NUCLEIC ACID CATALYSTS: INVESTIGATING THE EFFECT OF MONOVALENT IONS ON THE ACTIVITY OF DNA ENZYMES

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ABSTRACT

RNA and DNA enzymes often require divalent metal ion cofactors such as Mg$^{2+}$ for their function. However, numerous catalytic RNAs have been shown to also be active in high concentrations of monovalent ions such as Li$^+$. Given the similar chemical structures of RNA and DNA, the divalent ion requirement for DNAzymes has also come into question. Using gel electrophoresis, the activity of the 10-23 DNAzyme was tested in several monovalent salts in order to ascertain the exact function of metal ions in the function of the DNAzyme. Ultimately it was determined that monovalent ions were ineffective in aiding DNAzyme function, supporting the conclusion that divalent ions play a chemical, rather than simply structural, role as a cofactor.

INTRODUCTION

Enzymes play an integral role in the regulation and maintenance of biological processes. Enzymes catalyze chemical reactions via an active site, a small cleft in an otherwise large molecule that provides a specialized chemical environment to promote the reaction. Some enzymes additionally require cofactors, such as metal ions or vitamins, in order to perform functions that cannot be accomplished through the normal set of amino acids or nucleotides. During catalysis, specific substrate molecules will bind to the active site, often inducing a change in the shape of either the enzyme or substrate. Active sites facilitate chemical reactions by reducing the Gibbs free energy required for the formation of the transition state, a process known as transition state stabilization (Figure 1). Enzymes accomplish this stabilization through a multitude of interactions such as stressing chemical bonds or donating or accepting electrons to or from the substrate (1). This increases the percentage of molecules that fulfill the reaction's energy prerequisite, thus increasing the overall rate of the reaction.

Figure 1: Activation Energy Chart
Enzymes lower the activation energy required to achieve the transition state.
For many years it was commonly accepted that enzymatic activity was solely carried out by protein molecules. Studies conducted in the last few decades, however, have demonstrated that RNA and DNA can also function as enzymes. These enzymes, known as ribozymes and DNAzymes respectively, operate directly at the nucleotide level. As an example, the 10-23 DNAzyme, has an active site composed of 15 deoxynucleotides, with two associated segments approximately 8 nucleotides in length that flank each side. These linked sections are thought to play a role in substrate recognition, as well as in providing the binding energy necessary to hold the substrate within the active site of the enzyme (2). Together these units function in the cleavage of RNA substrate molecules, a reaction which occurs at a concentration of Mg\(^{2+}\) that can be easily found \textit{in vivo}. The mechanism by which the metal ions accelerate this reaction was originally suggested to be chemical in nature, with the divalent metal ion directly involved in the active site of the reaction. Such a chemical process would occur through either one of two proposed mechanisms.

As shown in Figure 2, the divalent metal ion serves in one proposed mechanism as a cofactor that induces hydroxide ions in solution to perform nucleophilic substitution general base catalysis on the 2’ hydroxyl group of ribose, activating it as a nucleophile to cleave the RNA phosphodiester backbone (3).

Alternatively, the divalent metal ion can act as a Lewis acid, as in Figure 3. The 2’ hydroxyl will donate an electron pair to the metal ion, facilitating its deprotonation, leading to a pathway similar to Mechanism A (3).

However, recent research has questioned the necessity of the magnesium cation in nucleic acid catalysis, suggesting that the effects of metal cations are largely structural rather than chemical (4). Studies exploring the usage of monovalent ions have challenged the general consensus that divalent metal ions, specifically magnesium, are necessary for nucleic acid catalysis. The relative success of ribozyme activity in the presence of monovalent cations suggests that the effect of cations on catalysis of RNA cleavage is to stabilize the catalytic structure rather than to play a specialized chemical role.

Figure 2: Mechanism A
Proposed mechanism with divalent metal inducing nucleophilic substitution on 2’ hydroxyl group of ribose (3).

