# Evidence that binding of C5 protein to P RNA enhances ribozyme catalysis by influencing active site metal ion affinity

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## ABSTRACT

**The RNA subunit (P RNA) of the bacterial RNase P ribonucleoprotein is a ribozyme that catalyzes the Mg-dependent hydrolysis of pre-tRNA, but it requires an essential protein cofactor (P protein) in vivo that enhances substrate binding affinities and catalytic rates in a substrate dependent manner. Previous studies of *Bacillus subtilis* RNase P, containing a Type B RNA subunit, showed that its cognate protein subunit increases the affinity of metal ions important for catalysis, but the functional role of these ions is unknown. Here, we demonstrate that the $Mg^{2+}$ dependence of the catalytic step for *Escherichia coli* RNase P, which contains a more common Type A RNA subunit, is also modulated by its cognate protein subunit (C5), indicating that this property is fundamental to P protein. To monitor specifically the binding of active site metal ions, we analyzed quantitatively the rescue by $Cd^{2+}$ of an inhibitory Rp phosphorothioate modification at the pre-tRNA cleavage site. The results show that binding of C5 protein increases the apparent affinity of the rescuing $Cd^{2+}$, providing evidence that C5 protein enhances metal ion affinity in the active site, and thus is likely to contribute significantly to rate enhancement at physiological metal ion concentrations.**

**Keywords: ribozyme; catalysis; ribonucleoprotein; RNase P; active site**

## INTRODUCTION

The biological functions of RNA and protein are intimately related and extensive structural and functional studies reveal that RNA binding proteins can modulate RNA function by binding to and stabilizing specific conformations (e.g., Weeks and Cech 1995; Ho and Waring 1999), by facilitating necessary conformational changes (Jankowsky and Bowers 2006; Caprara et al. 2007), as well as by providing unique surfaces for RNP targeting and regulation (Egea et al. 2005; Hopper 2006). The coordinated function of RNA and protein in enzyme catalysis is fundamental to ribosome catalyzed protein synthesis as well as numerous intron splicing and RNA processing reactions. Because of this essential functional interplay, understanding in detail how RNA and proteins form specific complexes and collaborate to achieve specific physiological function is an important goal.

A ribonucleoprotein enzyme in which the protein subunit plays a key role in specificity and catalysis is ribonuclease P, which catalyzes the 5′-end maturation of tRNA (Hsieh et al. 2004; Kazantsev and Pace 2006). In bacteria, RNase P is composed of an ~400 nucleotide (nt) RNA subunit (P RNA) and an ~100 amino acid protein subunit (termed C5 in *Escherichia coli*) (Fig. 1; Evans et al. 2006). The P RNA subunit is able to recognize pre-tRNAs and catalyze the hydrolysis reaction in vitro by itself; but substrate affinity, specificity, and catalytic rate are strongly dependent on assembly with its cognate protein subunit (Loria et al. 1998; Niranjanakumari et al. 1998; Kurz and Fierke 2000; Buck et al. 2005a; Sun et al. 2006). Binding of P protein dramatically increases pre-tRNA affinity, in part by making direct contact to the 5′-leader sequence (Niranjanakumari et al. 1998). However, the *E. coli* C5 protein subunit can enhance tRNA binding even though it is thought to only contact the 5′-leader sequence directly. Comparative equilibrium binding analyses of different *E. coli* pre-tRNAs indicate that the protein has differential

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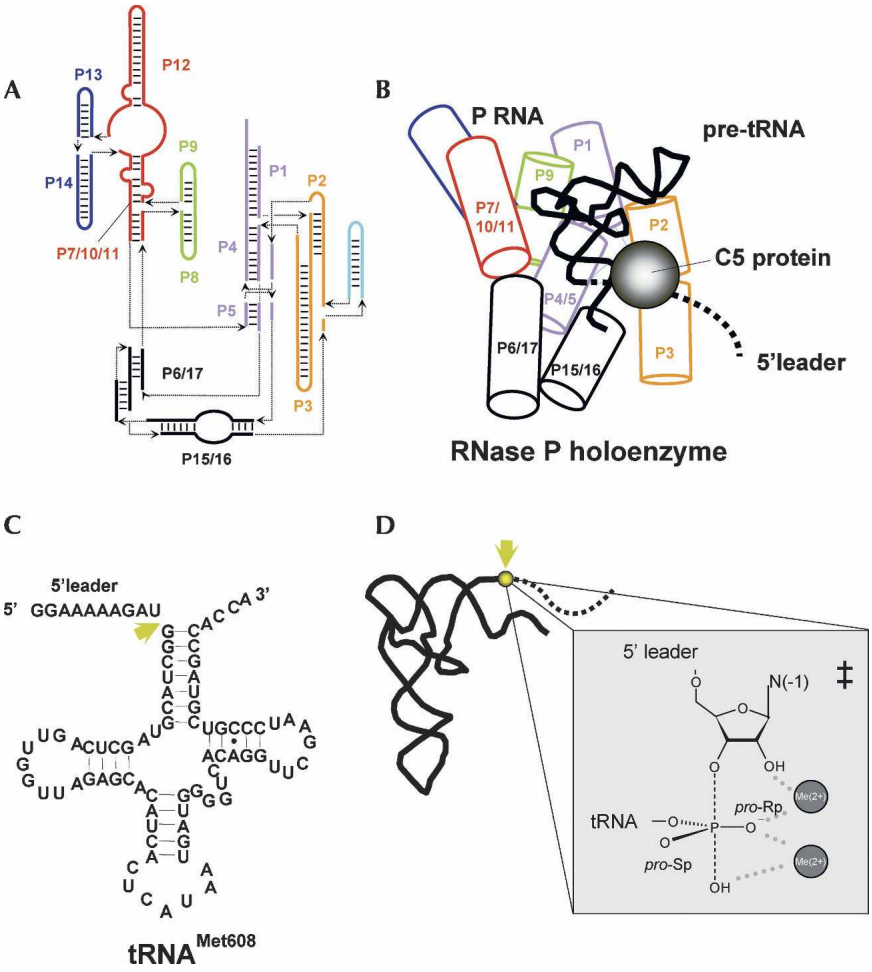


