Identification of the Hammerhead Ribozyme Metal Ion Binding Site Responsible for Rescue of the Deleterious Effect of a Cleavage Site Phosphorothioate†

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ABSTRACT: The hammerhead ribozyme crystal structure identified a specific metal ion binding site referred to as the P9/G10.1 site. Although this metal ion binding site is ∼20 Å away from the cleavage site, its disruption is highly deleterious for catalysis. Additional published results have suggested that the *pro*-R_P oxygen at the cleavage site is coordinated by a metal ion in the reaction's transition state. Herein, we report a study on Cd^{2+} rescue of the deleterious phosphorothioate substitution at the cleavage site. Under all conditions, the Cd^{2+} concentration dependence can be accounted for by binding of a single rescuing metal ion. The affinity of the rescuing Cd^{2+} is sensitive to perturbations at the P9/G10.1 site but not at the cleavage site or other sites in the conserved core. These observations led to a model in which a metal ion bound at the P9/G10.1 site in the ground state acquires an additional interaction with the cleavage site prior to and in the transition state. A titration experiment ruled out the possibility that a second tight-binding metal ion ($K_d^{Cd} < 10\ \mu M$) is involved in the rescue, further supporting the single metal ion model. Additionally, weakening Cd^{2+} binding at the P9/G10.1 site did not result in the biphasic binding curve predicted from other models involving two metal ions. The large stereospecific thio-effects at the P9/G10.1 and the cleavage site suggest that there are interactions with these oxygen atoms in the normal reaction that are compromised by replacement of oxygen with sulfur. The simplest interpretation of the substantial rescue by Cd^{2+} is that these atoms interact with a common metal ion in the normal reaction. Furthermore, base deletions and functional group modifications have similar energetic effects on the transition state in the Cd^{2+}-rescued phosphorothioate reaction and the wild-type reaction, further supporting the model that a metal ion bridges the P9/G10.1 and the cleavage site in the normal reaction (i.e., with phosphate linkages rather than phosphorothioate linkages). These results suggest that the hammerhead undergoes a substantial conformational rearrangement to attain its catalytic conformation. Such rearrangements appear to be general features of small functional RNAs, presumably reflecting their structural limitations.

The hammerhead ribozyme is a small RNA motif derived from several plant satellite RNA and viroid genomes that catalyzes phosphodiester cleavage to give products with a 2′,3′-cyclic phosphate and a 5′-hydroxyl group [Figure 1, panels A and B (*1*−*3*)]. Despite numerous mechanistic investigations and the determination of several X-ray crystal structures of ribozyme·inhibitor and ribozyme·substrate complexes, the catalytic mechanism has remained elusive (for review, see, e.g., refs *4*−*6*).

The crystal structure of the hammerhead revealed a metal ion binding site that involves the *pro*-R_P oxygen of the P9 phosphate and the N_7 nitrogen of G10.1, referred to herein as the P9/G10.1 metal ion binding site [Figure 1C (*7*), see also refs *8*−*10*]. Although catalysis of the hammerhead ribozyme is greatly enhanced by divalent metal ions (*11*, *12*), the P9/G10.1 metal ion was not expected to have a large impact on catalysis because its binding site is ∼20 Å away from the cleavage site (*7*−*10*). However, replacing the *pro*-R_P oxygen at the P9 phosphate with sulfur resulted in a 10^3-fold decrease in rate (*13*). This deleterious effect could be rescued by addition of Cd^{2+}, a thiophilic metal ion, and analysis of the rescue led to the suggestion that in the transition state this Cd^{2+} ion acquired one or more additional ligands within the ribozyme·substrate complex (*13*).

Early thio-substitution and metal ion rescue experiments also suggested an interaction of a catalytically important metal ion with the *pro*-R_P oxygen at the cleavage site (*14*−*16*). Although this interpretation of the functional data has been challenged (*17*), recent results provide strong support for the original interpretation (*18* and results herein).

As the previous results suggested that the P9/G10.1 metal ion acquires an additional ligand in the transition state and as the binding site for the metal ion rescuing the cleavage site thio-substitution had not been identified, we tested whether the P9/G10.1 metal ion could be responsible for

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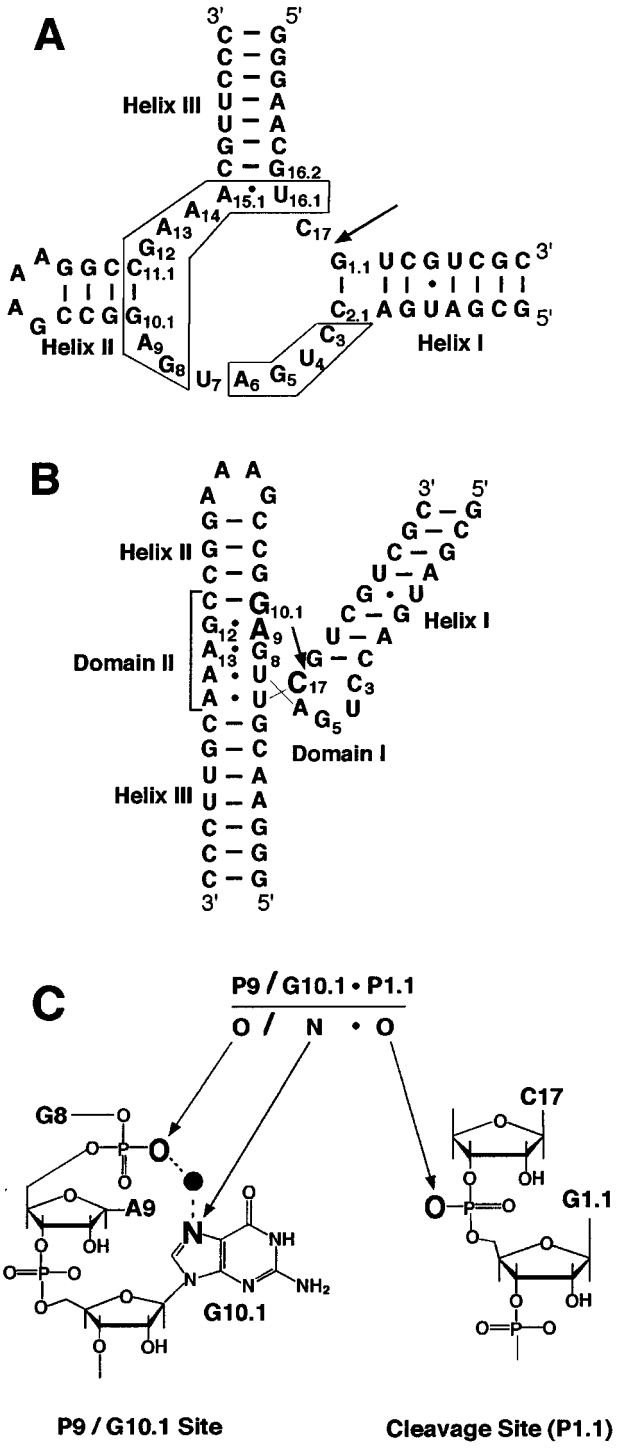


FIGURE 1: The structure and metal ion binding sites of the hammerhead ribozyme HH16. (A) The secondary structure of the E·S complex. The boxed bases depict the conserved core (*2*), and numbering is according to Hertel et al. (*74*). The cleavage site is marked with the arrow. The phosphate substrate used herein contained a U instead of a C at the 3′ terminal residue (see Experimental Procedures). (B) A two-dimensional representation of the tertiary structure observed in crystal structures (*7*−*10*). Domain I comprises a U-turn involving residues C3, U4, G5, and A6. (C) Sites of metal ion rescue (see text) and the three-letter nomenclature used herein to describe modifications at these positions. The metal ion is represented by the black sphere. For the nomenclature, the first two letters correspond to the ribozyme (P9/G10.1) and the last letter corresponds to the substrate (P1.1). Phosphate linkages are referred to as O, whereas S describes an R_P-phosphorothioate; G at position 10.1 is denoted by N, for the N_7 nitrogen, whereas 7-deazaG, in which the N is replaced with a C−H group, is denoted by C. For example, the unmodified E·S complex is described as O/N·O, whereas the complex with phosphorothioate substitutions at the P9 and the cleavage site is referred to as S/N·S.

rescue of the cleavage site thio-effect. The results reported herein strongly suggest that the P9/G10.1 metal ion indeed interacts with the scissile phosphoryl group, identifying the binding site for a catalytic metal ion. This implies that the hammerhead must undergo a substantial conformational rearrangement to achieve its catalytic conformation.

EXPERIMENTAL PROCEDURES

Materials. Ribozyme and substrate oligoribonucleotides were prepared by solid-phase synthesis (*19*), and sulfurization was performed by standard methods (*20*). The sequences for ribozyme and substrate are shown in Figure 1A. Figure 1C introduces the nomenclature used to refer to the ribozyme and substrate variants at positions P9, G10.1, and P1.1 (the cleavage site). Abasic and phenyl-containing ribozymes were prepared previously (*21*, *22*). The sequence of the phosphorothioate substrate differed from the phosphate substrate in the 3′-terminal nucleotide, with a C and U, respectively. Independent experiments have shown that the presence of U or C at this position has no effect on cleavage rate or Cd^{2+} affinity (k_{obs} and K_d^{Cd} values[1] were within 5% for the U-and C-containing substrate, for both O/N·O and S/N·O reactions; data not shown). Ribozymes with 7-deazaG10.1 also contained a 2′-deoxyribose substitution at this position. Independent control experiments showed that removal of the 2′-OH group at G10.1 to give deoxyG10.1 has at most small effects (<3-fold) on the cleavage rate and negligible effects on the Cd^{2+} affinity for O/N·O and O/N·S.

Stereoisomers of the S/N ribozyme and of the cognate phosphorothioate-containing substrate were separated by reversed-phase HPLC (*13*), and stereoisomers of ribozyme S/C and the mismatched phosphorothioate-containing substrates G16.2U and C17pyr were separated by ion-exchange HPLC (Dionex Nucleopac, PA-100 column, eluted with a 20 to 1000 mM NaCl gradient in 10 mM Tris-HCl, pH 9.3, and 10% ethanol). The R_P-thio-substrates were contaminated with ~3% phosphate or S_P-thio-substrate, whereas the contamination of the R_P-thio-ribozyme amounted to 20%, as judged by the burst in product formation with Mg^{2+} as the only divalent metal ion present. These contaminants were accounted for in obtaining the rate constants from single-turnover kinetic experiments (see below).

Stereoisomers at the P9 position were assigned by comparing the band pattern of the oligoribonucleotides after cleavage with stereoselective nucleases, RNase T1 and snake venom phosphodiesterase, which preferentially cleave a R_P-phosphorothioate linkage, and P1 nuclease, which prefers the S_P-stereoisomer. These assignments agree with previously published data on thio-effects in the hammerhead ribozyme

[1] Abbreviations: BisTris-propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; Tris, tris[hydroxymethyl]aminomethane; Pipes, piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]; Mops, 3-[*N*-morpholino]propanesulfonic acid; Mes, 2-[*N*-morpholino]ethanesulfonic acid; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; E·S, ribozyme·substrate complex; K_d^{Cd}, apparent dissociation constant for Cd^{2+} from the E·S complex; k_{max}, the cleavage rate in the presence of saturating Cd^{2+}; mutant ribozymes are denoted by the residue that is modified: pyr, 4-pyridinone; X, abasic nucleotide; e.g., A9X refers to a ribozyme with an abasic residue at position 9; S refers to phosphorothioate linkage 5′ to the named residue, i.e., P9S refers to the ribozyme with a phosphorothioate linkage between G8 and A9.

