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Bridging the Gap Between Theory and Experiment to Derive a Detailed Understanding of Hammerhead Ribozyme Catalysis

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Abstract

Herein we summarize our progress toward the understanding of hammerhead ribozyme (HHR) catalysis through a multiscale simulation strategy. Simulation results collectively paint a picture of HHR catalysis: HHR first folds to form an electronegative active site pocket to recruit a threshold occupation of cationic charges, either a Mg^{2+} ion or multiple monovalent cations. Catalytically active conformations that have good in-line fitness are supported by specific metal ion coordination patterns that involve either a bridging Mg^{2+} ion or multiple Na^+ ions, one of which is also in a bridging coordination pattern. In the case of a single Mg^{2+} ion bound in the active site, the Mg^{2+} ion undergoes a migration that is coupled with deprotonation of the nucleophile ($C17:O_2'$). As the reaction proceeds, the Mg^{2+} ion stabilizes the accumulating charge of the leaving group and significantly increases the general acid ability of $G8:O_2'$. Further computational mutagenesis simulations suggest that the disruptions due to mutations may severely impact HHR catalysis at different stages of the reaction. Catalytic mechanisms supported by the simulation results are consistent with available structural and biochemical experiments, and together they advance our understanding of HHR catalysis.

Keywords

simulation; free energy; RNA; enzyme; catalysis; mechanism; combined QM/MM; hammerhead ribozyme

1. INTRODUCTION

In the past two and a half decades, revolutionary changes have been seen in the original notion that the only function of RNA molecules was as only messenger intermediates in the pathway from the genetic code to protein synthesis. Now, the roles of RNA in cellular function are known to be considerably more diverse, ranging from regulation of gene expression and signaling pathways to catalyzing important biochemical reactions including protein synthesis.^{1–12} These discoveries have transformed our view of RNA as a simple messenger to a more profoundly central molecule in the evolution of life forms, our understanding and appreciation of which is still in its infancy.¹³ Ultimately, the elucidation of the mechanisms of RNA catalysis¹⁴ will extend our understanding of biological processes and facilitate the design of new RNA-based technologies.^{15–17}

Simulations of biological systems at the atomic level could potentially offer access to the most intimate mechanistic details that may aid in the interpretation of experiments and provide predictive insight into relevant drug design or therapeutic efforts.^{18–22} In particular, a quantum mechanical description is ultimately required for reliable study of chemical reactions, including reactions catalyzed by biological macromolecules such as RNA. At the same time, a high-level fully quantum mechanical treatment of these systems in molecular simulations is not yet feasible.

Practical simulation approaches involve the use of so-called multiscale models^{23–25} that integrate a hierarchy of models to work together to provide a computationally tractable representation of a complex biochemical reaction in a realistic environment. The most simple and widely applied multi-scale model to study biochemical reactions is the use of a combined quantum mechanical/molecular mechanical (QM/MM) potential.^{25–31} As a specific example, for enzyme systems, one typically treats the reactive chemical events in a region localized around the active site with a sufficiently accurate high-level QM model, the microscopic solvent fluctuations and changes in molecular conformation using MM force field model, and the macroscopic dielectric relaxation using a continuum solvation model.

Combined QM/MM methods have been widely applied to various biological systems.^{25–31} RNA catalysis simulations, however, are particularly laden with challenges not apparent for most other biological systems such as protein enzymes. RNA molecules are highly negatively charged and exhibit strong and often specific interactions with solvent.^{20–22,32} This requires special attention to the microscopic *in silico* model that requires consideration of a very large number of water molecules, counter-ions, and co-ions to be included. Electrostatic interactions need to be treated rigorously without cutoff, and long simulation times are typically needed to insure that the ion environment is properly equilibrated.^{33–36} These issues are further complicated by the fact that RNA molecules bind divalent metal ions that play an important role in folding, and that may also contribute actively to the catalytic chemical steps.

The hammerhead ribozyme (HHR)^{37–39} is an archetype system to study the fundamental nature of RNA catalysis^{40–45} and is arguably the best characterized ribozyme due to its small size, known crystal structures, and the wealth of biochemical and biophysical studies. HHR catalyzes the site-specific attack of an activated 2'OH nucleophile to the adjacent 3' phosphate, resulting in cleavage of the P-O5' phosphodiester linkage to form a 2',3' cyclic phosphate and a 5' alcohol. A detailed understanding of the structure–function relationships in the HHR^{38,41} will ultimately aid in the understanding of other cellular RNA catalysts such as the ribosome. The HHR has gained attention as a potential anti-HIV-1 therapeutic agent,^{46–49} an inhibitor of *BCR-ABL1* gene expression,⁵⁰ an inhibitor of hepatitis-B virus gene expressions,^{51,52} and as a tool in drug design and target discovery for other diseases.^{50,53,54} The hammerhead-like motif is distributed throughout various genomes,⁵⁵ and recently a discontinuous HHR motif has been found embedded in the 3' untranslated regions of a mammalian messenger RNA, suggesting HHR's possible common role in posttranscriptional gene regulation.⁵⁶

However, the detailed reaction mechanism of HHR is still elusive despite significant experimental and theoretical work.^{38,39,41,44,57–61} One aspect of the catalytic mechanism that has perplexed the community involves the specific role of divalent metal ions in catalysis. Specifically, one of the main puzzles involves the apparent inconsistency between the interpretation of thio and metal ion substitution^{62–66} and mutational^{61,67–69} experiments with available crystallographic structural information of the minimal HHR (mHHR) sequence.^{70–72} Biochemical experiments have been interpreted to suggest that a pH-dependent conformational change must precede or be concomitant with the catalytic chemical step, including a possible metal ion bridge between the A9 and scissile phosphates. This is inconsistent with the interpretations of crystallographic data for the mHHR motif,^{70–72} where A9 and scissile phosphates are found to be about 20 Å apart. Moreover, the function of the 2'-OH group of G8 remains unclear from the data.^{38,41} Recent crystallographic studies of the extended HHR (eHHR) have characterized the ground state active site architecture⁷³ and its solvent structure,⁷⁴ including the binding mode of a presumed catalytically active divalent metal ion in the active site. These findings, together with molecular simulation studies,^{75–79} have reconciled a longstanding controversy between structural and biochemical studies for this system.⁸⁰

In this chapter, we summarize our recent efforts to unveil the detailed mechanisms of HHR catalysis, with emphasis on the characterization of metal ion-binding modes and their relationship with structure and catalysis. Through molecular dynamics (MD) simulations, we first examined metal ion-binding modes in the HHR at various stages of progression along the reaction coordinate, and the characterization of the electrostatic environment of the HHR and its ability to recruit cationic charge, as well as the relationship between the threshold occupancy of metal ions and the formation of catalytically active conformations. Using QM/MM techniques, we identified the most plausible reaction path of the HHR enzymatic reaction, and the correlation between the metal ion coordination and the reaction path. To further clarify and verify the proposed mechanisms, a systematic computational mutagenesis study of HHR on three key residues was performed to provide atomic-level explanation of experimentally observed mutational effects, as well as prediction of possible rescuing mutations.

2. A METAL ION STORY WITH A HAPPY ENDING

2.1. Discrepancies between biochemical and crystallographic data

It is well known that mHHR sequences of only 43 residues retain almost full catalytic activity, compared to the naturally found eHHR sequences with ~63 residues, if the conserved core region is kept. The mHHR sequence consists of three base-paired stems (stems I, II, and III) flanking a central core of around 15 highly conserved nucleotide residues,^{81–83} as shown in Fig. 2.1. The conserved central bases, with few exceptions, are essential for the ribozyme catalytic activity. The mHHR exhibits a turnover rate (k_{cat}) of about 1 molecule/min and a k_m in the order of 10 nM. The rate for cleavage and ligation for the naturally occurring eHHR motifs are around 1000 and 2000 times faster, respectively, than the corresponding rates for mHHR.^{84–86}

Due to its size, the mHHR had originally been the main target to study HHR and has been exhaustively studied by biochemists and enzymologists as well as by X-ray crystallographers, NMR spectroscopists, and other experimentalists using various biophysical techniques. The first detailed X-ray crystal structure of mHHR, an RNA/DNA hybrid, was published in 1994 by Pley et al.,⁸⁷ followed shortly thereafter by an all-RNA mHHR structure published in 1995 by Scott et al.⁸⁸ In a pioneering series of papers,^{70–72} crystallographic structures of the mHHR were solved at different stages along the reaction coordinate, including unbound and Mg^{2+} -bound systems, early and late intermediates, and a ribozyme product structure.

These mHHR crystal structures were the departure point for many initial theoretical investigations, and initiated a significant amount of effort toward detailed understanding of HHR mechanism. On the other hand, it also ignited numerous mechanistic debates among research groups⁸⁹ that had conflicting interpretations of structural and biochemical data. As the crystal structures did provide the global positions of the distal termini of all three flanking helical stems, many biochemical experiments designed to probe transition-state interactions and the chemistry of catalysis appeared to be irreconcilable with the crystal structures.^{61,90} Molecular simulations based on the mHHR only served to fuel speculation and further debate without providing new insights. Some of the key issues that remained unresolved are summarized in the next sections.

2.1.1 Positions of the A9 and scissile phosphates were inconsistent with interpretations from thio/rescue effect experiments—Thio-substitution and soft metal ion rescue experiments^{63,91,92} suggest that both the two oxygen atoms (A9:O2P) and the scissile phosphate oxygen (C1.1:O2P) acquire key direct metal ion interactions in the transition state for catalysis, and may be bound to a single active Mg^{2+} ion simultaneously in the transition state, requiring these residues to be in close proximity. In the mHHR structure, however, the distance between these two oxygen atoms is almost 20 Å. Hence, it is not possible to explain the related thio-substitution data if only one Mg^{2+} ion is considered,⁹³ unless a fairly large-scale conformational change takes place or unless two Mg^{2+} ions are involved and bind to A9:O2P and C1.1:O2P separately.

2.1.2 Crystal structures did not provide straightforward interpretation of mutagenesis data—It has been shown that four totally conserved residues, G5, G8, G12, and C3, were critical for the HHR catalysis, and they are extremely sensitive to any modification. Even a small chemical modification of a single exocyclic functional group on any one of these nucleotides could result in the nearly total loss of catalytic activity.^{83,94,95} Nevertheless, these mutational effects cannot be explained from the mHHR structure based on the interruption of key indices, such as Watson–Crick hydrogen bond interactions.⁶¹

2.1.3 Lack of structural support for general acid and base candidates identified from biochemical experiments—It has been recently suggested that the conserved residues G8 and G12 act as the general acid and the general base, respectively, of the HHR reaction mechanism.⁹⁶ However, it was not clear from available crystallographic data how these two residues could play such roles given they are distant from the cleavage site in the mHHR structure.

2.1.4 Other concerns with the mHHR structure—NMR data and chemical mutation data suggest that U4 and U7 bases must approach one another within about 6 Å, although it did not appear to be possible from the mHHR crystal structure.^{97,98} Furthermore, in all published mHHR structure, none has an in-line attack angle near 180 degrees, which is required by the first nucleophilic attacking step of the HHR reaction.

2.2. The crystal structures of the full-length (extended) HHR

The long-standing debate between structural and biochemical data of the mHHR was finally resolved with the determination of the first crystal structure of the eHHR (63 residues) by Martick and Scott,⁷³ and elucidation of the details of its solvent structure.⁷⁴ The eHHR crystal structure indicates a significant rearrangement from the mHHR structures that allow extensive new interactions between the loops of stem II and stem I, and almost all discrepancies are resolved in this structure. The distance between the oxygen atoms (A9:O2P and C1.1:O2P) is around 4 Å, C3 and G8 form a canonical Watson–Crick pair, G5 holds hydrogen bonds interacting with the active site (C1.1 and C17), G8 and G12 are in the ready positions as the general acid/base, U4 and U7 are nearby, and the nucleophile (C17:O2') has an in-line attacking angle around 160 degrees.^{68,99–101} The existing mutagenesis data also agree well with this eHHR crystal structure.⁶⁸

The eHHR structure successfully resolved a wealth of problems regarding the basic architecture of the active site that had been points of (often heated) contention. However, it also brought a new dimension to the problem regarding the identification of the role of divalent metal ions which, under physiological ionic strength conditions, were known to be critical for catalysis. In the first eHHR structure, no divalent metal ions were resolved,⁷³ and this left the burning question as to what was the specific role of divalent metal ions in catalysis. Moreover, the roles of G8 and G12 as plausible candidates as the general acid and the general base, respectively, as supported by their positions in the active site, still lacked compelling evidence as to how the ribozyme environment might induce the required shifts in pK_a values to be catalytically active. For example, in order for G12 to act as a general base, it must be deprotonated at the N1 position (solution pK_a of 9.2). Further, the crystal structure revealed that it was the 2'OH of G8 that was positioned to act as a general acid, which has been estimated to have considerably elevated pK_a values (>14). Consequently, the pK_a values of the implicated functional groups would require considerable shifts toward neutrality to be consistent with the apparent pK_a values derived from pH-rate profiles. Therefore, a detailed investigation of the roles of metal ions and the microenvironment around the active site is desperately needed.

An initial theoretical study was communicated⁷⁵ that offered a prediction that a divalent metal ion could stably occupy a position bridging the A9 and scissile phosphates in the transition state of the eHHR. A subsequent joint crystallographic and molecular simulation study of the ground state eHHR⁷⁴ revealed a single Mn^{2+} bound directly to the A9 phosphate in the active site, accompanying a hydrogen bond network involving a well-ordered water molecule spanning N1 of G12 (the general base) and 2'OH of G8 (previously implicated in general acid catalysis). The crystallographic data for the ground state solvent

structure, however, did not show a divalent ion in the bridging position in the transition state suggested by simulation which remained to be solved.

