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Crystallographic Analysis of a Hammerhead Ribozyme Variant and Its Impact on Catalytic Activity

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CRYSTALLOGRAPHIC ANALYSIS OF A HAMMERHEAD RIBOZYME VARIANT AND ITS IMPACT ON CATALYTIC ACTIVITY

A dissertation submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

CHEMISTRY AND BIOCHEMISTRY

by

Eric P. Schultz

March 2013

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ABSTRACT

Eric P. Schultz

Crystallographic Analysis of a Hammerhead Ribozyme Variant and its Impact on Catalytic Activity

Over the past two decades, the hammerhead ribozyme has been extensively studied leading to many new insights regarding the biochemical and structural properties of catalytic RNAs. Despite these insights, the role metal ions in catalysis is still of great debate. We have obtained a 1.55Å crystal structure of a hammerhead ribozyme from *Schistosoma mansoni* in conditions that permit detailed observation of Na$^{+}$ ions binding in the ribozyme’s active site. A Na$^{+}$ ion binds to the N7 of G10.1 and the adjacent A9 phosphate in a manner identical to that previously observed for divalent cations. A second Na$^{+}$ ion binds to the Hoogsteen face of G12, the general base in the hammerhead ribozyme cleavage reaction, thereby potentially dissipating the negative charge of the catalytically active enolate form of the base. We have also obtained a 2.2Å crystal structure of a hammerhead ribozyme variant containing adenine substituted for the general base that displays a structurally perturbed active site. Our results suggest that Na$^{+}$ directly and specifically substitutes for divalent cations in the hammerhead active site. Additionally, we suggest that hammerhead ribozyme substituted with adenine for the general base may compete between two conformational states: an active state in which A12 is situated for deprotonation of the 2’H of C17; and an inactive state in which A12 makes a non-canonical base pair A9, causing a perturbation in the active site, disrupting metal ion binding. These
results permit us to estimate the contribution that structural perturbation has on the catalytic activity of other hammerhead ribozyme variants containing nucleobase substitutions for the general base.
DEDICATION

I dedicate this thesis to my parents, Alan and Margaret Schultz.

The institution of a good education from an early age, along with constant love and support, provided the motivation to set high goals and to finish what I start.

The ability to think freely and pursue what truly makes me happy in life

I owe solely to them.
ACKNOWLEDGEMENTS

I would like to thank my advisor, William G. Scott, for allowing me to work freely and at my own pace. Having an advisor who supports my research and ability to succeed has allowed me to flourish in the lab, only strengthening my scientific drive. Your input and scrutiny were essential for our success.

I would like to thank the chair of my thesis committee, Ted Holman, for supporting me in my efforts, albeit from afar. Being a very reserved professional, I am very lucky to have someone so supportive. It has not been easy span for my graduate class at UCSC, both professionally and emotionally, but your outlook on life seems to consistently validate what we do and why we do it.

I would like to thank professor Seth Rubin, who has been one of my most valuable resources throughout my time at UCSC. Thank you for allowing me to pick your brain and obtain the insight needed for the progression of my projects. You, along with several members of your lab, shared your time and knowledge openly, significantly aiding my research.

I would like to thank my lab mate Michael Anderson for going through each step of training, failure, and success with me. Having a collaborative environment in the lab was essential for us to reach our goals. Your work on the highest-resolution RNA structure was truly profound and something we should be very proud of.

I would like to thank former members of the Scott laboratory, Monika Martick and Monica Lares, for welcoming me into the lab and providing training despite being incredibly busy. Your previous works provided the direction for my projects,
ultimately leading to this thesis. You are both truly great women of science and I wish you the best in all your endeavors.

Because there are too many to name individually, I would like to thank rest of my graduate class. It has not been easy and many of us did not make it through. Despite tragedy, we have all stuck together and always been there for one another. Without your friendships, I cannot say I would be in the same place I am today.

Finally, I must thank Ariann Miller-Rhoads, the love of my life. Without you all these years, I would most certainly not be the man I am today. You have been the light when things were dark, and the reason when I was irrational. You are one of the most caring and giving people I have ever met. Because of you, I forgot about what I was doing and began to focus on why I was doing it, providing me with the determination needed to accomplish my goals.
CHAPTER ONE
The Hammerhead Ribozyme
1.1 Introduction to Ribozyme Structure and Catalysis

Since the discovery of the RNase P \{Guerrier-Takada 1983\} and the Group I intron \{Zaug 1986\} ribozymes, the field of RNA biology has undergone a paradigm shift regarding the role of non-coding RNA in gene expression. The ability for RNA to catalyze specific enzymatic reactions has prompted molecular and structural biologists alike to investigate the biochemical requirements needed to perform such feats, as well as how these reactions fit into the long-accepted dogma of gene expression. By understanding how ribozymes function, one may also gain insights to how life originated. Because RNA can store genetic information as well as catalyze simple reactions, many who subscribe to the “RNA World” hypothesis \{Gesteland 1993\} suggest that RNA could have been the original self-replication molecule. Furthermore, ribozymes can be very valuable as they are uniquely well suited for design as targeted therapeutics towards many types of diseases \{Vaish 1998\}. It seems clear that RNA can adopt complex tertiary structures sufficient to make catalysis possible, but uncertainty remains about how these complex structures provide enzymatic behavior. In order to provide insights on the strategies ribozymes use to achieve their catalytic abilities, researchers need an ideal prototype that can be probed by various experimental techniques. In many ways, the hammerhead ribozyme is the ideal candidate for such experimentation due to its relatively small size compared to RNaseP and the Group I intron. Since its discovery, this relatively simple ribozyme has been subjected to intense experimental scrutiny in an effort to
understand the complex relationship between RNA structure and catalytic activity. 
{Scott 2008}

1.2 The Hammerhead Ribozyme

1.2.1 The Minimal Hammerhead Ribozyme

Hammerhead RNAs are small self-cleaving ribonucleic acids that have a conserved motif found in several viroids and satellite RNAs associated with plant viruses {Prody 1986} {Forster 1987} {Haseloff 1989} {Uhlenbeck 1987}, as well as other species that replicate via a rolling circle mechanism. The catalytically active part of the ribozyme contains a minimal sequence that consists of three base-paired stems flanking a central core of fifteen conserved nucleotides. {Uhlenbeck 1987} {Ruffner 1990} {Sheldon 1989} The conserved central bases (Figure 1A) are mostly invariant and essential for the ribozyme’s catalytic activity. The hammerhead ribozyme is perhaps the best-characterized ribozyme, as its small size, known crystal structure, highly-investigated cleavage chemistry, and biological significance make it ideal for biochemical investigations into the nature of RNA catalysis. Despite extensive analysis of the hammerhead ribozyme’s structure and chemistry {McKay 1996} {Wedekind 1998} {Scott 1999}, many questions remain concerning how the ribozyme’s structure enables its catalytic properties.
Figure 1. Schematic diagrams of the secondary structures of two hammerhead ribozyme constructs.

The minimal hammerhead (A) consists of a conserved, mostly invariant core region that contains the cleavage site, as well as three flanking A-form RNA helices whose sequence is only restricted by the need to maintain base-pairing. The full-length hammerhead (B) contains additional nucleotides as depicted in grey. Although the sequences of the stem II loop (L2) and stem I bulge (B1) are not restricted in any obvious way that is apparent from the RNA sequence, the tertiary interaction that forms between these two regions is critically important and enhances catalysis by approximately three orders of magnitude. {Scott 2008}
1.2.2 Mechanism of Catalysis

The hammerhead ribozyme catalyzes a phosphodiester isomerization reaction that is initiated by the abstraction of the 2’-hydroxyl proton from the 2’-oxygen. The deprotonated 2’-oxygen becomes the attacking nucleophile for an “in-line” or $S_{N2}(P)$-like reaction \{van Tol 1990\} \{Slim 1991\} \{Koizumi 1991\}, however it is not yet known whether the proton is removed before or during the chemical step. Despite the reaction not being bimolecular, it does undergo inversion of configuration subsequent to forming a transition state consisting of a pentacoordinated oxyphosphorane. \{Scott 2008\} In addition, the attacking and leaving group oxygens both occupy the two axial positions in the trigonal bipyramidal transition state like other $S_{N2}(P)$-like reaction mechanisms.

