

# Structural Roles of Monovalent Cations in the HDV Ribozyme

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## SUMMARY

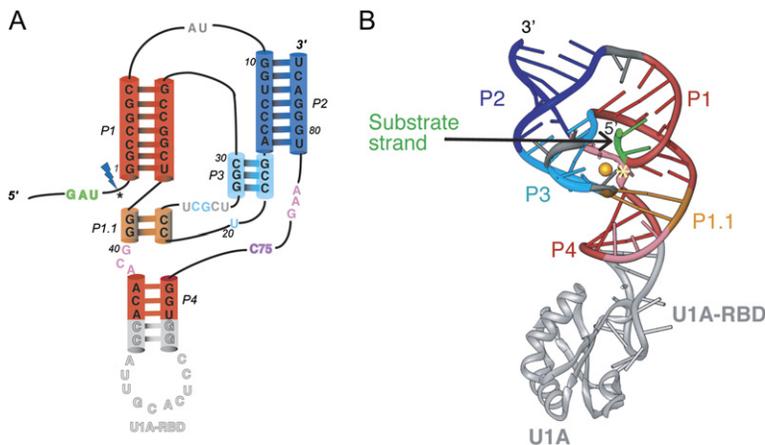
The hepatitis delta virus (HDV) ribozyme catalyzes viral RNA self-cleavage through general acid-base chemistry in which an active-site cytidine and at least one metal ion are involved. Monovalent metal ions support slow catalysis and were proposed to substitute for structural, but not catalytic, divalent metal ions in the RNA. To investigate the role of monovalent cations in ribozyme structure and function, we determined the crystal structure of the precursor HDV ribozyme in the presence of thallium ions ( $Tl^+$ ). Two  $Tl^+$  ions can occupy a previously observed divalent metal ion hexahydrate-binding site located near the scissile phosphate, but are easily competed away by cobalt hexamine, a magnesium hexahydrate mimic and potent reaction inhibitor. Intriguingly, a third  $Tl^+$  ion forms direct inner-sphere contacts with the ribose 2'-OH nucleophile and the *pro*- $S_p$  scissile phosphate oxygen. We discuss possible structural and catalytic implications of monovalent cation binding for the HDV ribozyme mechanism.

## INTRODUCTION

The 85 nucleotide hepatitis delta virus (HDV) ribozyme, found as two closely related genomic and antigenomic variants in HDV, catalyzes efficient site-specific cleavage of the viral RNA phosphodiester backbone through transesterification (Figure 1) (Perrotta and Been, 2006; Shih and Been, 2002). The ribozyme cleaves at the 5' edge of its catalytic core with little sequence preference upstream of the cleavage site, and generates 2',3'-cyclic phosphate and 5'-OH termini. Solvent isotope (Nakano et al., 2000; Shih and Been, 2001), proton inventory (Nakano and Bevilacqua, 2001; Shih and Been, 2001), as well as pH-profile analysis (Nakano et al., 2000; Perrotta et al., 1999, 2006) all suggest that a chemical step involving proton transfer is rate limiting in the HDV ribozyme. The cleavage reaction

displays a bell-shaped pH-rate profile, suggesting two titratable functional groups with apparent  $pK_a$ s of  $\sim 6.5$  and  $\sim 9$ , respectively (Shih and Been, 2002). Mutagenesis (Tanner et al., 1994), chemical rescue, and substrate-modification studies (Das and Piccirilli, 2005; Perrotta et al., 2006; Shih and Been, 2001) revealed that a cytidine nucleotide contained within the ribozyme sequence, C75 (C76 in the viral antigenomic version), is essential to the catalytic activity. The HDV ribozyme has a nonspecific requirement for divalent cations, a variety of which, including  $Mg^{2+}$ ,  $Ca^{2+}$ ,  $Mn^{2+}$ ,  $Sr^{2+}$ ,  $Ba^{2+}$ , and  $Co^{2+}$ , can support catalysis (Nakano et al., 2003; Shih and Been, 1999; Suh et al., 1993; Wu et al., 1989). The cleavage reaction is competitively inhibited by  $Co(NH_3)_6^{3+}$  (Nakano et al., 2000), a mimic of  $Mg^{2+}$  hexahydrate in size and geometry but inert for solvent exchange, such that it does not shuttle protons and only forms outer-sphere coordination with RNA.