2.3. Probing metal ion-binding modes in HHR with molecular simulation

In the first eHHR crystal structure, the two presumably negatively charged oxygen atoms, A9:O2P and C1.1:O2P, are around 4 Å away, an ideal distance for direct coordination of a Mg^{2+} ion in a bridging “B-site” position. Hence, placing a divalent metal ion between them would be a reasonable first attempt to explore the possible roles of the required metal ion, although there is no metal ion resolved in the first eHHR crystal structure.⁷⁵ Nevertheless, the solvent structure of eHHR⁷⁴ suggests a different site for Mn^{2+} , which is defined as the “C-site” (Fig. 2.2). Hence, the first set of simulations^{75,76} was performed to explore the possible positions and roles of the Mg^{2+} .

2.3.1 Simulation setup

2.3.1.1 Mg^{2+} ion-binding modes in the active site: As mentioned, the Mg^{2+} -binding sites were not resolved in the original eHHR RNA structure.⁷³ To explore possible Mg^{2+} -binding modes in the active site, two initial positions were selected based on biochemical, theoretical, or crystallographic sources.^{63,74,75} The first initial Mg^{2+} -binding site, designated the “C-site,” is an implicated metal ion-binding site based on the solvent structure of eHHR,⁷⁴ in which a Mn^{2+} cation directly coordinates to both A9:O2P and G10.1:N7. The second initial Mg^{2+} -binding site, designated the “bridging” site or “B-site,” is one in which the Mg^{2+} ion bridges the A9 and scissile phosphates, directly coordinating the two nonbridging O2P atoms that are 4.3 Å apart in the first eHHR crystal structure.⁷³ This type of coordination was inferred both from the O—O distance in the crystal structure and also the thio/rescue effect experiments that suggest a single divalent metal ion might bridge these positions in the transition state.^{63,74,75} No metal ion at this position, however, has yet been observed crystallographically.

2.3.1.2 Simulations performed: A total of eight classical force field MD simulations were performed that involved different points along the catalytic reaction pathway including the reactant state (RT), reactant state activated precursor where the 2'OH nucleophile is deprotonated (dRT), early transition state (ETS), and late transition state (LTS) mimics. In addition, the presence and initial position of the Mg^{2+} ion in the active site was also varied so as to include Mg^{2+} ion bound at the C-site or in a bridging position (B-site) as described above. The parameters for the Mg^{2+} ion and the reactive intermediates in the simulations have been described in detail elsewhere,^{75,102} and a summary of the different simulations and their abbreviations are provided in Table 2.1. All the simulations were performed with CHARMM¹⁰⁶ (version c32a2) using the all-atom CHARMM27 nucleic acid force field^{103,104} with extension to reactive intermediate models (e.g., transition state mimics)¹⁰² and TIP3P water model.¹⁰⁷

2.3.1.3 System preparation: Initial structures used in the simulations were based on the crystallographic structure of the first eHHR structure.⁷³ The positions of hydrogen atoms were determined using the HBUILD facility in the program CHARMM¹⁰⁶ (version c32a2). With hydrogen atoms built and the Mg^{2+} cation position established, the ribozyme was

immersed in a rhombic dodecahedral cell of 10,062 pre-equilibrated TIP3P water molecules centered about the active site, and pruned such that any water molecule within 2.8 Å from the solute was removed. The ion atmosphere consisted of Na⁺ and Cl⁻ ions that were added at random positions to neutralize the system and reach the physiologic extracellular concentration of 0.14 M. The ion positions were kept initially at least 4.7 Å away from any solute atoms. The resulting system (the reactant state) contained 9053 water molecules, 82 Na⁺ and 23 Cl⁻ ions, and 2021 RNA atoms.

2.3.2 Simulation protocols—Periodic boundary conditions were used along with the isothermal-isobaric ensemble (NPT) at 1 atm and 298 K using extended system pressure algorithm¹⁰⁸ with effective mass of 500.0 amu and Nosé–Hoover thermostat^{109,110} with effective mass of 1000.0 kcal/mol-ps², respectively. The smooth particle mesh Ewald method^{111,112} was employed with a λ value of 0.35 Å⁻¹, 80 FFT grid points for each of the lattice directions, and a B-spline interpolation order of six. Nonbonded interactions were treated using an atom-based cutoff of 10 Å with shifted van der Waals potential. Numerical integration was performed using the leap-frog Verlet algorithm with 1 fs time step.¹¹³ Covalent bond lengths involving hydrogen were constrained using the SHAKE algorithm.¹¹⁴

2.3.2.1 Solvent/ion equilibration: The following equilibration procedures (total 1 ns) were applied to the system prior to the production simulations in order to insure reasonable relaxation of the solvent and ion environment. The positions of the solute atoms, including the Mg²⁺ ion, were held fixed in the equilibration stages.

2.3.2.1.1 Pre-annealing stage: Water and ion molecules were first energy-optimized and then underwent a constant volume simulation annealing for 50 ps. The temperature was increased from 0 to 298 K in a 7.5 ps period and then was kept at 298 K. The annealing simulations were repeated twice with temperature increased from 298 to 498 K and then back to 298 K.

2.3.2.1.2 Annealing stage: Four steps of constant volume simulations (50 ps each) were performed in this stage. First step: The temperature increased from 298 to 498 K in 7.5 ps and then was kept at 498 K. Second step: The temperature increased from 498 to 698 K in 7.5 ps and then was kept at 698 K. Third step: The temperature decreased from 698 to 498 K in 7.5 ps and then was kept at 498 K. Fourth step: The temperature decreased from 498 to 298 K in 7.5 ps and then was kept at 298 K. The whole annealing stage was repeated three times before the post-annealing stage.

2.3.2.1.3 Post-annealing stage: Three steps of constant volume simulations were performed in this stage. First step (50 ps): The temperature increased from 298 to 498 K in 7.5 ps and then was kept at 498 K. Second step (50 ps): The temperature decreased from 498 to 298 K in 7.5 ps and then was kept at 298 K. Third step (150 ps): The temperature was kept at 298 K for 150 ps.

2.3.2.1.4 Solute relaxation stage: The solute atoms were energy-optimized and then were allowed to move under harmonic restraints over a 50 ps simulation at 298 K under constant

pressure of 1 atm. The harmonic force constant (in kcal/mol/Å²) 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 50 ps with a half-life decay parameter of 10 ps. At the end of the 50 ps simulation, the restraints were reduced to about 3% of the initial restraint values. Three harmonic restraints of 20 kcal/mol/Å² were added to keep the Mg²⁺ ion in the middle of the C1.1:O2P and A9: O2P positions. Another harmonic restraint of 20 kcal/mol/Å² was used to force the distance between G8:H O2P and C1.1:O5' to be around 1.8 Å, which is to ensure that the HO2P of G8 is initially hydrogen bonded. All restraints were then released prior to the production simulation.

2.3.2.2 Production simulation: After the 1 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. A total of 12 ns of unconstrained dynamics was performed for each of the eight simulations (reactant with and without Mg²⁺, ETS mimic, and LTS mimic), the last 10 ns of which were used for the analysis. The motions and relaxation of solvent and counter-ions are notoriously slow to converge in nucleic acid simulations,¹¹⁵ and careful equilibration is critical for reliable simulations. In summary, for each simulation, a total of 3 ns of equilibration (1 ns of solvent relaxation and 2 ns of solvent and structure relaxation) was carried out before 10 ns of data sampling.

2.3.2.2.1 QM/MM simulation setup: QM/MM simulations on the ETS and LTS mimics were set up as follows. Initial structures were taken from snapshots of the classical MD simulations after 2 ns production simulation, and the C17:O2'-P and P—C1.1:O5' distances of the scissile phosphate were harmonically restrained with a force constant of 1000 kcal/mol/Å² and equilibrium distances of 2.010 and 1.850 Å, respectively, for the ETS mimic and 1.856 and 2.382 Å, respectively, for the LTS mimic. The system is partitioned into a QM region constituting the active site that is represented by the AM1/d-PhoT Hamiltonian¹⁰⁵ and the modified AM1 magnesium parameters of Hutter and coworkers.¹¹⁶ The total number of solute and solvent atoms, setup of periodic boundary conditions, etc. were identical to the classical simulations. The QM subsystem was defined as the 43 atoms around the active site, and included the scissile and A9 phosphates, parts of the nucleophilic and leaving ribose rings, and Mg²⁺ ion and coordinated waters. The generalized hybrid orbital method¹¹⁷ is used to cut a covalent bond to divide the system into the QM and MM region. Full electrostatic interactions were calculated using a recently introduced linear-scaling QM/MM-Ewald method.¹¹⁸

2.4. Results

2.4.1 A diverse set of Mg²⁺-binding modes along the reaction coordinate—The key heavy-atom distances around the active site are listed in Table 2.2 for the simulations with Mg²⁺ placed at the bridging position (B-site) and in Table 2.3 for the simulations with Mg²⁺ initially placed at the C-site position. In both tables, a comparison with the crystallographic values is provided. Figure 2.3 shows a series of snapshots that illustrate the

migration of the Mg^{2+} . The Mg^{2+} ion clearly migrates from the C-site to the bridging position between the A9 and scissile phosphates (i.e., directly coordinating A9:O2P and C1.1:O2P) in both ETS and LTS mimic simulations (ETS-C and LTS-C) and in the reactant state simulation where the nucleophilic O2' has been deprotonated (dRT-C). However, in the parent reactant state system (RT-C), where C17:O2' is protonated, the divalent metal ion stays in the C-site in the course of the 12 ns MD simulation. The Mg^{2+} ion directly coordinates to A9:O2P with a distance less than 2.5 Å for all simulations (data not shown).

2.4.2 Mg^{2+} binding and migration in the reactant state—In the reactant simulations with Mg^{2+} at the bridging position (RT-B), the Mg^{2+} coordination between the C1.1 and A9 phosphate oxygens fluctuates between axial–axial and axial–equatorial modes, resulting in a shorter average oxygen–oxygen distance (3.36 Å) than that observed in the X-ray structure (around 4.3 Å; Table 2.2). The reactant simulation with the Mg^{2+} ion initially placed at the C-site (RT-C) shows that, although Mg^{2+} does not move to the bridging position during simulation time (12 ns), its distance to G10.1:N7 varies from around 2.0 to around 4.5 Å. The distance between the A9 and scissile phosphates jumps to 6 Å at the beginning of the simulation and returns to around 4 Å, resulting in an average of 5.06 Å (Table 2.3).

In the deprotonated reactant state simulation (dRT-C), Mg^{2+} migrates from the C-site to the bridging B-site position after about 2 ns while maintaining coordination in the axial–equatorial mode. This results in a shorter average in the A9 and scissile phosphate distance of 2.92 Å (Table 2.3). In all three reactant simulations with Mg^{2+} ion present, the distances between the A9 and scissile phosphates remain within 1.5 Å of the crystallographic value of 4.3 Å.

In the RT-B simulation, the Mg^{2+} bridges the A9 and scissile phosphates, effectively tethering them at a distance of 3–4 Å (Table 2.3), whereas in the RT-C simulation the average distance is 5.06 Å (Table 2.3). In the deprotonated dRT-C simulation, the negative charge facilitates the migration of the Mg^{2+} into a near-bridging position such that results are quite similar to those of the RT-B simulation. It is also noteworthy that the interaction of the implicated general acid and base becomes stronger with Mg^{2+} in a bridging position (either in the RT-B simulation, or after migration in the dRT-C simulation). These results suggest that in the reactant state the preferred binding mode of Mg^{2+} is at the C-site, which is between A9 and N7 of G10.1 (through a water molecule)^{74,92,95} and that the negatively charged environment near the scissile phosphate, formed after the initial pH-dependent general base reaction, brings the Mg^{2+} into a bridging position between A9 and scissile phosphates leading to the transition state.

2.4.3 Mg^{2+} binding in the transition state—In both ETS and LTS mimic simulations with Mg^{2+} at the bridging position (ETS-B and LTS-B), the distance between the A9 and scissile phosphates is around 4 Å and the Mg^{2+} coordination between the C1.1 and A9 phosphate oxygens keeps an axial–axial position along the whole simulations (Table 2.2). The distance between A9 and scissile phosphates in the crystallographic structure is around 4.3 Å, which is well suited for Mg^{2+} bridging coordination. However, in both ETS and LTS mimic simulations with Mg^{2+} initially at the C-site position (ETS-C and LTS-C), the Mg^{2+} ion migrates from the C-site to the bridging B-site position in less than 0.5 ns and remains at

the B-site (bridging position) for the remainder of the simulation. A similar situation is found in the deprotonated dRT-C simulation (see above). The migration is also indicated by the broken coordination between the Mg^{2+} ion and G10:N7 (details not shown here). This observation may again suggest that the B-site is the preferred position for Mg^{2+} when an additional negative charge is accumulated at the scissile phosphate in formation of the transition state.

2.5. The big picture

In this section, we present a series of MD simulations of the eHHR in solution to study the Mg^{2+} -binding mode and conformational events at different stages along the catalytic pathway. Our results suggest an HHR model whereby the active site forms a region of local negative charge that requires electrostatic stabilization to preserve its structural integrity, and that this stabilization can be effected by divalent metal ion binding at the C-site or the B-site in the ground (prereactive) state. A Mg^{2+} ion is observed to weakly bind at the C-site position at solvent separation with G10.1, facilitating the formation of near in-line attack conformations, particularly when in the bridging position where there is increased interaction between the nucleophile (C17:O2') and the implicated general base (G12).

