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A re-investigation of the thio effect at the hammerhead cleavage site

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ABSTRACT

The effect of introducing a phosphorothioate at the hammerhead cleavage site was investigated using a kinetically well-characterized hammerhead. In buffers containing Mg ion, the R_P-phosphorothioate isomer cleaved 2000- to 80 000-fold slower than the S_P isomer or the unmodified RNA substrate. Addition of low concentrations of several thiophilic metal ions, especially Cd^{2+}, to these reactions is sufficient to fully restore the cleavage rate of the R_P substrate without affecting cleavage rate of the all-oxygen or S_P substrate. Thus, a model proposing coordination of a divalent metal ion to the pro-R oxygen at the hammerhead cleavage site appears justified.

INTRODUCTION

The hammerhead ribozyme is a small catalytic RNA molecule that catalyzes the cleavage of its backbone at a specific phosphodiester bond to generate products with 2′,3′-cyclic phosphate and 5′-hydroxyl termini (1,2). The reaction proceeds at a rate of ~1/min in millimolar concentrations of divalent metal ion, typically Mg^{2+}. When the hammerhead is prepared with a phosphorothioate linkage at the scissle phosphodiester bond, the cleavage rate is drastically altered, but only for the R_P isomer (3). The S_P isomer cleaves well and proceeds with inversion of configuration about the phosphorothioate bond (4–6). Although the cleavage rates of the R_P isomers reported in the previous studies vary, it is clear that the rate is reduced by more than a factor of 30. Studies of phosphorothioate containing dinucleotides, mononucleotides and uridine 3′-aryl derivatives show that there is no intrinsic difference in the reactivity of a phosphate versus a phosphorothioate linkage (7–10). Therefore, the slow rate of cleavage of the R_P isomer reflects disruption of the catalytic mechanism, similar to several protein enzymes catalyzing phosphoryl transfer reactions (reviewed in 11).

An important observation is that the cleavage rate of a hammerhead containing an R_P-phosphorothioate at the cleavage site is substantially increased in the presence of Mn^{2+} ion (3,4,6,12). Such a ‘manganese rescue’ phenomenon can be explained by the relative preference for different divalent metal ions to bind sulfur and oxygen. Experiments using nucleotide mono-, di- and tri-phosphates demonstrate that Mg^{2+} binds poorly to sulfur, whereas Mn^{2+} binds better (13,14). Restoration of hammerhead activity in Mn^{2+} led to a model proposing that Mg^{2+} binds to the pro-R_P oxygen at the cleavage site (3). This model is consistent with the observation that a crystal of an all-RNA hammerhead frozen shortly after the addition of Mg^{2+} showed a Mg^{2+} ion bound to pro-R_P oxygen of the cleavage site phosphate (15).

While restoration of hammerhead activity by manganese has been known for several years, a re-investigation was needed to address several concerns. The hammerhead substrate (and possibly the ribozyme) used for the original experiments folds into several stable alternate conformations (16). As a result, the K_m for this hammerhead is high (2.3 μM) meaning the original experiments were performed under subsaturating conditions (3). Thus, there is uncertainty as to the magnitude of the thio effect, as well as the step in the reaction pathway that is affected. Also, the original experiments were performed in Tris–HCl (pH 7.5) at varying concentrations of Mn^{2+}, with or without 0.5 mM spermine. These reaction conditions can potentially complicate interpretation of the results in several ways. First, since Mg^{2+} and Mn^{2+} differ in their propensity to bind the oxygen and nitrogen ligands present in RNA (17), a hammerhead in Mn^{2+} may fold slightly differently than a hammerhead in Mg^{2+}, and thus have different cleavage properties that are unrelated to the reaction occurring at the cleavage site phosphate. Secondly, at the pH used in the previous experiments, the Mn^{2+} concentration approached or exceeded its solubility limit (18). Finally, polyamines and Tris buffer bind Mg^{2+} and Mn^{2+} differently, also potentially complicating the interpretation of the experiment (19).

