Evidence for a hydroxide ion bridging two magnesium ions at the active site of the hammerhead ribozyme.
Evidence for a hydroxide ion bridging two magnesium ions at the active site of the hammerhead ribozyme

Thomas Hermann, Pascal Auffinger, William G. Scott and Eric Westhof

Institut de Biologie Moléculaire et Cellulaire du CNRS, 15 rue René Descartes, F-67084 Strasbourg, France and Institute of Chemistry, Indiana University, Bloomington, IN, USA

Received May 30, 1997; Revised and Accepted July 10, 1997

ABSTRACT

In the presence of magnesium ions, cleavage by the hammerhead ribozyme RNA at a specific residue leads to 2'-cyclic phosphate and 5'-OH extremities. In the cleavage reaction an activated ribose 2'-hydroxyl group attacks its attached 3'-phosphate. Molecular dynamics simulations of the crystal structure of the hammerhead ribozyme, obtained after flash-freezing of crystals under conditions where the ribozyme is active, provide evidence that a µ-bridging OH–ion is located between two Mg2+ ions close to the cleavable phosphate. Constrained simulations show further that a flip from the C3'-endo to the C2'-endo conformation of the ribose at the cleavable phosphate brings the 2'-hydroxyl in proximity to both the attacked phosphorous atom and the µ-bridging OH–ion. Thus, the simulations lead to a detailed new insight into the mechanism of hammerhead ribozyme cleavage where a µ-hydroxo bridged magnesium cluster, located on the deep groove side, provides an OH–ion that is able to activate the 2'-hydroxyl nucleophile after a minor and localized conformational change in the RNA.

INTRODUCTION

The hammerhead ribozyme is a catalytically active RNA that cleaves a phosphodiester bond within its own backbone (1). The hammerhead RNA comprises three stems connected by single-stranded regions that contain conserved bases that are required for ribozyme activity (Fig. 1a) (2). Self-cleavage of the hammerhead RNA depends on divalent cations such as Mg2+. There is evidence (3) that a hydroxide ion bound to a divalent cation abstracts the proton from the cleavage-site 2'-hydroxyl, which then attacks the adjacent 3'-phosphate (Fig. 1b and c) (4,5). A second divalent cation may be necessary for stabilizing the pentacoordinated phosphate transition state or, as was suggested recently (3,6), one metal ion may provide both functions. The positions of five Mg2+ ions bound to the hammerhead RNA were determined by X-ray crystallography (Fig. 1a) (6,7). One of the Mg2+ (site 6) was found bound to the cleavage-site phosphate in a hammerhead ribozyme captured by flash-freezing crystals after soaking with Mg2+ at pH 8.5, conditions under which the ribozyme cleaves in the crystal at room temperature (6). This phosphate-bound Mg2+ ion at site 6 is thus considered part of the active site of the ribozyme (6). A second Mg2+ (site 1) is located 4.25 Å apart from the site 6 cation (Fig. 1a) (6). In the crystal structure of the hammerhead RNA the cleavage-site 2'-hydroxyl is not in a favourable position for the attack at the adjacent phosphate. A conformational change in the hammerhead RNA during the cleavage reaction was therefore proposed (6–9). However, the nature and extent of the required conformational changes are unknown.

The catalytic mechanism for hammerhead cleavage still contains controversial components (reviewed in 10). Based on several experiments (11,12), a double-metal-ion mechanism has been put forward in which the metal ions bind directly to the attacking 2'-OH and leaving 5'-Oxygen, without direct coordination to the pro-Rp oxygens of the cleavable phosphate (13, K. Taira, private communication). Our working hypothesis here was to analyze the crystal structure of a catalytic hammerhead intermediate obtained after flash-freezing in presence of Mg2+ ions at pH 8.5 (6). The crystal structure shows two Mg2+ ions bound in the deep groove with one ion directly bound to the pro-Rp oxygen of the cleavable phosphate (Fig. 1d).