The result of the cleavage reaction is a 5’-product containing 2’,3’-cyclic phosphate terminus, while the 3’-product contains a 5’-hydroxyl terminus. \{Buzayan 1986\} \{Hutchins 1986\} The reaction is therefore reversible, in principal, as the scissile phosphate remains a phosphodiester. The product of the cleavage reaction may thus act as a substrate for hammerhead RNA-mediated ligation requiring neither ATP nor any other exogenous energy sources. Unlike non-enzymatic alkaline cleavage of RNA, the hammerhead ribozyme cleavage reaction is highly sequence specific with a typical turnover rate of one molecule of substrate per molecule of enzyme per minute at pH 7.5 in 10mM Mg$^{2+}$. This represents roughly a 10,000-fold increase in reaction rate over non-enzymatic cleavage. \{Scott 2008\}
1.2.3 Hammerhead Ribozyme Structure

The first hammerhead ribozyme three-dimensional structure was published in 1994 {Pley 1994} and consisted of a 34 nucleotide enzyme strand containing unmodified RNA. The substrate for the ribozyme was an analog consisting of DNA in order to inhibit ribozyme cleavage due to the missing 2’-hydroxyl in the active site that acts as the nucleophile. (Figure 2A) Two subsequent hammerhead ribozyme crystal structures were published: the first with a 2’-OMe replacing the nucleophile at the active site {Scott 1995}; the second with an unmodified nucleotide at the active site {Scott 1996}. These ribozymes both displayed the same tertiary structure for the invariant core nucleotides (Figure 2B) despite having significant differences in their nonessential regions, divalent metal concentrations, and crystal packing schemes.

The hammerhead ribozyme containing all unmodified nucleotides remains catalytically active in the crystalline state, allowing for crystallographic analysis of the ribozyme in multiple catalytic states. {Scott 1996} {Murray 2002} In fact, the hammerhead ribozyme construct designed for growing crystals rather than optimum catalytic activity cleaves faster in the crystalline form than in solution, suggesting that the crystal lattice did more to promote cleavage than disrupt the active structure of the ribozyme. {Murray 2002} Although these structures helped rationalize much of the previously published biochemical data, they also presented some important problems regarding the catalytic strategy of the ribozyme. The greatest of these problems was the scissile phosphate in all structures reported was in a conformation incompatible with an “in-line” $S_N2$-like reaction mechanism. {Scott 2001}
Figure 2. Three crystallographically independent hammerhead ribozymes (A) occupied the asymmetric unit in the first hammerhead crystal structure; one of these is displayed in what has become the conventional orientation (B) such that the RNA enzyme strand (green) and DNA substrate-analogue (cyan) are clearly visible. Two crystallographically independent all-RNA hammerheads occupied the asymmetric unit (C) of the second hammerhead ribozyme crystal structure. One of these (D) is chosen and displayed to facilitate comparison with (B). Here, the shorter strand is (nominally) the enzyme strand and the longer strand (cyan) is the substrate, where a 2′-OMe nucleotide occupies the cleavage site. A subsequent crystal form having but one molecule per asymmetric unit (not shown) is otherwise identical, despite the absence of the 2′-OMe modification. {Scott 2008}
1.2.4 Irreconcilable Data

Although the hammerhead ribozyme appears catalytically active in the crystalline form, many biochemical experiments designed to look at transition-state interactions as well as the chemistry of catalysis provide results that are irreconcilable with crystal structures. For example, the invariant core nucleotides G5, G8, G12, and C3 in the ribozyme do not tolerate even a single exocyclic functional group change without causing a dramatic reduction in catalytic activity {McKay 1996}, yet there are few hydrogen bonds involving the Watson-Crick faces of these nucleotides in any of the crystal structures. As soon as the G8 and G12 residues were identified as the acid and base, respectively, {Han 2005} for acid-base catalysis and that no divalent metal ions were required for catalysis to occur {Murray 1998} {Scott 1999}, it became apparent that the RNA itself must have a direct chemical role in any such chemistry in the active site of the ribozyme. {Scott 2008} NMR characterization of the hammerhead ribozyme cleavage products {Simorre 1997} resulted in a NOE between U4 and U7 suggesting that these residues must approach within 6Å of one another; however, such close approach of these bases does not seem plausible from the crystal structure. Perhaps the most troublesome data comes from soft metal ion rescue experiments {Wang 1999} suggesting that the A9 and scissile phosphate come within 4Å of one another in the transition-state, yet they are 18Å apart in the crystal structure. These structural and biochemical analyses together suggest that a relatively large conformational change is required to reach the transition-state within these constructs.
1.3 The Full-Length Hammerhead Ribozyme

As concern grew over the disagreement between biochemical and structural data, it became apparent that researchers must have been missing something fundamental regarding the general strategy for RNA catalysis. In order to solve this complex biological puzzle, researchers would need to escape the dogmas of molecular biology and look at the hammerhead ribozyme in a more physiological context. {Scott 2008}

The hammerhead ribozyme was first discovered as 370 nucleotide single-stranded genomic satellite RNA, most of which could be truncated without losing catalytic activity. {Forster 1987} {Haseloff 1989} {Uhlenbeck 1987} In 2003, it was finally pointed out that for optimal catalytic turnover, the ribozyme requires the presence of sequences in stems I and II that interact to form tertiary contacts. {De la Pena 2003} {Khvorova 2003} (Figure 1B) This revelation caused the field’s attention to shift towards these extended constructs or “full-length” ribozymes, making crystal structures of hammerhead ribozymes containing these distal tertiary contacts highly valued targets for structural biologists.

The first crystal structure of the full-length hammerhead ribozyme (Figure 3) was solved to 2.2Å in 2006 {Martick 2006}, and appeared to resolve much of the troublesome discrepancies previously reported. Most importantly, the C17 appears to be positioned for in-line attack on the scissile phosphate, and the invariant residues C3, G5, G8, and G12 all appear to make crucial interactions relevant to catalysis. Furthermore, the A9 and scissile phosphate are observed to be 4.3Å apart, which is consistent with the notion that, when modified, could bind a single thiophilic metal
Figure 3. Secondary (A) and tertiary (B) structure of the full-length hammerhead ribozyme. The color-coding of the secondary structure corresponds to that of the tertiary structure. The cleavage-site nucleotide, C-17, is depicted in green in both figures. {Martick 2006}
ion. The crystal structure also reveals how the invariant residues G8 and G12 are positioned in the active site. (Figure 4) The results are consistent with their previously suggested role in acid-base catalysis. {Han 2005} G12 is positioned within hydrogen bonding distance of the 2’-O of C17, the nucleophile in the cleavage reaction, and the ribose of G8 hydrogen bonds to the leaving group 5’-O, while the nucleotide base of G8 forms a Watson-Crick pair with the invariant C3. This configuration suggests that G12 is the general base in the cleavage reaction while G8 may function as the general acid, and idea that is consistent with previously reported biochemical observations {Han 2005}. Additionally, residue G5 hydrogen bonds to the furanose oxygen of C17, helping to position it for in-line attack. As a consequence of the base pair between G8 and C3, residues U4 and U7 are close enough to explain the NOE signal obtained from previous NMR experiments {Simorre 1997}.

The initial crystal structure of the full-length hammerhead ribozyme clearly reconciled all major biochemical and structural data previously reported, but questions still remain regarding how the ribozyme achieves catalysis. Most importantly, the mechanistic role that Mg$^{2+}$ ions might play is still being highly investigated within the field. Whether Mg$^{2+}$ ions are directly involved during the chemical step {Wang 1999}, or if they merely play a structural role {Murry 1998}, has become the main focus of structural and biochemical studies alike. In the past decade, great strides have been made towards elucidating the intricacies of hammerhead ribozyme catalysis and many mechanistic proposals involving the
Figure 4. A three-dimensional model of the full-length hammerhead active site. The 2’O of C17 appears to be approaching the “in-line” coordination (cyan dashed line) required for a $S_N2(P)$-like reaction mechanism. The N1 of residue G12 and the 2’O of residue G8 appear appropriately situated for acid-base catalysis (orange dashed lines). The A9 and scissile C17 phosphates are roughly 4.3Å apart (magenta dashed lines) consistent with soft metal ion rescue data {Wang 1999}. 
participation of Mg$^{2+}$ ions have been suggested {Vogt 2006} {Lee 2008} {Wong 2011}.

1.4 References for Chapter One


34. Lee T. S., Lopez C. S., Giambasu G. M., Martick M., Scott W. G., York D. M.,

CHAPTER TWO
Active-Site Monovalent Cations Revealed in a 1.55Å Resolution Hammerhead Ribozyme Structure
2.1 Introduction

The hammerhead ribozyme is found in satellite RNAs of various plant RNA virus genomes {Prody 1986} {Symons 1997}, in the 3'-UTRs of mammals {Martick 2008}, and within introns of many eukaryotes {GarciaRobles 2012} {Hammann 2012}. The ribozyme consists of a conserved core of about 15 mostly invariant residues {Ruffner 1990} and, for optimal activity, requires the presence of sequences in stems I and II that interact to form tertiary contacts {De la Pena 2003} {Khvorova 2003}. (The optimal form has been termed the “natural” or “full-length” hammerhead.) The hammerhead ribozyme catalyzes an RNA self-cleavage phosphodiester isomerization reaction that involves nucleophilic attack of the C17 2’O upon the adjacent scissile phosphate, producing two RNA product strands.