**FIGURE 1.** (*A*) Secondary structure diagram of *E. coli* P RNA. Helices are designated as P (paired) and numbered from the 5′ end of the RNA. Segments of the RNA connecting helices are designated J (joining) and numbered according to the helices they connect. The helices P1–P18 that make up the secondary structure of *E. coli* P RNA are labeled. The secondary structure is organized according to the three-dimensional structure of the P RNA subunit, and arrows depict connections that define the path of the RNA chain. (*B*) Cartoon diagram of the three-dimensional structure of a Type A P RNA with pre-tRNA bound. The structure diagram is based on the crystal structure of *Thermotoga maritime* P RNA (Torres-Larios et al. 2005). Individual helices are shown as cylinders that are colored according to the secondary structure diagram in panel *A*. The bound pre-tRNA is depicted by a black ribbon and the P protein subunit is shown as a sphere. The 5′-leader sequence of pre-tRNA is shown as a dashed line. (*C*) Sequence and secondary structure of pre-tRNA$^{Met608}$. The location of the RNase P cleavage site between nucleotides N(+1) and N(−1) is indicated by an arrow. (*D*) Detail of the proposed active site metal ion interactions and the reactive phosphate of pre-tRNA in the transition state. As described in the text, two metal ions are proposed, both of which interact with the pro-Rp phosphate oxygen of the reactive phosphate. This position is substituted by a sulfur atom in the Rp(+1) pre-tRNA substrate.

effects on the thermodynamic contribution of the tRNA and leader portions of the substrate and that low affinity of some tRNA bodies is compensated for by combining them with 5′ leaders that increase affinity more than others in order to reach uniformity of binding (Sun et al. 2006). Additionally, the protein subunit of *E. coli* RNase P was shown to significantly enhance the rate of the substrate cleavage step, which for some substrates can be >800-fold. Such effects on tRNA interactions, catalysis, and specificity likely result both from direct contacts to the 5′ leader as well as from protein-induced conformational changes that occur in the catalytic core of the ribozyme (Buck et al. 2005b). Our understanding of the mechanistic basis for these effects is currently limited by a lack of detailed information on the structural consequences of protein binding, and as well as a quantitative description of the accompanying changes in enzyme specificity and catalysis.

P RNA is considered to be a metalloenzyme and a key insight has been the demonstration that the protein subunit of *Bacillus subtilis* RNase P increases the apparent affinity of metal ions important for catalysis (Kurz and Fierke 2002). Although it is a foundational structural and kinetic system, the *B. subtilis* enzyme represents a minor subclass of RNase P enzyme in that it contains a variant Type B RNA subunit (Haas et al. 1994). Relatively small changes in RNA structure can result in significantly different $Mg^{2+}$ requirements for folding and catalysis (Lehman and Joyce 1993; Frank and Pace 1997; Nelson et al. 2005; Roychowdhury-Saha and Burke 2006). Thus, an important question is whether the previously documented contributions of RNase P protein to catalysis by the *E. coli* enzyme, which contains the more common Type A RNA subunit, are due to an enhancement of $Mg^{2+}$ binding. If so, it follows that this enhancement could be due to a direct effect on increasing affinities of active site metal ions.

Indeed, several lines of evidence indicate that P RNA positions active site metal ions. Detailed structural and functional studies of protein endoribonucleases that catalyze the same chemistry as P RNA show the presence of one or more active site metal ions (Cowan 1998). Additionally, the presence of active site metal ions in the Group I intron ribozyme, which catalyzes similar intermolecular nucleophilic attack of a phosphodiester, is strongly supported by detailed biochemical and high resolution structural data (Hougland et al. 2005; Stahley and Strobel 2006). Indeed, rate enhancement by P RNA is clearly dependent on the $Mg^{2+}$ ion concentration under conditions in which the

RNA subunit is folded and enzyme concentrations are saturating (Smith et al. 1992; Beebe et al. 1996). Furthermore, phosphorothioate modifications at the pre-tRNA cleavage site inhibit P RNA catalysis, and the effects of pro-Rp phosphate oxygen modification by sulfur can be rescued in part by addition of the thiophilic metal ion $Cd^{2+}$ (Warnecke et al. 1996). A general two-metal ion mechanism for RNase P catalysis is assumed (see Fig. 5, below) in which the two catalytic metal ions are positioned to coordinate the pro-Rp oxygen of the reactive phosphate (Steitz and Steitz 1993; Warnecke et al. 1996). One of the ions helps to position a water molecule as the nucleophile and assist its deprotonation, and the second is proposed to coordinate and stabilize the 3′O leaving group. Analyses of the bonding environment of the nucleophilic water in the transition state using kinetic isotope effects is consistent with direct metal ion catalysis in nucleophilic activation (Cassano et al. 2004). Thus, if the previously observed effects of C5 protein on enhancing catalytic rates results from a general property of P protein on influencing the metal ion dependence of catalysis, then it raises the possibility that these effects could be related to enhanced active site metal ion affinity.

However, the contributions of metal ions to RNA function are complex, and relating the binding of specific ions to specific functional roles continues to be one of the most vexing problems in the field. RNAs bind numerous metal ions in solution, which can interact site specifically by direct coordination and H-bonding or by electrostatic interactions (DeRose 2003; Draper et al. 2005). Both classes can make significant contributions to function that are reflected in $Mg^{2+}$ titration experiments, which are difficult to interpret in terms of the thermodynamics of individual metal ion interactions. Additionally, it is well established in other ribozyme systems that $Mg^{2+}$ binding can increase catalytic rate constants indirectly by stabilizing active RNA conformations or by promoting substrate docking steps (e.g., Shan and Herschlag 2002; Tinsley et al. 2004). Thus, while the contribution of the protein subunit to P RNA catalysis appears to be related to the binding of $Mg^{2+}$ ions, the binding sites and functional roles of these ions are fundamentally difficult to establish.

To address these issues, we compared the pH and $Mg^{2+}$ dependence of the catalytic step for the P RNA subunit of *E. coli* RNase P in the presence and absence of its cognate protein subunit (C5 protein) under identical reaction conditions. Additionally, we used quantitative analysis of the $Cd^{2+}$ rescue of a cleavage site Rp phosphorothioate (PS) modification to isolate the binding thermodynamics of active site metal ions (Peracchi et al. 1997; Shan et al. 2001). The comparative kinetic analyses show that C5 protein reduces the apparent affinity of $Mg^{2+}$ ion essential for catalysis without changing the rate-limiting step. Importantly, binding of the C5 protein to P RNA results in higher apparent affinity for the binding of rescuing $Cd^{2+}$ ions to a substrate phosphorothioate. Thus, these data provide evidence that an important function of the bacterial RNase P protein subunit is to enhance the rate of catalysis by increasing metal ion affinity in the active site of the catalytic RNA subunit.