Structural, chemical rescue and substrate modification studies indicate that the HDV ribozyme carries out general acid-base catalysis by using the C75 base and a divalent metal ion to activate the 2'-OH nucleophile and stabilize the 5'-oxygen leaving group (Ferre-D'Amare et al., 1998; Das and Piccirilli, 2005; Perrotta et al., 2006; Shih and Been, 2001). The exact roles of the C75 nucleobase and the metal ion in the HDV ribozyme reaction mechanism, however, have been difficult to establish through biochemical studies. Crystal structures of the genomic HDV ribozyme precursor in which the critical C75 base was mutated to a uracil revealed the substrate RNA strand to be sharply kinked at the cleavage site. The geometrically strained substrate is surrounded by two functional groups at the cleavage site: the nucleotide at position 75 that can form hydrogen bonds with the scissile phosphate, and a hexa-hydrated divalent metal ion coordinated to the 5'-oxygen leaving group. Comparison of the precursor ribozyme structure with that of the postcleavage, or product, form (Ferre-D'Amare et al., 1998) revealed a localized conformational change that results in loss of the divalent metal ion from the active site and downshifting of nucleotide 75 to form a hydrogen bond with the 5'-OH of the cleavage product. These crystal structures together with then-available biochemical data led to a model in which C75 deprotonates the 2'-OH nucleophile and the



**Figure 1. Secondary and Tertiary Structure of the HDV Ribozyme**

(A) Sequence and secondary structure of the precursor-form HDV ribozyme crystallization constructs; structural elements are colored as follows to match the 3-D representation in subsequent figures: P1, P4 (red); J1/2, L3 (gray); P2 (blue); P3 (light blue); J1.1/4, J4/2 (pink), U1A-RBD and binding site (silver). The cleavage site is indicated with an asterisk.

(B) Ribbon-stick representation of the precursor HDV ribozyme, color-coded as in (A); a divalent metal ion observed in the active site is colored in gold. The  $-2$  base is modeled in to show the U-turn conformation of the substrate strand more clearly.

magnesium hexahydrate ion protonates the 5' hydroxyl leaving group during ribozyme-catalyzed transesterification (Ke et al., 2004). However, subsequent sulfur atom substitution experiments seemed to support an alternative model, in which a divalent cation is responsible for 2'-OH activation and C75 functions as a general acid to protonate the 5' oxygen leaving group (Das and Piccirilli, 2005). This model was originally proposed based, in part, on experiments using high monovalent ion concentrations to "unmask" the direct catalytic contribution of the RNA (Nakano et al., 2000). However, there has been no crystallographic evidence for metal ion coordination to the 2'-OH nucleophile.

Although the HDV ribozyme is most active at low millimolar concentrations of divalent metal ions, low-level activity is observed in the presence of monovalent ions. Its cleavage rate in 1 M NaCl is  $\sim 1000$ -fold slower than that measured in the presence of 10 mM magnesium (Nakano et al., 2003, 2000; Perrotta and Been, 2006; Wadkins et al., 2001). This observation suggests that monovalent cations may weakly replace divalent cations that stabilize the active structure of the ribozyme and/or contribute directly to catalysis.

To further examine the role of monovalent cations in HDV ribozyme catalysis, we have determined the structure of the HDV ribozyme precursor in the presence of  $Tl^+$ , which has an atomic radius and coordination geometry similar to potassium. Of the 15 RNA-bound  $Tl^+$  ions observed at 2.4 Å resolution, 3 occur near the active site. Two of these localize to the 5'-leaving oxygen side of the active site that is occupied by a hexahydrated divalent cation under physiological conditions, and both are easily competed away by cobalt hexammine. Intriguingly, a third  $Tl^+$  is positioned near the attacking 2'-OH side of the active site, where it can form inner-sphere contacts with both the 2'-OH and the *pro*-Sp scissile phosphate oxygen. Consistent with its inner-sphere coordination to the RNA, cobalt hexammine does not compete for this site. Extensive experiments using manganese-soaked ribozyme crystals failed to provide any evidence for a divalent metal ion at or close to this position. Physiological concentrations of various monovalent ions, similar to or greater than the