We have used molecular dynamics (MD) simulations to investigate the dynamical flexibility of the hammerhead RNA. MD calculations, using an explicit solvent model along with an efficient treatment of the long-ranging electrostatic interactions by the Ewald summation method (14), have recently been demonstrated to be successful for reliable simulations of RNA molecules (15,16). The calculations were done within the AMBER4.1 force field (17) using the previously adjusted and validated parameters (18). It was the aim of this work to examine how dynamically occurring conformational changes in the hammerhead RNA could be integrated into a consistent mechanistic model for hammerhead ribozyme cleavage. We appreciate the fact that the halftime of the cleavage reaction is in the minute timescale, while the MD simulations cover the picosecond range, and we thus do not pretend to simulate the catalytic reaction in real-time. The simulation of a chemical cleavage reaction itself within a complex structural framework such as the hammerhead RNA is not feasible with currently available computational methods. However, it was our aim to demonstrate that careful MD simulations based on the freeze-trapped crystal structure of an active hammerhead ribozyme are helpful for defining pathways.
Figure 1. Three-dimensional structure of the hammerhead RNA as determined by crystal structure analysis after flash-freezing at pH 8.5 (6). (a) The positions of four Mg$^{2+}$ ions (1, 3, 4, 6) in proximity to the cleavable phosphate (gold) are shown. Two sites were omitted, namely the ion at site 2 in stem II which is 15 Å apart from the active site and site 5 that binds Mn$^{2+}$ but not Mg$^{2+}$ (6). (b and c) General mechanism previously proposed for hammerhead cleavage (3–5). Hydroxide coordinated to a Mg$^{2+}$ ion that is bound to the cleavable phosphate activates the cleavage-site 2′-hydroxyl group, which then attacks the adjacent 3′-phosphate (b). The cleavage gives rise to a 2′,3′-cyclic phosphate and a 5′-hydroxyl end (c). (d) Stereoview of a snapshot taken after 400 ps of MD simulation illustrating the hydration pattern of Mg$^{2+}$ ions at sites 1, 3 and 6. Shown are nucleotides G$_{2.2}$–G$_{8}$ of the connection between helices I and II and C$_{17}$–C$_{1.2}$ in the cleavable strand. Mg$^{2+}$ (6) is bound to the pro-Rp phosphate oxygen of A$_{1.1}$. The µ-bridging OH$^-$ ion is shown in ball-and-stick representation between the two metals. Water molecules in the first coordination sphere around the Mg$^{2+}$ ions are depicted as red spheres. Hydrogen bonds of metal coordinated water molecules stable during the simulations are found to N7 of A$_{1.1}$, to phosphate oxygens of U$_7$ (pro-Rp and O5′) and G$_8$ (pro-Sp), and to O4 of U$_{2.1}$. For clarity, the water molecules solvating the system are not shown.

around the equilibrium conformation which could lead towards an activated transition state where the proximity of the reactive groups would allow the cleavage reaction to proceed.

MATERIALS AND METHODS

The AMBER4.1 package (17) was used for MD simulations. RNA atomic coordinates were from a previously published crystal structure analysis of an active hammerhead ribozyme freeze-trapped at pH 8.5 (6). Parameters for Mg$^{2+}$ ions were from Åqvist (19), those for OH$^-$ from Lee et al. (20). The RNA was placed in a rectangular box of SPC/E water (21) containing ~6300 solvent molecules. Fifty Na$^+$ and 18 Cl$^-$ ions, consistent with a concentration of 380 mM Na$^+$ and 140 mM Cl$^-$, were placed according to the electrostatic potential around the solute such that no ion was closer than 4.5 Å to any solute atom.