(*13*−*15*). The cleavage site stereoisomers (P1.1) were assigned based on differential reactivity and previous stereochemical assignments (*14*−*16*).

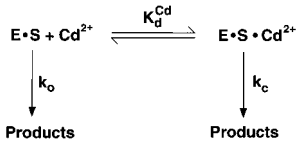
Substrates were 5′-end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase and were purified by electrophoresis on a nondenaturing 24% polyacrylamide gel. EDTA was omitted in the gel purification and subsequent storage to prevent contamination with low amounts of metal chelator. Concentrations of radiolabeled substrates were determined from specific activity. Concentrations of nonradioactive oligonucleotides were determined from absorbance using $\epsilon_{260} = 3.2 \times 10^5$ and 1.6×10^5 M^{-1} cm^{-1} for ribozymes and substrates, respectively.

$MgCl_2$, $CdCl_2$, and $CaCl_2$ were from Aldrich (>99.99%). Stock solutions were made with water and stored at −20 °C no longer than three months. BisTris-propane (>99%) was purchased from Sigma.

Methods. (1) Kinetics. All reactions were single-turnover and were carried out at 25 °C essentially as described previously (*13*, *23*). A trace amount of labeled substrate (final concentration <0.1 nM) and a large excess of ribozyme (50−600 nM) were heated together at 95 °C for 2 min in 50 mM BisTris-propane-HCl or Tris-HCl at the experimental pH and then equilibrated for 15 min at 25 °C to allow formation of the ribozyme·substrate complex. Increasing the ribozyme concentration from 50 to 600 nM did not change the cleavage rate constant k_{obs}, indicating that the substrate was fully bound to the excess ribozyme. The reaction was initiated by addition of metal ions and 6−9 aliquots were removed at specified times and quenched with 86% formamide and an excess of EDTA. The 5′-labeled cleavage product was separated from substrate on 20% acrylamide/7 M urea gels, and the fraction of cleaved substrate at each time point was quantitated using a PhosphorImager (Molecular Dynamics). For fast reactions the time courses were followed to completion, and as observed previously for HH16 (*13*, *23*), the data fit well to a single-exponential function with endpoints of ∼80%. Values of k_{obs} for the R_P-isomer were obtained by fitting the time courses with the sum of two independent single exponential equations to account for the ∼20% contamination by the S_p-isomer and/or phosphate-containing substrate, as described previously (*13*). For slower reactions, the time courses were followed for up to 10 days and the values of k_{obs} were obtained from the initial rates, assuming an endpoint of 80%. Product formation increased linearly with time, and independent control experiments showed that the ribozyme is fully active after these incubation times. In all cases, good fits to the appropriate kinetic model were obtained, with $R^2 > 0.99$ (Kaleidagraph, Synergy Software).

A subset of the abasic ribozymes (C3X, G5X, G8X, and A15.1X) gave lower endpoints with increasing concentrations of Cd^{2+}. In these cases, initial time points, with linear time dependence, were fit and an endpoint of 75% assumed, which would account for a Cd^{2+}-dependent partitioning of the ribozyme·substrate complex between reaction and formation of an inactive complex. In contrast, native gel analysis (*24*) with the G1.1U oxygen- and sulfur-containing substrates indicated that the endpoint of 40% for these substrates arises from the presence of an unreactive conformer of the substrate, which does not exchange with the active conformation over the experimental time scale, so the endpoint of 40% was used in kinetic analyses. Analysis of the mutant sequence indicates that this conformation is likely to be a hairpin. The inability to separate stereoisomers of the phosphorothioate-containing G1.1U substrate by HPLC is presumably due to this conformation, so these reactions were carried out with a mixture of isomers. About half of the reactive thio-substrate reacted similarly to the normal oxygen-containing substrate, and half exhibited a substantial thio-effect when Mg^{2+} was the only metal ion present, consistent with previous observations for the matched substrate (*14*−*16*).

Scheme 1



Experiments using oligonucleotides containing phosphorothioate substitutions within the conserved core were carried out with the mixture of stereoisomers of P3S, P4S, and P6S. The kinetics could be fit with a single exponential and an endpoint of ∼75% in all cases, suggesting that both stereoisomers behaved similarly. Nevertheless, small differences in k_{obs} would not be detected without separation of the isomers. Thus, there is an uncertainty in the K_d^{Cd} values obtained, which is estimated to be 2-fold.

(2) Apparent Dissociation Constants for Cd^{2+}. The dependences of k_{obs} on the concentration of Cd^{2+} up to 2 mM were well described by binding of a single stimulatory Cd^{2+} ion (Scheme 1, eqs 1 and 2), except for cases noted below.

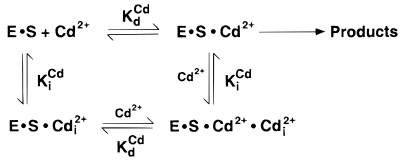
$$k_{obs} = k_o \frac{K_d^{Cd}}{K_d^{Cd} + [Cd^{2+}]} + k_c \frac{[Cd^{2+}]}{K_d^{Cd} + [Cd^{2+}]} \quad (1)$$

$$\log k_{obs} = \log\left\{k_o \frac{K_d^{Cd}}{K_d^{Cd} + [Cd^{2+}]} + k_c \frac{[Cd^{2+}]}{K_d^{Cd} + [Cd^{2+}]}\right\} \quad (2a)$$

$$\log k_{obs} = \log\left\{k_o + k_c \frac{[Cd^{2+}]}{K_d^{Cd}}\right\}; [Cd^{2+}] \ll K_d^{Cd} \quad (2b)$$

Equation 2b shows that, on a log−log scale, k_{obs} increases linearly with increasing concentrations of Cd^{2+} at Cd^{2+} concentrations below K_d^{Cd}. Linear (nonlogarithmic) plots are also presented in order to facilitate comparison of K_d values (eq 1). The data were fit using Kaleidagraph (Synergy Software; $R^2 > 0.99$). Competition of the rescuing Cd^{2+} with the background metal ion was observed, so the observed dissociation constants, K_d^{Cd}, are apparent values. For ribozymes containing phosphorothioate substitutions at the P9/G10.1 or the cleavage site, k_o, the cleavage rate in the absence of Cd^{2+} varied between experiments by up to 10-fold. However, addition of 1 mM EDTA did not change the cleavage rate, suggesting that the variability is not due to contamination of heavy metal ions but results from larger errors when the cleavage rate is very slow ($<10^{-4}$ min^{-1}) and the reaction is not followed to completion. Furthermore, between individual experiments there was some variation in the observed data at the highest Cd^{2+} concentrations. Above 2 mM Cd^{2+}, a second phase was sometimes observed that resulted in a stimulatory effect of 2-fold or inhibitory effect

Scheme 2



of up to 2-fold, apparently depending on the age of the Cd^{2+} stock. However, the effect on K_d^{Cd} values is small because these dissociation constants were substantially smaller than 2 mM and thus well separated from these secondary effects. Furthermore, even though some variation in K_d^{Cd} was observed from experiment to experiment, the relative values of K_d^{Cd} between the ribozyme·substrate complexes under comparison did not change. Finally, these effects at high Cd^{2+} concentrations were observed independent of the sulfur substitutions, suggesting that these effects are not involved in rescue of the deleterious effects from the sulfur substitutions. Thus, these variations at high concentrations are not expected to affect the conclusions drawn herein.

Side-by-side experiments in Na^+-Pipes, Na^+-Mops, and BisTris-propane at pH 6.5, as well as with Na^+-Mes and BisTris-propane at pH 6.0, suggest that buffer specific effects on the observed K_d^{Cd} are less than 2-fold.

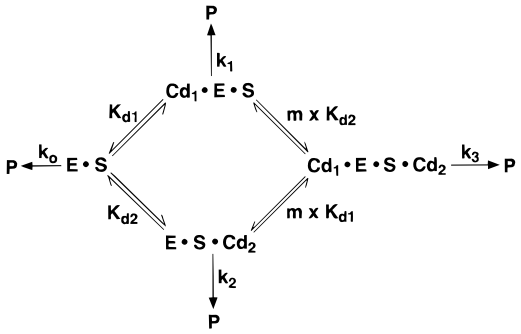
Cd^{2+} concentration dependences with the phosphorothioate-containing ribozymes (except for P9S) and some of the abasic ribozymes reproducibly gave modest inhibition (<20%) above 2 mM Cd^{2+}. These concentration dependences were therefore fit to a model analogous to that in Scheme 1, but with a second metal ion that is inhibitory and can bind to the E·S and E·S·Cd^{2+} complexes (Scheme 2, eq 3). These

$$k_{obs} = k_o \frac{[Cd^{2+}]}{\{K_d^{Cd} + [Cd^{2+}]\}\left\{1 + \frac{[Cd^{2+}]}{K_i^{Cd}}\right\}} \quad (3)$$

fits gave values of $R^2 > 0.98$, and the higher Cd^{2+} affinity was assigned to the stimulatory metal ion. The error in k_{max} and K_d^{Cd} for reactions that were inhibited is about 2-fold and thus significantly higher than the uncertainty of ~20% for a reaction in which no inhibition is observed. Fits to eq 1 for a single metal ion gave K_d^{Cd} values within the 2-fold error range, but the fits were markedly worse ($R^2 \approx 0.94$). Representative plots are available as Supporting Information.

(3) Control for Desulfurization. The 5′-cleavage products from phosphate and phosphorothioate substrates migrate slightly differently on denaturing acrylamide gels (20% acrylamide/7 M urea, data not shown), providing strong evidence that the product does not arise from cleavage of the phosphate substrate after desulfurization. In the presence of Cd^{2+}, a second band that runs slightly faster formed from the product of the phosphorothioate-containing substrate, but not from the phosphate substrate. This was shown both by the kinetics of formation of this band and also by generation of this band from the phosphorothioate product upon addition of Cd^{2+}. Thus, formation of this band apparently arises from Cd^{2+}-catalyzed hydrolysis of the cyclic phosphorothioate product after its formation in the ribozyme-catalyzed reaction. In kinetic experiments, both bands were appropriately quantitated as products of the hammerhead reaction.

Scheme 3



	Binding	Catalysis
Independent	I m = 1	A $k_3/k_o = k_1/k_o \times k_2/k_o$
Cooperative	II m < 1	B $k_3/k_o > k_1/k_o \times k_2/k_o$
Anticooperative	III m > 1	C $k_3/k_o < k_1/k_o \times k_2/k_o$

(4) Cd^{2+} Titration. To test for the existence of a potential second tight-binding metal ion, a titration experiment was carried out with the S/N·S complex. This complex was chosen because it has both phosphates of interest replaced with phosphorothioates so that it would be most likely to reveal the involvement of a potential second Cd^{2+} ion in the rescue. This experiment was carried out in 10 mM Mg^{2+} and 50 mM Tris-HCl buffer, pH 7.5, with varying concentrations of Cd^{2+} as described above, except that 20 μM of a stoichiometric E·S complex was used. The fraction of the complex containing R_P-phosphorothioates at both P9 and P1.1 is calculated to be 16 μM, as the thio-substrate stock contained ~97% of the R_P-isomer, but the ribozyme stock contained 20% contamination from the S_P-isomer (see above). A control experiment using 50 nM ribozyme and 80 pM of the thio-substrate was carried out side-by-side with the titration experiment.

