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Involvement of a Specific Metal Ion in the Transition of the Hammerhead Ribozyme to Its Catalytic Conformation*

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Previous crystallographic and biochemical studies of the hammerhead ribozyme suggest that a metal ion is ligated by the *pro*-R_p oxygen of phosphate 9 and by N_7 of G10.1 and has a functional role in the cleavage reaction. We have tested this model by examining the cleavage properties of a hammerhead containing a unique phosphorothioate at position 9. The R_p-, but not S_p-, phosphorothioate reduces the cleavage rate by 10^3-fold, and the rate can be fully restored by addition of low concentrations of Cd^{2+}, a thiophilic metal ion. These results strongly suggest that this bound metal ion is critical for catalysis, despite its location ~20 Å from the cleavage site in the crystal structure. Analysis of the concentration dependence suggests that Cd^{2+} binds with a K_d of 25 μM in the ground state and a K_d of 2.5 nM in the transition state. The much stronger transition state binding suggests that the P9 metal ion adopts at least one additional ligand in the transition state and that this metal ion may participate in a large scale conformational change that precedes hammerhead cleavage.

The catalytic cleavage of an RNA phosphodiester bond to a 2′,3′-cyclic phosphate by the hammerhead ribozyme requires the participation of divalent metal ions. McKay and co-workers (1) observed a single bound metal ion when Mn^{2+} or Cd^{2+} was soaked into hammerhead crystals, with the metal ion in close proximity to the *pro*-R_p-oxygen of the P9 phosphate and N_7 of the guanine base at position 10.1 (Fig. 1). Previous biochemical experiments suggested that both of these groups were important in hammerhead cleavage: the P9 *pro*-R_p-oxygen was identified in phosphorothioate interference experiments (2, 3), and a role for a purine at position 10.1 was implicated in nucleotide substitution experiments (4, 5). More recently, the N_7 of this purine was implicated by the ability of guanine, but not 7-deazaguanine, to efficiently rescue the activity of a ribozyme with an abasic nucleotide at position 10.1 (6).

These results, taken together, support a model in which binding of a metal ion to the P9 *pro*-R_p-oxygen and the N_7 of G10.1 affects catalysis. However, the metal ion site identified in the crystal structure is ~20 Å from the cleavage site phosphodiester, with no obvious connection to this site. In addition, there are no data directly demonstrating a functional role for the structurally identified metal ion, nor are there quantitative data that indicate how important this metal ion might be for catalysis. Finally, coordination of a metal ion to the *pro*-S_p-oxygen of P9 rather than the *pro*-R_p-oxygen was suggested from crystallographic data with a different ribozyme construct (7).

We have therefore tested this model and quantitated the functional consequences of perturbing this site by substituting the *pro*-R_p- and *pro*-S_p-phosphoryl oxygen atoms at position P9 with sulfur and following catalysis in the presence and absence of Cd^{2+}, a thiophilic metal ion. The results provide strong support for the model and indicate that a metal ion coordinated at the *pro*-R_p position is critical for efficient catalysis.

EXPERIMENTAL PROCEDURES

Materials

The ribozymes and substrates were prepared by solid phase synthesis (8). Variants of each ribozyme containing a phosphorothioate in position 9 were produced by published sulfurization methods (9), which result in a nearly racemic mixture of R_P and S_P diastereomers (10). For HHα1 (Scheme 1), the two isomers of the P9 phosphorothioate (referred to as thio-P9$_{Rp}$ and thio-P9$_{Sp}$) were separated by reverse phase HPLC[1] (11). When each 5′-^{32}P-labeled oligonucleotide was digested with snake venom phosphodiesterase, a 12-nucleotide species accumulated, consistent with a phosphorothioate at position 9. Furthermore, the 12-nucleotide species from the second HPLC peak was cleaved more slowly than that from the first peak, suggesting that the second peak is the S_P-phosphorothioate isomer (12). For HH16 (Scheme 1), the ribozyme length (38 nucleotides) prevented efficient large scale separation of the thio-isomers.

Substrates were 5′-end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase and purified by nondenaturing polyacrylamide gel electrophoresis. Oligonucleotide concentrations were determined using specific activities for radioactive RNAs and assuming a residue extinction coefficient of 8.5×10^3 M^{-1} for nonradioactive RNAs.

