2015

The Effects of Hg2+ on Secondary Structures Formed by T-Rich DNA

Zachary Boynton

University of Mississippi. Sally McDonnell Barksdale Honors College

Follow this and additional works at: https://egrove.olemiss.edu/hon_thesis

Recommended Citation

https://egrove.olemiss.edu/hon_thesis/969
The Effects of Hg$^{2+}$ on Secondary Structures Formed by T-Rich DNA

By
Zachary Michael Boynton

A thesis submitted to the faculty of the University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College

Oxford, Mississippi
May 2015

Approved By:

[Signatures]

Advisor: Dr. Randy Wadkins

Reader: Dr. Tracy Brooks

Reader: Dr. Susan Pedigo
The Effects of Hg$^{2+}$ on Secondary Structures Formed by T-Rich DNA

By
Zachary Michael Boynton

A thesis submitted to the faculty of the University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College

Oxford, Mississippi
May 2015

Approved By:

______________________________________________
Advisor: Dr. Randy Wadkins

______________________________________________
Reader: Dr. Tracy Brooks

______________________________________________
Reader: Dr. Susan Pedigo
Acknowledgements

I would like to acknowledge the Department of Chemistry and Biochemistry at the University of Mississippi for granting me access to their numerous resources, facilities and equipment during the time spent working on this project. First, I would like to thank my advisor, Dr. Randy Wadkins, for his guidance throughout the duration of this project. I have worked with Dr. Wadkins since my freshman year and this project would not be possible without his advice and commitment to seeing this project succeed. Also, I would like to thank Dr. Tracy Brooks and Dr. Susan Pedigo for serving as readers on my thesis project.
Abstract

Single stranded DNA that forms secondary structures has been a popular area of research in recent years. Much research has also been done in the field of heavy metals binding to DNA. Recently, it has been discovered that mercury in the presence of thymines will form T-Hg^{II}-T bonds. It is our hypothesis that single stranded DNA rich in thymine residues will form into a structure similar to i-motifs, formed from C-rich single strands, in the presence of mercury. Based on spectral changes during a mercuric titration, it is evident that mercury induces a structural change in T-rich DNA. We then tested the thermal stability of this structure via heat-induced denaturation. Job Plots were constructed to determine the number of Hg^{2+} binding sites on the DNA and size-exclusion chromatography experiments were conducted to determine if T-I-motif’s are formed inter- or intra-molecularly.
# Table of Contents

1) **Introduction**: ............................................................................................................. 1  

2) **Experimental**: ........................................................................................................ 4  
   2.1) **Materials**: ....................................................................................................... 4  
   2.2) **Mercuric Titrations**: ...................................................................................... 4  
   2.3) **Thermal Denaturation**: ................................................................................... 5  
   2.4) **Job Plots**: ...................................................................................................... 5  
   2.5) **PAGE Gels**: ................................................................................................... 6  
   2.6) **Size Exclusion Chromatography**: ................................................................. 6  

3) **Results and Discussion**: ........................................................................................ 8  
   3.1) **Evidence of Conformational Change**: ......................................................... 8  
   3.2) **Reversibility of Secondary Structure Formation**: ...................................... 10  
   3.3) **Binding Sites**: ............................................................................................... 12  
   3.4) **Structural Formation**: .................................................................................. 14  

4) **Conclusion**: ........................................................................................................... 16  

5) **Bibliography**: ....................................................................................................... 17
1) Introduction

The interactions between various metal ions and DNA have become a popular area of study in recent years. Due to their inherent toxicities, it is generally regarded that the introduction of heavy metal ions (Hg^{2+}, Pb^{2+}) are destructive to the nature of DNA. However, recent studies have shown that the presence of some heavy metals can have stabilizing effects on DNA under certain conditions. When heavy metals are present near DNA, the hydrogen bonds between base pairs are broken. The heavy metal will form a linkage between 2 nucleotides resulting in non-complementary base pairs. Research has suggested that many of the heavy metals can alter various secondary structures of DNA when introduced to the system (Onyido, 2004). In biological systems this can have varying implications as the manipulation of DNA structures can inhibit or promote DNA replication, protein expression and other biological processes.

