $S_{3'S,P-S}$ reaction with G. Nevertheless, the Cd^{2+} concentration dependence of the $S_{3'S,P-S}$ reaction with G_N has the same slope as that of the $S_{3'S,P-S}$ reaction with G; this concentration dependence can be entirely accounted for by two rescuing Cd^{2+} ions (Figure 5A, solid line). In contrast, rescue by three Cd^{2+} ions is predicted to give a steeper concentration dependence (Figure 5A, dashed lines).

The steep Cd^{2+} concentration dependence predicted for the model involving three Cd^{2+} ions (Figure 5A, dashed lines) relies on the assumption that Cd^{2+} does not saturate any of the three metal sites. Is it possible that a Cd^{2+} ion is already bound at site C at the lowest Cd^{2+} concentrations used in this experiment, thereby giving rise to an apparent dependence on two Cd^{2+} ions even though three Cd^{2+} ions are required for rescue? To test this possibility, the Cd^{2+} occupancy of site C was determined by measuring the effect

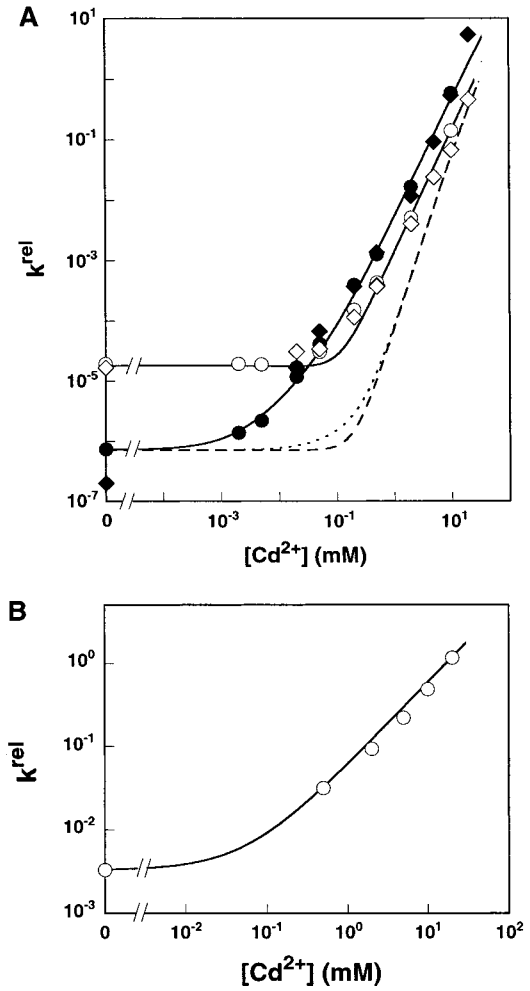

FIGURE 5: Test for the involvement of site C in the rescue of the $S_{3'S,P-S}$ reaction. (A) $[Cd^{2+}]$ dependence for the reaction of $S_{3'S,P-S}$ with G_N relative to the reaction of −1r,dS with G (closed symbols), determined with 50 mM Mg^{2+} present. The different symbols represent determinations from two independent experiments. The solid line is a fit of the data to a model in which two Cd^{2+} ions are responsible for rescue; the dashed lines are fits of the data to a model in which three Cd^{2+} ions are required for rescue (see Materials and Methods). The longer dashed line uses a value of 200-fold for the rescuing effect from the Cd^{2+} ion at site C, obtained from the observed rescuing effect of Cd_C^{2+} relative to the control substrate −1r,dS (part B). The shorter dashed line uses a value even greater, 1000-fold, for the rescuing effect from Cd_C^{2+}. The $[Cd^{2+}]$ dependence for the $S_{3'S,P-S}$ reaction with G relative to the −1r,dS reaction with G (open symbols) was from Figure 3. (B) The $[Cd^{2+}]$ dependence for reaction of −1r,dS with G_N relative to the reaction of −1r,dS with G (k^{rel}), determined under the same conditions as in part A. The solid line is a fit of the data to a model in which a single Cd^{2+} ion rescues the G_N reaction (see Materials and Methods).

of Cd^{2+} on the reaction of G_N relative to G for the substrate without thio modifications, −1r,dS (Figure 5B). Addition of Cd^{2+} increases the rate of the −1r,dS reaction with G_N relative to G (k^{rel}), and the Cd^{2+} concentration dependence for k^{rel} is consistent with rescue of the G_N reaction by a single Cd^{2+} ion, the Cd^{2+} at site C. The k^{rel} value continues to increase log-linearly at the highest Cd^{2+} concentrations, suggesting that Cd^{2+} does not saturate site C over the entire concentration range. Thus, if a metal site other than site C were responsible for rescuing the $S_{3'S,P-S}$ reaction, a steeper concentration dependence would indeed be predicted for rescue of the $S_{3'S,P-S}$ reaction with G_N than the $S_{3'S,P-S}$ reaction with G. As this was not observed (Figure 5A), we conclude that the Cd^{2+} bound at site C is responsible for the rescue.