Recent work has questioned whether the Mn^{2+} rescue phenomenon is due to Mn^{2+} binding to the R_P-phosphorothioate at all (20). The authors propose that the effects of Mn^{2+} on the cleavage rate of thio-substituted hammerheads, derive solely from the lower pK_a of water molecules bound to Mn^{2+}, and not from the propensity of Mn^{2+} to bind sulfur. Their conclusion is based on the observation that, under certain conditions, replacing Mg^{2+} with Mn^{2+} increases the cleavage rate of an unmodified hammerhead by as much as the corresponding thio-modified hammerhead. A subsequent mechanistic proposal for hammerhead catalysis therefore did not include a metal ion coordinated to the pro-R oxygen of the cleavage site phosphate (21). In this paper, we re-investigate the rescue experiment using a kinetically well-characterized hammerhead under saturating conditions. A high concentration of Mg^{2+} or Ca^{2+} together with a much lower

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concentration of several different thiophilic metal ions were used. The reaction buffer was changed to ensure complete solubility of the metal ion and omit components that would compete for metal ion binding.

MATERIALS AND METHODS

RNA synthesis

Ribozyme 8 and substrate 8 (Fig. 1) were made by *in vitro* transcription and chemical synthesis, respectively, as described by Fedor and Uhlenbeck (22). The phosphorothioate linkage was introduced into the substrate molecule during chemical synthesis using the Beaucage reagent (3H-1,2-benzodithiole-3-one 1,1-dioxide) (23). Separation of R_P and S_P isomers of the thio-modified substrate was achieved by reverse phase HPLC (Waters 60F pump with 600E controller, and 400E UV/visible detector) on a C18 column (Beckman, ultrasphere, C18, 10 mm × 25 cm, semi-preparative) with the following buffers and buffer gradient: 0–5 min, 100% buffer A (0.1 M ammonium acetate, pH 6.8); 5–10 min, 95% buffer A, 5% buffer B (20% buffer A, 80% acetonitrile); 10–90 min, 89% buffer A, 11% buffer B; 90–120 min, column wash of 100% buffer B followed by 100% buffer A. The first peak eluted at 64.2 min and the second at 67.8 min. The leading peak was designated R_P due to its lower retention time (11). The identity of each isomer was also consistent with a hammerhead cleavage performed in previous studies (3,4,6,12) in which the R_P isomer cleaves more slowly than the S_P isomer. The phosphorothioate containing RNAs were stored in filter sterilized, distilled and deionized water at –20°C. No degradation due to repeated freezing and thawing was observed.

Metal ion stock solutions

Stock solutions of 2 M $MgCl_2$ and $CaCl_2$ were prepared by dissolving the salt in water through which argon had been bubbled for 1 h to displace the dissolved CO_2, and by adding concentrated nitric acid to a final concentration of 100 mM. The completed stock solutions were filter sterilized using a 0.2 µm cellulose acetate membrane. The HNO_3 ensured that the pH of the stock solution remained low and the metal ions were completely solubilized. Stocks of 0.1 M $MnCl_2$, $CoCl_2$ and $CdCl_2$ were similarly prepared. The presence of HNO_3 in the metal stocks was not enough to effect the pH of the well-buffered reactions after dilution to reach the final metal ion concentrations used. All metal ions were stored at –20°C and thawed for use in the reaction buffers. The metal-chloride salts were the purest grade available from Aldrich Chemical Co. (Milwaukee, WI) and contained the following trace impurities: $MgCl_2$ [(99.995%) Na 2 p.p.m.]; $CaCl_2$ [(99.99%) Pb 60 p.p.m.]; $MnCl_2$ [(99.99%) Pb 35 p.p.m., Co 3 p.p.m.]; $CoCl_2$ [(99.999%) no trace impurities]; $CdCl_2$ [(99.99%) Au 20 p.p.m.].

Kinetics

Single turnover reactions were performed as described previously (22) with minor changes. Aliquots of 50 mM MOPS and PIPES buffers were used at pH 6.5–7.0. The RNAs were heated to 95°C for 1 min in buffer without metal ions in order to dissociate aggregates formed upon freezing. Following cooling of the ribozyme to room temperature, both the ribozyme and substrate

