CATALYTIC ABILITIES OF THE *SCHISTOSOMA MANSONI* HAMMERHEAD RIBOZYME WITH MUTATED SUBSTRATES IN ICE

A Thesis
Presented to
The Academic Faculty

by

Audrey E. Calvird

In Partial Fulfillment
of the Requirements for the Degree
Bachelors of Science in the
School of Biological Sciences, College of Sciences

Georgia Institute of Technology
December 2016

CATALYTIC ABILITIES OF THE *SCHISTOSOMA MANSONI* HAMMERHEAD RIBOZYME WITH MUTATED SUBSTRATES IN ICE

Approved by:

Dr. Roger M. Wartell, Advisor
School of Biological Sciences
Georgia Institute of Technology

Dr. Loren D. Williams
School of Chemistry and Biochemistry
Georgia Institute of Technology

Dr. Michael Goodisman
School of Biological Sciences
Georgia Institute of Technolog

Date Approved: [Date Approved by Committee]

ACKNOWLEDGEMENTS

I would like to thank Lively Lie and Roger M. Wartell for their invaluable guidance and assistance in and out of the lab. I would also like to thank the members of the Williams and Hud labs for their help and patience as this study was conducted with their help.

TABLE OF CONTENTS

LIST OF TABLES & FIGURES

SUMMARY

The synthesis of biomolecules in an environment similar to a pre-biotic Earth within the field of evolutionary chemistry has applications in understanding abiogenesis and the evolution of early biological systems on Earth. The RNA world is a prebiotic environment proposed and subsequently heavily studied in effort to better understand how biochemical reactions started on Earth. This study proposes to assess the catalytic ability of a Hammerhead Ribozyme (HHRz) isolated from the protozoa *Schistosoma mansoni* (*Schist* HHRz). It has been previously shown that the *Schist* HHRz can catalyze the synthesis of extended sequences of ribonucleic acid (RNA) by ligating two RNA substrates in ice, as well as cleave the same substrate back to the original separate substrates in the presence of Mg^{2+} (Lie et al. 2016). The same study showed that the *Schist* HHRz is able to catalyze the ligation with mutated substrates (Lie et al. 2016). This study aims to evaluate *Schist* HHRz's ability to ligate a mutated substrate in comparison to the wild type substrate in ice, as well as compare the kinetics of the *Schist* HHRz to cleave the mutated and wild type substrates in the presence of Mg^{2+}. This study will utilize the techniques specified by Lie et al. (2016) to further investigate the extent of the ligation of a mutated substrate catalyzed by the *Schist* HHRz compared to that of the wild type substrate. This study also assays the kinetics of the cleavage reaction catalyzed by the same *Schist* HHRz of both the wild type substrate and mutated substrate. This study hopes to improve the understanding of pre-biotic biochemistry in efforts to identify possible mechanisms or models for RNA polymerization by ribozymes on early Earth.

CHAPTER 1

INTRODUCTION

3.8 billion years ago, evidence shows life emerged on Earth and, following extrapolation of Darwin's Theory of Evolution, states that from then on, life grew to become more and more complex (1872). Since Darwin's theory, extensive research has been conducted, collecting evidence explaining the evolution of archaic history and present organisms (1872). This research has lead to the development of a field predicting the pre-biotic world and how it facilitated the very first organisms. Evolutionary chemistry has seen the birth of many theories of early earth, giving rise to one theory called the 'RNA world' (Gilbert 1986).

The 'RNA world' theory predicts that the current biochemistry of life was preceded by ribonucleic acid molecules (RNA) working not just as RNA, but also as proteins and deoxyribonucleic acid (DNA) to carry out metabolic functions in a pre-biotic world (Gilbert 1986). The ability of RNA to work like a catalyst is paramount, and evidence has been substantial in assaying RNA's ability to interact with other molecules in intra- and extracellular environments (Stortz 2002; Bartel & Unrau 1999; Cech 2011).