## RESULTS

To quantify the contribution of the P protein subunit to rate enhancement by RNase P, we determined the effect of C5 protein binding on the single turnover rate constant for cleavage of *E. coli* pre-$tRNA^{Met608}$. The pre-$tRNA^{Met608}$ substrate was chosen because it is representative of a ''typical'' *E. coli* pre-tRNA substrate in that it contains all of the optimal recognition elements that have been defined by sequence comparisons and by structure function studies. Thus, the mechanistic features of substrate recognition and catalysis defined by using this substrate are anticipated to reflect general features of RNase P enzymology. Analyses were performed typically under conditions of 100 mM NaCl, 17.5 mM $Mg^{2+}$, which was determined to be optimal for the holoenzyme multiple turnover reaction (Guo et al. 2006). Reaction pH and $Mg^{2+}$ concentration were varied as described below. As shown in Figure 2A, under identical reaction conditions the holoenzyme has an ~10-fold higher rate constant for cleavage of pre-$tRNA^{Met608}$ compared to P RNA alone (0.14 $min^{-1}$ versus 1.3 $min^{-1}$). The lower rate constant for P RNA alone under these conditions could be due to a slower, noncatalytic rate-limiting step that is enhanced by the protein subunit. In this scenario the protein subunit could change the mechanism to one in which substrate cleavage, rather than a slower upstream step, is rate limiting. Alternatively, the protein subunit could enhance catalysis in a more direct fashion by altering the affinity of metal ions necessary for catalysis as suggested previously by results from *B. subtilis* RNase P.

To first assess if the protein changes the rate-limiting step of the pre-tRNA cleavage reaction, we compared the pH dependence of the single turnover rate constants for P RNA in the presence and absence of C5 protein. Analysis of the pH dependence of the cleavage rate constant as a probe for the rate limiting step of the RNase P reaction is based on the rationale that deprotonation to yield a hydroxide ion nucleophile is an essential step in the RNase P catalyzed phosphodiester bond hydrolysis reaction. Accordingly, the rate of substrate cleavage increases with increasing hydroxide ion concentration and follows a rate law for specific base catalysis (Smith and Pace 1993; Cassano et al. 2002; Persson et al. 2003). Thus, plots of $\log(k_{obs})$ versus pH that are linear with a slope of unity suggest that the chemical step is rate limiting, whereas an alteration in the rate-limiting step of the reaction is likely to result in the absence of or a significant decrease in pH dependence. As shown in Figure 2B, the rates of both P RNA and holoenzyme cleavage are pH sensitive with a log-linear relationship

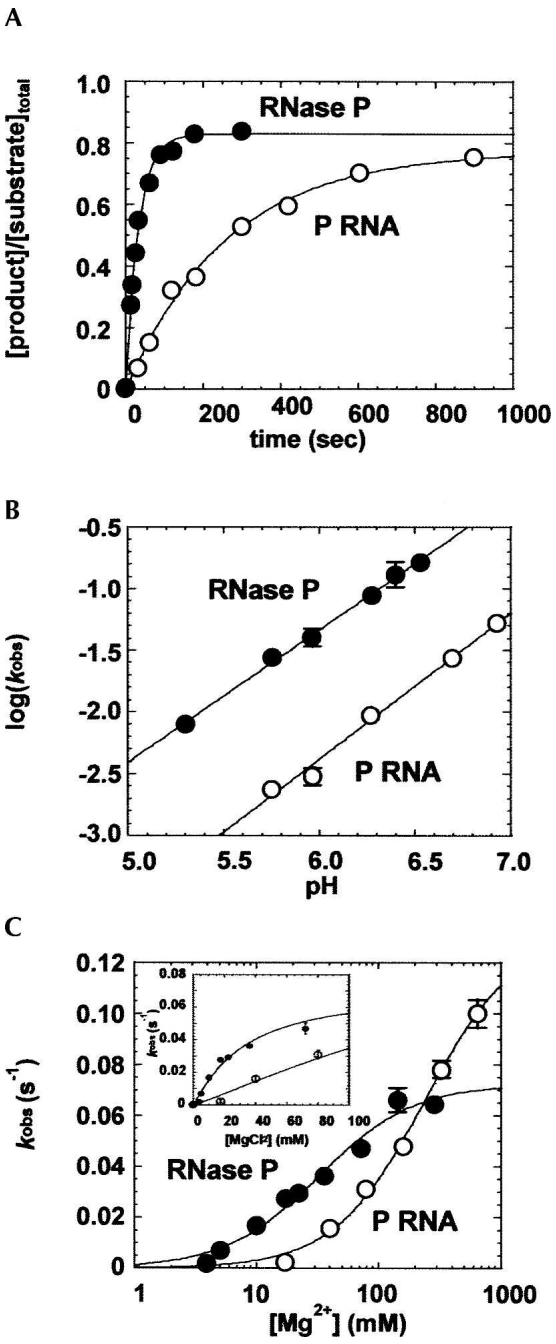


**FIGURE 2.** Kinetic analyses of the pH and $Mg^{2+}$ dependence of the single turnover rate constants for P RNA and RNase P holoenzyme cleavage of pre-$tRNA^{Met608}$. (*A*) Single turnover reactions for RNase P holoenzyme (●) and P RNA alone (○) are compared at saturating enzyme concentration in: MES 50 mM (pH 5.75), NaCl 100 mM, $MgCl_2$ 17.5 mM, and Triton 0.005%. (*B*) Plots of $\log(k_{obs})$ versus pH for RNase P (●) and P RNA (○) are fit to a linear equation (slope=1.1 and 1.2 for RNase P and P RNA alone reactions, respectively). (*C*) Plot of $k_{obs}$ values for P RNA and RNase P holoenzyme versus $Mg^{2+}$ concentration. The data are fit to the equation for a cooperative binding mechanism (Equation [2], Materials and Methods). The Hill coefficients are unity for both reactions; however, the apparent dissociation constant for holoenzyme is smaller than that for P RNA alone ($K_{Mg}$ [RNase P]=32 mM and $K_{Mg}$ [P RNA alone] >250 mM). The *inset* shows a closeup of the data at low $Mg^{2+}$ concentrations.

between $k_{obs}$ and pH. The slopes for both reactions are approximately equal to 1 (1.1 and 1.2 for RNase P and P RNA, respectively), consistent with the deprotonation of a functional group in the transition state for both reactions. Thus, these data indicate that there is essentially no difference in the rate-limiting steps in the holoenzyme and P RNA reactions under these reaction conditions.