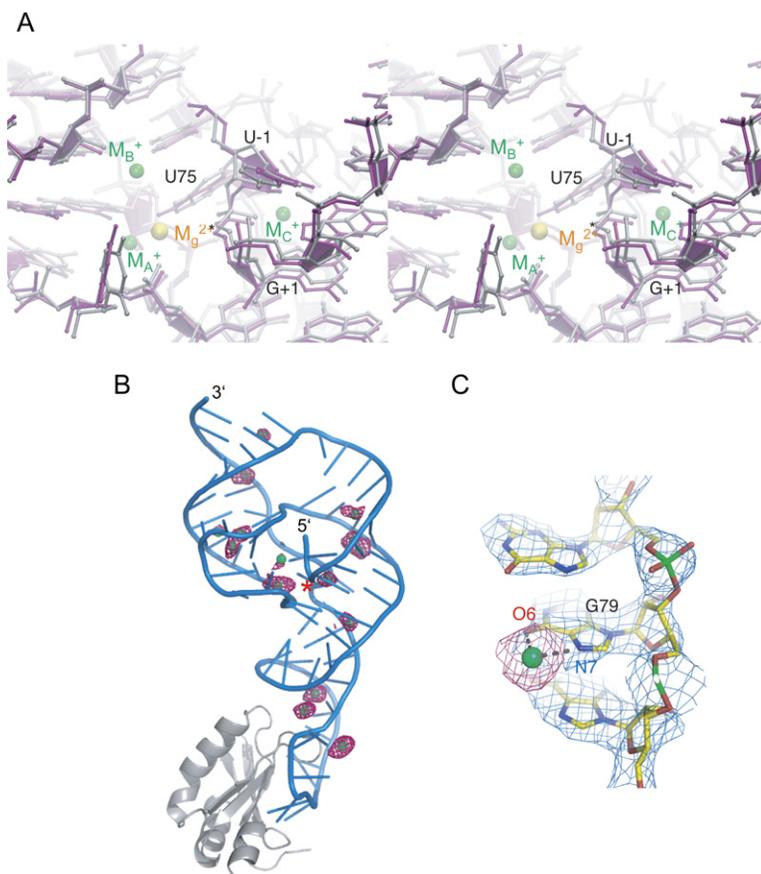
concentrations used in our crystallographic analysis, neither support HDV ribozyme catalysis nor hinder it. These results imply that a monovalent, but not divalent, ion can coordinate to the 2'-OH nucleophile in the ribozyme, but that this ion is not directly relevant to ribozyme structural stability or catalytic mechanism.

## RESULTS AND DISCUSSION

### $Tl^+$ -Binding Sites in the HDV Ribozyme Precursor

Monovalent cations, which are primarily  $K^+$  under physiological conditions, can play important roles in RNA folding and catalysis (Basu et al., 1998; Draper et al., 2005). However, locating these ions in experimental electron density maps is complicated by their low electron density, low occupancy, and lack of strong anomalous X-ray absorption.  $Tl^+$  has an ionic radius and enthalpy of hydration similar to  $K^+$  (Feig and Uhlenbeck, 1999), but contains 4-fold more electrons and exhibits strong anomalous X-ray diffraction, making it ideal for crystallographic identification of monovalent cation positions in macromolecules (Basu et al., 1998; Zhou and MacKinnon, 2004).

$Tl^+$ -containing HDV ribozyme precursor crystals were obtained by transferring crystals grown in the presence of 30 mM strontium chloride to a solution containing 30 mM  $Tl$  acetate for 2 hr. Diffraction data measured using X-rays tuned to the wavelength of the  $Tl$  L<sub>III</sub> absorption edge enabled calculation of anomalous difference electron density maps. Overall, the resulting 2.4 Å crystal structure of the  $Tl^+$ -soaked HDV ribozyme precursor (containing a C75U mutation to prevent the cleavage reaction during crystallographic analysis) is very similar to the 2.1 Å  $Mg^{2+}$ -bound structure (rmsd of 0.55 Å for all phosphorus atoms) (Ke et al., 2004). The RNA substrate adopts the previously observed kinked conformation at the active site due to a rotation of 180° about the 5'-O-P bond of the scissile phosphate. Consistent with the  $Mg^{2+}$ -bound structure, the RNA substrate preceding the cleavage site is disordered, with only the sugar phosphate backbone of the U-1 nucleotide (refined with high temperature [B] factors) visible in the experimental electron density map. The partial negative charge on the *pro*-Rp scissile



**Figure 2. TI Ion-Binding Sites in the HDV Ribozyme**

(A) Stereo view of the superposition of the TI<sup>+</sup>-bound (magenta) and the Mg<sup>2+</sup>-bound (silver) C75U mutant precursor ribozyme active sites. TI<sup>+</sup> and Mg<sup>2+</sup> are colored in green and yellow, respectively. The cleavage site is indicated with an asterisk.

(B) Position of the fifteen TI<sup>+</sup> ions modeled in the precursor structure, superimposed with the magenta anomalous difference density contoured at 3.5  $\sigma$ .