One catalytic RNA motif with particularly robust catalytic function is the Hammerhead Ribozyme (HHRz) (Kruger et al. 1982). The HHRz has been shown to self-cleave, as well as cleave and ligate substrate target strands in varying divalent cation concentrations (Kruger et al. 1982, Khvorova et al. 2003). Crystallizations of the active site conserved in many HHRz's revealed a 'wishbone' structure, comprising of three stem structures meeting at a catalytic core (Figure 1; Doudna 1995). The catalytic core is a highly conserved CUGA sequence (cytosine-uracil-guanine-adenine) located between stems I and II (Doudna 1995). Crystal structures also exposed a lack of Watson-crick base-pairing, but rather 'non-canonical pairing' of bases,

allowing the formation of a 'uridine-turn' in the structure very similar to that of a tRNA isolated from yeast (Doudna 1995). Stems I and II of the HHRz can bind to form alpha helices with complementary RNA strands, typical of those formed by double stranded DNA (Doudna 1995).

Despite extensive studies concerning the HHRz motif, structure, and catalytic functions, the evaluation of HHRz activity without the presence of divalent cations has only been researched very recently by Lie et al. (2016). Lie et al. (2016) focused on a 49 nucleotide long HHRz isolated from *Schistosoma mansoni* (*Schist* HHRz; figure 1). Lie et al. (2016) were able to assess the *Schist* HHRz's ability to ligate substrate strands in ice without the presence of divalent cations and also showed that the *Schist* HHRz can ligate mutated substrate strands in the same environment.

In order to assay the *Schist* HHRz's ability to ligate mutated stands, Lie et al. (2016) had an assay of 1040 different mutant strands denoted as P2-5N with point mutations at 5 specific locations along the 16 nucleotide substrate (Table 1). After ligation, the ligated substrate and mutated substrate pool were analyzed by high throughput sequencing in order to compare the mutated sequences presence in the substrate pool to the ligated product strands, giving an 'enrichment value' (Lie et al. 2016). The mutated substrate with the highest enrichment value had a 52X fold enrichment value over the wild type P2 substrate, which was only 90th most enriched (Table 2; Lie et al. 2016). The significant increase of enrichment over the wild type P2 identified the substrate known as Highly Enriched 1, referred to as HE1 (Table 1; Lie et al. 2016).

The ability of a HHRz to ligate substrate strands is important in fitting into the 'RNA World' theory because the larger a strand of RNA is, the more secondary structures it can form each of which could have a potential catalytic function (Gilbert 1986). Through ligating in ice

without divalent cations, Lie et al. showed the potential of an 'RNA world' in the frozen regions of a pre-biotic earth (2016; Gilbert 1986; Bada & Lazcano 2002). At the same time, through showing ligation of mutant strands, Lie et al. (2016) showed that the *Schist* HHRz lacks a certain level of specificity in substrates, suggesting the potential of ligating together and synthesizing novel strands of RNA.

This project proposes to take Lie et al.'s studies (2016) a step further, measuring the *Schist* HHRz's catalytic ability with mutated substrates. Specifically, this project will evaluate the *Schist* HHRz's ability to ligate a mutated substrate strands in frozen solution as well as the *Schist* HHRz's ability to cleave the same mutated substrate in the presence of Mg^{2+}, with both reactions compared to the wild type reaction. Both reactions will follow the in-ice ligation protocol and cleavage in the presence of Mg^{2+} as specified by Lie et al. (2016).

Through assessing the kinetics of the *Schist* HHRz in ligating mutated substrates, we can show that HHRz's have the ability to bind relatively un-specifically to substrate strands to create potentially novel, longer, and therefore more complicated, strands of RNA. The more strands that HHRz's can bind and ligate, the more likely that they had pivotal role in the 'RNA World' in synthesizing biochemically functional RNA strands and biogenesis (Gilbert 1986).

CHAPTER 2

MATERIALS AND METHODS

This study used assays to evaluate the catalytic ability of the *Schist* HHRz to cleave and ligate the wild type substrate and a mutated substrate, previously identified by Lie et al. (2016). The mutated substrate is referred to here as P1-HE1 for the 26-nucleotide long substrate for cleavage and as HE1 for the 16-nucleotide long substrate to be ligated to the 10-nucleotide P1>p. The HE1 substrate gave the highest fold enrichment value among an ensemble of P2 substrates that were ligated from the P2-5N library. The nucleotide sequences for each substrate strand are given in Table 1.