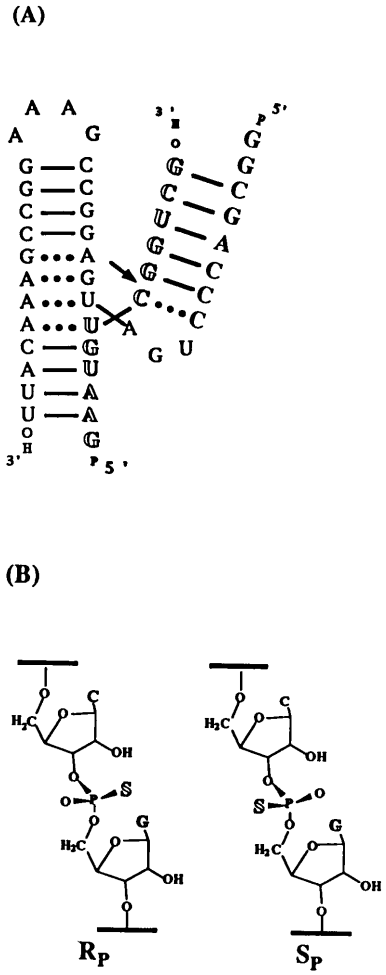

Figure 1. HH8 and phosphorothioate isomers. (**A**) HH8 is comprised of a 12 nt substrate (open letters) and a 34 nt ribozyme. The arrow indicates the cleavage site where the phosphorothioate linkages were incorporated. (**B**) The R_P- and S_P-phosphorothioate isomers.

strands were incubated briefly in separate tubes with $MgCl_2$ and the appropriate thiophilic metal. Reactions were initiated by adding ribozyme solution to substrate solution. Reaction rates at saturating ribozyme concentrations varied by <30% when the reaction rate exceeded 0.01/min. All values are the averages of at least two determinations.

In order to measure the very slow cleavage rate of R_PS8, 40 µl reactions were prepared containing 50 mM MOPS (pH 7.0), 1.5 or 0.5 µM ribozyme, and trace (<1.0 nM) 5′-^{32}P-R_PS8. The reactions were heated to 95°C for 1 min and then cooled to 25°C for 15 min. The reactions were initiated by adding $MgCl_2$ to a final concentration of 3, 10, 33, 90 or 500 mM. A 1 µl aliquot from each reaction was immediately quenched in 10 µl of stop-buffer (80% formamide, 100 mM EDTA, 0.02% xylene cyanol and bromophenol blue dyes). The remaining reaction was then drawn up into 5 µl capillary tubes in 1 µl aliquots and sealed at both ends. The capillaries were placed in water and incubated at 25°C. At appropriate time intervals over a period of 3 days, capillaries were opened and the contents quenched in 10 µl of stop-buffer. The capillaries ensured that no loss of reaction volume occurred as a

result of evaporation during the long incubation. The quenched aliquots were analyzed on a 20% polyacrylamide gel and the amounts of cleaved and uncleaved substrate determined by using a Molecular Dynamics radioanalytical scanner. A total of 3–5% of the substrate was cleaved in the first 3 h, reflecting contaminating substrate molecules with either oxygen or S_P-phosphorothioate linkages. From 3 h to 3 days, 13–18% additional substrate was cleaved. The data for the slower cleaving fraction of substrate was fit to a linear equation (Kaleidagraph) to determine the rate of cleavage. No detectable cleavage at other phosphate linkages occurred during the 72 h incubation. Reaction rates are the averages of two determinations which varied by <3-fold.

RESULTS

The kinetically well-characterized hammerhead 8 (HH8) was used to quantitate the effects of replacing the cleavage site phosphate of the hammerhead ribozyme with an R_P- or S_P-phosphorothioate linkage (Fig. 1). HH8 is an excellent system in which to perform such experiments. The short substrate simplifies the separation of R_P- and S_P-phosphorothioate isomers. Native gel analyses do not reveal stable alternate conformations of HH8 which could potentially interfere with kinetic analysis (22). Its reaction pathway is well characterized and it has a k_{cat} of 1/min at pH 7.5, 10 mM $MgCl_2$ and 25°C, which reflects the chemical step (k_2) (22). Under these conditions, the binding affinity of substrate to ribozyme is 50 nM (16) and thus, saturation is easily achieved. HH8 has been used to study how modifications of residue 17 affect the kinetics and thermodynamics of the reaction (24). For the reactions reported here, single turnover conditions using a saturating concentration of ribozyme (0.5 or 1.5 μM) with <1 nM substrate were used, since the available amounts of purified R_P and S_P thio-modified substrates were limiting.