Figure 3: Mechanism B
Proposed mechanism with divalent metal inducing a Lewis base to donate an electron pair and deprotonate the 2’ hydroxyl group of ribose (3).
Previous studies demonstrated that the hammerhead, VS, and hairpin ribozymes were all shown to work in the presence of high concentrations of the monovalent cations lithium ($\text{Li}^{+}$), sodium ($\text{Na}^{+}$), and ammonium ($\text{NH}_4^+$). Furthermore, in a study conducted to test the effect of High Hydrostatic Pressure (HHP) on the activity of DNAzymes, it was found that DNAzymes could operate in the absence of $\text{Mg}^{2+}$ at high pressures (Figure 4). High pressure compacts the structure of the enzyme-substrate complex, performing the same function that metal cations would if the mechanism were strictly structural. Separate studies have also shown that the Hepatitis Delta Virus (HDV) ribozyme can also work with high concentrations of monovalent cations, though at a significantly reduced rate. This combined data suggests that in high concentrations, monovalent ions can partially or fully replace the function of $\text{Mg}^{2+}$ in ribozyme-catalyzed reactions (5).

This experiment will compare the performance of the 10-23 DNAzyme in the presence of monovalent ions with its performance in the presence of divalent cations. By conducting DNAzyme-catalyzed RNA-cleaving reactions in salt solutions and then examining the results through gel electrophoresis, the effectiveness of monovalent ions in the function of the DNAzyme will be determined. If the cleaving action of the DNAzyme is successful under high concentrations of monovalent cations, it can be concluded that the role of metal ion cofactors in DNAzyme activity is ultimately structural rather than chemical. However, if the monovalent ions are shown to be ineffective in this regard, it is highly probable that metal ion cofactors play a fundamentally chemical rather than structural role. Due to the success of monovalent cations in reactions involving ribozyme catalysis, we hypothesize that the monovalent cations $\text{Li}^{+}$, $\text{Na}^{+}$, and $\text{NH}_4^+$ will prove as effective as $\text{Mg}^{2+}$ in deoxyribozyme-catalyzed reactions, demonstrating that the function of metal ion cofactors is fundamentally structural in nature. Ultimately, understanding how DNAzymes function will aid in design of future catalysts that may be used against viral RNA.

Figure 4: The Effects of High Hydrostatic Pressure
HHP forces the enzyme and substrate into a structure conducive to the catalytic reactions, without any ion cofactors (5).
MATERIALS AND METHODS

Preparing the gel electrophoresis

Gel electrophoresis was run using pre-cast 15% Tris-Urea gels (BioRad) in a Tris-Borale EDTA (TBE, 89 mM Tris, 89 mM Boric acid, 2 mM EDTA) buffer system. Gels were run at 90 V per gel for 30 to 75 minutes. A loading buffer consisting of formamide, EDTA, and bromophenol blue dye was created as well. The formamide acts as a "stop" solution that arrests the RNA-cleaving activity of the DNAzyme in the various timepoint samples to be loaded into the gels. To test for the lowest efficient dye concentration in the electrophoresis, bromophenol blue and xylene cyanol were compared in their ability to stain an 11-nucleotide RNA product in various concentrations. After running the electrophoresis for forty minutes at 180 V, the gels were analyzed. Nucleic acids were visualized by staining them with SYBR® Green II (which was diluted 1:10,000 in TBE solution) and using Hitachi© FMBIO II Multi-View. After analyzing the resulting images, it was determined that at low concentrations, bromophenol blue was more efficient than xylene cyanol at staining the 11-nucleotide RNA product. A new loading buffer containing 4.32 mL of 98% formamide, .25 mL of .2 M EDTA, and .005 g of .1% bromophenol blue was prepared without xylene cyanol and with half the original bromophenol blue concentration.

Calculating the RNA product standard

The concentration of the RNA product standard solution was calculated in order to determine the optimum amount of RNA required for effective visibility in gel electrophoresis. Data of absorbance (ε260) from NJGSS 2008’s Nucleic Acid Team was used; ε260 for DNA was 3.0 · 10^5 and RNA was 2.1 · 10^5 M^-1 cm^-1. Desired absorbance was set at .35 A · M^-1 cm^-1 with a target range of 1>x>.1 A · M^-1 cm^-1. Calculating in accordance with Beer’s Law, theoretical DNA and RNA concentration was found. A light spectroscopy scan using the Beckman Coulter DU 640 spectrophotometer confirmed concentrations to be within the target range. For confirmation, ε260 was recalculated using the found absorbance and given concentration; ε260 for DNA was 3.1 · 10^5 and RNA was 2.0 · 10^5 M^-1 cm^-1. The final concentration for RNA product standard was found to be 2.23 · 10^-6 M.

