

Explanation by a putative triester-like mechanism for the thio effects and Mn^{2+} rescues in reactions catalyzed by a hammerhead ribozyme

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Abstract Divalent metal ion-dependent hammerhead ribozymes can cleave any RNA with a NUX triplet, wherein the N can be any residue and X can be C, U or A. In recent literature on the mechanism of action of hammerhead ribozymes, one important role of divalent metal ions is generally suggested to be an electrophilic catalyst by directly coordinating with the *pro*-Rp oxygen of the scissile phosphate to stabilize the transition state. This proposal was made on the basis of thio effects and the proposed electrophilic catalyst is very attractive as an explanation for the catalytic activity of metalloenzymes. Reexamination of thio effects with substrates having a GUA triplet at the cleavage site shows that, in agreement with the previous finding, the cleavage rate, in the presence of Mg^{2+} ions, is significantly reduced in the case of the phosphorothioate substrate (RpS), wherein the *pro*-Rp oxygen at the scissile phosphate is replaced by sulfur, while the cleavage rate is reduced to a much lesser extent for the other isomer (SpS), wherein the *pro*-Sp oxygen at the scissile phosphate is replaced by sulfur. However, more careful examination of the rescue ability of Mn^{2+} ions with these isomers demonstrates that more thiophilic Mn^{2+} ions rescue the reaction not only with the RpS isomer but also with the SpS isomer and, importantly, to a greater extent for the SpS isomer. These results argue against the previous conclusion that a metal ion is directly coordinating with the *pro*-Rp oxygen of the scissile phosphate to stabilize the transition state. In this paper we try to elucidate the possible origin of the thio effects and propose a 'triest-er-like' mechanism in reactions catalyzed by hammerhead ribozymes.

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Key words: Double-metal-ion mechanism; Hammerhead ribozyme; Metalloenzyme; Thio effect; Triester-like mechanism

1. Introduction

It is now well established that metal ions are absolutely required in hammerhead ribozyme reactions, as structural folding agents [1–3] and also as catalytic cofactors [4–11]. Thus, hammerhead ribozymes have been recognized as metalloenzymes [1–15]. Recent X-ray structure by Scott's and Uhlenbeck's group identified a metal-ion binding site at position 9 (Fig. 1). Binding of a metal ion at the P9 *pro*-Rp oxygen and the N7 of G_{10.1} was proposed to be required for a conformational change that leads to an activated complex [16,17]. Original X-ray structures determined by Scott's and Klug's group

identified five potential metal-ion binding sites [18,19]. Among the five metal ions, one was close to the scissile site [20] and it was proposed to bind to the Rp-oxygen of the scissile phosphate (Fig. 1, in the expanded box) [4,20]. This direct coordination with the *pro*-Rp oxygen providing an electrophilic catalyst was in accordance with previous biochemical studies [4,21,22], as follows: thio substitution at the *pro*-Rp oxygen at the cleavage site of a substrate (RpS) for a hammerhead ribozyme resulted in a large thio effect that was relieved by replacement of Mg^{2+} by Mn^{2+} ions, which have a higher affinity for sulfur than do Mg^{2+} ions [4,21,22].

Although the proposed electrophilic catalyst was very attractive as an explanation for the catalytic activity of metalloenzymes, our recent data of thio effects by the use of a GUC-triplet-cleaving hammerhead ribozyme could not be explained by the direct coordination of a metal ion with the *pro*-Rp oxygen [23]: thio effects of 540 and 28, respectively, for RpS and SpS isomers were obtained experimentally in our case [23], in agreement with previous biochemical studies showing a greater thio effect for the RpS isomer by the use of other GUC-triplet-cleaving hammerhead ribozymes [4,21,22]. However, quantitative analysis of the rescue ability of Mn^{2+} ions with these isomers demonstrated that Mn^{2+} ions could rescue the reaction not only with the RpS isomer but also with the SpS isomer, and to a similar extent ($k_{\text{Mn}^{2+}}/k_{\text{Mg}^{2+}} = \sim 23$) [23]. Moreover, more recent, further analysis by Scott indicated the probable invalidity of the earlier postulate [20] that the Mg^{2+} ion bound to the *pro*-Rp oxygen of the scissile phosphate in the ground state might move, together with the phosphate, into a conformation more suitable for in-line attack: Scott recently trapped an intermediate with an advanced conformational change, where the phosphate had moved considerably. However, the metal ion (this time a Co^{2+} ion) remained associated with N7 of A_{1.1} and did not move with the *pro*-R oxygen (personal communication).