$MgCl_2$ and $CdCl_2$ (>99.99%) were purchased from Aldrich. Buffers were from Sigma (molecular biology grade). $CdCl_2$ solutions were used immediately after preparation or were made as concentrated, acidic stocks (pH 2) and diluted into buffer immediately prior to use.

Methods

General Kinetic Methods—All reactions were single turnover and were carried out with ribozyme in excess of 5′-end-labeled substrate at 25 °C, in 50 mM buffer (pH 6.5) (PIPES·Na for experiments with HHα1 and BisTris-propane·HCl for experiments with HH16) and 10 mM $MgCl_2$, unless otherwise indicated. The reaction protocols were essentially as described previously (13, 14). Ribozyme and substrate were annealed prior to initiating reactions by the addition of divalent metal ions. Control reactions varying the final concentration of ribozyme indicated that the substrate was completely bound in all cases. Data

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[1] The abbreviations used are: HPLC, high performance liquid chromatography; PIPES, 1,4-piperazinediethanesulfonic acid; BisTris-propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane.

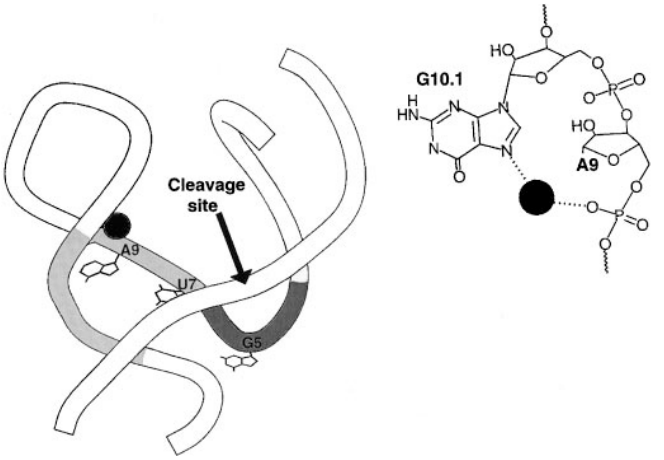
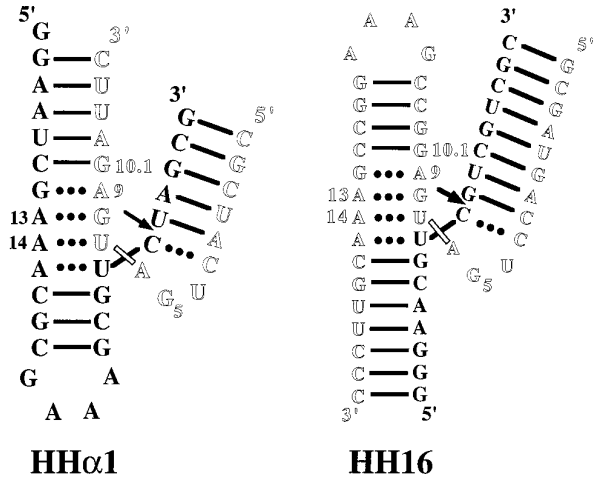


FIG. 1. **Metal ion binding site in the hammerhead ribozyme.** Schematic drawing of the three-dimensional structure of the hammerhead ribozyme (after (1)). The metal bound near position P9 is shown as a black sphere, and the *inset* shows the ribozyme ligands that coordinate this metal ion, N_7 of G10.1 and the *pro-*R_p-phosphoryl oxygen of P9 in the McKay structure (1). Residues referred to in the text are numbered according to the standard hammerhead nomenclature (31). Domain I of the conserved core is shown in *dark gray* and domain II in *light gray*.



SCHEME 1. **Hammerhead ribozymes.**

were fit to the appropriate kinetic equation using KaleidaGraph (Synergy Software) or SigmaPlot (Jandel Scientific) and gave fits with $R^2 > 0.99$ in all cases. Values of k_2 varied <25% between independent experiments.