Analogous results were obtained with Zn^{2+}-mediated rescue. The Zn^{2+} concentration dependence of the $S_{3'S,P-S}$ reaction with G_N is not steeper than the $S_{3'S,P-S}$ reaction with G, consistent with rescue of the three substrate modifications by two Zn^{2+} ions but inconsistent with rescue by three Zn^{2+} ions (Supporting Information, Figure S4). These results provide strong independent evidence that M_C, the metal ion that coordinates the 2′-OH of G (Figure 1), is one of the two metal ions responsible for rescuing the thio effect in the dithioate substrate.

A direct or indirect interaction between M_C and the reactive phosphoryl group has previously been suggested from studies of the Mn^{2+} effect on reaction of G_N. It was found that Mn^{2+} can rescue the binding of G_N relative to G, but at 30 °C this rescuing effect requires the presence of the reactive phosphoryl group within the active site (*28*). The results herein provide evidence that M_C coordinates directly to the *pro-*S_P oxygen.

The Metal Ion at Site A Also Interacts with the pro-S_P *Oxygen.* With the identification of M_C as the metal ion coordinating the *pro-*S_P oxygen, it was curious that rescue of the single thio substitution at the *pro-*S_P oxygen (S_{P-S}; Table 1) had not been observed previously with soft metal ions such as Mn^{2+}, Zn^{2+}, and Cd^{2+} (*30*, *32*). Only in the presence of an additional thio substitution at the 3′-bridging oxygen ($S_{3'S,P-S}$) was the deleterious thio effect at the *pro-*S_P oxygen efficiently rescued by binding of Cd^{2+} or Zn^{2+} to both sites A and C (Figure 6, **vii**; *30* and this work). Below we describe how these and additional observations can be accounted for by a model in which the *pro-*S_P oxygen makes interactions with both M_A and M_C. We then describe experiments that provide strong support for this model.

Why could Mn^{2+} provide reasonable rescue for the thio substitution at either the 3′-bridging oxygen of S or G [Figure 6, **iii**; the model for Mn^{2+} interaction with the 3′-oxygen of G is not shown (*3*, *8*)] but not at the *pro-*S_P oxygen? One simple model is that the *pro-*S_P oxygen interacts with two metal ions, rather than one, for example with the metal ions at both sites A and C. Although Mn^{2+} has a greater thiophilicity than Mg^{2+}, it is important to recognize that Mn^{2+} still interacts considerably more favorably with oxygen than with sulfur (*1*, *41*, *42*). These relatively unfavorable Mn^{2+}−sulfur interactions are depicted by the small dots in Figure 6. Therefore, Mn^{2+} rescue for the S_{P-S} reaction would be expected to be less efficient than previously observed for the $S_{3'S}$ reaction, as two unfavorable Mn^{2+}−sulfur interactions are present with S_{P-S} (Figure 6, **i**).

In addition, if M_C is the only metal ion coordinating the *pro-*S_P oxygen, then efficient rescue for the $S_{3'S,P-S}$ reaction would be expected by the combination of a Mn^{2+} at site A and a Cd^{2+} at site C (Figure 6, **vi**). This is because the Mn^{2+} at site A gives reasonable rescue for the thio substitution at the 3′-bridging oxygen, only 10-fold less than rescue by the Cd^{2+} at site A (**iii** vs **iv**). However, rescue of the $S_{3'S,P-S}$ reaction by Mn_A^{2+} and Cd_C^{2+} is at least 100-fold less efficient than rescue with Cd^{2+} at both sites (**vi** vs **vii**). Although this difference is modest and could arise from a number of effects,