Therefore, whether or not an electrophilic catalysis, mediated by a metal ion, is operative in reactions catalyzed by hammerhead ribozymes remains obscure. In order to generalize our finding that Mn^{2+} ions could rescue the reaction not only with the RpS isomer but also with the SpS isomer, we reexamined thio effects and Mn^{2+} rescues by the use of a new substrate that contained a GUA triplet at the cleavage site since, in the past, all the thio effects were examined using substrates that contained exclusively a GUC triplet. The advantage of using the GUA triplet is that it can also become a substrate for the DNzyme selected by Joyce's group [24]. We found that the DNzyme could cleave the normal substrate and its SpS isomer in the presence of Mn^{2+} ions with a half-life of less than 1 min, while the RpS isomer could survive

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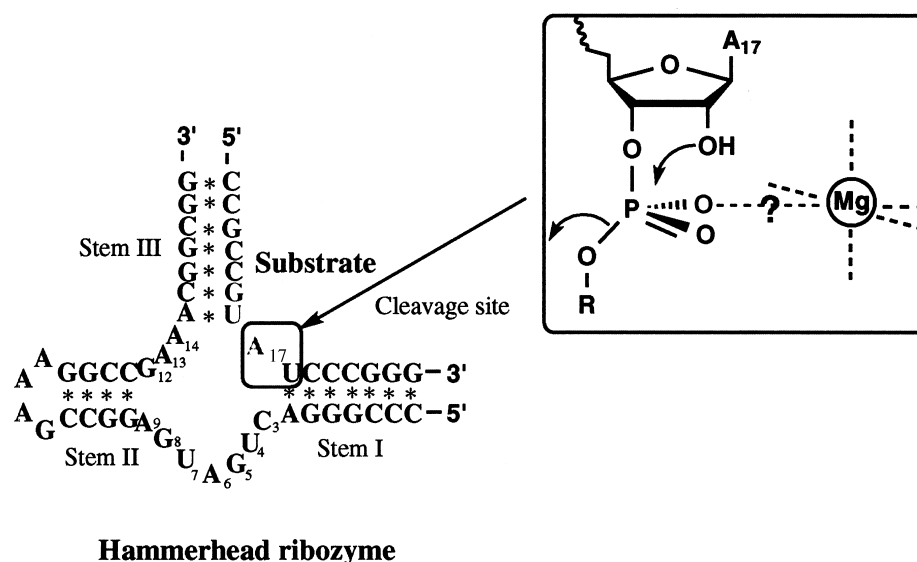


Fig. 1. The sequence and the secondary structure of the hammerhead ribozyme and substrate complex used in this study, with Watson-Crick base pairs indicated by asterisks. The previously proposed direct coordination of a Mg^{2+} ion with the *pro*-Rp oxygen atom at the cleavage site, acting as an electrophilic catalyst, is shown in the expanded box.

more than 2 h under identical conditions (manuscript in preparation). This high preference of the DNAzyme for the SpS isomer enabled us to completely remove the residual SpS isomer after HPLC purification of the RpS isomer (see Section 2) and, thus, we could accurately measure kinetic parameters for the less reactive RpS isomer in hammerhead ribozyme-catalyzed reactions.

By reexamination of the thio effect using the newly synthesized GUA-containing substrate and reconsideration of previous kinetic data including pH profiles and inverse correlation between the pK_a of metal ions and the ribozyme activity, we in this paper revisit the role of metal ions, speculate on the origin of the thio effect, and propose a putative 'triest-er-like' mechanism in reactions catalyzed by hammerhead ribozymes.

2. Materials and methods

2.1. Synthesis of ribozyme and substrates

Thiosubstrates (RpS and SpS isomer) and the ribozyme were chemically synthesized on a DNA/RNA synthesizer (394; Applied Biosystems, Division of Perkin Elmer Co. (ABI), California) and purified by reverse-phase HPLC as described previously [22,25]. In order to remove the small amount of contaminating normal and SpS substrates in the RpS isomer, the separated RpS substrate by HPLC was incubated with a two-fold excess of a DNAzyme in the presence of 25 mM $MnCl_2$ at 37°C for 2 h. We found that this DNAzyme selected by Joyce's group [24] could cleave the normal substrate and its SpS isomer in the presence of Mn^{2+} ions with a half-life of less than 1 min, while the RpS isomer could survive more than 2 h under identical conditions (manuscript in preparation). This high preference of the DNAzyme for the SpS isomer enabled us to completely remove the residual SpS isomer after HPLC purification of the RpS isomer.

