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Two active site divalent ions in the crystal structure of the hammerhead ribozyme bound to a transition state analog

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Abstract

The crystal structure of the hammerhead ribozyme bound to the pentavalent transition state analog vanadate reveals significant rearrangements relative to the previously solved structures. The active site contracts, bringing G10.1 closer to the cleavage site, and repositioning a divalent metal ion such that it could, ultimately, interact directly with the scissile phosphate. This ion could also position a water molecule to serve as a general acid in the cleavage reaction. A second divalent ion is observed coordinated to the O6 of G12. This metal ion is well-placed to help tune the pK_A of G12. Based on this crystal structure as well as a wealth of biochemical studies, we propose a mechanism in which G12 serves as the general base and a magnesium-bound water serves as a general acid.

Graphic Abstract

The hammerhead ribozyme was the first of the small nucleolytic ribozymes to be described¹ and the first to have its crystal structure determined^{2, 3}. While these initial structures were found to be trapped in an inactive conformation,^{4, 5} structures of the extended hammerhead ribozymes inhibited by placement of a deoxy- or methoxy- modification at the cleavage site were largely in agreement with solution biochemical experiments^{6–9}. Nevertheless, there remains a significant body of data that cannot be reconciled with the crystal structures of the trapped, inhibited versions of the extended ribozyme previously

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Author Contributions

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Notes

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Supporting Information. The Supporting Information is available free of charge on the ACS Publications website. Detailed materials and methods, Figure S1 Electron density for the hammerhead ribozyme active site, Figure S2 Anomalous diffraction from a Mn^{2+} -soaked crystal, and Figure S3 Electron density near G8. (Word file)

studied^{9–11}. We therefore sought to obtain a crystal structure of the hammerhead ribozyme bound to a transition state analog.

We started with the sequence of the artificial hammer-head RzB ribozyme.¹² To obtain the crystal structure of the transition state analog, we mixed the three RNA strands shown in Figure 1. The resulting complex lacks the scissile phosphate but retains the 2′-,3′- and 5′-hydroxyls at the cleavage site. This complex was co-crystallized with NH_4VO_3 . Details are provided in the accompanying Supporting Information and in Table S1 and electron density for the active site is shown in Figure S1. We obtained crystal structures for crystals soaked in Mg^{2+} or Mn^{2+} ions at 3.0 and 3.2 Å resolution respectively. Both of these divalent ions support hammerhead ribozyme activity.^{13, 14}

We first examined the structure for evidence that vanadate was indeed present at the position corresponding to the scissile phosphate. Anomalous difference maps calculated with Mg^{2+} or Mn^{2+} soaked crystals reveal a 4–5 σ peak at the cleavage site centered on the position normally occupied by the scissile phosphate (Figure S2). While the phosphorus atoms in the RNA backbone can potentially give rise to an anomalous signal, there is no density in the anomalous difference map for any of the backbone phosphate groups. We therefore attribute the peak in the anomalous map to vanadium. Distances between the 2′-, 3′- and 5′-oxygens and the vanadium atom are consistent with direct coordination between the oxygen atoms and the vanadium atom. Finally, the structure of the active site is distinctly different than that observed for the previous structures containing a tetravalent phosphate at the active site (Figure 2). The description of the active site described here will focus on the structure of the ribozyme in Mn^{2+} ion as locations of the divalent metal ion binding sites can be unambiguously characterized using anomalous diffraction.

The first major difference between the inhibited structures and the transition state analog involves the G10.1-C11 base pair. A significant body of work suggests that there is an active site divalent metal ion whose ligands include the N7 of G10.1, the pro- R_P oxygen of A9 and the pro- R_P oxygen of the scissile phosphate^{10, 15}. This metal ion, which we call Mn1, is observed in crystals to bind near the N7 of G10.1 and the pro- R_P oxygen of A9, however, is it always distant from the cleavage site^{8, 9, 16}. For example, in the crystal structure of the RzB ribozyme at pH 5.0,⁹ Mn1 is 4.6 Å away from the pro- R_P oxygen of the scissile phosphate.

In the crystal structure of the transition state analog, the active site has, in fact, undergone small, but significant conformational change that results in rotation of the oxygens on the scissile phosphate. As a result, Mn1 is now observed to be only 3.9 Å from the oxygen atom from the vanadate that would correspond to pro- S_P oxygen of the scissile phosphate (Figure 2C, Table 1). The oxygen atom corresponding to the pro- R_P oxygen of the scissile phosphate is 5.3 Å distant. While the position of Mn1 in the crystal structure of the transition state analog still does not allow direct interactions with all of the predicted ligands to occur simultaneously, there is net movement of the metal ion towards the scissile phosphate providing better agreement with the available solution biochemical data.^{10, 11, 15} It is possible that there are additional changes in the active site structure that occur during the cleavage reaction that allow all three predicted ligands to interact with Mn1. Alternately,

it is possible that vanadate is not a sufficiently good mimic of the pentavalent phosphate intermediate to observe these interactions in what is still a trapped and unreactive crystal structure.