The simulations were run with a time step of 2 fs at a constant temperature of 298 K and a constant pressure of 1 atm. The SHAKE algorithm (22) was used to constrain the X-H bond lengths. Van der Waals interactions were truncated at 9.0 Å, while not cut-off was applied on the electrostatic term. The electrostatic interactions were calculated with the Particle Mesh Ewald method (23) with a charge grid spacing close to 1.0 Å. An elaborate equilibration protocol was refined from protocols used in our preceding work (15,16) to allow for a careful accommodation of the water structure around the RNA and Mg$^{2+}$ ions. Three ps of water equilibration at 298 K with all ions and RNA fixed were followed by a restart at 10 K and heating to 298 K in steps of 50 K with 2 ps at each temperature where water, Na$^+$ and Cl$^-$ ions were allowed to move. After 10 ps at 298 K a restart at 10 K was performed while the RNA non-hydrogen atoms were constrained with 20 kcal and Mg$^{2+}$ ions with 10 kcal to their positions in the crystal structure. The system was heated to 298 K in steps of 50 K with 5 ps at each temperature. Subsequently the constraint on the Mg$^{2+}$ ions was gradually removed during 40 ps simulation at
the crystal is stabilized by the packing of two hammerhead molecules which stack their stem blunt ends against each other leading to a pseudocontinuous helical packing scheme (6). In the crystal structure of a different RNA/DNA hammerhead molecule this contact between stem ends is absent and, consequently, stems I and II are tilted (8). Similarly, in our simulations the absence of crystal contacts lead to tilting of the stems. However, no base-pair breaking was observed in the stem regions.

In a first set of simulations we observed that the Mg\(^{2+}\) ion at site 1 was not stable at the position of the crystal structure (Fig. 2a) despite the fact that this ion is the most tightly bound one, as suggested by its appearance in the early electron density maps. When the positional constraints on the Mg\(^{2+}\) ions were removed during the equilibration procedure, the cation at site 1 moved rapidly away from its original location in proximity to the Mg\(^{2+}\) ion at site 6 while the latter stayed at its original position bound to the phosphate of A\(_{1.1}\) during the simulation (bottom). (c) Distances between the Mg\(^{2+}\) ions at sites 3 and 4 and atoms of the RNA. Mg\(^{2+}\)(3) remained stably bound to the RNA (left two panels), while Mg\(^{2+}\)(4) dissociated after 200 ps (right).

Interactions of Mg\(^{2+}\)(3) with the RNA are mediated via water bridges (Fig. 1d). Rearrangement of water bridges between Mg\(^{2+}\)(3) and the G\(_{8}\) phosphate at 120 ps leads to a shifting of the distance between this ion and the pro-RP oxygen of G\(_{8}\). The resulting hydration pattern remains stable until the end of the simulation. The position of Mg\(^{2+}\)(3), relative to the RNA, is not affected by this rearrangement as indicated by the distances to three reference atoms [O4 of U\(_{2.1}\), N6 of A\(_{1.1}\) and Mg\(^{2+}\)(6)]. O4 of U\(_{2.1}\) forms a contact to Mg\(^{2+}\)(3) via a water bridge while A\(_{1.1}\) is the nucleotide stacking on top of C\(_{17}\) both of which are linked by the cleavable phosphate (Fig. 1d).

RESULTS AND DISCUSSION

Evidence for a bridging OH\(^{-}\) ion between two Mg\(^{2+}\) ions at the hammerhead active site

We have gathered evidence from MD simulations of the crystalized hammerhead RNA that at least one bridging OH\(^{-}\) is required to stabilize the two Mg\(^{2+}\) ions at sites 6 and 1. We performed simulations on the fully hydrated and neutralized hammerhead RNA in presence of the Mg\(^{2+}\) ions located at the sites observed in the pH 8.5 crystal structure (Fig. 1a) (6). Special care was taken when equilibrating the system of RNA, ions and solvent. An elaborate heating protocol was employed to ensure relaxation of the RNA and at the same time a favourable arrangement of solvent molecules around both RNA and Mg\(^{2+}\). First, solvent water was allowed to equilibrate, while RNA and ions were fixed followed by a simulation phase where the constraints on the ions and the RNA were successively removed (see Materials and Methods). The quality of the trajectories obtained in our MD calculations is attested by the fact that the hammerhead RNA retained its structural integrity in all simulations. The RMS deviation was in the range of 2.0 Å after 500 ps of calculation. Stems I and II, roughly parallel in the starting crystal structure, tilted towards each other during the simulations. The parallel stem geometry in