2.2. Kinetic measurements

Reactions were carried out at 37°C in 10 mM metal ions and 50 mM MES (pH 6.0) under single-turnover conditions with 10 μ M ribozymes and 1.0 μ M substrate. Reactions were initiated by addition of metal ions to a solution that contained both ribozyme and substrate, and stopped by removal of aliquots from the reaction mixture at appropriate intervals and mixing them with an equivalent volume of a solution that contained 100 mM EDTA, 9 M urea, 0.1% xylene

cyanol, and 0.1% bromophenol blue. Substrates and 5'-cleaved products were separated by electrophoresis on a 20% polyacrylamide/7 M urea denaturing gel and were detected by autoradiography. The extent of cleavage was determined by quantitation of radioactivity in the bands of substrate and product with a Bio-Image Analyzer (BA100 or BA2000; Fuji Film, Tokyo).

3. Results and discussion

3.1. Thio effect and rescue effect

As described in detail in Section 2, we synthesized epimeric SpS and RpS isomers of substrates, which contained a GUA triplet at the cleavage site [26], and they were successfully separated by HPLC and the residual contaminants (SpS isomer) in the less reactive RpS isomer in the ribozyme reactions were completely removed by the use of a DNAzyme ([24], manuscript in preparation). Kinetic measurements of thio effects were performed under single-turnover conditions at pH 6.0 in the presence of 10 mM $MgCl_2$, 10 μ M ribozyme and 1.0 μ M substrate. Under these conditions, the concentration of the ribozyme was well above the K_m for this GUA substrate. Thus, the afforded k_{obs} solely reflected the chemical cleavage step [25]. Time courses of the hammerhead ribozyme-mediated cleavage of thiosubstrates are shown in Fig. 2 and the corresponding kinetic parameters are listed in Table 1. As can be seen, the SpS isomer in that the *pro*-Sp oxygen of the scissile phosphate was replaced by sulfur could be cleaved by the hammerhead ribozyme with a cleavage rate constant of 0.06 min^{-1} . When the *pro*-Rp oxygen atom was substituted by a sulfur atom (RpS isomer), the hammerhead ribozyme-mediated reaction was significantly inhibited, with a k_{obs} of 0.0008 min^{-1} . The large thio effect in the RpS isomer, as has been observed previously with GUC-cleaving ribozymes [4,21,22], suggests that the *pro*-Rp oxygen plays a much more critical role than does the *pro*-Sp oxygen atom, in hammerhead ribozyme-mediated cleavage of RNA. This thio effect was previously suggested to be attributable to a blockage of the direct coordination of a Mg^{2+} ion with the *pro*-Rp oxygen atom [4,21,22], resulting in a loss of electrophilic cat-