Reactions with the HH16 Thio-P9$_{Rp}$ and Thio-P9$_{Sp}$ Substitutions—Reactions of the HH16 construct containing a phosphorothioate in position 9 yielded biphasic reaction time courses, with each phase corresponding to about half of the total reaction. These time courses were fit to the sum of two independent exponentials, giving independent k_2 values for each phase. The fast phase, characterized by a k_2 value nearly identical to that of the unmodified ribozyme, was attributed to the thio-P9$_{Sp}$ isomer, and the slower phase was attributed to the thio-P9$_{Rp}$ isomer based on the results with the resolved HHα1 thio-isomers. The rates and relative amplitudes of the two phases did not change when the annealed ribozyme-substrate complex was diluted by 150-fold (decreasing the ribozyme concentration from 600 to 4 nM) immediately after starting the reaction, arguing against kinetic complexities arising from multimeric ribozyme complexes. Also, the reaction time course did not change when the ribozyme-substrate complex was diluted and chased at the start of the reaction with a large molar excess of an HH16 variant (with G5 replaced by an abasic residue) that binds substrate normally but does not react (6).[2] This suggests that there is no dissociation of the pre-annealed ribozyme-substrate complex even over the longest time courses (48–96 h). Each phase of the time course was ~10-fold faster at pH 7.5 than at pH 6.5, as expected if each process were limited by the chemical step (15). Finally, purification of this phosphorothioate-substituted HH16 by anion exchange HPLC (8) resulted in partial separation of ribozyme forms such that the two phases had identical rate constants to those observed in the racemic mixture but different relative amplitudes (one fraction gave 0.8 of the fast component and 0.2 of the slow, whereas a second fraction gave 0.2 of the fast and 0.8 of the slow).

Rates and relative amplitudes of the two phases for reactions in 10 mM Mg^{2+} did not change upon addition of 0.2 mM EDTA or 2 mM dithiothreitol to the reaction mixture, suggesting that neither kinetic process depended on the presence of contaminating metal ions. In reactions with added Cd^{2+}, the concentration of EDTA carried over from the ribozyme and substrate stocks was <15 nM.

RESULTS

We have used two different hammerhead ribozyme constructs, HHα1 and HH16 (Scheme 1), in testing the role and importance of the metal ion identified in the x-ray crystallographic structure. Each of these ribozymes is kinetically and thermodynamically well characterized (13, 14), allowing the chemical cleavage step to be followed. In addition to the added confidence provided by obtaining parallel results with different constructs, specific attributes of each ribozyme were exploited in the experiments described below.

An R_p-phosphorothioate at Position 9 Substantially Reduces Catalysis—The R_P- and S_P-phosphorothioate isomers at position 9 formed during solid phase synthesis of HHα1 (referred to as thio-P9$_{Rp}$ and thio-P9$_{Sp}$ ribozyme, respectively) could be separated by HPLC to give fractions with high enrichment of each isomer. In single turnover reactions with saturating ribozyme, cleavage by the thio-P9$_{Sp}$ ribozyme proceeded at the same rate, within error, as that of the unmodified ribozyme (k_2 = 0.7 ± 0.1 min^{-1}; 10 mM Mg^{2+}, 25 °C (pH 6.5)). In contrast, catalysis by the thio-P9$_{Rp}$ ribozyme was substantially reduced, with an observed cleavage rate constant of k_2 = 0.028 min^{-1}. This supports the previous qualitative observations of compromised ribozyme function upon substitution of an R_P-phosphorothioate at position P9 (2, 3) and is consistent with a functional interaction with the *pro-*R_p-, but not *pro-*S_p-, oxygen at position P9.

The P9 R_p-phosphorothioate Slows the Chemical Step by 10^3-Fold—The observed rate decrease of 25-fold upon substitution of the P9 *pro-*R_P-oxygen of HHα1 with sulfur represents a lower limit for the effect of this change on the chemical step. This limit arises because the ribozyme preparation could contain a small amount of phosphate or S_P-phosphorothioate contaminant. Because dissociation of bound substrate from HHα1 is fast on the time scale of the reaction (k_{off}^{S} = 0.4 min^{-1}; (14)), the substrate can exchange between different ribozyme molecules, even in a single turnover experiment performed with saturating concentrations of ribozyme. A thio-P9$_{Rp}$ preparation contaminated with only 4% of unmodified or thio-P9$_{Sp}$ ribozyme would show a 25-fold rate decrease, even if the thio-P9$_{Rp}$ ribozyme was completely inactive. Consistent with this possibility, lowering the temperature to slow the exchange of substrate between different ribozymes gave a much larger observed effect from the thio-P9$_{Rp}$ substitution (≥500-fold at 4 °C; data not shown).