Deprotonation of the nucleophile is correlated with the migration of the Mg^{2+} from the C-site into a bridging position (B-site), and with the formation of the dianionic transition state, suggesting that the accumulation of negative charge around the scissile phosphate center is sufficient to induce a change in the binding mode of the Mg^{2+} . Once in the bridging position in the transition state, the Mg^{2+} ion interacts with the O5' leaving group of C1.1 and the 2'OH of G8, the implicated general acid catalyst. The B-site Mg^{2+} ion can act both as a Lewis acid catalyst to stabilize directly the accumulating charge on the O5' leaving group and can induce a pK_a shift on the 2'OH of G8 to facilitate general acid catalysis. Upon proton transfer from G8:O2' to C1.1:O5', the Mg^{2+} is poised to directly stabilize the resulting 2' alkoxide (which could occur synchronously). Combined QM/MM simulations on two points (LTS and ETS) along the reaction coordinates suggest that the barrier for the general acid proton transfer step may be sufficiently low so as to occur on the nanosecond timescale.

The mechanistic model evinced by these simulations is consistent with a considerable body of experimental work, including (1) thio/rescue effect experiments^{63,92} that support a mechanism in which a single metal ion bound at the C-site in the ground state acquires an additional interaction with the scissile phosphate in proceeding to the transition state; (2) kinetic studies,⁹⁶ photocrosslinking experiments,¹¹⁹ and mutational data^{61,90} that implicate G8 and G12 as possible general acid and base, respectively; and (3) recent metal ion titrations suggesting that the pK_a of the general acid is down-shifted by around 4–7 pK_a units in a metal ion-dependent manner, correlated with the metal ion pK_a ,¹²⁰ and that divalent metal ions may play a specific chemical role in catalysis.^{120,121} The direct coordination of Mg^{2+} and the 2'OH of G8 has been confirmed experimentally.⁶⁰

Although our simulation results are consistent with most available experimental evidence, there remain caveats that have not yet been fully resolved. The present work is suggestive that a bound divalent metal ion at the C-site migrates to the B-site between the A9 and

scissile phosphates in proceeding to the transition state (one metal ion mechanism). However, at this point, one cannot fully discount an alternate mechanism whereby there is no direct participation of a metal ion at the scissile phosphate in the transition state (no metal ion mechanism), or a mechanism whereby the metal ion at the C-site does not migrate, but rather the scissile phosphate acquires an additional metal ion interaction in proceeding to the transition state (the two metal ion mechanism). In order to fully explore these alternate mechanistic scenarios, full free-energy profiles of the chemical reaction steps are necessary, as summarized in the next section.

3. FINDING THE CATALYTIC REACTION PATH THROUGH MULTIDIMENSIONAL FREE-ENERGY SURFACES

In the previous section, we showed that the Mg^{2+} metal ion may migrate from the C-site to the B-site when C17:O2' is activated (deprotonated), and may be directly involved in the general acid step by coordinating with the general acid, the 2'OH group of G8. Two 1 ns QM/MM simulations on fixed reaction coordinate points, representing early and LTSs, support such a picture.^{75,76} In this section, we report our effort to further extend our QM/MM calculations to obtain the free-energy profile along the relevant reaction coordinates to explore the proposed metal ion-assisted phosphoryl transfer and general acid catalysis mechanism in HHR. A preliminary communication of these results has been presented elsewhere.⁷⁹

The reaction mechanisms considered here, (see Fig. 2.4), assume that the 2'OH group of C17 has already been activated (i.e., deprotonated) and act as a nucleophile to undergo in-line attack on the adjacent scissile phosphate, passing through a pentavalent phosphorane intermediate/transition state, followed by acid-catalyzed departure of the O5' leaving group of C1.1. The general acid is assumed to be the 2'OH group of G8. The goal of the work is to provide insight into two fundamental questions regarding the catalytic mechanism: (1) to characterize and quantify the degree to which the phosphoryl transfer step and general acid step are coupled (i.e., occur via a stepwise or a concerted mechanism) and (2) to identify and quantify the specific role of a key divalent metal ion in each chemical step of the reaction.

In order to answer these questions, we have determined a minimum free-energy reaction pathway by simulating a series of six two-dimensional (2D) potential of mean force (PMF) or free-energy profiles, and 1D PMF refinements along the minimum free-energy paths (MFEPs) that require an aggregate of over 100 ns of QM/MM simulation.

3.1. Results

3.1.1 Phosphoryl transfer and general acid steps follow a stepwise mechanism and depend on Mg^{2+} coordination—Our initial attempts to study the chemical steps of the HHR reaction from 2D PMF profiles using phosphoryl transfer and proton transfer reaction coordinates, but not considering a reaction coordinate associated with Mg^{2+} ion-binding mode, led to barriers that were unexpectedly high (~37 kcal/mol). We extended the calculations so as to include 3D-PMF profiles with a coarse-grained reaction coordinate associated with the Mg^{2+} -binding mode, and confirmed the sensitivity of

the barriers to the Mg^{2+} ion position along the reaction coordinate. A common feature of the reaction mechanism derived from the 3D profile was that the phosphoryl transfer and general acid steps were stepwise, demonstrated in Fig. 2.5A, allowing these steps to be decoupled.

Since both the phosphoryl transfer and general acid steps of the reaction were coupled with Mg^{2+} -binding mode, two separate 2D profiles were generated for each step with a reaction coordinate corresponding to the Mg^{2+} -binding mode as a second dimension. Table 2.4 summarizes key average geometrical parameters and free-energy values for stationary points along the minimum free-energy reaction paths derived from the two separate 2D PMF profiles, which are the dark arrow in Fig. 2.5B and the white arrow in Fig. 2.5C, respectively.

3.1.2 Phosphoryl transfer is rate limiting and facilitated by electrostatic stabilization by Mg^{2+} —Figure 2.5B depicts the 2D free-energy profile for the Mg^{2+} -binding mode during the phosphoryl transfer step. Comparing to the free-energy barrier in another 2D PMF profile for the general acid catalysis (Fig. 2.5C), we conclude that this phosphoryl step is rate controlling. To further refine the samplings in MD simulations, we generated a 1D PMF profile following the minimum free-energy reaction path (Fig. 2.5B). The computed free-energy barrier for this rate-controlling step is approximately 24.4 kcal/mol.

The minimum free-energy reaction path indicates that the position of the Mg^{2+} ion follows the negative charge along the phosphoryl transfer reaction coordinate, in order to provide electrostatic stabilization. The change in the Mg^{2+} position is continuous and monotonic throughout the phosphoryl transfer step (Fig. 2.5B), although it is most pronounced in the initial and final stages when the nucleophile and leaving group have the greatest negative charge. The transition state is late (Table 2.5 and Fig. 2.5), having a P—C1.1:O5' distance of 2.11 Å. As the P—C1.1:O5' bond breaks, the Mg^{2+} ion forms an inner-sphere coordination, leading to a Mg^{2+} -bound O5' alkoxide intermediate.

3.1.3 General acid catalysis is concerted with changes in Mg^{2+} -binding mode—The 2D free-energy profile for the Mg^{2+} -binding mode during the general acid catalysis is shown in Fig. 2.5C. The general acid step considered here assumes that the 2'OH of G8 acts as a general acid catalyst to transfer a proton to the C1.1:O5' leaving group. Table 2.5 and Fig. 2.5C indicate that proton transfer occurs after the formation of Mg^{2+} -coordinated cleaved intermediate, but that proton transfer is concerted with changes in the Mg^{2+} -binding mode. Unlike the phosphoryl transfer step, participation of the Mg^{2+} along the reaction coordinate is most pronounced not at the endpoints of the step but near the midpoint where the proton transfer occurs.

To further enhance the samplings in MD simulations, we generated a 1D-PMF profile following the minimum free-energy reaction path. The simulated free-energy value for the transition state of the general acid step in Fig. 2.5C is 13.7 kcal/mol with respect to the activated precursor state. The intermediate is only 6.7 kcal/mol lower in free energy than the activated precursor, and has a 20.4 kcal/mol barrier to break down into the product state with

a proton transferred to the C1.1:O5' leaving group. We note that this barrier is effectively reduced if proton-tunneling effects are included.

3.2. Summary

The QM/MM free-energy profile work presented here explores a specific mechanistic scenario, departing from the activated precursor state, which assumes the catalytic metal ion is in a position bridging the A9 and scissile phosphates, and the 2'OH of G8 acts as a general acid catalyst. The former metal ion-binding mode has not yet been observed experimentally, but has been inferred from biochemical experiments, and predicted by molecular simulations summarized in Section 2. The QM/MM results provide further computational support for the plausibility of a cleavage mechanism where phosphoryl transfer and general acid catalysis are stepwise, and that the catalytic divalent metal ion plays an active role in the chemical steps of catalysis. The results of these QM/MM simulations are of interest to compare with very recent reports that use thio/Cd²⁺-rescue experiments to probe the ground state coordination of a catalytic metal ion to the scissile phosphate of an eHHR motif¹²² in a bridging mode identical to that proposed in the current theoretical investigation. It is thus of great interest to extend our future theoretical studies to be more directly comparable to these experiments.

3.3. Simulation setup and protocol

The free-energy simulations performed here use the same settings and protocols as the QM/MM simulations in Section 2.3.2, with the following additions and exceptions: The QM region includes G8, A9, C1.1, C17, and an Mg²⁺ ion with coordinated water molecules (81 atoms). For Mg²⁺, the AM1/d model parametrized by Imhof was utilized.¹²³ For the MM region, the all-atom AMBER parmbsc0 force field was employed,¹²⁴ along with the TIP4P-Ewald water model¹²⁵ and the consistent set of monovalent ion parameters.¹²⁶ Multidimensional PMF profiles were generated along reaction coordinates corresponding to phosphoryl transfer, proton transfer from the general acid to the leaving group, and divalent metal ion-binding mode.

3.3.1 Free-energy profile—To shed light on the HHR reaction mechanisms of the phosphorylation and protonation steps in which the binding mode of Mg²⁺ continuously changes, we explicitly simulated the enzymatic reactions by generating a series of six 2D PMF or free-energy profiles. Three of these 2D PMFs were combined to form a coarse-grained 3D PMF (the coarse-grained third dimension representing the coordinate associated with the Mg²⁺-binding mode).

Before the QM/MM simulations, we first performed classical MD simulations (i.e., the whole system was described by an MM force field) to further equilibrate the entire system for more than 1 ns from the structure which had already been equilibrated for more than 2 ns in the previous classical MD simulations.^{75,76} Next, a series of umbrella samplings¹²⁷ using QM/MM MD simulations were carried out to span the entire range of the 2D or 3D reaction coordinates.

3.3.2 Enhanced sampling efficiency—Harmonic biasing potentials along each reaction coordinate were applied with a force constant ranging between 20 and 45 kcal/mol/Å² in accordance with different regions of the configuration samplings. The choices of the force constants and the number of windows were made to ensure sufficient overlap of the probability distributions with neighboring windows. Following the initial 1 ns equilibration in classical MD simulations, each window was further equilibrated with the QM/MM potential for more than 5 ps after the system was equilibrated in the adjacent windows. Subsequently, a 10 ps of QM/MM configuration sampling was performed in each window.

3.3.3 Weighted histogram analysis method—Finally, the weighted histogram analysis method^{128,129} was used to generate *unbiased* PMFs as a function of different sets of reaction coordinates by combining all the sampled configurations. All the configurations were collected at every MD time step in a bin size of 0.1 Å except for the third reaction coordinate in the 3D PMF. In the 3D PMF, configurations along z_3 were collected in a bin size of 0.4 Å at every time step. After determining the MFEP from the 2D PMF profiles, we simulated a series of 1D PMFs following the MFEP, in which 100 ps sampling was performed in each window and the bin size of collecting configurations is 0.01 Å.

4. HHR USES SPECIFIC CATION-BINDING MODES TO REACH ITS CATALYTICALLY ACTIVE CONFORMATION

Simulations summarized in Sections 2 and 3 suggested that the divalent metal ion can serve to stabilize active in-line conformations and interact with key residues to facilitate acid/base catalysis. However, it is known that under high non-physiological salt concentrations, the HHR and other ribozymes remain catalytically proficient.^{130–132} Hence, a new question arises whether 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.

As already mentioned, 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.¹¹⁵

In this section, we summarize the results of a series of five 300-ns simulations of the eHHR, 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.¹³³ The results indicate that the HHR 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 possibly common feature that might be exploited by other ribozymes: the engineering of electrostatically pre-organized active sites that are able to recruit solvent components to facilitate catalysis.

4.1. Results

MD simulations were set up to explore the metal ion-binding modes in eHHR and their relationship to structure and catalysis. Specifically, we consider the “C-site” (see also Section 2) derived from a recent joint crystallographic/molecular simulation study⁷⁴ of the solvent structure of the eHHR that indicates a Mn^{2+} ion is coordinated to the O2P atom of the A9 phosphate and the N7 atom of G10.1, and the “B-site” inferred from thio/rescue effects^{62,63} and also predicted from molecular simulations^{75,76} where a divalent metal ion bridges A9:O2P and the scissile phosphate (C1.1:O2P). Simulations, in the absence of divalent metal ions, were also performed since HHR activity can be recovered by high concentrations of monovalent ions.^{130–132,134} Furthermore, the specific metal ion-binding modes in the reactant state and the activated precursor state (with the nucleophile C17:2'OH deprotonated) are also explored. Compared to the simulations performed in Section 2, here we significantly extended the simulation time in order to further study the long-time dynamics of eHHR. The detailed differences of simulation setup and protocols are discussed in Section 4.2.

