Published in final edited form as:

J Mol Biol. 2009 April 24; 388(1): 195–206. doi:10.1016/j.jmb.2009.02.054.

Threshold occupancy and specific cation binding modes in the hammerhead ribozyme active site are required for active conformation

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Abstract

The relationship between formation of active in-line attack conformations and monovalent (Na^+) and divalent (Mg^{2+}) metal ion binding in the hammerhead ribozyme has been explored with molecular dynamics simulations. To stabilize repulsions between negatively charged groups, different requirements of threshold occupancy of metal ions were observed in the reactant and activated precursor states both in the presence or absence of a Mg^{2+} in the active site. Specific bridging coordination patterns of the ions are correlated with the formation of active in-line attack conformations and can be accommodated in both cases. Furthermore, simulation results suggest that the hammerhead ribozyme folds to form an electronegative recruiting pocket that attracts high local concentrations of positive charge. The present simulations help to reconcile experiments that probe the metal ion sensitivity of hammerhead ribozyme catalysis and support the supposition that Mg^{2+} , in addition to stabilizing active conformations, plays a specific chemical role in catalysis.

Introduction

It remains an open question how RNA molecules can catalyze a broad range of reactions with efficiency that rivals many protein enzymes, with fairly limited chemical functional groups available from the sugarphosphate backbone and four relatively inert nucleobases.¹ One hypothesis is that RNA enzymes (*ribozymes*) may recruit metal ions and other solvent components to facilitate catalysis through electrostatic engineering. In the case of the hammerhead ribozyme (HHR),² it is generally accepted that, under physiological conditions, divalent metal ions are required for the chemical steps of catalysis. Recent crystallographic studies of a full length hammerhead ribozyme have characterized the ground state active site architecture³ and solvent structure,⁴ including the binding mode of a presumed catalytic divalent metal ion in the active site, and reconciled a long-standing controversy between structural and biochemical studies for this system.⁵ Based on these new crystal structures, molecular simulations studies^{6, 7} have suggested a role for the divalent metal ion in the

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chemical reaction. From these simulations, it has been proposed that the divalent metal ion can serve to stabilize active in-line conformations, and interact with key residues to facilitate acid/base catalysis. Further studies have explained the origin of mutational effects on catalysis.⁸ Nonetheless, it is known that, under high non-physiological salt concentrations, the hammerhead and other ribozymes remain catalytically proficient,^{9–11} bringing into question the extent to which divalent metal ion occupancy in the active site is strictly necessary, or just a sufficient condition to achieve efficient catalysis that might be affected by other cations and solvent components.

The modeling of ions in highly charged systems such as RNA affords tremendous challenges with regard to simulation time scales. The reason is that the equilibration and sampling times of mass transport/diffusion limited processes (such as ion migration) and subsequent macromolecular structural relaxation are notoriously slow.¹² In the present study, we report results of a series of five 300 ns simulations of the full length hammerhead ribozyme, in both the reactant ground state and activated precursor state, in order to explore the relationship between cation binding modes and active in-line conformation. Results indicate that the hammerhead ribozyme has an electronegative cation recruiting pocket in the active site that, upon threshold occupancy, stabilizes catalytically active in-line attack conformations via specific cation binding patterns. These results provide a first glimpse into a possible common feature that might be exploited by other ribozymes: the engineering of electrostatically preorganized active sites that are able to recruit solvent components to facilitate catalysis.

Results and Discussion

Simulations performed

A recent joint crystallographic/molecular simulation study⁴ of the solvent structure of the full length HHR indicates that an Mn^{2+} ion is coordinated to the O2P atom of the A9 phosphate and the N7 atom of G10.1. This binding site is designated as the “C-site”. An alternate binding site where a divalent metal ion bridges A9:O_{2P} and the scissile phosphate (C1.1:O_{2P}), designated the “B-site” has been inferred from thio/rescue effects^{13, 14} and also predicted from molecular simulations.^{6, 7} In the absence of divalent metal ions, HHR activity can be recovered by high concentrations of monovalent ions.^{9–11} The specific metal ion binding modes at different stages along the HHR reaction coordinate, and their relation to formation of catalytically active structures has not yet been determined. The present work reports results of a series of molecular dynamics simulations aimed at providing atomic level insight into these questions.