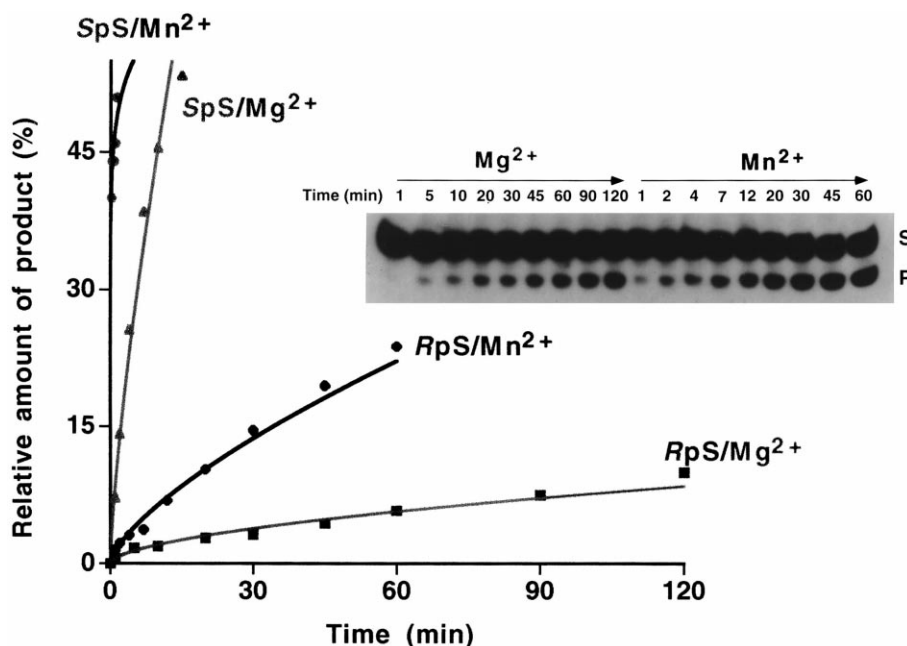


Fig. 2. Time courses of hammerhead ribozyme-catalyzed reactions, in the presence of either Mg^{2+} or Mn^{2+} ions, with *RpS* or *SpS* substrates in that either *pro-Rp* or *pro-Sp* oxygen at the cleavage site is replaced by sulfur. The autoradiographs show time courses for the cleavage of the *RpS* isomer catalyzed by the hammerhead ribozyme in the presence of Mg^{2+} ions or Mn^{2+} ions.

alysis, since the Mg^{2+} ion is coordinated 30 000-fold less strongly to sulfur than to oxygen [27,28].

This explanation might be plausible if and only if the significant thio effect could be completely rescued by replacement of Mg^{2+} ions by Mn^{2+} ions, as the strength of affinity of a Mn^{2+} ion to oxygen atom and to sulfur atom is more or less equal [27,28]. Replacement of Mg^{2+} ions by Mn^{2+} ions rescued the cleavage of *RpS* isomer catalyzed by the hammerhead ribozyme, increasing the cleavage rate constant to the level of 0.0047 min^{-1} . Although Mn^{2+} ions could enhance the hammerhead ribozyme-mediated cleavage of *RpS* isomer more than six times relative to the cleavage mediated by Mg^{2+} ions, this rescue by Mn^{2+} ions was only partial because the cleavage rate was still much more lower than that of the cleavage of *SpS* isomer promoted by Mg^{2+} ions (Fig. 2). Moreover, replacement of Mg^{2+} ions by Mn^{2+} ions also increased the ribozyme-mediated cleavage of the *SpS* isomer, yielding a k_{obs} of 0.85 min^{-1} , a value more than 14 times larger than that obtained with Mg^{2+} ions. Therefore, the Mn^{2+} rescue was not specific to the *RpS* isomer, as demonstrated previously in the case of GUC-cleaving ribozymes [22,23]. The higher reactivity of hammerhead ribozymes in Mn^{2+} -containing solutions than in Mg^{2+} -containing solutions originates from the fact that the Mn^{2+} ion is a stronger Lewis acid than the Mg^{2+} ion and, therefore, Mn^{2+} ions can better stabilize the transition state [9–11,15], no matter whether the substrate is *RpS* isomer or *SpS* isomer. If, indeed, the Mn^{2+} ion had directly coordinated to the sulfur atom in the hammerhead ribozyme-mediated cleavage of the *RpS* isomer, a complete rescue by Mn^{2+} ions should have been observed as had been demonstrated in the case of *Tetrahymena* ribozyme-catalyzed reactions [29] and also in hammerhead ribozyme-catalyzed reactions with a phosphorothioate linkage at the P9 position [16]. The much too slow cleavage rate for the *RpS* isomer in the presence of either Mg^{2+} or Mn^{2+} ions,

compared with the *SpS* isomer, argues against the previous conclusion that a metal ion is directly coordinating with the *pro-Rp* oxygen of the scissile phosphate in hammerhead ribozyme-catalyzed reactions. This is the third independent example that demonstrates that the rescue by Mn^{2+} ions is not specific to the *RpS* isomer [22,23].

