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Thermodynamic Evidence for Negative Charge Stabilization by a Catalytic Metal Ion within an RNA Active Site

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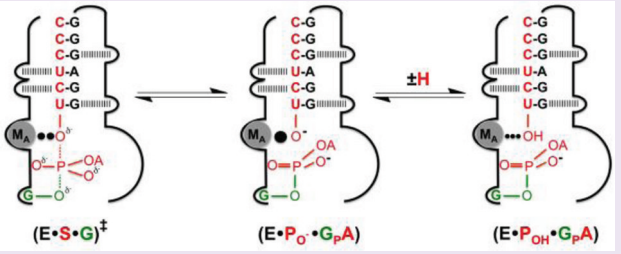
Supporting Information

ABSTRACT: Protein and RNA enzymes that catalyze phosphoryl transfer reactions frequently contain active site metal ions that interact with the nucleophile and leaving group. Mechanistic models generally hinge upon the assumption that the metal ions stabilize negative charge buildup along the reaction coordinate. However, experimental data that test this assumption directly remain difficult to acquire. We have used an RNA substrate bearing a 3′-thiol group to investigate the energetics of a metal ion interaction directly relevant to transition state stabilization in the *Tetrahymena* group I ribozyme reaction. Our results show that this interaction lowers the pK_a of the 3′-thiol by 2.6 units, stabilizing the bound 3′-thiolate by 3.6 kcal/mol. These data, combined with prior studies, provide strong evidence that this metal ion interaction facilitates the forward reaction by stabilization of negative charge buildup on the leaving group 3′-oxygen and facilitates the reverse reaction by deprotonation and activation of the nucleophilic 3′-hydroxyl group.



Phosphoryl transfer reactions are central to the performance and regulation of essentially every cellular process, and many protein and RNA enzymes mediating these reactions employ metal ions in catalysis. In their classic review, Benkovic and Schray envisaged a number of roles for catalytic metal ions in biological phosphoryl transfer, including (a) serving as a template for organizing the active site and orienting substrates and (b) neutralizing negative charge buildup.[1] Supported by precedent established through analysis of well-defined model systems, these catalytic modes implicitly underlie most models for metalloenzyme-catalyzed phosphoryl transfer.[2−4] The templating mode follows logically from ground state metal ion interactions with substrates and chelating ligands, usually inferred from structural inspection and spectroscopy.[2,4] However, negative charge frequently borne by the chelating ligands (*e.g.*, anionic side chains in proteins and phosphate oxygen atoms in RNA) that mediate these ground state interactions may attenuate the electropositive potential of the active site metal ions and thereby render the catalytic contribution of charge neutralization in the transition state ambiguous. Moreover, assessing the ability of active site metal ions to neutralize negative charge poses significant experimental challenges, and there exist limited data that support this mode of metal ion catalysis directly (*e.g.*, see ref 5).

Metal ions at the active site of the *Tetrahymena* ribozyme were initially revealed from metal ion rescue experiments that take advantage of the preference for interactions of soft metal ions with sulfur and nitrogen relative to oxygen.[6] Early functional work identified metal ion interactions in the transition state involving the guanosine 3′-oxygen nucleophile and the adjacent 2′-hydroxyl group, the U(−1) 3′-oxygen leaving group, and a nonbridging oxygen atom of the transferred phosphoryl group (Figure 1). Thereafter, applica-

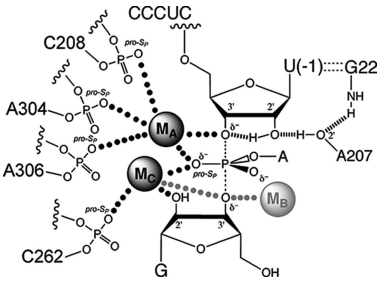


Figure 1. Model of the transition state of the group I self-splicing reaction from functional and structural data.[6] Dotted and hatched lines denote metal ion interactions and hydrogen bonds, respectively. Metal ion interactions supported by functional and structural evidence are colored black. Functional data suggest that the 3′-oxygen atom of G interacts with a metal ion (M_B, shown in gray) distinct from M_C. The structural data provide no evidence for M_B and suggest that the 3′-oxygen atom of G coordinates to M_C (represented by gray dots), the same metal ion that interacts with the 2′-OH group of G.