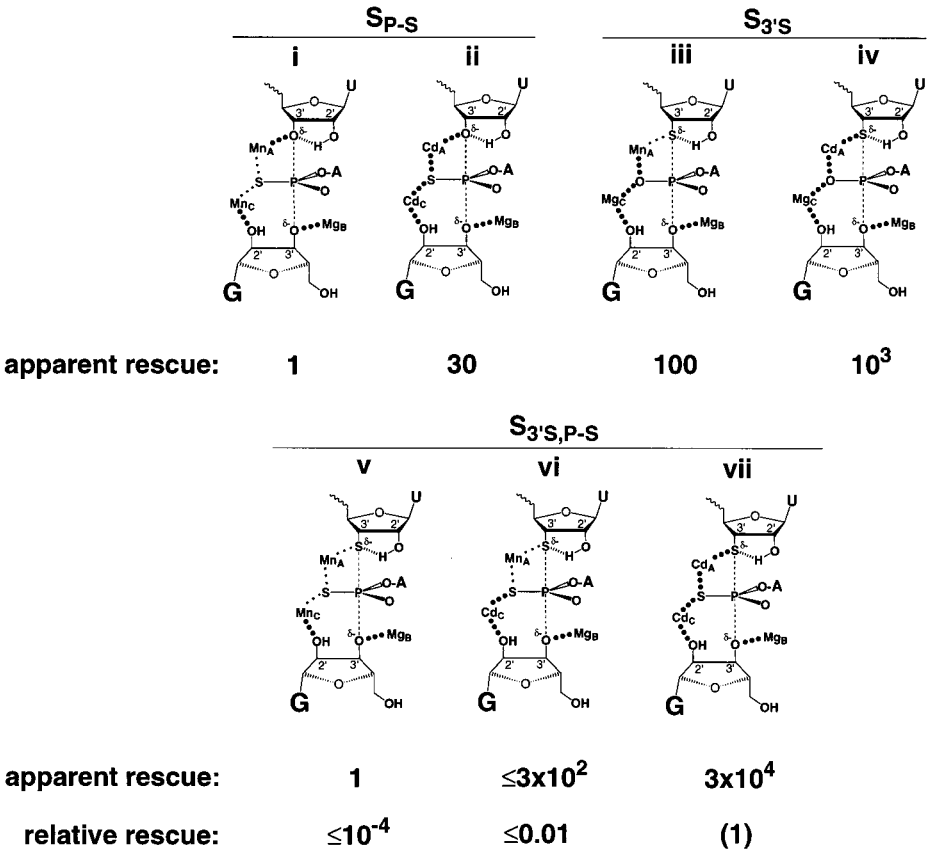

FIGURE 6: Summary of metal ion rescue and models for metal ion interactions with modified oligonucleotide substrates. "Apparent rescue" refers to the amount of increase in k^{rel} for the modified substrates at the highest Mn^{2+} or Cd^{2+} concentration used in the experiments relative to that in the presence of Mg^{2+} alone. For Mn^{2+}, the apparent rescue is equal to the rescue with Mn^{2+} bound at the sites of interest, because these sites can be saturated by Mn^{2+}. For Cd^{2+}, the apparent rescue is a lower limit for the rescue with Cd^{2+} bound, because Cd^{2+} does not saturate these metal sites (see text). "Relative rescue" refers to the amount of rescue provided by a single Cd^{2+} ion for species **vi** relative to the rescue by two Cd^{2+} ions for species **vii**. For reactions with S_{P-S} and $S_{3'S}$, the amounts of apparent rescue are obtained directly from experimental determinations (*3*, *28*, *32*). For reactions with $S_{3'S,P-S}$, the amounts of apparent rescue for **v** and **vii** are from experimental determinations (Figures 2 and 3; ref *30*; data not shown); the relative rescue is defined as equal to 1 for **vii**, and the relative rescue for **v** is obtained from comparison of the rescue in this species relative to that of **vii**. For species **vi**, the steep $[Cd^{2+}]$ dependences for rescue of the $S_{3'S,P-S}$ reaction in the presence of added Mn^{2+} (Figure 4) indicate that the rescue provided by Mn_A^{2+} and Cd_C^{2+} at the two rescuing sites is at least 100-fold less than the rescue by two Cd^{2+} ions (relative rescue $\leq$ 0.01; see Supporting Information for a detailed description). The apparent rescue for **vi** is obtained from this relative rescue value and the apparent rescue for **vii**. The smaller dots between Mn^{2+} and the sulfur atoms depict that these Mn^{2+}−sulfur interactions are less favorable than the corresponding Mn^{2+}−oxygen or Cd^{2+}−sulfur interactions.

it is a lower limit and can most simply be accounted for by the model in which both M_A and M_C coordinate the S_P or *pro*-S_P atom.