(C) Typical inner-sphere contacts between TI<sup>+</sup> and O6 and N7 of guanine in the HDV ribozyme. The structural model was superimposed with experimental electron density in blue and TI<sup>+</sup> anomalous difference density in magenta. Ten of the fifteen identified TI<sup>+</sup> ions form similar contacts.

phosphate oxygen is apparently stabilized by forming a hydrogen bond with the N3 of U75 (Figure 2A).

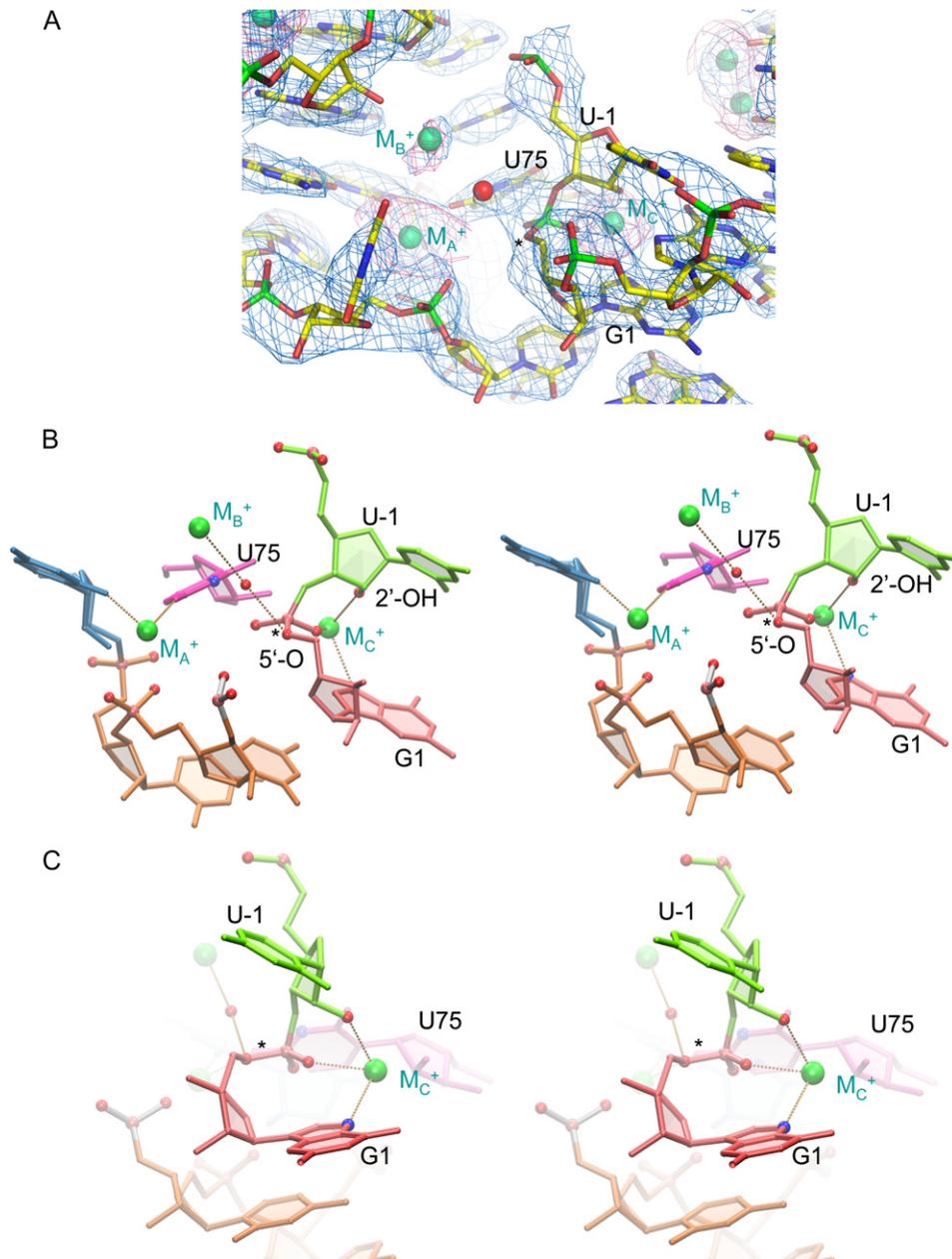
A total of 15 TI<sup>+</sup> ions were identified in the TI<sup>+</sup>-soaked C75U HDV ribozyme precursor (Figure 2B). Each of them produced a strong anomalous signal at least 3.8  $\sigma$  above the noise level in the anomalous difference map. These TI<sup>+</sup> ions tend to coordinate RNA functional groups through direct inner-sphere contacts, as opposed to the outer-sphere electrostatic contacts with RNA frequently observed for divalent cations (Misra and Draper, 1998). Of the 12 TI<sup>+</sup> ions found outside the active site, 10 of them form inner-sphere contacts with N7 and O6 of guanines on the major groove side of the RNA (Figure 2C); one forms inner-sphere contacts with the 2'-OH and O2 of C68; and one mediates contacts between O2 of C69 and O6 of G10 from a symmetry-related molecule. The affinity of TI<sup>+</sup> for these sites is probably weak, given their low occupancy and high temperature factors in crystallographic refinement. Three TI<sup>+</sup> ions were also found near the active site of the ribozyme, as discussed in detail below.