Next, we tested whether binding of C5 protein influences the $Mg^{2+}$ concentration dependence of the single turnover rate constant for cleavage of pre-$tRNA^{MET608}$. As in the pH titration experiments, reactions were performed at a saturating concentration of enzyme in order to minimize the contribution of binding steps to the observed rate constant. Additionally, titrations were performed at $Mg^{2+}$ concentrations greater than the concentration required for P RNA folding (>5 mM) as indicated by sensitivity to chemical and oligonucleotide probing (Zarrinkar et al. 1996; Pan and Sosnick 1997; Kent et al. 2000). As shown in Figure 2C, the observed single turnover rate constants for both the holoenzyme and the P RNA alone reactions increase as $Mg^{2+}$ concentration increases. The concentrations of monovalent ions were not adjusted to maintain constant ionic strength, and thus both site-specific and diffuse metal ion interactions will contribute to the observed metal ion dependence. For P RNA alone, the apparent $Mg^{2+}$ affinity is comparatively low, and thus only an estimate of the apparent affinity ($K_{d,Mg}$) and the rate at saturating $Mg^{2+}$ concentrations ($k_{obs(max)}$) could be obtained. Also, binding of the protein subunit is sensitive to very high ionic strength (>0.5 M), limiting the upper range at which data can be collected (Day-Storms et al. 2004). Nonetheless, these data indicate that the $k_{obs(max)}$ values for the P RNA and holoenzyme reactions differ by less than twofold ($\sim$0.07 $s^{-1}$ for holoenzyme; $\sim$0.13 $s^{-1}$ for P RNA alone). Importantly, the $Mg^{2+}$ titration data for both reactions fit to a noncooperative binding model and the *E. coli* holoenzyme displays a significantly lower overall $K_{d,Mg}$ (32 mM) compared to the P RNA-alone reaction (>250 mM). Since the highest concentration tested for P RNA was only twofold higher than the apparent $K_{d,Mg}$ this value represents a lower limit. At 10 mM $Mg^{2+}$, the rate enhancement due to the presence of C5 protein becomes >55-fold, and, at lower concentrations, such as those encountered in a cellular environment ($\sim$1 mM) (e.g., Froschauer et al. 2004; Farruggia et al. 2005), the extent of enhancement will be greater.

While these data are important for establishing the contributions of C5 protein to RNase P function in modulating apparent metal ion affinity, they raise obvious questions regarding the specific role (or roles) these metal ion interactions play in enzyme catalysis and where they bind in the enzyme–substrate complex. Previous studies of both large and small ribozymes clearly demonstrate that metal ions that bind at a distance from the active site and promote folding can provide a large apparent contribution

to catalysis (DeRose 2003; Draper et al. 2005). At the concentrations of divalent ions used in Figure 2 (17.5 mM), the $Mg^{2+}$ requirement for P RNA global folding is satisfied (~5 mM) (Zarrinkar et al. 1996; Fang et al. 2000); however, it is possible that C5 protein enhances the affinity of $Mg^{2+}$ ions necessary for a local folding transition that affects the active site or one that is necessary for substrate positioning (Christian et al. 2006). Additionally, as described above, a simple model would be that C5 protein directly affects the affinity of metals within the ribozyme active site.

A strategy for observing the effect of the protein subunit on the specific binding of catalytic metal ions is suggested by the observation that replacing the pro-Rp oxygen of the substrate phosphate with sulfur results in a >1000-fold defect in catalysis, and that adding a "thiophilic" metal ion, like $Mn^{2+}$ or $Cd^{2+}$, into the reaction rescues cleavage (Warnecke et al. 1996; Chen et al. 1997). Under the appropriate conditions such a result can be interpreted as indicating that the rescuing ion is coordinated directly to the site-specific sulfur modification and by analogy that $Mg^{2+}$ ions are coordinated to the analogous oxygen atom in the unmodified RNA (Pecoraro et al. 1984; Piccirilli et al. 1993). For the Group I intron, Group II intron, and hammerhead ribozymes, quantitative analysis of metal ion dependent PS rescue has provided a means for analyzing the relative affinity of specific active site metal ions (Peracchi et al. 1997; Shan et al. 1999; Gordon and Piccirilli 2001). In this analysis, nonspecific effects on reaction rate caused by the presence of $Cd^{2+}$ is accounted for by evaluation of $k_{rel}$, which is the ratio of the observed rates for the oxygen-containing substrate and sulfur-containing substrate ($k_{rel}=k^{S}_{obs}/k^{O}_{obs}$). Plotting $k_{rel}$ versus the concentrations of rescuing $Cd^{2+}$ provides a thermodynamic signature for binding of these ions to the substrate cleavage site (Shan et al. 1999, 2001). Thus, comparison of the concentration dependence of $Cd^{2+}$ rescue for the holoenzyme and the P RNA alone reactions, in principle, should permit the contribution of C5 protein to active site metal affinity to be measured.

To interpret such data in terms of specific metal ion binding, several criteria must be met: First, the addition of $Cd^{2+}$ must provide significant rescue for the modified substrate in the background of $Mg^{2+}$ ions; second, the added $Cd^{2+}$ should not cause miscleavage that limits the accuracy of measured rate constants; third, there must be no change in the rate limiting step in the presence and absence of rescuing metal ion, as this would invalidate the quantitative evaluation of $Cd^{2+}$ binding thermodynamics (Christian 2002, and references therein). To first test whether $Cd^{2+}$ can rescue the catalytic defect caused by substrate modification, single turnover cleavage reactions were performed in the presence or absence of $Cd^{2+}$ in the background of 17.5 mM $Mg^{2+}$ to ensure appropriate RNA folding and to minimize the effects of $Cd^{2+}$ on folding. For these experiments, a substrate containing a Rp phosphorothioate modification at the N(+1) position of pre-$tRNA^{Met608}$ (Rp[+1] pre-tRNA) was created by ligating an 11 nt oligo encompassing the cleavage site to a 3′ fragment containing the remainder of the tRNA portion of the substrate. As shown in Figures 3 and 4, the PS modification results in a significant defect in the rate of substrate cleavage ($k^{O}_{obs}/k^{S}_{obs}>1000$) for the Rp(+1) pre-tRNA substrate. This defect is not due to a deleterious effect on substrate binding, which was assessed by measuring substrate dissociation constants by gel filtration. Apparent $K_d$ were ~1 nM for binding of pre-tRNA and Rp(+1) pre-tRNA, respectively, to RNase P. The modification had only a minor effect on binding for P RNA alone as well ($K_d$ of 1 μM and 3 μM for pre-tRNA and Rp[+1] pre-tRNA, respectively). Importantly, this catalytic defect can be rescued by addition of $Cd^{2+}$ into both P RNA and holoenzyme reactions. These data further illustrate that the second criterion is also met in that the presence of $Cd^{2+}$ does not result in significant miscleavage (Fig. 3). However, the presence of the phosphorothioate modification in the Rp(+1) pre-tRNA does engender some miscleavage in the absence of $Cd^{2+}$. This result is important, since miscleavage arises from parallel reactions that would by definition obscure the intrinsic cleavage rate (Loria and Pan 1999; Zahler et al. 2005). Thus, the rate constants determined in the absence of $Cd^{2+}$ contain an inherent inaccuracy.