The model that the *pro*-S_P oxygen coordinates both M_A and M_C predicts that rescue of the single thio substitution at this oxygen would require two Cd^{2+} ions, one at site C and one at site A (Figure 6, **ii**). This model also predicts that a high concentration of Cd^{2+} would be needed in order for rescue to be observed. This is because Cd^{2+} does not saturate either metal site, so that the probability of having two Cd^{2+} bound at both sites is low. In addition, rescue for the S_{P-S} reaction would be expected to be less efficient than for the $S_{3'S,P-S}$ reaction, because the S_{P-S} reaction involves only two Cd^{2+}−sulfur interactions instead of the three Cd^{2+}−sulfur interactions made in the $S_{3'S,P-S}$ reaction (**ii** vs **vii**).

To test this possibility, we explored rescue of the S_{P-S} reaction by Cd^{2+} and determined the number of Cd^{2+} ions required for rescue. As described above, the reaction E·S·G → products was followed as a function of Cd^{2+} concentration for S_{P-S} and dS, and the rate constant of the S_{P-S} reaction relative to dS was plotted (Figure 7). Addition of Cd^{2+}, up to 15 mM, provides 30-fold rescue for the S_{P-S} reaction (Figure 7, ●; control experiments strongly suggest that the observed rescue does not arise from desulfurization of S_{P-S} (see Supporting Information)). This rescue has not been detected previously, presumably because of the modest amount of rescue and the inhibitory effects of Cd^{2+} at high concentrations that must be controlled for (Figure 2). The relative reactivity of S_{P-S} has a steep dependence on Cd^{2+} concentration, consistent with rescue by two Cd^{2+} ions (Figure 7, solid line). In contrast, rescue by a single Cd^{2+} ion is not expected to give as steep a concentration dependence (dashed line). Thus, even with a single thio substitution at the *pro*-S_P oxygen, two Cd^{2+} ions are required for rescue.

As expected, the reaction of S_{P-S} with G_N is also rescued by Cd^{2+} with a concentration dependence on two Cd^{2+} ions, analogous to that for the S_{P-S} reaction with G (Supporting Information). This is consistent with the involvement of site C in rescuing the thio effect at the *pro*-S_P oxygen as described above. Further, the substrate $S_{3'S,P-S}$ studied above, which

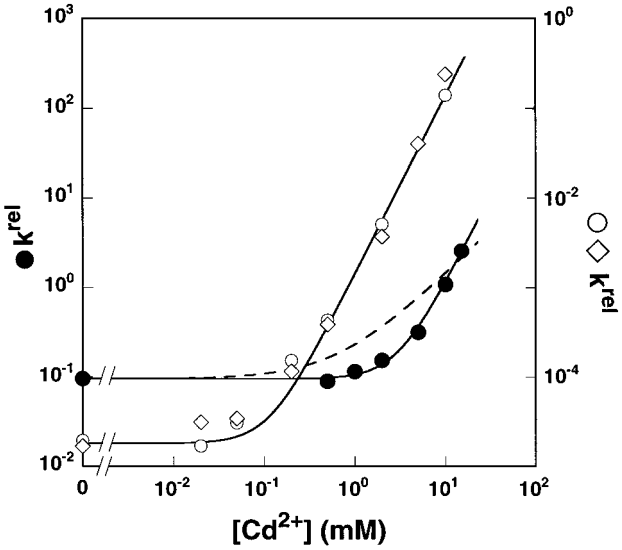

FIGURE 7: Two Cd^{2+} ions are required to rescue the S_{P-S} reaction. The [Cd^{2+}] dependence for the reaction E·S·G → products was determined for S_{P-S} and dS as described in Materials and Methods (pH 7.0, 50 mM Mg^{2+}), and the rate constant of the S_{P-S} reaction relative to dS was plotted (k^{rel}; ●). The [Cd^{2+}] dependence for rescue of the $S_{3'S,P-S}$ reaction relative to −1r,dS (○, ◇) was from Figure 3. The solid lines are fits of the data to a model in which two Cd^{2+} ions are required to rescue the S_{P-S} and the $S_{3'S,P-S}$ reactions, and the dashed line is a fit of the S_{P-S} data to a model in which a single Cd^{2+} rescues the reaction (see Materials and Methods).

has an additional thio substitution at the 3′-bridging oxygen, does not give rise to a steeper concentration dependence than that for the S_{P-S} reaction (Figure 7, ○ and ◇). This strongly suggests that the same two metal ions responsible for rescuing the $S_{3'S,P-S}$ reaction, M_A and M_C, are also required for rescuing the single thio substitution in the S_{P-S} reaction (Figure 6, **ii**). These results provide strong evidence that the *pro*-S_P oxygen interacts with both of the metal ions at sites A and C.