Extensive studies on thio effects have been performed to probe the rate-limiting step of ribozyme-catalyzed reactions and to identify the coordination site of a metal ion with a phosphoryl oxygen [29,30]. The evidence that a Mg^{2+} ion being directly coordinated with the *pro-Rp* oxygen of a scissile phosphate in hammerhead ribozyme reaction [4,21,22] and that a Mg^{2+} ion being directly coordinated with the *pro-Sp* oxygen of a scissile phosphate in *Tetrahymena* ribozyme reaction [31–33] was reported on the basis of the thio effects. However, care must be taken as this experimental approach relies on the different affinity between divalent metal ions: the discrimination by Mn^{2+} ions between oxygen and sulfur atoms is poor, while the much lower affinity of Mg^{2+} ions to sulfur results in a very large thio effect [27,28]. Therefore, unless rescue values can be evaluated quantitatively, it is dangerous to conclude that a metal ion is coordinated directly to one of the non-bridging oxygen. Such a conclusion can be drawn only when substitution of Mg^{2+} by Mn^{2+} ions significantly and specifically rescues the lowered activity of phosphorothioates. Otherwise, since the two proximal phosphoryl oxygens are not equivalent, minor structural differences could result in different stereospecificities due to changes in interaction with the active site of each enzyme [34]. The previous conclusion that a metal ion is coordinated directly to the *pro-Sp* oxygen in *Tetrahymena* ribozyme-catalyzed reactions [31–33] has also been questioned recently [15,35] because of the poor rescue by Mn^{2+} ions, despite the fact that a significant thio effect was observed when the *pro-Sp* oxygen of the scissile phosphate was substituted by a sulfur atom.