(5) Simulations of Metal Ion Binding. We explored theoretical models in which two metal ions give Cd^{2+} concentration dependences consistent with the observed binding data, which as noted above, fit well to a single binding curve. Scheme 3 depicts a general kinetic scheme for binding of two metal ions, and eq 4 describes the

$$k_{obs} = \frac{k_o + k_1\frac{[Cd^{2+}]}{K_{dl}} + k_2\frac{[Cd^{2+}]}{K_{d2}} + k_3\frac{[Cd^{2+}]^2}{mK_{dl}K_{d2}}}{1 + \frac{[Cd^{2+}]}{K_{dl}} + \frac{[Cd^{2+}]}{K_{d2}} + \frac{[Cd^{2+}]^2}{mK_{dl}K_{d2}}} \quad (4)$$

predicted dependence of the observed rate constant on the Cd^{2+} concentration. Binding of the metal ions in the ground state can be independent (case I, $m = 1$), mutually enhancing (cooperative, case II, $m < 1$), or mutually weakening (anticooperative, case III, $m > 1$). Simulations were carried out with $m = 1$, 0.1, or 10, representing cases I−III, respectively. Similarly, the interdependence of the two metal ions in catalysis can also be described as independent, cooperative, or anticooperative (Scheme 3, cases A, B, and C, respectively). If the metal ions are independent in their stimulatory effect, the product of their individual rate enhancements equals the observed rate enhancement ($k_2/k_o \times k_1/k_o = k_3/k_o$; case A), whereas cooperative and anticooperative effects give products of the individual rate enhance-

Table 1: Cd^{2+} Rescues the Deleterious Effect of Phosphorothioates at P9/G10.1 and at the Cleavage Site

E·S complex, P9/G10.1·P1.1	k_{obs} (min^{-1})			
	10 mM Mg^{2+} [a]		10 mM Ca^{2+} [b]	
	$-Cd^{2+}$	2 mM Cd^{2+}	$-Cd^{2+}$	2 mM Cd^{2+}
O/N·O	0.045	0.64	5.1×10^{-3}	1.8
O/N·S_{R_P}	1.1×10^{-5}	0.58	5.4×10^{-5}	0.92
O/N·S_{S_P}	0.021	0.33	2.8×10^{-3}	0.98
S_{R_P}/N·O	1.6×10^{-4}	1.2	3.3×10^{-4}	1.8
S_{S_P}/N·O	0.046	1.2	3.4×10^{-3}	2.6

[a] Reactions were performed side-by-side in 50 mM BisTris-propane, pH 6.5, 25 °C. [b] Reactions were performed side-by-side in 50 mM BisTris-propane, pH 6.9, 25 °C. Cd^{2+} (2 mM) is saturating because it is more than 8-fold above the K_d^{Cd} value. The subscripts "R_P" and "S_P" refer to the substitution of the *pro*-R_P or *pro*-S_P oxygen by sulfur.

ments that are larger and smaller than the observed rate enhancement, respectively (cases B and C).

For the simulations, each ground-state model (cases I−III) was combined with each transition-state model (cases A−C) and eq 4 was used to simulate the expected dependence of rate on Cd^{2+} concentration. For example, IC represents the case in which the metal ions bind independently and have anticooperative effects in catalysis. We obtained k_3/k_o from the experimental data and assigned various values to k_1/k_o and k_2/k_o, spanning 3 orders of magnitude. Further, we assigned values of K_{d1} and K_{d2} covering 3 and 4 orders of magnitude, respectively and, using eq 4, calculated Cd^{2+} concentration dependences on the cleavage rate constant for these cases (Excel, ~250 calculated concentration dependences). The resulting curves were plotted with the experimental data to assess which classes of models could provide reasonable fits to the observed data.

RESULTS

Figure 1C summarizes the three-letter shorthand nomenclature used throughout the Results to describe the ribozyme and substrate variants used in this study. Unless noted otherwise, phosphorothioates have R_P stereochemistry.

Characterization of Cd^{2+} Rescue of the Cleavage Site Phosphorothioate Substitution. We first confirmed the observations of Scott and Uhlenbeck concerning the R_P-phosphorothioate at the cleavage site (*18*) with the hammerhead construct HH16 used in this study. Consistent with the previous results, this phosphorothioate substitution at the cleavage site slowed the chemical step ~10^4-fold in the HH16 reaction, and the addition of 2 mM Cd^{2+} completely alleviated this deleterious effect ($k_{O/N\cdot S}/k_{O/N\cdot O} = 2.4 \times 10^{-4}$ and 0.9 in 10 mM Mg^{2+} in the absence and presence of 2 mM added Cd^{2+}, respectively; Table 1). There was no significant thio-effect from the S_P-phosphorothioate at this position and the Cd^{2+} concentration dependence with this substrate was the same within error as that with wild-type substrate (data not shown), again consistent with the previous results (*15*, *17*, *18*, *25*).

To better understand the nature of this rescue, we investigated its Cd^{2+} concentration dependence. A constant background of 10 mM Mg^{2+} was maintained, and the effect of Cd^{2+} on reaction of the O/N·S complex was compared to that on the O/N·O complex in an attempt to learn about the Cd^{2+} ion or ions responsible for the rescue (Figure 2). The

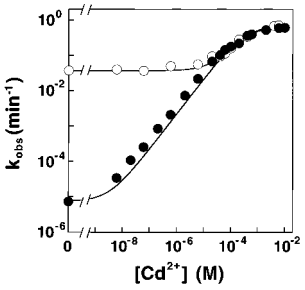


FIGURE 2: The affinity for Cd^{2+} rescuing the thio-effect at the cleavage site in a Mg^{2+} background is not affected by the phosphorothioate substitution. Reactions of O/N·O (○) and O/N·S (●) were carried out in 50 mM BisTris-propane, pH 6.5, and 10 mM Mg^{2+}, 25 °C, with varying concentrations of Cd^{2+}. Data were plotted on a log−log scale and the lines represent nonlinear least-squares fits for binding of a single rescuing Cd^{2+} ion (eq 2) and give apparent values of $K_d^{Cd} = 509$ and 280 μM O/N·O and O/N·S, respectively. The K_d^{Cd} values of 282 and 241 μM, respectively, reported in Table 2 were obtained from five to six independent experiments, including the experiment shown here. As described in the Experimental Procedures, at the highest Cd^{2+} concentrations variations of the experimental data between individual experiments resulted in variations of the K_d^{Cd} values. The uncertainty in the observed K_d^{Cd} value is especially large for O/N·O in a Mg^{2+}-background, because the rate enhancement that Cd^{2+} provides is only ~10-fold.

observed dependence is consistent with a simple model in which rescue is achieved by binding of a single Cd^{2+} ion.[2] This can be seen in the log−log plot of Figure 2, in which the log of the rate constant increases linearly with slope 1 over a range of Cd^{2+} concentration of nearly 4 orders of magnitude. The simplest interpretation of these data is the loss of an important interaction with a Mg^{2+} ion when the *pro*-R_p phosphate oxygen at the cleavage site is substituted with a sulfur. This loss can then be rescued by the addition of a single Cd^{2+} ion that interacts directly and stereospecifically with the R_P-phosphorothioate at the cleavage site in the reaction's transition state. The O/N·O and O/N·S complexes follow the same dependence on Cd^{2+} concentration with apparent Cd^{2+} affinities of 282 ± 131 and 214 ± 60 μM, respectively (Table 2).

The absence of a significant increase in the apparent binding affinity upon introduction of the cleavage site phosphorothioate introduced the possibility that the rescuing Cd^{2+} does not interact with the cleavage site in the ground state. However, the precision of this comparison is compromised by the small rate enhancement upon Cd^{2+} binding to the O/N·O complex of only ~10-fold and by the additional small effects at the highest Cd^{2+} concentrations (see Experimental Procedures). We therefore sought other conditions in which Cd^{2+} binding to the wild-type O/N·O complex would give a larger signal. With a background of 10 mM Ca^{2+}, the rates of reaction of the O/N·O and O/N·S complex

[2] Although in some experiments the data at the highest Cd^{2+} concentrations reveal small stimulatory or inhibitory effects relative to the concentration dependence expected from an effect by a single Cd^{2+} ion, these small effects were common to all ribozyme·substrate complexes investigated side-by-side (see Experimental Procedures). Thus, these effects appear to be independent of the rescue, which occurs at lower Cd^{2+} concentrations and follows a concentration dependence expected for rescue by a single metal ion (see Results). To maximize the precision of the comparisons all comparative data shown were obtained in side-by-side experiments.

Table 2: Modifications at the P9/G10.1 Site, but Not the Cleavage Site, Affect the Apparent Cd^{2+} Affinity[a]

E·S complex; P9/G10.1·P1.1	K_d^{Cd} (μM)			
	$[Mg^{2+}]$ (mM)		$[Ca^{2+}]$ (mM)	
	10	100	10	100
O/N·O	282 ± 131	$\geq 10^4$	104	4400
O/N·S	214 ± 60	$\geq 10^4$	133	2800
S/N·O	73 ± 24	$\geq 10^4$	36	840
S/N·S	79 ± 26	$\geq 10^4$	28	1400
O/C·O	*b*	*b*	*b*	*b*
O/C·S	$\geq 10^4$	$\geq 10^4$	$\geq 10^4$	$\geq 10^4$
S/C·O	*b*	*b*	2500	*b*
S/C·S	4300	$\geq 10^4$	2400	$\geq 10^4$

[a] Reactions were performed in 50 mM BTP, 25 °C. Different pH values were used with different background metal ions to ensure that the rates of reactions could be reliably measured. Control experiments showed that pH did not affect the observed values of K_d^{Cd} (see Methods). Errors are reported as standard deviations from five or six independent experiments. [b] K_d^{Cd} values could not be determined because of the small rate enhancement observed upon addition of Cd^{2+} (0−10 mM).

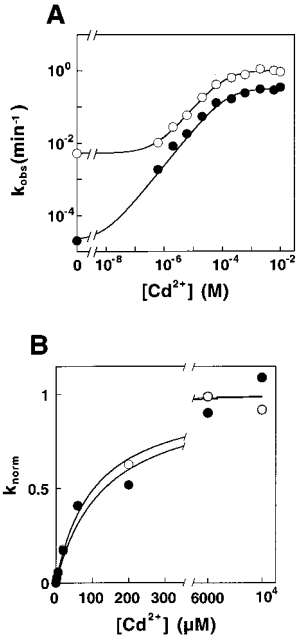


FIGURE 3: The affinity for the Cd^{2+} ion rescuing the thio-effect at the cleavage site in a Ca^{2+} background. The reactions were carried out in 50 mM BisTris-propane, pH 6.9, 10 mM Ca^{2+}, 25 °C and varying concentrations of Cd^{2+} with O/N·O (○) or O/N·S (●). Data were plotted on a log−log scale (A) or were normalized and plotted on a linear scale (B). The curves represent nonlinear least-squares fits for binding of a single rescuing Cd^{2+} ion (eqs 1 and 2a) and give apparent values of K_d^{Cd} = 104 and 133 μM for O/N·O and O/N·S, respectively.

were increased 350- and 10^4-fold, respectively, by the addition of 2 mM Cd^{2+} (Table 1). We therefore investigated the Cd^{2+} concentration dependence of the reaction rate for the O/N·O and O/N·S complexes in the presence of 10 mM Ca^{2+} (Figure 3). The Cd^{2+} dependence fit well to a single binding curve and the affinity of the Cd^{2+} rescuing both O/N·O and O/N·S was identical (Table 2, K_d^{Cd} = 104 and 133 μM, respectively), consistent with rescue by a single metal ion that does not interact with the cleavage site in the ground state.