It is interesting that direct inner-sphere interaction between TI<sup>+</sup> and N6 and O6 of guanines appears to be the predominant mode of contact between monovalent cations and RNA at physiological conditions, since TI<sup>+</sup> mimics K<sup>+</sup> in many respects (charge, ionic radius, energy of hydration, etc.). A total of 10 of the 23 structured guanine resi-

dues were found to form such contacts with TI<sup>+</sup> in the 2.4 Å HDV ribozyme precursor structure. The tendency of TI<sup>+</sup> to form direct inner-sphere interactions, as opposed to the through-water outer-sphere RNA contacts frequently found for divalent cations, most likely reflects their difference in hydration enthalpy ( $\Delta H_{\text{hyd}}$  [kJ/mol]) are 320.9 and 405.4 for K<sup>+</sup> and Na<sup>+</sup>, respectively, similar to that for TI<sup>+</sup> [325.9], but significantly smaller than that for Mg<sup>2+</sup> [1922.1] (Feig and Uhlenbeck, 1999). This finding supports the idea that monovalent cations assist RNA folding differently from divalent cations.

#### TI<sup>+</sup> Occupies the Divalent Cation-Binding Cavity in the Active Site

Interestingly, the highest peak in the anomalous difference map corresponds to one of the two TI<sup>+</sup> ions located in the divalent metal ion-binding pocket in the ribozyme active site, close to the 5'-leaving oxygen. This site was previously shown to accommodate a single, hydrated, divalent cation (Ke et al., 2004). Divalent cations that support the ribozyme cleavage reaction, including Mg<sup>2+</sup>, Sr<sup>2+</sup>, Ba<sup>2+</sup>, and Mn<sup>2+</sup>, have all been observed crystallographically to occupy this site in the HDV ribozyme precursor (Ke et al., 2004). Co(NH<sub>3</sub>)<sub>6</sub><sup>3+</sup> and Ir(NH<sub>3</sub>)<sub>6</sub><sup>3+</sup>, structural mimics of magnesium hexahydrate that cannot shuttle protons due to their exchange-inert amine groups, efficiently compete for binding at this site. In the absence of divalent ions



**Figure 3. Active Site Configuration in the  $\text{Ti}^+$ -Bound C75U Mutant Precursor Ribozyme Structure**

(A) The 2.4 Å sigma A-weighted  $2|F_o| - |F_c|$  map (contoured at 1  $\sigma$ ) (blue) was calculated with the  $\text{Ti}^+$  (in green) and water (in red) molecules omitted. In magenta, anomalous difference map contoured at 3.5  $\sigma$ ; the scissile phosphate (asterisk) and U-1 and U75 residues are indicated here and in panels (B) and (C).

(B) Stereo view of the active site configuration in the  $\text{Ti}^+$ -bound C75U mutant precursor ribozyme.  $\text{Ti}^+$  at the  $M_A^+$  position forms inner-sphere contacts with O2 of U20 and O4 of U75;  $\text{Ti}^+$  at the  $M_B^+$  position coordinates a water, which is hydrogen bonded with the 5'-leaving oxygen at the active site. (C) Stereo view of the  $M_C^+$  coordination at the attacking 2'-OH.  $\text{Ti}^+$  at this site forms inner-sphere contacts with the 2'-OH of U-1, the *pro*-Sp phosphate oxygen of the scissile bond, and N7 of G1.

or hexammines, this active site pocket is occupied by two  $\text{Ti}^+$  ions (Figure 3A). Site  $M_A^+$ , with an occupancy of 0.65, is located close to the divalent cation position in the active site (2 Å away from the position of  $\text{Mg}^{2+}$  in the  $\text{Mg}^{2+}$ -bound structure [Figure 2A]). In contrast to divalent ions, which form entirely outer-sphere contacts at this site, this  $\text{Ti}^+$

ion directly contacts the O2 of U20 and O4 of U75, thus shifting further away from the 5'-leaving oxygen (Figure 3B). The other  $\text{Ti}^+$  site,  $M_B^+$ , is located 4.9 Å above the  $M_A^+$  site, and has a much lower occupancy of 0.17. These two  $\text{Ti}^+$  ions may share one or more water ligands that are not resolved at the current structure resolution.