Perhaps the most substantial of the controversies {Mckay 1996} {Blount 2005} {Nelson 2008} remaining subsequent to elucidation of the full-length hammerhead ribozyme structures {Martick 2006} {Chi 2008} is the mechanistic role that metal ions might play in the chemistry of catalysis. At one extreme, it has been proposed, based on observed cleavage in the presence of very high ionic-strength monovalent cations but in absence of divalent metal ions, that Mg$^{2+}$, when present, plays a purely structural role, and is not a required participant in the chemical mechanism of catalysis {Murray 1998a}. At the other extreme are proposals in which one or more Mg$^{2+}$ ions participate directly in the transition-state of the hammerhead self-cleavage reaction. The first and arguably most important of these hypotheses is one in which a single Mg$^{2+}$ ion is proposed to coordinate directly two
non-bridging phosphate oxygens simultaneously, one belonging to the A9 phosphate, and the other belonging to the scissile phosphate of the cleavage site \{Wang 1999\}. Many other mechanistic proposals involving participation of monovalent and divalent metal cations that reside between these mechanistic extremes have also been suggested \{Vogt 2006\} \{Lee 2008\} \{Wong 2011\} \{Ward 2012\}.

The first crystal structure of the natural, full-length hammerhead ribozyme did not reveal any metal ions bound near the active site \{Martick 2006\}, even while revealing the A9 and scissile phosphates, previously implicated in binding a single metal ion, to be only 4.3Å apart, a distance that would be easily bridged by a single Mg\[^{2+}\] ion. Even more puzzling is the mode of Mn\[^{2+}\] binding (a divalent metal ion known to substitute for Mg\[^{2+}\] that possesses a distinct X-ray absorption signature enabling its unambiguous identification even at moderate resolution). Despite the A9 and scissile phosphates forming an apparently perfect potential divalent metal ion binding-site, Mn\[^{2+}\] is observed to bind exclusively to the A9 phosphate and the adjacent N7 of A10.1 \{Martick 2008a\}, in a manner essentially identical to that observed for the minimal hammerhead \{Pley 1994\}, in which the scissile phosphate is 18Å away. This in turn has lead to proposals that include migration of the Mg\[^{2+}\] ion from the A9-only position to the bridging position, and binding of an additional bridging Mg\[^{2+}\] ion during formation of the transition-state \{Lee 2008\} \{Wong 2011\}. This is a report the highest-resolution ribozyme structure to date. New crystallization conditions were obtained in the presence of a high concentration of Na\(^{+}\) ions, and also produced better-diffracting crystals. The new structure reveals Na\(^{+}\) ions at the active
site whose catalytic relevance is quite suggestive.

2.2 New Crystallization Conditions

Hammerhead ribozyme crystals prepared using 1.7M sodium malonate, i.e. \( \text{Na}_2(\text{CH}_2(\text{COO})_2) \), buffered to pH 7.5 as a precipitating agent, in place of \((\text{NH}_4)_2\text{SO}_4\), and 35% PEG 3350, possessed the same space group and unit cell dimensions as our previous crystals \{Martick 2006\}, but yielded significantly improved diffraction (1.55Å resolution vs. 2.2Å). These crystals permit visualization of specifically bound \( \text{Na}^+ \) ions under nearly ideal conditions. Data collection and refinement statistics are reported in Table 1. The quality of the near-atomic resolution electron density is shown in Figure 6A (a close- up of G12, the general base in the hammerhead cleavage reactions, and Figure 7 (a network of RNA-water-\( \text{Na}^+ \) ion interactions near the active site). This is currently the highest resolution ribozyme structure available in the PDB database, and thus provides the best opportunity to identify metal ion interactions relevant to hammerhead ribozyme catalysis. Sixteen \( \text{Na}^+ \) ion-binding sites were identified based on coordination number and distances to adjacent water molecules. While most are in non-conserved regions of the ribozyme, two \( \text{Na}^+ \) ion-binding sites reside within the hammerhead ribozyme active site and are comprised of invariant residues. Additionally, a third potential site is apparent, consistent with either \( \text{Na}^+ \) ion or a water molecule. We focus on these sites primarily due to their potential mechanistic relevance to the chemistry of hammerhead ribozyme catalysis.
Figure 5: An all-bond representation of the 1.55 Å refined crystal structure of the full-length hammerhead ribozyme with bound Na\(^+\) ions. The enzyme strand is depicted in red, the substrate strand in yellow, the cleavage-site nucleotide in green, and the various Na\(^+\) sites are represented as large purple spheres. Coordinates are available in the PDB (3ZP8).
Figure 6: (A) Representative 1.55Å resolution 2Fo-Fc electron density contoured at 2.0 r.m.s.d. reveals a hole in the six-membered ring of the general base, G12, in the active site. (B) The canonical A9 divalent metal ion binding-site is occupied by Na⁺, which forms inner-sphere interactions with the pro-R phosphate oxygen of A9, the N7 of G10.1, and four water molecules, three of which in turn form bridging interactions with other RNA atoms. (C) The Hoogsteen face of the general base G12, within the active site, forms a second potentially catalytically relevant Na⁺ binding site. The O6 of G12 forms an inner-sphere interaction with the Na⁺, and one of the three unambiguously resolved water molecules it coordinates bridges to the N7 of G12. The other observed water molecules bridge to various phosphates near the active site. (D) A third potentially catalytically relevant Na⁺ ion coordinates the pro-R oxygens of A9 and scissile phosphates, the 2'O nucleophile of the cleavage site base, the 2'O of G8 implicated in general acid catalysis, and a water molecule in a somewhat distorted octahedral complex.
2.3 A Na⁺ Ion Binds to the A9 Phosphate Equivalent to Divalent Cations

A single strong divalent metal ion-binding site has been observed in every minimal hammerhead ribozyme structure containing divalent metal ions {Scott 1999a}, including the first structure reported {Pley 1994}. Because of the modest resolution of these initial structures, employment of Mn²⁺ or other divalent metal ions with a characteristic X-ray absorption signature greatly facilitated unambiguous identification {Pley 1994} {Murray 1998}. This divalent metal ion-binding site, which has come to be known as the “McKay site,” is consistently characterized by direct coordination of the pro-R non-bridging phosphate oxygen atom of A9 with the metal ion, and direct coordination of the N7 of G10.1, the conserved nucleotide 3’-adjacent to the A9 phosphate at the proximal end of helical Stem II. The remaining four ligand positions in the octahedral complex are occupied by water molecules. This same mode of Mn²⁺ binding is also observed in the full-length hammerhead structure {Martick 2008a}.

The 1.55Å hammerhead ribozyme structure reveals that Na⁺ binds to the A9 phosphate and the N7 of G10.1 in the same way that Mn²⁺ binds (Figure 6B). The distance between Na⁺ and the pro-R phosphate oxygen of A9 is 2.36Å, and the distance between Na⁺ and the N7 of G10.1 is 2.56Å. The angle between these three atoms is109°, indicating that the octahedral complex is somewhat distorted. Bond distances to the four water molecules range between 2.4Å and 2.6Å, with the other bond angles rather more close to 90°. (See Table 2) Three of the coordinated water
**Figure 7:** Wall-eyed stereo diagram illustrating the network of hydrogen bonding interactions and other close contacts within the active site, as well as Na⁺ ions, shown as green spheres, and well-resolved water molecules, whose oxygen atoms are shown as red spheres. Here the carbon atoms within the enzyme strand are white, and those of the substrate strand are yellow. The blue mesh of the top figure is a 1.55Å resolution $2F_o-F_c$ electron density contoured at 1.2 r.m.s.d. The bottom figure is the same view, with the electron density map removed for clarity.
molecules form bridging interactions with other RNA atoms, including a hydrogen bond between water and the pro-R phosphate oxygen atom of G10.1, a hydrogen bond between water and the O6 of G10.1, and a hydrogen bond between water and O4 of U10.2. The remaining water molecule does not appear to make additional hydrogen bonding interactions. The G10.1/A9 phosphate metal binding site thus serves as an important positive control in the current structural analysis. Table II reports the coordination distances to Na\(^+\) in the current structure and to Mn\(^{2+}\) in the previous full-length hammerhead structure, as well as average coordination distance values for these and Mg\(^{2+}\) from the literature and databases cited. Based upon the chemical identities of the ligands in the octahedral complex, the bond distances and angles, identification of the ion as Na\(^+\) is reasonably certain. Hence, Na\(^+\) is observed to bind to the A9 phosphate in a manner essentially identical to that of previously observed divalent cations, including Mn\(^{2+}\) and Mg\(^{2+}\).