The ligation reaction consisted of three main components: the *Schist* HHRz, the P1>p, and either the P2 or the HE1 substrate. The reaction was measured through radiolabeling the P1>p substrate and calculating the amount that was un-ligated and ligated as separated by size by gel electrophoresis. While the ligation reaction used a radioactively labeled P1>p, the cleavage reaction used either a FAM labeled substrate (wild type cleavage) or a SYBR gold stain (HE1 cleavage) to allow for fluorescent detection of the cleaved substrate products and un-cleaved substrate RNA strands. The experiments varied in time from 30 minutes to 24 hours, depending on the reaction being studied.

2.1 Synthesis of *Schist* HHRz E-Strand

The *Schist* HHRz enzyme or e-strand was transcribed from a duplex DNA that contained the template sequence and the T7 RNA polymerase promoter sequence. The DNA duplex strands were each ordered from Integrated DNA Technologies (idtdna.com). The transcription protocol and reagents were taken from the Biorad MEGAshortscript kit (bio-rad.com). The RNA transcription product was then gel purified using a 12% polyacrylamide denaturing gel and cut

from the gel using UV shadowing on a TLC plate. The gel pieces containing the *Schist* HHRz e-strand were macerated before eluting in 300 uL of 0.3 M NaCl by shaking overnight at 4°C. The elution buffer was then exchanged with an additional 100 uL of 0.3 M NaCl, and left to shake at 4°C for at least 6 hours. The second elution buffer was extracted and combined with the previous elution solution stored at -20°C. The transcribed RNA was then ethanol precipitated using 2.5x volume of ethanol and 0.2 ug/uL of purified glycogen. The RNA is left to precipitate overnight. The RNA precipitation solution was centrifuged down to form an RNA pellet and the supernatant decanted and discarded. Any remaining ethanol was allowed to evaporate for two hours, and the RNA was re-dissolved in RNAse free water. An approximate final concentration of purified *Schist* HHRz e-strand was determined from the UV absorbance value at 260 nm evaluated using the Nanodrop 2000 spectrophotometer. The isolated enzyme strand was stored at -80°C.

2.2 Synthesis of P^{32}-P1>p

In order to replicate Lie et. al (2016)'s results, it was necessary to generate a radiolabeled P1 substrate with the 3' cyclic phosphate required for the P1 substrate to be ligated. This was done by attaching a P^{32}-ATP molecule to the 5' end of a 26 nucleotide long P1P2 RNA oligomer using T4 Polynucleotide kinase and protocol from New England Biolabs (neb.com). The P1P2 strand was obtained from Integrated DNA Technologies, Iowa. The P^{32} labeled P1P2 was cleaved using the *Schist* HHRz e-strand at a 1:10 ratio of substrate to enzyme strand. After adding the *Schist* HHRz e-strand to the kinase reaction mixture, the mixture remained at room temperature for 30 minutes before being heated to 68°C for 2 minutes and returned to room temperature for 20 minutes. This heat cycling was repeated 3 times. After the cleavage reaction

had occurred, the P^{32} labeled P1 with a cyclic phosphate on the 3' end (P1>p) was gel purified using the same technique as used for purifying the *Schist* HHRz enzyme strand.

2.3 Synthesis of P2-HE1

The synthesis of the P2-HE1 followed the same protocols as synthesizing the *Schist* HHRz, following methodology as described for the transcription protocol and reagents were taken from the Biorad MEGAshortscript kit (bio-rad.com). The only alteration was that due to the short length, transcription with fewer run-off transcriptions or abortive initiations was achieved through using 1:4 ratio of UTP to other NTP's in the transcription reaction. The P2-HE1 was gel purified and precipitated using isopropanol. The RNA was re-dissolved in RNAse free water. An approximate final concentration of purified P2-HE1 strand was determined from the UV absorbance value at 260 nm evaluated using the Nanodrop 2000 spectrophotometer. The isolated P2-HE1 was stored at -80°C.