To explore the divalent and monovalent metal ion binding modes (Fig. 1) and their relation to formation of catalytically active, in-line attack conformations in both the neutral reactant and activated precursor (deprotonated 2'OH nucleophile) states, we set up the following series of simulations (summarized in Table 1):

1. RT-C-Mg, the reactant state with Mg^{2+} at the C-site.
2. RT-B-Mg, the reactant state with Mg^{2+} at the bridging position.
3. dRT-C-Mg, the activated precursor with Mg^{2+} at the C-site.
4. dRT-B-Mg, the activated precursor with Mg^{2+} at the bridging position.
5. RT-Na, the reactant state in the absence of Mg^{2+} (presence only of NaCl).
6. dRT-Na, the activated precursor in the absence of Mg^{2+} (presence only of NaCl).

The dRT-C-Mg simulation, the activated precursor with Mg^{2+} initially placed at the C-site, quickly (less than 200 ps) lead to migration of the Mg^{2+} ion into the B-site position, as observed

in a previously simulation study,⁷ and afterward exhibits nearly identical behavior as if the Mg^{2+} ion was initially placed at the B-site (dRT-B-Mg). Hence, we only extended the dRT-B-Mg simulation to 300 ns, and designate it simply as dRT-Mg.

All 5 simulations have been extended to 300 ns in a background of 0.14MNaCl. The equilibration of each simulation was monitored by the root-mean-square-deviation (RMSD) and had reached steady states after 30–50 ns (see Supporting Information). Hence all analysis was performed over the last 250 ns of trajectories for each simulation.

Threshold occupancy and specific coordination patterns of ions are highly correlated with catalytically active conformations

To understand the relationship between the active in-line conformation and the metal ion binding patterns, we first examine the metal occupation and coordination numbers in the active site. Four key coordination sites are used for characterizing metal binding (Figure 1): G8:O_{2'}, A9:O_{2P}, C1.1:O_{2P}, and C17:O_{2'}. The averages, denoted by $\langle \rangle$, of the following three quantities were calculated for both Mg^{2+} and Na^+ : N: the number of ions with at least one coordination to any one of the four key coordination sites, CN: the total coordination number of ions with at least one coordination to any one of coordination sites, and NB: the number of ions which coordinate to at least two of the four coordination sites.

The active conformation can be examined by the distribution of the nucleophile in-line attack angle and distance. Their distributions from all simulations are shown in Figure 2 and manifest two k-mean clusters:¹⁵ cluster A (red) containing structures in the near in-line attack conformations, and cluster B (blue) containing structures not in the near in-line attack conformations, with the exception of the dRT-Mg simulation, where only cluster A is found.

The metal occupation and coordination numbers were then analyzed for each cluster and the results are listed in Table 2. During all simulations, when present in the active site, one Mg^{2+} ion remained stably bound at full occupancy ($\langle N_{\text{Mg}^{2+}} \rangle = 1.00$). In the reactant state simulations RT-C-Mg and RT-B-Mg, there is no significant population of Na^+ ions in the active site ($\langle N_{\text{Na}^+} \rangle \leq 0.1$), which suggests that the Mg^{2+} ion is sufficient to neutralize the local charge of the A9 and scissile phosphates. In the RT-C-Mg (Mg^{2+} at the C-site) simulation, the Mg^{2+} ion directly coordinates only A9:O_{2P} of the RNA ($\langle \text{CN}_{\text{Mg}^{2+}} \rangle = 1.00$), and thus not involved in a bridge ($\langle \text{NB}_{\text{Mg}^{2+}} \rangle = 0.00$). Cluster A (in-line conformation) represents approximately 21% of the sampled data over the last 250 ns of simulation, whereas cluster B (not in-line conformation) represents the remaining 79%.

The RT-B-Mg (Mg^{2+} at the bridging position) simulation, on the other hand, is dramatically different. In this simulation, the Mg^{2+} ion directly coordinates both A9:O_{2P} and the scissile phosphate C1.1:O_{2P} of the RNA ($\langle \text{CN}_{\text{Mg}^{2+}} \rangle = 2.00$) as a stable bridge ($\langle \text{NB}_{\text{Mg}^{2+}} \rangle = 1.00$). Cluster A, containing a high degree of in-line near attack conformations, represents the vast majority of the sampled data (over 99%), whereas cluster B is observed less than 1% of the time. This suggests that a bridging Mg^{2+} ion contributes to stabilization of catalytically active conformations in the reactant simulations.

This feature is even more pronounced in the dRT-Mg simulations (activated precursor state with Mg^{2+}). There is only a single cluster with in-line conformation ($\theta = 155^\circ$). For dRT-Mg, an additional negative charge in the active site is arising from deprotonation of the nucleophile, and a single Na^+ ion is observed in the active site ($\langle N_{\text{Na}^+} \rangle = 0.97$) that makes direct coordination to only one RNA ligand ($\langle \text{CN}_{\text{Na}^+} \rangle = 1.01$) with essentially no bridging interactions.