To circumvent this problem, we determined the thio effect using a different hammerhead construct, HH16 (Scheme 1). Substrate dissociation is immeasurably slow for this ribozyme, with a calculated $t_{½}$ of ~10,000 years (13). Because exchange does not occur on the time scale of the reaction, each substrate molecule is cleaved by whichever ribozyme it initially binds, the thio-P9$_{Sp}$ or thio-P9$_{Rp}$. Single turnover substrate cleavage should therefore occur in two independent phases, each corresponding to the reaction of one isomer population. As expected,

[2] A. Peracchi, L. Beigelman, E. C. Scott, O. C. Uhlenbeck, and D. Herschlag, unpublished results.

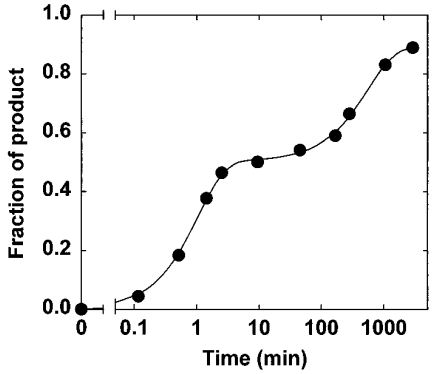


FIG. 2. **Two reaction phases are observed for cleavage by a mixture of the thio-P9$_{Sp}$ and thio-P9$_{Rp}$ HH16 ribozymes (0.6 μM HH16 with 0.1 nM substrate; 10 mM $MgCl_2$, BisTris propane (pH 7.5), 25 °C).** Time is plotted on a logarithmic scale to allow both reaction phases to be viewed. The line represents a nonlinear least squares fit to the sum of two exponentials, representing two simultaneous independent first-order reactions, and gives k_2 = 1.2 min^{-1} and 2.5 × 10^{-3} min^{-1}, with 0.52 and 0.39 of the substrate reacting in the fast and slow phase, respectively.

two separate kinetic phases, each corresponding to reaction of about half of the substrate, were observed (Fig. 2). The first phase occurs at essentially the same rate as the wild type reaction (Table I) and was assigned as reaction of the thio-P9$_{Sp}$ ribozyme, based on the results with the defined isomers of HHα1 described above. The slow phase was similarly assigned as reaction of the thio-P9$_{Rp}$ ribozyme. (Control experiments supporting this interpretation are described under "Methods.") The cleavage rate for the slow, thio-P9$_{Rp}$ isomer is 500-fold slower than that for the unmodified ribozyme (Table I). Thus, the effect of this single atom substitution is much larger than was determined using HHα1 under the same conditions. Indeed, it was this paradoxical result that led us to propose the fast exchange model for HHα1 and test it at low temperature, as described above. The results indicate that catalysis by both ribozymes is greatly compromised by this thio substitution.

The Deleterious Effect of the P9 R_p-phosphorothioate Is Fully Rescued by a Thiophilic Metal Ion—The large deleterious effect of the R_P-phosphorothioate at position 9 was obtained in reactions carried out in the presence of Mg^{2+}, which is a "hard" divalent metal ion having a low affinity for sulfur (16–18). To determine if the slow reaction of the thio-P9$_{Rp}$ ribozyme resulted from loss of a metal ion bound at this site, reactions were carried out with low concentrations of Cd^{2+}, a strongly thiophilic metal ion, and with 10 mM Mg^{2+} present to minimize the effect of Cd^{2+} at other sites. As shown in Table I, the addition of 100 μM Cd^{2+} increased the cleavage by the thio-P9$_{Rp}$ ribozyme over 10^4-fold, while having only ~5-fold effects on the unmodified and thio-A9$_{Sp}$ ribozymes. In addition, in the presence of Cd^{2+}, the thio-P9$_{Rp}$ ribozyme reacts at nearly the same rate as the unmodified and thio-P9$_{Sp}$ ribozymes. Thus, the "rescue" is complete. Similar results were obtained with HHα1 (data not shown). Analogous metal ion rescue has been observed with protein enzymes and other ribozymes (*e.g.* Refs. 16 and 19–24). The ability to observe efficient rescue strongly suggests that the deleterious effect from the P9 R_P-phosphorothioate arose from disruption of a Mg^{2+} site and indicates that this metal ion is of critical importance for hammerhead function.[3]

TABLE I

Effect of phosphorothioate substitution at position 9 on the hammerhead cleavage reaction in the presence and absence of Cd^{2+}

Single-turnover reactions with HH16 in 50 mM BisTris-propane·HCl (pH 6.5), 25 °C.

