Biochemistry **2000,** *39,* 4947−4954

Hammerhead Cleavage of the Phosphorodithioate Linkage

Wesley B. Derrick, Charles H. Greef,‡ Marvin H. Caruthers, and Olke C. Uhlenbeck*

Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309-0215

Received January 21, 2000

ABSTRACT: Under standard reaction conditions, a hammerhead ribozyme with a phosphorodithioate linkage at the cleavage site cleaved to the expected products with a rate about 500-fold slower than the corresponding phosphodiester linkage. When the greater stability of the dithioate linkage to nonenzymatic nucleophilic attack is taken into account, the hammerhead is remarkably effective at cleaving the dithioate linkage considering that the R_P-phosphoromonothioate linkage is virtually inactive. On the basis of experiments determining the Mg^{2+} concentration dependence of the cleavage rate and the stimulation of cleavage by thiophilic Cd^{2+} ion, the lesser catalytic rate enhancement of the dithioate linkage is primarily due to the loss of a single Mg^{2+} ion bound near the cleavage site. These results are qualitatively similar to, but quantitatively different from, similar experiments examining the hammerhead cleavage properties of the R_P-phosphoromonothioate linkage. The dithioate linkage thus promises to be a valuable alternative phosphate analogue to the monothioate linkage in studying the mechanisms of RNA catalysis.

The hammerhead is an RNA motif that catalyzes the cleavage of a specific phosphodiester to yield 5′ hydroxyl and 2′,3′-cyclic phosphate products (*1*). This cleavage activity represents an approximately 10^6-fold rate enhancement over the uncatalyzed background rate under standard conditions (*2*). Exactly how this RNA motif enhances the cleavage is not fully understood (*3*, *4*), although the sequence and base substituent requirements for hammerhead activity have been studied extensively (*5*).

An important clue to the mechanism of hammerhead catalysis involves experiments where one of the nonbridging oxygens of the scissile phosphodiester was substituted with sulfur, forming either the R_P- or S_P-phosphorothioate linkage. Hammerhead cleavage of the R_P isomer was reduced by more than 10^4-fold (*6*−*9*), while cleavage of the S_P isomer is unaffected and occurs at the same rate as the all-oxygen phosphodiester. As there is no intrinsic difference in susceptibility to intramolecular transesterification between the two phosphorothioates and the unmodified phosphodiester (*10*−*13*), the lower cleavage rate of the R_P isomer must represent some disruption of the catalytic mechanism of the hammerhead. An important observation is that the introduction of low concentrations of a thiophilic metal ion such as Cd^{2+}, Co^{2+}, or Mn^{2+} restores the ability of the hammerhead to cleave the R_P-phosphorothioate (*6*−*9*, *14*). This “rescue” of cleavage by thiophilic metal ions suggests that the mechanism of cleavage of the R_P-phosphorothioate substrate involves direct coordination of the thiophilic metal ion to the sulfur. This led to the proposal that a similar interaction between the *pro*-R_P oxygen and a Mg^{2+} ion occurs in the mechanism of cleavage for oxygen substrates (*7*). Since Mg^{2+} cannot coordinate well to sulfur (*15*, *16*), the activity of the R_P-phosphorothioate hammerhead is reduced.

A divalent metal ion bound to the *pro*-R_P oxygen of the scissile phosphate can promote catalysis in several ways. The most straightforward is that it acts as an electrophilic catalyst by neutralizing the negative charge on the nonbridging oxygen, making the phosphate more susceptible to attack by the 2′-oxygen, much like Lys41 in the RNase A mechanism (*17*, *18*). However, the metal ion could have additional roles in the mechanism as well, including acting as a base to increase the nucleophilicity of the 2′-oxygen or as a Lewis acid to stabilize the leaving oxygen (*3*, *4*, *19*). Several have suggested that the *pro*-R_P oxygen may bind more than one metal ion in performing these functions (*20*−*22*). Recently, Wang et al. (*23*) have presented evidence that a single metal ion bound to the *pro*-R_P oxygen also has an important structural role in helping to stabilize the hammerhead in a catalytically active configuration.

Given that the bulk of the evidence for the importance of metal bound to the *pro*-R_P oxygen in hammerhead catalysis is based a single derivative of phosphate, it is valuable to examine the hammerhead cleavage properties of a different derivative of phosphate where both of the nonbridging oxygens were substituted with sulfur. The dithioate linkage (*24*), like the R_P-phosphoromonothioate, should also block the coordination of magnesium. However, unlike the phosphoromonothioate (*25*, *26*), the dithioate has a more symmetric charge distribution about the phosphorus and thereby more closely resembles phosphate. By examining hammerhead catalysis with this different derivative, the importance of the metal ion in catalysis could be verified and any effect of the asymmetric sulfur substitution of the R_P-phosphorothioate could be evaluated.

MATERIALS AND METHODS

Oligonucleotides. R8 and R16, the ribozymes for the well-characterized HH8 (*27*) and HH16 (*28*), were synthesized by in vitro transcription of partially duplex synthetic DNA by T7 RNA polymerase (*29*). Transcription products were

---

* Corresponding author: email: Olke.Uhlenbeck@Colorado.EDU; Telephone: (303) 492-6929.

‡ Present address: Nanogen, 10398 Pacific Center Ct., San Diego, CA 92121.

10.1021/bi000146a CCC: $19.00

purified on denaturing polyacrylamide gels, concentrated by absorbing to DEAE-650S resin and eluting with 300 mM sodium acetate, and desalted by twice ethanol-precipitating. RNA was quantified by measuring its absorbance at 260 nM, assuming a residue $E_{260} = 8 \times 10^3\ M^{-1}$. All reagents and solvents utilized were of the highest purity due to concern about the inclusion of extraneous metal ions, particularly thiophilic metal ions. Water was twice glass-distilled. Buffers were made from the highest purity reagents available. $MgCl_2$ stocks were 99.999% pure from Alpha Aesar. Gels were made from ultrapure acrylamide and were preelectrophoresed extensively prior to use.