On the other side, the simulations without an active site Mg^{2+} ion are considerably different than those with the divalent ion present. In the reactant RT-Na simulation, cluster A is dominant

(87% of the time) and shows a high degree of in-line conformations ($\theta=153^\circ$). Most of cluster A population contains a single Na^+ ion in the active site ($\langle N_{\text{Na}^+} \rangle = 1.15$), less frequently two Na^+ ions, and the average number of bridging Na^+ ions is 0.88. Cluster B, on the other hand, has a slightly higher active site Na^+ occupation ($\langle N_{\text{Na}^+} \rangle = 1.38$), but a lower average number of bridging ions (0.66).

In the activated precursor dRT-Na simulation, the average Na^+ occupancy ($\langle N_{\text{Na}^+} \rangle$) increases to approximately 3 and 2.5 for clusters A and B, respectively. Cluster B (not in-line conformation) is the dominate population, occurring 76% of the time. Cluster A (in-line conformation) occurs 24% of the time.

A striking feature that distinguishes cluster A from B is that it exhibits a very high degree of bridging ion character, in addition to higher Na^+ occupancy. For cluster A, the average number of these ions that coordinate at least two RNA ligands ($\langle N_{\text{B}_{\text{Na}^+}} \rangle$) is 2.68 while the number is only 1.36 for cluster B.

These results suggest that the bridging coordination patterns are highly correlated with formation of inline conformations for both cases with and without Mg^{2+} ions. Besides the above ion occupation and coordination number analysis, we further look into the specific binding patterns for both cases with or without the Mg^{2+} ion.

A bridging Mg^{2+} ion maintains rigid coordination patterns that stabilize in-line attack conformations

In this section we compare the effect of different Mg^{2+} binding modes in both the neutral reactant and activated (deprotonated 2'OH) precursor states on the active site structure and fluctuations. Table 3 lists the averages of key in-line indexes, the A9/scissile phosphate-phosphate distance and Mg^{2+} coordination distances for the RT-C-Mg, RT-B-Mg and dRT-Mg simulations. Figure 1 shows a general schematic view of the active site metal ion coordination from the simulations. The distances and standard deviations indicate that the Mg^{2+} ion retains rigid coordination with the phosphate oxygens over the course of the simulation, being directly coordinated to A9:O_{2P} in all simulations. In the RT-C-Mg simulation, the Mg^{2+} ion coordinates G10.1:N₇ indirectly through one of four inner-sphere water molecules. However, this coordination pattern is not highly conducive to formation of an in-line attack conformation. The RT-B-Mg simulation, on the other hand, shows a more rigid Mg^{2+} coordination with both the A9 and scissile phosphate oxygens, and sustains a considerably population of in-line attack conformations. These results suggest that the coordination pattern found in the RT-B-Mg simulation is able to stabilize in-line attack conformations more readily than Mg^{2+} binding at the C-site as in the RT-C-Mg simulation. The dRT-Mg simulation is similar to the RT-B-Mg simulation with regard to exhibiting rigid coordination with the A9 and scissile phosphate oxygens and stabilization of in-line attack conformations.

With the Mg^{2+} ion at the bridging position (RT-B-Mg and dRT-Mg simulations), there is considerably reduced interaction with G10.1:N₇, which are compensated by interactions with the C17:O_{2'} that occur through two water molecules in the inner sphere of the Mg^{2+} ion. This interaction is most pronounced in the dRT-Mg simulation where the C17:O_{2'} is deprotonated. In the ground state reactant simulations with Mg^{2+} (RT-C-Mg and RT-B-Mg), no Na^+ ions were observed to infiltrate the active site. In the activated precursor simulation, dRT-Mg, a single Na^+ ion was observed to be bound at high occupancy to the deprotonated C17:O_{2'} in a manner similar to the M₃ position in Figure 1.