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tion of a quantitative approach termed Thermodynamic Fingerprint Analysis (TFA) provided information about the affinity of the rescuing metal ions and ascribed the identified interactions to three distinct metal ions: M_A, M_B, and M_C (Figure 1).[7] Subsequent X-ray analysis of crystals derived from the *Azoarcus* group I intron revealed the locations of two metal ions, corresponding to M_A and M_C, within the active site.[8] In the structure-derived model, M_C supplants the role of the missing metal ion (M_B). This unresolved difference may reflect limitations in either the functional or structural results or possibly idiosyncratic differences associated with distinct evolutionary subclasses of introns.[6]

The structural and functional data for M_A show perfect agreement, and in this work we investigate an important and potentially general mechanistic role for this metal ion (Figure 1). M_A contacts the U(−1) 3′-oxygen atom, which is the leaving group in the forward reaction and the nucleophile in the reverse reaction (eq 1). Mechanistic models inferred from this contact

$$\underset{(S)}{\overset{-3\ -1}{CCCUCU_pA + G_{OH}}} \underset{k_{reverse}}{\overset{k_{forward}}{\rightleftharpoons}} \underset{(P_{OH})}{\overset{-3\ -1}{CCCUCU_{OH} + G_pA}} \quad (1)$$

implicitly assume that M_A imparts catalysis *via* electrostatic charge neutralization, stabilizing the negative charge that develops on the leaving group en route to the transition state and activating the nucleophile by increasing the amount of the corresponding anion. Nevertheless, M_A ranks among the mostly highly chelated metal ions observed in an RNA structure, with three negatively charged phosphate ligands from the catalytic core (C208, A304, and A306) making direct inner sphere coordination (Figure 1).[8,9] To assess whether M_A can function in leaving group stabilization and nucleophile activation, we have used an extension of TFA to evaluate its capacity for electrostatic stabilization within the active site.

We first tested whether a metal ion forms a ground state contact with the U(−1) 3′-OH group of P_{OH} by using two analogues containing in place of the 3′-OH group either a thiol group (-SH, herein referred to as "P_{SH}") or a hydrogen atom (-H, herein referred to as "P_H"). Neither of these oligonucleotides serves as a substrate for the reverse reaction (eq 1, $k_{reverse}$), P_H because it lacks a nucleophile and P_{SH} because sulfur has extremely low nucleophilicity toward phosphorus.[10] To characterize these substrates, we took advantage of a previously studied miscleavage reaction that is catalyzed by the ribozyme and G_{OH} in the absence of added G_pA. We were able to use this ribozyme-catalyzed miscleavage reaction to obtain a readout of active site binding strength and interactions, as illustrated in Figure 2A and described below.

P_{OH} binds to the *Tetrahymena* ribozyme (E) in two distinct steps: P_{OH} first makes base-paring interactions with the internal guide sequence of E to form the "open" $(E{\cdot}P_{OH})_O$ complex, and the resulting P1 duplex then docks *via* tertiary interactions into the catalytic core of the ribozyme to form the "closed" $(E{\cdot}P_{OH})_C$ complex (Figure 2a).[6] Binding of G_{OH} to the $(E{\cdot}P_{OH})_C$ complex does not lead to a cleavage product directly, but the P1 duplex can undock from the $(E{\cdot}P_{OH})_C$ complex and redock transiently in a different, less stable register to form a misdocked complex, $(E{\cdot}P_{OH})_C'$ (Figure 2a).[11] In a misdocked complex, the P1 duplex is displaced by one or more base pairs with respect to the tertiary interactions, placing a different base pair at the active site, thereby leading to aberrant cleavage by G_{OH}, most prominently at the U(−3) position of P_{OH} (Figure 2a).[11,12]