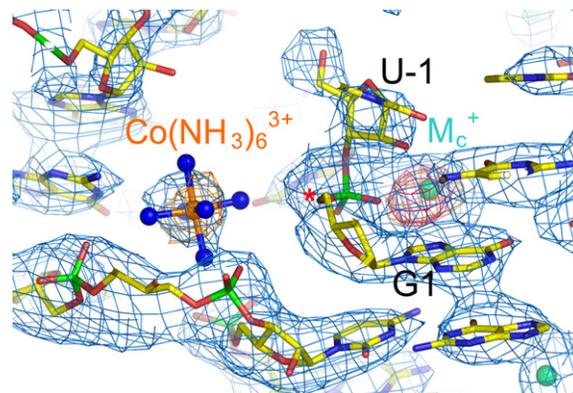
In the presence of higher  $\text{Ti}^+$  concentrations (50 or 500 mM),  $\text{Ti}^+$  starts to populate the  $\text{M}_B^+$  site with occupancy similar to that of the  $\text{M}_A^+$  site, as evidenced by the increased size of its electron density and anomalous difference signal (data not shown). A low-occupancy water molecule bridges an outer-sphere contact between  $\text{M}_B^+$  and the 5'-leaving oxygen (Figure 3B). The position of the water becomes better resolved in the simulated omit map contoured at 0.8  $\sigma$  level (data not shown). Consistent with these crystallographic observations, a recently published  $\sim 200$  ns molecular dynamics simulation (Krasovska et al., 2006) suggested that two monovalent cations can localize to the 5'-leaving oxygen-binding pocket, but are readily replaced by  $\text{Mg}^{2+}$ .

#### Direct Contacts between a $\text{Ti}^+$ Ion and the 2'-OH Attacking Nucleophile

The most interesting finding was a monovalent ion-binding site located at the attacking 2'-OH side of the active site. This site ( $\text{M}_C^+$ ), as revealed by an 8.4  $\sigma$  anomalous difference peak measured at the  $\text{Ti}$  L-III edge, forms direct inner-sphere contacts with the 2'-OH nucleophile, the *pro*-Sp scissile phosphate oxygen, and the N7 of G+1 (Figure 3C). While the coordinated waters are not resolved at this resolution, this  $\text{Ti}^+$  ion could potentially form water-mediated outer-sphere contacts with the 2'-OH of U75 and the O6 of G+2. In the crystallographic refinement, the occupancy of  $\text{Ti}^+$  at site  $\text{M}_C^+$  was estimated to be around 0.4.

#### Cobalt Hexammine Competes $\text{Ti}^+$ from the 5'-O-Binding Site, but Not from the 2'-OH Site

Cobalt hexammine, a potent competitive inhibitor of the HDV ribozyme, binds near the 5'-leaving oxygen in the precursor structure at the site normally occupied by magnesium hexahydrate (Ke et al., 2004). Two  $\text{Ti}^+$  ions ( $\text{M}_A^+$  and  $\text{M}_B^+$ ) bind at this site in the absence of hexammines or divalent metal ions. To test their relative affinity for this site, a competition experiment was performed in which HDV ribozyme precursor crystals were soaked in 20 mM  $\text{Ti}$  acetate and 1 mM cobalt hexammine prior to diffraction data collection. Anomalous diffraction data were measured at the absorption edge of both  $\text{Ti}$  and cobalt. The resulting  $\text{Ti}^+/\text{Co}(\text{NH}_3)_6^{3+}$ -bound structure is very similar to the  $\text{Ti}^+$ -bound structure, with an rmsd of 0.46 Å for all phosphorus atoms. In the  $\text{Ti}^+/\text{Co}(\text{NH}_3)_6^{3+}$ -bound structure, the two  $\text{Ti}$  anomalous difference peaks disappeared from the 5'-oxygen-binding site, coupled with the appearance of a strong anomalous difference peak for cobalt, 2.2 Å away from the high-occupancy  $\text{Ti}$  site (Figure 4). The position of the new cobalt anomalous signal coincides exactly with a spherical electron density peak, presumably belonging to  $\text{Co}(\text{NH}_3)_6^{3+}$ , in the  $2F_o - F_c$  electron density map calculated with metal ions omitted. These data suggest that, at a concentration as low as 1 mM,  $\text{Co}(\text{NH}_3)_6^{3+}$  efficiently competes away both  $\text{Ti}^+$  ions from the 5'-oxygen-binding pocket. In contrast, the location and intensity of the  $\text{Ti}$  anomalous difference peak at the attacking 2'-OH side of the active site remains the same (only 0.2 Å shift in