2.4 Purchase of Wild Type P2 & P2-HE1

The wild type P2 substrate and the P2-HE1 was obtained from Integrated DNA Technologies, Iowa. The chemically synthesized P2 and P2-HE1 were dissolved in RNAse free water and an approximate final concentration of each strand was verified using the UV absorbance value at 260 nm evaluated using the Nanodrop 2000 spectrophotometer. The P2 and P2-HE1 was stored at -80°C.

2.5 Ligation in Ice

Lie et al. showed that the *Schist* HHRz is capable of ligating in ice without the presence of divalent cations, namely Mg^{2+} (2016). For this study's assay, the same final reaction concentrations of HHRz (0.5 uM), P^{32}-P1>p (trace, <0.05 uM), and mutated P2 (2.5 uM) strands were used, as well as the buffer (1x NTE buffer) and stopping solutions (100% formamide,

20uM EDTA) (Lie et al. 2016). The reaction was then flash frozen in -80°C Ethanol to start freezing-induced ligation, before being stored at -18±1°C for 24 hours before analysis. The reaction was stopped with an equivolume amount of stopping solution and flash frozen before being stored at -80°C until analyzed. And identical master mix was used in the ligation reaction containing the HHRz (0.5 uM), P^{32}-P1>p (trace, <0.05 uM) and the wild type P2 (2.5 uM), and was carried out in tandem with the HE1 ligation reactions, ensuring identical reaction and analysis conditions.

2.6 Cleavage in the Presence of Mg^{2+}

Also adapted from Lie et al. the cleavage assays consisted of a buffer (1x NTE buffer), and a 5:1 ratio of HHRz (1.25 uM) to P1P2 target strand (.25 uM). Prior to starting the reaction, the strands were annealed by heating to 70°C for two minutes and cooling to 25°C in air for 5 minutes. The reaction was initiated by the addition of Mg^{2+} (5 mM) and allowed to sit at 25°C for 30 minutes. To assay single-turnover reaction kinetics for the cleavage reaction, 5 equivolume samples were taken at different time points: prior to the addition of Mg^{2+}, then after at 30 seconds, 60 seconds, 5 minutes, and 30 minutes. The reaction was stopped with equivolumes of stopping solution (100% formamide, 20 uM EDTA) and chelating beads. The reaction was then stored at -80°C until analyzed.

2.7 Data Analysis

Each reaction was analyzed using a 12% denaturing urea-polyacrylamide gel. The gel was then be either imaged overnight at -80°C on a phosphorescent imaging plate or stained for 40 minutes with SYBR gold, the former if the substrates were radiolabeled and the latter if they were not. The gel image was taken with a Typhoon FLA 9500 imager (GE Life Sciences). This

image was then analyzed for band composition percentages using Multi Gauge image quantifying software (Fuji Film).

For reactions containing Mg^{2+} the samples were run on a 15% denaturing polyacrylamide gel with 2 mM EDTA. 2 mM EDTA was also added to the buffer. The added EDTA and higher polyacrylamide percentage gel ensure that the reaction results are not heavily altered by the presence of Mg^{2+} and increase the amount of separation between the 26-mer, 16-mer, and 10-mer products.

CHAPTER 3

RESULTS

Preliminary pilot results showed that the Schist HHRz does catalyze the cleavage and ligation of the HE1 mutated substrate stand.

3.1 Ligation

Ligation of the HE1 to the P^{32}-P1>p in ice was observed in several replicates of a reaction in frozen solution, each carried out in tandem with a wildtype ligation reaction in ice (figure 1). The results showed similar ligation product percent yield after 24 hours for the wildtype P2 (average product yield 21.5%, $n=3$) substrate and with the HE1 substrate average product yield 16.9%, $n=3$). A two-tailed unpaired t-test showed that there was no statistically significant difference in the product yield of ligation across all three trials ($p=0.61$, $\alpha \leq 0.05$).