The following series of long-time simulations (300 ns each) were set up (Table 2.3):

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 B-site
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 B-site
5. RT-Na, the reactant state in the absence of Mg^{2+} (presence of only Na^+)
6. dRT-Na, the activated precursor in the absence of Mg^{2+} (presence of only Na^+)

The dRT-C-Mg simulation of the activated precursor with Mg^{2+} initially placed at the C-site quickly (less than 200 ps) leads to migration of the Mg^{2+} ion into the B-site position, as was observed in a previously simulation study (Section 2 and Ref.^{75,76}), and afterward exhibited nearly identical behavior as if the Mg^{2+} ion was initially placed at the B-site (dRT-B-Mg). Therefore, we only extended the dRT-B-Mg simulation to 300 ns and designated it simply as dRT-Mg. All five simulations were carried out to 300 ns in a background of 0.14 M NaCl. The equilibration of each simulation was monitored by the root-mean-square-deviation (RMSD) and reached steady states after 30–50 ns. Hence, all analyses were performed over the last 250 ns of trajectories for each simulation.

4.1.1 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 ion occupation and coordination numbers in the active site. Four key coordination sites are used for characterizing metal ion binding (Fig. 2.6): G8:O2', A9:O2P, C1.1: O2P, and C17:O2'. The averages of the following three quantities were calculated for both Mg^{2+} and Na^+ ions: N is the number of ions with at least one coordination to any one of the four key coordination sites, CN is the total coordination number of ions with at least

one coordination to any coordination site, and NB is 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 Fig. 2.7 and manifest two k-mean clusters¹³⁶: cluster A (light gray) containing structures in the near in-line attack conformations, and cluster B (dark gray) 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 ion occupation and coordination numbers were then analyzed for each cluster and the results are listed in Table 2.6. During all simulations, when present in the active site, one Mg^{2+} ion remained stably bound at full occupancy ($\text{NMg}^{2+}=1.00$). In the reactant state simulations (RT-C-Mg and RT-B-Mg), there is no significant population of Na^+ ions in the active site ($\text{NNa}^+>0.1$), 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:O2P of the RNA ($\text{CNMg}^{2+}=1.00$), and thus is not involved in a bridge ($\text{NBMg}^{2+}=0.00$). 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 B-site) simulation, on the other hand, is dramatically different. In this simulation, the Mg^{2+} ion directly coordinates both A9:O2P and the scissile phosphate C1.1:O2P of the RNA ($\text{CNMg}^{2+}=2.00$) as a stable bridge ($\text{NBMg}^{2+}=1.00$). 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 ($\theta=155$). For dRT-Mg, an additional negative charge in the active site arises from deprotonation of the nucleophile, and a single Na^+ ion is observed in the active site ($\text{NNa}^+=0.97$) that makes direct coordination to only one RNA ligand ($\text{CNNa}^+=1.01$) 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 ($\theta=153$). Most of cluster A population contains a single Na^+ ion in the active site ($\text{NNa}^+=1.15$), less frequently two Na^+ ions, and the average number of bridging Na^+ ions is 0.88. On the other hand, cluster B has a slightly higher active site Na^+ occupation ($\text{NNa}^+=1.38$), but a lower average number of bridging ions (0.66).

In the activated precursor dRT-Na simulation, the average Na^+ occupancy (NNa^+) 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 (NBNa⁺) 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 in-line conformations for both cases with and without Mg²⁺ 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 Mg²⁺ ion.

4.1.2 A bridging Mg²⁺ ion maintains rigid coordination patterns that stabilize in-line attack conformations—In this section, we compare the effect of different Mg²⁺-binding modes in both the neutral reactant and activated (deprotonated 2'OH) precursor states on the active site structure and fluctuations. Table 2.7 lists the averages of key in-line indexes, the A9/scissile phosphate-phosphate distance, and Mg²⁺ coordination distances for the RT-C-Mg, RT-B-Mg, and dRT-Mg simulations. Figure 2.6 shows a general schematic view of the active site metal ion coordination from the simulations. The distances and standard deviations in Table 2.7 indicate that the Mg²⁺ ion retains rigid coordination with the phosphate oxygens over the course of the simulation, being directly coordinated to A9:O2P in all simulations. In the RT-C-Mg simulation, the Mg²⁺ 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. On the other hand, the RT-B-Mg simulation shows a more rigid Mg²⁺ coordination with both the A9 and scissile phosphate oxygens and sustains a considerable 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 Mg²⁺ 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 Mg²⁺ ion at the bridging position (RT-B-Mg and dRT-Mg simulations), there is considerably reduced interaction with G10.1:N7, which is compensated by interactions with the C17:O2' that occur through two water molecules in the inner sphere of the Mg²⁺ ion. This interaction is most pronounced in the dRT-Mg simulation where the C17:O2' is deprotonated. In the ground-state reactant simulations with Mg²⁺ (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:O2' in a manner similar to the M3 position in Fig. 2.6.

4.1.3 Na⁺ ions bind nonspecifically 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 in the active site

(RT-Na and dRT-Na), binding of Na^+ ions to the coordination sites exhibits larger variation and exchange events occur giving rise to a fairly broad array of coordination patterns.

Simulation results¹³³ suggest that two Na^+ ions are present in the active site and bind to both A9:O2P and C1.1:O2P at the same time in the RT-Na simulation. Hence, two Na^+ ions collectively act like a single bridging Mg^{2+} ion to hold the negatively charged A9 and scissile phosphates together to maintain an in-line conformation. In the dRT-Na simulation, 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, the in-line angle comes to a reaction-competent value (150°). Figure 2.8 illustrates the different Na^+ binding patterns for cluster A (defined in Table 2.6, in-line conformation, Fig. 2.8 lower panels) and cluster B (not in-line conformation, Fig. 2.8 upper panels) from the dRT-Na simulation. The in-line cluster A clearly exhibits three Na^+ bridges that involve C17:O2'/C1.1:O2P, C1.1:O2P/G8:O2', and C1.1:O2P/A9:O2P. For cluster B, on the other hand, 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^+ that bring them together, is necessary to keep the in-line conformation in the deprotonated activated precursor state, although the binding patterns are not as rigid as those of Mg^{2+} .

4.1.4 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 (Fig. 2.9) show that the Na^+ ion density at a medium contour level (left panels, Fig. 2.9) is located near the RNA's phosphate backbone, whereas at high contour level (right panels, Fig. 2.9) 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 during simulations. This suggests that the HHR folds to form a strong local electronegative pocket that attracts Mg^{2+} or Na^+ ions. A similar case has been observed in the tetraloop-receptor complex analyzed by NMR, where the divalent ions were experimentally found to be located at strong electronegative positions formed by the RNA fold.¹³⁷ 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 the formation of active conformations discussed in detail in the previous sections.

4.2. Discussion

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

one to two Na^+ ions. In the activated precursor state, this is accomplished by an 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 HHR retains activity at high concentration of monovalent ions.¹³¹ The properties of HHR cleavage in high concentrations of monovalent ions are similar to those in the presence of divalent metal ions, which have been interpreted to indicate that the major role of the cations is simple electrostatic stabilization of the phosphates to allow folding into an active conformation. However, there remains some smaller contribution to the rate enhancement that 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 monovalent ions¹³⁰ or exchange inert ions.¹³²

There are notable exceptions whereby HHR cleavage differed significantly in the presence and absence of divalent metal ions. Disruption of implicated divalent metal ion-binding sites at G10.1:N7, A9:O2P, and C1.1:O2P has significant deleterious effects on HHR cleavage in the presence of divalent ions, but not in high concentrations of Li^+ . For example, both A9:O2P and C1.1:O2P exhibit significant catalytic thio effects that can be rescued by thiophilic ions such as Cd^{2+} . Moreover, both G10.1: N7 and A9:O2P form a divalent metal ion-binding site as pinpointed by electron spin-echo envelope modulation¹³⁸ and as observed crystallographically for the eHHR.⁷⁴ The present simulation results are consistent with 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 HHR activity to divalent ions is reduced upon introduction of tertiary stabilizing motifs.^{121,139–142} A recent study of the tertiary stabilized RzB HHR has led to the suggestion that HHR catalysis may occur through a multichannel mechanism that has available both a divalent-dependent and divalent-independent pathways.¹²¹ These experiments, together with previous measurement of pH-rate profiles,¹⁴¹ are consistent with the interpretation that the divalent ion may play a specific nonstructural 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 (Sections 2 and 3) that a 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').

4.3. Simulation setup and protocols

The simulation setup and protocols are similar to those in Sections 2.3.1 and 2.3.2, except the following modification and extension:

The all-atom Cornell et al. force field (parm94)¹⁴³ in CHARMM format is provided in the AMBER 9 package.^{144–146} The equilibration procedures have been extended to total 10 ns

as follows: 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.

4.3.1 Pre-annealing stage—Water and ion molecules were first energy-optimized for 2000 steps and then underwent a constant volume simulation annealing: The temperature was increased from 0 to 298 K at the rate of 1 K per ps. The system then was kept at 298 K for 500 ps.

4.3.2 Solvent annealing stage—First step: The temperature increased from 298 to 600 K at the rate of 1 K/ps and then was kept at 600 K for 500 ps with constant volume. Second step: The temperature decreased from 600 to 298 K at the rate of 1 K/ps and then was kept at 298 K for 1500 ps with constant volume. Third step: The system was kept at 298 K for 3000 ps at a constant pressure (1 atm). The whole annealing stage was repeated twice before the post-annealing stage.

4.3.3 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/Å²) 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% of the initial restraint values.

4.3.4 Production simulation—After 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/Å² were added to keep the Mg^{2+} ion binding to G10.1:N7 and A9:O2P position. Another three harmonic restraint of 20 kcal/mol/Å² were used: the distances between G8:HO2P and C1.1:O5', and between G12:H1 and C17:O2', were kept around 1.8 Å to ensure the initial hydrogen bonding; the distance between A9: O2P and C1.1:O2P was kept at 4.3 Å (crystal distance). After 10 ns, all restraints were removed. The motions and relaxation of solvent and counter-ions are notoriously slow to converge in nucleic acid simulations,¹¹⁵ and careful equilibration is critical for reliable simulations. In summary, each simulation was carried out to 300 ns beginning with a total of 20 ns of equilibration (10 ns of solvent/ion relaxation and 10 ns of solvent and structure relaxation). Analysis was performed over the last 250 ns with data collected every 10 ps.

5. COMPUTATIONAL MUTAGENESIS OF KEY RESIDUES OF HHR

In this section, we report MD simulations to elucidate the origin of mutational effects in the HHR. In a series of 24 100-ns molecular MD simulations, we explore the structure and dynamics of eHHR mutants involving C3, G5, U7, and G8 positions in both the reactant and activated precursor (deprotonated 2' OH) states. Simulations for each mutation are compared

with the wild-type (WT) simulation results. The activated precursor state, distinguished by the deprotonation of the 2'OH group of C17, is already described in previous sections.

The U7 mutation has been known as a benign mutation, and hence is used as a control simulation in this study. Single mutations at both the C3 and G8 positions (C3U, G8A, G8I, and G8D) are explored, where “D” indicates 2,6-diaminopurine, and “I” indicates inosine. In addition, double mutants that exhibit a partial rescue effect have been examined, including the isosteric C3U/G8D and hydrogen bond-preserving C3G/ G8C double mutation. A simulation of C3U/G8D mutations, for which there currently exists, to our knowledge, no experimental measurement, predicts an almost complete rescue effect. Finally, a series of single mutations at the G5 position have been studied, including G5I, G5A, and G5D.

The general structure of the HHR active site, including identification of indexes used to characterize key hydrogen bond networks and base stacking interactions involving conserved residues, is shown in Fig. 2.10. Representative hydrogen bond patterns observed in the simulations for the C3 and G8 mutants are shown in Fig. 2.11, and for the G5 mutants are shown in Fig. 2.12. Averages and fluctuations for key indexes used to characterize the active site are listed in Tables 2.8 and 2.10 for the reactant state and Tables 2.9 and 2.11 for the activated precursor state. Table 2.12 lists indexes used to characterize the base stacking interactions between G8 and C1.1 in the wild type, benign U7C and G8I single mutants, and double mutant simulations. A summary of the overall mutational effects inferred from the MD simulations are provided in Table 2.13 and compared to experimental values for the relative catalytic rates.

In the following discussion, we will apply certain mechanistic assumptions in our analysis, in particular regarding the role of G12 and G8 2'OH as the general base and acid, respectively. These roles are supported by structural data,^{73,74} mutagenesis,^{61,68} and biochemical^{59,60} studies, but have not been definitively proven. Assuming this plausible mechanistic hypothesis, we then ask whether our simulation results can explain the origin of the mutational effects. It should be emphasized that the mechanistic assumptions regarding G12 and G8 be incorrect, so must be the interpretations of the simulation data that invoke this model. Further study is needed to resolve these details, for example, using molecular simulation of the catalytic chemical steps of the reaction with combined QM/MM methods.

5.1. Results

5.1.1 Control simulations: wild type and U7C—As a precursor to the discussion of the origin of mutation effects on reaction rate, a characterization of the key elements of the WT simulation that affect catalysis is needed. Moreover, to lend credence to our simulation methodology and our mechanistic interpretation of simulation results, we perform a control simulation of a U7C mutation that has been observed experimentally to have no adverse effect on the relative rate of reaction as determined by the ratio of rate constants for WT and mutant reactions, $k_{\text{rel}} = k_{\text{mut}}/k_{\text{wt}}$.¹⁵¹ The WT and U7C control simulation results are included in all of the tables for reference and comparison.