In 10 mM Mg^{2+}, where all catalytic metal sites (Figure 1) are occupied by Mg^{2+},[13] P_H and P_{SH} undergo miscleavage by G_{OH} at similar rates, approximately 50- and 75-fold faster than the rate of P_{OH} miscleavage, respectively (Figure 2b, black bars). According to the model illustrated in Figure 2a, the faster miscleavage of P_H and P_{SH} results from an increase in the fraction of time in which the $(E{\cdot}P_X)$ complex is in the misdocked state. Higher occupancy of the misdocked state by P_H and P_{SH} relative to P_{OH} indicates a decrease in stability of the correctly docked $(E{\cdot}P_H)_C$ and $(E{\cdot}P_{SH})_C$ complexes relative to the $(E{\cdot}P_{OH})_C$ complex.[11] This difference in stability with the different versions of P presumably reflects the presence of a favorable interaction with the 3′-OH that stabilizes the $(E{\cdot}P_{OH})_C$ binding mode but is weakened or absent in the $(E{\cdot}P_H)_C$ and $(E{\cdot}P_{SH})_C$ complexes due to the absence of the 3′-OH group. The decrease in the stability of the $(E{\cdot}P)_C$ complex shifts the docking equilibrium toward $(E{\cdot}P)_O$ (see below), which allows the P_{SH} and P_H substrates to sample the alternative misdocked $(E{\cdot}P)_C'$ binding mode more frequently, resulting in faster miscleavage in the presence of G_{OH}.

Considering that the U(−1) 3′-oxygen interacts with M_A in the transition state (Figure 1), destabilization of the $(E{\cdot}P_X)_C$ binding mode by P_H or P_{SH} could reflect disruption of the interaction between the 3′-OH and this active site metal ion in the ground state: P_H lacks a ligand altogether and sulfur coordinates much more weakly to Mg^{2+} than oxygen does.[14] To test the possibility that the 3′-OH interacts with a metal ion in the ground state $(E{\cdot}P_{OH})_C$ complex, we monitored miscleavage of P_{OH}, P_H, and P_{SH} in the presence of 1 mM Mn^{2+}. Mn^{2+} decreased the rate of miscleavage of P_{SH} but did not affect the miscleavage rates for P_{OH} or P_H (Figure 2b, gray bars). We attribute the specific inhibition of P_{SH} miscleavage by Mn^{2+} to stabilization of the $(E{\cdot}P_{SH})_C$ complex. As Mn^{2+} coordinates sulfur more strongly than Mg^{2+} does,[14] stabilization of the $(E{\cdot}P_{SH})_C$ complex by Mn^{2+} provides strong evidence that Mn^{2+} interacts with the U(−1) 3′-sulfur in the $(E{\cdot}P_{SH})_C$ complex.

To further explore the Mn^{2+} stabilization of correct docking that we observed by following P_{SH} miscleavage, we directly determined dissociation rate constants (k_{off}) for P_{SH} as a function of Mn^{2+} concentration in a 10 mM Mg^{2+} background using pulse-chase methods. In the absence of Mn^{2+}, P_{SH} and P_H dissociate from the ribozyme approximately 20- to 30-fold faster than does P_{OH} (Figure 2c and Supplementary Table S1), consistent with the 50- to 75-fold greater miscleavage rates observed (Figure 2b) and with previous results for P_H.[15] Addition of Mn^{2+} reduced k_{off} for P_{SH} by approximately 200-fold, whereas the effect of Mn^{2+} on P_{OH} and P_H dissociation was ≤2-fold (Supplementary Table S1). The observed decrease in P_{SH} dissociation by Mn^{2+} provides additional support for the model in which a metal ion interacts with the U(−1) 3′-SH to stabilize correct docking of the $(E{\cdot}P_{SH})$ complex. We infer that a metal ion interacts with U(−1) 3′-OH analogously to stabilize correct docking of P_{OH}.