### 2.4 A Na\(^+\) Ion Binds to the Hoogsteen Face of G12

Based upon proximity to the attacking nucleophile \{Martick 2006\}, in addition to compelling biochemical evidence \{Han 2005\}, G12 has previously been identified as the general base in the hammerhead ribozyme cleavage reaction. Deprotonation of the N1 of G12 is thought to lead to the transient formation of a negatively charged enolate form of guanine, in which the negative charge becomes dispersed between N1 and O6. Upon initiation of the cleavage reaction by G12’s
\[ R_{\text{merge}} = \sum | I - \langle I \rangle | / \Sigma I \], where I is the intensity of measured reflection and \( \langle I \rangle \) is the mean intensity of all symmetry-related reflections.

b Model-building and validation, and identification of Na⁺, was performed using COOT\(^{33}\).

c Refinement and analysis was carried out using Phenix (phenix.refine and phenix.xtriage)\(^{32}\).

d \[ R_{\text{cryst}} = \sum | F_{\text{calc}} - F_{\text{obs}} | / \Sigma F_{\text{obs}} \] where \( F_{\text{obs}} \) and \( F_{\text{calc}} \) are observed and calculated structure factors, respectively.

e \[ R_{\text{free}} = \sum T| F_{\text{calc}} - F_{\text{obs}} | / \Sigma F_{\text{obs}} \] where T is a test data set of about 7.5% of the total unique reflections randomly chosen and set aside prior to refinement.

f TLS groups\(^{34}\) were identified and assigned employing the TLS Server\(^{35}\).
abstraction of the 2’-proton from C17, the uncharged keto form of guanosine is restored. A Na⁺ ion (Figure 6C) directly coordinates the O6 of G12 in the plane of the base, with a bond distance of 2.4Å, and directly coordinates a water molecule, with a distance of 2.6Å. This latter water molecule bridges to the N7 of G12, with a hydrogen bonding distance of 2.8Å. Two additional water molecules are well resolved, with bond distances of 2.3Å and 2.5Å, and angles consistent with an octahedral complex. These also form hydrogen bonding bridges to phosphate oxygen atoms. Weaker electron density corresponds to the two remaining ligand sites in the presumed octahedral complex; water molecules were not assigned to these positions in the PDB coordinates due to their rather high temperature factors. This is the first crystallographic observation of a metal ion interaction with G12, although a similar interaction has been predicted previously, based upon biochemical evidence {RoychowdhurySaha 2006; RoychowdhurySaha 2007}.

2.5 A Potential Ion-Binding Site Bridging the A9 and Scissile Phosphates

Another potential ion-binding site, observed in the hammerhead ribozyme active site, corresponds to a well-ordered electron density peak at water position 1, bridging the pro-R non-bridging phosphate oxygen atoms of the A9 and scissile phosphates with distances of 3.1Å and 2.5Å, respectively, and a bond angle of 85.6°. Additional contacts include the 2’O of C17, the cleavage-site nucleotide at 3.2Å, the 2’O of G8 implicated in general acid catalysis at 3.3Å, and water molecule 2131 at 3Å, which is also coordinated by the Na⁺ ion bound at the canonical A9 phosphate
Table II

Metal Ion Binding Site Bond Distances (Å)

<table>
<thead>
<tr>
<th>G10.1/A9 phosphate site</th>
<th>current structure 3ZP8</th>
<th>Mähler &amp; Persson (^f)</th>
<th>MESPEUS db(Na) (^g)</th>
<th>MESPEUS db(Mg) (^g)</th>
<th>Mn(II) structure 20EU (^h)</th>
<th>MESPEUS db(Mn) (^g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Me-N7</td>
<td>2.61</td>
<td>---</td>
<td>2.64 (^a)</td>
<td>2.77 (^a)</td>
<td>2.08</td>
<td>2.42 (^a)</td>
</tr>
<tr>
<td>Me-OP(_R)</td>
<td>2.33</td>
<td>---</td>
<td>2.77 (^c)</td>
<td>2.04 (^c)</td>
<td>2.01</td>
<td>2.03</td>
</tr>
<tr>
<td>Me-OH(_{2}) (3)</td>
<td>2.36</td>
<td>2.43 (^f)</td>
<td>2.52 (^e)</td>
<td>2.18 (^e)</td>
<td>2.41</td>
<td>2.26 (^e)</td>
</tr>
<tr>
<td>Me-OH(_{2}) (4)</td>
<td>2.51</td>
<td>2.43</td>
<td>2.52</td>
<td>2.18</td>
<td>2.24</td>
<td>2.26</td>
</tr>
<tr>
<td>Me-OH(_{2}) (5)</td>
<td>2.33</td>
<td>2.43</td>
<td>2.52</td>
<td>2.18</td>
<td>2.02</td>
<td>2.26</td>
</tr>
<tr>
<td>Me-OH(_{2}) (6)</td>
<td>2.56</td>
<td>2.43</td>
<td>2.52</td>
<td>2.18</td>
<td>1.70</td>
<td>2.26</td>
</tr>
</tbody>
</table>

\(^a\) Me-N7 Guanosine contact in DNA and RNA
\(^b\) All Me-N contacts
\(^c\) Me-O for non-bridging phosphate oxygens
\(^d\) Me-O for all oxygens
\(^e\) Me-O for water only
\(^f\) Solution values obtained from large angle X-ray scattering and double difference infrared spectroscopy.\(^2\)
\(^g\) MESPEUS data base\(^2\)
\(^h\) Mn identified with unambiguous anomalous X-ray scattering signature\(^1\).

Numbers in italics are average values from solution scattering experiments (f) and MESPEUS database (g). Non-italics numbers are from hammerhead ribozyme crystal structures (3ZP8 for Na\(^+\) and 20EU for Mn\(^{2+}\)(h)).
site. The final position in the slightly distorted octahedral complex is occupied by the exocyclic amine of G12, forming a close contact at 2.9Å. We have chosen to model this position as a water molecule rather than as a sodium ion in the PDB file 3ZP8 as the more conservative interpretation of the data. Although the observed octahedral coordination of the atom or ion at position 1 is consistent with assignment of a Na\textsuperscript{+} ion rather than water, the close contact with the exocyclic amine of G12 and the longer bond distances are more consistent with a hydrogen bond to a water molecule. The position was refined assuming the identity of water with an occupancy of 1.0 and 10 electrons. (Na\textsuperscript{+} also has 10 electrons, and therefore an experimentally indistinguishable X-ray scattering factor.) The identity of this feature is addressed further in the discussion section.

2.6 Further Details Regarding Active-Site Geometry

The high-resolution diffraction data permits us to measure the most critical non-bonded inter-atomic distances and associated angles in the hammerhead ribozyme active site with unique precision. The ribozyme-substrate complex includes a 2’OMe C17 at the active site that functions as a substrate analogue-inhibitor in that the active 2’-H is replaced with an inert methyl ether linkage that can only function as a hydrogen bond acceptor. When G12 is protonated at N1, the N1 proton forms a hydrogen bond with the 2’O of C17 that is 3.1Å in this structure. The corresponding bond angles with respect to C2 and C6 in G12 are 106° and 129° (An ideal symmetric hydrogen bond would possess angles of about 117°, given the internal ring angle of
guanine). The inter-atomic distance between the attacking nucleophile, i.e., the 2’O of C17, and the adjacent scissile phosphorus atom is 3.3Å, and the accompanying in-line attack angle is 156.8°. The ideal transition-state (or reaction intermediate) in-line attack angle will approach 180° as the scissile phosphate approaches a pentacoordinated trigonal-bipyramidal oxyphosphorane geometry, and the final bond distance between the 2’O and the phosphorus atom in the final reaction product will be about 1.59Å. The 2’OH of G8 makes a 3.2Å hydrogen bond to the 5’O of C1.1, the leaving group in the cleavage reaction. These and several additional contacts are illustrated in Figure 7.

2.7 Discussion

Although the hammerhead ribozyme is catalytically active in the absence of divalent metal ions {Murray 1998a}, it does require a high concentration of positive charge in the form of molar quantities of monovalent cations such as Na⁺, or exchange-inert trivalent complexes such as cobalt hexaamine, i.e., Co(NH₃)₆³⁺, for activity {RoychowdhurySaha 2007}. Under physiological conditions, the most likely source of high cationic strength is Mg²⁺. It is thus of considerable importance to understand how Na⁺ may substitute for Mg²⁺ in hammerhead ribozyme catalysis, in terms of its structural interactions. Crystallographic identification of Na⁺ binding-sites is not always straightforward, especially when dealing with moderate diffraction resolutions typically associated with RNA crystals. Na⁺, like Mg²⁺ and water, has only 10 electrons, and unlike divalent metal ions such as the more electron-rich Mn²⁺
often used to identify metal binding sites indirectly, Na\(^+\) does not possess a useful X-ray absorption edge that would facilitate unambiguous identification. Hence a combination of coordination geometry, biochemistry, and known propensities to form nucleotide complexes must be used together with high-resolution X-ray diffraction data to identify Na\(^+\) binding sites unambiguously. Hence, obtaining hammerhead ribozyme crystals that diffract to 1.55Å resolution in crystallization conditions that include a high concentration of Na\(^+\) ions presents a unique opportunity to identify monovalent cation binding-sites that may have mechanistically significant implications.

The single most prominent divalent metal ion-binding site in both the minimal and full-length hammerhead structures, the “McKay Site,” involves inner-sphere coordination of the pro-R phosphate oxygen of A9, and inner-sphere coordination of the N7 of G10.1, the adjacent nucleotide base. The binding mode appears identical for Mn\(^{2+}\) in both the minimal and full-length structures, despite their very different active site conformations {Pley 1994} {Martick 2008a}. Because of its proximity to the scissile phosphate in the full-length hammerhead structure, and because of biochemical evidence suggesting that the A9 and scissile phosphate are bridged by a single metal ion in the transition-state of the cleavage reaction {Wang 1999}, the McKay Site metal ion has received considerable attention.