It has been determined that in the presence of mercury, single stranded, T-rich DNA will often form T-Hg^{II}-T bonds (Figure 1) (Miyake, 2005). The research done by Miyake et al. has lead to the development of many practical applications surrounding heavy metals. Molecular beacon probes, which contain DNA-fluorophores, have been developed to detect the presence of mercury (Park, 2014). Other research has shown that mercury can induce enzymatic reactions. It has been shown that the formation of T-Hg^{II}-T bonds can elongate primers and trigger the
activity of DNA Polymerase in replication processes (Urata, 2010). Also, heavy metal ions can induce hairpins in single-stranded DNA, which can trigger the activity of various endonucleases (Li, 2011). Little is known about the conformational changes that can occur when mercury is introduced into a solution containing DNA that forms complex secondary structures.

![Figure 1: The proposed structure of a cis-binding thymine-Hg\textsuperscript{II}-thymine linkage.](image)

Extensive research has been done on secondary structures that form in single stranded DNA. G-quadruplexes and I-motifs form in strands of DNA that are rich in G and C nucleotides respectively. G-quadruplexes and I-motifs have been found serve as targets for gene regulation and gene expression in biological systems (Collie, 2011). The knowledge of these structures has led to clinical applications such as targets for new cancer therapies and practical applications like the development of pH sensors. Due to the understanding about secondary structures in single stranded DNA, like G-quadruplexes and I-motifs, it is possible that DNA rich in
thymine residues could form a secondary structure in the presence of mercury. The presence of Hg$^{2+}$ ions could cause a single stranded, T-rich DNA to fold back on itself and form a structure similar to an I-motif. The purpose of my research is to determine if mercury in the presence of T-rich DNA induces intermolecular or intramolecular structures. Also, this study hoped to determine the stability of these structures while learning about the characteristics of these metal-ion induced structures. The knowledge gained throughout this research will lead to a better understanding of the inherent toxicities that heavy metal ions pose to biological systems. It can also be used to further the knowledge used to generate practical applications of DNA secondary structures. A better understanding of the stability of T-Hg$^{II}$-T bonds can further the industries surrounding heavy metal ion probes and allow researchers to incorporate T-I-motifs, intramolecular structures formed by T-rich DNA in the presence of Hg$^{2+}$, into DNA-based nanostructures.
2) Experimental

2.1 Materials

The DNA used throughout this experiment is referred to as TiMo (sequence 5’-TTTCTTTCTTTCTTT-3’) and was purchased from the Midland Reagent Company (Midland, TX). The DNA was mixed into solution at 6.3 μM in 20 mM MOPS buffer at pH 7.1. The Hg(ClO₄)₂ was purchased from Alfa Aesar and dissolved in the same 20 mM MOPS buffer.

2.2 Mercuric Titrations

A 5.5 μM sample of TiMo was prepared and the concentration was checked by heating the sample to 90 °C for 5 minutes and then collecting the absorbance at 260 nm on a Cary 100 UV/VIS spectrometer. After an appropriate DNA sample was prepared, a mercury titration was conducted and analyzed using an Olis DSM 20 Circular Dichroism (CD) spectrometer. Hg(ClO₄)₂ was added in 5 μM increments from 0 μM – 95 μM or until there was no change in the spectrum. The spectrum was generated between wavelengths 240-350 nm. This test was used to confirm that there were conformational changes in the structure of TiMo. Also, by conducting a series of titrations, an understanding of the necessary concentration of Hg²⁺ ions that must be present in order for there to be structural changes in TiMo.
2.3 Thermal Denaturation