We used TFA to assess whether M_A mediates the stabilizing interaction in the closed complex. The Mn^{2+} concentration dependence of k_{off} for P_{SH} dissociation allowed us to determine the apparent affinity of the Mn^{2+} that stabilizes the $(E{\cdot}P_{SH})_C$ complex ($K^{Mn,app}_{(E{\cdot}P_{SH})}$ = 3.2 ± 0.6 μM, Figure 2c). With this measurement, we could use the thermodynamic cycle shown in Figure 2d to calculate the apparent affinity of this Mn^{2+} for binding to E in the *absence* of the -SH ligand ($K^{Mn,app}_{(E)}$ = 0.58 ± 0.1 mM). This value is consistent with the apparent affinity of Mn^{2+} for binding to the M_A site in the ribozyme in the absence

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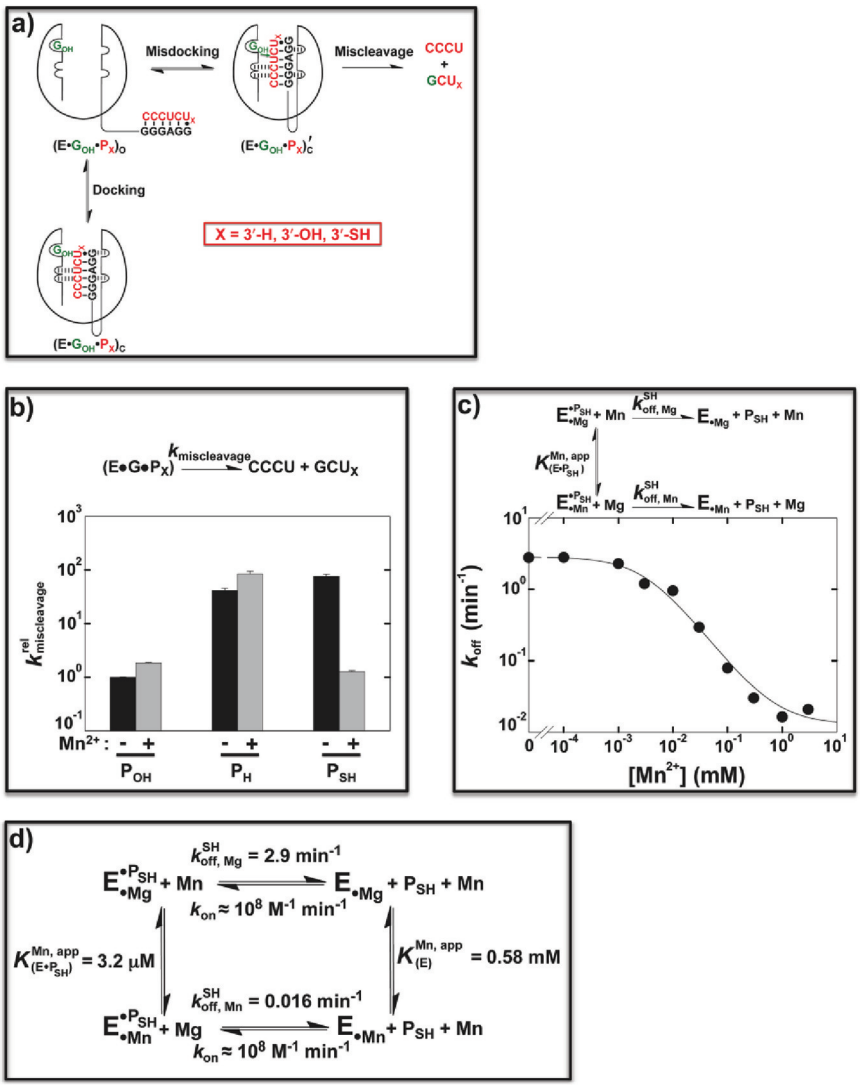