Values of k_2 were initially determined by using ^{32}P-labeled, unmodified (S8) or thio-modified (S_PS8 or R_PS8) substrates at saturating ribozyme concentrations (Table 1). The value of k_2 = 0.18/min for S8 at pH 7.0 agrees well with k_2 = 1/min at pH 7.5 reported by Dahm *et al.* (25). The k_2 of 0.17/min for S_PS8, essentially the same as S8. In contrast, R_PS8 has a k_2 of only 1×10^{-5}/min, which is ~18 000-fold slower than S8 and only slightly faster than 10^{-6}/min obtained as the background rate of cleavage of RNA in a similar buffer (26,27). Since the values of k_2 for all three substrates are essentially the same at 0.5 and 1.5 μM ribozyme, all three hammerheads are at saturation.

Table 1. Cleavage rates of HH8 assembled with unmodified, S_PS8 or R_PS8 thio-modified substrates as a function of [$MgCl_2$]

$MgCl_2$ (mM)	k_2[a] (/min) for substrates			Thio effect[b]
	Unmodified S8	S_PS8	R_PS8	
3	0.12	0.06	1.5×10^{-6}	80 000
10	0.18	0.17	1.0×10^{-5}	18 000
33	0.69	0.40	7.0×10^{-5}	10 000
90	2.4	2.5	1.4×10^{-4}	16 000
500	3.0	–	1.2×10^{-3}	2000

[a]Cleavage rates of HH8 with 1.5 μM ribozyme, trace (<1.0 nM) 5′ end labeled substrate, 50 mM PIPES (pH 7.0) and varying amounts of $MgCl_2$.
[b]The thio effect is the ratio of the cleavage rates of the unmodified and R_PS8 substrates.

In order to isolate the effect at the cleavage site from more general effects of Mn^{2+} on hammerhead structure, the rescue experiment should be performed in the presence of a high concentration of Mg^{2+} ions so that a majority of the metal binding sites are filled with Mg^{2+}. Since some reports suggested that the R_P isomer can cleave at high Mg^{2+} concentrations (3,4), the cleavage rates of R_PS8 and S_PS8 with S8 were first compared at several concentrations of Mg^{2+}. The results of these experiments are shown in Table 1. The cleavage rate for S8 increases with the Mg^{2+} concentration in a manner similar to that observed previously (25). The cleavage behavior of the S_P substrate is essentially the same as S8. In contrast, the R_P S8 reaction remains slow at all Mg^{2+} concentrations. Even at 0.5 M Mg^{2+}, the cleavage rate of R_PS8 is 2000-fold slower than S8.

Since any concentration of Mg^{2+} can be used for the rescue experiment, we chose 50 mM, which is near saturation for HH8 (25). At this concentration, it is less likely that the rescue metals will compete for other binding sites for metals on the hammerhead which could perturb the reaction in a manner unrelated to the thio-modification. Mn^{2+}, Co^{2+} and Cd^{2+} were tested as rescue metals. All are known to prefer sulfur over oxygen ligands relative to Mg^{2+}, but each possess different coordination properties and could potentially fit into the cleavage site differently (14). Lower concentrations of thiophilic metals were used so that they would not compete with Mg^{2+} at other sites in the hammerhead and not contribute significantly to the total concentration of hydroxy cation in the reaction. At these concentrations, each of the metal ions is completely soluble at ≤pH 7.0 (18). The temperature of the reaction was also lowered to 10°C in order to slow the cleavage rate so that it could be measured accurately. The results of the initial rescue experiments are shown in Table 2. The addition of only 200 μM Mn^{2+} increases the rate of R_PS8 cleavage by >500-fold, which agrees with the trend observed in previous experiments (3,4,12). In contrast, the oxygen substrate only increases by ~5-fold when 200 μM Mn^{2+} is added. Thus, it is clear that low concentrations of Mn^{2+} increase the rate of R_PS8 substrate cleavage much more than S8 cleavage. As the concentration of Mn^{2+} is further increased, the cleavage rates of S8 and R_PS8 both slowly increase, suggesting that Mn^{2+} may participate at an additional site, as well. The data for adding various Co^{2+} concentrations are virtually identical to the Mn^{2+} data; however, the Cd^{2+} data are somewhat different. At 200 μM Cd^{2+}, the cleavage rate for S8 is not affected while the rate of R_PS8 is slightly higher than for S8. At Cd^{2+} concentrations >200 μM, the cleavage rates of both S8 and R_PS8 increase slowly. Therefore, low concentrations of all three thiophilic metal ions differentially stimulate the rate of R_PS8 with respect to S8, even in the presence of a large excess of Mg^{2+}. Of the three thiophilic metal ions, Cd^{2+} is the most effective at rescuing the cleavage rate of R_PS8, which correlates well with its greater affinity for sulfur, compared with Mn^{2+} and Co^{2+} (14).