After conducting a series of mercuric titrations of TiMo, thermal melts were conducted on this strand under the presence of mercury. A 5.5 μM solution of TiMo was made up in 20 mM MOPS buffer. The concentration of the solution was found by heating the sample to 90°C for 5 minutes and then collecting the absorbance at 260 nm on a Cary 100 UV/VIS spectrometer. Next, 65 μM of Hg(ClO₄)₂ was added to this solution. This concentration of mercury was chosen because this was the concentration of mercury where changes in the spectra during the titration ended. A thermal denaturation experiment was then conducted on the sample by taking a spectra from 240-350 nm. The temperature was increased from 20 °C-90 °C by 1 °C and held for 1 minute before the spectra were taken by the CD. Thermal denaturations provide information on the thermal stability of the structure formed by the T-Hg²⁺-T bonds. These tests also provide information on whether or not the reaction between thymines and mercury ions is reversible.

2.4 Job Plots

After conducting a series of t-melts to better understand the thermal stability of the T-I-motif structure, a Job Plot was constructed. Job plots provide information regarding the number of binding sites present in a structure. Nine different samples of DNA were made at concentrations, 1.6 μM, 2.9 μM, 4.3 μM, 5.6 μM, 6.8 μM, 8.2 μM, 9.5 μM, 10.9 μM and 12.3 μM. Hg(ClO₄)₂ was added to the solutions in concentrations of 10.7 μM, 9.4 μM, 8.0 μM, 6.7 μM, 5.5 μM, 4.1 μM, 2.8 μM, 1.4 μM and 0.0 μM respectively. It is necessary for the total molar concentration (the sum of the molarity of TiMo and the molarity of Hg(ClO₄)₂ to be the same for all samples.
used in the Job Plot. Once the UV/Vis spectra for all of these samples were collected, the difference in absorbance for the spectrum generated without mercury and the spectrum generated with mercury was plotted against the mole fraction of both mercury and DNA. The number of binding sites present in the TiMo DNA can be inferred from this plot.

2.5 PAGE Gels

Polyacrylamide gel electrophoresis (PAGE) was used to analyze potential conformational changes in the structures formed from the addition of Hg$^{2+}$ to TiMo. 12% PAGE gels were cast in the BioRad gel casters. A 2-Log DNA ladder purchased from New England BioLabs was loaded into the first lane of the gel to determine how big the strands of DNA were after the addition of Hg$^{II}$. A 5.5μM solution of TiMo was taken and varying concentrations of mercury were added to the solution. Concentrations of 0, 10, 20, 30, 40, 50, 60, 70, and 80 μM Hg(CLO$_4$)$_2$ were added to the solutions and then loaded into the gel. The gel was electrophoresed at 100 volts for approximately 10 hours. After the gels had finished running, they were stained in SYBER GOLD for 20 minutes and then imaged using a BioRad Gel Doc 2000 camera. The results from the PAGE gel experiments should be able to determine if the T-I-motifs are forming intermolecularly or intramolecularly. Also, by running several samples with varying concentrations of mercury, the concentration necessary to form a T-I-motif can be determined.

2.6 Size Exclusion Chromatography

Due to the poor, unreadable results generated by PAGE gels, size exclusion chromatography experiments were conducted in an attempt to determine if the
structure formed in the presence of mercury is intermolecular or intramolecular. A 20 mM MOPS, 100 mM NaCl buffer at pH 7.1 was used to equilibrate a Superose 12 10/300 GL column. Roughly 150 mL of buffer was run across the column during the equilibration. Next 50 μL of a 5.5 μM solution of TiMo was run through the column and the elution volume was recorded. Then 50 μL of a 5.5 μM solution of TiMo containing 65 μM of Hg(ClO₄)₂ was run through the column. The elution volumes were compared in order to determine if the secondary structure of TiMo in the presence of mercury is intermolecular or intramolecular.
3) Results and Discussion