Substrate RNAs, S8 and S16, were synthesized chemically with standard ribonucleotide phosphoramidites and procedures (*30*). Phosphorothioate-substituted RNAs were synthesized chemically by modified phosphoramidite chemistry (*31*) where the growing nucleotide chain was sulfurized with Beaucage reagent (*32*). The RNA phosphorodithioate substrates were synthesized by a modification of H-phosphonothioate chemistry (*24*). ^{31}P NMR analysis of the dithioate substrate S8 revealed a single dithioate linkage (116 ppm downfield shift) and no appreciable monothioate linkages. Previously, shifts of 110−115 ppm have been observed for dithioate DNA (*33*, *34*). Each of the substrates was purified as described above. Separated R_P and S_P stereoisomers for kinetic characterization were obtained by reverse-phase HPLC as described previously (*8*, *26*). Phosphorodithioate S8 RNA was subjected to further purification by anion-exchange chromatography on a pa-100 HPLC column at 55 °C with a gradient from 0.2 to 0.8 M NH_4Cl at 1.2 mL/min which separates, with baseline resolution, the product from any contaminating phosphorothioate S8 RNA.

Cleavage Reactions. Cleavage of the all-oxygen and mono- and dithioate substrates of HH8 were performed by preincubating 2 μM R8, a trace concentration (<1 nM) of (5′-^{32}P) labeled substrate, 100 mM tricine (pH 8.0), and 1 mM EDTA at 25 °C for 10 min. Reactions (final volume = 20 μL) were initiated by addition of 11 mM $MgCl_2$. Reactions at other pH values employed 100 mM MES (pH 6.0), Bis-Tris (pH 6.5), PIPES (pH 7.0), HEPES (pH 7.5), and TAPS (pH 8.5). At least 10 1-μL aliquots were removed at desired times and quenched by addition of 10 volumes of stop dye (7 M urea, 10 mM EDTA, 0.05% xylene cyanol, and 0.05% bromophenol blue). Unless loaded directly onto gels, quenched samples were frozen and stored at −70 °C. Labeled substrate and product were separated on 20% denaturing polyacrylamide gels and quantified by phosphorimaging as previously described (*35*). Duplicate reactions gave rates that differed by less than 20%.

Large-scale (100 μL) cleavage reactions for the HPLC analysis of products were performed with 10 μM substrate and 1 μM R8 (for all-oxygen and phosphorothioate substrates) or 5 μM R8 (for dithioate) in 10 mM $MgCl_2$ and 50 mM Tris-HCl (pH 8) incubated at 25 °C for 20 h. Substrates and products were analyzed by use of anion-exchange HPLC as described previously for purification of substrates. The 5′ product of the cleavage of dithioate S8, P1, was collected, desalted, and analyzed by zero time-of-flight electrospray mass spectrometry.

Cleavage reactions utilizing a thiophilic ion-specific chelator, diethyl dithiocarbamate, were carried out in a manner similar to the normal kinetic reactions but without EDTA. Reaction mixtures contained trace concentrations (<1 nM) of end-labeled phosphorodithioate S8, 100 mM PIPES (pH 7.0), and 10 mM $MgCl_2$ and were preincubated with or without 2 mM diethyl dithiocarbamate for 10 min or with 0.5 mM $CdCl_2$ for 5 min followed by addition of 2 mM diethyl dithiocarbamate and preincubated for an additional 10 min. All cleavage reactions were then initiated by the addition of 2 μM R8.

Rates of uncatalyzed cleavage of S8 and the mono- and dithioate analogues were determined by preincubation of trace concentrations of (5′-^{32}P) labeled oligonucleotides in 100 mM Tricine (pH 8.0) and 1 mM EDTA for 10 min at 25 °C. The reactions were initiated by the addition of 11 mM $MgCl_2$, and time points were taken over a period of several days, frozen, and subsequently analyzed on polyacrylamide gels. The cleavage rate with inactive ribozyme was measured for each substrate by incubating it with 2 μM of the G5A mutation of R8. In the case of the phosphorothioate and phosphodiester RNAs, an approximately equal amount of cleavage was seen at each internucleotide linkage, indicating that the linkage at the hammerhead cleavage site was no different than that of a typical phosphodiester bond. The rate of uncatalyzed cleavage was calculated from the rate of increase as the fraction of P1, the 7-mer corresponding to the 5′ hammerhead cleavage product. However, in the case of the uncatalyzed cleavage of the dithioate substrate, it was evident from the polyacrylamide gel of the products that the dithioate linkage was more stable than neighboring phosphodiester linkages. This made it necessary to correct the rate of increase of the fraction of P1 by the amount of disappearance of S and P1 due to comparatively rapid cleavage at one of the other phosphodiester linkages. Failure to use this correction leads to an overestimate of the phosphorodithioate cleavage rate by a factor of 2−3.

Rates of hammerhead cleavage at different Mg^{2+} concentrations were used to determine the apparent metal ion binding affinity for the unmodified and dithioate hammerheads. Reactions containing S8(oxy) were initiated by addition of appropriate Mg^{2+} stock solutions to 100 nM R8 and 4 μM S8 in 50 mM HEPES (pH 7.5). Cleavage reactions to probe the apparent Mg^{2+} affinity for HH8 cleavage of S8(dithioate) were initiated by addition of trace radiolabeled substrate to 3.8 μM R8 and 40 mM HEPES (pH 7.5) with appropriate concentrations of Mg^{2+}. Cd^{2+} "rescue" experiments were performed by preincubating 2 μM R8, 100 mM Bis-Tris (pH 6.5), and an appropriate mixture of $CdCl_2$ and $MgCl_2$ prior to initiating the reaction by addition of trace (5′-^{32}P) labeled substrate. In each case, 1 μL aliquots were taken at appropriate times and quenched in 15 μL of 7 M urea/150 mM EDTA stop buffer with dyes.