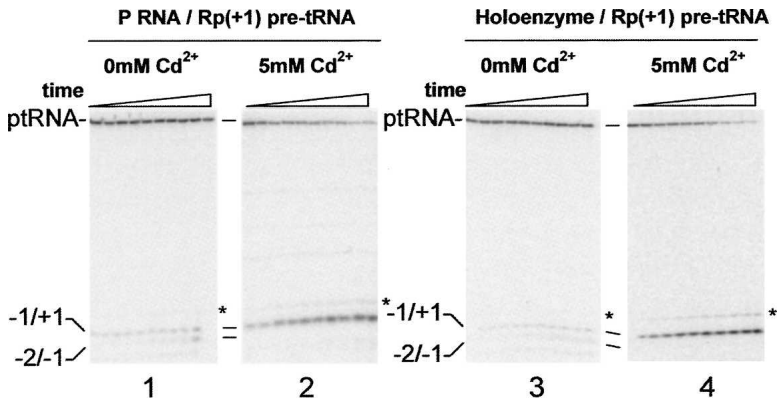


**FIGURE 3.** $Cd^{2+}$ rescues the cleavage of a N(+1) PS substrate modification by P RNA and RNase P holoenzyme. Single turnover reactions were performed under standard conditions (50 mM MES at pH 5.75, 100 mM NaCl, 17.5 mM $MgCl_2$, and 0.005% Triton X-100) in the absence (panels *1*,*3*) or presence of 5 mM $Cd^{2+}$ (panels *2*,*4*) for RNase P holoenzyme (panels *3*,*4*) and P RNA (panels *1*,*2*) reactions. Aliquots are removed at predetermined time points and analyzed in 20% PAGE. The positions of products resulting from correct cleavage (−1/+1) and miscleavage (−2/−1) are indicated. The product marked by an asterisk results from correct cleavage of a minor population of substrate containing an additional nucleotide at its 5′ end.

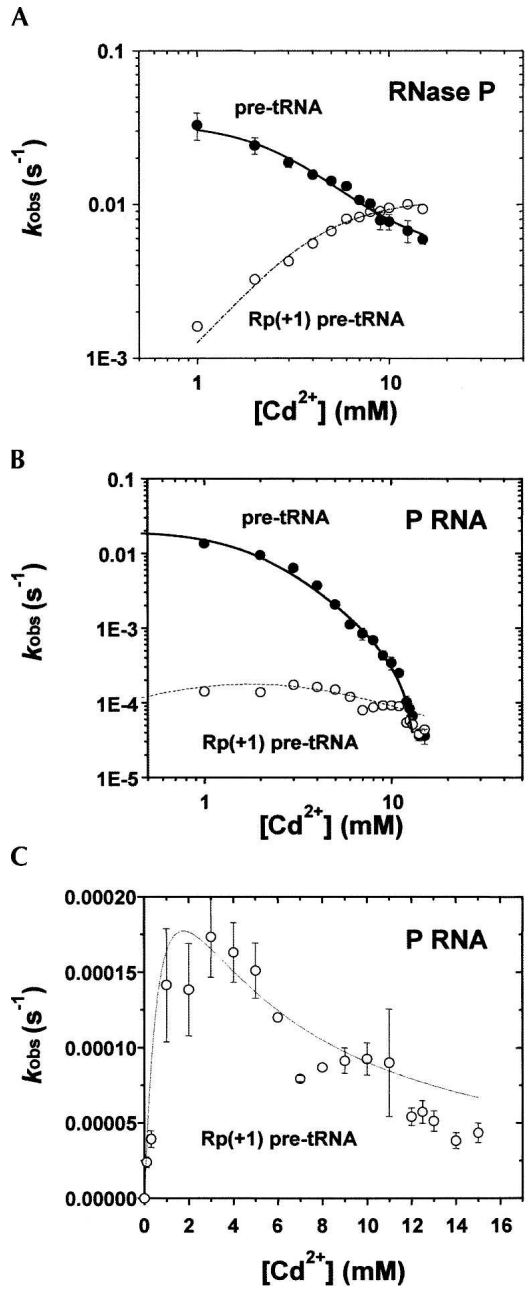


**FIGURE 4.** Quantitative analysis of $Cd^{2+}$ dependent rescue of the (+1 Rp) PS substrate modification. Single turnover rate constants were determined at a series of increasing $Cd^{2+}$ concentrations in the background of 17.5 mM $Mg^{2+}$. (*A*) Plots of $k_{obs}$ (RNase P) versus $Cd^{2+}$ concentration are shown for PS modified (○) and unmodified (●) substrates. (*B*) Plots of $k_{obs}$ (P RNA alone) versus $Cd^{2+}$ concentration are shown for modified (○) and unmodified (●)substrates. (*C*) The $k_{obs}$ (P RNA alone) data for PS modified substrates are shown separately for a better view of $Cd^{2+}$ stimulation at low $Cd^{2+}$ concentrations. Data represent the average values for at least three independent trials.

Nonetheless, because these rate constants are so small relative to the rate constants for cleavage of the unmodified substrate (>1000-fold), this inaccuracy in the 0 mM $Cd^{2+}$ data point has little effect on the subsequent $k_{rel}$ analysis. Moreover, micromolar concentrations of $Cd^{2+}$ entirely suppress miscleavage; therefore, comparison of rate constants determined under these conditions will accurately reflect the intrinsic substrate cleavage rate. In addition, to test whether the presence of $Cd^{2+}$ changes the rate limiting step for cleavage of Rp(+1) pre-tRNA and unmodified pre-tRNA, the effect of pH on the rate constants for these substrates was examined. In these experiments the catalytic rate constant is still pH dependent in the presence of $Cd^{2+}$ up to 15 mM for both reactions, indicating that there is no change in rate limiting step (data not shown).