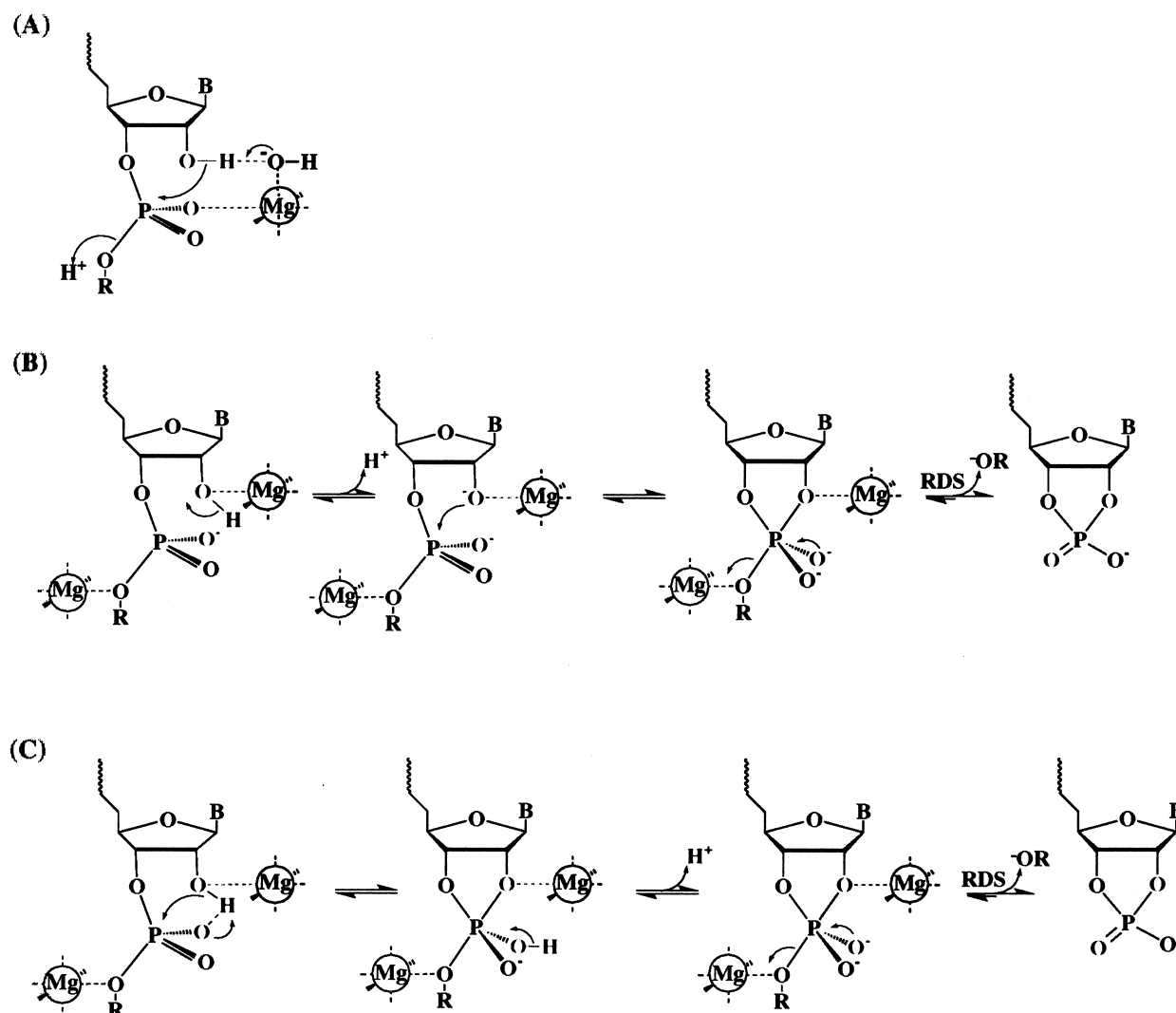


Fig. 3. Three possible mechanisms in cleavage of RNA catalyzed by hammerhead ribozymes. A: The generally accepted 'single-metal-ion' mechanism of catalysis for hammerhead ribozyme reactions in that a solvated Mg^{2+} -hydroxide acts as a base to deprotonate the 2'-OH, meanwhile the same metal ion is coordinated directly with the *pro*-Rp oxygen of the scissile phosphate to stabilize the intermediate/transition state. B: The 'double-metal-ion' mechanism in that two metal ions are coordinated with the attacking 2'-hydroxyl oxygen and the leaving 5'-oxygen. C: The 'triester-like' mechanism proposed newly in this paper. Similar to the mechanism shown in B, the first metal ion, acting as a Lewis acid, is coordinated directly with the 2'-oxygen to promote deprotonation of the 2'-hydroxyl group, meanwhile the second metal ion is coordinated with the 5'-oxygen leaving group to neutralize the developing negative charge on the 5'-oxygen as it departs from the phosphorus. The hydrogen bonding interaction between the *pro*-Rp oxygen and the 2'-OH plays an important role in ribozyme-catalyzed reactions. The favorable hydrogen bonding interaction between the 2'-oxygen and the *pro*-Rp oxygen assists in the deprotonation of the 2'-OH, increasing its nucleophilicity, but also in neutralization of the negative charge on the nonbridging *pro*-Rp oxygen, accelerating the formation of the pentacoordinate intermediate/transition state.

3.2. Factors leading to thio effects in hammerhead ribozyme-catalyzed reactions

If it is not the blockage of the direct coordination of a Mg^{2+} ion with the *pro*-Rp oxygen by the introduction of sulfur at the Rp position of the scissile phosphate, what might be the factor(s) that lead(s) to such a significant thio effect? Why does such a significant 'thio effect' appear only in the RpS isomer in hammerhead ribozyme-catalyzed reactions?

The hydrolysis of RNA at a particular site is initialized by a nucleophilic attack of the 2'-oxygen on the adjacent phosphorus atom, forming a pentacoordinate intermediate or a transition state, P(V), and the reaction is completed by the release of the 5'-oxygen leaving group from P(V), generating a cyclic 2',3'-phosphodiester and a free 5'-hydroxyl group.

Cleavage of RNA can be accelerated by a number of factors, including: an acidic (Lewis acid) or a basic group to aid in deprotonation of the 2'-hydroxyl; an acidic group that can neutralize and stabilize the leaving 5'-oxyanion group; any environment that can stabilize the pentavalent species which is either a transition state or a short-lived intermediate [36]. Generally, metal ions or protons play these roles in the above processes. Evidence for the double-metal-ion mechanism of catalysis [6,7] has been provided, in reactions catalyzed by hammerhead ribozymes, in that the first metal ion, acting as a Lewis acid, is coordinated directly with the 2'-oxygen to promote deprotonation of the 2'-hydroxyl group, meanwhile the second metal ion is coordinated with the 5'-oxygen leaving group to neutralize the developing negative charge on the

