Hammerhead Ribozyme-Mediated Cleavage of a Substrate Analogue Containing an Internucleotidic Bridging 5′-Phosphorothioate: Implications for the Cleavage Mechanism and the Catalytic Role of the Metal Cofactor

Robert G. Kuimelis and Larry W. McLaughlin*

Department of Chemistry, Merkert Chemistry Center
Boston College, Chestnut Hill, Massachusetts 02167

Received July 13, 1995

The hammerhead ribozyme, derived from a structural motif present in the RNA genomes of several plant pathogens,[1] consists of three helical stems and includes nine conserved nucleotides that are nominally single-stranded and appear to be responsible for the formation of a catalytically active domain (Figure 1a).[2] Recent structural studies[3,4] have provided detailed information about the ground state conformation, and base analogue substitutions[5] have identified specific functional groups critical for catalysis. Autolytic cleavage of the RNA occurs via internal transesterification involving the 2′-hydroxyl adjacent to the scissile phosphodiester, generating terminal 5′-hydroxyl and 2′,3′-cyclic phosphate fragments.[2c] Divalent metal ions (Mg^{2+} or Mn^{2+}) are required for efficient cleavage, and more than one metal cofactor may be necessary for activity. Although essentially metalloenzymes,[6] the precise role of the metal(s) remains obscure.[7] Solvated metal hydroxide has been implicated as the base in the hammerhead complex.[8] Substitution of the Rp and Sp phosphorothioate diesters into the cleavage site has confirmed that the reaction follows an in-line S_N2 mechanism with inversion of configuration at the phosphorus center, and that the *pro-R* nonbridging oxygen of the scissile phosphodiester is bound to the metal cofactor in the transition state.[9] To investigate the cleavage mechanism of the hammerhead ribozyme, we have prepared an oligonucleotide substrate containing an internucleotide 5′-bridging phosphorothioate diester (**2**, Figure 1b) and compared hammerhead-mediated substrate cleavage of the native (**1**) and thio (**2**) ribo linkages.

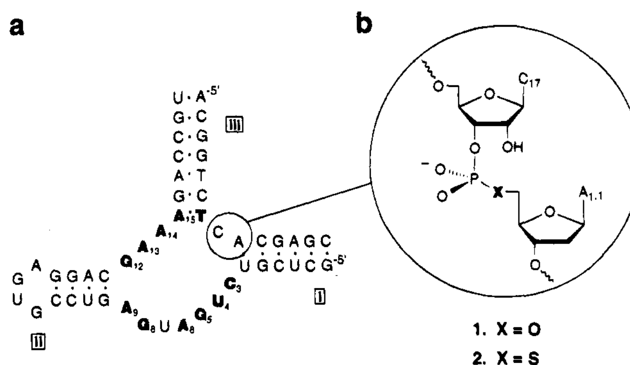

Figure 1. (a) Sequence and secondary structure of the hammerhead complex. Bold letters represent conserved nucleotides. Circled portion indicates the cleavage site. (b) Expansion of the cleavage site between C_{17} and $A_{1.1}$, showing native substrate **1** and the 5′-bridging phosphorothioate substrate analogue **2**.

The two substrates partially illustrated in Figure 1b consist entirely of deoxynucleotides, except for the single mandatory ribonucleotide (C_{17}) at the cleavage site.[10] Such substrates are effectively cleaved by the hammerhead ribozyme.[10,11] We have used single-turnover conditions, where the ribozyme is in large excess over the radiolabeled substrate, to assure that (i) the observed rate constants represent the actual chemical cleavage step[12] and (ii) all the substrate is complexed to the ribozyme. We examined the ability of the hammerhead ribozyme to cleave **2** in the presence of a series of divalent metals: Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, and Cd^{2+} (previously shown to support the cleavage of a native substrate[13]). We also investigated the effect of spermine on the cleavage rate. In all cases examined, the expected cleavage products were obtained: the 5′-thiol fragment (8-mer) and the 2′,3′-cyclic phosphate fragment (6-mer), identified by both nucleoside composition analysis[14] and analytical PAGE using authentic standards for comparison (see Figure 2).