Perturbations of the P9/G10.1 Binding Site Affect Cd^{2+} Rescue of the Cleavage Site Thio-Effect. As previous

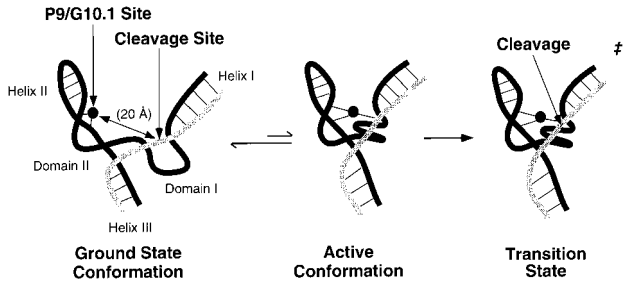


FIGURE 4: Model for the hammerhead reaction involving a large conformational change that brings together the cleavage site and P9/G10.1 metal ion in the active conformation. The ribozyme is shown in black, the substrate in gray, and the metal ion is represented as a black sphere. The cartoon of the ground state conformation is based on the crystal structures, which show the metal ion at the P9/G10.1 site ~20 Å away from the cleavage site (*7*, *8*). The proposed transition state interaction of the metal bound at the P9/G10.1 site with the *pro*-R_P phosphoryl oxygen atom at cleavage site therefore requires a large conformational change. This model also illustrates the speculative proposal that domain II remains relatively unchanged whereas domain I rearranges in forming the catalytically active structure (see Discussion).

experiments had provided evidence for binding of a functionally important metal ion to the P9/G10.1 site (*13*), we tested whether Cd^{2+} bound at that site was responsible for rescuing the deleterious effect of the cleavage site phosphorothioate. Indeed, analysis of the Cd^{2+} rescue of a phosphorothioate substitution at the P9/G10.1 site (S/N·O) and of the large effect of this metal ion on reactivity had led us to propose that the metal ion at this site adopts at least one additional ligand in the transition state (*13*).

We investigated the affinity of the Cd^{2+} rescuing the cleavage site phosphorothioate using hammerhead variants with a 7-deazaguanine residue at position 10.1, with an R_P-phosphorothioate at position 9, and with both substitutions together (Figure 1C). These residues were shown biochemically (*13*, *21*) and structurally (*7*, *8*) to provide ligands to the metal ion at the P9/G10.1 site, so perturbations at this site would be predicted to change the affinity of a metal ion bound there in the ground state. The Cd^{2+} concentration dependence of the cleavage rate was determined with the thio-substrate and the normal substrate for each of these ribozyme variants in backgrounds of 10 and 100 mM Mg^{2+} and 10 and 100 mM Ca^{2+}. In each case, the results were consistent with a single Cd^{2+} ion providing rescue according to Scheme 1. The results are summarized in Table 2 and are described below. Representative data are shown graphically to convey the precision of the comparisons (Figures 5 and 6). The remainder of the plots are available as Supporting Information.

If the rescue of the cleavage site phosphorothioate substitution were due to a metal ion bound at the P9/G10.1 site in the ground state, the affinity of the rescuing Cd^{2+} would be decreased by exchanging the N_7 nitrogen ligand at G10.1 with a hydrophobic methylene group. Figure 5 shows that this prediction is met; k_{obs} for the O/C·S complex increases linearly with Cd^{2+} concentration, showing no sign of saturation even at 6 mM Cd^{2+}, the highest concentration added, whereas the rate of the O/N·S reaction levels with K_d^{Cd} = 280 μM, as discussed earlier.

The second ligand of the P9/G10.1 metal ion site was also altered by replacing the *pro*-R_P phosphoryl oxygen between G8 and A9 with sulfur to give the S/N hammerhead. The

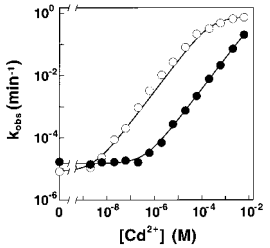


FIGURE 5: The apparent affinity for the metal ion rescuing the cleavage site thio-substitution decreases when the N_7 nitrogen ligand at the P9/G10.1 site is removed. Reactions were carried out in 50 mM BisTris-propane, pH 6.5, and 10 mM Mg^{2+}, 25 °C, and varying concentrations of Cd^{2+} with O/N·S (○) or O/C·S (●). To allow direct comparison, the binding curve for O/N·S shown here, was obtained side-by-side with the data for O/C·S in a different experiment than that shown in Figures 2 and 6. The lines represent nonlinear least-squares fits for binding of a single rescuing Cd^{2+} ion (eq 2a) and give apparent values of $K_d^{Cd} = 240$ and $\geq 10\,000$ μM for O/N·S and O/C·S, respectively.

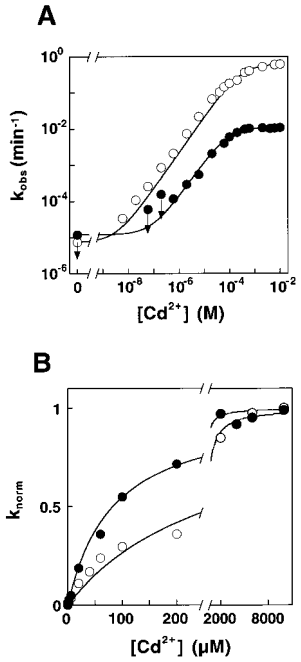


FIGURE 6: The apparent affinity for the metal ion rescuing the cleavage site thio-substitution increases when the *pro*-R_P oxygen at P9 is replaced by sulfur. Reactions were carried out in 50 mM BisTris-propane, pH 6.5, and 10 mM Mg^{2+}, 25 °C, and varying concentrations of Cd^{2+} with O/N·S (○) or S/N·S (●). Data were plotted on a log−log scale (A) or were normalized and plotted on a linear scale (B). The lines represent nonlinear least-squares fits for binding of a single rescuing Cd^{2+} ion (eqs 1 and 2a) and give apparent values of $K_d^{Cd} = 280$ and 83 μM for O/N·S and S/N·S, respectively. The arrows indicate that the values obtained are upper limits (see Experimental Procedures).

stronger intrinsic affinity of Cd^{2+} for sulfur than for oxygen (*26*, *27*) would be expected to increase the affinity of the rescuing Cd^{2+} ion. Figure 6A shows the Cd^{2+} dependence of the reaction rate for the S/N·S and O/N·S complexes in a background of 10 mM Mg^{2+}. To facilitate direct comparison, these rate constants have been normalized and replotted in Figure 6B. Even though the rescue is incomplete (see Discussion), the apparent Cd^{2+} affinity is greater for the S/N·S complex than for the O/N·S complex, as expected. The affinity increase of ~4-fold observed in the 10 mM Mg^{2+} background was also observed in backgrounds of 10 and 100 mM Ca^{2+} (Table 2), establishing the generality of this effect. The increase in affinity of ~4-fold was less than the ~100-fold Cd^{2+} affinity increase observed with 5′-adenosine monophosphorothioate compared to monophosphate (ref *26*; see also ref *27*). This modest increase in Cd^{2+} binding affinity for the ribozyme with the P9 phosphorothioate and the incomplete Cd^{2+} rescue for S/N·S ($k_{S/N\cdot S}/k_{O/N\cdot O} \approx 0.01$ at saturating Cd^{2+}; Figure 6A) are consistent with geometrical constraints within the P9/G10.1 metal ion site that influence affinity and reactivity (see Discussion). Alternatively or additionally, the absence of a large binding preference for the phosphorothioate-containing ribozyme could arise from a smaller intrinsic effect on Cd^{2+} affinity from sulfur substitution in a phosphate diester (RNA) than in the reference phosphoryl compounds AMP and ATP (*26*, *27*).

Model for Cd^{2+} Rescue. The change in the apparent affinity of the Cd^{2+} ion that rescues the cleavage site thio-effect with changes at the P9/G10.1 site suggests that the rescuing metal ion binds at this site, as depicted in the model of Figure 4. Conversely, the absence of an effect on the Cd^{2+} affinity from the cleavage site phosphorothioate suggests that the cleavage site interaction is not made in the ground state, also depicted in Figure 4. All of the Cd^{2+} concentration dependences are consistent with the involvement of a single rescuing metal ion, suggesting that there is a large-scale conformational transition that allows the metal ion bound at the P9/G10.1 site to interact with the cleavage site phosphoryl group in the reactive conformation and in the subsequent transition state. Such a large-scale conformational change is consistent with the available biochemical and structural data, as described in the Discussion.

Further Tests of the Single Metal Ion Model for Cd^{2+} Rescue.

(1) Cd^{2+} Affinity is Unchanged by Other Perturbations in the Conserved Core. The effects on the affinity of the rescuing Cd^{2+} ion could arise directly from perturbations of the P9/G10.1 site as depicted in Figure 4 or, alternatively, result from an indirect effect of this site on another site. This alternative model was of particular concern as substitution of G10.1 to 7-deazaguanine weakens binding of the rescuing Cd^{2+}, a deleterious effect that could arise directly or indirectly, and because the P9 phosphorothioate substitution gave only a modest increase in apparent Cd^{2+} affinity. It was therefore important to determine if effects on the affinity of the rescuing metal ion were localized to the P9/G10.1 site or if effects were also observed elsewhere in the hammerhead core. To accomplish this, we determined the Cd^{2+} concentration dependence of the cleavage rate of the thio-substrate with hammerhead variants containing abasic residues throughout the catalytic core (*21*, *22*) and with variants containing phosphorothioate substitutions at several positions.

The results with abasic substitutions are summarized in Figure 7. The apparent K_d^{Cd} for all abasic ribozymes, except G8X, A9X, and G10.1X, is between 100 and 450 μM, within error of the wild-type value of 280 μM.[3] The U4X, G5X, U7X, and A13X ribozymes were modestly (<20%) inhibited above 2 mM Cd^{2+}, leading to a higher uncertainty in these K_d^{Cd} values (see Experimental Procedures). For G8X and G10.1X, only limits ($K_d^{Cd} > 2.5$ and 2.7 mM, respectively) could be obtained, as no saturation was observed even at the highest Cd^{2+} concentrations. The lower limit for A9X

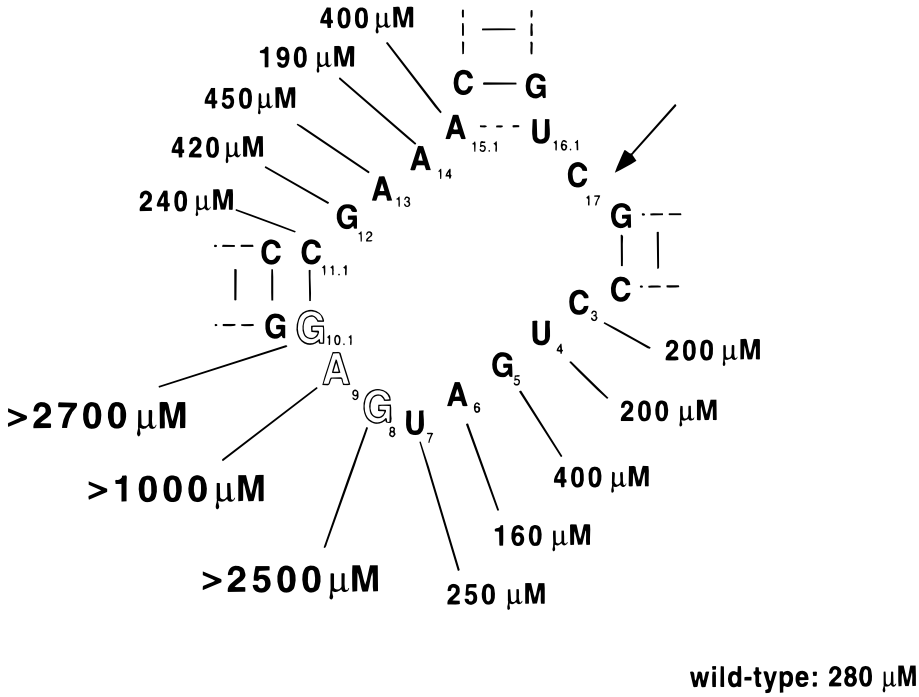


FIGURE 7: The affinity for the metal ion rescuing the cleavage site phosphorothioate substitution is affected only by ablation of bases at the P9/G10.1 site. Reactions of a series of ribozymes containing single abasic residues were carried out in 50 mM BisTris-propane, pH 7.5, and 10 mM Mg^{2+}, 25 °C, with varying concentrations of Cd^{2+}. Values of K_d^{Cd} were obtained from Cd^{2+} concentration dependences as described in the Experimental Procedures, and representative plots are available as Supporting Information.