Figure 2. M_A facilitates proper docking of the (E·P) complex. (a) The ribozyme-catalyzed miscleavage reaction. Hatched lines denote tertiary contacts between the ribozyme and 2′-OH groups on P_X. The green arrow indicates nucleophilic attack by the 3′-OH of G. (b) Miscleavage rate constants for P_H, P_{OH}, and P_{SH} in the absence (black) and presence (gray) of 1 mM $MnCl_2$. $k^{rel}_{miscleavage}$ is the rate constant for miscleavage of P_X with or without Mn^{2+} present relative to the P_{OH} miscleavage rate constant in the absence of Mn^{2+}. (c) Plot of k_{off} vs $[Mn^{2+}]$ for P_{SH}. The line is a fit of the data to binding of a single Mn^{2+} ion and gives an apparent Mn^{2+} affinity ($K^{Mn,app}_{(E\cdot P_{SH})}$) of 3.2 ± 0.6 μM. (d) Thermodynamic cycle showing coupling between P_{SH} and Mn^{2+} binding to metal site A. $k^{SH}_{off,Mg}$, $k^{SH}_{off,Mn}$, and $K^{Mn,app}_{(E\cdot P_{SH})}$ were obtained from the data in Figure 2c. $K^{Mn,app}_{(E)}$ was calculated from the thermodynamic cycle shown in this panel. An association rate constant (k_{on}) of 10^8 M^{-1} min^{-1} was assumed for P_{SH} (Supplementary Table S2).[13]

of bound substrate, obtained from independent experiments using substrate cleavage assays as described previously (R.N.S, J.A.P., and D.H., unpublished data).[7] This agreement suggests that the metal ion that stabilizes docking of P in the ground state also provides stabilizing interactions to the U(−1) 3′-oxygen atom in the transition state (*i.e.*, M_A, Figure 1).[7] These results, in conjunction with prior structural and functional data,[9] link M_A to ground and transition state interactions with the U(−1) 3′-oxygen atom.

Interactions with M_A may stabilize the negative charge that builds up on the leaving group and nucleophile as they traverse the reaction coordinate in the forward and reverse directions, respectively. However, such stabilization for formation of an oxyanion from a hydroxyl group frequently eludes direct measurement because RNA's 3′-hydroxyl group (pK_a ~13) generally ionizes at pH values higher than the range observable for a biomolecule.[16] Moreover, ribozymes can exhibit complex pH−rate profiles, rendering it difficult to ascribe pK_a values to specific functional groups.[6] In contrast, thiols generally ionize with pK_a values significantly lower than the corresponding alcohols,[17] raising the possibility that substitution of the hydroxyl group with a thiol could lower the pK_a of the enzyme-bound species to within an observable range.

To probe the electrostatic character of M_A in the context of its RNA active site, we assessed whether binding of P_{SH} to the ribozyme affects the pK_a of the U(−1) 3′-SH. First, we measured the solution pK_a of the P_{SH} thiol by monitoring alkylation by iodoacetamide,[18] which reacts with the thiolate anion significantly faster than with the neutral thiol (Figure 3a). The pH-dependence of P_{SH} alkylation gave a solution pK_a of 7.6 ± 0.1 (Figure 3a, $pK_a^{P_{SH}}$). Next, we measured k_{off} for dissociation of P_{SH} from the ribozyme as a function of pH. With Mg^{2+} as the only divalent cation present, P_{SH} dissociated from the ribozyme with a rate constant of approximately 3 min^{-1}

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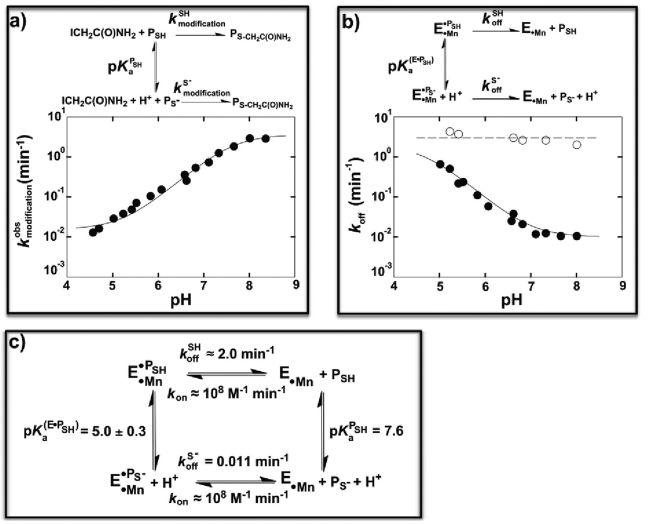