Metal ions	k_2		
	Unmodified	Thio-P9$_{Sp}$	Thio-P9$_{Rp}$
		min^{-1}	
10 mM Mg^{2+}	0.07	0.06	1.4×10^{-4}
10 mM Mg^{2+} + 100 μM Cd^{2+}	0.4	0.3	1.2
Ratio	6	5	8500

The Affinity of the P9 Metal in the Ground State and in the Transition State—The dependence of the cleavage rate for the thio-P9$_{Rp}$ and wild type ribozyme was measured as a function of [Cd^{2+}] to determine the apparent metal ion affinity for the P9 site (Fig. 3*A*). The marked dependence for the thio-P9$_{Rp}$ ribozyme and the shallow dependence for the wild type and thio-P9$_{Sp}$ ribozymes (Fig. 3*A* and data not shown) indicate that this large effect is specific for the P9 R_P-thio-isomer. The Cd^{2+} concentration dependence of $k_{2(obs)}$ for the thio-P9$_{Rp}$ ribozyme suggests that binding of a single Cd^{2+}, which has an equilibrium constant for dissociation from the ribozyme-substrate complex of K_d = 25 μM, is responsible for increasing the activity by ~10^4-fold.[4]

The association of this metal ion and its rate effects are summarized in Fig. 3*B*. Transition states can be considered as if they were species in equilibrium with ground states, according to transition state theory. This allows the Cd^{2+} affinity of the transition state to be calculated: the 10^4-fold faster reaction with Cd^{2+} bound indicates that Cd^{2+} binds 10^4-fold stronger to the transition state than to the ground state, corresponding to a dissociation constant, $K_d^{\ddagger}$, of 2.5 nM.

DISCUSSION

The functional importance of a distinct metal ion observed in the x-ray crystallographic structure of the hammerhead ribozyme (Fig. 1*A*; Ref. 1) has been tested. There is a large deleterious effect from substituting the *pro-*R_p-phosphoryl oxygen at position 9 with sulfur for reactions carried out in Mg^{2+} alone, and small amounts of Cd^{2+}, a thiophilic metal ion, restore the activity to unmodified levels. These observations provide strong support for an important functional role of this metal ion. Consistent with this interpretation, modification of G10.1, which contains the other ligand observed in the structure, decreases the ability of Cd^{2+} to rescue the deleterious effect of the thio substitution at P9.[5]

The rate decreases by 10^3-fold upon replacing the P9 *pro-*R_p-oxygen with sulfur when Mg^{2+} is the only divalent metal ion. This corresponds to a loss of 4 kcal/mol in transition state

[3] While this manuscript was in preparation, Knoll *et al.* (25) reported that a hammerhead containing the thio-P9$_{Rp}$-phosphoryl group cleaved 10-fold slower than the corresponding unmodified ribozyme and showed that this deleterious effect could be rescued in 2–3 mM Cd^{2+}. The small effect relative to that observed herein could reflect exchange during the reaction, as observed with HHα1, that leads to an overestimate of the cleavage rate of the thio-P9$_{Rp}$ ribozyme. In addition, it is not known if the chemical step is rate-limiting for the three-part hammerhead used by Knoll *et al.* (25). Kinetic analysis could be further complicated because one of the oligonucleotides of the three-part ribozyme can adopt alternative structures (14, 26) and because Cd^{2+} may have solubility problems at concentrations of 2–3 mM at pH 8 (27).

[4] The data of Fig. 3*A* also suggest that binding of Cd^{2+} to a single site on the unmodified ribozyme increases the cleavage rate, but with a 10-fold lower affinity and a 10^3-fold smaller rate enhancement than observed with the thio-P9$_{Rp}$ ribozyme. Preliminary results suggest that the same metal ion binding site is responsible (A. Peracchi, S. Wang, L. Beigelman, and D. Herschlag, unpublished results). The full observed rate enhancement from Cd^{2+} addition to the thio-P9$_{Rp}$ ribozyme is therefore used in Fig. 3*B* to calculate the affinity of the Cd^{2+} for the P9 site in the transition state ($K_d^{Cd\ddagger}$).