The second major difference between the inhibited structures and the structure of the transition state analog centers around G12. The nucleobase of G12 appears well placed to serve as the general base in the cleavage reaction. In all of the inhibited structures, G12 is positioned such that N1 can hydrogen bond with the 2'-hydroxyl of C17, the nucleophile in the cleavage reaction. In the structure of the transition state analog, the position of G12 is distinctly different from that observed previously. There are three major changes. First, G12 has shifted slightly such that the 2'-oxygen of C17 is positioned by a bifurcated bond involving both the N1 and exocyclic N2 of G12 (Figure 2, Table 1). Second, a metal ion, referred to here as Mn2, is observed to interact directly with the O6 of G12 and indirectly with the pro-R_p oxygen of C17. Third, the base-pairing between G12 and A9 has been broken, allowing G12 to move towards the cleavage site. Interestingly, the position of G12 in this transition state analog is similar to that previously observed at high pH and in structures of the G12A mutants.^{9, 17, 18}

The conformation of G12 as observed in the structure of the transition state analog is supported by a significant body of solution biochemical studies. While the sheared base pair between G12 and A9 is observed in structures of the inhibited ribozyme, replacement of A9 by a purine nucleobase, which cannot base pair with G12, is only modestly detrimental to catalysis^{19, 20}. This suggests this base-pairing interaction is not critical for ribozyme activity and could, in fact, be inhibitory. The exocyclic amine of G12, however, does seem to play an important role in catalysis as there is a significant difference in activity when G12 is replaced by inosine, a nucleobase that resembles guanosine but lacks the N2 exocyclic amine^{19–21}. These data point to a significant role for the exocyclic amine of G12. The hydrogen bond between the N2 of G12 and the 2'-oxygen of C17 could play critical roles in catalysis. This interaction likely serves to help position the nucleophile and it could also facilitate deprotonation of the 2'-hydroxyl of C17.

While monovalent and divalent ions have previously been observed to interact with the Hoogsteen face of G12 both at the N7 and O6 positions, the interaction with O6 was water-mediated and the metal ions were distant from the active site^{8, 9}. A new divalent metal ion binding mode at G12 is observed in transition state analog. In this structure, Mn2 is observed to directly coordinate to the O6 of G12 (Figure 2, Table 1). Anomalous scattering experiments clearly demonstrate Mn²⁺ binding at this position and a 3σ peak is observed at this position in the F_o-F_c map from the crystal containing Mg²⁺. The importance of this divalent ion has been demonstrated using a G12A mutant of the hammerhead ribozyme. When the keto oxygen of G12 is replaced with the exo-cyclic amine of A12, a metal specificity switch is observed, with the G12A mutant exhibiting a clear preference for Zn²⁺ ion⁹. This suggests that there is a direct interaction between the Zn²⁺ ion and the exocyclic amine of A12 and, by analogy, an interaction between a divalent metal ion and the O6 of G12 in the wild-type ribozyme. The pro-R_p oxygen of C17 also interacts with this metal ion, likely through an outer-sphere, water-mediated interaction consistent with the lack of thio effect at this phosphate.²²

It has been suggested that the pK_A of G12 is shifted from the ~9.4 observed for free guanosine to ~8.0 in the active site of the hammerhead ribozyme^{9, 23}. Coordination of the Mn^{2+} ion at O6 has the potential to lower the pK_A of G12 either through electrostatic effects or through favoring the formation of tautomeric species that help distribute the negative charge on N1.

It remains unclear how, or even if, the ribozyme activates the 5'-oxygen leaving group. In most structures of the inhibited ribozyme,^{6, 8, 9} the 2'-hydroxyl of G8 interacts with the 5'-hydroxyl and was proposed to serve as a general acid in the cleavage reaction.⁶ Solution biochemical experiments clearly demonstrate that the 2'-hydroxyl of G8 plays a significant role in the catalytic reaction.^{24, 25} In the crystal structure of RzB at pH 8.0 and in the transition state analog⁹, however, the 5'-oxygen leaving group is pointed away from the 2'-hydroxyl of G8 (Figure S3). In these structures, the 2'-hydroxyl of G8 is engaged in a hydrogen bond with the vanadate oxygen that would correspond to the pro- R_P oxygen of the scissile phosphate. Thus, it is possible that the 2'-hydroxyl of G8 plays a role other than serving as the general acid. The structure of the transition state analog suggests that the 2'-OH of G8 could help organize the active site by positioning the scissile phosphate and dissipating any negative charge that builds up on the non-bridging oxygen.