(K_d^{Cd} > 1 mM) was obtained by fitting the data below 1.5 mM Cd^{2+} to a single metal ion model. Up to 1.5 mM Cd^{2+}, only slight curvature was observed and above 2 mM inhibition prevented a more precise determination of the K_d^{Cd}, as the affinity of the inhibitory metal was very similar to the affinity of the stimulatory metal. The K_d^{Cd} value obtained from fitting to a model that includes an inhibitory metal ion is higher than the lower limit given here. The maximal rates obtained from these concentration dependences are presented and interpreted below.

G8 and A9 neighbor the phosphate ligand of the P9/G10.1 metal ion binding site and G10.1 provides the N_7 ligand of the metal ion (Figure 1C). Removal of G8 and A9 is expected to disrupt local stacking interactions (*7*) and could thus result in defective Cd^{2+} binding. This interpretation is strongly supported by the fact that a hammerhead mutant that has a phenyl residue at position 9, and therefore can support stacking, has wild-type Cd^{2+} affinity (data not shown) and its reactivity is increased more than 100-fold relative to the abasic residue at A9 (*22* and data not shown). In contrast, a phenyl at position G10.1 did not rescue affinity (data not shown), further reinforcing the view that the metal ion interacts with G10.1 via the N_7 nitrogen. Surprisingly, ablation of residues G12 and A13 which form reverse Hoogsteen base pairs with G8 and A9 (*7*) did not have a significant effect on the apparent affinity of the rescuing Cd^{2+} ion. One explanation for this result is that the stacking interactions that are formed by G8 and A9 are sufficient to position the phosphodiester backbone to allow metal binding.

The absence of an effect of base ablation on Cd^{2+} affinity for most residues shows that the Cd^{2+} affinity does not change widely with substitutions throughout the hammerhead conserved core. On the contrary, changes in affinity are localized to the P9/G10.1 site. These observations substantially strengthen the conclusion that the changes in apparent affinity for the rescuing Cd^{2+} from perturbations in the P9/G10.1 site arise because this site is directly responsible for the rescue (Figure 4).

To further test whether the small increase in Cd^{2+} affinity observed for the S/N ribozyme was due to binding to the P9/G10.1 site, the Cd^{2+} affinities for ribozymes containing phosphorothioate substitutions at P3, P4, P6, P9, and P14 were determined from Cd^{2+} concentration dependences of the rate of reaction. Mixtures of stereoisomers were used for all ribozymes except P9S (see above) and P14S, where the isomers were investigated separately. Of these 10 different ribozymes, only the ribozyme with the R_P-phosphorothioate at P9 had an increased Cd^{2+} affinity. All the other ribozymes exhibited Cd^{2+} affinities within 2-fold of the wild-type affinity (data not shown). This further supports the interpretation that the tighter Cd^{2+} binding observed for the S/N ribozyme is due to binding of the Cd^{2+} ion to the *pro*-R_P sulfur at P9 and not the result of a nonspecific effect upon phosphorothioate substitution within the conserved core.

[3] Contrary to what is observed for the phosphorothioate-containing substrate free in solution, the cleavage rate levels off at 2 mM Cd^{2+} for all abasic ribozymes except those with ablations at the P9/G10.1 site. Thus, it appears that the binding affinities for Cd^{2+} report on binding to this site, even though the cleavage rate does not exceed the rate of the uncatalyzed reaction for some of the abasic ribozymes. Presumably, the Cd^{2+} ion is bound and sequestered at the P9/G10.1 site and occasionally comes in contact with the cleavage site phosphoryl group to promote cleavage. As this cleavage may largely be the result of the proximity of the P9/G10.1 site without relationship to the normal catalytic mechanism, the cleavage rates with the highly compromised abasic ribozymes cannot be used to rule out the involvement of a second metal ion in the rescue and in the normal reaction. Nevertheless, the data suggest that the Cd^{2+} affinity for the P9/G10.1 site is determined by local interactions and provide no indication of a second metal ion binding site.

(2) Consideration of Alternative Models That Involve a Second Bound Cd^{2+} Ion. All of the data above are consistent with the model of Figure 4 in which a single metal ion bound at the P9/G10.1 site is responsible for rescuing the cleavage site phosphorothioate substitution. There is no indication of a second rescuing metal ion from any of the data. Nevertheless, under certain circumstances, two metal ions can give a concentration dependence consistent with a single metal ion model. It was therefore important to explore the possibility that two metal ions were involved in the rescue. As described below, all models involving two rescuing metal ions are directly ruled out, with one exception, and other experiments indicate that the remaining model for two metal ion rescue is unlikely. It should be noted that these results strongly suggest the involvement of only a single metal ion in the rescue of thio-effects at the P9/G10.1 and the cleavage sites, but provide no information about the presence or absence of other functional metal ions.

Scheme 3 shows a general two metal ion rescue model that was used to simulate Cd^{2+} concentration dependences as described in the Experimental Procedures. Many of the models could be ruled out immediately as the simulated graphs did not provide reasonable fits to the experimental data. However, the simulations showed that if one metal ion bound tightly, with $K_d^{Cd} < 10$ μM and did not give a significant rate effect until a second Cd^{2+} bound at a lower affinity site, the concentration dependence could be indistinguishable from that expected for a single rescuing metal ion (Figure 8A).

To investigate whether such a high-affinity metal ion binding site exists, we performed a titration experiment. If the concentration of ribozyme·substrate complex were higher than the dissociation constant K_{d1} for the tight-binding Cd^{2+}, then added Cd^{2+} would be titrated by the E·S complex, leaving little free Cd^{2+} in solution. To fit the data, the model requires that the tightly bound metal ion does not provide catalysis until the second metal ion is bound. Thus, a significant rate enhancement would be observed only when the concentration of added Cd^{2+} exceeded the concentration of E·S complex. Conversely, if there were only a single metal ion responsible for rescue of the phosphorothioate substitutions, the concentration dependence of the observed cleavage rate with high E·S concentration would be indistinguishable from the data with low E·S concentration. As shown in Figure 8B, the Cd^{2+} concentration dependences with high and low concentrations of E·S are indistinguishable, providing strong evidence against a model with a second Cd^{2+} binding with $K_d^{Cd} < 10$ μM.

The titration experiment ruled out most alternative two metal ion models that predicted Cd^{2+} concentration dependences that could fit the data. The remaining class of two metal ion models requires anticooperative effects in catalysis, such that binding of the second metal ion reduces the stimulatory effect of the first metal ion (see Experimental Procedures). That is, the product of the individual rate enhancements is larger than the observed rate enhancement (Scheme 3, case C). More specifically, as shown in Figure 9, models with two such anticooperative metal ions that bind independently (Scheme 3, case IC) or strengthen binding of each other (case IIC) can fit the observed data, when similar dissociation constants between 10 and 100 μM and the appropriate rate enhancements are assumed. The models can fit the data because the similar metal ion affinities would prevent the binding curve from being biphasic and the anticooperative catalytic effect of the two metal ions could reduce the slope from a slope of 2, which is expected for two metal ions with independent energetic effects on catalysis, to the observed slope of 1. We first provide evidence against the model involving independent binding

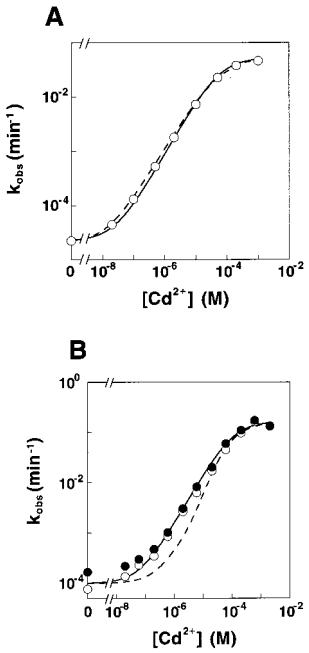


FIGURE 8: A titration experiment demonstrates that no tight binding metal ion is involved in the rescue. (A) The observed data for 50 nM S/N ribozyme and 80 pM thio-substrate (○) obtained in 50 mM BisTris-propane, pH 6.5, and 10 mM Mg^{2+} at 25 °C were fit by a single metal ion model (—; eq 2a). The dashed line shows an example of a two metal ion model (Scheme 3) with a tight binding site that can fit the observed data, with $K_{d1} = 1$ μM and $k_1/k_o = 50$ and $K_{d2} = 60$ μM and $k_2/k_o = 44$ and 80 pM E·S complex. (B) The Cd^{2+} concentration dependences for the reactions of S/N·S with 80 pM E·S (○) or 16 μM E·S (●, titration) (50 mM Tris-HCl, pH 7.5, and 10 mM Mg^{2+}, 25 °C). Using eq 2a, the observed data at both E·S concentrations were fit by a single metal ion model (solid line). The dashed line shows data simulated for independent binding and catalytic effects (Scheme 3, case IA) with $K_{d1} = 10$ μM and $k_1/k_o = 50$ and 16 μM E·S. Tighter binding or a smaller rate enhancement by the first metal ion would result in even larger deviations from the observed data (not shown).

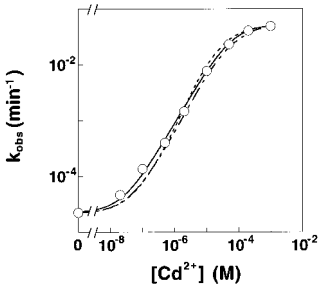


FIGURE 9: A model with two metal ions that have anticooperative effects in catalysis could fit the observed data.The observed data for S/N·S (○) from Figure 8A were fit to eq 2a (—). Simulated data for two metal ions that bind independently and have anticooperative effects in catalysis (case IC) with $K_{d1} = 10$ μM, $k_1/k_o = 200$, $K_{d2} = 60$ μM, $k_2/k_o = 100$ (— - —), and two metal ions that bind cooperatively and have anticooperative effects in catalysis (Scheme 3, case IIC) with $K_{d1} = 10$ μM, $k_1/k_o = 200$, $K_{d2} = 300$ μM, $k_2/k_o = 100$, and $m = 0.1$ (— —).

in the ground state and then provide additional data that suggest that the remaining model invoking cooperative ground-state binding also does not hold.

