Ribozyme Mechanism Revisited: Evidence against Direct Coordination of a Mg^{2+} Ion with the *pro-R* Oxygen of the Scissile Phosphate in the Transition State of a Hammerhead Ribozyme-Catalyzed Reaction

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Over the last a few years, ribozymes have been recognized as metalloenzymes.[1−11] In current research, much emphasis is being placed on the identification of both the metal-binding site and the number of catalytic metal ions involved in the reaction. In studies of enzymatic processes, sulfur-containing analogs of biological phosphoric esters are often used as substrate analogs.[12−14] Substitution of sulfur for one of the diastereotopic oxygens of a phosphate moiety can be conveniently achieved by automated solid-phase synthesis with standard phosphoramidite building blocks. The normal iodine-promoted oxidation is replaced by a sulfur-transfer step,[15] and the reaction generates the epimers *R*pS and *S*pS, wherein either *pro-R*p or *pro-S*p of a nonbridging phosphoryl oxygen is replaced by sulfur. The reaction products can be easily separated by reverse-phase HPLC.[11] These compounds, which exhibit varying degrees of stereoselectivity in their interactions with enzymes, especially when the chiral phosphorothioate group is located at the reaction center, have been widely used in efforts to obtain details of the stereochemical configurations.[16−18] The effect on the rate of the reaction of the substitution of oxygen by sulfur is commonly referred to as the "thio effect" (i.e., thio effect = $k_{\text{phosphate}}/k_{\text{phosphorothioate}}$).[19] Recently, studies of the thio effect have frequently been used to probe the rate-limiting step of ribozyme-catalyzed reactions and to identify the coordination site of a metal ion with a phosphoryl oxygen.[1,9−11,20,21] In the latter case, the experimental approach relies on the previous measurements of the affinity of divalent ions for ATPβS, since the Mg^{2+} ion is coordinated 30000-fold more strongly to oxygen than to sulfur while the Mn^{2+} ion is coordinated to both oxygen and sulfur more or less equally.[22,23] Thus, the discrimination by Mn^{2+} ions in binding to either an oxygen or a sulfur atom is poor, while the poor binding of the Mg^{2+} ion to sulfur results in a very large thio effect.[1]

Extensive examinations of thio effects have been performed in the case of reactions catalyzed by hammerhead ribozymes.[9−11] The products of the magnesium-dependent cleavage reactions catalyzed by hammerhead ribozymes[24−26] are 5′-hydroxyl and 2′,3′-cyclic phosphate termini, and each reaction proceeds with inversion of the configuration at the phosphorus atom.[10,11] Thus, the cleavage reaction occurs by *trans*-esterification, with the oxygen atom of the 2′-hydroxyl group attacking the scissile phosphate by an *in-line* S_N2 mechanism. Nucleophilic attack by the 2′-oxygen on the phosphorus yields either a pentacoordinated transition state or a short-lived intermediate. This transition state/intermediate should be stabilized by direct coordination of a metal ion to the negatively charged nonbridging phosphoryl oxygen (electrophilic catalysis; Figure 1b).[6] The direct evidence for binding of a metal ion to the *pro-R*p oxygen is derived from a Mn^{2+} "rescue" experiment.[9−11] Thio substitution at the *pro-R*p oxygen at the cleavage site of the substrate (*R*pS) for a hammerhead ribozyme resulted in a large thio effect that was relieved by replacement of Mg^{2+} by Mn^{2+}, which has a higher affinity for sulfur than does Mg^{2+}.[9−11] This observation led to the general conclusion that a Mg^{2+} ion is directly coordinated with the *pro-R*p oxygen.[6] In this arrangement, the bound metal ion can act as an electrophilic catalyst, and thus, the proposed mechanism is very attractive as an explaination of the catalytic activity of metalloenzymes.