The A9 and scissile phosphates are separated by 4.2Å in the full-length hammerhead ribozyme structure, and form a nearly geometrically ideal potential divalent metal ion binding-site for a metal ion favoring formation of an octahedral
complex. Thus it is quite puzzling that A9 phosphate metal ion binding favors coordination with N7 of G10.1 rather than the pro-R oxygen of the scissile phosphate in the full-length hammerhead. This has lead to suggestions that this metal ion migrates and bridges the two phosphates only as the reaction approaches the transition-state, or that the metal ion’s binding mode is somehow disrupted by the presence of the 2’-OMe at the cleavage site, or that the corresponding Mg$^{2+}$ ion simply binds differently compared to the observed mode of Mn$^{2+}$ binding, as has been witnessed in the context of tRNA \{Jovine 2000\}. These proposals have been tested computationally \{Lee 2008\} \{Martick 2008a\}, and in the case of unmodified substrate structures, crystallographically, for both the minimal \{Scott 1996\} and full-length \{Chi 2008\} hammerhead ribozyme structures. We are now able to add direct high-resolution crystallographic observations.

We observe that a Na$^+$ ion binds to the McKay site via exactly the same mode as observed in all previous hammerhead crystal structures with more electron-rich divalent cations, i.e., via inner-sphere coordination of the pro-R phosphate oxygen of A9, and inner-sphere coordination of the N7 of G10.1, the adjacent nucleotide base (Figure 2b), despite the fact that the uncharged N7 is a softer ligand than oxygen, a more typical ligand for a hard divalent metal ion such as Mg$^{2+}$ ion. In other words, this Na$^+$ ion prefers a coordination environment consisting of one hard and one soft ligand (in addition to four bound water molecules, as one might propose for a softer divalent metal ion such as Mn$^{2+}$), rather than a coordination environment consisting instead of two hard ligands (the pro-R oxygens of the A9 and scissile phosphates). A
second active site Na\(^+\) ion forms an inner-sphere interaction with the exocyclic O6 keto oxygen of G12, the nucleotide implicated as the general base in the hammerhead cleavage reaction (Figure 6C). This Na\(^+\) ion is ideally positioned on the Hoogsteen face of G12 to counter-balance (or disperse) the transient negative charge accumulated at O6 as an enolate ion accompanying deprotonation of the N1 of G12, in turn required to initiate the cleavage reaction via abstraction of the 2’H of C17. This Na\(^+\) ion, in other words, may favorably perturb the pKa of G12 to enhance general base catalysis. This sort of Na\(^+\) ion interaction with G12 has in fact been suggested previously, based upon biochemical evidence {RoychowdhurySaha 2006; RoychowdhurySaha 2007}.

A third, but more ambiguous, potential ion-binding site forms a distorted octahedral complex that bridges the pro-R oxygens of the A9 and scissile phosphates, in addition to forming interactions with the 2’O attacking nucleophile of C17, and the 5’O leaving group of C1.1, and a bridging water molecule that spans between it and the McKay Site Na\(^+\) ion (Figure 6D). We have conservatively modeled the bridging entity as a water molecule because its identity is somewhat ambiguous. Specifically, it forms a close contact with the exocyclic amine of G12 at the sixth and final octahedral coordination position. The coordination distance is rather long, and the chemical identity of the ligand is unexpected, as noted in the results section. It is therefore unlikely to be a very stable interaction, even by the standards of Na\(^+\). The distance is more consistent with a hydrogen bond to water, but modeling it as water does not permit us to account for more than one of the other five observed
coordination interactions. The scattering center in question refines well to 10 electrons, equally consistent with either Na$^+$ ion or water. We therefore chose the more cautious interpretation of the data, and suggest that if indeed this is the predicted bridging metal ion, the coordination environment may change somewhat as the transition-state geometry is approached. At the very least, the pro-R oxygen of the scissile phosphate must move to form the required trigonal bipyramidal oxyphosphorane transition-state.

2.8 Methods

Full-length hammerhead ribozyme crystals were obtained via vapor diffusion, as described previously {Martick 2006} {Martick 2008a}, except the crystallization conditions were modified as follows: The reservoir contained 1.7M sodium malonate, buffered to pH 7.5, and 1mM MgCl$_2$. The hanging drop contained ½ the concentration of the reservoir solution mixed with the RNA solution prepared as described previously {Martick 2006}. Crystals were stabilized in a mother liquor containing 1.7M sodium malonate, pH 7.5, and 10mM MgCl$_2$, and flash-frozen, using the sodium malonate as a cryoprotectant. The data collection is summarized in Table I. The data were processed using iMosflu {Battye 2011} and CCP4 {Winn 2003}, and refined using Phenix, beginning with rigid-body refinement using 2GOZ (now 3ZD5) after substituting ribouridine for 5’-bromouridine. This was followed by simulated annealing and TLS refinement using default phenix.refine protocols. Model building and adjustment was performed within COOT {Emsley 2010},
including identification and rejection of water molecules and Na⁺ ions. The refined structural coordinates and accompanying F_{obs} are currently available in the Protein Data Bank as 3ZP8.

2.9 References for Chapter Two


24. Murray J. B., Terwey D. P., Maloney L., Karpeisky A., Usman N., Beigelman L.


CHAPTER THREE
Structural and Catalytic Effects of General Base Substitution in the Hammerhead Ribozyme
3.1 Introduction

Like other RNA enzymes, the hammerhead ribozyme catalyzes RNA cleavage via acid-base catalysis. G12, when deprotonated, is thought to initiate the cleavage reaction by abstracting a proton from the 2’OH of the cleavage-site nucleotide, C17. Although G12 is an invariant nucleotide in the natural hammerhead sequence, synthetic constructs with purine substitutions at position 12 show a general trend of decreasing catalytic prowess with a decrease in the pKa of the substituted purine corresponding to protonation or deprotonation at position N1 {Han 2005}. Because N1 of G12 is within hydrogen-bonding distance (3Å) of the 2’O nucleophile, the pKa trend of the G12 purine substitutions is considered strong evidence in support of the hypothesis that a deprotonated G12 functions as the general base in hammerhead ribozyme catalysis {Martick 2006}, in the same manner that a deprotonated His12 is thought to function in general base catalysis in the analogous RNase A reaction.

However, substitution of G12 with other purine nucleotide analogues with different functionality may also attenuate the reaction rate by perturbing the hammerhead ribozyme’s structure, so in principle, some or all of the observed reduction in the rate of catalysis may be due to structural changes. These two effects may nonetheless give rise to the same rate law; the principle of kinetic equivalence entails that we cannot distinguish between the extremes of a mechanism of general base catalysis, in which G12 functions as a Brønsted base, and a mechanism of specific base catalysis, in which the role of G12 is purely structural, and water functions as a Brønsted base, on the basis of rate measurements alone. {Fedor 2009}
To better understand the contributions of structural perturbations that might potentially disrupt catalysis, we have obtained a crystal structure of a G12A substitution in a well-characterized full-length hammerhead ribozyme crystal form that, in the case of the wild-type G12 ribozyme, diffracts to 1.55 Å resolution (3ZP8). We have also measured the effect of several purine analogue substitutions at G12 with varying pK\(_A\) values in the context of the crystallized hammerhead ribozyme. Our results permit us to estimate the contribution of a structural perturbation observed in the G12A substituted hammerhead, and thus the relative contributions of the \(\Delta pK_A\) and structural changes to the reaction rate.

3.2 Crystal Structure of a G12A Substituted Hammerhead Ribozyme

Crystals of the hammerhead ribozyme containing a G12A substitution were prepared using 1.7M sodium malonate, i.e. \(\text{Na}_2(\text{CH}_2(\text{COO})_2)\), buffered to pH 7.5 as a precipitating agent, and possessed the same space group and unit cell dimensions as the crystals reported in the previous chapter. Crystallographic data and refinement statistics are shown in Table III. The 2.2Å structure displays an active site perturbation in which the N1 of A12, in place of the general base G12, is not positioned within hydrogen bonding distance from the 2’O of C17. The substituted purine A12 presents an altered hydrogen-bonding interface with residue A9 in which the N3 and 2’OH of A12 hydrogen bond to the 6-amino functionality of A9 (Figure 8A). The misalignment results in the 6-amino group of A12 forming a hydrogen-
Table III
Crystallographic data and refinement statistics