With the criteria of $Cd^{2+}$ dependent rescue, accurate cleavage, and no change in mechanism for the Rp(+1) pre-tRNA substrate established, it is possible to employ analysis of $k_{rel}$ (see above) to probe active site metal ion affinities for P RNA in the presence and absence of C5 protein. Therefore, we determined the effect of $Cd^{2+}$ on the rate constant for cleavage of the native oxygen containing substrate ($k^{O}_{obs}$) by P RNA in the presence and absence of C5 protein. As shown in Figure 4, $Cd^{2+}$ inhibits both reactions in a concentration-dependent, saturable fashion. The tritation data for both RNase P and P RNA fit to a cooperative binding mechanism in which two classes of $Cd^{2+}$ ions bind simultaneously and inhibit RNase P catalysis. Furthermore, the apparent affinity for the inhibitory $Cd^{2+}$ ions is similar for both P RNA and RNase P ($K_{d,Cd}$=1.9 mM and 3.0 mM for P RNA and RNase P, respectively). In contrast, $Cd^{2+}$ results in an increase in the rate constant for cleavage of Rp(+1) pre-tRNA ($k^{S}_{obs}$) for both P RNA and holoenzyme. Because of the large degree of inhibition of the P RNA alone reaction, the stimulatory effect of $Cd^{2+}$ on $k^{S}_{obs}$ is most clearly observed at lower concentrations (Fig. 4C). Thus, the effect of $Cd^{2+}$ ions on the rate constant for cleavage of Rp(+1) pre-tRNA is the combination of both the site-specific rescue effect and inhibition.

To isolate the thermodynamics of the binding of the rescuing metal ions, $k_{rel}$ is utilized as described above ($k_{rel}=k^{S}_{obs}/k^{O}_{obs}$), which normalizes the inhibitory effect of $Cd^{2+}$ on the cleavage reactions catalyzed by P RNA in the presence and absence of C5 protein. A plot of $k_{rel}$ versus the concentration of rescuing $Cd^{2+}$ ions fits well to the cooperative binding model for both holoenzyme and P RNA alone reactions (Fig. 5A). The apparent dissociation constant for catalytic metal ion binding ($K_{d,app}$) for the holoenzyme reaction is 9.3 mM and the Hill coefficient ($n$) is 2, indicating that two metal ions interact with the Rp sulfur modification in Rp(+1) pre-tRNA, consistent with the two metal ion model as described above (Fig. 5B). For P RNA alone, a $Cd^{2+}$ concentration significantly higher than 15 mM is required to reach saturation. However, precipitation of RNA in higher $Cd^{2+}$ concentrations limits the precise measurement of the metal ion affinity and cooperativity, and the $K_{d,app}$ is estimated to be significantly >30 mM. Thus, the apparent dissociation constant for

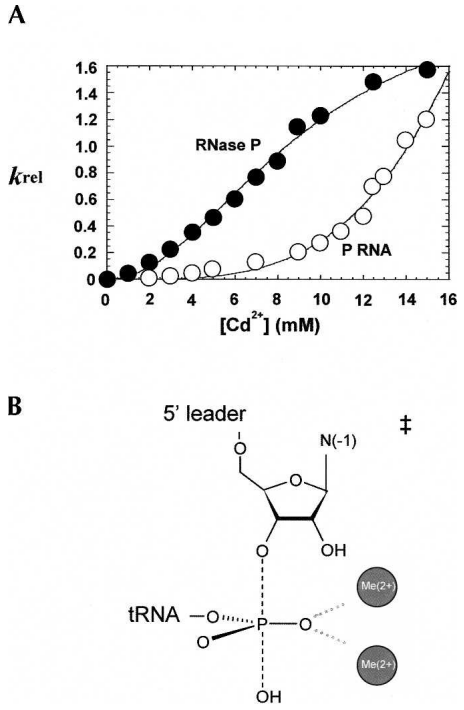


**FIGURE 5.** Comparison of $k_{rel}$ as a function of $Cd^{2+}$ concentration for P RNA and RNase P holoenzyme shows that C5 enhances the binding affinity of catalytic metal ion. (*A*) Relative reaction rates ($k_{rel}$) calculated from the ratio of observed reaction rates for substrates with and without the sulfur substitution (i.e., $k_{rel}=k^{S}_{obs}/k^{O}_{obs}$) are plotted versus $Cd^{2+}$ concentrations. Data for the holoenzyme reaction is represented by (●) and P RNA alone is represented by (○). Fitting to the Hill equation reveals that $n$=2 for the holoenzyme reaction, and $K_{Cd}$ (holo)=9 mM and $K_{Cd}$ (P RNA alone)>30mM. (*B*) Interpretation of the concentration dependent rescue of the Rp phosphorothioate modification by $Cd^{2+}$. Because the $k_{rel}$ analysis controls for non-specific effects of $Cd^{2+}$, the cooperative nature of the $Cd^{2+}$-dependent rescue suggests that two rescuing metal ions interact with the single sulfur modification, which reflects two metal ions binding in the P RNA active site for the native, unmodified substrate.

active site metal ion binding is at least threefold higher for P RNA than the RNA–protein complex.

## DISCUSSION

If the $Cd^{2+}$ rescue experiments accurately reflect the interactions of $Mg^{2+}$ interactions with the reactive phosphate, then the observed increase in apparent $Mg^{2+}$ affinity observed in Figure 2 in part reflects active site metal ion binding. The close correspondence in the metal ion interactions in the Group I intron ribozyme active site identified by quantitative metal ion rescue experiments and high resolution X-ray crystallography indicates that this assumption is likely to be valid (Shan et al. 1999; Stahley and Strobel 2005). Thus, a simple interpretation of these data supports a model for RNase P catalysis in which two divalent metal ions bind to the reactive phosphate in the enzyme–substrate complex that are essential for catalysis. In this model the protein subunit has a significant effect on catalysis by influencing the affinity of these active site metal ions, thus increasing the apparent rate constant for pre-tRNA cleavage (Fig. 6).