Na⁺ ions bind non-specifically and exhibit different coordination patterns in the reactant and activated precursor states

In this section we explore the monovalent metal ion binding modes that are correlated with formation of catalytically active in-line attack conformations. For the simulations with no Mg²⁺ ions (RT-Na and dRT-Na) in the active site, binding of the Na⁺ ions to the coordination sites exhibits larger variation, and exchange events occur giving rise to a fairly broad array of coordination patterns. In order to characterize the distribution and frequency of this array of coordination patterns, a binary-coded coordination index is used (Figure 1). This index is defined as follows. When a Na⁺ is within a cutoff distance (3.0 Å) to a ligand, it is classified as bound to the ligand, and assigned a unique coordination score for binding to that particular ligand. The coordination scores for the four possible coordination sites (ligands) are 1 for G8:O_{2'}, 2 for A9:O_{2P}, 4 for C1.1:O_{2P}, and 8 for C17:O_{2'}. The coordination index of an ion is the sum of all coordination scores from its bound sites. In this way, the coordination pattern of a Na⁺ can be uniquely represented by a single number. For example, an index of 12 means a Na⁺ directly coordinates to both C1.1:O_{2P} and C17:O_{2'} simultaneously (4+8=12). Through this coordination index, the coordination patterns of Na⁺ ions in the active site can be traced as a time series over the course of the simulation as shown in Figure 3. Distinct colors are also used to distinguish individual Na⁺ ions present in the active site such that transitions between coordination patterns (indexes) can be monitored.

Figure 3 shows that in the RT-Na simulation, two Na⁺ ions (colored as red and green) are present in the active site and, most of time, they both have a coordination index of 6, indicating binding to both A9:O_{2P} and C1.1:O_{2P} at the same time (2+4=6, refer to Figure 1). Hence, two Na⁺ ions collectively act like a single bridging Mg²⁺ ion to hold the negatively charged A9 and scissile phosphates together to maintain an in-line conformation. During the period from approximately 210–240 ns, only one Na⁺ ion (red) with coordination index of 6 is present in the active site. During this period the in-line angle drops suddenly from around 155 to 120 degrees. In those periods, the in-line conformation is no longer held.

In the dRT-Na simulation, the in-line angle is less well preserved than in the dRT-Mg simulation. This is consistent with the lower activity of the ribozyme in the absence of Mg²⁺. Nonetheless, there are several periods (e.g., 25–50 ns and 210–270 ns) where an in-line conformation is visited, and again we observe a high correlation between the Na⁺ ion coordination index and in-line conformation. When less than three Na⁺ ions bind to the active site ligands, the in-line conformation is no longer held, which happens during most of the simulation. During the periods of simulation where three Na⁺ ions bind to different ligand sites simultaneously and the in-line angle comes to a ready-to-react value (≥ 150 degrees).

Figure 4 illustrates the different Na⁺ binding patterns for clusters A, (defined in Table 2, in-line conformation, lower panels) and cluster B (not in-line conformation, upper panels) from the dRT-Na simulation. The in-line cluster A clearly exhibits three Na⁺ bridges that involve C17:O_{2'}/C1.1:O_{2P}, C1.1:O_{2P}/G8:O_{2'}, and C1.1:O_{2P}/A9:O_{2P}. On the other hand, for cluster B, the first two of these bridges are absent with the third one being significantly less pronounced.

The above analysis suggests that the compensation of the negative charges of these three coordination sites, as well as the bridging binding patterns of Na⁺ to bring them together, are necessary to keep the in-line conformation in the deprotonated activated precursor state, although the binding patterns are not as rigid as those of Mg²⁺.

The HHR active site forms a local electronegative recruiting pocket for cation occupation

In this section we examine the preferential occupation of cations in the HHR active site. The 3D density contour maps for the Na^+ ion distribution determined over the last 250 ns of simulation (Figure 5) show that the Na^+ ion density at a medium contour level (left panels, Figure 5) is located near the RNA's phosphate backbone, whereas at high contour level (right panels, Figure 5) the highest probability Na^+ occupation sites were all concentrated in the active site for both the reactant and activated precursor. No explicit Na^+ ions were initially placed in the active site, and Na^+ ion exchange events were observed to occur. This suggests that the HHR folds to form a strong local electronegative pocket that attracts Mg^{2+} or Na^+ ions. As analyzed by NMR, a similar case has been observed in the tetraloop-receptor complex, where the divalent ions were experimentally found to be located at strong electronegative positions formed by the RNA fold.¹⁶ Together with the known divalent metal ion binding at the C-site, these results provoke the speculation that perhaps the active sites of some ribozymes such as the HHR have evolved to form electrostatic cation binding pockets that facilitate catalysis. In the case of the HHR, this speculation is further supported by the simulated correlation of cation binding mode with formation of active conformations discussed in detail in the previous sections.

Discussions

Our simulations suggest that in order to maintain the active in-line conformation, the highly negative charged environment of the active site needs to be balanced by a threshold cation occupancy. This can be accommodated in the reactant state by either a single Mg^{2+} ion, or 1 to 2 Na^+ ions. In the activated precursor state, this is accomplished by a Mg^{2+} ion and an additional Na^+ ion, or three Na^+ ions. Moreover, to form active in-line conformations, these ions must adopt specific bridging coordination patterns: either a bridging Mg^{2+} ion, or specific patterns of bridging Na^+ ions.