Data collection

<table>
<thead>
<tr>
<th>Data Processing Software</th>
<th>iMosflm(Batty 2011), CCP4 suite, phenix.xtriage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>Monoclinic, C2</td>
</tr>
<tr>
<td>Unit cell parameters</td>
<td>a=50.14Å  b=68.45Å  c=60.22Å  β = 112.56°</td>
</tr>
<tr>
<td>Solvent Content, V_s (%)</td>
<td>49.2</td>
</tr>
<tr>
<td>Matthews Coef., V_m (Å³/M_r)</td>
<td>2.42</td>
</tr>
</tbody>
</table>

| Resolution range (Å)    | 20.22 - 2.20                                      |
| No. unique reflections  | 9162                                              |
| Redundancy              | 3.9                                               |
| I/σ                     | 16.80                                             |
| Completeness (%)        | 95.42                                             |
| R_merge a               | 0.03                                              |

Overall | Highest res. shell
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.32-2.20</td>
</tr>
</tbody>
</table>

Structure refinement

<table>
<thead>
<tr>
<th>Model building software</th>
<th>COOT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Refinement software</td>
<td>phenix.refine</td>
</tr>
<tr>
<td>Target</td>
<td>Maximum Likelihood (ML)</td>
</tr>
</tbody>
</table>

R-factors

| Rкуп     | 0.1820                      |
| R_free  | 0.2360                      |
| Test set size (%)        | 9.9                         |
|                      | 10.3                        |

Geometry

| r.m.s.d. bond lengths (Å) | 0.005                        |
| r.m.s.d. bond angles (°)  | 0.938                        |
| r.m.s.d. planarity (°)    | 0.007                        |
| r.m.s.d. torsion angles (°) | 14.735                       |
| ML coordinate error (Å)   | 0.33                         |
| ML phase error (°)        | 30.80                        |
| β-factor from Wilson plot (Å²) | 53.23                       |
| No. TLS groups f          | 10                           |

a $R_{\text{merge}} = \Sigma |I - \langle I \rangle| / \Sigma I$, where I is the intensity of measured reflection and $\langle I \rangle$ is the mean intensity of all symmetry-related reflections.

b Model-building and validation, and identification of Na⁺, was performed using COOT. {Emsley 2010}

c Refinement and analysis was carried out using Phenix (phenix.refine and phenix.xtriage). {Adams 2002}

d $R_{\text{cryst}} = \Sigma |F_{\text{calc}} - F_{\text{obs}}| / \Sigma F_{\text{obs}}$ where $F_{\text{obs}}$ and $F_{\text{calc}}$ are observed and calculated structure factors, respectively.

e $R_{\text{free}} = \Sigma |F_{\text{calc}} - F_{\text{obs}}| / \Sigma F_{\text{obs}}$, where T is a test data set of about 7.5% of the total unique reflections randomly chosen and set aside prior to refinement.

f TLS groups {Painter 2006} were identified and assigned employing the TLS Server {Painter 2006a}.
bond with the 2’O of C17. Figure 8B shows a close-up of this same region, with the same color-coding, and the corresponding 2.2Å sigma-A-weighted 2F₀-Fᵉ electron density map shown as a blue mesh. The data and coordinates for the structure are currently available in the Protein Data Bank as 3ZD4.

### 3.3 Comparison to Wild-Type Structures

The two crystal structures of the G12 hammerhead, obtained in the same crystal form but under different crystallization conditions, are very similar. The 1.55Å resolution structure (3ZP8) was obtained in the presence of a high concentration of Na⁺, and Na⁺ is observed to coordinate the N7 of G10.1 and the pro-R oxygen of the A9 phosphate in a manner similar to what has been previously observed for various divalent cations. The 2.2Å structure (3ZD5, formerly 2GOZ) was obtained in the presence of NH₄⁺, and no metal ions are observed to coordinate at the A9 phosphate site {Martick 2006}. Both structures contain a modified substrate wherein C17 is replaced by 2’OMe-C17, which prevents the cleavage reaction. The N1 of G12 donates a hydrogen bond to the 2’O of C17 in both structures. (Presumably in an active ribozyme, the 2’O is a hydrogen bond donor, and a deprotonated N1 will be a hydrogen bond acceptor. Abstraction of the 2’H⁺ by G12 presumably initiates the cleavage reaction.)
Figure 8: The crystal structure of a G12A-substituted hammerhead ribozyme. (A) An overall view of the full-length G12A hammerhead ribozyme crystal structure (3ZD4), with the G12A purine substitution and its two hydrogen-bonding partners, A9, and C17, shown with atom color-coding. The A9 phosphorus is highlighted in magenta. The substrate strand, which contains C17, is depicted as a light-blue ribbon, and the enzyme strand as a grey ribbon. The remaining nucleotides are shown as stylized representations. (B) Wall-eyed stereo view of the G12A hammerhead ribozyme (3ZD4) active site. The 2Fo-Fc electron density map contoured at 1.0 r.m.s.d. is shown as a blue mesh. A12, A9 and C17 are highlighted as before, including the A9 phosphorus, which is highlighted in magenta.
3.4 The G12A-C17 Interface

The crystal structure of the corresponding G12A modification, 3ZD4, reveals several structural perturbations. Because of the absence of a hydrogen bond donor, the N1 of A12 cannot make a hydrogen bond with the 2’O of C17; instead this distance is 4.3Å, corresponding to a van der Waals contact (Figure 9A) rather than ~3Å observed in the G12 structures (Figures 9B and 9D). The exocyclic 6-amine instead donates a hydrogen bond to the 2’O of C17. This interaction is almost certainly an artifact created by the presence of the 2’OMe-C17. In the case of the previously-solved G12A hammerhead structure (2QUS)\{Chi 2008\}, in which an unmodified C17 is present, the N1 of A12 receives a nearly ideal hydrogen bond from the 2’OH of C17 (Figure 9C).

3.5 The G12A-A9 Interface

In the 3ZD4 structure, the N3 on the minor-groove face of A12, along with the 2’OH of A12, forms hydrogen bonds with the exocyclic 6-amine of A9 (Figure 9A), similar to the hydrogen bonds formed between the corresponding G12 and A9 (Figure 9B and 9D). Because A12 lacks a 2-amine group, the hydrogen bond between it in G12 and the N7 of A9 does not exist. Hence it appears that A12 in 3ZD4 interfaces with A9 in a manner as analogous as possible with G12 in 3ZP8 and 3ZD5. In the 2QUS G12A structure, a single 3.3Å hydrogen bond between A12 and A9 more tenuously mediates a weakened interface between these purines \{Chi 2008\}.
**Figure 9**: G12 and A12 hydrogen-bonding networks. Hydrogen-bonding networks involving G12 and A12 are shown for four structures. Those for the two G12 structures (3ZP8 and 3ZD5) are essentially identical. The hydrogen-bonding network involving A12 in the presence of the 2'-OMe-C17 (3ZD4) differs from that in the presence of the unmodified substrate (2QUS). The latter makes a good hydrogen bond between N1 of A12 and the 2’OH of C17, whereas the N1 of 3ZD4 is 4.3Å from the 2’O of the methylated substrate, due to the fact that no hydrogen bond can form between these atoms. Instead, the 6-amino group appears to make a hydrogen bond donation to the 2’O of the methylated substrate in 3ZD4, and A12 hydrogen bonds to A9 in a manner similar to what is observed in the G12 structures, apart from the absence of the 2-amino group.
3.6 The A9 Phosphate Potential Metal Ion Binding Site

Least-squares superposition of G8, A9, and G10.1 of the two G12 structures and the two A12 structures, using the high-resolution G12 structure (3ZP8) as the reference (Figure 10), reveals the two G12 structures are nearly identical, despite the presence of a metal ion in one structure and absence in the other. The two G12A substituted structures, despite being from two very different sequences and crystal forms, and despite the presence (2QUS) vs. absence (3ZD4) of an active substrate, show rather similar distortions at the A9 phosphate, suggesting the G12A substitution perturbs the A9 phosphate, and thus metal ion binding. Indeed, unlike the G12 structure in which a partially hydrated, octahedrally-coordinated Na$^+$ ion is bound to the N7 of G10.1 and the pro-R oxygen of the A9 phosphate, the G12A structure obtained under identical crystallization conditions does not reveal the presence of a bound Na$^+$ ion at the A9 phosphate (Figure 10). Hence it appears that this structural perturbation is not only a consequence of the G12A substitution itself, but results in the interference with the most prominent metal ion binding sites in the hammerhead ribozyme, one that is known to have structural and catalytic importance.

3.7 Discussion

The primary structural perturbations observed in 3ZD4 due to the G12A include misalignment of N1 of A12 with the 2’O of C17, the altered hydrogen-bonding interface between the nucleotide bases of A12 and A9 relative to G12 and A9 of 3ZP8 and 3ZD5, and the movement of the A9 phosphate relative to that of 3ZP8 and
Figure 10: Conformations of the A9 Phosphate. A superposition of G12 and G12A hammerhead ribozyme nucleotides including and adjacent to A9. The superpositions were performed in COOT using 3ZP8, the highest-resolution structure, as a reference. The color-coding shows two G12A structures (3ZD4: Red 2QUS: Orange) and two G12 structures (3ZD5: Light Blue and 3ZP8: Blue). The blue sphere represents the position of Na⁺ in 3ZP8. The distance between this ion and the A9 pro-R phosphate oxygen of 3ZP8 is 2.36Å, of 3ZD4 is 3.22Å, and of 2QUS is 3.81Å (see Table IV).
3ZD5. The question of whether these changes are the consequence of the G12A substitution, the presence of the 2’OMe on C17, or the absence of metal ions must be addressed for each change.