In order to more carefully investigate Cd^{2+} rescue, a more extensive titration was performed, using Cd^{2+} concentrations ranging from 2.5 to 600 μM. The reaction conditions were identical except that 25°C was used, since all the rates can be accurately measured at this temperature. As shown in Figure 2A rescue by Cd^{2+} is even more clearly demonstrated. When the concentration of Cd^{2+} is <200 μM, the rate of the S8 substrate does not change significantly. In contrast, the cleavage rate of R_PS8 increases with increasing Cd^2 with a K_{app} for Cd^{2+} of ~50 μM. Thus, at 66 μM Cd^{2+}, the cleavage rate of R_pS8 is

identical to that of S8 with (or without) the same concentration of Cd^{2+}. Thus, one can conclude that Cd^{2+} promotes full rescue of the phosphorothioate substrate.

Table 2. Cleavage rate (k_2) for S8 and R_PS8 at high concentrations of thiophilic metal ions

	k_2/min	
[$MnCl_2$] mM	**S8**	**R_PS8**
0.0	0.2	$<3 \times 10^{-4}$
0.2	1.1	0.1
0.6	1.4	0.1
2.0	2.1	0.2
6.6	2.8	0.6
[$CoCl_2$] mM	**S8**	**R_PS8**
0.0	0.2	$<3 \times 10^{-4}$
0.2	1.2	0.1
0.7	1.5	0.3
2.0	(–)	0.5
6.6	2.9	(–)
[$CdCl_2$] mM	**S8**	**R_PS8**
0.0	0.2	$<3 \times 10^{-4}$
0.2	0.2	0.5
0.7	0.4	1.0
2.0	0.4	1.5
6.6	0.9	1.2

Cleavage rates (k_2) were determined at 1.5 µM ribozyme, trace 5′ end labeled substrate, in 50 mM PIPES (pH 7.0), 50 mM $MgCl_2$, and the indicated concentrations of $CdCl_2$, $CoCl_2$ and $MnCl_2$ at 10°C. Cleavage of the R_PS8 substrate was not carried out for as long as in Table 1.

A similar Cd^{2+} titration experiment was performed with 100 mM Ca^{2+} instead of 50 mM Mg^{2+} (Fig. 2B). Ca^{2+} supports hammerhead cleavage in a manner similar to Mg^{2+}, but the rate is 15-fold slower at a given concentration. The slower rate has been attributed to the 15-fold lower concentration of $[Ca(OH)]^+$ in the reaction (25), but could be due to a slightly altered structure of the hammerhead in Ca^{2+} or to the binding of Ca^{2+} instead of Mg^{2+} to the 2′ oxygen (28) or another oxygen in the transition state. In 100 mM $CaCl_2$, the cleavage rate of S8 is 0.06/min and R_PS8 is 8.5×10^{-5}/min giving a 700-fold thio effect. The general features of the rescue experiment are quite similar to when Mg^{2+} was used. Complete rescue of the R_pS8 substrate is achieved between 44 and 66 µM Cd^{2+}. However, at these concentrations of Cd^{2+}, the cleavage rate of the S8 substrate is also slightly enhanced. Either Cd^{2+} is slightly more effective at competing with Ca^{2+} than with Mg^{2+} or the slower absolute rate in Ca^{2+} makes the reactions more sensitive to the added Cd^{2+}.

DISCUSSION

The thio-modification of the cleavage site pro-R_P oxygen reduces the cleavage rate by ≥1000-fold under various reaction conditions. Considering the total catalytic potential of the hammerhead, the effect of the thio-modified substrate is very large. At 10 mM Mg^{2+}, the hammerhead enhances the cleavage rate ~1 000 000-fold over the non-catalyzed rate, whereas the phosphorothioate hammerhead enhances the cleavage rate by ~10-fold. Thus, the R_P-phosphorothioate substitution at the cleavage site is one of a growing number