Preparing the reactions

The reactions testing for RNA cleavage by DNAzyme were prepared in 4 M monovalent salt concentrations of LiCl, NaCl, and NH₄Cl. The first reaction set consisted of 48 µL of 5 M salt solution, 4 µL of 10-23 DNAzyme, 1 µL of 216 µM RNA substrate, 4 µL of .5 M EPPS buffer, and 3 µL of .5 M EDTA, intended to chelate any divalent ions present in solution and thus to prevent false positive results. Reactions containing Na⁺, NH₄⁺, and Li⁺ were incubated at 37°C, and a reaction containing Li⁺ was held at room temperature. The four 60 µL reactions were each stopped after 2 hours, 24 hours, and 48 hours by adding 9 µL of the reaction solution to 9 µL of the stop solution. Gels were loaded with six of the reaction solutions and five controls: a positive control containing 50 mM Mg²⁺ among the same nucleic acid concentrations, a
negative control in the form of the same reaction solution containing distilled water in place of a salt solution, RNA substrate only (6 µL RNA substrate and 54 µL distilled water), RNA product standard only (6 µL RNA product standard and 54 µL distilled water), and DNAzyme only (6 µL DNAzyme and 54 µL distilled water).

For the second trial, three reactions were each prepared with a higher concentration of RNA but a lower concentration of DNA (48µL salt, 4µL EPPS buffer, 3µL EDTA, 2.5µL DNAzyme, and 2.5µL RNA substrate). The same three salts were used, but all reactions were run at room temperature. Furthermore, 4.5µL of each reaction solution was added to 4.5µL of distilled water and 9µL stop solution. Each control was prepared with 3.6µL of 5 M LiCl and 5.4 µL of reaction mix in order to balance the well's ion concentrations and facilitate gel electrophoresis. One gel was run with a stop solution containing both bromophenol blue dye and formamide. A second gel was run with a stop solution containing only formamide.

The third set of reactions replicated exactly the composition of the second set. The three reaction solutions were incubated at room temperature for 72 hours because earlier experimentation indicated that the degree of cleavage is independent of time after 24 hours have passed. The "RNA product only" and "RNA substrate only" control solutions were each recreated with a higher concentration of RNA (7µL RNA in 21µL distilled water) to guarantee visibility. Additionally, the samples containing DNA were incubated at 95°C for three minutes before being loaded into the gel.

The three sets of gels were scanned using the FMBIO analysis software. By measuring the degree of photon intensity emitted from the suspected substrate bands, the volume of RNA in each band was quantified.

RESULTS

The first trial tested the effect of 4 M salt solutions on the cleavage of a 20-nucleotide RNA strand by the 10-23 DNAzyme. The reaction solution contained 1.08·10⁻⁵ M DNAzyme combined with 3.60·10⁻⁶ M RNA substrate, 0.033 M EPPS, and 0.0250 M EDTA in the presence of this salt concentration. The first PAGE gel from this trial (Figure 5) displayed the results derived from allowing this reaction to run with lithium ions for one hour, one day, and two days at both room temperature and 37°C. The uppermost set of bands correlated to the DNAzyme, and direct comparison of these bands demonstrated that the DNA in the lithium solution moved through the gel slower than the DNA control. Horizontal movement of the bands was also seen. Due to this asymmetric movement of the nucleic acids through the gel, the resulting image was rendered uninterpretable. This discrepancy was attributed to the excessively high concentrations of salt present in the wells in the duration of the electrophoresis. Therefore, the next gel electrophoresis was preceded by diluting the reaction solutions to lower salt concentrations and increasing the amount of salt in the controls to create a more unilateral movement.