An important observation regarding the differential functional effects of RNA metal ion interactions is the significant inhibition of both P RNA and RNase P holoenzyme by increasing concentrations of $Cd^{2+}$. When the PS-modified substrate is used, both activation and inhibition contribute to the observed $Cd^{2+}$ dependence and the $k_{rel}$ approach permits the affinity of activating ion binding to be deconvoluted. However, the underlying quantitative relationship between the observed inhibitory $Cd^{2+}$ binding affinity and active site metal affinity for the PS-modified substrate is difficult to evaluate. Inhibition could arise from competition with active site metal ions or indirectly by affecting active site geometry at a distance. Figure 4 also shows that there is a significant different in the magnitude of the effect of increasing $Cd^{2+}$ concentration on the rate constants for the P RNA and RNase P reactions. A first order interpretation is that the geometry of the RNA with $Cd^{2+}$ bound is different when C5 protein is also bound to P RNA, and this difference in structure accounts for the difference in the degree of inhibition that is observed.

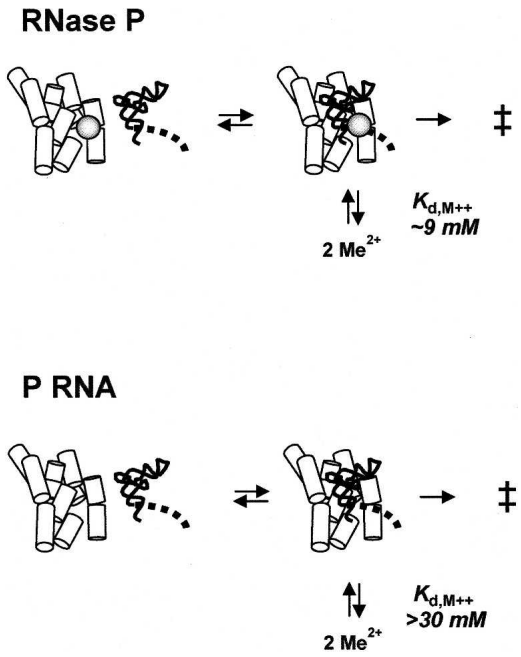


**FIGURE 6.** P protein increases the affinity of metal ions in the active site of P RNA. Interpretation of the $Cd^{2+}$ rescue data supports a model in which two $Mg^{2+}$ ions bind to the active site of the enzyme–substrate complex in the ground state. The apparent affinities of the rescuing ion interactions are indicated. However, the affinities of corresponding active site $Mg^{2+}$ ions may be significantly different. However, as indicated in the text, evaluation of the $Mg^{2+}$ titration data are consistent with cooperative binding of a high affinity class of metal ions that contributes to catalysis as indicated in the diagram.

Additionally, titration data alone do not distinguish whether there is a single inhibitory $Cd^{2+}$ ion bound or multiple $Cd^{2+}$ ions binding noncooperatively with similar affinity. Thus, a second possibility is that the P RNA has additional inhibitory $Cd^{2+}$ binding interactions that are distinct from the RNase P holoenzyme.

A further illustration of the multicomponent nature of RNA–metal ion interactions is provided by the differences in apparent binding thermodynamics of "catalytic" metal ion binding for total $Mg^{2+}$ titration versus active site metal ion binding obtained by $k_{rel}$ analysis. The apparent binding thermodynamics of the rescuing $Cd^{2+}$ ions is cooperative, while metal ion binding in bulk $Mg^{2+}$ titration experiments, which should contain a significant contribution from active site metal ion binding, are not. In this regard it is important to note that if additional $Mg^{2+}$ ions are necessary for catalysis, even if they bind within the active site, they will not be detected in the PS rescue experiments, which only report on the interaction of metal ions with the introduced sulfur modification. Based on physical studies of model systems, it is likely that the bulk $Mg^{2+}$ titration results reflect the binding of multiple classes of metal ions, including diffuse ions that do not interact with the RNA in a site-specific fashion. The data in Figure 2 for bulk $Mg^{2+}$ titration fit to a model for binding of a single class of activating $Mg^{2+}$ ions in a noncooperative manner. Inspection of the titration data at low $Mg^{2+}$ ion concentrations reveals that the observed rate constants could also be accounted for by a more complex model in which two classes of ions are necessary for catalysis: a high affinity class that binds cooperatively and a lower affinity class that binds in a noncooperative fashion. However, inaccuracies in the current data set for measuring small rate constants and in controlling for depletion of $Mg^{2+}$ by the high concentrations of P RNA required to achieve saturation make it difficult to distinguish between these models. Additionally, differences in the size and charge distribution of $Cd^{2+}$ versus $Mg^{2+}$ or changes in the electrostatic environment of the active site due to introduction of the PS modification could also result in differences in apparent $Mg^{2+}$ and $Cd^{2+}$ affinity. Nonetheless, these results provide the first evidence for a specific effect of the RNase P protein subunit on active site metal ion binding that at physiological divalent metal ion concentrations (0.5–1 mM) (e.g., Froschauer et al. 2004; Farruggia et al. 2005) will be very large.

Given the importance of understanding the cooperative function of RNA and protein, the mechanism by which RNase P protein acts to enhance active site metal ion affinity is of significant interest. The effect of protein binding on active site metal ion affinity could in principle result from P protein contributing protein functional groups that serve as additional ligands for metal ions in the active site. This possibility seems unlikely given that there are substrates and reaction conditions under which C5 protein can make relatively little contribution to catalysis (Beebe and Fierke 1994; Sun et al. 2006) and the low degree of sequence conservation of P protein compared to the catalytic core of P RNA (Haas and Brown 1998; Jovanovic et al. 2002). However, due to the presence of unique structural motifs in the protein cofactor, it is conceivable that the polypeptide backbone itself might contribute to metal ion binding. Alternatively, P protein could act indirectly, inducing a conformational change in P RNA that results in a more optimal alignment of RNA functional groups that contact metal ions. Previous studies have indeed shown that the protein decreases the $Mg^{2+}$ requirement for folding (Buck et al. 2005a). However, these effects are manifest at lower concentrations than the titration analyses presented here and chemical and oligonucleotide probing confirm that the P RNA is folded over the range of $Mg^{2+}$ concentrations tested here (Zarrinkar et al. 1996; Sun et al. 2006). Further, the single turnover analyses were performed at saturating concentrations of enzyme that should control for differences in the active fraction of enzyme at different metal ion concentrations. Alternatively, the protein could contribute to a substrate docking step, after initial substrate binding, in which the binding sites for active site metal ions are formed. If catalysis is slow relative to equilibration between the docked and undocked forms, then the pH dependence will be the same for the P RNA and holoenzyme reactions since phosphodiester bond cleavage is rate limiting in both cases (Loria and Pan 1999), consistent with the data reported here. Since in this model docking results in formation of metal ion binding sites associated with catalysis, then shifting the equilibrium to the docked state should result in a decrease in the concentration of $Mg^{2+}$ required to reach saturation, again consistent with the observations reported herein. Previous studies by Fierke and colleagues showed that shortening the leader sequence of a model pre-tRNA substrate decreased the apparent affinity of metal ion interactions necessary for cleavage (Kurz and Fierke 2002). However, preliminary results show that truncation of the 5′-leader sequence has no significant effect on the extent of catalytic enhancement due to C5 protein binding to P RNA (L. Sun, F.E. Campbell, and M.E. Harris, in prep.) consistent with an indirect effect of the protein rather than a direct effect due to contribution of functional groups or by substrate positioning interactions.