In the presence of the ribozyme and the absence of a metal cofactor (or spermine), there is no increase in the cleavage rate of **2** over the control rate[15] (0.12×10^{-3} vs 0.15×10^{-3} min^{-1}, Table 1). The ribozyme-mediated cleavage of **2** is substantially accelerated in the presence of a metal cofactor. A 10 mM concentration of Mg^{2+} increases the ribozyme-catalyzed cleavage rate 540-fold, but the same metal (in the absence of ribozyme) increases the control rate by only a factor of 10. Other divalent cations also increase the cleavage rate of **2**, most notably Mn^{2+}, which accelerates the cleavage rate more than 680-fold over the no-metal, ribozyme-mediated cleavage. In the case of the two metals Zn^{2+} and Cd^{2+}, the ribozyme actually affords moderate *protection* from cleavage, compared to the control rates. This implies that the ribozyme does not bind Zn^{2+} or Cd^{2+} and use them effectively as catalytic cofactors, and the labile phosphorothioate linkage is somewhat shielded from the bulk solution by the tertiary structure of the folded complex.

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(15) The control rate is defined here as the rate of spontaneous cleavage of **2** in the absence of ribozyme, which varies dramatically depending upon the reaction conditions employed; increasingly "soft" Lewis acids help facilitate the transesterification of **2**, in accordance with Pearson's HSAB theory: (a) Pearson, R. G. *Coord. Chem. Rev.* **1990**, *100*, 403–425. (b) Pearson, R. G. *Acc. Chem. Res.* **1993**, *26*, 250–255. (c) Huheey, J. E. *Inorganic Chemistry: principles of structure and reactivity*; Harper and Row: New York, 1983.

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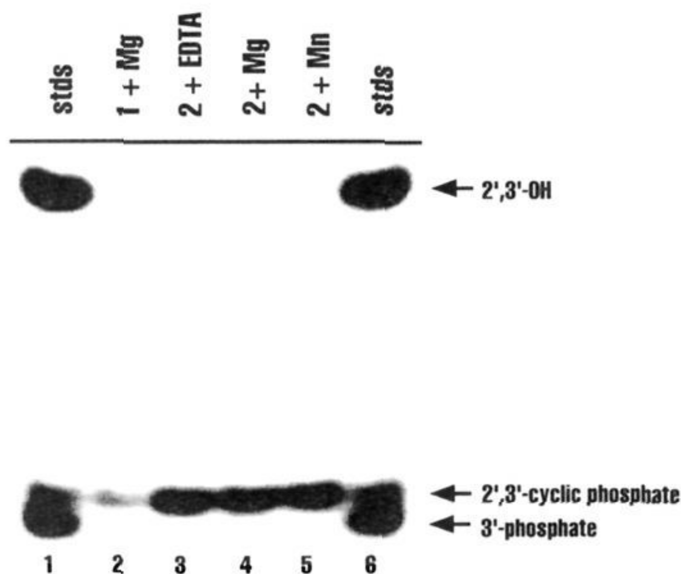

Figure 2. Analytical PAGE analysis of the ribozyme-mediated 8-mer 5′-cleavage products. Lanes 1 and 6 (standards) contain a mixture of 5′-ACGGTCTrC, where rC is 2′,3′-dihydroxy, 2′,3′-cyclic phosphate or 3′-monophosphate (authentic standards). Lane 2 is the 5′-cleavage product of **1**. Lanes 3–5 are the 5′-cleavage products of **2** in the presence of EDTA, Mg, or Mn.

Perhaps the most striking finding revealed in Table 1 is that the addition of 0.5 mM spermine (known to stabilize higher-order nucleic acid structure[16]) to the ribozyme reaction mixture increases the cleavage rate nearly 200-fold *in the absence of a metal cofactor.* Moreover, the rate of this reaction is comparable to the metal-containing ribozyme-mediated cleavage reactions. In contrast, when spermine was added to the metal-containing ribozyme-mediated cleavage reactions, only very moderate rate enhancements were observed. NaCl and spermidine are also capable of stabilizing nucleic acid structures, but neither of these (at 0.5 M or 0.5 mM, respectively) increased the rate of the nonmetal ribozyme-promoted cleavage. These observations suggest a special role for spermine and show that a metal cofactor is not necessary for efficient cleavage by the hammerhead ribozyme when the substrate possesses a sufficiently good leaving group at the cleavage site.[17] Spermine did not support cleavage of **1** in the absence of metal ions. Some hammerheads have been reported to support minimal cleavage in the absence of a metal cofactor, but only if a polycation is present in the reaction mixture.[13,18]