**Figure 4. Experimental Electron Density Map at the Active Site of the  $\text{Ti}^+/\text{Co}(\text{NH}_3)_6^{3+}$ -Bound C75U Mutant Precursor Ribozyme, Superimposed on the Structural Model**

The 2.9 Å sigma A-weighted  $2|F_o| - |F_c|$  map (contoured at 1  $\sigma$ ) (blue) was calculated with the  $\text{Ti}^+$  (in green) and  $\text{Co}(\text{NH}_3)_6^{3+}$  (in orange) molecules omitted. The anomalous difference maps of  $\text{Co}(\text{NH}_3)_6^{3+}$  (orange) and  $\text{Ti}^+$  (magenta) were both contoured at 5.0  $\sigma$ . The presence of  $\text{Co}(\text{NH}_3)_6^{3+}$  anomalous density at  $\text{M}_C^+$  position is explained in the text.

the all phosphorus atom alignment). Due to the strong anomalous absorbance of  $\text{Ti}$  at the K edge of cobalt, this peak is also present in the cobalt anomalous difference map. However, the metal ion position remains the same and forms multiple inner-sphere contacts with surrounding functional groups, ruling out the possibility that a cobalt hexammine could occupy this site. Together with a previous crystallographic analysis showing that divalent cations do not occupy the  $\text{M}_C^+$  site at concentrations as high as 20 mM (Ke et al., 2004), we conclude that the HDV ribozyme prefers a monovalent cation at the 2'-OH site.

These results demonstrate that the metal ion-binding pocket at the 5'-leaving oxygen side of the active site can accommodate both monovalent and divalent cations, but with a strong preference for hydrated divalent cations. In contrast, the binding site at the 2'-OH side of the active site has a distinct preference for monovalent metal ions.

#### Contributions of Monovalent Cations to HDV Ribozyme Catalysis

The HDV ribozyme catalyzes self cleavage via general acid-base catalysis in which C75 and a hydrated divalent cation are thought to contribute directly to rate enhancement. Although magnesium hexahydrate was observed to bind near the 5' oxygen of the leaving group in the precursor structure, a catalytic mechanism invoking divalent ion binding to the 2'-OH nucleophile remains a formal possibility (Das and Piccirilli, 2005; Nakano et al., 2000). However, no corresponding divalent cation density near the 2'-OH nucleophile was observed in the HDV ribozyme precursor crystal structures (Ke et al., 2004). The observation that  $\text{Ti}^+$  binds to the 2'-OH site with the loss of three waters from its hydration shell suggests that the smaller size and preferred octahedral coordination geometry of  $\text{Mg}^{2+}$  may disfavor its binding at this site.

The directly coordinated monovalent cation could contribute to ribozyme catalysis as a Lewis acid to promote deprotonation of the 2'-OH group or to neutralize the developing negative charge on the nonbridging phosphoryl oxygen in the transition state. If this is the case,  $M_C^+$  is equivalent to divalent metal ion  $M_A^+$  in the two-metal ion catalytic mechanism used by DNA and RNA polymerases (Steitz and Steitz, 1993), and M1 in the group I intron structure (Adams et al., 2004). In both polymerases and the group I intron, however,  $Mg^{2+}$  (with higher charge density) is preferred over monovalent cations.