3.1 Evidence of conformational change

A series of mercuric titrations showed us that this strand of DNA is undergoing some conformational change when mercury is present. It is apparent that around 65 μM of Hg(ClO$_4$)$_2$, the 5.5 μM solution of TiMo has become fully saturated (Figure 2a). After some data analysis, plotting the absorbance at 264 nm for all spectrum generated during the titration against the logarithm of the concentration of Hg(ClO$_4$)$_2$ added to the TiMo solution (Figure 2b), the $K_d$ of this reaction was determined to be around 21.3 μM ± 3.3 by looking at the half way point of the titration curve generated and confirmed with the data analysis program Igor Pro (Figure 2c). This suggests that the equilibrium of this reaction will fall to the right and a lot of product will be formed. It is important to note that the lack of an isobestic point during the titration could suggest that there are more than two species being formed during this reaction. Having a transition point during a reaction is not entirely uncommon, but it would change our understanding of the potential T-I-motif folding mechanism. Also, this would suggest that there are multiple Hg$^{2+}$ binding sites. If we assume the intrinsic affinity of the binding sites is equal then they interact with a cooperativity factor of 300. This cooperativity between Hg$^{2+}$ binding sites can indicate that the binding of one mercury can
increase the affinity of another binding action. Also, this cooperativity can suggest a favorable conformational change such as the T-I-motif formation. However, these titrations do not provide a complete understanding of these reactions and other experiments will need to be conducted in order to fully understand what happens when Hg$^{2+}$ is added to TiMo.
Figure 2: (a) Shows the changes in absorbance of TiMo as Hg(ClO$_4$)$_2$ is added to the solution. The changes in spectra suggest that there is a conformational change in the structure of the DNA. (b) This graph is the absorbance at 264 nm plotted against the logarithm of the concentration of Hg(ClO$_4$)$_2$. From this graph we were able to determine the $K_d$ value of this reaction. (c) Shows the table that was constructed during the data analysis of this experiment.

3.2 Reversibility of Secondary Structure Formation

Little was known about the properties surrounding the process of the formation of the T-Hg$^{II}$-T linkage. At first, it was unclear if the Hg$^{2+}$ ions would release from thymine residues or if this was a permanent linkage. However, due to a very basic, preliminary thermal test, it was observed that there is a shift in the spectra when the sample is heated. A standard solution of 5.5 μM TiMo was saturated with 65 μM of Hg(ClO$_4$)$_2$ and the spectrum was taken at 20 °C. The solution was then heated to 90 °C and the spectrum experienced a red shift, the spectrum at 90 °C was very similar to the spectrum generated without the presence of Hg$^{2+}$ ions. When the solution was cooled back down to 20 °C, it recreated the original spectra (Figure 3a). After this preliminary test showed that the reaction was reversible, a complete thermal denaturation experiment revealed that the actual melting point of this
structure lies somewhere above 70 °C (Figure 3b). It is difficult to find an exact melting point of this structure because as the temperature approaches 100 °C concerns about boiling of the solution began to play a role. However, 70 °C appears to be a close approximation of the melting point of this structure. This figure is interesting as it appears that the structure could be biphasic as there is a decrease in the spectra between 20 – 30 °C. This would support the evidence shown in the mercuric titrations. The thermal denaturation provided evidence to support the theory that these structures are easily formed and stable, and that the reaction is reversible.
Figure 3: (a) Confirms that the linkage formed between TiMo and Hg(ClO$_4$)$_2$ can be denatured at high temperatures and will reform when the temperature decreases. (b) Thermal denaturation scan of TiMo in the presence of the Hg$^{2+}$ ion. The distinct increase in absorbance at 70 °C suggests that this is the temperature where the T-Hg$^{II}$-T formation denatures.

3.3 Binding Sites

Several Job Plots were conducted to try and estimate the number of Hg$^{2+}$ binding sites that are present on the TiMo strand. This information can lead to a better understanding of the possible structure that is formed in the presence of mercury.

By looking at the peak of the Job Plot, the number of binding sites in the structure can be inferred by comparing the mole fraction of Hg(ClO$_4$)$_2$ to the mole fraction of TiMo. However, the exact location of the peak can be difficult to determine because as the number of binding sites in the structure increases, the difference in the ratios (say determining six vs. seven binding sites) decreases exponentially (this is why
Job Plots are best to analyze structures that have a maximum of four binding sites.