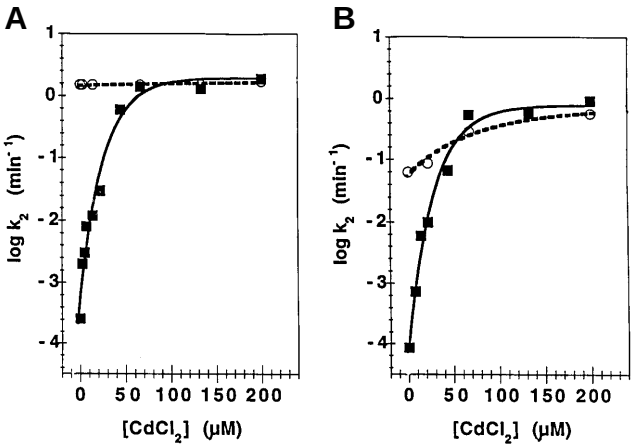

Figure 2. The effect of $CdCl_2$ concentration on the cleavage rate of HH8 in 50 mM PIPES (pH 7.0) and either 50 mM $MgCl_2$ (**A**) or 100 mM $CaCl_2$ (**B**) at 25°C with the oxygen substrate (○) and the R_P-phosphorothioate substrate (■). The lines are drawn to fit the data points.

of structurally moderate mutations that cause very large transition state effects in the hammerhead. For instance, an R_P-phosphorothioate at position 9 reduces k_2 1000-fold, and replacement of G5 with an adenosine or abasic residue causes a 100 000-fold effect (29,30). It should be noted, however, that not all structurally moderate mutations give large transition state effects in the hammerhead. For example, in a recent analysis of functional group mutations on nucleotide C17, most mutations produced much smaller transition state effects (24).

Although qualitatively in agreement with previous studies using different hammerheads, our data for HH8 indicates that an R_P-thiophosphate at the cleavage site is much more deleterious than was previously indicated. Six other studies report reductions in cleavage rate varying from 25-fold to 1000-fold (Table 3). The discrepancies among the studies exist both in the reported rates for the R_P-phosphorothioate substrates and in the rates of the unmodified substrates. It is unlikely that these discrepancies reflect an intrinsic difference of the R_P isomer on the chemical step for the different hammerhead sequences. In recent reports, the same hammerhead mutation was examined in two or more kinetically well-characterized systems, and a very similar effect was observed (24,31). Instead, the variation among the reported thio effects probably derives from the substantial experimental difficulties in obtaining reliable kinetic data for many hammerhead ribozymes (32). One such difficulty is that some of the studies used hammerheads that formed alternate conformations, making measurement of the chemical step more difficult, even for the unmodified control hammerhead. For example, in the previous measurements of the cleavage-site thio effect from this laboratory (3), the hammerhead used contained alternate conformations in the ribozyme, and perhaps in the substrate strand, resulting in an unusually high K_m (2.3 µM) (16). Thus, the reported rates were obtained under subsaturating conditions that partially reflect rate limiting association of ribozyme and substrate. Several other of the hammerheads in Table 3 also form alternate conformers, as is discussed in greater detail elsewhere (32). However, alternate conformations would not generally be expected to greatly alter the thio effect, unless the phosphorothioate substitution changed the proportion of alternate conformers.

Table 3. Comparison of the cleavage site thio effects in various hammerheads

Hammerhead	$k_{observed}$/min^{-1}[a] substrates			Thio effect[b]	Reference
	Oxygen	R_P	S_P		
HH8	0.18	10^{-5}	0.17	18 000	This work
HH10	0.15	0.003	(n.d.)	50	(3)
R37	0.5[c]	0.015[d]	0.25	33	(4)
CL-2,4	0.25	0.0045	0.70	55	(6)
R32	0.086	0.00016	0.003	540	(20)
HH3	0.93	0.00093	0.85	1000	(12)
R34	0.25	0.01	0.12	25	(37)

[a]$k_{observed}$ refers to the rates reported in the various works for an unmodified hammerhead substrate and substrates containing either R_P or S_P thiophosphate at the cleavage site. The reported values do not necessarily reflect the chemical step in the hammerhead reaction.
[b]The thio effect is the ratio of $k_{observed}$ for the oxygen and R_P-phosphorothioate substrates.
[c]Multiple turnover reactions containing a 1:30 ratio of ribozyme to substrate gave a $t_{1/2}$ of 32/min, from which an estimated cleavage rate of 0.5/min was calculated.
[d]Calculated from 12% substrate cleaved in 240 min.
(n.d.) indicates that the value was not determined.