It has been well-established that, in the absence of divalent ions, the hammerhead ribozyme retains activity at high concentration of monovalent ions.¹⁰ The properties of hammerhead cleavage in high concentrations of monovalent ions are similar to those in the presence of divalent metal ions, which has been interpreted as indicative of the major role of the cations is simple electrostatic stabilization of the phosphates and allow folding into an active conformation. However, there remains some smaller contribution to the rate enhancement can be effected through a more active role played by at least one divalent metal ion, as observed by the reduction in rate in the presence of only monovalent⁹ or exchange inert ions.¹¹

There are notable exceptions whereby hammerhead cleavage differed significantly in the presence and absence of divalent metal ions. Disruption of implicated divalent metal binding sites at G10.1:N7, A9:O_{2P} and C1.1:O_{2P} has significant deleterious effects on hammerhead cleavage in the presence of divalent ions, but not in high concentrations of Li^+ . For example, both A9:O_{2P} and C1.1:O_{2P} exhibit significant catalytic thio effects that can be rescued by thiophilic ions such as Cd^{2+} . Moreover, both G10.1:N7 and A9:O_{2P} form a divalent metal ion binding site as pinpointed by electron spin-echo envelope modulation¹⁷ and observed crystallographically for the full length hammerhead ribozyme.⁴ The present simulation results are consistent with those experimental evidence and indicate that threshold occupancy and specific coordination patterns of either Mg^{2+} or Na^+ can electrostatically stabilize the active site and facilitate active in-line conformations. These results provide detailed insight into the specific roles played by divalent and monovalent ions.

It has been demonstrated that the sensitivity of hammerhead ribozyme activity to divalent ions is reduced upon introduction of tertiary stabilizing motifs.^{18–22}

A recent study of the tertiary stabilized RzB hammerhead ribozyme has led to the suggestion that hammerhead ribozyme catalysis may occur through a multichannel mechanism that has available both a divalent-dependent and divalent-independent pathways.²¹ These experiments, together with previous measurement of pH-rate profiles,²⁰ are consistent with the interpretation that the divalent ion may play a specific non-structural role in catalysis. The present simulation results provide insight into the nature of the different metal ion binding patterns that give rise to catalytically active conformations, and further support the previous supposition that Mg^{2+} ion, in a bridging position, plays an active chemical role by interacting with the leaving group (C1.1:O5') and general acid (G8:O2').⁷

The present study provides direct evidence for the involvement of threshold cation occupation in the active site and the formation of specific ion binding patterns as a requirement for formation of catalytically active conformations. The implications of these results support the hypothesis that ribozymes may exploit electrostatic engineering of the active site to recruit ions in order to facilitate catalysis. This does not imply that the recruitment of such ions is a necessary condition for catalysis, but is suggestive that it may be one successful strategy that ribozymes have evolved to exploit. In the case of other ribozymes, such as the hairpin ribozyme, there has been recent evidence based on combined quantum mechanical/molecular mechanical simulations that suggests the electrostatic environment of the active site alone may account for a considerable percent of the observed rate enhancement without explicit involvement in metal ions.^{23, 24} That the pK_a s of implicated nucleobase functional groups may be shifted into the physiological pH range by electrostatic and ionic environment has recently been demonstrated for the *Neurospora* VS ribozyme.²⁵ It remains an open question as to the full extent to which ribozymes may use electrostatic organization, either directly or indirectly by establishment of a specific ionic environment, as a general strategy to facilitate catalysis. An understanding of these and other general principles that govern ribozyme activity is a central goal that would enable design of new RNA-based technology.

Material and Methods

Initial structures used in the simulations were based on a 2.0 Å crystal structure with divalent Mn^{2+} ions and solvent resolved (PDB: 2OEU),⁴ with Mn^{2+} ions replaced by native Mg^{2+} ions in the simulations. The positions of hydrogen atoms were determined using the VMD program (version 1.8.6).²⁶ Simulations were performed in a cubic cell of 60 Å by 60 Å by 100 Å filled with pre-equilibrated TIP3P²⁷ waters. The ion atmosphere consisted of Na^+ and Cl^- ions that were added to neutralize the system and reach a concentration of 0.14 M. The resulting system contains 36,534 atoms: 11,463 water molecules, 5 Mg^{2+} , 83 Na^+ , 29 Cl^- , and 2,021 RNA atoms.