As described above, for two metal ions binding independently and giving anticooperative effects in catalysis (case IC), similar Cd^{2+} affinities between 10 and 100 μM are required to fit the observed data. If such a model were to account for the observed data, the second metal ion would be expected to be uncovered with substituted ribozymes that changed the affinity for the P9/G10.1 metal ion. For example, the large decrease in the affinity of this metal ion, e.g., by the 7-deaza substitution at P9/G10.1 would give a biphasic Cd^{2+} concentration dependence, with the effect from the second "hidden" metal ion giving a rate increase and plateau below 1 mM Cd^{2+} ($K_d^{Cd} \leq 100$ μM). Strengthening binding of the P9/G10.1 metal ion with the P9 phosphorothioate substitution might similarly deconvolute the single binding curve into a biphasic binding curve if there were a second Cd^{2+} involved in the rescue. However, the Cd^{2+} concentration dependences for the 7-deazaG10.1 and P9S ribozymes follow a single binding curve with a slope of 1.

The remaining two metal ion rescue model involves a metal ion that binds cooperatively with the metal ion at the P9/G10.1 site and has anticooperative effects in catalysis (case IIC). The interrelationship in binding between this putative metal ion and the P9/G10.1 metal ion would have been expected to be uncovered in the experiments with at least one of the abasic ribozymes. This is because ablation of at least one of the bases would have been expected to decrease the affinity of the second metal ion, and this in turn would also decrease the affinity for the P9/G10.1 metal ion, as the model requires cooperative binding of two rescuing metal ions in order to fit the data. As only base ablations at the P9/G10.1 site affected the Cd^{2+} affinity, the involvement of a second metal ion that binds cooperatively with the P9/G10.1 metal ion is unlikely.

In summary, under all conditions, with Ca^{2+} and Mg^{2+} backgrounds and with all the ribozymes investigated, all of the data are consistent with a single rescuing metal ion. The data strongly support the involvement of a single metal ion in the rescue of phosphorothioate substitutions at both the P9/G10.1 and the cleavage site. It should be recognized that, despite the strength of these results, a single rescuing metal ion is not proven by the data herein.

Core Functional Group Modifications Have Similar Effects in the Phosphate and Cd^{2+}-Rescued Phosphorothioate Reactions, Suggesting Analogous Reaction Mechanisms. To investigate whether the phosphate- and phosphorothioate-containing ribozyme·substrate complexes follow the same mechanism and involve the same molecular interactions, we compared the importance of functional groups on the ribozyme in the normal reaction with the importance of these groups for the Cd^{2+}-rescued reaction with phosphorothioates.

Figure 10 compares the effect of abasic substitutions on cleavage of the normal phosphate-containing substrate with the effect on cleavage of the R_P-phosphorothioate substrate in the presence of saturating Cd^{2+}. In both cases, large deleterious effects were observed for C3X, G5X, A6X, G12X, A13X, A14X, and A15.1X. The observed cleavage rates of $\sim 10^{-6}$ min^{-1} for the phosphate substrate in the absence of Cd^{2+} and $\sim 10^{-4}$ min^{-1} for the phosphorothioate substrate in the presence of 2 mM Cd^{2+} were similar to the background cleavage rates under these respective conditions, as indicated by the dashed lines in Figure 10. Similarly, the abasic ribozymes U4X, U7X, G10.1X, and C11.1X retained significant activity in both cases. Thus, with the exception of G8X and A9X, this comparison shows that the effects of base removal are analogous for reaction of the phosphate-containing substrate and reaction of the phosphorothioate substrate in the presence of Cd^{2+}.

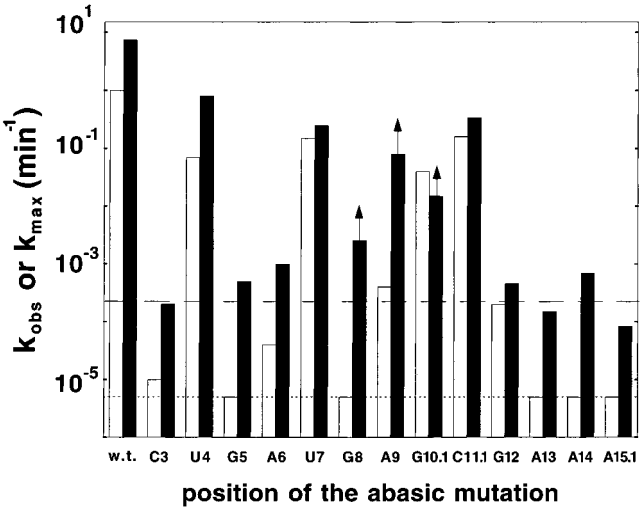


FIGURE 10: Comparison of the cleavage rates of abasic ribozymes with phosphate and phosphorothioate substrate bound. All reactions were carried out in 50 mM BisTris-propane, pH 7.5, 25 °C in 10 mM Mg^{2+} and varying concentrations of Cd^{2+}. k_{max} with phosphorothioate substrate in Mg^{2+} and saturating Cd^{2+} (except for cases noted with an arrow) is shown in the dark columns, the cleavage rate with phosphate substrate bound in Mg^{2+} in the absence of Cd^{2+} is shown in the light columns. Experiments with some of the faster reacting abasic ribozymes showed that with the phosphate substrate bound Cd^{2+} gives 5−10-fold rate enhancement, comparable to the wild-type ribozyme (O/N·O). However, the slow cleavage rates together with the small rate enhancement by Cd^{2+} prevented determination of reliable K_d^{Cd} values for these ribozymes so these data are not shown. The dashed line represents the uncatalyzed cleavage rate of the phosphorothioate substrate at 10 mM Mg^{2+} and 2 mM Cd^{2+}, pH 7.5, whereas the background rate for the phosphate substrate in the absence of Cd^{2+} is shown as the dotted line. The rates observed for the G8X, A9X and G10.1X ribozymes are lower limits, as they were not saturated with Cd^{2+}; this is depicted by the arrows. Representative Cd^{2+} concentration dependences are available as Supporting Information.

For the G8X and A9X ribozymes, the Cd^{2+}-rescued reaction rate with the phosphorothioate substrate is substantially greater than the reaction rate with the phosphate substrate. Thus, ablation of the G8 and A9 bases, which flank the phosphate ligand of the rescuing Cd^{2+}, enhances rescue. This enhanced rescue may arise because geometrical constraints on the binding site are relieved (see Discussion).

The base pairs flanking the cleavage site and the C at the cleavage site (position 17) were shown previously to be important for hammerhead catalysis (*28*−*30*). We tested the effect of base changes that abolished base pairing at these positions on Cd^{2+} rescue, again probing the correspondence of the Cd^{2+}-rescued reaction with the phosphorothioate substitutions to reaction of the phosphate-containing substrate and ribozyme. The Cd^{2+} concentration dependences of the observed reaction rate were obtained for the wild-type and P9S ribozyme with the normal substrate and with the G1.1U,

Table 3: Groups Required for the Wild-Type Hammerhead Reaction Are Also Required for the Cd^{2+}-Rescued Reaction with Phosphorothioates[a]

A

	k_{obs} (min^{-1})							
	cognate[b]		G1.1U		G16.2U		C17pyr	
P9/G10.1·P1.1	$-Cd^{2+}$	$+Cd^{2+}$	$-Cd^{2+}$	$+Cd^{2+}$	$-Cd^{2+}$	$+Cd^{2+}$	$-Cd^{2+}$	$+Cd^{2+}$
O/N·O	1.1	3.7	5.7×10^{-3}	2×10^{-2} [c]	1.8×10^{-2}	4.0×10^{-2}	1.6×10^{-3}	2.4×10^{-3}
O/N·S	8×10^{-4}	7.4	$<1 \times 10^{-5}$	$>1 \times 10^{-2}$ [d]	$<1 \times 10^{-5}$	2.4×10^{-2}	2×10^{-6}	1.9×10^{-3}
S/N·O	1×10^{-3}	4.3	2×10^{-5}	1.9×10^{-2}	1×10^{-4}	7.1×10^{-2}	5×10^{-6}	3.8×10^{-3}
S/N·S	1×10^{-4}	7.6×10^{-2}	$<1 \times 10^{-6}$	$>3 \times 10^{-4}$ [c]	$<1 \times 10^{-6}$	7.8×10^{-4}	$<1 \times 10^{-6}$	4.2×10^{-4}

B

	k_{rel}^{Cd} [e]		
P9/G10.1·P1.1	G1.1U	G16.2U	C17pyr
O/N·O	4×10^{-3}	1×10^{-2}	7×10^{-4}
O/N·S	$>2 \times 10^{-3}$	3×10^{-3}	3×10^{-4}
S/N·O	4×10^{-3}	2×10^{-2}	9×10^{-4}
S/N·S	4×10^{-3}	1×10^{-2}	6×10^{-3}

[a] Reactions at 25 °C in 50 mM BisTris-propane, pH 7.5, with 10 mM Mg^{2+}. Reactions with the phosphorothioates were carried out with the purified R_P-stereoisomer, except for G1.1U, for which the rate for the R_P-stereoisomer was obtained from reaction of the mixed isomers (see Experimental Procedures). Cd^{2+} (2 mM for O/N ribozymes or 500 μM for S/N ribozymes) was present for reactions designated by "+ Cd^{2+}". As K_d^{Cd} values are <350 and <80 μM for complexes with O/N and S/N ribozymes, respectively, these values are within 15% of k_{max} (see footnotes *c* and *d* for exceptions). [b] Cognate denotes that the sequence of the oligonucleotide substrate is unchanged; experiments with this substrate were performed at pH 6.5 and the rate extrapolated assuming a log-linear pH dependence to the rate expected at pH 7.5. [c] Plots of cleavage rate versus Cd^{2+} concentration exhibited a small amount of inhibition at 2 mM Cd^{2+} so that the highest observed rate is listed. Fits of the Cd^{2+} concentration dependencies suggest that these values are within 2-fold of the rate at saturating Cd^{2+}. [d] This rate is a lower limit obtained at 50 μM Cd^{2+}; the use of higher concentrations was prevented by inhibition. [e] $k_{rel}^{Cd} = k_{obs,modified}/k_{obs,cognate}$ in the presence of Cd^{2+}.

G16.2U, and C17pyr substrates, and each substrate was investigated with and without a phosphorothioate at the scissile bond (Table 3). The cleavage rates for the phosphate-containing ribozyme and substrate (Table 3A, O/N·O) agreed reasonably well with values obtained previously (*28*, *30*). Upon introduction of the phosphorothioate substitution at P1.1 (cleavage site) or P9, there was a large thio-effect for each of the mutant substrates, analogous to the effects for the substrate with the cognate sequence (Table 3A, O/N·O vs O/N·S and S/N·O, "$-Cd^{2+}$"). Further, saturating Cd^{2+} rescued the cleavage of the modified substrates when a phosphorothioate substitution was present at either the cleavage site or the P9/G10.1 site (Table 3A, O/N·S and S/N·O, "$-Cd^{2+}$" vs "$+Cd^{2+}$"). As for the substrate with the wild-type sequence, the Cd^{2+}-rescued rates for the modified substrates in the O/N·S and S/N·O complex were nearly the same as the cleavage rate for O/N·O in the presence of Cd^{2+} (Table 3A, O/N·O vs O/N·S and S/N·O, "$+Cd^{2+}$"). Table 3B summarizes the correspondence of the effects of the substrate modifications on the normal reaction (O/N·O) and the Cd^{2+}-rescued reaction with phosphorothioate substitutions. The values of k_{rel}^{Cd} represent the deleterious effect from these substrate modifications in the presence of saturating Cd^{2+}. The similarity of k_{rel}^{Cd} for the O/N·O and the phosphorothioate-containing complexes indicates that the cleavage site base and the base pairs flanking this site are of similar importance in the Cd^{2+}-rescued phosphorothioate reactions as in the normal reaction. The 10-fold smaller effect of the C17pyr modification for S/N·S may arise because of the limit imposed by the background cleavage rate.