It remains possible that a Mn^{2+} -bound water serves as the general acid in the cleavage reaction. Mn1 remains about 3 Å distant from a position that would allow direct coordination to the pro- R_P oxygen of the vanadate while solution biochemical experiments suggest it becomes directly coordinated to this atom. If Mn1 does move to coordinate the pro- R_P oxygen of the scissile phosphate as predicted, the 5'-hydroxyl leaving group is in a conformation that would be within hydrogen bonding distance of the water molecules coordinated to the divalent metal ion. Thomas and Perrin demonstrated that a ribozyme modified to contain a 5'-sulfur leaving group, a hyperactive substrate, was insensitive to metal ion substitutions, and retained activity even in the absence of divalent metal ions²⁵. This observation is consistent with an active site divalent metal ion that is important for stabilization of the 5'-O leaving group.

We therefore propose a mechanism for the hammer-head ribozyme shown in Figure 3. In this model, G12, activated by a divalent metal ion, serves as the general base. A second divalent metal ion, coordinated to the N7 of G10.1, the pro- R_P oxygen of A9, and the pro- R_P oxygen of the scissile phosphate, is positioned such that one of its water ligands can serve as the general acid. A general base with a pK_A of 8–9 and a Mg^{2+} -bound water ($pK_A \sim 11.4$)²⁶ would be consistent with the observed pH-rate profiles for this ribozyme.^{9, 13} While there is significant experimental data available to support this model, it will clearly require further validation, both to determine if G12 is, in fact, a general base, and to learn if and how the hammerhead ribozyme stabilizes the leaving group.

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Figure 1.

Secondary structure of the RNA used to determine the structure of the transition state analog. The ribozyme strand is shown in green. The strand corresponding to the substrate (pink) is synthesized in two fragments. The free 2', 3'- and 5'-hydroxyls at the cleavage site bind and position the vanadate ion.

Figure 2.

Rearrangements in the active site of the hammerhead ribozyme. Nucleotides in the ribozyme active site are shown in green, the cleavage site dinucleotide is shown in pink, and the Mn^{2+} ions in the active site are shown in purple. The three structures shown represent the ribozyme inhibited by a deoxy substitution at C17 at pH 5.0 (A), pH 8.0 (B), and the transition state analog at pH 8.2 reported here (C). The pdbid's for these structures are 5DI4, 5DI2 and 5EAQ respectively. Dashed lines represent base-pairing interactions and the distances observed in Table 1. (A) At pH 5.0, G12 forms a sheared base pair with A9 and an active site Mn^{2+} , Mn1, is 5.5 Å from the pro- R_p oxygen of the scissile phosphate. (B) When the pH is raised to 8.0, the sheared base pair between G12 and A9 is broken, allowing G12 to stack upon C17. A second Mn^{2+} ion, Mn2, is observed to interact with the Hoogsteen face of G12 and Mn1 is observed to be 4.6 Å distant from the pro R_p -oxygen of the scissile phosphate. (C) In the structure of the transition state analog, G12 is positioned such that both N1 and N2 can hydrogen bond with the nucleophile. Mn2 is observed to directly ligand the O6 keto oxygen of G12 and Mn1 is 3.9 Å distant from the position of the pro- S_p oxygen of the scissile phosphate.

Figure 3.

Proposed mechanism for general acid/general base catalysis by the hammerhead ribozyme. G12 serves as the general base. Mn2 binds at the O6 of G12 where it can lower the pK_A of G12 and help to organize the active site. Mn1 becomes positioned near the 5'-oxygen leaving group, positioning one of its bound water molecules such that it could serve as a general acid.

Table 1

Atomic distances in the hammerhead ribozyme active site.

		<u>pH 5.0^a</u>	<u>pH 8.0</u>	<u>TS</u>
G12-N2	C17-2'OH			3.0 Å
G12-N1	C17-2'OH			2.9 Å
G10.1-N7	Mn1	3.2 Å	3.3 Å	2.7 Å
A9-proR _p O	Mn1	1.9 Å	2.6 Å	2.2 Å
U1.1-proR _p O	Mn1	5.5 Å	4.6 Å	5.3 Å
U1.1-proS _p O				3.9 Å
G12-O6	Mn2	3.3 Å	2.6 Å	
C17-proR _p O	Mn2	6.2 Å	3.8 Å	
U1.1-proR _p O	G8-2'-OH		2.7 Å	

^aDistances were obtained from structures with the pdbid's 5DI4 (pH 5.0), 5DI2 (pH 8.0) and 5EAQ (TS).