The second set of gels provided more interpretable results than the first, proving that high salt concentration in the gel was an issue for electrophoresis. The positive control lane with Mg²⁺ showed cleavage of the RNA substrate into two smaller fragments. However, all lanes with monovalent salt produced band patterns differed from that containing Mg²⁺, implying that the
DNAzymes cannot catalyze RNA with the aid of monovalent ions. There was also an additional band in the DNA-only lane, possibly due to the DNA folding into a secondary structure. Another set of reactions and gel was run to confirm that the DNAzyme cleaved the RNA substrate in Mg\(^{2+}\) but not in Li\(^{+}\), Na\(^{+}\), and NH\(_4\)^{+}.

Figure 5: Trial 1
RNA cleavage by DNAzyme in the presence of 4 M lithium cations at room temperature (R) and 37°C (I). Reactions were carried out for one hour (1), one day (2), and two days (3). A positive control utilizing Mg\(^{2+}\) ions was used along with a negative control that lacked salts altogether. Solutions containing only DNAzyme, RNA substrate, and RNA product molecules were also used as controls for comparison to the reaction solutions. Lateral movement towards the right is seen in all bands. Controls did not run as predicted: Mg\(^{2+}\) did not exhibit characteristic reactant and product bands, RNA substrate and product were indistinguishable. Lithium appeared to move slower than DNA control and showed no product yield.
Figure 6 is a PAGE gel of sodium and ammonium salt solution reactions. The bands demonstrated a noticeable right lateral movement out of their respective lanes. Additionally, the DNA in the salt solutions traveled slower than the DNA control. Extended reaction times did not produce any noticeable change in product and substrate bands for any salt solution. The “no salt” negative control exhibited similar distance traveled as the positive control, Mg$^{2+}$. The RNA substrate and product also travelled similar lengths despite having clear molecular weight differences.

Figure 6: Trial 1
The gel showed the effect of sodium and ammonium ions on RNA cleavage at 37°C. Reactions were carried out for one hour (1), one day (2), and two days (3). Lateral movement of the bands is observed, with a shift to the right. As in the case of Figure 3.0, nucleic acids in the reaction solutions containing high salt concentrations moved slower than the DNA control. RNA substrate and product moved the same length, suggesting little separation.
Figure 7 shows the effects of the salts on RNA cleavage. All the wells show a significant amount of the substrate band with the exception of the positive control and DNAzyme control; however, the RNA substrate and RNA product moved the same distance making the two bands indistinguishable. In addition, the DNAzyme appears to run in two separate bands. Figure 7 also displays lateral movement in the gel distorting some of the bands.

Figure 7: Trial 2
This image shows LiCl, NaCl, and NH₄Cl solutions at two time stops, 1A (24 hours) and 2A (48 hours). It also shows the control solutions which include the positive control (MgCl₂ solution), the negative control (a no salt solution), RNA substrate solution, RNA product solution, and DNAzyme. All solutions contain the staining dye without xylene cyanol.
Figure 8 shows the effect of the salts on RNA cleavage without the addition of a dye. Compared to Figure 7, the solutions ran a greater distance without the gels. There is also a greater gap between the second DNAzyme band and the RNA substrate band. In this gel, the RNA substrate and RNA product ran the same distance as well causing difficulty in distinguishing the product band. Lateral movement also occurs in this gel.

Figure 8: Trial 2
This image shows LiCl, NaCl, and NH₄Cl solutions at two time stops, 1 (24 hours) and 2 (48 hours). It also shows the control solutions which include the positive control (MgCl₂ solution), the negative control (a no salt solution), RNA substrate solution, RNA product solution, and DNAzyme. No solutions contain the staining dye.
Figure 9 shows the activity of DNAzyme at room temperature for 72 hours with the addition of dye. Each salt solution was loaded into two wells. Like in the last trail, the RNA substrate band and the RNA product band traveled the same distance. The positive control solution of Mg\(^{2+}\) showed an absence of the lower band, and therefore it was determined that the absence of this band indicated that the DNAzyme had cleaved the substrate. All monovalent salt solutions showed the presence of the lower dark band, indicating no RNA substrate cleavage.