5.1.1.1 Characterization of the active site structure and dynamics of the WT

simulation: The active site scaffold and hydrogen bond networks for the WT simulation are

depicted in Fig. 2.10. The eHHR ribozyme catalyzes the site-specific cleavage by transesterification of the phosphodiester bond with a rate enhancement up to 10^6 -fold relative to the rate of non-catalyzed cleavage.³⁹ The rate for cleavage and ligation for the naturally occurring eHHR motifs are around 1000 and 2000 times faster, respectively, than the corresponding rates for mHHR ribozymes.^{84–86}

Catalysis is generally believed to proceed by a general acid and base mechanism. In this mechanism, the endocyclic amine of G12 (G12:N1) in deprotonated form acts as the general base to abstract a proton from the 2'OH of C17 (the nucleophile) to form an activated precursor. The activated precursor then proceeds by in-line attack on the adjacent scissile phosphate to form a pentacovalent phosphorane transition state. The 2'OH group of G8 (G8:O2') acts as a general acid catalyst to donate a proton to the 5' oxygen of C1.1 (the leaving group) to facilitate breakdown of the phosphorane and phosphodiester bond cleavage. The pK_a of the general acid is believed to be shifted toward neutrality through interaction of a divalent metal ion that bridges the phosphoryl oxygens of A9 and the scissile phosphate. These oxygens are positioned approximately 4.3 Å away from one another in the crystal structure, and both exhibit significant catalytic thio effects in the presence of Mg^{2+} ions that can be rescued by titration with thiophilic Cd^{2+} ions. Simulation results indicate that the divalent metal ion migrates from a distal binding site involving A9 and G10 in the reactant state to a bridging position between A9 and scissile phosphates upon formation of the activated precursor.

From this mechanistic picture, several conditions for catalytic competency of the HHR can be inferred. First, the general base must be correctly positioned to abstract a proton from the nucleophile to form the activated precursor. Second, the structure of the active site must allow the activated nucleophile to be in-line with the scissile phosphate, and fluctuations must sample conformations that have a high degree of in-line fitness. Third, the integrity of the active site, and in particular, the proximity of the A9 and scissile phosphates must be conducive to binding a bridging divalent metal ion. Fourth, the general acid must be poised to donate a proton to the leaving group to facilitate cleavage. In order to satisfy these conditions, a specific network of hydrogen bonds and base stacking interactions must be in place. Indexes correlated with each of these conditions are depicted in Fig. 2.10. The average values and fluctuations of these indexes for the WT simulation are listed in the tables for reference, and representative hydrogen bond networks involving conserved residues are shown in the figures.

5.1.1.2 U7C control simulation satisfies all of the conditions for WT catalysis: The U7C mutant has a catalytic rate virtually identical to that of the WT ($k_{rel} = 1.1$).¹⁴⁷ Comparison of the WT and U7C control simulations shows no major differences in the indexes likely to be key for catalysis (Tables 2.8–2.13). The general base forms a stable hydrogen bond with the nucleophile in the reactant state ($r_{HB} = 2.1$ Å), the distance between the A9 and scissile phosphates (d_0) in the reactant state is around 4.3 Å as in the crystal structure, and the activated precursor maintains in-line fitness comparable to the WT simulation and significant hydrogen bonding between the general acid and the leaving group. In addition, the base stacking interactions are very similar between the WT and U7C simulations (Table 2.12).

Perhaps, the most notable difference is that the U7C simulation of the reactant state does not exhibit a strong hydrogen bond between the general acid and leaving group ($r_{\text{HA}} = 3.41 \text{ \AA}$ in the U7C simulation, whereas the corresponding value is $2.75 \text{ \AA}$ in the WT simulation). However, the general acid step occurs at a point farther along the reaction coordinate from the reactant state, and examination of the hydrogen bond of the general acid in the activated precursor state indicates a comparable, slightly stronger hydrogen bond interaction ($r_{\text{HA}} = 2.50$ and $2.61 \text{ \AA}$ in the U7C and WT activated precursor simulations, respectively). Overall, the U7C simulation results indicate very comparable integrity of the active site, in-line fitness, and positioning of the general base and acid that are conducive for catalysis.

5.1.2 Single mutations at the C3 and G8 positions—The C3 and G8 positions form a Watson–Crick base pair in the eHHR structure^{73,74} and they are important in stabilizing the active site structure and positioning the 2'OH of G8 for acid catalysis. Here, we consider a series of single mutations (C3U, G8A, G8I, and G8D) for which representative hydrogen bond patterns are shown in Fig. 2.11.

5.1.2.1 C3U mutation disrupts the active site in the reactant: The C3U mutation reduces the catalytic rate by a factor $\sim 3 \times 10^{-4}$.¹⁴⁸ Simulation results indicate this mutation disrupts the normal Watson–Crick hydrogen bonding with G8 (Fig. 2.11 and Table 2.8), causing a base shift that disrupts the active site structure in the reactant state. The average distance between the A9 and scissile phosphates (d_0) increases by $2.67 \text{ \AA}$ relative to the WT simulation, breaking key hydrogen bonds between the O2' nucleophile of C17 and N1 of G12 (the implicated general base). These perturbations in the reactant state would prevent activation of the nucleophile and progress toward the transition state.

5.1.2.2 G8I mutation is relatively benign: The G8I mutation does not significantly alter catalytic activity, where the measured rate reduction is less than a factor of two.^{94,96} The removal of the exocyclic amine at the C2 position weakens the hydrogen-bonded base pair with C3, but does not alter the structure (Fig. 2.11 and Table 2.8). None of the structural features derived from the simulations in either the reactant state or activated precursor state show any marked differences from the WT simulations. Overall, the marginal effect on catalysis is likely a consequence of modest weakening, but not disruption of the base pair between C3 and G8.

5.1.2.3 G8A mutation blocks the general acid step in the activated precursor: The catalytic rate of the G8A mutant is reduced to less than 0.004.⁸³ Simulation results indicate that G8A mutation considerably weakens the base pair with C3 such that only one weak hydrogen bond remains intact (Fig. 2.11 and Table 2.8). In the reactant state simulation, G8A does not appear to dramatically alter the active site contacts relative to the WT simulation, with the exception that A8:N1—C3:N3 distance increases due to a shift in the hydrogen bond pattern.

In the activated precursor state (Table 2.9), however, the hydrogen bond between G8:H2' and C1.1:O5' is significantly disrupted relative to the WT simulation. The breaking of this key hydrogen bond ($r_{\text{HA}} = 4.13 \text{ \AA}$) in the G8A mutation and movement of the general acid away from the leaving group effectively block the general acid step of the reaction. The

G8A mutation has the largest effect on catalysis of all the single mutations at the G8 position considered here.

5.1.2.4 G8D mutation alters the active site structure and weakens the interaction of the nucleophile and general base: Mutation of G8 to 2-aminopurine^{61,96} or to 2,6-diaminopurine (G8D)⁹⁶ reduces the reaction rate by approximately three orders of magnitude. The effect of this mutation in the reactant state is to weaken the hydrogen bonding between G8D and C3 relative to the WT simulation. This results in corruption of the active site structure (d_0 increases by 2.89 Å relative to the WT simulation) and impairs hydrogen bonding of the 2'OH nucleophile with the general base.

5.1.3 Double mutations at the C3/G8 positions—In order to better understand the role played by individual residues on ribozyme structure and function from mutagenesis data, it is useful to consider the effects of double mutations that are able to restore, at least in part, activity lost by deleterious single mutations. Here, we consider a series of double mutations (C3G/G8C, C3U/G8A, and C3U/G8D), for which representative hydrogen bond patterns are shown in Fig. 2.11.

5.1.3.1 C3G/G8C double mutation has a partial rescue effect: The C3G/G8C double mutation preserves strong hydrogen bond interactions between the 3 and 8 positions and has a partial rescue effect relative to the C3G and G8C single mutations. It, nonetheless, still reduces the activity 150–200-fold relative to the WT.^{70,149} The purine/pyrimidine substitutions alter the local stacking environment and lead to subtle structural changes in the active site. As a consequence, the average distance between the A9 and scissile phosphates increases by 0.29 Å relative to the WT simulation, and the hydrogen bond between the 2'OH nucleophile and general base is weakened (r_{HB} decreases from 2.07 Å in the WT to 2.50 Å in the C3G/ G8C mutant).

5.1.3.2 C3U/G8A double mutation is relatively benign: The C3U/G8A double mutation is an isosteric substitution that slightly weakens the base pair hydrogen bonding, and it has a relatively benign effect on activity (k_{rel} around 0.5–0.012).^{67,149} The simulation results for C3U/G8A are similar in hydrogen bond pattern to the relatively benign G8I single mutation and the WT base pair. The C3U/G8A mutation is a slightly larger perturbation than G8I, and the C3U/G8A simulation results indicate correspondingly more significant deviations from the WT. The C3U/G8A double mutation is overall similar to the C3G/G8C double mutation. Although the pyrimidine/purine pattern is reversed in C3G/G8C, it preserves the same number of base pair hydrogen bonds as the WT. The average distance between the A9 and scissile phosphates increases by 0.15 Å in the C3U/G8A mutant relative to the WT simulation, and the average hydrogen bond distance between the 2'OH nucleophile and general base is increased by 0.37 Å. This is expected to only moderately retard the efficiency of the general base step of the reaction. No other notable effects on the structure or dynamics were observed as a result of this mutation.

5.1.3.3 C3U/G8D double mutation is predicted to have a rescue effect: The results of the hydrogen bond-preserving C3G/G8C double mutation and isosteric C3U/G8A double mutation both show partial to strong rescue effects on HHR activity. Computationally, we

performed simulations of an isosteric C3U/G8D double mutation that also preserved the number of native base pair hydrogen bonds. Despite the corresponding single mutations having significant deleterious effects on activity, the double mutation is predicted by our simulations to have a rescue effect.

The only significant deviation from the WT was in d_0 in the reactant state simulation, which increased by around 0.7 Å, but did not lead to disruption of any of the key hydrogen bonds or in-line fitness indexes. However, a closer examination reveals some differences in the base stacking of C3U/ G8D relative to the WT and control simulations (Table 2.12). The alteration of the relative position between C1.1 and G8, in particular the increase in the r_7 stacking distance, shifts the position of the A9 phosphate leading to the modest increase in d_0 . Consequently, the simulation results predict this double mutation to have a significant rescue effect, but not necessarily fully rescue to the WT activity.

5.1.4 Single mutations at the G5 position—The G5 position forms an important element in the structural scaffold of the active site that helps hold the scissile phosphate in position via hydrogen bond interactions with the sugar and base functional groups of C17, and with the nucleobase of A14 (Fig. 2.10). These include hydrogen bonds between the G5:N1 endocyclic amine and C17:O2 (r_4), and the G5:N2 exocyclic amine and C17:O4' (r_5) and A14:N1 (r_6). Here, we consider a series of single mutations (G5I, G5A, and G5D), representative hydrogen bond patterns for which are shown in Fig. 2.12.

5.1.4.1 G5I mutation retards the general acid step: Experimental rate measurements for the G5I mutation suggest a reduction of the catalytic rate by 0.006⁹⁴ to $<10^{-3}$,⁹⁶ respectively. This mutation eliminates the exocyclic amine of guanine, and hence the hydrogen bond with C17:O6 and A14:N1. As a result, in the reactant state (Table 2.10), the integrity of the active site is somewhat disrupted (d_0 increases by 0.67 Å). In the activated precursor state (Table 2.11), the hydrogen bond between G8:H2' and C1.1:O5' is lost ($r_{\text{HA}} = 3.6$ Å), which would severely impair the general acid step of catalysis.

5.1.4.2 G5A mutation severely disrupts the active site integrity and in-line fitness: The G5A mutation leads to no detectable activity in the HHR.⁸³ This mutation eliminates both the endocyclic and exocyclic amines at the 1 and 2 positions of guanine that hydrogen bond with C17 and A14, and replaces the O6 carbonyl group with a new exocyclic amine at the 6 position. The result (Table 2.10) is that the nucleobase shifts such that the exocyclic amine at the 6 position of inosine hydrogen bonds with C17:O4', previously hydrogen bonded to the endocyclic amine at the 1 position. The other hydrogen bonds to C17 and A14 are eliminated. This results in considerable degradation of the active site (d_0 increases by 0.98 Å in the reactant state), poor in-line fitness in the activated precursor state (F decreases by 50%), and greatly reduces hydrogen bonding interaction between G8:H2' and C1.1: O5' (r_{HA} increases by 1.1 Å). Overall, the active site structure and positioning of the general acid in the activated precursor are not conducive for catalysis.

5.1.4.3 G5D mutation severely disrupts the active site integrity and in-line fitness: The G5D mutation leads to reduction of catalytic rate by four orders of magnitude.⁹⁶ The hydrogen bond pattern for this mutation is not as severely perturbed as that for the G5A

mutation. Nonetheless, in the reactant state (Table 2.10), the average distance between the A9 and the scissile phosphates (d_0) increases to 5.2 Å, and the hydrogen bond between the 2'OH nucleophile and G12:N1 (the implicated general base) is less pronounced. In the activated precursor state (Table 2.11), the general base hydrogen bond is dramatically weakened ($r_{\text{HB}} = 2.62$ Å), and the average in-line attack angle, θ_{incl} , is reduced to 139.7 degrees. These structural deviations are expected to hinder the attack of the nucleophile on the scissile phosphate.

5.2. Discussion

5.2.1 In the reactant state, shifted residue positions stabilized by hydrogen bond networks disrupt the active site integrity—Simulation results (Table 2.8) show that among the C3 and G8 mutants, d_0 exhibits the greatest deviation for C3U ($d_0 = 6.64$ Å) and G8D ($d_0 = 5.86$ Å) compared to the WT ($d_0 = 3.97$ Å) and U7C control simulation ($d_0 = 4.27$ Å). In these two mutants, the C3/G8 base pairs are held together by two hydrogen bonds and the bases are shearer relative to the WT (Fig. 2.11). In other mutants, including C3U/G8A, G8I, and C3G/G8C, all d_0 values are similar to WT and U7C. In the case of the C3U/G8D double mutation, subtle changes in base stacking produce a slight shift in the relative positions of C1.1 and G8 (Table 2.12), and this deviation is stabilized by a strong hydrogen bond network leading to a modest increase in d_0 ($d_0 = 4.68$ Å). In the case of mutations at G5, the G5D mutant has the largest value for d_0 ($d_0 = 5.2$ Å). The G5D mutant also displays a significant shift in the orientation of D5 with respect to C17 (Fig. 2.12) that is stabilized by a fairly strong hydrogen bond network.