In summary, the effects on the cleavage rate from removal of individual bases or functional groups correlate strongly in the phosphate and Cd^{2+}-rescued phosphorothioate reactions, suggesting that the Cd^{2+}-rescued reaction with phosphorothioates uses the same groups on the ribozyme that are required for the normal reaction with the wild-type substrate. Thus, the results suggest that the same catalytic interactions are made and, specifically, that a metal ion bridges the P9/G10.1 and the cleavage sites in the wild-type reaction, analogous to the Cd^{2+}-rescued phosphorothioate reaction.

Metal Ion Specificity at the P9/G10.1 Site. In a background of Ca^{2+} ions, the cleavage rate for the O/N·O complex is about 30-fold lower than in a Mg^{2+} background. Nevertheless, when Cd^{2+} is added at saturating concentrations, the cleavage rates are the same within error (Table 1). The results described above suggest that the Cd^{2+} ion responsible for this effect binds at the P9/G10.1 site. Thus, Cd^{2+} binding at the P9/G10.1 site appears to completely rescue the deleterious effect of the Ca^{2+} background.[4] This suggests that any other sites important for structure and catalysis do not differentiate between Mg^{2+} and Ca^{2+}. Furthermore, Cd^{2+} bound at the P9/G10.1 site appears to give a ~10-fold stimulation in a background of Mg^{2+} (Figure 2). These results suggest that the catalytic activity of the wild-type ribozyme changes with the metal ion bound at the P9/G10.1 site, following the order $Cd^{2+} > Mg^{2+} > Ca^{2+}$.

DISCUSSION

A Single Cd^{2+} Is Responsible for Rescue of the Phosphorothioate Substitutions at the P9/G10.1 and Cleavage Sites. All of the Cd^{2+} concentration dependences of the cleavage

[4] The different activity of the hammerhead ribozyme in 10 mM Mg^{2+} and 10 mM Ca^{2+} could also arise if the P9/G10.1 site was not saturated with Ca^{2+}. However, the 8−10-fold Cd^{2+} affinity change from 1 to 10 mM Mg^{2+} and Ca^{2+} suggests that the P9/G10.1 site is essentially saturated at 10 mM Mg^{2+} or Ca^{2+}. The more than 10-fold increase in the K_d^{Cd} value observed between 10 and 100 mM Mg^{2+} or Ca^{2+} (Table 2) could result from a general electrostatic effect from increased ionic strength or from competitive binding at a nearby low-affinity Mg^{2+} or Ca^{2+} site that precludes binding of Cd^{2+} to the P9/G10.1 site.

rates described herein, both in Mg^{2+} and Ca^{2+}, are consistent with the involvement of a single common Cd^{2+} ion in the rescue of the thio-effects at the P9/G10.1 and the cleavage site. The affinity of the Cd^{2+} ion rescuing the phosphorothioate substitution at the cleavage site changes with perturbations at the P9/G10.1 site but not at the cleavage site. This led to the model depicted in Figure 4, in which a single metal ion, bound at the P9/G10.1 site in the ground state, acquires the *pro*-R_p phosphoryl oxygen at the cleavage site as an additional ligand in the transition state. Thus, prior to the transition state the hammerhead ribozyme appears to undergo a global conformational change to bring the two sites from 20 Å away in the ground state to within 7 Å, allowing both sites to interact with a single Cd^{2+} ion in the active conformation and the transition state.

This model was tested in experiments using ribozyme variants containing base ablations or phosphorothioate substitutions throughout the hammerhead core. Effects on binding of the rescuing Cd^{2+} are localized to the P9/G10.1 site, with other sites having no significant effect. These results further support the involvement of a single Cd^{2+} ion that is bound at the P9/G10.1 site in the ground state.[5]

The potential of models with two rescuing metal ions to predict Cd^{2+} concentration dependences indistinguishable from those predicted for a single rescuing metal ion was also explored. Most of the alternative two metal ion models that could reasonably fit the observed data required tight binding of one of the rescuing Cd^{2+} ions, with $K_d^{Cd} \leq 10\ \mu M$. Such models were ruled out by a titration experiment carried out at a high concentration of the E·S complex. The only alternative class of models not directly ruled out requires the second metal ion to be anticooperative in catalysis; the effect of the two metal ions would be less than the product of the effects from each alone (Scheme 3, case C). If this class of models were to hold, then perturbing the metal ion binding sites would have been expected to affect the observed concentration dependence for Cd^{2+} rescue. However, the P9 phosphorothioate and the G10.1 7-deazaG substitutions strengthen and weaken the apparent affinity of the rescuing Cd^{2+} but do not uncover a second rescuing Cd^{2+} ion. Further, base deletions throughout the core, which would have likely perturbed the binding of a second rescuing Cd^{2+} ion, did not reveal such a site (see Results). Thus, the observation of concentration dependences reflecting a single Cd^{2+} ion, with all of the modified ribozymes under all conditions tested, suggests that a single, common Cd^{2+} ion is responsible for the rescue of the effects from phosphorothioate substitutions at the P9/G10.1 and cleavage sites.

Does the Cd^{2+}-Rescued Reaction with the Phosphorothioate Ribozyme and Substrate Reflect the Wild-Type Reaction? Whenever properties of a molecule are inferred from studies with its modified analogue, it is crucial to know whether the behavior of the variant reflects the behavior of the unmodified molecule of interest. This question is especially pertinent to the present study, because rescue of the S/N·S complex is incomplete, with $k_{S/N\cdot S}/k_{O/N\cdot O} \approx 0.01$ at saturating Cd^{2+}. The incomplete rescue highlights the following question: is there a metal ion bridging the P9/G10.1 site and the cleavage site in the normal reaction with wild-type E·S complex, or does Cd^{2+} simply exploit its high affinity toward sulfur to bridge these sites when phosphorothioates are present, allowing a reaction that is not analogous to the normal reaction? We first describe observations that suggest the existence of the bridging metal ion in the normal hammerhead reaction and then explain how geometrical constraints within the metal ion binding site of the active complex may result in the observed incomplete Cd^{2+} rescue.

The large stereospecific thio-effects at the P9/G10.1 and the cleavage site suggest that there are interactions with these oxygen atoms in the normal reaction that are compromised by replacement of oxygen with sulfur. The simplest interpretation of the substantial rescue by the thiophilic Cd^{2+} ion is then that these atoms interact with a metal ion in the normal reaction. It remains possible that the rescue could be indirectly mediated through the ribozyme if, as a result of rearrangements caused by the thio substitutions, the larger Cd^{2+} coincidentally were to fit better than Mg^{2+} in the active hammerhead conformation. Such a model would also be expected to require an active conformation that differs substantially from the observed ground-state structure, as the crystal structure shows no obvious connection between the P9/G10.1 and the cleavage site.

To test whether the rescue by a single bridging Cd^{2+} reflects the behavior of the normal reaction, we compared the molecular requirements for reactions of phosphate- and the Cd^{2+}-rescued phosphorothioate-containing ribozyme and substrate. The observed correlation of the effects on base removal and functional group modifications with phosphate versus Cd^{2+}-rescued phosphorothioate-containing ribozyme and substrate suggests that the catalytic conformations and reaction mechanisms are analogous and that the metal-bridged interaction between the P9/G10.1 and the cleavage site is made in both the phosphate and the Cd^{2+}-rescued phosphorothioate reactions.

The functional importance of a metal ion bound at the P9/G10.1 site for normal hammerhead catalysis is further supported by the differential ability of metal ions bound at that site to promote catalysis in the wild-type ribozyme reaction. The cleavage rate with different metal ions at the P9/G10.1 site appears to follow the order $Cd^{2+} > Mg^{2+} > Ca^{2+}$. Further, the model of Figure 4 can account for previous biochemical and structural data, including data that are difficult to reconcile without invoking a substantial conformational change (see the following section).

We now return to the question raised above. If the wild-type and Cd^{2+}-rescued reactions are indeed analogous, why then is Cd^{2+} rescue incomplete? According to the model of Figure 4, the cleavage rate with Cd^{2+} bound at the P9/G10.1 site and a phosphorothioate at the cleavage site (i.e., O/N·S) is expected to be higher than the rate with a phosphate at the cleavage site. This is because of the greater affinity of Cd^{2+} for sulfur than for oxygen (*26*, *27*) and because the Cd^{2+} phosphorothioate interaction is absent in the ground state but made in the transition state. However, the relative reaction rate of the O/N·S complex is about 1 ($k_{O/N\cdot S}/k_{O/N\cdot O} = 0.9$) with saturating Cd^{2+}. Thus, it appears that the ability

[5] A charge-transfer band from a phosphorothioate at the cleavage site to a bound Hg^{2+} ion has been observed by UV−vis spectroscopy (*31*). The extremely strong interaction between Hg^{2+} and sulfur may result in binding of Hg^{2+} to the cleavage site phosphorothioate in the ground state even in the absence of such a metal ion interaction in the ground state of the wild-type ribozyme·substrate complex.

of Cd^{2+} to promote catalysis is compromised with sulfur substitution at the cleavage site. In addition, the introduction of a second phosphorothioate (S/N•S) further reduces the relative rate about 100-fold, as noted above. A metal ion binding site within a folded RNA, especially one of functional importance, would be expected to have distinct geometrical constraints. Thus, the catalytic function of the hammerhead may be compromised by substitution of a Mg^{2+} ion with a larger Cd^{2+} ion [0.86 vs 1.09 Å diameter (*32*)] and substitution of oxygen atoms with larger sulfur atoms [1.40 vs 1.85 Å van der Waals radii, 1.51 vs 1.95 Å bond lengths for P−O and P−S bond in $H_2PO_4^-$ and $H_2PO_3S^-$, respectively (*33*, *34*)]. Steitz and co-workers have shown that the metal ion binding sites of the Klenow exonuclease are perturbed by thio-substitution of the 3′-bridging and the *pro*-S_P oxygens of the DNA substrate, substitutions that greatly decrease the cleavage activity (*35*, *36*).

Given the above, it is possible that disruption of the Cd^{2+} binding site could relax steric restrictions and allow Cd^{2+} bound at the P9/G10.1 site to take better advantage of its high intrinsic affinity for a sulfur at the cleavage site. Indeed, this has been observed for the G8X and A9X ribozymes, which lack bases in the vicinity of the P9/G10.1 site. In the presence of Cd^{2+}, the complexes of these ribozymes with phosphorothioate substrate react faster than the complexes with phosphate substrate. A similar result is observed with the S/C•S complex, in which the N_7 nitrogen ligand is removed (unpublished data).

The Catalytically Active Tertiary Structure of the Hammerhead Is Only Transiently Formed. Unlike protein catalysts, the hammerhead ribozyme has no active-site cleft lined with multiple catalytic groups to bind and activate the substrate. Instead, the scissile bond faces solution. The data presented herein suggest that the hammerhead ribozyme undergoes a large conformational change prior to catalysis. This rearrangement might then create an active-site pocket akin to the active sites found in protein and larger RNA enzymes (*37*−*39*). This poses the following question: how does the hammerhead conformation change to create an active site? On the basis of an analysis of the available functional data, we propose a crude, speculative model for this active conformation. It is suggested that domain I rearranges and docks onto a largely unperturbed domain II to give an interconnected core that forms a catalytic pocket occupied by the reactive phosphoryl group.