The G12 wild-type structures (3ZP8 and 3ZD5) were obtained from the same sequence in two different crystallization conditions, but belong to the same crystal form as 3ZD4. The 3ZP8 structure was obtained in a high concentration of Na\(^+\) ions, and reveals a Na\(^+\) ion bound to the N7 of G10.1 and the pro-R oxygen of the A9 phosphate, binding in a manner previously identified for divalent metal ion binding. The 3ZD5 structure was obtained in a high concentration of NH\(_4^+\) and does not appear to have a metal ion bound to the N7 of G10.1 and the pro-R oxygen of the A9 phosphate. Nevertheless, the two structures are almost identical; the positional difference of the A9 phosphate pro-R oxygen between the two structures upon least-squares superposition is about 0.7 Å, little more than the sum of the positional errors of the two structures. The absence of a metal ion binding to this site is thus of minimal structural significance in the context of the wild-type structure.

The positional difference between the A9 phosphate pro-R oxygen of the G12A substituted structure, 3ZD4, and 3ZP8, is 2.1Å, and the conformation of the phosphate appears much less amenable to cation binding. Similarly, the distance in the case of the G12A substituted structure 2QUS is 1.7Å, with the phosphate conformation similarly distorted \{Chi2008\}. Based upon these comparisons, the structural perturbation of the A9 phosphate appears to be primarily a consequence of the G12A substitution rather than the presence vs. absence of the unmodified 2’OH of
Table IV  
A9 Phosphate Geometry

<table>
<thead>
<tr>
<th>Structure</th>
<th>R12</th>
<th>Me\textsuperscript{n+}</th>
<th>C17</th>
<th>N7 -- O\textsubscript{R}P</th>
<th>Na\textsuperscript{+} -- O\textsubscript{R}P</th>
</tr>
</thead>
<tbody>
<tr>
<td>3ZP8</td>
<td>G12</td>
<td>Na\textsuperscript{+}</td>
<td>2'-OCH\textsubscript{3}</td>
<td>4.02</td>
<td>2.36</td>
</tr>
<tr>
<td>3ZD5</td>
<td>G12</td>
<td>-</td>
<td>2'-OCH\textsubscript{3}</td>
<td>4.71</td>
<td>2.53</td>
</tr>
<tr>
<td>3ZD4</td>
<td>A12</td>
<td>-</td>
<td>2'-OCH\textsubscript{3}</td>
<td>6.06</td>
<td>3.22</td>
</tr>
<tr>
<td>2QUS</td>
<td>A12</td>
<td>-</td>
<td>2'-OH</td>
<td>5.74</td>
<td>3.81</td>
</tr>
</tbody>
</table>

Table IV displays the distances between the Na\textsuperscript{+} ion density form 3ZP8 and the pro-R oxygen of the A9 phosphates of other wild-type and G12A crystal structures. Additionally, the total distances between the N7 of G10.1 and pro-R oxygen of A9 are shown for all structures. The identity of the general base (R), any identified metal ion (Me\textsuperscript{n+}), as well as the presence or absence of the 2’OMe group of C17 are also listed.
C17. It is noteworthy that 3ZD4 and 2QUS are two completely different hammerhead ribozyme sequences belonging to two different tertiary structural contact classes, and the crystal forms and crystallization conditions have little in common. Nevertheless, the observed structural perturbation is apparent in both G12A structures. Figure 10 shows a superposition of all four structures, with 3ZP8 and its accompanying Na$^+$ ion and electron density as the reference. Because there is little change in the position of the N7 of G10.1 in the superposed structures, potential coordination distances and angles between the Na$^+$ ion and the other pro-R A9 phosphate oxygens can be estimated (Table IV).

Comparison of the two G12A structures reveals that the presence vs. absence of the unmodified C17 2’OH has a pronounced effect upon the disposition of the purine base. The N1 of A12 is deprotonated near neutral pH, so a hydrogen bond can form only if the 2’O of C17 donates a hydrogen. Thus the 3ZD4 structure is incapable of forming the hydrogen bond relevant to catalysis due to the presence of the methyl group, and instead the exocyclic amine of A12 forms a presumably irrelevant hydrogen bond with the 2’O. In the 2QUS structure, the relevant hydrogen bond is able to form, due to the presence of a 2’H in the active, unmodified substrate. (The G12 structures can also form a hydrogen bond with the 2’O of the methylated substrate because the N1 is protonated near neutrality) Comparison of the A12/A9 nucleotide base hydrogen-bonding interface in the two G12A structures, however, reveals that the 3ZD4 structure forms a more stable interaction, with two hydrogen bonds present, mimicking the G12 structures, whereas the 2QUS structure reveals a
less stable interaction, with only one hydrogen bond between A12 and A9 present. This suggests two competing A12 active site conformations may exist, in which the stabilizing interface between A12 and A9 must be disrupted for catalysis to take place. In other words, the hydrogen bonding network that helps to stabilize G12 and position it favorably for catalysis may instead have an inhibitory effect upon other purines, especially those lacking the 2-amino group present in G12. This would explain the pK$_A$ trend anomaly observed for G12I substitutions. Inosine is similar to guanosine, lacking only the 2-amino group. The absence of the 2-amino group lowers the pK$_A$ by only one unit, suggesting a $\sim$10-fold reduction in catalytic activity. Instead, the observed reduction is $\sim$10$^4$-fold, implying a rather more dramatic effect, due to the absence of the 2-amino group. This implies a 10$^3$-fold reduction in catalytic activity due to structural perturbations alone (assuming an approximately additive energetic effect). A similar violation of the pKa trend, with a pronounced inhibitory effect for the G12I substitution, was previously observed in the context of a minimal hammerhead sequence {Han 2005}, so this is unlikely to be simply an artifact due to the peculiarities of our particular full-length hammerhead sequence. This will be addressed further in the next chapter.

### 3.8 Materials and Methods

Full-length G12A hammerhead ribozyme crystals were obtained via vapor diffusion, as described previously {Martick 2006} {Martick 2008}, except the crystallization conditions were modified as follows: The reservoir contained 1.7M
sodium malonate, buffered to pH 7.5, and 1mM MgCl$_2$. The hanging drop contained 1/2 concentration of the reservoir solution mixed with the RNA solution prepared as described previously {Martick 2006}. Crystals were stabilized in a mother liquor containing 1.7M sodium malonate, pH 7.5, and 10mM MgCl$_2$, and flash-frozen, using the sodium malonate as a cryoprotectant. The data collection is summarized in Table III. The data were processed using iMosflm {Battye 2011} and CCP4 {Winn 2003}, and refined using Phenix {Adams 2002}, beginning with rigid-body refinement using 3ZP8 after substituting A12 for G12 in the model. This was followed by simulated annealing and TLS refinement using default phenix.refine protocols. Model building and adjustment was performed within COOT{Emsley 2010}. Superposition calculations for structural comparisons were performed using least-squares superposition within COOT. Figures were created with PyMOL{Delano 2003}. The refined structural coordinates and accompanying $F_{obs}$ are currently available in the Protein Data Bank as 3ZD4.

3.9 References for Chapter Three


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Molecular Graphics System.

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CHAPTER FOUR
Kinetic Analysis of Full-Length Hammerhead Ribozyme Variants
4.1 Introduction

The phosphodiester bond isomerization catalyzed by the hammerhead ribozyme is a reversible cleavage reaction similar to those of the hairpin, hepatitis delta, and VS ribozymes {Takagi 2001}. It has been well supported that the hammerhead ribozyme utilizes a general acid-base mechanism during catalysis in which the N1 of residue G12 and the 2’OH of G8 function as the base and acid, respectively {Martick 2006} {Han 2005}. Unlike that of other ribozymes utilizing acid-base chemistry {Bevilacqua 2003}, the hammerhead ribozyme shows a characteristic log-linear relationship between catalytic rate and pH that does not level off until very high alkalinity. Early kinetic and mechanistic studies rationalized this behavior with metal ion-hydroxide complexes being required for catalysis {Takagi 2001} {Dahm 1993}, but it was later revealed that inner-sphere coordination of metals is not required for hammerhead catalysis {Murray 1998} {Curtis 2001} {O’Rear 2001}.

In order to probe the key forces driving hammerhead ribozyme catalysis, nucleobase substitutions for either the acidic G8 or basic G12 (or both) with 2,6-diaminopurine, 2-aminopurine, and inosine have been kinetically analyzed {Han 2005}. Results suggest that the substitution of G12, the general base, with nucleobases of lower basicity (Figure 11) can reduce a minimal hammerhead ribozyme’s catalytic activity while not perturbing their structure {Han 2005}. However, the extent of catalytic rate reduction is not always proportional to the loss
Figure 11. Structure and N1 pKₐ of guanine and substituted analogues 2,6-diaminopurine, 2-aminopurine, inosine, and adenine. Purine ring numbering for guanosine is also displayed. {Han 2006}
of basicity. Chapter two of this dissertation suggests that specifically coordinated metal ions can help stabilize the catalytic core of the hammerhead ribozyme as well as coordinate directly to the general base at position 12 providing a countercharge to the negatively charged enolate form of the base. The new A12 hammerhead ribozyme structure described in chapter three suggests that structural perturbations within the active sites of hammerhead ribozymes may cause a significant reduction in catalytic activity, possible to the same extent as a reduction of basicity.