Simulations were performed with the NAMD simulation package (version 2.6)²⁸ using the all-atom Cornell *et al.* force field (parm94)²⁹ in CHARMM format, provided in the AMBER 9 package.^{30,31} Periodic boundary conditions were used along with the isothermal-isobaric ensemble (*NPT*) at 1 atm and 298 K using extended system pressure algorithm³² with effective mass of 500.0 *amu* and Nose-Hoover thermostat^{33,34} with effective mass of 1000.0 kcal/mol- ps^2 , respectively. The smooth particle mesh Ewald (PME) method^{35,36} was employed with a B-spline interpolation order of 6 with κ value of 0.2579. FFT grid points of 60, 60, and 100 were used for the lattice directions x, y, and z, respectively. Non-bonded interactions were treated using an atom-based cutoff of 12 Å with switching of non-bond potential beginning at 10 Å. Numerical integration was performed using the leap-frog Verlet algorithm with 1 fs time step.³⁷ Covalent bond lengths involving hydrogen were constrained using the SHAKE algorithm.³⁸

The following equilibration procedures (total 10 ns) were applied to the system prior the production simulations. The positions of the solute atoms, including the Mg^{2+} ion, were restrained by a harmonic potential of 50 kcal/mole/Å² in the equilibration stages.

Pre-annealing stage

Water and ion molecules were first energy-optimized for 2,000 steps then underwent a constant volume simulation annealing: The temperature was increased from 0 K to 298K at the rate of 1 K per ps. The system then was kept at 298 K for 500 ps.

Solvent annealing stage

First step: The temperature increased from 298K to 600 K at the rate of 1 K/ps, then was kept at 600 K for 500 ps with constant volume. Second step: The temperature decreased from 600 K to 298K at the rate of 1K/ps, then was kept at 298K for 1,500 ps with constant volume. Third step: The system was kept at 298K for 3,000 ps at constant pressure (1 atm). The whole annealing stage was repeated twice before the post-annealing stage.

Solute relaxation stage

After the annealing stage, the solute atoms were energy-optimized and then were allowed to move under harmonic restraints over 500 ps simulation at 298 K with a constant pressure of 1 atm. The harmonic force constant (in kcal mol⁻¹ Å⁻²) on each heavy atom was obtained from the empirical formula $k_i = 25 + 2 \times 10^3 / B_i$ where k_i is the force constant for atom i and B_i is the corresponding crystallographic B-value. The restraints were exponentially released over 500 ps with a half-life decay parameter of 100 ps. At the end of the 500 ps simulation, the restraints were reduced to about 3 percent of the initial restraint values.

Production simulation

After the 10 ns of solvent equilibration, the whole system was energy-optimized and unconstrained dynamics simulation began from 0 K under constant pressure of 1 atm. The temperature was increased to 298 K at the rate of 1 K/ps and then kept fixed at 298 K. The same equilibration process was applied for each simulation. At the first 10 ns production simulation, two harmonic restraints of 20 kcal mol⁻¹ Å⁻² were added to keep the Mg^{2+} ion binding to G10.1:N7 and A9:OP₂ position. Another three harmonic restraint of 20 kcal mol⁻¹ Å⁻² were used: the distances between G8:H_{OP2} and C1.1:OP₅, and between G12:H₁ and C17:O_{2'}, were kept around 1.8 Å to ensure the initial hydrogen bonding; the distance between A9:OP₂ and C1.1:OP₂ was kept at 4.3 Å (crystal distance). After 10 ns, all restraints were removed. The motions and relaxation of solvent and counterions are notoriously slow to converge in nucleic acid simulations,¹² and careful equilibration is critical for reliable simulations. In summary, for each simulation, a total of 20 ns of equilibration (10 ns of solvent/ion relaxation and 10 ns of solvent and structure relaxation) and carried out to 300 ns. Analysis was performed over the last 250 ns with data collected every 10 ps.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

TL is grateful for financial support from the University of Minnesota through the Consortium for Bioinformatics and Computational Biology. The authors are grateful for financial support provided by the National Institutes of Health (GM62248 to DY and AI043393 to WS). GMG is grateful for financial support from the University of Minnesota Training Program in Bioinformatics and Computational Biology (BICB). This work was supported in part by the University of Minnesota Biomedical Informatics and Computational Biology program (DY) and by a generous allocation on an IBM Blue Gene BG/P with 4,096 850 Mhz CPUs at the IBM Advanced Client Technology Center in

Rochester, Minnesota with further thanks to Cindy Mestad, Steven Westerbeck, and Geoffrey Costigan for technical assistance. This research was also performed in part using the Molecular Science Computing Facility (MSCF) in the William R. Wiley Environmental Molecular Sciences Laboratory, a national scientific user facility sponsored by the U.S. Department of Energy's Office of Biological and Environmental Research and located at the Pacific Northwest National Laboratory, operated for the Department of Energy by Battelle.