298 K. Finally, after a restart at 10 K, the system was again heated to 298 K in 5 ps steps of 50 K without applying any constraints.
In the subsequent simulations of the hammerhead RNA the OH− ion was constrained during the first phase of equilibration as were the Mg2+ ions. Yet, after complete removal of the constraints, the complex of the two Mg2+ ions at sites 1 and 6 along with the bridging OH− maintained its geometry over the whole 500 ps simulation period (Fig. 2a). Thus, the OH− successfully moderates the electrostatic repulsion between the two proximate Mg2+ ions. In the presence of the bridging OH− ion, the Mg2+ ion at site 1 remained very stable at its crystal structure position in line with the observation that this cation is always seen in X-ray analysis. Despite the fact that site 1 is adjacent to a non-conserved region of the hammerhead RNA, specific binding of cations could be favoured independently of the sequence because this site is located in the deep groove of stem I which displays the lowest electrostatic potential around an RNA helix (24).

The metal ion at site 1 was not stabilized when instead of an OH− ion either one or two water molecules were placed bridging the Mg2+ ions at sites 1 and 6 (data not shown). In all such instances, the repulsion between the two adjacent metal ions rapidly lead to breaking of the water bridges, expelling the Mg2+ ion at site 1 from its original position. However, the Mg2+ ion at site 1 was not expelled from its original position in simulations where the ion at site 6 was not present (Fig. 2b). This corresponds to the situation observed in crystals of the hammerhead RNA grown at pH 5.0 where a Mg2+ ion is bound at site 1 but not at site 6 which is occupied by a water molecule (6). In agreement with this finding, we observe in such simulations a water molecule linking both the pro-Rp anionic oxygen of the cleavable phosphate and a water molecule close to the cleavable phosphate of site 1. In the second state, corresponding to the crystal at pH 8.5, two Mg2+ ions are bound close to the cleavable phosphate at sites 1 and 6 with an OH− ion coordinated to both Mg2+ ions. The OH− ion required for stabilizing the dimer of Mg2+ ions is bridging these cations similarly to the situation in hydroxo complexes formed by olation of metal hydroxides (25,26). In line with the assumption of a µ-bridging OH− ion is the observation that in X-ray analysis some density maps show a water molecule close to the cleavable phosphate at sites 1 and 6 with an OH− ion coordinated to both Mg2+ ions. The OH− ion stabilizing the dimer of Mg2+ ions is bridging these cations similarly to the situation in µ-hydroxo complexes formed by olation of metal hydroxides (25,26).

During the different simulations the Mg2+ ion at site 3 stayed bound to the RNA while the ion at site 4 dissociated from the RNA (Fig. 2c). The Mg2+ at site 3 stabilizes the turn between helices I and II by interacting with the phosphates of G5 and U7′ and with the side-chain of U2′ (Fig. 1d). The distance between the Mg2+ at site 3 to the ion at site 6, which is bound to the cleavable phosphate, remained in the range found in the crystal structure (8–10 Å) (Fig. 2c). Therefore, it is unlikely that the Mg2+ ion at site 3 is directly involved in the chemistry of the cleavage reaction. Following our simulations we have no clues regarding the Mg2+ ion at site 4, which is bound to G5, a nucleotide essential for catalysis (2), since this ion dissociated in the present simulations.

**Experimental results in line with the proposal of a bridging OH− ion**

While for X-ray crystal structure analysis it is difficult to distinguish between water molecules and hydroxide ions, it is hoped that MD simulations can help to elucidate such details of the solvation structure of metal ions. This could be especially meaningful for the hammerhead RNA where one of the crystallographically identified Mg2+ ions is only observed after freeze-trapping at high pH (site 6) (6). In the present MD simulations, the binuclear metal center was not stable, but could be stabilized as a µ-hydroxo-bridged metal complex. Following the prevalent mechanism for catalytic cleavage (6) which involves a metal hydroxyl activating the 2′-hydroxyl group for nucleophilic attack and where the reaction rate increases as pH is increased (4), it is tempting to suggest that the µ-hydroxo-bridged metal complex is implicated in the chemistry of the ribozyme catalysis. It was proposed (6) that the Mg2+ ion at site 6 provides two basic functions required for ribozyme cleavage. First, by binding of Mg2+ close to the cleavable phosphate a conformational change is induced that is required for in-line attack of the active-site 2′-hydroxyl. Second, the same Mg2+ carries an OH− ion that is necessary for the deprotonation at the cleavage-site 2′-hydroxyl in the base-catalyzed step of the cleavage reaction.