In a recent comprehensive biochemical study, molar concentrations of monovalent cations were required to support slow rates of  $Mg^{2+}$ -independent HDV ribozyme activity. Furthermore, similar levels of monovalent ions were necessary to partially inhibit  $Mg^{2+}$ -dependent ribozyme catalysis (Perrotta and Been, 2006). These data show that the functions of monovalent and divalent metal ions in HDV ribozyme catalysis do not completely overlap, consistent with the crystallographic observation that monovalent and divalent ion-binding sites are not identical. Numerous factors could contribute to this lack of interchangeability between mono- and divalent metal ions, including differences in  $pK_a$  values, charge density, ionic radii, and preferred coordination geometries. Importantly, at physiological monovalent ion concentrations (~100 mM), no HDV ribozyme rate enhancement or inhibition occurs (Perrotta and Been, 2006), a result that was replicated with the HDV ribozyme crystallization construct used in this study (data not shown). Together, these findings fail to provide evidence that a metal ion at position  $M_C^+$  near the 2'-OH nucleophile in the HDV ribozyme stimulates the self-cleavage reaction. It is possible that a monovalent ion normally occupies this site under physiological conditions, but that this ion does not affect ribozyme reactivity. The results presented here also imply that monovalent ion occupation of the  $M_A^+$  and  $M_B^+$  sites is insufficient to support efficient ribozyme self cleavage, even at higher monovalent ion concentrations, where the occupancy of sites  $M_A^+$  and  $M_B^+$  are similarly high. It remains possible that metal ions are not involved directly in the catalytic step of the HDV ribozyme self-cleavage reaction, but instead play essential roles in RNA structure stability and conformational dynamics that are integral to ribozyme reactivity.

## EXPERIMENTAL PROCEDURES

### Sample Preparation and Crystallization

The HDV ribozyme precursor containing a cytidine 75-to-uridine mutation and a GAU trinucleotide sequence preceding the cleavage site was transcribed and purified following published protocols (Ke and Doudna, 2004). The RNA was mixed with the RNA-binding domain of U1A protein (U1A-RBD) at 1:1 molar ratio. Before crystallization, the RNA-protein complex was refolded using a published urea-refolding protocol, with minor modifications (Ke and Doudna, 2004). Briefly, the complex was diluted to ~50  $\mu$ M, and dialyzed overnight into the refolding buffer containing 100 mM sodium acetate (pH 6.0), 5 mM magnesium acetate, and 8 M urea. Three 2 hr-long dialysis steps were then carried out, in which the urea concentration was dropped to 4 M, 2 M, and 0 M, respectively. Crystals as large as  $100 \times 75 \times 75 \mu\text{m}^3$  were grown by the hanging drop vapor diffusion method over the course

of 7 days at 4°C in optimized conditions containing 5%–10% (v/v) MPD, 50 mM sodium cacodylate (pH 6.0), 40–80 mM NaCl, 30 mM  $\text{SrCl}_2$ , and 15–50 mM spermine-HCl. Crystals used for  $\text{TI}^+$  data collection were soaked overnight in a solution containing 75 mM sodium acetate (pH 6.0), 20 mM TI acetate, and 10% MPD. Crystals containing both  $\text{TI}^+$  and cobalt hexammine were soaked in the same solution, with the addition of 1 mM  $\text{Co}(\text{NH}_4)_3^{3+}\text{Cl}_3$ . All crystals were subsequently cryoprotected by adding sucrose to 35% (w/v) in four steps before flash cooling in liquid nitrogen.

### Diffraction Data Collection and Structure Refinement

The  $\text{TI}^+$  data set was collected at both inflection (0.97910 Å) and peak (0.97810 Å) wavelengths of the TI  $L_{-III}$  anomalous absorption edge using the inverse-beam method. The peak data set was used to generate the anomalous difference map using CNS (Brunger et al., 1998). The native data set suffered radiation decay, so the anomalous signals in the inflection data set were merged to generate a native data set for structure refinement. The  $\text{TI}^+/\text{Co}(\text{NH}_4)_3^{3+}$  data were collected at the K edge of cobalt (1.5998 Å) and  $L_{-III}$  edge of TI (0.97788 Å). The native data set was generated by averaging the cobalt K edge data set.

The crystal structures were solved by molecular replacement using the 2.1 Å C75U HDV ribozyme precursor structure (PDB entry 1SJ3) as the search model (with metal ions and waters deleted). Refinement was carried out by CNS with 10% of the reflections removed for  $R_{\text{free}}$  factor calculation. Following rigid-body refinement, simulated annealing was performed on each refined structure to remove model bias. Ribozyme-bound metal ions were identified in anomalous difference electron density maps. Alternating rounds of positional (Powell minimization), individual B-factor refinement, and manual rebuilding were carried out until the R and R factors were below 30%.

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#### Accession Numbers

The coordinates for the  $Tl^+$ -bound HDV ribozyme precursor structure have been deposited with RCSB ID [rcsb041176](#) and PDB ID [2OIH](#); the  $Tl^+/Co(NH_3)_6^{3+}$ -bound HDV ribozyme precursor structure has been deposited with RCSB ID [rcsb041198](#) and PDB ID [2OJ3](#).