In an attempt to quantitate the rescue capability of Mn^{2+} ions, we re-examined the thio effects for two epimeric thio substrates, *R*pS and *S*pS, in which the *pro-R*p and the *pro-S*p oxygen at the cleavage site had been replaced, respectively, by sulfur. The sequences of a 32-mer hammerhead ribozyme (R32) and its 11-mer substrate (R11O) are shown in Figure 1a. In this system, the observed rate of the reaction (k_{cat}) represents the rate of the chemical cleavage step (k_{cleav}).[7,8] As can be seen in Figure 2 and in agreement with previous observations,[9−11] *R*pS was cleaved much more slowly than *S*pS in a solution that contained either Mg^{2+} or Mn^{2+} ions, and Mn^{2+} ions were able to enhance the cleavage of the thio substrates, providing a clear demonstration that the chemical cleavage step is indeed the rate-limiting step in this ribozyme system (Figure 1a).[7,8] The calculated rate constants for the cleavage step (k_{cleav}) are summarized in Table 1, together with the previously published rate constants from similar studies. The magnitude of the thio effect for the *R*pS thio substrate depends on the ribozyme system and the reaction conditions used; decreases in activity of about 34-fold, about 55-fold, and about 540-fold were observed, respectively, by Dahm and Uhlenbeck,[9] by Koizumi and Ohtsuka,[10] and in the present study. In the presence of Mn^{2+} ions, the thio effect can be partially overcome; with increases of about 4-fold,[11]

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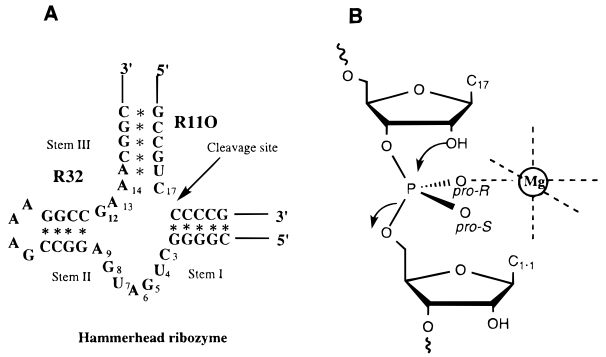

Figure 1. (a) Sequence and secondary structure of the hammerhead ribozyme (R32) and substrate (R11O) complex used in this study, with all Watson−Crick base pairs indicated by asterisks. (b) Previously proposed transition state or intermediate structure,[9] wherein a Mg^{2+} ion is coordinated directly to the *pro-R*p oxygen atom at the cleavage site, such that it can act as an electrophilic catalyst.[6]

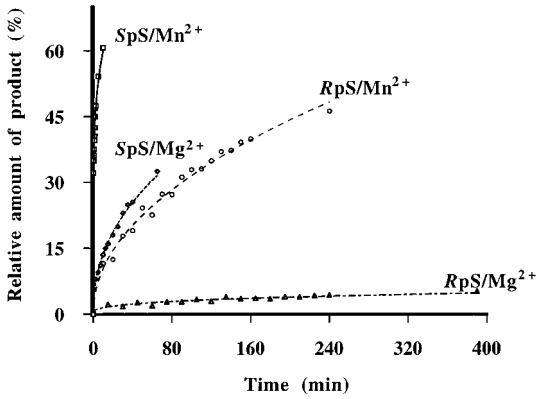

Figure 2. Time courses of hammerhead ribozyme-catalyzed reactions. Reactions were performed in the presence of either Mg^{2+} or Mn^{2+} ions, with the *R*pS or the *S*pS substrate, in which either the *pro-R*p or the *pro-S*p oxygen at the cleavage site had been replaced by sulfur (the autoradiograms for ribozyme-mediated cleavage of reactions used in Figure 2 are available as supporting information).[30]

about 20-fold,[9] and about 24-fold (Figure 2 and Table 1). These rescue values are much smaller than the 660-fold recovery that was observed in the reactions catalyzed by the *Tetrahymena* ribozyme when the 3′-bridging oxygen was replaced by a sulfur atom.[1]

It is noteworthy, however, that, although a small thio effect was observed with *S*p*S* in the presence of Mg^{2+} (about 1.9-fold[11] and 28-fold), the reaction could be enhanced to nearly the same extent as observed with *R*pS by the addition of Mn^{2+} ion (about 3.8-fold[11] and 23-fold; see Table 1). In addition to the observation of a rescue effect of the same magnitude by Mn^{2+} ions with the *R*pS and *S*pS substrates, a similar phenomenon can also be observed with the unmodified natural substrate (the rate of the ribozyme-mediated hydrolysis of R11O was about 20-fold higher in the presence of Mn^{2+} ions than in the presence of Mg^{2+} ions,[27] most probably because the pK_a of Mn^{2+}-bound water molecules was one unit lower than the pK_a of Mg^{2+}-bound water molecules[28]). Since Mn^{2+}-mediated cleavage occurs about 20-fold more rapidly than Mg^{2+}-mediated cleavage with *R*pS and *S*pS and also with the natural substrate, it seems unlikely that the previously observed rescue effects[9−11] support the proposed direct and specific coordination of a metal ion to the *pro-R*p oxygen in the transition state of a hammerhead ribozyme-catalyzed reaction. The higher catalytic power of Mn^{2+} than of Mg^{2+} ions at a specific pH is most probably a reflection of a lower pK_a of the hydrate of the former,[28] etc. and is not a result of rescue by Mn^{2+} ions.