Figure 3. M_A lowers the pK_a of the U(−1) 3′-SH group. a) Iodoacetamide modification of P_{SH} in solution as a function of pH. The line is a fit of the data to ionization of the -SH group and gives an apparent pK_a value (p$K_a^{P_{SH}}$) of 7.6 ± 0.1. b) Dissociation rate constant of P_{SH} as a function of pH in the absence (open circles) and presence (closed circles) of 1 mM Mn^{2+}. The dashed line passing through the open circles is a straight line, with k_{off} set to 3 min^{-1}. The line passing through the closed circles is a fit of the data to ionization of the -SH group in the (E·P_{SH}) complex assuming a value of 2 min^{-1} for k_{off}^{SH}, as described in the text. This fit gives a value of p$K_a^{(E•P_{SH})}$ = 4.7 ± 0.3. c) Thermodynamic cycle showing coupling between P_{SH} binding and ionization of the -SH group. p$K_a^{P_{SH}}$ was obtained from the data in Figure 3a. k_{off}^{SH} was approximated as described in the text. $k_{off}^{S^-}$ was obtained from k_{off} for P_{SH} in the presence of Mn^{2+} at high pH, as shown in Figure 3b. An association rate constant (k_{on}) of 10^8 M^{-1} min^{-1} was assumed (Table S2).[13] p$K_a^{(E•P_{SH})}$ = 5.0 ± 0.3 reflects the average of p$K_a^{(E•P_{SH})}$ = 5.3 calculated from the thermodynamic cycle and p$K_a^{(E•P_{SH})}$ = 4.7 determined from the fit of the data shown in Figure 3b.

from pH 5 to 8 (Figure 3b, open circles). This k_{off} value matches measurements for other substrates that primarily populate the open complex (Figure 2a).[12,19] This agreement and the P_{SH} miscleavage data presented above suggest that the (E·P_{SH}) complex exists predominantly in the open complex (Figure 2a), so that there is little or no direct interaction between the sulfur atom of P_{SH} and the Mg^{2+} ion that occupies site M_A. The pH-independence of dissociation indicates that binding in the open complex does not shift the pK_a of P_{SH} from its solution value. In contrast, when Mn^{2+} occupies the M_A site, increasing the pH from 5 to 8 decreases k_{off} for P_{SH} by 60-fold (Figure 3b, closed circles), whereas pH exerts little influence on dissociation of P_{OH} or P_H from the ribozyme (<4-fold; Supplementary Table S1). The pH-dependent decrease in k_{off} for P_{SH} conferred by Mn^{2+} can be explained if -SH ionizes to $-S^-$ over this pH range and if Mn^{2+} forms a more favorable interaction with the thiolate anion $-S^-$ than with the neutral thiol -SH. As described below, under this assumption, the pH-dependence of k_{off} allows measurement of the pK_a for P_{SH} in the (E·P_{SH}) complex in the presence of Mn^{2+}.

If Mn_A interacts more strongly with $-S^-$ than with -SH, then according to the thermodynamic cycle shown in Figure 3c the ribozyme must lower the pK_a of the U(−1) 3′-SH. Using this cycle, we can estimate the pK_a of the U(−1) 3′-SH in the (E·P_{SH}) complex with knowledge of three equilibrium values: the solution pK_a of P_{SH} and the equilibrium constants for dissociation of P_{SH} and P_{S^-} from the ribozyme. The limiting value for dissociation of P_{SH} at high pH gives k_{off} = 0.011 min^{-1} for the anionic ($-S^-$) form of P_{SH} (Figure 3b,c). In contrast, we could not obtain k_{off} for the neutral (-SH) form of P_{SH} from the pH dependence due to the lack of a plateau in the low pH regime (Figure 3b). Additionally, complications associated with ribozyme inactivation prevented k_{off} measurements below pH 5. Instead, we estimated the dissociation rate constant for P_{SH} as follows. Fits of the reaction-rate profile at low pH give k_{off} > 1