Figure 9: Trial 3
The gel shows the effect of Li\(^{+}\), Na\(^{+}\), and NH\(_4\)\(^{+}\) on RNA cleavage by DNAzyme. The reactions occurred for 72 hours at room temperature. Two samples (1 and 2) of each salt were loaded with formamide-only loading buffer. The Mg\(^{2+}\) lane exhibited cleavage but the No Salt lane did not. The six salt lanes did not show cleavage. RNA substrate and RNA product traveled approximately the same distance.
Figure 10 shows the effect of the salts on RNA cleavage after 72 hours at room temperature without the addition of dye. The RNA product band and the RNA substrate band traveled the same distance. The “no salt” negative control solution showed the presence of the lower dark band, which meant that the RNA was not cleaved, while the positive control solution of Mg$^{2+}$ showed the absence of the lower dark band, indicating that the RNA had been cleaved. All the salt lanes showed the presence of the lower dark band, and therefore there was no RNA cleavage.

Figure 10: Trial 3
The gel shows the effect of Li$^+$, Na$^+$, and NH$_4^+$ on RNA cleavage by DNAzyme. The reactions occurred for 72 hours at room temperature. Two samples (1 and 2) of each salt were loaded with bromophenol blue and formamide loading buffer. The Mg$^{2+}$ lane exhibited cleavage but the No Salt lane did not. The six salt lanes did not show cleavage. All lanes experienced minor smearing. RNA substrate and RNA product traveled approximately the same distance.
Figure 11: Volume of RNA Substrate per Band
This graph represents the average amount of RNA substrate in each lane’s band of the gels in Figures 9 and 10. The positive had the least amount of RNA. The negative control had nearly the same amount of RNA as the monovalent salt lanes, suggesting that Li+, Na+, and NH4+ do not aid DNAzyme in RNA cleavage.

Figure 11 quantifies the amount of RNA substrate in each lane’s band in the two gels of the third trial. This helped confirm our former qualitative analysis. The positive control with Mg2+ had the least amount of RNA substrate, indicating that DNAzyme with Mg2+ as a cofactor is most effective at cutting RNA. The negative control with no salt exhibited nearly the same amount of cleavage as the monovalent salt lanes, implying that Li+, Na+, and NH4+ does not aid DNAzyme in catalysis. The RNA substrate band contained the greatest amount of RNA as expected.
DISCUSSION

The most significant problem in interpreting the image of the PAGE gel was the smearing of bromophenol blue dye and the variable migration rates of nucleic acid. The difficulty in distinguishing nucleic acid stained by SYBR Green II called for an increase in the amount of RNA loaded into the gels. Therefore, the levels of the 10-23 deoxyribozyme and the RNA substrate were altered to achieve a 1:1 ratio for the second set of gels. For the second trial, one gel was prepared with both dye and formamide whereas the other gel contained solely formamide, in order to test whether the dye was responsible for the smearing. Since the 2 hour timepoint yielded insignificant RNA cleavage in the first trial, the second trial only used the timepoints of 24 and 48 hours. Additionally, differences between the reactions done at room temperature and those incubated at 37°C were noted to be negligible; thus the second set of reactions was carried out at room temperature.

The most significant problem with this trial was the variable rate of nucleic acid migration due to high salt concentrations. Since the control lanes with low salt concentrations (and greater resistivity) ran faster than the experimental lanes with high salt concentrations (and greater conductance), it was determined that the gels would run more effectively by equalizing the salt concentration of control lanes to that of experimental lanes by adding 1 M LiCl to control lanes to increase salt concentration. To counter the inhibitory effects of high salt concentration on electrophoresis, the salt concentration in the experimental lanes was diluted from 2 M to 1 M.

The second set of gels provided more interpretable results than the first, proving that high salt concentration in the gel was an issue for electrophoresis. The positive control lane with Mg²⁺ showed cleavage of the RNA substrate inferred from the disappearance of the substrate bond. However, all lanes with monovalent salt produced band patterns differed from that containing Mg²⁺, implying that the 10-23 DNAzyme cannot catalyze RNA with the aid of monovalent ions. The control lanes for the RNA substrate and RNA product lacked bands so it was unclear whether the bands in the experimental lanes were actually RNA product or RNA substrate. There was also an additional band in the DNA-only lane, possibly due to the DNA folding into a secondary structure. Another set of reactions and gel was run to confirm that the DNAzyme cleaved the RNA substrate in Mg²⁺ but not in Li⁺, Na⁺, and NH₄⁺.