The present simulations provide evidence for the existence of at least two stable states for magnesium binding to the hammerhead RNA. In one state, corresponding to the crystal at pH 5.0, a Mg2+ ion is bound at site 1 and there is a water molecule linking the pro-Rp anionic oxygen of the cleavable phosphate and a water molecule close to the cleavable phosphate at site 1. In the second state, corresponding to the crystal at pH 8.5, two Mg2+ ions are bound close to the cleavable phosphate at sites 1 and 6 with an OH− ion coordinated to both Mg2+ ions. The OH− ion required for stabilizing the dimer of Mg2+ ions is bridging these cations similarly to the situation in µ-hydroxo complexes formed by olation of metal hydroxides (25,26). In line with the assumption of a µ-bridging OH− ion is the observation that in X-ray analysis some density maps show a water molecule close to the cleavable phosphate at sites 1 and 6 with an OH− ion coordinated to both Mg2+ ions. The µ-hydroxo-bridged cluster is clearly superior to a single Mg2+ ion in stabilizing a high local concentration of OH− in proximity of the active site and the activity of OH− ions around the metal cluster may thus be higher.

Similar complexes of two metal ions bridged by OH− were also proposed to be part of the active sites of binuclear metallohydrolases (27). For inorganic pyrophosphatase from Escherichia coli, experimental findings strongly suggest that an OH− ion bridging two Mg2+ ions is involved in catalysis (28). Related situations are found in the crystal structures of mammalian phosphatase-1 and yeast enolase where two adjacent Mg2+ ions are bridged by a carboxylate oxygen (29,30). However, no calculations were performed on such systems. Interestingly, an anionic phosphate oxygen bridges two valent metal ions at the 3′–5′ exonuclease active site of Escherichia coli DNA polymerase I where one metal ion is directly bound to another phosphate oxygen (31). It has been proposed that in the 3′–5′ exonuclease the bridged metal
cluster provides an OH\(^{-}\) ion necessary for catalysis (31). Subsequently, a general mechanism for DNA and RNA polymerases has been set up, where a complex of two Mg\(^{2+}\) ions bridged by an anionic phosphate oxygen stabilizes the transition state in the catalysis (32). X-ray structure analysis revealed this kind of geometry for two Mg\(^{2+}\) ions bridged by an anionic phosphate oxygen at the active site of rat DNA polymerase \(\beta\) (Fig. 3) (33). As in the \(E. coli\) inorganic pyrophosphatase, where fluoride ions are known to inhibit activity (34), probably by competition with an OH\(^{-}\) ion implicated in catalysis (28), the hammerhead ribozyme should be tested for activity in presence of F\(^{-}\) ions. In this respect, it is interesting to note that F\(^{-}\) ions also inhibit splicing in group I introns (35) which are also metalloenzymes (36) and for which the positions of two Mg\(^{2+}\) ions at the catalytic site have been proposed (37).