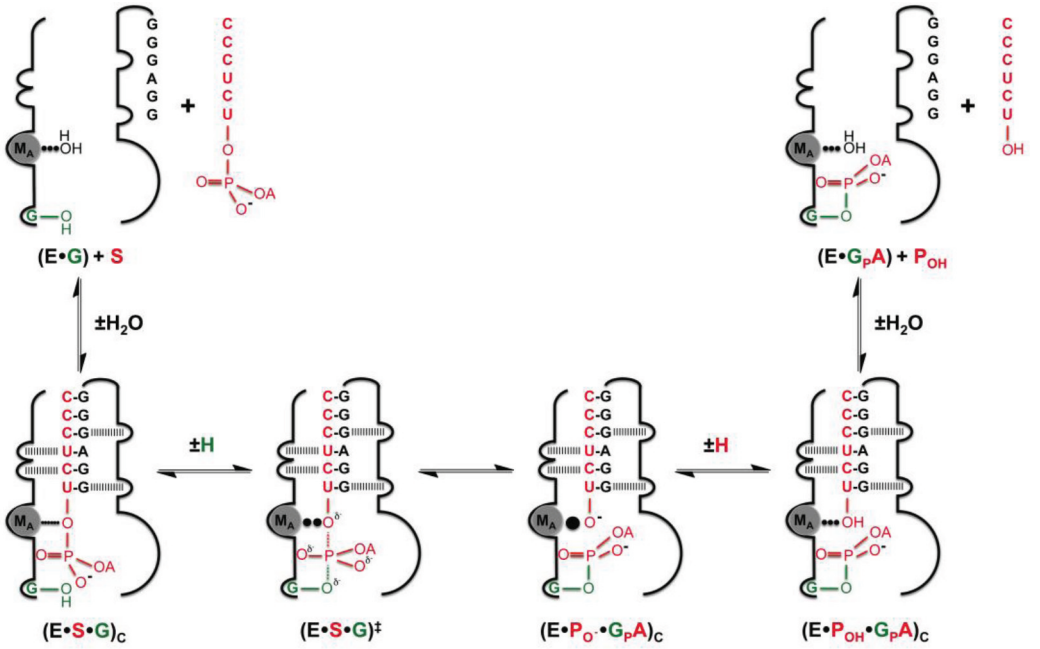

Figure 4. Model for the interaction between M_A and the U(−1) 3′-oxygen atom along the reaction cycle from the results of this study and previous work.[15] Dotted and hatched lines denote metal ion coordination and tertiary interactions, respectively. The size of the dots represents the extent of favorable electrostatic interaction between M_A and the U(−1) 3′-oxygen atom.

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min^{-1} as a lower limit for the value (Figure 3b, closed circles), and k_{off} = 3 min^{-1}, the pH-independent rate constant for dissociation of P_{SH} in Mg^{2+} alone, provides an upper limit for this dissociation constant (Figure 3b, open circles). As these limits are similar, their average of 2 min^{-1} likely represents a reliable approximation for the dissociation rate constant of the neutral form of P_{SH} from the ribozyme.