DISCUSSION

Catalytic Metal Ion Interactions at the Tetrahymena RNA Active Site. Functional analyses in this and previous studies define a novel set of metal ion interactions within the *Tetrahymena* group I ribozyme active site and suggest the identity of interactions that completely define the catalytic metal ion/substrate interactions within an enzyme active site for the first time.

Three metal ions, M_A, M_B, and M_C, contribute to catalysis by this RNA enzyme (Figure 8). M_A bridges the 3′-bridging oxygen of the oligonucleotide substrate and the *pro*-S_P oxygen of the reactive phosphoryl group; M_C bridges the 2′-OH of the guanosine nucleophile and the *pro*-S_P oxygen; M_B coordinates the 3′-OH of guanosine that attacks the reactive phosphorus (Figure 8A). The results herein strongly suggest that M_B is not involved in rescuing the thio effect at the *pro*-S_P oxygen, providing strong evidence against the classical two-metal-ion mechanism for this RNA enzyme (Scheme 1). Molecular modeling indicates that all of the identified interactions involving the three metal ions can be made simultaneously without steric clashes (Figure 8B).

This study and previous mechanistic work have defined all or nearly all of the interactions with substrate groups at

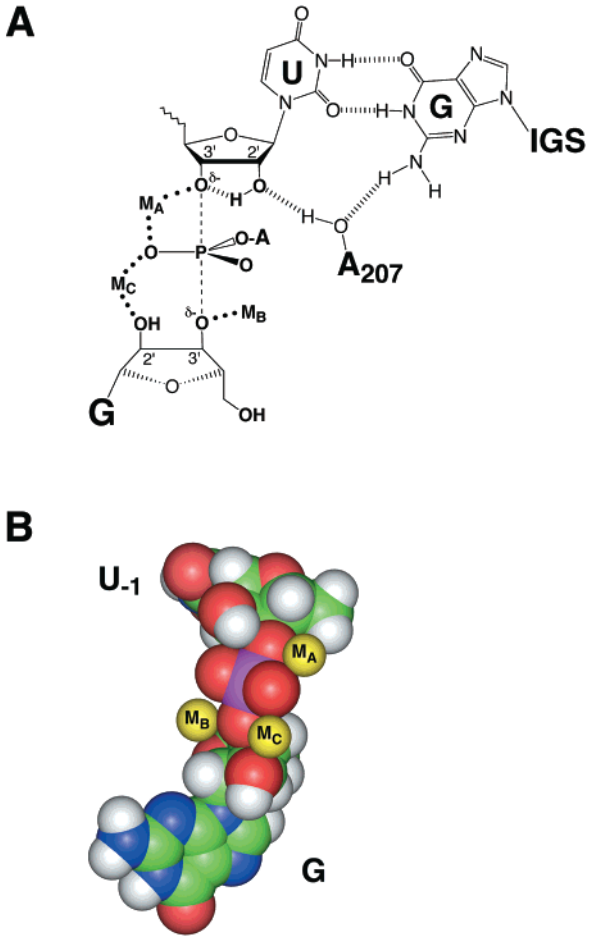

FIGURE 8: Model for catalytic interactions at the *Tetrahymena* ribozyme active site. (A) The transition state of the reaction is shown as in Figure 1. M_A, M_B, and M_C are the three previously identified metal ions that coordinate the 3′-bridging oxygens of S and G and the 2′-OH of G, respectively (*3*, *8*, *10*, *27*, *28*). The results of this work provide evidence that M_A and M_C also coordinate the *pro*-S_P oxygen of the reactive phosphoryl group. The 2′-OH of U(−1) donates a hydrogen bond to the neighboring 3′-bridging oxygen (*31*); there is evidence that this 2′-OH is part of a network of active site interactions that includes the 2′-OH of A_{207} and the exocyclic amine of the G·U pair that specifies the cleavage site (*43*, *45*). Interactions of metal ions with their proposed ligands are shown as dotted lines; hydrogen bonding interactions are shown as hashed lines. (B) Three-dimensional model of the proposed transition state interactions. Only the guanosine nucleophile, U(−1), and the catalytic metal ions are shown for clarity. The orientation is analogous to that of part A, with U(−1) on top; residue A(+1) esterified to the transferred phosphoryl group is not shown, and the *pro*-R_P oxygen of the reactive phosphoryl group is facing back and obscured in this view. The model was built with InsightII (Molecular Simulations Inc., San Diego, CA) using the P1 duplex structure determined previously (*50*). It is not intended to predict the precise positions within the active site, only to demonstrate that functionally identified metal ions and other interactions are sterically reasonable and can occur simultaneously.