4.2 Kinetic Analysis of G12R Substituted Hammerhead Ribozymes

The catalytic activity of the full-length hammerhead ribozyme from Schistosoma mansoni was obtained by radiolabeled PAGE analysis (see Methods). Additionally, the catalytic rates for four G12R substituted ribozymes were also obtained, where R was either 2,6-diaminopurine, 2-aminopurine, inosine, or adenine. The results of these analyses (Table V) suggest that in addition to lower basicity, differences in exocyclic functional groups may have a significant effect on catalytic activity of G12R substituted ribozymes. Substitutions with 2,6-diaminopurine (pKa 5.1) do in fact reduce the rate of catalysis roughly 1000-fold, consistent with the drop in basicity from guanosine (pKa 9.6). This result is consistent with other reported data using pyrrolo-cytosine fluorescence to measure hammerhead ribozyme kinetics {Buskiewicz 2012}. Like guanine, 2,6-diaminopurine has two exocyclic functional groups, albeit there is an amino group in the 6 position instead of a keto group. In contrast, inosine has much greater basicity (pKa 8.7) than 2,6-diaminopurine yet
Table V
Measured and predicted rates for G12R substitutions

<table>
<thead>
<tr>
<th>R12</th>
<th>pKₐ</th>
<th>rate kₑ (min⁻¹)</th>
<th>predicted relative rate</th>
<th>measured relative rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>9.6</td>
<td>~ 50*</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>I</td>
<td>8.7</td>
<td>0.02</td>
<td>1.3 X 10⁻¹</td>
<td>4 X 10⁻⁴</td>
</tr>
<tr>
<td>diAP</td>
<td>5.1</td>
<td>0.04</td>
<td>3.2 X 10⁻⁵</td>
<td>8 X 10⁻⁴</td>
</tr>
<tr>
<td>A</td>
<td>3.8</td>
<td>...</td>
<td>1.6 X 10⁻⁶</td>
<td>&lt; 10⁻⁷</td>
</tr>
<tr>
<td>2AP</td>
<td>3.5</td>
<td>...</td>
<td>8 X 10⁻⁷</td>
<td>&lt; 10⁻⁷</td>
</tr>
</tbody>
</table>

*rate for the wild-type (G12) extrapolated from pH 6.5 data

Table V: The catalytic rates for the wild-type and G12R substituted hammerhead ribozymes and their corresponding N1 pKₐs. Reactions were performed under “single-turnover” like conditions at pH 7.5 in which ³²P-labeled RNA substrate was mixed with excess hammerhead ribozyme and then annealed. Due to the high activity of the wild-type ribozyme, reactions were performed at pH 6.5 and extrapolated. The reactions were initiated by adding MgCl₂ for a final concentration of 10mM and reacted for 24 hours. Hammerhead ribozymes substituted with adenine and 2-aminopurine slowed cleavage so dramatically rates were impossible to determine. The predicted and measured rates relative to wild-type are also shown.
displays roughly equal rate reduction. Hammerhead ribozymes substituted with 2-aminopurine (pKa 3.8) and adenine (pKa 3.5) both nearly abolish cleavage, making it difficult to infer any specific rate differences between to two.

Consistent with the acid-base mechanism employed by various ribozymes, lowering the basicity of the general base does in fact lead to reduction of catalytic activity in the kinetic analysis of G12R substituted hammerhead ribozymes. However, the data suggests that the removal of the functional group at position 2 of the nucleobase can have a drastic effect on catalytic activity. The general base G12 uses its 2-amino functionality to make a crucial non-canonical base pair with the Hoogsteen face of residue A9 helping situate itself for abstraction of the 2’-hydrogen of residue C17. Additionally, as described in Chapter Two, a metal ion directly coordinates to the 6-keto functionality of the nucleobase in order to stabilize the negative charge built up by the enolate form. In this, it can be suggested that inosine, lacking a functional group at the 2 position, must compete between two conformational states: an active state in which the N1 of inosine is positioned for deprotonation of the 2’H of C17, or an inactive state in which inosine is base paired with residue A9. From the kinetic data presented in this chapter, this structural flocculation can account for an approximate 1000-fold reduction in activity, similar to that 2,6-diaminopurine. Such a base pair may cause a perturbation in the catalytic core of the ribozyme similar to the one we observe in the G12A structure described in Chapter Three, as adenine also lacks a functional group at the 2 position.
Figure 12. Catalytic activity of the full-length hammerhead ribozyme as well as G12R substituted analogues. Ribozyme and substrate strands were annealed in Tris Buffer (50mM Tris, 50mM NaCl, 100mM EDTA) adjusted to pH 7.5. Reactions were initiated with Mg$^{2+}$ for a final concentration of 10mM. Later time-points are omitted for better comparison. WT (red) cleaves extremely fast (~50min$^{-1}$) while diAP (green)(0.04min$^{-1}$) and I (purple)(0.02min$^{-1}$) are reduced roughly 1000-fold. A (black) and 2AP (blue) nearly abolish all catalytic activity.
The pronounced inhibitory effect of the G12A substitution in the hammerhead ribozyme is therefore likely due to the combination of two contributions, one that is structural (primarily the absence of the exocyclic 2-amino group), and the other that is a manifestation of the ΔpKₐ. The catalytic effect of the G12I substitution allows us to separate the contribution of the two effects to at least an order-of-magnitude approximation. I is in essence G with the 2-amino group removed. The pKₐ is very similar, and the hydrogen-bonding face presented to C17 is the same. A9, however, sees the minor groove edge of the purine that lacks the 2-amino group, and therefore presents the same hydrogen bonding potential as a G12A substitution. As a consequence, the observed ~10³-fold excess reduction in catalytic activity may be attributed to structural perturbations similar to those induced by the G12A substitution, and a similar in order-of-magnitude effect upon catalytic activity in the G12A hammerhead may therefore be attributed to perturbing structural effects.

4.3 Concluding Remarks

Based on comparison of four crystal structures, including the new structure (3ZD4) reported herein, and on the anomalously low cleavage rate we observe for the G12I substituted hammerhead ribozyme, it appears that the 2’-amine of G12 provides significant structural stabilization of the ribozyme catalytic core by the purine general base, resulting in up to 3 orders of magnitude catalytic enhancement. The structural basis for this appears to be found both at the hydrogen-bonding interface with A9 and the metal binding site formed in part by the participation of the phosphate of A9.
This in turn permits us to suggest that both pK\textsubscript{A} effects and primarily structural effects must be taken into account to explain how purine substitutions of the hammerhead general base influence ribozyme catalysis.

4.4 Materials and Methods

Hammerhead ribozyme constructs (43 nucleotides in length) containing both the wild-type G12 nucleobase and the A12 mutant nucleobase were created by chemically synthesizing DNA templates for T7 run-off transcription using an Expedite Nucleic Acid Synthesis System. In order to ensure proper polymerase termination as well as product length, the final two guanosine residues at the 5’-end of the DNA template contain methyl groups attached to the 2’-oxygen of the ribose ring. The cleavable substrate strand of RNA was synthesized and transcribed in the same matter. RNA products were purified on 20% polyacrylamide gels and eluted using 0.3M sodium acetate with 1mM EDTA at pH 8. RNA constructs were isolated via ethanol precipitation, filtered, and desalted. Hammerhead ribozyme constructs that contain non-natural nucleobases like 2,6-diaminopurine, 2-aminopurine and inosine at the 12 position were ordered from Dharamacon.

The maximum catalytic activity for all hammerhead constructs was obtained by radiolabeled PAGE analysis using the unmodified RNA substrate. Reactions were performed under “single-turnover” like conditions at pH 7.5 in which \textsuperscript{32}P-labeled RNA substrate was mixed with 100-fold excess hammerhead ribozyme and then annealed. Due to the high activity of the wild type ribozyme, reactions were
performed at pH 6.5 and extrapolated. The reactions were initiated by adding MgCl₂ for a final concentration of 10mM and reacted for 24 hours. Samples of the reactions were terminated at various time points using stop dye containing 47.5% formamide and 10mM EDTA. All time points as well as an uninitiated sample were run out on a 15% polyacrylamide gel. Gels were exposed to a radiograph and quantitated using a phosphoimager and ImageQuant software. Background and zero time normalization were performed to ensure accuracy of catalytic activity. The fraction of substrate cleaved per minute for the wild-type and G12R substituted hammerhead ribozymes is shown in Figure 12.

4.5 References for Chapter Four