A second experimental difficulty in obtaining a reliable estimate of the thio effect at the cleavage site, derives from the inevitable presence of a small amount of contaminating S_P-phosphorothioate or phosphate linkage in the R_p substrate oligonucleotide that either originates during purification or as a result of desulfurization. Thus, when measuring the cleavage rate of the R_p substrate, it is critical not to use data from the early time points which will primarily reflect cleavage of the faster cleaving impurities. This problem is compounded if the reactions are performed using substrate excess (4). In such a case, some of the impurities will not be able to bind the ribozyme and cleave until a previously bound R_p substrate cleaves or dissociates. Thus, even the faster cleaving impurities will cleave throughout the reaction, thereby overestimating the rate of cleavage of the phosphorothioate substrate. A final difficulty in obtaining a reliable measure of thio effects is that the buffer used in the reaction must not contain even trace amounts of thiophilic metals. As shown above, cleavage of the R_P substrate can be observed even when a metal-impurity is present at 2000-fold lower concentrations (e.g. 2.5 μM Cd^{2+} in 50 mM Mg^{2+}). In our experience, many sources of Mg^{2+} salts have sufficient impurities to stimulate the cleavage of the R_P-phosphorothioate substrate especially when used at higher concentrations. Presumably, one or more of the above problems have led to an overestimate of the R_P isomer cleavage rate in all of the previous studies. However, additional experiments would need to be done with each of the systems previously reported in order to be certain.

The very large reduction in the cleavage rate was obtained under conditions designed to avoid the problems described above. HH8 appears to be free of alternate conformations (16) and substrate dissociation is very rapid. Thus, by performing a cleavage reaction over the course of several days and only using the later time points, cleavage rate of the R_P substrate can be accurately determined. It is probably best to consider these data as an upper limit of the cleavage rate for the R_P isomer, since some desulfurization to oxygen probably occurs during the course of the long incubation. The rate of desulfurization for a dinucleotide phosphorothioate is 1×10^{-6}/min (7), or close to the background rate of hydrolysis of RNA. A ribozyme excess experiment with a hammerhead that cannot exchange its substrate, such as hammerhead 16 (29), would be a valuable system in which to confirm this large cleavage-site thio effect.

Unlike most previous rescue experiments, the experiments here were performed in the presence of a higher concentration of a 'folding' metal ion (either 50 mM $MgCl_2$ or 100 mM $CaCl_2$). This mixed metal ion approach has been used successfully in rescue experiments in the group I intron (10,33,34). It has several advantages over protocols which completely change the identity of the metal ion. The high concentration of the Mg^{2+} or Ca^{2+} ensures that the hammerhead is fully folded. Likewise, it ensures that most metal ion binding sites in both the thio-modified and unmodified hammerheads with Mg^{2+} are partially or fully saturated. As a result, the added thiophilic metal ions can only minimally interact with sites other than the one bearing the sulfur ligand. In addition, the low concentration of the thiophilic 'rescue' metal ion used in these experiments minimizes change in the total concentration of [$M(OH)^+$], that is believed to promote cleavage (25). The only disadvantage of using the high concentration of Mg^{2+} or Ca^{2+} is that they can potentially compete with the rescue metal ion, and thereby increase the amount needed for full restoration of activity.

All three thiophilic metal ions tested (Mn^{2+}, Co^{2+} and Cd^{2+}) promote clear rescue of the hammerhead containing an R_P-thiophosphate at the cleavage site. In each case, the cleavage rate increased from $<10^{-4}$/min to a value that approached the oxygen control, when 200 μM metal ion was added. At 200 μM, there were small differences in the cleavage rate of the oxygen control among the three metal ions. Mn^{2+} and Co^{2+} increased the cleavage rate, whereas Cd^{2+} did not. This result presumably reflects the different ability of these metal ions to either bind another site on the hammerhead, or to compete with Mg^{2+} for the pro-R_P oxygen at the cleavage site. More careful titration with Cd^{2+} in both Mg^{2+} and Ca^{2+} reinforces this conclusion. Cd^{2+} is capable of fully restoring cleavage activity for the R_P substrate at concentrations as low as 66 μM, and without affecting the activity of the unmodified hammerhead. The very large stimulation of the cleavage rate in the presence of Cd^{2+} may simply reflect the occupation of thiophilic metal in the transition state. If so, then the corresponding binding constant for Cd^{2+} in the transition state is much tighter. Therefore, a more favorable transition state binding of the cleavage-site metal ion helps promote catalysis. The much tighter binding of Cd^{2+} in the transition state suggests that the metal ion makes interactions in the transition state that are not made in the ground state.