or near the site of chemical transformation. The 3′-oxygen of S coordinates a metal ion, M_A, as described above; the remaining lone pair of electrons on this 3′-oxygen accepts a hydrogen bond from the neighboring U(−1) 2′-OH (Figure 8A; *31*). This then defines all of the transition state interactions surrounding this atom. The U(−1) 2′-OH donates a hydrogen bond to the 3′-oxygen of S, as noted above. The lone pair electrons on this 2′-OH does not appear to

coordinate a metal ion (*31*). Instead, there is evidence that this 2′-OH accepts a hydrogen bond from the 2′-OH of A_{207} (*43*). Although it remains possible that the other lone pair of electrons on the U(−1) 2′-OH accepts an additional hydrogen bond, the observation that replacing this 2′-OH group with $-NH_2$ has a less than 10-fold deleterious effect (*31* and data not shown) suggests that such an additional interaction is not present. The *pro-*S_P oxygen of the reactive phosphoryl group makes two metal ion interactions with M_A and M_C. This presumably accounts for all of the interactions surrounding this atom, as it is hard to imagine more than two metal ions around this atom on the basis of steric considerations. One of the lone pair electrons on the 3′-oxygen of G coordinates a metal ion, M_B (*8*). The remaining lone pair of electrons could coordinate M_C or another metal ion, could accept a hydrogen bond from the neighboring 2′-OH of G, or may not make additional active site interactions. The 2′-OH of G coordinates a third metal ion, M_C (*27*, *28*). It is not likely that the other lone pair of electrons on this 2′-OH makes additional active site interactions, because the reactivity of G_N is within 5-fold of that of G with Mn_C^{2+} present, despite the fact that the 2′-NH_2 group has only one lone pair of electrons (*28*). It is likely, however, that the 2′-OH of G donates a hydrogen bond to a ribozyme group, to a water molecule within the active site, or to the 3′-oxygen of G as noted above.

How do these active site metal ions provide catalysis? Mechanistic studies on the M_A interactions strongly suggest that M_A contributes to catalysis by stabilizing the developing negative charge on the 3′-leaving group oxygen in the transition state and may also contribute by electrostatic destabilization of the ground state (*3*, *44*). The interaction of M_B with the nucleophilic 3′-oxygen of G presumably helps to deprotonate the 3′-OH of G, thereby activating the nucleophile (*8*). Considering the geometrical requirements for attack of the 3′-OH of G on the reactive phosphorus, the bridging interactions of M_C between the 2′-OH of G and the *pro-*S_P oxygen of the reactive phosphoryl group may help to position the substrates with respect to one another and possibly with respect to other catalytic groups within the active site, thereby facilitating the reaction (*28*). The interaction of M_A with the *pro-*S_P oxygen may also help to organize the active site and position the substrates. Finally, the interactions of M_A and M_C with the *pro-*S_P oxygen could help to stabilize negative charge development on the *pro-*S_P oxygen in the transition state.

Besides metal ions, the *Tetrahymena* ribozyme uses additional active site interactions to provide catalysis. The hydrogen bond from the 2′-OH of U(−1) to the neighboring 3′-bridging oxygen may further stabilize the developing negative charge on the leaving group in the transition state (Figure 8A; *31*). The U(−1) 2′-OH also appears to be part of a network of active site interactions including the G•U wobble pair that specifies the cleavage site and the 2′-OH of A_{207} (*43*, *45*). Further away from the site of bond cleavage, tertiary interactions in the oligonucleotide substrate and guanosine binding sites help to position the substrates and may also contribute to electrostatic destabilization of the substrates (*39*, *44*, *46*). Together, these active site interactions provide an electrostatic and geometrical template for the reaction's transition state, allowing this RNA enzyme to achieve a 10^{11}-fold rate enhancement for reaction of its bound substrate relative to the corresponding solution reaction (*34*).