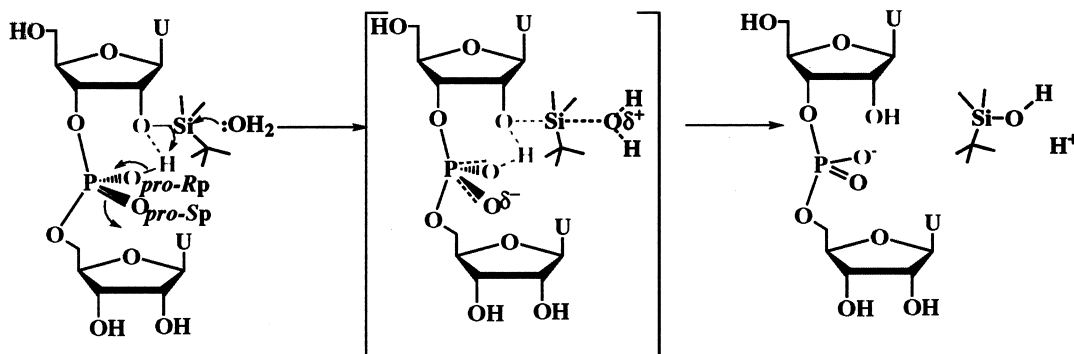


Fig. 4. Neighboring group participation of the internucleotidic phosphate residue in acid-catalyzed 2'-desilylation in that the *pro*-Rp oxygen atom is in a position favorable to form a hydrogen bond with the 2'-OH because the distance between the *pro*-Rp oxygen and the hydrogen of 2'-OH is short enough to form such a bond, whereas the distance between the *pro*-Sp oxygen atom and the hydrogen atom of 2'-OH is too long to form such a bond.

5'-oxygen as it departs from the phosphorus (Fig. 3) [6–11,15].

Only one explanation exists in the literature that attempts to explain the observed 'thio effect', in hammerhead ribozyme-catalyzed reactions, that is the direct coordination of a metal ion with the non-bridging *pro*-Rp oxygen, rendering the phosphorus more susceptible to nucleophilic attack [4,21,22]. It should also be possible that, alternatively, neutralization of the negative charge of the non-bridging oxygen be accomplished by a proton. According to Monte Carlo calculations, the *pro*-Rp oxygen atom is in a position favorable to form a hydrogen bond with the 2'-OH because the distance between the *pro*-Rp oxygen and the hydrogen of 2'-OH is short enough to form such a bond, whereas the distance between the *pro*-Sp oxygen atom and the hydrogen atom of 2'-OH is too long to form such a bond [37]. These Monte Carlo simulations have been verified recently by measuring kinetics of desilylation of 2'-O-silyl derivatives (Fig. 4) of phosphorothioate diastereomers in that desilylation of the Rp isomer proceeded more slowly than that of the Sp isomer due to the interruption of the potential hydrogen bonding interaction between the *pro*-Rp oxygen (of the parental native RNA) and the adjacent 2'-oxygen in the Rp isomer [37].

We propose that a similar hydrogen bonding interaction between the *pro*-Rp oxygen and the 2'-OH plays an important role in ribozyme-catalyzed reactions. The favorable hydrogen bonding interaction between the 2'-oxygen and the *pro*-Rp oxygen assists not only in the deprotonation of the 2'-OH, increasing its nucleophilicity, but also in neutralization of the negative charge on the non-bridging oxygen, accelerating the formation of the pentacoordinate intermediate/transition state (Fig. 3C). Such a migration of a proton to the pentacoordinate species should be energetically feasible because the second pK_a of the phosphorane is estimated to be above 11 [38], that is roughly the pK_a expected for the 2'-OH coordinated

directly with a divalent metal ion [36]. We expect a concerted cyclic transition state for the 2'-proton migration step: as the proton migrates toward the *pro*-Rp oxygen, the distance between the 2'-oxygen and phosphorus becomes shorter, then, as a result of more pentacoordinate-like character of the phosphorus, the pK_a of the *pro*-Rp oxygen becomes higher, more able to abstract the 2'-proton (Fig. 3C). This kind of proton migration during the formation of a pentacoordinate species and the subsequent deprotonation prior to the rate-limiting cleavage of the P-(5'-O) bond is in accordance with Breslow's mechanism, known as the 'triesters-like' pathway, in that a non-bridging phosphoryl oxygen atom is protonated to render the substrate triester-like [39–45]. The triester-like mechanism provides a more potential catalytic pathway because phosphate triesters are typically more than three orders of magnitude more reactive than the corresponding phosphate diesters [46]. Such a mechanism involving the nucleophilic attack of an oxyanion on the neutral phosphate is supported by the recent solution-phase *ab initio* calculations [47].