The ability of RNA-binding proteins to alter the apparent metal ion affinity of their cognate RNA ligands is well documented, yet providing direct experimental linkages between metal ion binding and function remains challenging. Both Group I and Group II self-splicing introns require essential $Mg^{2+}$ ion cofactors, and binding of maturase proteins essential for processing in vivo (Lambowitz and Zimmerly 2004; Stoddard 2006) results in significant decreases in the levels of $Mg^{2+}$ necessary to achieve optimal catalytic rates in vitro. While such effects are generally

attributable to metal ions involved in RNA folding, they are necessarily coupled to active site metal ion affinity, since formation of the active site obviously requires folding. While these proteins result in a single round of self-splicing, differential effects of P protein on the cleavage step for different substrates could provide a mechanism for substrate-dependent regulation. Clearly, the integral roles of P protein in both substrate recognition and catalysis rationalize the necessity of the protein subunit in vivo. However, the mechanism by which the protein alters catalytic metal ion affinity is not yet determined. Understanding this mechanistic basis is likely to help illuminate the important interplay between protein binding and RNA function.

## MATERIALS AND METHODS

The *E. coli* P RNA and pre-tRNA$^{Met608}$ were generated by in vitro transcription as previously described (Sun et al. 2006). The C5 protein was expressed in *E. coli* and isolated by affinity purification as described (Guo et al. 2006). The (+1 Rp) PS modified pre-tRNA (Rp[+1] pre-tRNA) was generated by ligation of a (−5, +6) 11 nt oligo (Dharmacon) and a (+7, +74) 3′ fragment of pre-tRNA$^{Met}$ generated by in vitro transcription (Zahler et al. 2005). As shown in Figure 3 this substrate gives rise primarily to cleavage at the correct phosphodiester bond 5′ to N(+1) of tRNA. An additional product migrating slower than the correct cleavage product was also observed, which is marked with an asterisk in Figure 3. We established that this product is an artifact arising from the ligation reaction. Observation of this specifies is independent of the presence of the phosphorothioate modification and is not observed with substrates generated by in vitro transcription. Formation of this product correlates with the appearance of the correct cleavage product. Because it is a minor product (<5%), rate determinations were not corrected to account of its formation.

For kinetic and thermodynamic studies, pre-tRNAs were 5′-end labeled with [$\gamma$-$^{32}$P]-ATP (MP Biomedicals) and T4 polynucleotide kinase after dephosphorylation by alkaline phosphatase. Single turnover kinetic analyses were performed essentially as described (Sun et al. 2006) with the following modifications. Rates were measured under the following conditions, with pH and metal ion concentration varied as indicated in text: 50 mM MES (pH 5.75), 100 mM NaCl, 17.5 mM $MgCl_2$, and 0.005% Triton X-100. Enzyme concentrations were determined to be saturating if the observed change in rate upon double the enzyme was less than 10%. Enzyme (1 μM RNase P or 10–30 μM P RNA) and 5′-$^{32}$P-end-labeled pre-tRNA (1–4 nM) were renatured separately in the absence of divalent metal ion by incubation at 95°C for 3 min, followed by decreasing the temperature to 37°C at 0.2°C per second and incubating for 10 min. $MgCl_2$ was then added to the desired concentration and the mixture was further incubated for 10 min. For holoenzyme reactions, C5 protein was then added (final concentration equal to P RNA), and the incubation was continued for another 10 min. Enzyme and substrate were mixed together to start the reaction, and aliquots were removed at predetermined intervals and quenched with EDTA at at least twice the concentration of divalent metal ion in the reaction. Reaction products were resolved by denaturing PAGE (20%). The conversion of substrate to product was quantified by phosphorimaging using a Molecular Dynamics system and ImageQuant software (Amersham). The data were plotted versus time using KaleidaGraph software (Synergy) and fit to a single exponential function:

$$F_c = A - Be^{-kt} \quad (1)$$

where $F_c$ is the fraction cleaved, $A$ is the maximal extent of the reaction, $B$ is the amplitude of the exponential, $k$ is the observed cleavage rate constant ($k_{obs}$), and $t$ is time. Rate constants reported are the average of at least three independent determinations, each with <10% error in the curve fit to the primary data.

Cleavage reactions in presence of increasing ($Mg^{2+}$) were performed essentially as described above. $k_{obs}$ was then plotted as a function of ($Mg^{2+}$) concentrations and fitted to a form of the Hill equation:

$$k_{obs} = \frac{k_{max}([Mg^{2+}]^n)}{(K_{Mg})^n + (Mg^{2+})^n} \quad (2)$$

where $k_{obs}$ is the rate constant measured at each $Mg^{2+}$ concentration, $k_{max}$ is the cleavage rate constant at saturating $Mg^{2+}$ concentration, ($Mg^{2+}$) is the $Mg^{2+}$ concentration, $K_{Mg}$ is the apparent dissociation constant, and $n$ is the Hill coefficient.

The pseudo-first-order rate constants were determined as described above in a series of increasing [$Cd^{2+}$]. The relative rate $k_{rel}$ was calculated from the ratio of the observed reaction rates of substrates with and without the sulfur substitution (e.g., $k_{rel} = k^S_{obs}/k^O_{obs}$). Plots of $k_{obs}$ versus ($Cd^{2+}$) were fit to the Hill equation as described above for $Mg^{2+}$ titration analyses.

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# Evidence that binding of C5 protein to P RNA enhances ribozyme catalysis by influencing active site metal ion affinity

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