The cleavage rates of the Mg^{2+}- and Mn^{2+}-containing reactions are almost identical at 93×10^{-3} and 83×10^{-3} min^{-1}, respectively. If a metal cofactor interacts with the 5′-leaving group of $A_{1.1}$ in the transition state, as proposed from solvent isotope effect experiments[19] and molecular orbital calculations,[20] one would expect a substantial rate increase when the "softer" Mn^{2+} cofactor is used in place of Mg^{2+}.[21] *The absence of an accelerated rate for this complex suggests that the metal cofactor does not interact with the 5′-leaving group, at least not in the transition state.* In the case of the *Tetrahymena* ribozyme, similar experiments have clearly established that a metal ion coordinates to the 3′-leaving group; in that case, the rate differential (Mg^{2+} vs Mn^{2+}) was 1000-fold.[6c] The hammerhead ribozyme-mediated cleavage rate of **1** in the presence of spermine and Mg^{2+} or Mn^{2+} is 40×10^{-3} or 90×10^{-3} min^{-1}, respectively. These rates are remarkably similar to those for cleavage of **2**, despite the drastic modification to the 5′-leaving group. We estimate that in the absence of ribozyme, **2** is nearly 10^6-fold more susceptible to transesterification than is **1**.[22] Despite this lability, the ribozyme-mediated cleavage rate of **2** is almost identical with the cleavage rate of **1** in the presence of Mg^{2+} or Mn^{2+}. This observation strongly suggests that the metal cofactor does not interact with the 5′-leaving group in the transition state and, further, that the departure of the 5′-leaving group is not the rate-limiting step of the cleavage reaction. Such a scenario would seem to rule out a concerted transesterification mechanism as the mode of cleavage and suggests that a two-step mechanism is operative, where attack by the 2′-oxygen of C_{17} on the phosphorus (the first step) is the rate-limiting step.

In summary, using a 5′-bridging phosphorothioate substrate, we have shown that (i) a metal cofactor is not necessary for efficient cleavage if the leaving group is sufficiently good, (ii) in contrast to the *Tetrahymena* ribozyme, a metal cofactor does not interact with the leaving group in the transition state, and (iii) the departure of the 5′-leaving group is not the rate-limiting step of the cleavage reaction.

Table 1. Pseudo-First-Order Rate Constants, k_{obs}, for the Ribozyme-Mediated Cleavage of Phosphorothioate **2** under Various Conditions

	$k_{obs} \times 10^{-3}$ (min^{-1})[a]						
	EDTA	Mg^{2+}	Ca^{2+}	Mn^{2+}	Co^{2+}	Zn^{2+}	Cd^{2+}
spermine[b]	23	93	66	83	39	25	290
no spermine	0.12	65	45	82	24	13	150
control[c]	0.15	1.5	3.7	11	15	57	530

[a] Ribozyme reactions contained 0.5 μM ribozyme and 0.5 nM radiolabeled substrate in 50 mM Tris-HCl (pH 7.5) at 25 °C containing 10 mM metal dichloride or 5 mM EDTA. [b] 0.5 mM spermine added to the ribozyme reaction. [c] Rate constants for cleavage of the substrate in the absence of ribozyme (50 mM Tris-HCl, pH 7.5, 5 mM metal or EDTA, 25 °C).

Acknowledgment. This work was supported by a grant from the NIH (GM47660). L.W.M. is the recipient of a Faculty Research Award (FRA-384) from the American Cancer Society.

Supporting Information Available: Synthetic procedures for the mercapto monomer and details on the synthesis, purification, and characterization of **1** and **2**; 5′-cleavage product PAGE analysis for the complete metal series; representative autoradiograms showing the time course of product formation; pH–rate profiles for **1** and **2** (11 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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