If this is the case, when the *pro*-Rp oxygen is replaced by sulfur, such migration of a proton becomes more difficult because sulfur is less able to abstract a proton as evidenced by the lower pK_a of thiol relative to the pK_a of the corresponding alcohol [48], with resultant inhibition of hammerhead ribozyme-mediated cleavage. By contrast, when the *pro*-Sp oxygen is replaced by sulfur, the very important hydrogen bonding interaction between the *pro*-Rp oxygen and the 2'-OH is maintained, and, thus, the thio substitution is not expected to have any effects on the hammerhead-dependent RNA hydrolysis. This expectation has been demonstrated experimentally [22,23]. Therefore, without the assumption of the direct coordination of a metal ion with the *pro*-Rp oxygen, the experimental observations can be reconciled with this proton-migration process. If the dianionic pentacoordinate species, which leads to the overall rate-limiting transition state (indicated by RDS in Fig. 3C), is the active species at steady-state concentrations, all the theoretical calculations [6,49–53] can also be reconciled to the experimental kinetic data. It is to be noted that, if the dianionic pentacoordinate species is the active species, the ribozyme-mediated cleavage rate should increase linearly with pH. Indeed, in the presence of various metal ions, the log of the cleavage rate was found to increase linearly with pH over a range from pH 6 to 9 with a slope of approximately unity, in agreement with the mechanism shown

Table 1

Kinetic parameters for cleavage of thiosubstrates catalyzed by a hammerhead ribozyme in the presence of Mg^{2+} or Mn^{2+} ions

Isomer	M^{2+}	k_{obs} (min^{-1})	Rescue value
RpS	Mg^{2+}	8.1×10^{-4}	5.8
	Mn^{2+}	4.7×10^{-3}	
SpS	Mg^{2+}	6.0×10^{-2}	14
	Mn^{2+}	8.5×10^{-1}	

in Fig. 3C (although also in agreement with the mechanisms shown in Fig. 3A,B).

Substitution of Mg^{2+} by Mn^{2+} ions not only stimulated cleavage of the *Rp*-phosphorothioate substrate significantly but also stimulated cleavage of the *Sp*-phosphorothioate substrate (Fig. 2). These results are pertinent to our proposal that the rescue effects have originated from the intrinsic properties of metal ions and, in part, from the different pK_a values of the different metal ions used [10], without the specific interaction of a metal ion with the *pro-Rp* oxygen of the scissile phosphate in the transition state [10,15,23]. Therefore, the lower the pK_a of the metal ion that is bound directly with the 2'-hydroxyl group, the more polarizable the 2'-O-H bond and more favorable the proton migration to the *pro-Rp* oxygen, resulting in higher concentrations of pentacoordinate species. Neutral phosphates are up to 10^5 times more susceptible to nucleophilic attack by 2'-oxygen, compared with monoanionic phosphates [46]. Most importantly, the mechanism presented in Fig. 3C, involving proton migration from the 2'-OH to the *pro-Rp* oxygen during the formation of a pentacoordinate species and the subsequent deprotonation prior to the rate-limiting cleavage of the P-(5'-O) bond, can rationalize the observed thio effects and the absence of specific Mn^{2+} rescues (Fig. 2).

It is important to note that recent analysis by Scott's group indicates the probable invalidity of the earlier postulate [20] that the Mg^{2+} ion bound to the *pro-R* oxygen of the scissile phosphate in the ground state might move, together with the phosphate, into a conformation more suitable for in-line attack. They recently trapped an intermediate with an advanced conformational change, where the phosphate had moved considerably [54]. However, the metal ion (this time a Co^{2+} ion) remained associated with N7 of $A_{1.1}$ and did not move with the *pro-R* oxygen, demonstrating clearly that a ribozyme can cleave the phosphodiester bond with high efficiency without invoking an electrophilic catalysis by direct coordination of a metal ion with the *pro-Rp* oxygen.

Although the thio effect is a powerful tool in identifying the coordination site of a metal ion with a phosphoryl oxygen, care must be taken in the application of it in enzymatic reactions. In this paper, the roles of metal ions in hammerhead ribozyme reactions were revisited and the origin of the thio effect in ribozyme reactions was elucidated. The 'triest-er-like' mechanism proposed in this paper for reactions catalyzed by hammerhead ribozymes can rationalize the observed thio effects and the absence of specific Mn^{2+} rescues.

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