Thus, the mutants that exhibit large shifts in the relative positions of key residues in the active site scaffold and that are locked by hydrogen bonding networks (i.e., C3U, G8D, and G5D) generally display the largest disruptions of the active site architecture. Alternately, mutants that do not exhibit shifts of the relative positions of residues (i.e., G8I, C3U/G8A, and C3G/G8C) or that have weakened hydrogen bond networks (G8A, G5A, and G5I) have d_0 values more similar to the WT and control simulations.

5.2.2 In the activated precursor state, strong hydrogen bond networks stabilize the active site architecture—In all of the activated precursor simulations, the relative positions of the A9 and scissile phosphates are very similar (d_0 values of around 3.0 Å). This is a result of the bridging Mg^{2+} ion that locks the A9:O2P and C1.1 O2P positions. The general base indexes are also similar between the different simulations due to the strong hydrogen bond that forms between G12:N1 and the deprotonated C17:O2' nucleophile. The one exception is for d-G5D, where the loss of a hydrogen bond between D5 and the C17:O4' causes the position of the sugar to shift and the hydrogen bond between the activated nucleophile and G12 to be less populated.

The orientation of the implicated general acid, G8:O2', which is poised to donate a proton to the C1.1:O5' leaving group, is important for catalytic competency. Alterations in the hydrogen bond network that disrupt the position of G8:O2', particularly the base pair hydrogen bonds between C3 and G8, will have an adverse effect on the general acid step. These alterations can be tracked by monitoring the hydrogen bond distance r_{HA} (Tables 2.9

and 2.11) and indicate that activated precursor simulations generally have similar general acid indexes (r_{HA} and θ_{HA}) with a few notable exceptions. In the d-G8A simulation, the C3–G8 hydrogen bond network is lost. In the d-C3U/G8D simulation, the C1.1–G8 base stacking is significantly different from other mutants. In the d-G5I and d-G5A simulations, the hydrogen bond network between G5 and C17 is disrupted.

Hence, in the activated precursor state, the two strands are brought together by the bridging divalent metal ion and a strong C3–G8 hydrogen bond network is necessary to keep G8 in position so that the general acid (G8:O2') is poised for catalysis. The interaction between G5 and C17 is important since G5 stabilizes the orientation of both the nucleobase and ribose of C17, and it helps position the nucleophile in-line with the scissile phosphate. Disruption of G5–C17 hydrogen bonds will either prevent in-line attack (d-G5A and d-G5D) or alter the position of the general acid (d-G5I). For the double mutant d-C3U/G8D, although all U3–D8 hydrogen bonds are maintained (Table 2.9), the C1.1–G8 base stacking distance is slightly elongated as indicated by larger r_7 and r_8 in Table 2.12. As a result, the general acid, G12:O2' is shifted away from the leaving group C1.1:O5' (r_{HA} in Table 2.9).

5.2.3 C3/G8 mutations may induce structural changes that have remote consequences at the general base step—There is evidence that mHHR motifs are conformationally variable, and that there is an equilibrium between active and inactive conformational states.^{84,149,152} It has been hypothesized that mHHR and eHHR may utilize a similar dynamic reaction mechanism for catalysis, and that observed correlations between mutations at C17 and C3/G8 may be explained by transient pairing between residues 3 and 17 and 8 and 13 arising from interactions in the inactive conformation.¹⁴⁹ This supposition was based in part on the observation that C3/G8 are distant from C17 in the eHHR structures, making it difficult to rationalize the observed correlations between mutations at C17 and C3/G8 in an active conformational state, as in the inactive mHHR structures, C3 pairs with C17 and G8 pairs with A13.^{2,72,87}

Our simulation results of the active eHHR, on the other hand, suggest that mutations at C3/G8 in some cases may induce remote changes in the structure and dynamics at C17 that affect the general base step of the reaction. Recent kinetics studies have indicated that the native eHHR constructs exhibit less conformational variability than the mHHR motifs, and they maintain a higher population of active conformations.^{84,152} Hence, the present simulation results afford an alternative interpretation of experimentally observed correlations between mutations at C17 and C3/G8 positions that do not rely, at least as heavily, on the assumption that mutants of the eHHR are in a dynamic equilibrium that populate an inactive conformation with contacts resembling that of the mHHR.

5.2.4 Variation of base stacking interactions of G8 and C3 with C1.1 and G2.1 may lead to new HHR motifs with modified activity—Our results suggest that the identities of nucleotides at C3 and G8, including some double mutants that preserve base pairing, can adversely effect HHR catalytic activity. This implies that the environmental effect due to base stacking interactions between G8/C1.1 and C3/G2.1 may be important to hold G8/C1.1 in the proper relative position for the general acid step of HHR catalysis (Table 2.12). In recent studies on various HHRs, it has been shown that the tolerance of

mutations at C3 and G8 is dependent on the identities of surrounding nucleotides, including C1.1 and G2.1. Residues C1.1 and G2.1 are not conserved,^{69,83,153} and hence it may be possible to exploit variations in C1.1 and G2.1, along with correlated mutations at G8 and C3, to design new HHR motifs with modified activity.

5.2.5 Long-time simulations are required to understand some mutational effects

—The current study reports 100 ns simulations for a series of HHR mutants in the reactant and activated precursor states. As previously discussed,^{77,133} long-time simulations are necessary to relax the mutations and sufficiently sample the conformational space required to observe the mutational effects. As shown in Fig. 2.13, the simulation of the activated precursor state of G8A reaches a stable relaxed state after 30 ns. However, on this timescale, we do not observe for the eHHR an equilibrium between proposed active and inactive states that involve changes in stem contacts, as has been inferred from biochemical studies.¹⁴⁹

Recent time-resolved NMR spectroscopic investigations have indicated that an ensemble of conformations sensitive to the presence of Mg^{2+} are accessible to mHHR, and that dynamic transitions localized around the catalytic core occur on the ms timescale.¹⁵² This timescale extends beyond that which has been probed in the current work for the eHHR. Nonetheless, it remains important to address the question of dynamic conformational equilibrium and its role in catalysis in future work.

The present molecular simulation results provide insight into the origin of mutational effects in the eHHR that are not easily derived from available experimental structural data. We report results of MD simulations of the native and mutated eHHR in the reactant state and in an activated precursor state. A key result from this work is that in performing computational mutagenesis simulations of ribozymes, one must consider timescales greater than 30 ns for relaxation of mutant structures and in some instances look beyond the reactant state along the catalytic reaction coordinate in order to reconcile the origin of mutational effects. The present study makes predictions and offers new insights into the understanding of the HHR mechanism as interpreted through mutational data.

5.3. Simulation setup and protocol

MD simulations were set up with protocols identical to that described in Section 4.3. The mutants are generated from the VMD package.¹⁵⁴

6. CONCLUSION

In this chapter, we summarized our progress toward understanding HHR catalysis through a multiscale simulation strategy that employs MD simulations with classical MM and combined QM/MM potentials, using linear-scaling electrostatic methods and specialized MM residues and semi-empirical QM models derived from density-functional calculations of phosphoryl transfer reactions.

So far, simulations collectively paint a picture of HHR catalysis that includes a novel role for a catalytic metal ion: the HHR first folds to form an electrostatic negative pocket to

recruit a threshold occupation of cationic charge, either an Mg^{2+} ion or multiple monovalent ions when Mg^{2+} ions are not present. The position and coordination pattern of these ions are important for formation of active in-line attack conformations. In the case of single Mg^{2+} ion bound in the active site, the Mg^{2+} ion initially stays at the C-site in the reactant state and migrates to the B-site after the nucleophile (C17:O2') is deprotonated. As the reaction proceeds, the Mg^{2+} ion can stabilize the accumulating charge of the leaving group and bind to the general acid (G8:O2'), significantly increasing its ability to act as a general acid catalyst to transfer a proton to the leaving group (C1.1:O5'). Our QM/MM studies demonstrate that the Mg^{2+} ion not only facilitates the protonation of the leaving O5', but it also plays an important role in the final dissociation step of catalysis.

The mutational simulation results are consistent with observed mutational data and suggest that the active site fold is well tuned for the reaction, and most disruptions due to mutations have severe impact on HHR catalysis that can affect different stages of the reaction. Our simulation results propose mechanisms consistent with available experimental evidence, and have motivated new experimental work. The insight gained from these studies provides guiding principles into catalysis of an archetype ribozyme that may ultimately facilitate the design of new RNA based technologies.

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Figure 2.1.

The minimal functional HHR sequence (mHHR) with conserved core region labeled with bold and italic letters. The letter N represents any RNA nucleotide (under the complimentary sequence constraint).

Figure 2.2.

Possible catalytic role of Mg^{2+} in the eHHR. The C-site in the prereactive state involves Mg^{2+} binding at the N7 of G10.1 and the A9 pro-R phosphate oxygen. Activation of the 2'OH may occur through interactions with G12, the proposed general base. Migration from the C-site to a bridging position between A9 and the scissile phosphates occurs in proceeding to the transition state in which the Mg^{2+} acquires additional interaction with the O5' leaving group and the 2'OH of G8, the implicated general acid.

Figure 2.3.

The Mg^{2+} positions from snapshots of simulations with Mg^{2+} initially placed at the C-site position. Snapshots shown are for the initial C-site position (upper left), the reactant state with C17:O2' protonated (upper right), the reactant state with C17:O2' deprotonated (lower left), and the ETS mimic (lower right). The Mg^{2+} position in the LTS mimic is similar to the ETS mimic (not shown). The Mg^{2+} ion migrates from the C-site to the position bridging the A9 and scissile phosphates (i.e., directly coordinated with the A9:O2P and C1.1:O2P) in the transition state mimic simulations and in the reactant state simulation with Mg^{2+} initially placed at the C-site position and with C17:O2' deprotonated, but not in the reactant state simulation with C17:O2' protonated. The distances shown are distances to Mg^{2+} from A9:O2P, C1.1:O2P, and G10.1:N7.

Figure 2.4.

The proposed HHR reaction pathway derived from the free-energy profiles obtained in Section 3.1.

Figure 2.5.

(A) Selected 2D surface in 3D free-energy profile simulations, harmonically restrained along the coarse-grained metal ion-binding coordinate at $d(\text{Mg}, \text{G8:O2}') = 2.5 \text{ \AA}$, where $z_1 = d(\text{C1.1:O5}', \text{P}) - d(\text{P}, \text{G8:O2}')$, $z_2 = d(\text{G8:O2}', \text{G8:Ho2}') - d(\text{G8:Ho2}', \text{C1.1:O5}')$. (B) 2D PMF for Mg^{2+} -binding mode in phosphoryl transfer step, where $z_4 = d(\text{Mg}, \text{O5}') + d(\text{Mg}, \text{G8:O2}')$. (C) 2D PMF for Mg^{2+} -binding mode in general acid step, where $z_5 = d(\text{Mg}, \text{O5}') - d(\text{Mg}, \text{G8:O2}')$. $d(x, y)$ denotes distance between x and y . TS is the acronym of transition state.

Figure 2.6.

Schematic view of the coordination sites in the HHR active site. Left: The coordination pattern of Mg^{2+} in the “C-site” coordinated to G10.1:N7 and A9:O2p. Middle: The coordination pattern of Mg^{2+} in the “B-site” bridging A9:O2p and C1.1:O2p of the scissile phosphate. Right: Coordination sites for Na^+ in the HHR 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:O2p and C1.1:O2p and indirect binding to G10.1:N7 through a water molecule. M2 involves direct binding to C17:O2' and C1.1:O2p. M3 involves direct binding to C17:O2' and is positioned toward the outside of the active site.

Figure 2.7.

Plot of the C17:O2' $\cdots$ P—C1.1:O5' angle versus C17:O2'—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 that the Mg^{2+} was initially placed at the C-site position, while B-Mg means the Mg^{2+} ion was initially placed in the B-site position. 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 2.6: cluster A (light gray) and cluster B (dark gray). The light gray lines at 3.25 Å and 150 degrees indicate the near in-line attack conformation (NAC) region defined by Torres and Bruice.¹³⁵

Figure 2.8.

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 show 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 2.6). The axes are the distances (in Å) to different metal ion coordination sites. The light gray lines indicate the regions where Na^+ ions have distances less than 3.0 Å to both sites indicated by the axes.

Figure 2.9.

The 3D density contour maps (white) 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 HHR is shown in dark gray. The figure shows that, although the Na^+ ions distribute around the RNA phosphate backbone (left panels), the HHR 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.

Figure 2.10. Schematic representations of the HHR active sites and the two potentially important hydrogen bond networks between C3 and G8 and between G5 and C17. All key structural indexes calculated are also labeled.

Figure 2.11.

Schematic representations of the mutants and the hydrogen bonding network of the C3 and G8 positions. k_{rel} is the experimental cleavage constant relative to the wild type. The relevant references are listed in Table 2.13.

Figure 2.12.

Schematic representations of the mutants of the G5 position and the hydrogen bonding network between the G5 and C17 positions. k_{rel} is the experimental cleavage constant relative to the wild type. The relevant references are listed in Table 2.13.

Figure 2.13.

The time series of general acid hydrogen bond indexes, r_{HA} and θ_{HA} , as well as the hydrogen bond distance r_3 (see Figs. 2.10 and 2.11), from wild type and G8A activated precursor state simulations. The distances are in Å and the angles are in degrees. These results indicate that the timescale for equilibration and stable fluctuation in some cases exceed 30 ns (shown by vertical dashed line).