RESULTS

Hammerhead Cleavage of the Dithioate Linkage. The well-characterized HH8 (*27*) was used to compare cleavage of the phosphorodithioate linkage with unmodified and both R_P- and S_P-phosphorothioate controls (Figure 1A). In an initial experiment, cleavage of a trace concentration of the four (5′-^{32}P) substrates was determined at saturating ribozyme concentrations in 50 mM Tris-HCl (pH 8.3) and 10 mM $MgCl_2$ (Figure 1B). As had previously been found, the all-oxygen substrate and S_P-phosphorothioate substrates were

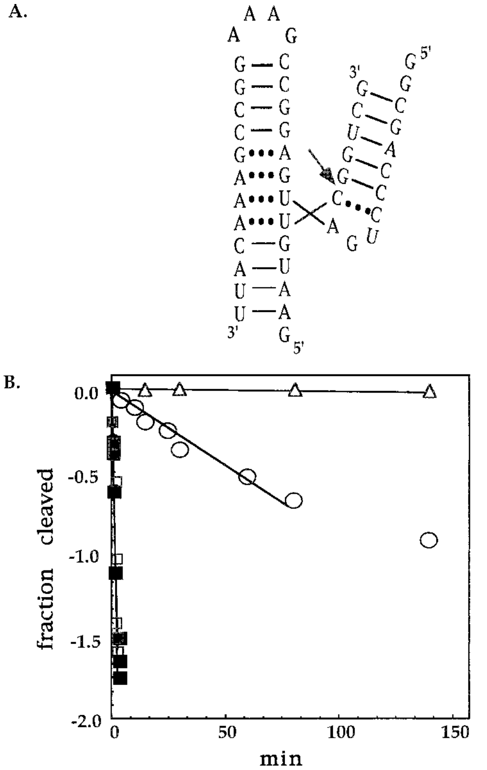

FIGURE 1: (A) Secondary structure of hammerhead 8 (HH8) consisting of a 34-nucleotide ribozyme (R8) and a 12-nucleotide substrate (S8). The arrow indicates the site of cleavage and the site of phosphate modification. (B) Cleavage of HH8 in 10 mM $MgCl_2$ and 50 mM Tris, pH 8.3, at 25 °C with unmodified (■), S_P-phosphorothioate (□), R_P-phosphorothioate (△), and dithioate (○) linkage at the cleavage site.

Table 1: Hammerhead 8 Cleavage Rates and Substrate Binding Constants

substrate	$k_2{}^a$ (min^{-1})	$K_d{}^b$ (nM)	K_d R8C3U^c (nM)
phosphodiester	1.3	50	130
R_P-phosphorothioate	7×10^{-5}	nd	160^d
S_P-phosphorothioate	1.2	nd	160^d
phosphorodithioate	2.7×10^{-3}	180	400

[a] Cleavage rates (k_2) were determined in 10 mM $MgCl_2$ and 100 mM Tricine, pH 8.0, at 25 °C. [b] K_d for the phosphodiester substrate was determined by determining K_M in the same buffer. K_d for dithioate substrate was determined by gel mobility shift assay. [c] K_ds of substrates to inactive C3U ribozyme were determined by gel mobility shift assay. [d] A mixture of the R_P- and S_P-phosphorothioate isomers was used.

both cleaved to completion within a few minutes, while R_P-phosphorothioate substrate was not cleaved to an appreciable extent. Unexpectedly, the dithioate substrate, which also has the *pro*-R_P oxygen substituted by sulfur, cleaved at an intermediate rate, which could be described by a single rate constant of 1.3×10^{-2} min^{-1}. Although cleavage of the dithioate substrate was only 60% complete after 150 min (Figure 1B), incubation for 10 h gave >90% cleavage, indicating that the majority of the molecules were a substrate for hammerhead cleavage.The cleavage rates of the four substrates at pH 8 are compared in Table 1. In each case, the rates were the same at approximately 4-fold higher enzyme concentration, indicating that each substrate was saturated by ribozyme. In the case of the dithioate and oxygen substrates, cleavage rates were measured at six different pH values, and as previously reported for the oxygen substrate (*35*), the log of the cleavage rate was a linear function of pH with a slope of 1 (data not shown). This confirms that the rate reflects the chemical step of the cleavage reaction.

Cleavage of a second RNA substrate, S16, with dithioate at the cleavage site by its corresponding ribozyme, R16, was examined in 2 mM $MgCl_2$ and 50 mM PIPES (pH 7.0). Under these conditions, the all-oxygen substrate cleaved with a single rate constant. However, the cleavage of the dithioate S16 exhibited biphasic character. Thirty percent of the molecules cleaved rapidly (0.2 min^{-1}) and the remainder cleaved slowly (2×10^{-4} min^{-1}). Unlike the dithioate S8 substrate, the dithioate S16 did not resolve well from the oxygen and phosphorothioate substrates during reverse-phase HPLC purification, so it is likely that the fast-cleaving component is due to contaminating all-oxygen or S_P-phosphorothioate and the slow-cleaving component is the authentic dithioate substrate. Thus, while it was inconvenient to use HH16 to characterize the cleavage of dithioate RNA, it is apparent that the dithioate linkage cleaves at approximately the same rate in HH16 as in HH8 under similar conditions. Importantly, this indicates that the cleavage of the dithioate linkage is not dependent upon sequence or structure beyond the conserved core region of the hammerhead.

The effect of modification of the cleavage site phosphate upon substrate binding was determined (Table 1). In the case of the slowly cleaving dithioate substrate, a native gel (*36*) was used to determine a $K_d = 180$ nM, which is only slightly weaker than the $K_M = 50$ nM for the oxygen substrate [$K_M = K_d$ for HH8 (*27*)]. Native gels were also used to compare the K_ds of the oxygen, dithioate, and a racemic mixture of monothioate substrates to the catalytically inactive C3U mutation of R8 (*37*). While all three substrate binding affinities are slightly weaker to this mutant ribozyme, the dithioate substrate is only about 3-fold weaker than the other two. Taken together, these data indicate that although the weaker binding of the dithioate substrate is significant, the difference is very small.