**Could the bridging OH\(^{-}\) ion initiate hammerhead cleavage?**

In the simulated MD trajectories, where the hammerhead conformations are always close to the crystal structure, the \(\mu\)-hydroxo bridge was never seen in a favourable position for the direct activation of the cleavage-site 2'-hydroxyl. As such, this observation is not surprising since the conformational sampling is done around the equilibrium state, from which input of activation energy is required in order to reach the transition state. Simulations performed at higher temperatures up to 350 K followed by rapid cooling led at times to only slightly shorter distances between the 2'-hydroxyl oxygen atom and the cleavable phosphate (data not shown). However, when a flip of the ribose pucker from C3'-endo to C2'-endo at the C17 residue which holds the cleavable 3'-phosphate was induced during the simulations, the reactive-site 2'-hydroxyl moved in close proximity to both the attacked phosphorous atom and the \(\mu\)-bridging OH\(^{-}\) ion (Fig. 4). At the same time, the 2'-hydroxyl comes within hydrogen bonding distance of two water molecules bound to the Mg\(^{2+}\) ion at site 6 (Fig. 4b). Although the present study does not show evidence for it, the sugar flip could constitute a conformational rate-limiting step. At room temperature, only a few hammerhead molecules could probably enter the thermally-driven conformational change from the inactive C17-C3'-endo to the active C17-C2'-endo pucker. This makes the pucker flip a rare process which is beyond the nanosecond time scale of the present MD simulations. It was speculated earlier that among other conformational changes such a flip of the C17 ribose pucker could be necessary for the hammerhead self-cleavage (9,38).

The Mg\(^{2+}\) ion at site 4 could also participate in the conformational change from ground state to the transition state. In the MD simulations, this ion dissociated from its site in the crystal structure in proximity to the base of G5, a nucleotide essential for catalysis (3). It might be possible that the release of the Mg\(^{2+}\) ion at site 4 is required for facilitating the conformational change. Interestingly, dissociation of Mg\(^{2+}\) ions from the hammerhead RNA upon cleavage is experimentally observed (39).
After the change in sugar pucker from C3′-endo to C2′-endo, we speculate that proton shuffling could occur within the hydration sphere of the bound Mg2+ ions leading to proton abstraction from the 2′-hydroxyl and to proton donation to the 5′-hydroxyl of one cleavage product (Fig. 5) so that the final and initial states are identical, as expected for a catalytic mechanism. Within this proposed scheme, the same Mg2+ ion binds via water molecules to both the attacking and leaving oxygens. This proton shuffling mechanism does not arise from the simulations, although it is compatible with the distances and geometries within the simulated states. In the course of the proton shuffling mechanism, the μ-hydroxo bridge stores transiently a proton, a capacity facilitated by the lower basicity of hydroxide when coordinated to metal ions (40). The moderate basicity of the μ-OH− ion might allow for a favourable equilibrium between both proton transfer steps, first in the deprotonation of a metal-bound water molecule by the μ-OH− ion and secondly in the final deprotonation of the μ-H2O by an OH− ion bound to the metal (Fig. 5).

Protonation of the bridging OH− ion and the suggested ensuing proton shuffling are expected to have much shorter timescales than that of the present simulations, and the instability on the 10 picosecond timescale of the binuclear metal center with a bridging water molecule transiently formed during proton shuffling does not constitute a contradiction. The sluggish rate of the overall catalytic reaction (minute timescale) is probably related to activation energies of the μ-hydroxo-bridged Mg2+ cluster which provides the OH− ion activating the 2′-hydroxyl nucleophile (Fig. 4a). A minimal conformational change in the RNA restricted to a single sugar pucker change from C3′-endo to C2′-endo is sufficient for initiating the cleavage (Fig. 4b).

CONCLUSIONS

The present simulations suggest that, during hammerhead ribozyme catalysis, the 2′-hydroxyl nucleophile which attacks the cleavable phosphate is activated from the deep groove side of stem I where Mg2+ ions 6 and 1 are located in the crystal structure (Fig. 1d). The two-metal-ion mechanism proposed here for hammerhead ribozyme cleavage is defined by the role of the μ-hydroxo-bridged Mg2+ cluster which provides the OH− ion activating the 2′-hydroxyl nucleophile (Fig. 4a). A minimal conformational change in the RNA restricted to a single sugar pucker change from C3′-endo to C2′-endo is sufficient for initiating the cleavage (Fig. 4b).

ACKNOWLEDGEMENTS

W.G.S. thanks S. Lippard for useful discussions. We thank C. Massire for help with the DRAWNA program in preparation of Figure 1a and K. Taira for sending E.W. unpublished work. We are much indebted to Peter Kollman and his group at UCSF for making available to us the latest version of the AMBER MD package used in the present study. T.H. is supported by an EMBO long-term fellowship.

REFERENCES