Table 2.1

Simulations performed in Section 2.3

	Mg ²⁺ ion	State	Time (ns)	Potential
RT-B	B-site	Reactant	12	CHARMM
RT-C	C-site	Reactant	12	CHARMM
dRT-C	C-site	Deprotonated Reactant	12	CHARMM
ETS-B	B-site	ETS mimic	12	CHARMM
LTS-B	B-site	LTS mimic	12	CHARMM
ETS-C	C-site	ETS mimic	12	CHARMM
ETS-C	C-site	LTS mimic	12	CHARMM
QM-ETS-B	B-site	ETS	1	QM/MM
QM-LTS-B	B-site	LTS	1	QM/MM

Summary of simulations discussed in Section 2. Simulations for eHHR differ by their initial placement of the Mg²⁺, protonation state of the 2'OH of C17, and progression along the reaction coordinate. A total of eight 12-ns simulations were performed with the all-atom CHARMM27 nucleic acid force field^{103,104} with extension to reactive intermediate models,¹⁰² and two 1-ns simulations using a QM/MM potential using the AM1/d-PhoT Hamiltonian¹⁰⁵ for phosphoryl transfer. Initial Mg²⁺ placement was either in the B-site (bridging position)⁷⁵ coordinated to the A9 and scissile phosphates, or the “C-site” position⁷⁴ coordinating the A9 phosphate and N7 of G10.1.

Table 2.2

Comparison between crystallographic and simulation data for selected heavy-atom distances in the HHR active site with Mg²⁺ initially placed at the bridging position (B-site, Fig. 2.2)

	X-ray	Simulation			
		2GOZ	2OEU	RT-B	ETS-B LTS-B
C1:1:O2P ... A9:O22P	4.33	4.28	3.36 (49)	4.00 (60)	4.01 (70)
Mg ²⁺ ... G8:O2'	3.04	3.14	3.97 (102)	2.24 (13)	3.21 (23)
Mg ²⁺ ... C1:1:O5'	3.84	4.01	4.22 (21)	3.68 (35)	2.09 (50)
G8:O2' ... C1:1:O5'	3.19	3.51	4.29 (77)	4.41 (65)	2.91 (17)
C17:O2' ... C1:1:P	3.18	3.3	3.61 (23)	1.89 (12)	1.76 (40)
G12:N1 ... C17:O2'	3.54	3.26	3.02 (27)	3.14 (28)	2.97 (13)
A9:N6 ... G12:N3	2.63	3.22	3.27 (58)	3.15 (21)	3.17 (21)
A9:N6 ... G12:O2'	3.21	2.98	3.36 (86)	3.01 (18)	2.99 (16)
A9:N7 ... G12:N2	2.9	2.9	3.42 (93)	3.85 (44)	3.66 (33)

Average values are shown with standard deviations in the parentheses (divided by the decimal precision). For simulation summary and abbreviations, see Table 2.1.

2GOZ: The eHHR crystallographic structure at 2.2 Å resolution that was also used in this chapter as the starting structure.⁷³ 2OEU: The eHHR crystallographic structure at 2.0 Å resolution with resolved Mn²⁺ sites and solvent.⁷⁴

Table 2.3

Comparison between crystallographic and simulation data for selected heavy-atom distances in the HHR active site with Mg^{2+} initially placed at the C-site (Fig. 2.2)

	X-ray	Simulation				
		2GOZ	2OEU	RT-C	dRT-C	ETS-C
C1.1:O2P ... A9:O22P	4.33	4.28	5.02 (97)	2.92 (26)	4.02 (6)	3.78 (27)
Mg ²⁺ ... G8:O2'	3.04	3.14	5.92 (28)	4.84 (36)	3.66 (61)	2.9 (85)
Mg ²⁺ ... C1.1:O5'	3.84	4.01	7.01 (79)	4.23 (41)	3.59 (16)	2.09 (6)
G8:O2' ... C1.1:O5'	3.19	3.51	4.54 (59)	3.21 (35)	5.26 (72)	3.66 (73)
C17:O2' ... C1.1:P	3.18	3.3	3.55 (20)	3.57 (16)	1.86 (4)	1.76 (4)
G12:N1 ... C17:O2'	3.54	3.26	4.92 (81)	2.99 (16)	2.95 (14)	3.66 (84)
A9:N6 ... G12:N3	2.63	3.22	3.11 (17)	3.12 (21)	3.14 (18)	3.14 (21)
A9:N6 ... G12:O2'	3.21	2.98	3.07 (19)	3.36 (36)	3.03 (18)	3.09 (22)
A9:N7 ... G12:N2	2.9	2.9	3.00 (14)	3.07 (20)	3.60 (44)	3.06 (23)

Average values are shown with standard deviations in the parentheses (divided by the decimal precision). For simulation summary and abbreviations, see Table 2.1.

2GOZ: The eHHR crystallographic structure at 2.2 Å resolution that was also used in this chapter as the starting structure.⁷³ 2OEU: The eHHR crystallographic structure at 2.0 Å resolution with resolved Mn^{2+} sites and solvent.⁷⁴

Table 2.4
Relative free energies and internuclear distances at various states of RNA self-cleavage in HHR

	Reactant	TS1	Intermediate	TS2	Product
C17:O2'—P	3.50 (04)	1.76 (05)	1.66 (03)	1.67 (03)	1.68 (03)
P—C11:O5'	1.65 (03)	2.11 (05)	4.51 (04)	4.24 (48)	3.63 (23)
G8:O2'—H	0.96 (00)	0.96 (00)	0.96 (00)	1.78 (04)	3.75 (04)
H—C11:O5'	2.57 (51)	4.07 (47)	4.13 (73)	1.03 (03)	1.00 (03)
Mg ²⁺ —C11:O5'	3.99 (18)	3.61 (17)	2.02 (05)	2.83 (86)	4.48 (05)
Mg ²⁺ —G8:O2'	4.56 (18)	4.03 (18)	4.33 (06)	3.38 (86)	2.03 (05)
G	0.0 (4)	24.4 (6)	−6.7 (3)	13.7 (7)	−13.6 (9)

Free energies (*G*) are in kcal/mol, which were extracted from 1D PMF profiles along the minimum free-energy path in the 2D profiles. Average distances (*X* – *Y*) are in Å. Standard deviations are listed in parentheses divided by the decimal precision of the average values.

Table 2.5

Index of simulations performed in Section 4

Abbr.	State of C17:2/OH	Mg ²⁺ position	Simulation (ns)	Analysis (ns)
RT-C-Mg	Neutral reactant	C-site	300	250
RT-B-Mg	Neutral reactant	B-site	300	250
dRT-C-Mg	Deprotonated precursor	C-site	<1	–
dRT-B-Mg	Deprotonated precursor	B-site	300	250
RT-Na	Neutral reactant	Absent	300	250
dRT-Na	Deprotonated precursor	Absent	300	250

Each simulation is given an abbreviation (Abbr.) that is referenced in the text. The simulations differ in the protonation state of the 2′OH nucleophile (State of C17:2/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 the 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 2.6

Coordination patterns of Mg^{2+} and Na^+ in active site

	Cluster	Percentage	R	θ	$\langle \text{NMg}^{2+} \rangle$	$\langle \text{CNMg}^{2+} \rangle$	$\langle \text{NBMg}^{2+} \rangle$	$\langle \text{NNa}^+ \rangle$	$\langle \text{CNNa}^+ \rangle$	$\langle \text{NBNa}^+ \rangle$
RT-C-Mg	A	20.78	3.3	144.05	1	1	0	0.05	1	0
	B	79.22	4.13	122.66	1	1	0	0.03	1	0
RT-B-Mg	A	99.54	3.27	151.1	1	2	1	0	1	0
	B	0.46	4	129.76	1	2	1	0.09	1	0
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	3.64	154.89	1	2	1	0.97	1.01	0.01
dRT-Na	A	23.99	3.5	144.72	–	–	–	2.96	2.29	2.68
	B	76.01	4.3	115.16	–	–	–	2.46	1.69	1.36

Distances and angles are in Å and degrees, respectively. The average values, denoted as $\langle \rangle$, are obtained by averaging 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 2.7

Characterization of the Mg²⁺ coordination in the active site

	<i>R</i>	<i>θ</i>	O—O	A9:O2P	C1.1:O2P	C17:O2'	G8:O2'	G10:N7
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 are in Å and degrees, respectively. Standard deviations (SDs) are listed in parentheses 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). *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'); "O—O" is the distance between A9:O2P and C1.1:O2P; all others are distance between the Mg²⁺ and the indicated ligand site.

Characterization of the active site structure and fluctuations for the C3 and G8 mutants in reactant states

Table 2.8

	WT	U7C	C3U	G8A	C3U/G8A	G8I	G8D	C3U/G8D	C3G/G8C
r_{Nu}	4.07 (0.25)	3.78 (0.43)	3.88 (0.42)	3.2 (0.1)	3.35 (0.36)	3.96 (0.35)	4.1 (0.17)	3.63 (0.45)	4.2 (0.13)
θ_{int}	124.3 (8.6)	133.3 (15.1)	132 (12.8)	155.5 (7.6)	150.2 (14.1)	125.6 (12)	123.7 (8.6)	140.4 (16.3)	122 (4.2)
F	0.25 (0.11)	0.38 (0.2)	0.34 (0.18)	0.68 (0.09)	0.6 (0.19)	0.29 (0.15)	0.23 (0.06)	0.46 (0.22)	0.21 (0.03)
d_0	3.97 (0.39)	4.27 (0.63)	6.64 (1.59)	4.26 (0.34)	4.12 (0.45)	4.17 (0.57)	5.86 (1.65)	4.68 (0.63)	4.26 (0.44)
r_{HB}	2.07 (0.25)	2.1 (0.28)	2.88 (0.85)	2 (0.15)	2.44 (0.57)	2.05 (0.26)	2.54 (1.00)	2.11 (0.37)	2.5 (0.72)
θ_{HB}	152.7 (13.4)	153.6 (13.4)	136.9 (22.5)	163.9 (8.5)	150 (15.7)	155.7 (12.1)	149.5 (18.4)	158.8 (12.8)	145.2 (19)
%	98	97	52	100	82	98	77	95	76
%	62	64	34	93	51	71	55	78	46
r_{HA}	2.75 (0.45)	3.41 (1.02)	5.88 (2.4)	2.97 (0.34)	3.61 (0.6)	2.85 (0.71)	5.74 (1.81)	4.3 (1.38)	2.84 (0.5)
θ_{HA}	115.7 (18.6)	107.4 (36.3)	68.9 (47.5)	118.1 (14.9)	136.7 (23.8)	115.9 (26.2)	58.3 (38.3)	83.7 (48.7)	108.5 (16.2)
%	28	19	19	33	6	32	5	15	17
%	4	2	10	1	1	7	0	1	2
r_{NN}	2.98 (0.09)	2.96 (0.09)	3.65 (0.22)	5.39 (0.57)	2.94 (0.14)	2.96 (0.12)	3.69 (0.28)	3.1 (0.18)	2.93 (0.09)
r_1	2.01 (0.17)	2.02 (0.18)	–	–	–	–	2.58 (0.74)	2.32 (0.4)	1.9 (0.13)
θ_1	162.9 (9)	163.2 (8.8)	–	–	–	–	156.7 (15.4)	157 (13.4)	163.5 (8.8)
r_2	2.01 (0.1)	2 (0.1)	2.17 (0.27)	–	1.97 (0.16)	1.99 (0.13)	2.30 (0.67)	2.14 (0.22)	1.98 (0.11)
θ_2	162.1 (8.7)	161 (8.8)	156.4 (12.8)	–	161.9 (9.8)	163.1 (8.4)	151.3 (17.0)	160.7 (11.6)	158.9 (11.8)
r_3	1.89 (0.13)	1.88 (0.12)	1.89 (0.13)	2.08 (0.31)	2.1 (0.22)	2.01 (0.21)	–	1.91 (0.14)	2.05 (0.2)
θ_3	163.5 (8.8)	164.4 (8.3)	162.1 (9.5)	149.3 (14.8)	162.7 (9.6)	163.8 (8.9)	–	155.9 (10.4)	161.3 (10.2)

This table lists key structural indexes fluctuations for the C3 and G8 mutants, along with the control mutant U7C in reactant states. Data analysis was performed over the last 65 ns of each simulation with a 10 ps sampling frequency. Distance and angles (Fig. 2.10) are in Å and degrees, respectively. SDs are listed in parentheses. Boldface font is used to highlight key quantities that are significantly altered with respect to the wild-type (WT) simulation upon mutation and that are discussed in the text. F is the in-line fitness index.¹⁵⁰ The r_{NN} distance is between nucleobases in the 3 and 8 positions. r_{HB} and θ_{HB} are the hydrogen bond length and angle for the general base step; defined by G12:NI—C17:HO2'—C17:O2'. r_{HA} and θ_{HA} are the hydrogen bond length and angle for the general acid step; defined by C1.1:O5'—G8:HO2'—G8:O2'. The hydrogen bond contact percentage for the above entries, defined as the percentage of the snapshots in which r 3.0 Å and θ 120°. The hydrogen bond contact percentage for the above entries, defined as the percentage of the snapshots in which r 2.5 Å and θ 150°.