[5] A. Peracchi, L. Beigelman, and D. Herschlag, unpublished results.

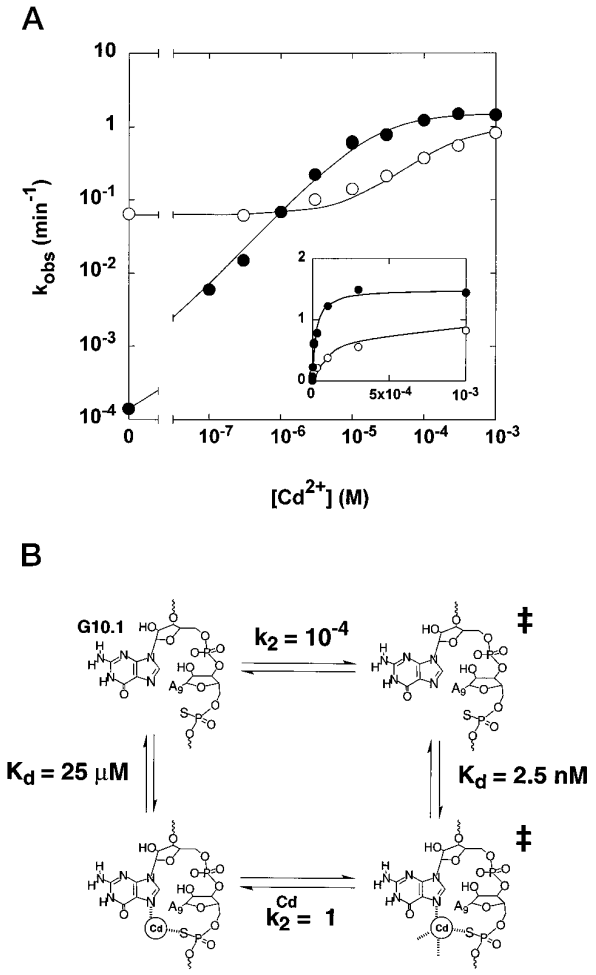


FIG. 3. **Concentration dependence for Cd^{2+} rescue.** *A*, effect of Cd^{2+} on cleavage by the thio-P9$_{Rp}$ (●) and unmodified (○) ribozymes. Single turnover reactions with saturating ribozyme in BisTris-propane (pH 6.5), and 10 mM $MgCl_2$, with varying concentrations of added Cd^{2+}. The *lines* represent nonlinear least squares fits to the data to a simple binding isotherm and give apparent dissociation constants of K_d^{Cd} = 25 and 220 μM for the thio-P9$_{Rp}$ and unmodified ribozymes, respectively. The logarithmic scales are used to show the large effect of Cd^{2+} on the thio-P9$_{Rp}$ ribozyme reaction; the *inset* shows the same data plotted on a linear scale. *B*, thermodynamic cycle depicting the effect of Cd^{2+} on the thio-P9$_{Rp}$ ribozyme. The equilibrium constant for dissociation of Cd^{2+} from the ribozyme-substrate complex in the ground state, K_d^{Cd}, combined with the relative rate constant for cleavage in the presence and absence of bound Cd^{2+}, allow calculation of the dissociation constant for Cd^{2+} in the transition state ($K_d^{Cd‡}$) according to transition state theory: $K_d^{Cd‡} = K_d^{Cd}\cdot(k_2/k_2^{Cd})$. (See also Footnotes 4 and 6). The additional *dashed lines* in to Cd^{2+} in the transition state represent the model described in the text in which there is one or more additional ligands in the transition state.

stabilization upon loss of a bound metal ion,[6] rivaling the effects from nucleotide substitution or excision of bases from the conserved hammerhead core (6, 28).