3.2 Cleavage

Cleavage results showed that the Schist HHRz was capable of catalyzing the cleavage of a mutated P2-HE1 26-nucleotide strand of RNA at intial rates similar to that of the wildtype cleavage reaction in the presence of 5 mM Mg^{2+}. The initial rate for the wild type P1P2 cleavage is 43% cleaved after 30 seconds and the initial rate for P1P- HE1 is 58% cleaved after 30 seconds. Analysis of the pilot reaction showed that the wild type P1P2 substrate was cleaved to a maximum yield of 55% and the P1P2-HE1 substrate was cleaved to a maximum yield of 75% before equilibrating at 70% (Figure 2).

CHAPTER 4

DISCUSSION AND FUTURE STUDIES

The results from this pilot study support that the Schist HHRz's catalytic functions are robust to the HE1 mutations at rates comparable to the wildtype.

The pilot ligation studies showed evidence for the Schist HHRz to be able to catalyze ligation in ice, without the presence of Mg^{2+}, of a mutated substrate at similar rates to the wildtype substrate after 24 hours. While statsitcs analyzing the *similarity* in rates were unable to be performed, the lack of a statistically significant difference between the rates on ligation could be used to drive further studies to more distinctly analyze the rate of ligation of the HE1 substrate, as well as other mutated strands.

The pilot cleavage rate studies showed that the Schist HHRz could be more efficient at cleavage of the P2-HE1 strand than the P1P2 wildtype strand. Because I lacked replicates for this assay, I am unable to definitively make that conclusion, but the results from the pilot cleavage rate study supports this claim. If the Schist HHRz is more effective at cleaving the P2-HE1 strand than the wildtype, the ability for it to ligate at comparable rates is even more impressive, as the shift towards the 'forward' ligation reaction over the 'backward' cleavage reaction is increased by the reaction in ice, the reaction without Mg^{2+}, or a combination of the two factors. Lie et al. (2016) showed that in prolonged presence of the Schist HHRz and Mg^{2+}, the cyclic phosphate on the P1 substrate could be broken open, preventing the strand from being ligated. Further studying this phenomenon and how it could alter the reaction dynamics would be of interest, especially in light of the pilot results from this study.

The Schist HHRz having a non-specific catalytic ability contributes the study of ribozymes and search for evidence of an RNA world because it provides a potential pathway for novel RNA strand synthesis before the emergence of a self-polymerizing RNA. The initial results shown in this study build off of what was proven by Lie et al. (2016) by showing that the

Schist HHRz maintains catalytic ability with mutant strands, and that catalytic ability is similar to that of the wildtype for at least one mutant sequence.

Future studies should foremost focus on collecting more definitive and statistically significant data on the HE1 ligation in ice. From there, ligation of the other Highly Enriched substrates can be studied (Table 2). The culmination of these studies should be a total assay of how well the Schist HHRz can ligate these mutated substrates compared to each other and compared to the wildtype P2. Quantifying the Schist HHRz's catalytic ability to ligate substrates could aid in the search for potential pathways for novel RNA synthesis in a prebiotic earth, an essential part of piecing together an RNA world.

REFERENCES

Bada JL & Lazcano A. Origin of Life - Some Like It Hot, but Not the First Biomolecules. Science 2002; 296(5575):1982-1983.

Bartel DP & Unrau PJ. Constructing an RNA World. Trends in Biomedical Sciences 1999; 24(12):M9-M13

Crick F. Central Dogma of Molecular Biology. Nature 1970; 227: 561-563.

Cech T. The RNA Worlds in Context. Cold Spring Harbor Perspectives in Biology 2011; 1-6.

Darwin C. The Origin of Species. Murray 1872. 6th Edition.

Doudna JA. Hammerhead Ribozyme Structure: U-Turn for RNA Structural Biology. Structure 1995; 3:747-750.

Gilbert W. Origin of Life – the RNA World. Nature 1986; 319:618

Khvorova A, Lescoute A, Westhof E, & Jayasena SD. Sequence Elements Outside the Hammerhead Ribozyme Catalytic Core Enable Intracellular Activity. Nature Structural Biology 2003; 10(9):708-712.