Product Identification. The unexpectedly rapid cleavage of the dithioate substrate mandated that we confirm that the reaction products of cleavage had the appropriate 2,3′-cyclic dithioate and 5′-hydroxyl termini. As the 5′ cleavage products of all the substrates comigrated on polyacrylamide gels, the products were analyzed by anion-exchange HPLC where the mobility of oligonucleotides was sensitive to the presence of sulfur atoms. As shown in Figure 2, S8 with a dithioate linkage elutes at 23 min, which is later than the mixed isomers of S8 phosphorothioate (21.5 min) or oxygen S8 (20 min). When each substrate is subjected to cleavage, all produce the same 3′ product (P2) as expected, and the 5′ products migrate in the same order as the substrates but at 16, 19, and 21.5 min, respectively (Figure 2). Importantly, the 5′ product of the dithioate substrate migrates as a single peak with a distinct mobility, indicating that no desulfurization had occurred. To confirm that the 5′ cleavage product of the dithioate substrate was indeed the expected 2′,3′-cyclic phosphorodithioate, it was collected and analyzed. Mass spectrometry of the oligonucleotide gave a mass of 2293.5 Da. This agrees well with the predicted mass of the product of 2294.5 and is significantly different from that of the 2′,3′-cyclic phosphorothioate (2278.4) or the hydrolytic product, 2′(3′)-phosphorodithioate (2312.5). Thus, we can conclude

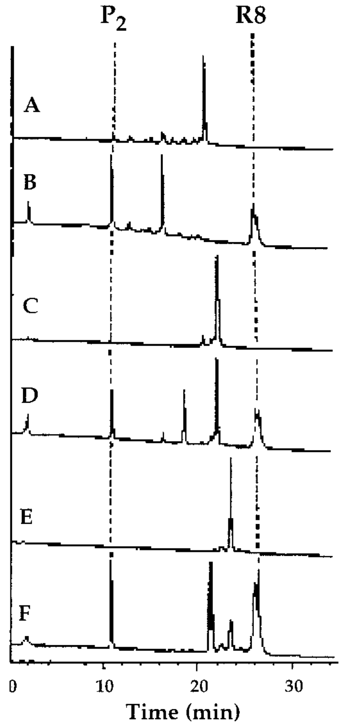

FIGURE 2: HPLC analysis of S8 (A, B), the mixed isomers of S8 phosphorothioate (C, D), and S8 dithioate (E, F), without ribozyme (A, C, E) and after cleavage by ribozyme (B, D, F). The positions of the 3′ product at 11 min (P_2 = GGUCG) and the ribozyme at 26 min (R8) are indicated by dashed lines. The 5′ products (P_1 = GAAUGUC-cyclic-P) migrate at 16 min (B), 19 min (D), and 21.5 min (F).

that the hammerhead cleaves the phosphorodithioate linkage by transesterification, just as with the normal phosphodiester.

Catalytic Enhancement of Dithioate Hammerhead. To gauge the effectiveness of hammerhead cleavage of the dithioate linkage, it is essential to determine the uncatalyzed rate of cleavage so that the rate enhancement can be calculated. The uncatalyzed cleavage of the oxygen, R_P- and S_P-phosphorothioate, and dithioate linkages of S8 at 25 °C were compared in the same buffer as used for measuring cleavage by the hammerhead. Reactions were performed on the substrate alone and in the presence of saturating concentrations of the catalytically inactive G5A ribozyme. Over the course of 6 days, significant cleavage could be detected at all internucleotide linkages in all reactions. Cleavage rates at the hammerhead cleavage site were calculated, taking into account the cleavage at other sites (see Materials and Methods). Uncatalyzed cleavage rates of the unmodified and phosphorothioate linkages were similar (Table 2) and were not significantly altered by the presence of G5A ribozyme. The dithioate linkage was observed to cleave approximately 15-fold more slowly than the normal phosphodiester under hammerhead cleavage reaction conditions.

The catalytic rate enhancement by the hammerhead with different substrates was estimated by dividing the cleavage rate by the corresponding uncatalyzed rate (Table 2). These data indicate that while the rate enhancement for cleavage of the dithioate substrate (2300-fold) is less than for all-oxygen substrate (72 000-fold), it is significantly greater than for cleavage of R_P-phosphorothioate substrate (<10-fold). Thus, the dithioate linkage is cleaved by the hammerhead only about 30-fold less effectively than the normal all-oxygen substrate under the same conditions.

Table 2: Background Rates of Cleavage and Rate Enhancement by HH8

substrate	background cleavage rate[a]	rate enhancement[b]
phosphodiester	1.8×10^{-5}	72 000
R_P-phosphorothioate	1×10^{-5} [c]	<10
S_P-phosphorothioate	1.1×10^{-5} [c]	110 000
phosphorodithioate	1.2×10^{-6}	2300

[a] The background cleavage rate (per minute) is the average of the rates observed in 10 mM $MgCl_2$ and 100 mM Tricine (pH 8) with and without the inactive hammerhead variant R8A5. [b] The rate enhancement is the degree to which the addition of active R8 was observed to increase the cleavage over the background rate under the same conditions. [c] Rates for cleavage of phosphorothioates are upper limits, as some desulfurization is known to occur under these conditions to yield unmodified linkages.

Cleavage of Dithioate Substrate Is Not the Result of Thiophilic Metal Ion Contamination. Although ultrapure $MgCl_2$ was used in cleavage reactions, there was a concern that cleavage of the dithioate substrate was stimulated by the presence of trace amounts of a contaminating thiophilic metal ion such as Cd^{2+}, Zn^{2+}, or Cu^{2+}. If present, these metal ions might bind the dithioate linkage selectively and thereby "rescue" cleavage in the same way as low concentrations of thiophilic metal ions can restore cleavage of the R_P-phosphorothioate substrate. Presumably due to their ability to chelate, dithioate linkages are known to bind thiophilic metal ions more tightly than monophosphorothioates (*38*). Thus, it seemed possible that a thiophilic metal ion may have remained associated with dithioate oligonucleotides during synthesis and purification.