Table 1. Kinetic Parameters for Cleavage of Substrates by Ribozyme in the Presence of Mg^{2+} or Mn^{2+} Ions and the Corresponding Thio Effects in the Presence of Mg^{2+} Ions and the Rescue Values Obtained in the Presence of Mn^{2+} Ions

ribozyme	substrate	M^{2+}	k_{cleav} (min^{-1})	thio effect	rescue value
R32	R11O	Mg^{2+}	8.6×10^{-2}		
	*R*pS	Mg^{2+}	1.6×10^{-4}	540	
		Mn^{2+}	3.9×10^{-3}		24
	*S*pS	Mg^{2+}	3.0×10^{-3}	28	
		Mn^{2+}	6.9×10^{-2}		23
R37[a]	R13O				
	*R*pS	Mg^{2+}		62	
		Mn^{2+}			4.3
	*S*pS	Mg^{2+}		1.9	
		Mn^{2+}			3.8
R33[b]	R13O	Mg^{2+}	0.17		
	*R*pS	Mg^{2+}	5×10^{-3}	34	
		Mn^{2+}	9×10^{-2}		18
	*S*pS	Mg^{2+}			
		Mn^{2+}			
tetrahymena[c]	3′O	Mg^{2+}	8.9×10^{-4}		
	3′S	Mg^{2+}	9×10^{-7}	1000	
		Mn^{2+}	6×10^{-4}		667

[a] The thio effects and rescue values were estimated from the time course and the half-life on the basis of the results in ref 11. [b] Kinetic parameters were estimated from a graph of the cleavage rate *versus* concentration of the metal ions in ref 9 at the point where the concentration of metal ions was 25 mM. [c] Results taken from ref 1.

Although examination of cleavage of *R*pS and *S*pS substrates demonstrated that *R*pS is much less reactive than *S*pS,[9−11] the difference in reactivity does not appear to originate from the direct coordination of a metal ion to a specific nonbridging oxygen. Substitution by sulfur of one of the diastereotopic oxygen atoms at the cleavage site could, in principle, perturb the active site because an atom of sulfur is larger than an atom of oxygen, because of a longer P−S bond (the P−S bond is 1.9 Å long, whereas the P−O bond is 1.4 Å long) and because of localization of a negative charge on the sulfur that is singly bonded to phosphorus.[29] This kind of perturbation (asymmetric reaction center) might be responsible, at least in part, for the higher reactivity of *S*pS than of *R*pS.

Although examination of thio effects is a valuable tool for attempts at the elucidation of the mechanism of action of various enzymes, including ribozymes, care must be taken in interpreting the rescue of a reaction by Mn^{2+} ions. We demonstrated in this report that the generally accepted mechanism of action of ribozymes (Figure 1b), wherein a Mg^{2+} ion is directly coordinated with the *pro-R*p oxygen such that the bound metal ion can act as an electrophilic catalyst, is unlikely to be operative.

Supporting Information Available: Autoradiograms for ribozyme-mediated cleavage of reactions used in Figure 2 (2 pages). See any current masthead page for ordering and Internet access instructions.

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(30) *R*pS and *S*pS substrates were prepared by automated solid-phase synthesis[15] and separated by reverse-phase HPLC.[11] The separated *R*pS substrate was incubated with a 2-fold excess of ribozyme and 25 mM Mg^{2+} at 37 °C for 1 h, and then it was purified by HPLC again to remove the small amount of contaminating normal and *S*pS substrates. All reactions were carried out under single-turnover conditions, in a solution that contained 10 μM ribozyme, 1 μM substrate, and 25 mM $MgCl_2$ or $MnCl_2$ in 50 mM MES buffer (pH 6.0) at 25 °C. In the pH range of 6−8, k_{cat} represents the rate of chemical cleavage (k_{cleav}).[7] Reactions were initiated by addition of metal ions to a solution that contained both ribozyme and substrate, and products were quantitated as described previously.[7,8,27]