Kruger K, Grabowski PJ, Zaug AJ, Sands J, Gottschling DE, & Cech TR. Self-Splicing RNA: Autoexcision and Autocyclization of the Ribosomal RNA Intervening Sequence of Tetrahymena. Cell 1982; 31(1):147-157.

Lie L, Biliya S, Vanneberg F, & Wartell RM. Ligation of RNA Oligomers by the Schistosoma mansoni Hammerhead Ribozyme in Frozen Solution. Journal of Molecular Evolution 2016; 82(2-3):81-92.

Storz G. An Expanding Universe of Noncoding RNAs. Science 2002; 296(5571):1260-1263.

Figures and Tables

Name	# of bases	Sequence 5'->3'
P1P2	26	GGAGGGCAUCCUGGAUUCCACUCGCC
P1P2-FAM	26	GGAGGGCAUCCUGGAUUCCACUCGCC-FAM
P1P2-HE1	26	GGAGGGCAUCCUGUAUUCCACGCACC
P2	16	CUGGAUUCCACUCGCC
HE1	16	CUGUAUUCCACGCACC
P2-5N*	16	CUGNNUUCCACNNNCC
P1	10	GGAGGGCAUC
P1>p	10	GGAGGGCAUCp
* N denotes one of the four nucleotide bases (A,G,C,U)		

Table 1: RNA Substrate Sequences
Lie et al. 2016

Name	Enrichment Value	Sequence 5'->3'
Wild Type P2	0.5	CUGGAUUCCACUCGCC
P2-HE1	26	CUGGAUUCCACGCACC
P2-HE2	20	CUGUAUUCCACUGUCC
P2-HE3	15	CUGGGUUCCACUGGCC
P2-HE4	14	CUGUAUUCCACUUUCC
P2-HE5a	13	CUGUAUUCCACGUUCC
P2-HE5b	13	CUGAAUUCCACUUACC
P2-HE6	12	CUGGAUUCCACGCGCC
P2-HE7	11	CUGAAUUCCACGCACC

Table 2: Highly Enriched P2 Substrate Strand Sequences
The red bases indicate where the mutations points are within each of the mutated strands
Lie et al. 2016

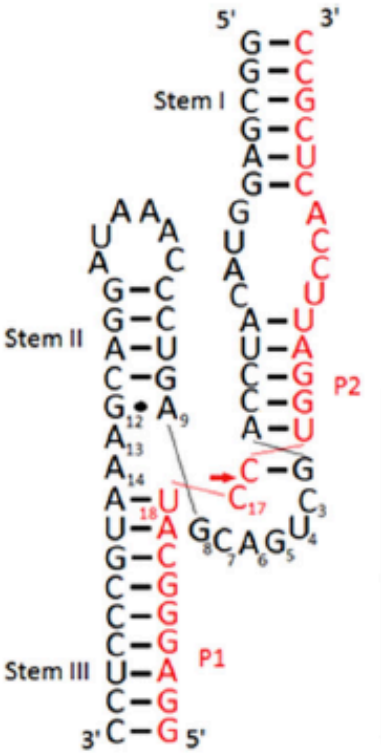

Figure 1: Schist HHRz E-Strand Bound to Wild Type P1P2
The Schist HHRz e-strand is shown in black, with the each stem labeled. The P1P2 strand is shown bound to the Schist HHRz by Watson-Crick base pairing. The arrow shows the cleavage site on the P1P2, and each resulting substrate strand is labeled with the corresponding P1 or P2.