In contrast to the large difference in k_{off} for the neutral and anionic forms of P_{SH}, both forms bind to the ribozyme with similar association rate constants (k_{on}, Supplementary Table S2). Continuing with the thermodynamic analysis in Figure 3c, our values of k_{on} and k_{off}, together with the P_{SH} solution pK_a, give three equilibrium constants in the cycle and allow calculation of the pK_a for bound P_{SH} as 5.3 ($pK_a^{(E\cdot P_{SH})}$, Figure 3c). This value is similar to the pK_a of 4.7 ± 0.3 obtained from the fit of the data in Figure 3b (closed circles), assuming a dissociation rate constant of 2 min^{-1} for the -SH form of P_{SH}. Taking the average of $pK_a^{(E\cdot P_{SH})}$ obtained from the fit (4.7) and from the thermodynamic cycle (5.3), we estimate an apparent pK_a of 5.0 ± 0.3 for P_{SH} in the ($E\cdot P_{SH}$) complex (Figure 3c). We thus conclude that Mn^{2+}-induced docking of P_{SH} into the ribozyme active site allows the electropositive character of the active site to manifest as a pK_a lowering of the U(−1) 3′-SH group by approximately 2.6 units, corresponding to stabilization of the thiolate anion by 400-fold or 3.6 kcal/mol.

Our analysis shows that M_A, although highly chelated by anionic active site phosphoryl oxygen atoms (Figure 1), retains sufficient electropositive character to mediate a stabilizing interaction with the thiolate anion. These findings have important implications for catalysis throughout the reaction cycle (Figure 4). Beginning with the (E·G) + S ground state, Narlikar *et al.* have proposed that docking of S to form the $(E\cdot S\cdot G)_C$ complex results in a destabilizing interaction with M_A because the electron-withdrawing phosphoryl group likely renders the 3′-oxygen electropositive relative to an oxygen from water.[15] Proceeding along the reaction coordinate, the M_A interaction becomes favorable in the transition state ($(E\cdot S\cdot G)^{\ddagger}$, Figure 4) due to the substantial negative charge that develops on the leaving group in phosphoryl transfer reactions.[20] The results herein show that when the 3′-atom bears a full negative charge, M_A-mediated substrate docking can provide approximately 3.6 kcal/mol of stabilization. A significant fraction of this stabilization likely occurs in the transition state, although the exact value may differ when Mg^{2+} and a leaving group oxygen are present instead of Mn^{2+} and sulfur. Following complete transfer of the phosphoryl group to form the ($E\cdot G_pA\cdot P_{O^-}$) complex, the immediate product of the ribozyme reaction, P_{O^-}, bears a full negative charge, and would experience further stabilization by the electrostatic environment. Subsequently, P_{O^-} becomes protonated to form P_{OH} in an energetically downhill reaction at neutral pH (Figure 4). In the reverse reaction (eq 1 and Figure 4), the electropositive character of M_A activates P_{OH} by increasing the amount of the more reactive P_{O^-} species, an effect expected to outweigh the decrease in reactivity due to metal ion coordination.[3]

Our results directly demonstrate that a metal ion has the capacity to impart catalysis through classic charge neutralization within a highly complex, active site environment, even one formed by a polyanionic RNA enzyme. The findings provide fundamental mechanistic insight into metallohydrolase function and metal-catalyzed phosphoryl transfer and represent an essential step toward quantitative accounting of metal ion catalysis of phosphoryl transfer by RNA and protein enzymes.

METHODS

RNA Synthesis. L-21 *ScaI Tetrahymena* ribozyme was prepared as described previously.[21] All oligonucleotides, unless otherwise noted, were purchased from Dharmacon (Lafayette, CO) and deprotected according to manufacturer's protocol. Details for the synthesis of P_H and P_{SH} as well as the steps taken to ensure the integrity of the -SH group are described in the Supporting Information. All RNA substrates were 5′-^{32}P-end-labeled with [γ-^{32}P]ATP (Perkin-Elmer) and T4 polynucleotide kinase (New England Biolabs), as previously described.[21]

General Kinetics. All reactions were carried out at 50 °C in 10 mM $MgCl_2$ with the following buffers (50 mM): sodium acetate, pH 4.6−5.5; NaMES, pH 5.4−6.6; NaMOPS, 6.6−7.3; NaEPPS, 7.7−8.4 (pH values were determined at 50 °C). Details on the iodoacetamide modification assay, the miscleavage reaction, and pulse-chase experiments can be found in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

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