The precise coordination properties of this metal ion at the cleavage site of the hammerhead are not yet fully defined. In the freeze-trapped RNA hammerhead (15), a Mg^{2+} ion was observed within 2.2–2.4 Å of the pro-R_P oxygen, which is consistent with direct coordination. No other atoms within the RNA are close enough to imply direct coordination to the Mg^{2+}, suggesting that the remaining coordination sites are occupied by water molecules. However, the resolution of the structure is sufficiently low that additional ligands to the Mg^{2+} may be revealed upon further refinement. In addition, it is possible that the structure in the transition state is significantly different than in the crystal structure (29), and that additional ligands to the Mg^{2+} are formed in the transition state.

In many respects, our analysis of Cd^{2+} rescue of the phosphorothioate at the cleavage site reported here, closely resembles the recent analysis of Cd^{2+} rescue at phosphate 9 in the hammerhead

(29). In both cases, the cleavage rate was reduced >1000-fold by the substitution of a sulfur atom. This decrease in the cleavage rate was fully rescued by Cd^{2+} with a K_{app} in the range of 25–50 μM in the presence of relatively high Mg^{2+} concentrations. The presence of metal ion at phosphate 9 is also supported by X-ray crystallographic data (35,36), but the interaction is quite different, with coordination to the pro-R_P oxygen and also the N7 of G10.1. While the binding of a metal ion to the cleavage site phosphate is consistent with numerous mechanistic proposals, a similar role for phosphate 9 is much less clear, considering that it is very far from the cleavage site. A large scale conformational change has been proposed that places the metal at P9 closer to the cleavage site (29).

The conclusions presented in this paper agree with a recent report examining the interaction of the very large and very thiophilic divalent ion Hg^{2+} with a hammerhead containing an R_P-phosphorothioate at the cleavage site (37). Full rescue of the cleavage rate is observed with very low concentrations of Hg^{2+}. Evidence for direct coordination of the Hg^{2+} with the sulfur is suggested by a UV absorption change that may be a charge-transfer band.

The conclusions presented in this paper disagree with those of Zhou *et al*. (20) who argued that all the published Mn^{2+} rescue data could be explained in terms of the higher concentration of metal hydroxides present in the rescue reactions due to the lower pK_a of Mn^{2+}-bound water molecules. This argument implies that Mn^{2+} binding to the phosphorothioate at the cleavage site does not occur. Indeed, experiments where the cleavage rate of the phosphorothioate hammerhead is measured solely in the presence of Mn^{2+} ion are difficult to interpret, because of the requirement for divalent ions to promote hammerhead folding. However, by using mixed metal ion protocol, we show that complete rescue can be achieved without significantly changing the total metal hydroxide concentration. For example, in the presence of 50 mM Mg^{2+} ion, the addition of 66 μM Cd^{2+} ion fully rescued the cleavage rate, while only increasing the total metal hydroxide concentration from 1.8 to 2.5 μM. As expected, no detectable change in the cleavage rate of the unmodified hammerhead was observed. Thus, the earlier conclusion that a metal ion must be bound to the R_P phosphorothioate for hammerhead cleavage to occur remains valid. It is, however, important to point out that the presence of a metal ion coordinated to the pro-R oxygen in the unmodified hammerhead is only inferred. It is possible that the presence of the sulfur 'recruits' the metal ion to the site and the reaction proceeds with a different mechanism than when oxygen is present. However, especially in light of the crystallographic evidence, the earlier suggestion that a metal ion is bound to the pro-R_P oxygen remains reasonable and should be part of any mechanistic model for hammerhead catalysis.

The role of this metal ion in the catalytic mechanism remains unexplained. It may act as a Lewis acid to increase the susceptibility of the phosphate to nucleophilic attack by the 2′ oxygen. It may also help position the phosphate in its transition state structure in the way similar to that proposed for several protein enzymes (38). Additional rescue experiments with hammerheads containing sulfurs at other positions near the cleavage site phosphate should help to clarify the location and role of metal ions in the transition state structure of the hammerhead.

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