For the final set of gels, the RNA controls were recreated because RNA bands did not stain clearly in the past two runs. To avoid the formation of the secondary structure of DNA, the DNA control and reaction solutions containing DNA were incubated at 95°C to denature all of the DNA into its primary structure so only a single band would appear in the DNA control lane.

The third gel produced the clearest results because all lanes ran at a constant rate and each band was fully distinguishable. Although the DNA was heated to denature all DNA to a uniform size, two bands still appeared in lanes with DNA. Like previous trials, the RNA substrate and RNA product control bands traveled the same distance. Due to non-optimized electrophoresis conditions, this recurring issue may be attributed to improperly labeled samples because last year's experiments were beset with the same issue. As a result of this discrepancy, it is unknown whether the lower dark band contains RNA substrate, RNA product, or both. However, it is more likely that the lower dark band contains substrate because the positive...
control of Mg$^{2+}$ lacked the band, implying that the RNA substrate was cleaved as expected. Furthermore, the negative control of "no salt" contained the lower dark band, implying that RNA substrate was not cleaved. All six experimental lanes containing the three salts were identical to the "no salt" lane because they contained the lower dark band, indicating that cleavage did not occur despite the high monovalent salt concentrations.

**CONCLUSION**

In conclusion, monovalent ions are not viable cofactors for the 10-23 DNAzyme. Although the divalent ions ended up being more effective than the monovalents, the experiment did give insight into the structure of the deoxyribozyme. The observation that monovalent ions have little effect suggests that divalent ions, and not merely charge, are crucial to the function of the 10-23 DNAzyme. This suggests that the magnesium plays more of a catalytic role rather than a structural role as a cofactor. This observation is in contrast with the RNAzyme where high concentrations have been found to work effectively in inducing enzymatic properties (6). The research suggests the mechanism is chemical. This contrasts Fedoruk-Wyszomirska's paper which shows that 10-23 can function under HHP. Therefore, since magnesium presence and HHP both produce the same result, finding the common ground between these two methods is essential for future research in order to define exactly what mechanism is activated. Understanding how the DNAzyme works will require much research but holds much promise in hopes to learn how they function and why. Once understood it is possible that the DNAzyme can be manipulated and designed to perform specific tasks. Not only will a better understanding allow us to manipulate DNAzymes themselves, but will also lead to the development of new applications in which we can use these enzymes.

DNAzymes have been found to be just as effective as ribozymes in addition to being more stable, less costly, and easier to synthesize (4). Deoxyribozymes are also highly selective, allowing scientists to target specific nucleotides even among large chains (4). These characteristics make DNAzyme a viable and applicable venue for research. For example, many viruses, such as HIV, utilize genetic strains consisting of RNA, so a DNAzyme may be designed to select for and cleave the pathogenic RNA (7). Another practical application of the DNAzyme is in the treatment of cardiovascular disease, specifically with the 10-23 DNAzyme. Researchers have taken the 10-23 DNAzyme and have targeted the TNF alpha mRNA in mice. This mRNA codes for the TNF alpha complex which has found to be involved with heart failure after infarction. After treatment with the DNAzyme, the rats showed significant improvement (8). DNAzyme also has the potential to aid in cancer treatment by use disrupting the translation of the mutated K-Ras(G12V) mRNA sequence. Mutated K-Ras(G12V) is responsible for the proliferation and metastasis of many types of cancers. DNAzyme can help in cancer treatment by cleaving the mRNA K-Ras(G12V) and therefore reducing the number of K-Ras(G12V) synthesized (2). Another practical application for DNAzyme is in the creation of miniature lead detecting devices that could be used for such purposes as the detection of lead in the drinking water supply. Lead has been shown to successfully activate RNA enzyme cleavage by DNAzyme, a property that can be utilized to detect lead. Due to its potentially smaller size as compared to other lead detectors currently available, DNAzyme lead detectors could provide a fast, efficient, and low cost method with less waste product produced for lead detection.
REFERENCES