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Figure 1.

Schematic view of the coordination sites in the hammerhead ribozyme active site.

Left: The coordination pattern of Mg^{2+} in the “C-site” coordinated to G10.1”N₇ and A9:O_{2P}.

Middle: The coordination pattern of Mg^{2+} in the “B-site” bridging A9:O_{2P} and C1.1:O_{2P} of the scissile phosphate.

Right: Coordination sites for Na^+ in the hammerhead ribozyme active site found in the RT-Na and dRT-Na simulations. Red numbers next to the coordination sites are the scores used to calculate the coordination index (see text). M₁ involves direct binding to A9:O_{2P} and C.

1:O_{2P} and indirect binding to G10.1:N₇ through a water molecule. M₂ involves direct binding to C17:O_{2'} and C.1:O_{2P}. M₃ involves direct binding to C17:O_{2'} and is positioned toward the outside of the active site.

Figure 2.

Plot of the $O_2'-P-O_5'$ angle versus $O_2'-P$ distance for the approach of the 2'-hydroxyl of residue C17 to the phosphate of residue C1.1 for the reactant state (RT) and the activated state (dRT) simulations. C-Mg indicates the Mg^{2+} was initially placed at the C-site position⁴ while B-Mg means the Mg^{2+} ion was initially placed in an equatorial bridging position.^{6,7} Data obtained from the last 250 ns of the simulations are shown with a frequency of 50 ps and points are colored according to the clustering results and Table 2: cluster A (red) and Cluster B (blue). Green lines at 3.25 Å and 150 degrees indicate the near in-line attack conformation (NAC) region defined by Torres and Bruice.³⁹

Figure 3.

Plot of the in-line attack angle ($\text{O}_{2'}\text{-P-O}_{5'}$) in degrees and the coordination index of Na^+ ions for the RT-Na (top) and dRT-Na (bottom) simulations. The coordination index is defined as follows: when a Na^+ ion has a distance less than a 3.0-Å cutoff value to a ligand, it is defined as bound to that ligand for indexing purposes. When an ion is bound, the scores for the four possible coordination sites are 1 for $\text{G8:O}_{2'}$, 2 for A9:O_{2P} , 4 for C11.1:O_{2P} , and 8 for $\text{C17:O}_{2'}$. The coordination index of a single Na^+ ion is the sum of all scores from its bound sites. Individual Na^+ ions are tracked using different colors (red, green, blue and yellow). Data obtained from the last 250 ns are shown in steps of 500 ps.

Figure 4.

Two dimensional radial distribution function of Na^+ ions in the active site for the activated precursor simulation without Mg^{2+} present in the active site (dRT-Na). The lower panels shows results for cluster A that contains population members that are in active in-line conformations, and the upper panels show results for cluster B that are not in-line (see Table 2). The axes are the distances (in Å) to different metal coordination sites. The green lines indicate the regions where Na^+ ions have distances less than 3.0 Å to both sites indicated by the axes.

Figure 5.

The 3D density contour maps (yellow) of Na^+ ion distributions derived from the RT-Na (upper panels) and dRT-Na simulations (low panels) at different isodensity contour levels (left panels: 0.1; right panels: 1.0). The hammerhead ribozyme is shown in blue with the active site highlighted red. The figure shows that, although the Na^+ ions distribute around the RNA phosphate backbone (left panels), the hammerhead ribozyme folds to form a local electronegative recruiting pocket that attracts a highly condensed distribution of the Na^+ ions (left panels) both in the reactant state and the deprotonated activated precursor state (deprotonated C17:O₂') simulations.

Table 1

Index of simulations.