To assess the possibility of thiophilic metal ion contamination, we made use of diethyl dithiocarbamate (DTC), a reagent that chelates and precipitates thiophilic metal ions with little or no affinity for Mg^{2+} (*39*). The log of equilibrium constants describing the ratio of metal ion in solution or removed from solution by precipitation ranges from −17 for Zn^{2+} to −40 for Hg^{2+} (*39*). Whenever this value is less than −7, the precipitation of metal ion can generally be considered to be quantitative (*40*). Thus, this reagent, if allowed to equilibrate with the reaction mixture, should effectively remove any trace thiophilic metal ions. The ability of this reagent to deplete thiophilic metal ions from cleavage reactions was tested in Figure 3. The cleavage of the dithioate substrate in 10 mM $MgCl_2$ is substantially enhanced by the addition of 0.5 mM Cd^{2+}. However, when 2 mM DTC was added as well, the reaction rate is the same as in 10 mM $MgCl_2$. This indicates that DTC treatment is sufficient to quantitatively remove the added thiophilic metal ion. Since the identical cleavage rates are obtained with and without DTC treatment, the cleavage of dithioate RNA by the hammerhead ribozyme in $MgCl_2$ is not due to contaminating thiophilic metal ion.

Dithioate Substrate Shows an Altered Mg^{2+} Ion Dependence of Cleavage. The Mg^{2+} dependence of the cleavage rate for the normal phosphodiester substrate was compared with dithioate substrate in Figure 4A. Cleavage rates for the normal phosphodiester fit well to a simple binding isotherm, giving an apparent K_d (Mg^{2+}) = 60 ± 10 mM. This apparent binding affinity is consistent with that observed earlier for

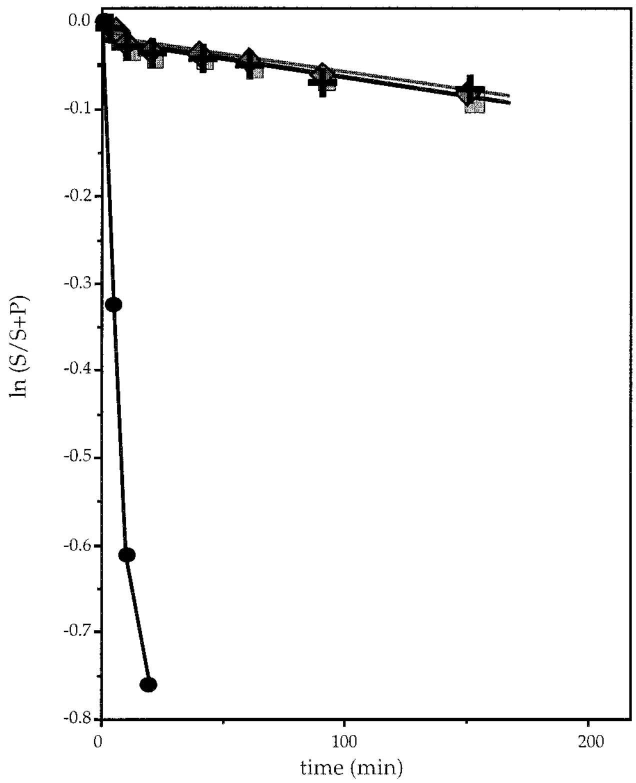

FIGURE 3: Test for thiophilic ion contamination. HH8 cleavage is shown for dithioate substrate in 10 mM $MgCl_2$, pH 7.0, at 25 °C with nothing added (+), with 2 mM diethyl dithiocarbonate (■), with 0.5 mM $CdCl_2$ (●), and after incubation in 0.5 mM $CdCl_2$ followed by addition of 2 mM diethyl dithiocarbamate (◆).

HH8, K_d (Mg^{2+}) = 180 ± 20 mM, at an ionic strength of 150 mM (A. Feig and O. Uhlenbeck, unpublished observations). It is also generally consistent with other hammerhead ribozymes (*41*, *42*). At subsaturating Mg^{2+}, the slope of Figure 4A yields a reaction order in Mg^{2+} of 0.9. In contrast, the cleavage rate for dithioate S8 never plateaus with respect to increasing $MgCl_2$ and the apparent reaction order of the dithioate substrate in Mg^{2+} is decreased to <0.3 (Figure 4A). Whereas additional Mg^{2+} binding to the hammerhead greatly enhanced hammerhead cleavage of the normal phosphodiester, it does not greatly enhance hammerhead cleavage of the dithioate. Thus, the presence of the dithioate linkage not only reduces the absolute rate of cleavage but also substantially changes the ability of the hammerhead to interact with Mg^{2+} ion.