Limitations in Interpreting Rescue Data. The ability of the Cd^{2+} ions at sites A and C to rescue the thio effect at the *pro-*S_P oxygen strongly suggests direct interactions of Cd_A^{2+} and Cd_C^{2+} with the S_P sulfur (Figure 6, **vii**). It is nevertheless possible that the interaction of M_A or M_C with the *pro-*S_P oxygen, or any other metal ion interaction identified by metal ion rescue experiments, is normally mediated by additional active site residues or bound water and that substitution of the oxygen atom and Mg^{2+} ion with the larger sulfur atom and Cd^{2+} ion changes a normally outer-sphere interaction to an inner-sphere interaction. More extensive rearrangement upon sulfur and Cd^{2+} substitution cannot be ruled out either. However, more extensive rearrangements would require there to be two or more different active site configurations, each giving substantial catalysis. Bearing these limitations in mind, we adopt the simplest, and we think the most probable, interpretation of the results in the model with metal ion/ligand interactions as shown in Figure 8 and described above.

Implications. Divalent metal ions are widespread in biological catalysis, particularly in catalysis of phosphoryl transfer reactions. Despite their functional importance, the mechanism by which metal ions are used by RNA and protein enzymes to facilitate phosphoryl transfer remains the subject of much debate (*11*, *15*, *17*, *23*, *47*−*49*). Because of multiple binding modes and the ability to rearrange within active sites, structural studies alone, while enormously valuable in suggesting models of catalytic interactions, cannot resolve these mechanistic questions. The atomic level substrate modification combined with in-depth mechanistic analysis described in this and previous work is a powerful functional approach to distinguish metal ion sites, define active site metal ion interactions, and isolate the effect of individual metal ions on each reaction step, thereby providing insights into the roles of active site metal ions.

ACKNOWLEDGMENT

We thank L. Beigelman for oligonucleotides, F. Eckstein for 2′-aminoguanosine, and members of the Herschlag and Piccirilli laboratories for comments on the manuscript.

SUPPORTING INFORMATION AVAILABLE

Six figures, two tables, and thirteen equations describing experiments with Zn^{2+} as a rescuing metal ion analogous to those described in the text using Cd^{2+}; Mn^{2+} competition of Cd^{2+} rescue for the dithioate substrate reaction at 50 mM Mg^{2+}; controls for the desulfurization reaction; Cd^{2+} rescue of the S_{P-S} reaction in the presence of G_N; derivations of eqs 2−5. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Jaffe, E. K., and Cohn, M. (1978) *J. Biol. Chem. 253*, 4823−4825.
2. Burgers, P. M., and Eckstein, F. (1979) *J. Biol. Chem. 254*, 6889−6893.
3. Piccirilli, J. A., Vyle, J. S., Caruthers, M. H., and Cech, T. R. (1993) *Nature 361*, 85−88.
4. Dismukes, G. C. (1996) *Chem. Rev. 96*, 2909−2926.