Abbrev.	State:2'OH	Mg ²⁺	[NaCl]	Temp., Press.	simulation	analysis
RT-C-Mg	neutral reactant	C-site	0.14 M	298 K, 1 atm	300 ns	250 ns
RT-B-Mg	neutral reactant	B-site	0.14 M	298 K, 1 atm	300 ns	250 ns
dRT-C-Mg	deprotonated precursor	C-site	0.14 M	298 K, 1 atm	< 1 ns	—
dRT-B-Mg	deprotonated precursor	B-site	0.14 M	298 K, 1 atm	300 ns	250 ns
RT-Na	neutral reactant	absent	0.14 M	298 K, 1 atm	300 ns	250 ns
dRT-Na	deprotonated precursor	absent	0.14 M	298 K, 1 atm	300 ns	250 ns

Each simulation is given an abbreviation (Abbrev.) that is referenced in the text. The simulations differ in the protonation state of the 2'OH nucleophile (State:2'OH) being either in the “neutral reactant”, or “deprotonated precursor” state. The simulations also differed from the presence/absence and initial placement of the Mg²⁺ ions in the active site. In the presence of a Mg²⁺ ion in the active site, initial placement could be in the “C-site” coordinated to O2P of the A9 phosphate and N7 of G10.1, or in the “B-site” position bridging between the O2P atoms of the A9 and scissile phosphates. In the “dRT-C-Mg” simulation of the deprotonated precursor with Mg²⁺ initially placed in the C-site, the Mg²⁺ ion quickly migrated to a B-site position, and exhibited dynamics essentially equivalent to the dRT-B-Mg simulation, and thus was not carried out further or analyzed. All simulations were carried out in 0.14 M NaCl at 298 K and 1 atm pressure, and with the exception of the “dRT-C-Mg” simulation mentioned above, were carried out to 300 ns, the last 250 ns of which was used for analysis.

Table 2

Coordination patterns of Mg²⁺ and Na⁺ in active site.

Cluster	Percentage	R*	θ†	⟨N _{Mg²⁺⟩‡}	⟨CN _{Mg²⁺⟩§}	⟨NB _{Mg²⁺} ⟩¶	⟨N _{Na} ⟩‡	⟨CN _{Na} ⟩§	⟨NB _{Na} ⟩¶
RT-C-Mg	A	20.78%	3.30	144.05	1.00	0.00	0.05	1.00	0.00
	B	79.22%	4.13	122.66	1.00	0.00	0.03	1.00	0.00
RT-B-Mg	A	99.54%	3.27	151.10	1.00	1.00	0.00	1.00	0.00
	B	0.46%	4.00	129.76	1.00	1.00	0.09	1.00	0.00
RT-Na	A	86.72%	3.23	152.91	-	-	1.15	1.99	0.88
	B	13.28%	4.12	122.82	-	-	1.38	1.54	0.66
dRT-Mg	A	100.00%	3.64	154.89	1.00	1.00	0.97	1.01	0.01
dRT-Na	A	23.99%	3.50	144.72	-	-	2.96	2.29	2.68
	B	76.01%	4.30	115.16	-	-	2.46	1.69	1.36

Distances and angles (Figure 1) are in Å and degrees, respectively. The average values, denoted as ⟨ ⟩, are obtained by averaging over all snapshots in the cluster.

* R is the in-line attack distance (C17:O2' to C1.1:P).

† θ is the in-line attack angle (between C17:O2', C1.1:P, and C1.1:O5').

‡ N is the number of ions with at least one coordination to any one of the four coordination sites.

§ CN is the total coordination number of all ions with at least one coordination to any one of the four coordination sites.

¶ NB is the number of ions which coordinate to at least two of the four coordination sites.

Table 3

Characterization of the Mg²⁺ coordination in the active site.

	R [*]	θ [†]	O–O [‡]	A9:O _{2P} [§]	C1.1:O _{2P} [§]	C17:O _{2P} [§]	G8:O _{2P} [§]	G10:N ₇ [§]
RT-C-Mg	4.01(34)	126.5(119)	4.14(49)	2.01(4)	4.40(30)	6.04(90)	5.76(46)	4.19(31)
RT-B-Mg	3.28(12)	151.2(79)	2.95(13)	2.02(5)	2.04(5)	4.25(24)	4.57(30)	4.38(25)
dRT-Mg	3.64(17)	155.0(80)	2.94(13)	2.01(4)	2.03(5)	3.76(17)	4.62(62)	5.05(26)

Analysis was performed over the last 250 ns (10 ps sampling frequency). Distances and angles (Figure 1) are in Å and degrees, respectively. Standard deviations (SD) are listed in parenthesis divided by the decimal precision of the average (e.g., if the average is reported to two digits of decimal precision, the SD is divided by 0.01).

* In-line attack distance (C17:O_{2P} to C1.1:P).

† In-line attack angle (between C17:O_{2P}, C1.1:P, and C1.1:O₅).

‡ Distance between A9:O_{2P} and C1.1:O_{2P}.

§ Distance between the Mg²⁺ and the indicated ligand site.