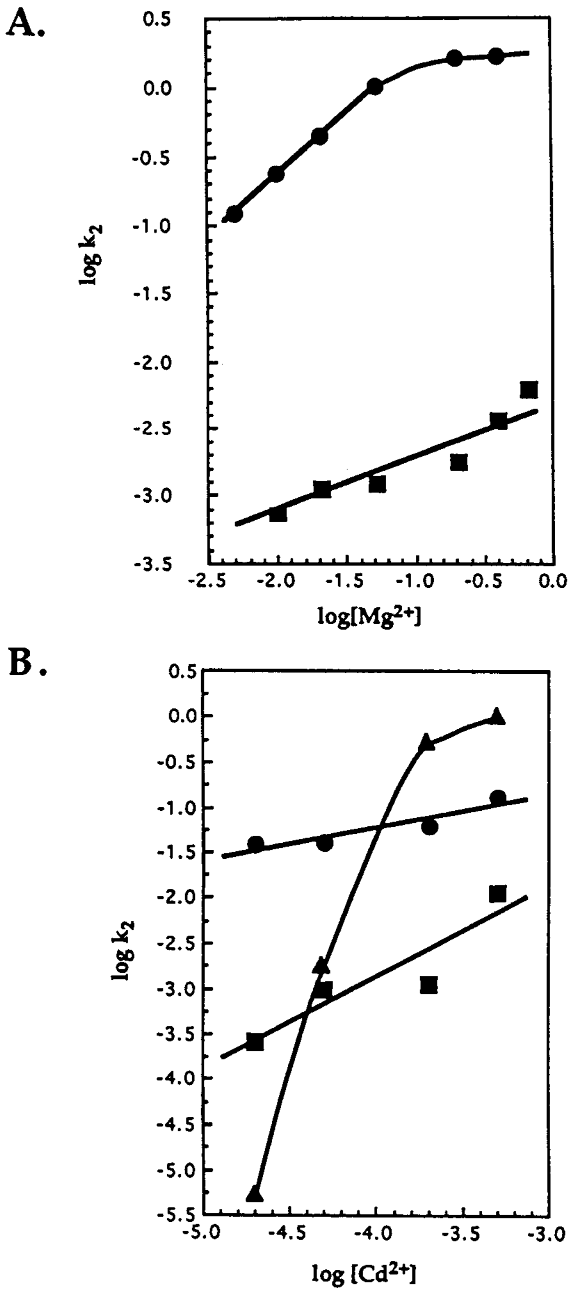

FIGURE 4: (A) Magnesium concentration dependence of hammerhead cleavage rate for oxygen (●) and dithioate (■) substrates. Slopes of 0.9 and 0.3 are observed for the linear portion. (B) Cadmium concentration dependence of hammerhead cleavage rate in the presence of 10 mM Mg^{2+} for oxygen (●), R_P-monothioate (▲), and dithioate (■) substrates. The slope for the dithioate is 1.0 and that for the monothioate is 5.0.

Thiophilic Metal Ion Rescue of Phosphorodithioate Cleavage Reaction. On the basis of the efficient rescue of hammerhead cleavage of the R_P-phosphorothioate substrate by thiophilic metal ions, it was expected that thiophilic metal ions would stimulate hammerhead cleavage of the dithioate linkage. Indeed, preliminary experiments in Figure 3 with Cd^{2+} suggested that this would be the case. However, analysis of cleavage reactions under those conditions was complicated by biphasic cleavage kinetics. In contrast to cleavage in 10 mM Mg^{2+}, where all the substrate cleaved slowly, the addition of Cd^{2+} to the reaction resulted in 10−50% of the substrate cleaving faster and the remaining cleaving slower or not at all. The proportions of fast- and slow-cleaving substrate varied significantly between experiments. It was subsequently discovered that the order in which metal ion, substrate, and ribozyme were added to the reaction mixture affected the proportion of fast- and slow-cleaving components. This suggested that the tight binding of thiophilic metal ions by diesters of dithiophosphoric acid (*38*) might cause this variability in cleavage kinetics. Such tight complexes between Cd^{2+} and one or more substrate molecules could bind to the ribozyme and inhibit cleavage. A new protocol was therefore developed that minimized the biphasic cleavage behavior of the dithioate substrates. The weakly chelating buffer Bis-Tris was used to reduce the rate of binding of Cd^{2+} to the dithioate linkage, and addition of the dithioate substrate was used to initiate cleavage.

Figure 4B compares the hammerhead cleavage rates of the all-oxygen, R_P- and S_P-phosphorothioate, and phosphorodithioate linkages in 5 mM $MgCl_2$ as a function of Cd^{2+} concentration. The oxygen and S_P-phosphorothioate substrates cleaved rapidly in the absence of Cd^{2+} and the cleavage rate increases only slightly with added Cd^{2+}. This indicates that hammerhead cleavage of these substrates used Mg^{2+} as the primary cofactor for catalysis. In contrast, as previously reported (*6*−*9*, *14*), the cleavage of the R_P-phosphorothioate is highly dependent upon the presence of the thiophilic metal ion. Unlike in other buffers, in Bis-Tris, the R_P-phosphorothioate substrate is cleaved slightly faster at higher concentrations of Cd^{2+} than either the corresponding oxygen or S_P-phosphorothioate substrates. The cleavage rate of phosphorodithioate RNA by the hammerhead also increases upon the addition of Cd^{2+}. Over the range of Cd^{2+} concentrations tested, hammerhead cleavage of the dithioate substrate is enhanced about 100-fold. While the absolute cleavage rates at higher concentrations of Cd^{2+} do not reach that of the unmodified substrate, it is important to recall that the uncatalyzed cleavage rate of the dithioate linkage is approximately 10-fold slower than the phosphodiester linkage. Thus, even though the absolute cleavage rate of dithioate at high Cd^{2+} is slower than the cleavage rate of the normal phosphodiester, hammerhead cleavage of dithioate linkage is still greatly enhanced by Cd^{2+}.

Support for the notion that the addition of Cd^{2+} restores a metal ion that was lost with the phosphorodithioate modification comes from examining the metal order of the cleavage reaction. As shown in Figure 4A, the hammerhead cleavage of unmodified RNA has a metal order of 0.9 with respect to Mg^{2+}. The dithioate substitution reduces this metal order substantially, presumably because it disrupts an interaction with a critical Mg^{2+} ion required for catalysis or for maintaining the ribozyme in a catalytically active conformation. As shown in Figure 4B, the metal order, with respect to Cd^{2+}, for cleavage of the dithioate substrate in the presence of Mg^{2+} is also about 1. This suggests that the binding of a single Cd^{2+} ion is sufficient to restore cleavage of dithioate substrate. Interestingly, the metal order, with respect to Cd^{2+}, for the *pro*-R_P monothioate substrate is considerably greater than 1. This result differs from previous reports (*6*, *23*) and may be an artifact resulting from competition between the chelating buffer and the monothioate substrate for the Cd^{2+} ion.

DISCUSSION

The phosphorodithioate linkage is a valuable alternative to the phosphorothioate linkage as a modified phosphodiester. Since both nonbridging oxygens are replaced by sulfur, the linkage is prochiral instead of chiral. In contrast to phosphorothioates, where there are distinct differences between the two nonbridging members (*25*, *26*), in phosphorodithioates the P−S bonds are more nearly equal in length, although not identical with respect to one another (*43*). One characteristic of the dithioate linkage is that while the charge distribution may still differ from that seen for the unmodified phosphodiester, it is more like the unmodified phosphodiester in this respect than the phosphorothioates.