Lie et al. 2016

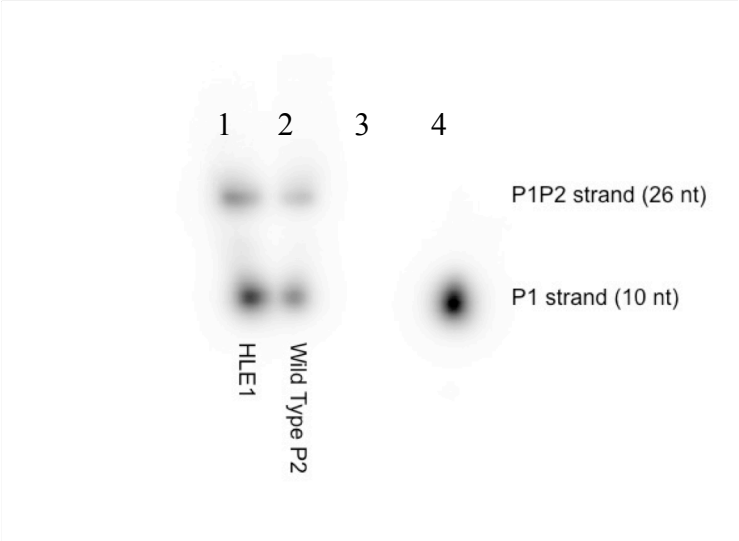

Figure 1: Wildtype P2 and HE1 Ligation
Lanes 1 & 2 contain the ligation reactions, labeled with which P2 strand was ligated. The third lane was left empty. The fourth lane contains the P1 marker strand that underwent the same treatment as both ligation reactions, minus the addition of the *Schist* e-strand, to ensure that there was no alteration to the P^{32}-P1 other than what was facilitated by the e-strand.

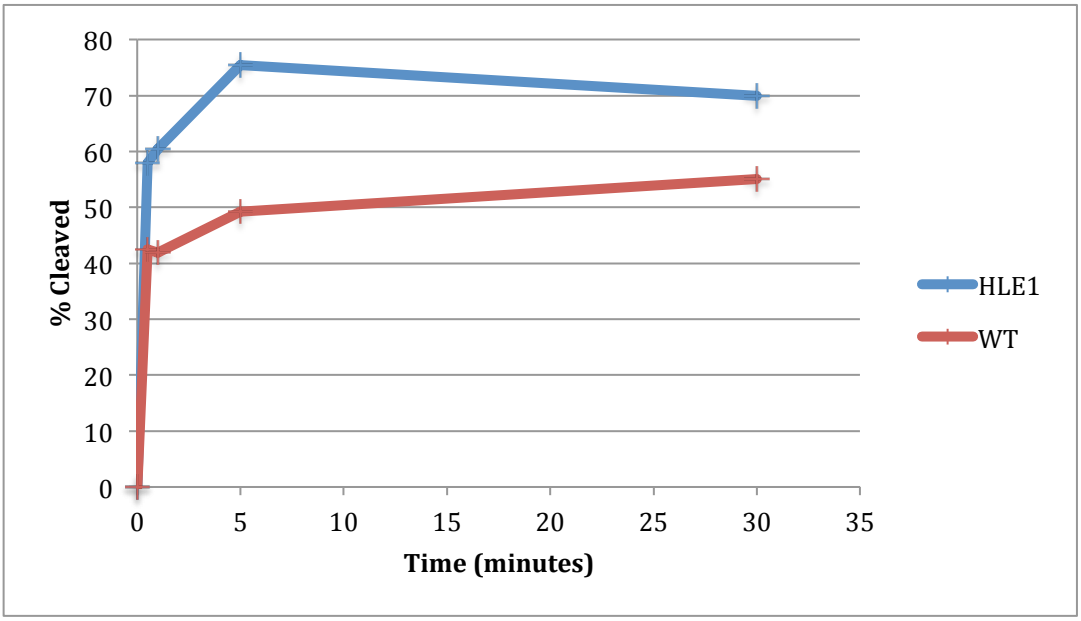

Figure 2: Wildtype and HE1 Cleavage Rate
The rate of cleavage for one cleavage cycle is shown for the wildtype P1P2 (WT) and the P1HE1 (HE1) 26-nucleotide oligomer substrates. The rate for the WT substrate maxes at 55% after 30 minutes at 25°C in the presence of 5 mM Mg^{2+}. The rate for the HE1 substrate maxes at 75% after 5 minutes and reaches equilibrium at 70% after 30 minutes at 25°C in the presence of 5 mM Mg^{2+}