5. Warnecke, J. M., Furste, J. P., Hardt, W. D., Erdmann, V. A., and Hartmann, R. K. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 8924−8928.
6. Wilcox, D. E. (1996) *Chem. Rev. 96*, 2435−2458.
7. Sontheimer, E. J., Sun, S., and Piccirilli, J. A. (1997) *Nature 388*, 801−805.
8. Weinstein, L. B., Jones, B. C., Cosstick, R., and Cech, T. R. (1997) *Nature 388*, 805−808.
9. Scott, E. C., and Uhlenbeck, O. C. (1999) *Nucleic Acids Res. 27*, 479−484.
10. Shan, S., Yoshida, A., Sun, S., Piccirilli, J. A., and Herschlag, D. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 12299−12304.
11. Smith, D., and Pace, N. R. (1993) *Biochemistry 32*, 5273−5281.
12. Steitz, T. A., and Steitz, J. A. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6498−6502.
13. Heikinheimo, P., Lehtonen, J., Baykov, A., Lahti, R., Cooperman, B. S., and Goldman, A. (1996) *Structure 4*, 1491−1508.
14. Kuimelis, R. G., and McLaughlin, L. W. (1996) *Biochemistry 35*, 5308−5317.
15. Cowan, J. A. (1997) *J. Biol. Inorg. Chem. 2*, 168−176.
16. Horton, N. C., Newberry, K. J., and Perona, J. J. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 13489−13494.
17. Keck, J. L., Goedken, E. R., and Marqusee, S. (1998) *J. Biol. Chem. 273*, 34128−34133.
18. Lott, W. B., Pontius, B. W., and von Hippel, P. H. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 542−547.
19. Steitz, T. A. (1998) *Nature 391*, 231−232.
20. Holtz, K. M., and Kantrowitz, E. R. (1999) *FEBS Lett. 462*, 7−11.
21. Sam, M. D., and Perona, J. J. (1999) *Biochemistry 38*, 6576−6586.
22. Tesmer, J. J., Sunahara, R. K., Johnson, R. A., Gosselin, G., Gilman, A. G., and Sprang, S. R. (1999) *Science 285*, 756−760.
23. Zhang, Y., Liang, J. Y., Huang, S., Ke, H., and Lipscomb, W. N. (1993) *Biochemistry 32*, 1844−1857.
24. Bone, R., Frank, L., Springer, J. P., and Atack, J. R. (1994) *Biochemistry 33*, 9468−9476.
25. Cooper, S. J., Leonard, G. A., McSweeney, S. M., Thompson, A. W., Naismith, J. H., Qamar, S., Plater, A., Berry, A., and Hunter, W. N. (1996) *Structure 4*, 1303−1315.
26. Klabunde, T., Strater, N., Frohlich, R., Witzel, H., and Krebs, B. (1996) *J. Mol. Biol. 259*, 737−748.
27. Sjögren, A. S., Pettersson, E., Sjöberg, B. M., and Strömberg, R. (1997) *Nucleic Acids Res. 25*, 648−653.
28. Shan, S., and Herschlag, D. (1999) *Biochemistry 38*, 10958−10975.
29. Rajagopal, J., Doudna, J. A., and Szostak, J. W. (1989) *Science 244*, 692−694.
30. Yoshida, A., Sun, S., and Piccirilli, J. A. (1999) *Nat. Struct. Biol. 6*, 318−321.
31. Yoshida, A., Shan, S., Herschlag, D., and Piccirilli, J. A. (2000) *Chem. Biol. 7*, 85−96.
32. Shan, S., and Herschlag, D. (2000) *RNA 6*, 795−813.
33. Sun, S., Yoshida, A., and Piccirilli, J. A. (1997) *RNA 3*, 1352−1363.
34. Herschlag, D., and Cech, T. R. (1990) *Biochemistry 29*, 10159−10171.
35. McConnell, T. S., Cech, T. R., and Herschlag, D. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 8362−8366.
36. Bevilacqua, P. C., Kierzek, R., Johnson, K. A., and Turner, D. H. (1992) *Science 258*, 1355−1358.
37. Herschlag, D. (1992) *Biochemistry 31*, 1386−1399.
38. Narlikar, G. J., and Herschlag, D. (1996) *Nat. Struct. Biol. 3*, 701−710.
39. Narlikar, G. J., Khosla, M., Usman, N., and Herschlag, D. (1997) *Biochemistry 36*, 2465−2477.
40. Szewczak, A. A., Ortoleva-Donnelly, L., Ryder, S. P., Moncoeur, E., and Strobel, S. A. (1998) *Nat. Struct. Biol. 5*, 1037−1042.
41. Martell, A. E., and Smith, R. M. (1977) in *Critical Stability Constants, Vol. 3: Other Organic Ligands*, Plenum Press, New York.
42. Pecoraro, V. L., Hermes, J. D., and Cleland, W. W. (1984) *Biochemistry 23*, 5262−5271.
43. Strobel, S. A., and Ortoleva-Donnelly, L. (1999) *Chem. Biol. 6*, 153−165.
44. Narlikar, G. J., Gopalakrishnan, V., McConnell, T. S., Usman, N., and Herschlag, D. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 3668−3672.
45. Knitt, D. S., Narlikar, G. J., and Herschlag, D. (1994) *Biochemistry 33*, 13864−13879.
46. Narlikar, G. J., and Herschlag, D. (1998) *Biochemistry 37*, 9902−9911.
47. Beebe, J. A., Kurz, J. C., and Fierke, C. A. (1996) *Biochemistry 35*, 10493−10505.
48. Groll, D. H., Jeltsch, A., Selent, U., and Pingoud, A. (1997) *Biochemistry 36*, 11389−11401.
49. Pontius, B. W., Lott, W. B., and von Hippel, P. H. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 2290−2294.
50. Allain, F. H., and Varani, G. (1995) *J. Mol. Biol. 250*, 333−353.

BI002887H