The dithioate linkage is somewhat more stable than the normal phosphodiester to nucleophilic attack by oxygen (*44*, *45*). In buffers used for hammerhead cleavage reactions, we estimated that the phosphorodithioate linkage is cleaved about 15-fold slower than the phosphodiester or the phosphorothioate. An extensive study of the nonenzymatic cleavage rate of a dithioate linkage embedded in an oligodeoxynucleotide at several pH values indicates that the dithioate linkage is cleaved about 5-fold slower than the corresponding phosphodiester (D. Sheehan and M. Caruthers, personal communication).

The phosphorodithioate linkage was an unexpectedly good substrate for the hammerhead ribozyme. In contrast to the R_P-monophosphorothioate, which is highly resistant to hammerhead cleavage, the phosphorodithioate linkage is cleaved at 2.7×10^{-3} min^{-1} under conditions where the normal phosphodiester is cleaved at 1.3 min^{-1}. However, as the dithioate linkage is intrinsically more stable, it is more appropriate to compare the enhancements rather than the absolute rates. In such a comparison, the hammerhead ribozyme enhances cleavage of the R_P-monothioate linkage less than 10-fold, the dithioate linkage 2300-fold, and the oxygen linkage 72 000-fold. Thus, the dithioate modification reduces catalysis by less than 2 orders of magnitude.

The major cause of reduced catalytic enhancement of cleavage of the dithioate linkage appears to be the disruption of an interaction between the linkage and a single divalent metal ion. This conclusion is supported by two observations. First, a study of the magnesium ion concentration dependence of the reaction rate indicates that the reaction order with respect to Mg^{2+} is changed from about 1 with the phosphodiester to less than 0.3 for the dithioate. Very slow cleavage made it impossible to obtain similar data with the monothioate. While a reaction order of 1 indicates that at least one magnesium ion must bind to the hammerhead to reach the transition state of the reaction, the simultaneous binding of one or more additional magnesium ions with similar binding affinity cannot be excluded. However, the large reduction in the magnesium reaction order when the dithioate linkage is introduced argues that only a single magnesium ion is directly involved in hammerhead catalysis. The fact that the cleavage of the dithioate hammerhead is so weakly dependent on magnesium ion concentration also excludes the possibility that the remaining rate enhancement is due to thiophilic metal ion contaminants in the Mg^{2+} buffer. If this occurred, a reaction order of 1 would be observed.

The second observation that suggests that loss of binding of a single metal ion is responsible for the reduced cleavage rate of the dithioate substrate is that the reaction rate can be effectively rescued by the addition of low concentrations of the thiophilic metal ion, Cd^{2+}. While this experiment was complicated by the need to use a chelating buffer to minimize the tendency of the dithioate linkage to chelate thiophilic divalent ions, the reaction rate could be stimulated by about 100-fold by the addition of Cd^{2+}. At the highest Cd^{2+} concentrations tested, the efficiency of the hammerhead to cleave the dithioate linkage approaches that of the phosphodiester linkage, when one takes into account the increased stability of the dithioate linkage. Thus, rescue of cleavage is nearly complete. The reaction order with respect to Cd^{2+} concentration with the dithioate substrate was 1 suggesting that only a single Cd^{2+} ion is involved in the stimulation of cleavage. A recent extensive study examining the rescue of several different phosphoromonothioate-substituted hammerheads in more normal buffers also concludes that only a single Cd^{2+} ion is required for rescue (*23*).

On the basis of the cleavage experiments with the dithioate hammerhead in Mg^{2+}, we conclude that the divalent ion coordinating to the scissile phosphodiester contributes only about 30-fold to the catalytic enhancement of the hammerhead. While this estimate is much less than the estimate from the phosphorothioate derivative, it agrees well with the recent report that the normal hammerhead ribozyme shows substantial catalytic activity in the absence of any divalent ions provided that a high concentration of monovalent cations are present (*46*). Indeed, experiments with the well-characterized hammerhead 16 indicate that the cleavage rate in 4 M LiCl is only 50-fold less than in 10 mM $MgCl_2$ (*46*; also A. Feig and O. Uhlenbeck, unpublished results) a reduction in rate enhancement quite similar to that caused by the dithioate substitution. Taken together, the experiments suggest that while a substantial portion of the catalytic rate enhancement of the hammerhead can occur without divalent ions, full activity requires participation of a Mg^{2+} ion binding to the scissile phosphodiester.

The hammerhead cleavage data presented here with the phosphorodithioate substrate suggests that the very slow cleavage of the phosphoromonothioate substrate is not only due to the substitution of oxygen by sulfur resulting in the loss of a magnesium ion. In addition, there must be some property of the asymmetric phosphorothioate linkage that cannot be accommodated in the structure of the hammerhead transition state and thereby further reduces the rate enhancement. It is unclear what feature of the R_P-phosphorothioate is so detrimental. One possibility is that the nearly full negative charge on the R_P sulfur atom cannot be tolerated in the transition state in the absence of a divalent ion. The half negative charge on the *pro*-R_P sulfur in the dithioate substrate would presumably not be as detrimental. Another possibility is that the shorter P═O double bond and/or the longer P—S single bond in the phosphorothioate cannot be accommodated in the transition state structure, while the more equivalent P—S bonds in the dithioate are less disruptive. The detrimental feature is unique to the R_P-phosphorothioate since the S_P-phosphorothioate shows a rate of hammerhead cleavage the same as for the phosphodiester.

Our experiments with the phosphorodithioate derivative have confirmed the presence of a metal ion coordinated to the scissile phosphodiester bond but have suggested that the contribution of the metal ion to the overall rate enhancement is less than had previously been thought. This emphasizes the importance of using different kinds of derivatives in deducing ribozyme mechanisms. It will be interesting to examine the cleavage properties of hammerheads that contain dithioate linkages at the other three sites known to have a phosphorothioate effect. Introducing the dithioate linkage into other RNA enzymes may also yield valuable insights into the mechanisms of RNA catalysis.

ACKNOWLEDGMENT

We thank W. Marshall for help with the mass spectrometry and D. Herschlag for comments on the manuscript.