Characterization of the active site structure and fluctuations for the C3 and G8 mutants in activated precursor states

Table 2.9

	d-WT	d-U7C	d-C3U	d-G8A	d-C3U/G8A	d-G8I	d-G8D	d-C3U/G8D	d-C3G/G8C
r_{Nu}	3.59 (0.17)	3.64 (0.17)	3.65 (0.16)	3.73 (0.2)	3.6 (0.16)	3.69 (0.23)	3.64 (0.21)	3.34 (0.13)	3.61 (0.17)
θ_{int}	156.8 (7.9)	153.6 (8.7)	154.9 (7.5)	148.1 (8.9)	155.7 (7.6)	149 (12.7)	152.5 (10.4)	164.1 (7.4)	155.4 (8.2)
F	0.49 (0.09)	0.46 (0.09)	0.46 (0.08)	0.41 (0.1)	0.48 (0.08)	0.43 (0.12)	0.46 (0.11)	0.65 (0.1)	0.48 (0.09)
d_0	2.94 (0.13)	2.93 (0.12)	2.93 (0.13)	2.95 (0.12)	2.93 (0.12)	2.93 (0.13)	3 (0.13)	2.97 (0.13)	2.95 (0.13)
r_{HB}	2.03 (0.37)	1.94 (0.14)	1.93 (0.14)	1.93 (0.2)	1.95 (0.14)	1.95 (0.18)	1.91 (0.13)	1.86 (0.1)	1.96 (0.15)
θ_{HB}	154.6 (11.9)	155.8 (9.4)	155.6 (9.4)	158.4 (9.2)	155.5 (9.3)	157.2 (9.8)	158.1 (9.3)	161.2 (8.4)	156 (9.2)
%	95	100	100	99	100	100	100	100	100
%	72	73	73	82	74	77	81	91	75
r_{HA}	2.61 (0.77)	2.5 (0.79)	2.63 (0.76)	4.13 (0.63)	2.6 (0.9)	2.44 (0.52)	2.46 (0.44)	3.53 (0.74)	2.42 (0.57)
θ_{HA}	131.8 (34.5)	135.5 (35.2)	122.3 (34.3)	82.6 (21.4)	130.5 (38)	137.3 (27.7)	139.6 (24.6)	117.2 (31.2)	138 (29.3)
%	69	75	57	1	69	74	81	20	77
%	35	44	24	0	41	37	34	1	41
r_{NN}	2.96 (0.1)	2.96 (0.09)	3.78 (0.21)	6.68 (0.42)	3.04 (0.15)	2.92 (0.11)	3.57 (0.19)	3.03 (0.12)	2.97 (0.09)
r_l	1.92 (0.15)	2.02 (0.17)	–	–	–	–	1.98 (0.11)	2.23 (0.28)	1.9 (0.13)
θ_l	160.9 (10)	163.8 (8.5)	–	–	–	–	163.1 (9.1)	157.9 (13.1)	163.7 (8.3)
r_2	1.98 (0.1)	1.99 (0.1)	1.91 (0.15)	–	2.08 (0.17)	1.94 (0.11)	2.06 (0.16)	2.06 (0.14)	2.01 (0.1)
θ_2	163.2 (8.3)	162.3 (8.8)	160.6 (10.7)	–	160.8 (10.1)	163.9 (8.3)	160.7 (10.8)	163.1 (9.8)	160.1 (8.6)
r_3	2 (0.19)	1.88 (0.11)	1.96 (0.17)	2.77 (0.4)	1.96 (0.17)	2.13 (0.24)	–	1.9 (0.13)	1.94 (0.15)
θ_3	163.7 (9)	164 (8.5)	161.1 (9.7)	106.8 (11)	160.3 (10.5)	162.8 (9.6)	–	156.1 (10.4)	162.2 (9.9)

This table lists key structural indexes fluctuations for the C3 and G8 mutants, along with the control mutant U7C in activated precursor states. Data analysis was performed over the last 65 ns of each simulation with a 10 ps sampling frequency. Distance and angles (Fig. 2.10) are in Å and degrees, respectively. SDs are listed in parentheses. Boldface font is used to highlight key quantities that are significantly altered with respect to the WT simulation upon mutation and that are discussed in the text. F is the in-line fitness index.¹⁵⁰ The r_{NN} distance between nucleobases in the 3 and 8 positions. r_{HB} and θ_{HB} are the hydrogen bond length and angle for the general base step; defined by G12: N1—C17:HO2'—C17:O2'. r_{HA} and θ_{HA} are the hydrogen bond length and angle for the general acid step; defined by C1.1:O5'—G8:HO2'—G8:O2'. The hydrogen bond contact percentage for the above entries, defined as the percentage of the snapshots in which $r < 3.0$ Å and $\theta < 120^\circ$. The hydrogen bond contact percentage for the above entries, defined as the percentage of the snapshots in which $r < 2.5$ Å and $\theta < 150^\circ$.

Table 2.10
Characterization of the active site structure and fluctuations for the G5 mutants in reactant states

	WT	U7C	G5I	G5A	G5D
r_{Nu}	4.07 (0.25)	3.78 (0.43)	4.18 (0.14)	3.84 (0.46)	3.94 (0.39)
θ_{ul}	124.3 (8.6)	133.3 (15.1)	118.3 (9.2)	129.8 (15.2)	128.1 (14.1)
F	0.25 (0.11)	0.38 (0.2)	0.2 (0.04)	0.35 (0.19)	0.31 (0.17)
d_0	3.97 (0.39)	4.27 (0.63)	4.64 (0.56)	4.95 (0.8)	5.2 (0.43)
r_{HB}	2.07 (0.25)	2.1 (0.28)	2.22 (0.76)	2.08 (0.33)	2.31 (0.64)
θ_{HB}	152.7 (13.4)	153.6 (13.4)	155.5 (14.4)	156.9 (12.7)	149.5 (18)
%	98	97	91	97	84
%	62	64	72	75	56
r_{HA}	2.75 (0.45)	3.41 (1.02)	5.1 (1.1)	3.48 (1.35)	5.22 (0.63)
θ_{HA}	115.7 (18.6)	107.4 (36.3)	40 (38)	93.8 (45.7)	43.9 (25.7)
%	28	19	5	33	1
%	4	2	1	11	0
r_4	2.11 (0.21)	2.17 (0.27)	4.99 (0.64)	2.04 (0.28)	2.18 (0.58)
θ_4	153.0 (12.9)	154.2 (12.5)	159.1 (10.7)	159.5 (11.0)	155.0 (13.4)
r_5	2.98 (0.58)	2.77 (0.67)	–	–	3.13 (0.96)
θ_5	119.1 (16.8)	130.2 (20.2)	–	–	129.4 (31.3)
r_6	2.08 (0.22)	2.07 (0.19)	–	–	2.31 (0.79)
θ_6	153.4 (10.9)	150.1 (11.3)	–	–	149.4 (16.0)

This table lists key structural indexes fluctuations for the G5 mutants, along with the control mutant U7C in reactant states. Data analysis was performed over the last 65 ns of each simulation with a 10 ps sampling frequency. Distance and angles (Fig. 2.10) are in Å and degrees, respectively. SDs are listed in parentheses. Boldface font is used to highlight key quantities that are significantly altered with respect to the WT simulation upon mutation and that are discussed in the text. F is the in-line fitness index.¹⁵⁰ r_{HB} and θ_{HB} are the hydrogen bond length and angle for the general base step; defined by G12:N1—C17:HO2'—C17:O2'. r_{HA} and θ_{HA} are the hydrogen bond length and angle for the general acid step; defined by C1.1:O5'—G8: HO2'—G8:O2'. The hydrogenbond contact percentage for the above entries, defined as the percentage of the snapshots in which r 3.0 Å and θ 120°. The hydrogen bond contact percentage for the above entries, defined as the percentage of the snapshots in which r 2.5 Å and θ 150°.

Table 2.11
Characterization of the active site structure and fluctuations for the G5 mutants in activated precursor states

	d-WT	d-U7C	d-G5I	d-G5A	d-G5D
r_{Nu}	3.59 (0.17)	3.64 (0.17)	3.63 (0.15)	4.15 (0.11)	3.58 (0.24)
θ_{ul}	156.8 (7.9)	153.6 (8.7)	156.5 (7.6)	128.6 (5.8)	139.7 (9.8)
F	0.49 (0.09)	0.46 (0.09)	0.47 (0.08)	0.24 (0.03)	0.43 (0.11)
d_0	2.94 (0.13)	2.93 (0.12)	3 (0.13)	2.9 (0.12)	2.88 (0.12)
r_{HB}	2.03 (0.37)	1.94 (0.14)	1.93 (0.13)	1.92 (0.15)	2.62 (0.96)
θ_{HB}	154.6 (11.9)	155.8 (9.4)	155.2 (9.4)	153.6 (8.9)	152.5 (11.9)
%	95	100	100	100	79
%	72	73	72	67	52
r_{HA}	2.61 (0.77)	2.5 (0.79)	3.6 (0.3)	3.67 (1.09)	2.66 (0.6)
θ_{HA}	131.8 (34.5)	135.5 (35.2)	62.2 (11.4)	74.2 (55.6)	128.1 (34)
%	69	75	0	30	62
%	35	44	0	12	24
r_4	1.97 (0.14)	1.97 (0.15)	1.99 (0.16)	2.11 (0.30)	2.66 (0.97)
θ_4	160.0 (10.5)	159.3 (9.8)	158.1 (10.2)	148.1 (13.7)	155.4 (14.8)
r_5	2.56 (0.46)	2.56 (0.49)	–	–	2.91 (0.87)
θ_5	135.3 (12.7)	135.6 (13.4)	–	–	130.7 (18.0)
r_6	2.12 (0.20)	2.09 (0.17)	–	–	2.09 (0.21)
θ_6	148.8 (10.5)	149.9 (10.8)	–	–	150.2 (13.0)

This table lists key structural indexes fluctuations for the G5 mutants, along with the control mutant U7C in activated precursor states. Data analysis was performed over the last 65 ns of each simulation with a 10 ps sampling frequency. Distance and angles (Fig. 2.10) are in Å and degrees, respectively. SDs are listed in parentheses. Boldface font is used to highlight key quantities that are significantly altered with respect to the WT simulation upon mutation and that are discussed in the text. F is the in-line fitness index. ¹⁵⁰ r_{HB} and θ_{HB} are the hydrogen bond length and angle for the general base step; defined by G12: N1—C17:HO2'—C17:O2'. r_{HA} and θ_{HA} are the hydrogen bond length and angle for the general acid step; defined by C1.1:O5'—G8:HO2'—G8:O2'. The hydrogen bond contact percentage for the above entries, defined as the percentage of the snapshots in which r 3.0 Å and θ 120°. The hydrogen bond contact percentage for the above entries, defined as the percentage of the snapshots in which r 2.5 Å and θ 150°.

Table 2.12

Structural indexes characterizing the relative positions between C1.1 and G8

	WT	U7C	G8I	C3U/G8A	C3U/G8D	C3G/G8C
r_7	4.17 (0.38)	4.15 (0.35)	4.19 (0.36)	4.70 (0.69)	4.89 (0.49)	4.24 (0.33)
θ_7	110.2 (11.3)	109.3 (9.6)	111.6 (9.8)	116.8 (13.1)	123.7 (9.0)	111.1 (8.1)
r_8	3.82 (0.34)	3.84 (0.37)	3.86 (0.36)	4.06 (0.45)	4.27 (0.50)	4.11 (0.43)
θ_8	47.6 (8.5)	45.4 (8.3)	43.8 (7.3)	61.1 (20.8)	61.4 (11.9)	32.5 (9.0)
	d-WT	d-U7C	d-G8I	d-C3U/G8A	d-C3U/G8D	d-C3G/G8C
r_7	4.04 (0.25)	3.98 (0.25)	4.13 (0.34)	4.00 (0.26)	4.40 (0.36)	4.23 (0.32)
θ_7	111.6 (7.1)	105.7 (7.9)	111.8 (8.7)	108.8 (7.9)	108.6 (10.5)	115.1 (9.1)
r_8	3.76 (0.31)	3.62 (0.29)	3.65 (0.34)	3.51 (0.27)	4.13 (0.38)	3.56 (0.27)
θ_8	49.4 (6.6)	47.6 (6.1)	54.1 (7.3)	51.2 (6.9)	54.0 (7.0)	55.6 (7.3)

This table lists key structural indexes to characterize base stacking between G8 and C1.1 for different mutants. Data analysis was performed over the last 65 ns of each simulation with a 10 ps sampling frequency. Distance and angles (Fig. 2.10) are in Å and degrees, respectively. r_7 is $\theta(\text{C1.1:N1, G8:N9})$; θ_7 is $\theta(\text{C1.1:C2, C1.1:N1, G8:N9})$; r_8 is $\theta(\text{C1.1:O4', G8:C1'})$; and the torsion angle θ_8 is $\theta(\text{C1.1:C1', C1.1: O4', G8:C1', G8:N9})$. SDs are listed in parentheses. Boldface font is used to highlight key quantities that are significantly altered with respect to the WT simulation upon mutation and that are discussed in the text. The notation “d-” denotes the activated precursor state simulations having the C17:O2' deprotonated. When G8 is mutated to a C, G8:N9 is replaced by C8:N1.

Table 2.13
Comparison of experimental evidence and simulation results for mutants studied

	U7C	C3U	G8A	C3U/G8A	G8I	G8D	C3U/G8D	C3G/G8C	G5I	G5A	G5D
Simulation											
Active site integrity	O	XX	O	O	O	XX	X	O	X	X	X
General base HB	O	XX	O	X	O	X	O	X	O	O	X
In-line angle	O	O	O	O	O	O	O	O	O	XX	X
General acid HB	O	O	XX	O	O	O	X	O	XX	X	O
Experimental k_{rel}											
Ref. [94]						0.68					
Ref. [147]						1.1					
Ref. [148]						0.0003					
Ref. [83]						<0.02					
Ref. [96]						<0.004					
Ref. [149]						0.012					
Ref. [73]											
Ref. [67]						~0.5					

This table summarizes the mutational effects on different stages of HHR reaction, derived from simulations (upper rows), and the relative experimental cleavage reaction constants (lower rows, relative to the WD). For simulation entries, “O” refers to benign effect, “X” mild deleterious effect; “XX” severe deleterious effect. For experimental entries, “nd” means “no catalysis activity detected.” The experimental rate constants are from various HHR sequences and may or may not be the same as the sequence in the simulations.