The P9 metal ion plays a critical role in hammerhead catalysis, despite its large distance from the labile phosphodiester group in the ground state. The ground state Cd^{2+} affinity of 25 μM is similar to that for adenosine 5′-*O*-thiomonophosphate (K_d = 24 μM (18)). The properties of this metal binding site change dramatically during catalysis. In the transition state, there is a 10^4-fold increase in Cd^{2+} affinity, relative to the ground state (Fig. 3*B*), corresponding to an additional 5.3 kcal/mol of binding free energy. This large increase suggests that there is at least one additional Cd^{2+} ligand in the transition state. For comparison, the additional carboxylate ligand of nitrilotriacetate relative to iminodiacetate increases Cd^{2+} affinity by 5.6 kcal/mol (K_d = $10^{-9.5}$ and $10^{-5.4}$ M, respectively (29)). An alternative model in which the Cd^{2+} ligands are better positioned in the transition state than in the ground state cannot be ruled out; however, this model would require the observed low nanomolar transition state affinity to be achieved with coordination by only two ligands, the sulfur at P9 and N_7 at G10.1.

How can this metal ion, which is ~20 Å from the reactive phosphoryl group in the hammerhead crystal structures (1, 7, 30), exert such a large effect, and what could an additional ligand(s) be? McKay pointed out that the ground state complex observed by crystallography would have to rearrange prior to cleavage to allow an in-line attack and also noted that the observed structure and structural variants with modest conformational rearrangements could not readily account for catalytic interactions or for roles of substituents that had been shown to be functionally important (1, 28). For example, the base of G5 is critical for catalysis, yet it engages in no interactions with the rest of the ribozyme (Fig. 1*A*). These observations suggest that a large scale conformational rearrangement may be required prior to cleavage. Such a conformational rearrangement could allow formation of additional transition state interaction(s) of the metal ion at P9 and could account for the importance of this metal ion in catalysis.[7]

We present the following speculative model for this conformational transition as a starting point for future discussions. We suggest that domain I rearranges and docks onto the major groove face of domain II. Several functional groups that are important for catalysis are located on the major groove face of domain II. In addition, the widened major groove face of this domain includes the *pro*-R_p-oxygens 5′ of P13 and P14, which are important in the transition state (3)[8] but appear to lack contacts in the ground state structure. The substantial network of interactions in domain II and the maintenance of the metal ion binding site at P9 and G10.1 in the transition state are consistent with domain II remaining largely unaltered in the transition state and serving as a "receptor" for domain I. A substantial rearrangement of domain I upon docking could account for the critical catalytic importance of functional groups such as those on G5 that do not make extensive ground state interactions. Finally, we suggest that the core is more packed in the active conformation with extensive interconnections between the conserved residues, consistent with the deleterious effects from removal of individual bases or 2′-hydroxyl groups that are large relative to overall catalysis (6, 28).[9]

In summary, a large scale conformational rearrangement may be required for the hammerhead to adopt its catalytic

[6] Experiments with varying concentrations of both Mg^{2+} and Cd^{2+} indicate that this site is not significantly occupied or blocked by the 10 mM Mg^{2+} used in the experiments herein (A. Peracchi, L. Beigelman, and D. Herschlag, unpublished results). Nevertheless, the slow reaction of the thio-P9$_{Rp}$ ribozyme in the absence of Cd^{2+} could arise from a small fraction of ribozyme with Mg^{2+} bound at this site. Thus, the value of k_2 in Fig. 3*B* is an upper limit, and correspondingly, the binding of Cd^{2+} to the transition state could be stronger than the value of $K_d^{Cd‡}$ = 2.5 nM calculated in Fig. 3*B*.

[7] In contrast, it has recently been suggested, based on structures of rapidly frozen ribozyme-substrate complexes, that only a small conformational rearrangement is required to achieve the catalytic conformation (30). However, since the crystals become disordered upon cleavage, the rearrangements observed in the crystals may not be on a reaction path that leads to cleavage; an off-pathway structure could also account for the very slow cleavage rate observed in the crystals. It is also possible that the observed structure represents an early intermediate that is on the reaction pathway but still differs substantially from the transition state structure.

[8] E. C. Scott and O. C. Uhlenbeck, unpublished results.

[9] A. Peracchi, L. Beigelman, and D. Herschlag, manuscript in preparation.

conformation. Subsequent to this conformational change, does the P9 metal ion interact directly at the cleavage site or does it exert its effect indirectly through the folded structure? Establishing the identity of the additional ligand to the metal ion bound at P9 would provide a test of the proposed model and would provide an important constraint for the active conformation of the hammerhead ribozyme.

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