REFERENCES

1. Prody, G. A., Bakos, J. T., Buzayan, J. M., Schneider, I. R., and Bruening, G. (1986) *Science 231*, 1577−1580.
2. Hertel, K. J., Peraccchi, A., Uhlenbeck, O. C., and Herschlag, D. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 8497−8502.
3. Kuimelis, R. G., and McLaughlin, L. W. (1998) *Chem. Rev. 98*, 1027−1044.
4. Zhou, D.-M., and Taira, K. (1998) *Chem. Rev. 98*, 991−1026.
5. McKay, D. B. (1996) *RNA 2*, 395−403.
6. Scott, E. C., and Uhlenbeck, O. C. (1999) *Nucleic Acids Res. 27*, 479−484.
7. Dahm, S. C., and Uhlenbeck, O. C. (1991) *Biochemistry 30*, 9464−9469.
8. Slim, G., and Gait, M. J. (1991) *Nucleic Acids Res. 19*, 1183−1188.
9. Koizumi, M., and Ohtsuka, E. (1991) *Biochemistry 30*, 5145−5150.
10. Almer, H., and Stromberg, R. (1991) *Tetrahedron Lett. 32*, 3723−3726.
11. Almer, H., and Stromberg, R. (1996) *J. Am. Chem. Soc. 118*, 7921−7928.
12. Herschlag, D., Piccirilli, J. A., and Cech, T. R. (1991) *Biochemistry 30*, 4844−4854.
13. Ora, M., Oivanen, M., and Lonnberg, H. (1996) *J. Org. Chem. 61*, 3951−3955.
14. Knoll, R., and Furste, J. (1997) *RNA 2*, 132−140.
15. Pecoraro, V. L., Hermes, J. D., and Cleland, W. W. (1984) *Biochemistry 23*, 5262−5271.
16. Sigel, R. K. O., Song, B., and Sigel, H. (1997) *J. Am. Chem. Soc. 119*, 744−755.
17. Cooperman, B. S. (1976) in *Metal Ions in Biological Systems* (Sigel, H., Ed.) pp 79−125, Marcel Dekker, New York.
18. Trautwein, K., Holliger, P., Stackhouse, J., and Benner, S. A. (1991) *FEBS Lett. 281*, 275−277.
19. Birikh, K. R., Heaton, P. A., and Eckstein, F. (1997) *Eur. J. Biochem. 245*, 1−16.
20. Steitz, T. A., and Steitz, J. A. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6498−6502.
21. Pontius, B. W., Lott, W. B., and von Hippel, P. H. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 2290−2294.
22. Lott, W. B., Pontius, B. W., and von Hippel, P. H. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 542−547.
23. Wang, S., Karbstein, K., Peracchi, A., Beigelman, L., and Herschlag, D. (1999) *Biochemistry 38*, 14363−14378.
24. Greef, C. H., Seeberger, P. H., and Caruthers, M. H. (1996) *Tetrahedron Lett. 37*, 4451−4454.
25. Frey, P. A., and Sammons, R. D. (1985) *Science 228*, 541−545.
26. Heidenreich, O., Pieken, W., and Eckstein, F. (1993) *FASEB J. 7*, 90−96.
27. Fedor, M. J., and Uhlenbeck, O. C. (1992) *Biochemistry 31*, 12042−12054.
28. Hertel, K. J., Herschlag, D., and Uhlenbeck, O. C. (1994) *Biochemistry 33*, 3374−3385.
29. Milligan, J. F., and Uhlenbeck, O. C. (1989) *Methods Enzymol. 180*, 51−62.
30. Usman, N., Ogilvie, K. K., Jiang, M.-Y., and Cedergren, R. J. (1987) *J. Am. Chem. Soc. 109*, 7845−7854.
31. Caruthers, M. H., Beaucage, S. L., Efcavitch, J. W., Fisher, E. F., Matteucci, M. D., and Stabinsky, Y. (1980) *Nucleic Acids Symp. Ser. 7*, 215−223.
32. Iyer, R. P., Egan, W., Regan, J. B., and Beaucage, S. L. (1990) *J. Am. Chem. Soc. 112*, 1253−1254.
33. Bjergaarde, K., and Dahl, O. (1991) *Nucleic Acids Res. 19*, 5843−5850.
34. Brill, W. K.-D., Nielsen, J., and Caruthers, M. H. (1991) *J. Am. Chem. Soc. 113*, 3972−3980.
35. Dahm, S. C., Derrick, W. B., and Uhlenbeck, O. C. (1993) *Biochemistry 32*, 13040−13045.
36. Clouet-d'Orval, B., and Uhlenbeck, O. C. (1996) *RNA 2*, 483−491.
37. Baidya, N., and Uhlenbeck, O. C. (1997) *Biochemistry 36*, 1108−1114.
38. Budnikov, G., K., Shakurova, N. K., Ulakhovich, N. A., Cherkasov, R. A., Ovchinnikov, V. V., Kutyrev, G. A., and Toropova, V. F. (1977) *Zh. Anal. Khim. 32*, 1326−1332.

39. Hulancki, A. (1967) *Talanta 14*, 1371−1392.
40. Ringbom, A. (1963) in *Complexation in analytical chemistry; a guide for the critical selection of analytical methods based on complexation reactions*, p 365, Interscience Publishers, New York.
41. Clouet-d'Orval, B., Stage, T. K., and Uhlenbeck, O. C. (1995) *Biochemistry 34*, 11186−11190.
42. Clouet-d'Orval, B., and Uhlenbeck, O. C. (1997) *Biochemistry 36*, 9087−9092.
43. Hall-Willis, M. (1995) Ph.D. Thesis, University of Colorado, Boulder, CO.
44. Nielsen, J., Brill, W. K. D., and Caruthers, M. H. (1988) *Tetrahedron Lett. 29*, 2911−2914.
45. Petersen, K. H., and Nielsen, J. (1990) *Tetrahedron Lett. 31*, 911−914.
46. Murray, J. B., Seyhan, A. A., Walter, N. G., Burke, J. M., and Scott, W. G. (1998) *Chem. Biol. 5*, 587−595.

BI000146A