This model arises first because several groups in domain I of the hammerhead core that have been shown to be critical for function do not appear to be engaged in interactions in the ground-state X-ray structures (*6*). Removal of the base at G5, which is ∼10 Å away from the cleavage site, decreases the observed cleavage rate $\sim 10^5$-fold (*21*), and substituting this base with inosine, which lacks the exocyclic amino group, or 2-aminopurine, which lacks the carbonyl oxygen at C_6 and deprotonates N_1, each reduce the cleavage rate $\sim 10^2$-fold (*40*, *41*). However, in the crystal structures, these functional groups are not within hydrogen-bonding distance of other residues (*7*). Further, removal of the 2′-OH of G5 has a deleterious effect of >300-fold, even though its hydrogen-bonding partner, the 2′-OH at C15.2, can be removed without affecting catalysis (*29*, *42*). Similarly, the 200-fold deleterious effect from substituting C3 with 2-pyridinone (unpublished results) and additional functional studies (*22*) suggest that the complete base pairing face of C3 is important for catalysis, whereas only N_3 is engaged in an interaction in the crystal structures. Finally, as described by Baidya et al., the crystal structures do not readily account for the importance of functional groups of the cleavage site base, C17 (*30*).

The converse observation, that certain groups engaged in interactions in the crystal structures do not appear to be critical for function, is further consistent with the rearrangements proposed in the active structure. The U-turn in domain I appears to be stabilized by a hydrogen bond between the 2′-OH of U4 and the N_7 nitrogen of A6 (*7*). Nevertheless, removal of the 2′-OH of U4 has less than a 4-fold effect on catalysis (*43*, *44*), whereas there is a larger effect of 30−100-fold from conversion of A6 to its N7-deaza variant (*45*). Further, O_2 and N_3 of U7 hydrogen bonded to A14 and G8, respectively. However, U7 is not conserved and can be removed or replaced by other bases without a large loss of activity (*46*, *47*, *21*). Finally, although the base at U4 is involved in several interactions within the domain I U-turn, removal of this base has only a 15-fold effect on catalysis (refs *39* and *21*; see also ref *48*). The simplest interpretation of these observations is that in the active conformation domain I is essentially inverted, bringing G5, C3, and C17 into a network of interactions within the core and placing U4 and U7 on the periphery.

A corollary of the proposed conformational change of the hammerhead ribozyme is that the active structure is thermodynamically unstable and thus only transiently adopted. This lack of stability of the active structure appears to be rather general for small RNAs. The binding of peptides has been observed to change the structure of their RNA partners in several cases (*49*−*53*). For example, the structure of RRE and TAR RNA change upon binding of peptides from Rev and Tat, respectively. This behavior of small RNAs is in contrast to that of proteins, for which conformational rearrangements upon ligand binding are typically limited.

The different ability of RNA and protein to stabilize a unique tertiary structure might be due to the limited structural information in nucleic acids (*54*−*56*). First, nucleic acids are composed of four side chains instead of 20 side chains, and the nucleic acid side chains lack the differences in size, shape, polarity, and charge exhibited by protein side chains. Further, RNA can hydrogen bond to form nonstandard base pairs, so that multiple pairing alternatives exist (*57*). In contrast, proteins use electrostatic and hydrophobic interactions, in addition to hydrogen bonds, to create particular environments that recognize specific side chains while excluding side chains that do not match the local shapes and patterns of polar and hydrophobic groups. RNA's problem of limited information content is further exacerbated because secondary structure formation hides most side-chain functional groups within the helix, whereas helices and sheets in proteins expose the side chains for interaction with one another.

From the above considerations it is not unreasonable that a small catalytic RNA such as the hammerhead would require a conformational rearrangement to create an active-site pocket. Several other catalytic RNAs appear to have solved the problem of achieving a ground-state conformation that is close to the active one by utilizing additional stabilizing interactions. The HDV ribozyme uses extensive base pairing

within a highly constrained double pseudoknot to achieve a compact fold with an active-site crevice with just 72 residues, and the still larger group I ribozymes appear to employ peripheral elements to enforce cooperative folding to an active conformation that contains a largely preformed active site (*37*−*39*, *58*).

Previous Structural Work Is Consistent with a Large Conformational Change. Several observations from structural work on the hammerhead ribozyme have led to the suggestion that the ribozyme undergoes a limited conformational change prior to catalysis (5, *10*). However, as described below, a thorough analysis of these data suggests that this structural work is compatible with a large conformational change, as suggested by the results herein.

A crystallized hammerhead ribozyme·substrate complex was shown to cleave its substrate faster in the crystal than in solution, with rate constants of 0.4 and 0.08 min^{-1}, respectively [1.8 M Li_2SO_4 and 50 mM $CoCl_2$, pH 8.5 (*10*)]. Further, for a modified substrate, a modest rearrangement into a structure that allows in-line attack was observed (*10*). These results have led to the suggestion that the structure within the crystal closely approximates the active structure and that no large-scale rearrangement is required for efficient catalysis (*10*). Uhlenbeck and co-workers have compiled data for a large number of hammerhead constructs (*59*). Their results show near uniform cleavage rates for different hammerhead ribozymes, except for constructs that apparently exhibit alternative conformational states that interfere with catalysis (*23*, *59*−*62*). The construct used in the crystallographic study mentioned above appears to be such an aberrant hammerhead·substrate complex, reacting with a rate constant of only 0.04 min^{-1} at pH 8.5 in the presence of 10 mM Mg^{2+} (*10*), ∼100-fold slower than other hammerheads under these conditions [e.g., 3 min^{-1} (*63*)]. Indeed, HH16, the ribozyme used in the present study cleaves its substrate with a rate constant of >10 min^{-1} under the salt and pH conditions used for the kinetic experiment with the crystalline ribozyme·substrate complex (1.8 M Li_2SO_4 and 50 mM $CoCl_2$, pH 8.5, unpublished results). Lowering the pH to slow cleavage to a measurable rate allows extrapolation to a rate of ∼500 min^{-1} for solution cleavage under these conditions [from $k_{obs} = 5$ min^{-1} at pH 6.5 assuming a log−linear pH dependence (*63*), unpublished results]. It is possible that for the construct used for crystallography, cleavage in the crystal proceeds faster than in solution because the crystal stabilizes the normal ground-state conformation relative to the aberrant conformer [the crystal structure closely resembles the structure of the original construct which exhibits standard solution kinetics (*7*)]. The observed 10^3-fold slower cleavage rate in the crystal compared to the expected solution rate is consistent with the requirement of a substantial conformational change that is impeded or prevented by lattice contacts or the crystal environment. However, it should be recognized that the above arguments do not provide evidence for the substantial conformational change proposed herein, as many factors can affect reactivity within the crystal environment.

In the same study, a *talo*-5′-C-methyl-modification was introduced at the leaving group position of the hammerhead substrate in an attempt to populate a rearranged, active conformer for observation by X-ray crystallography (*10*). After soaking the crystals in 50 mM Co^{2+} at pH 8.5, a modest rearrangement of this ribozyme·substrate complex was observed which brought the cleavage site in a conformation consistent with in-line attack (*10*). However, the observed conformational change occurred much more slowly than the reaction with the normal substrate; it was not observed after a 30 min soak in the Co^{2+}/high pH buffer, but only after 2.5 h, whereas the wild-type ribozyme·substrate complex cleaved in the crystals with a half-time of ∼2 min (*10*). Thus, there is no evidence that the normal reaction occurs from this slowly attained conformation. Further, it was assumed that the methyl substitution decreases the intrinsic reactivity of the substrate, allowing accumulation of the complex in its active conformation (*10*). Whereas such a strategy can be successful for an intermediate that is thermodynamically stable but kinetically labile (*64*, *65*), the thermodynamically *unstable* active hammerhead conformer is not predicted to accumulate upon decreasing the intrinsic reactivity (i.e., increasing the free-energy barrier for reaction *subsequent* to formation of the intermediate). Thus, the modification is not predicted to change the fraction of molecules in the rearranged conformation but rather decreases the fraction of molecules that partition to react from this active conformation rather than revert to the stable ground-state structure. Finally, there is no general expectation that complexes that accumulate under perturbed conditions represent the active species.

Ribozyme·substrate complexes with cross-links between C2.6 in helix I and L2.4 in the loop closing helix II remained active, whereas complexes with cross-links between the 2′-OH of C2.1 in helix I and C11.2 or C10.4 in helix II were inactive (*66*). These results have been interpreted as evidence against a requirement for a large conformational change to attain the active structure from the crystal structure (e.g., *5*, *66*). However, the inactive cross-linked species could be inactive because the cross-links constrain the helices in a geometry incompatible with the active conformation, as suggested previously, or because these cross-linkers sterically interfere with interactions adopted in the active conformation. The latter alternative may be especially pertinent in this case because both inactive cross-links involved residue C2.1, which adjoins the cleavage site and is involved in a base pair that provides a substantial contribution to catalysis (refs *28* and *29*; see also ref *67*). It should also be recognized that the active cross-linked species does not specify the distance between helices I and II, but rather places an upper limit of ∼16 Å on this distance. Substantial rearrangements from the observed crystal structures remain possible.

Are There Catalytically Important Divalent Metal Ions in the Hammerhead Ribozyme Reaction? In solutions containing very high concentrations of monovalent ions and no divalent metal ions, the hammerhead ribozyme is only 50-fold less active than under standard conditions with divalent metal ions (*68*). From this, it was inferred that divalent metal ions might only be needed for folding. However, it is possible that at such high ionic strength additional interactions formed in the active conformation are strengthened sufficiently so that the interaction between the P9/G10.1 and the cleavage site is dispensable. It is also possible that, at such high concentrations, a monovalent metal ion at the P9/G10.1 and other sites could perform the function of a Mg^{2+} ion that is normally present. Alternatively, the lower reactivity in monovalents could reflect the absence of a functional Mg^{2+}

interaction. Further experiments will be required to address these questions.

Roles for Metal Ions in the Hammerhead-Catalyzed Cleavage Reaction. Potential roles for metal ions in catalyzing phosphodiester cleavage have been discussed (*12*, *69*, *70*). The involvement of such metal ions in hammerhead catalysis has been proposed, and the number of metal ions involved in the reaction has been the subject of considerable controversy (e.g., *63*, *71*−*73*). The data shown herein strongly suggest that only a single metal ion is involved in the rescue of the thio-effects at the P9/G10.1 and the cleavage sites. The main role of this metal ion presumably is to stabilize the rearranged conformation. It could also stabilize negative charge that may develop on the nonbridging oxygen in the transition state. It is not known if this metal ion performs additional roles in catalysis such as aiding the deprotonation of the 2′-hydroxyl group or stabilizing the development of negative charge on the 5′-leaving group oxygen atom. It should be emphasized that the results herein provide no information about the presence or absence of additional catalytically important metal ions. There can be sites to which Cd^{2+} does not bind under the conditions of the experiments carried out herein or sites that give equal reactivity whether Cd^{2+}, Mg^{2+}, or Ca^{2+} is bound. We are aware of no unambiguous experimental data that distinguish whether additional metal ions are involved in the hammerhead reaction.

Summary and Implications. The data presented herein suggest that the metal ion bound at the P9/G10.1 site of the hammerhead ribozyme bridges this site and the cleavage site, thereby stabilizing the catalytically active structure. This observation suggests a substantial rearrangement of the conserved core in going from the stable ground-state conformation to a transiently adopted active conformation. The lack of thermodynamic stability observed here for the hammerhead active structure may represent a general feature of small RNAs, attributed to structural limitations of nucleic acids.

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SUPPORTING INFORMATION AVAILABLE

The remaining Cd^{2+} concentration dependences in 10 mM Mg^{2+} or Ca^{2+} from which the Cd^{2+} affinities in Table 2 were obtained, as well as representative plots of Cd^{2+} concentration dependences for abasic ribozymes are available as Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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