The data analysis of the Job Plot conducted on TiMo suggests that there could be at least six binding sites on TiMo. The peak in the curve generated in this study was roughly 0.12 (Figure 4). This data is slightly skewed because it is difficult to make solutions that accurately represent the varying, possible ratios of binding sites. This data suggests that the secondary structure that TiMo forms in the presence of mercury is intermolecular because six binding sites would be the maximum number in an intramolecular structure. A duplex could have as many as 12 binding sites for Hg$^{2+}$ ions. In practice it is difficult to distinguish a ratio of 1/6 from 1/12 because the room for error is essentially zero.

![Job Plot of TiMo and Mercury](image)

**Figure 4**: Job plot conducted on TiMo and Hg(ClO$_4$)$_2$. The peak of this graph falls at approximately 0.12 which suggests that there at least 6 Hg$^{2+}$ binding sites on the TiMo structure
3.4 Structural Formation

Due to the poor results generated by PAGE gels, size exclusion chromatography experiments were conducted to try and determine if T-I-motifs were forming as a result of intramolecular folding of secondary structures or if duplexes were forming as a result of intermolecular folding. Because samples in PAGE gels move due to their electrostatic attraction, it is believed that PAGE gels did not work in this experiment because as each Hg$^{2+}$ ions binds to DNA it equilibrates two of the negative charges associated with the phosphate backbone of DNA. If the charge on DNA were neutralized, there would be no force to move the DNA through the PAGE gel (Figure 5a). Therefore, a 5.5 μM solution of TiMo was run through the size exclusion chromatography column. The elution volume for this sample was approximately 13.49 mL. Next a 5.5 μM solution of TiMo with 65 μM Hg(ClO$_4$)$_2$ was run through the same column. The elution volume for this sample was approximately 13.57 mL (Figure 5b). These results are relatively inconclusive. There is no distinct shift in the elution volume between the sample without heavy metals and the solution that has Hg$^{2+}$ ions. The one interesting portion of the results generated from the size exclusion chromatography experiment was the slight peak that was present in the sample containing mercury that exists between 21.33 mL – 23.57 mL. This suggests that there may be some intramolecular structure forming in solution. By including some concentration of Hg(ClO$_4$)$_2$ in the buffer, the latter peak may be maximized. When the sample encounters the buffer in the column, the concentration of mercury decreases drastically which may break the linkages that are forming in the intramolecular structure.
Figure 5: (a) Shows results from a PAGE gel. The ladder on the right is smeared and indistinguishable. The lane immediately to the left shows some signs of DNA, but the remaining lanes all appear to be without sample. (b) This graph shows that are results in this experiment are relatively inconclusive. There is no distinct shift in the elution volume.
4) Conclusions

This research was an attempt to determine if the presence of Hg$^{2+}$ could induce the formation of an intramolecular secondary structure in T-rich DNA. Determining the nature of the structure that was formed in the presence of mercury was not possible at this junction in this research. There is evidence that suggests an intramolecular structure forms, but until further testing is conducted these results remain unclear. While a definitive answer surrounding our hypothesis was not found, a lot was learned about this reaction. Various experiments helped to quantify $K_d$ values, the temperature at which thermal denaturation occurs and the number of binding sites present on the TiMo structure. These experiments also lead us to believe that there could be more than 2 species being formed in the presence of mercury. This suggests that there could be a formation of a transition state prior to the folding of the T-I-motif structure. These sets of experiments also showed that the T-Hg$^{	ext{II}}$-T linkage could be formed in non-complementary strands of DNA. Previous research has all been done on complementary strands that had mismatched T-T nucleotides and the T-Hg$^{2+}$-T linkage was confined to these mismatched base pairs. Research could be continued by adding Hg(ClO$_4$)$_2$ to the running buffer in the size exclusion chromatography experiments as well as altering the sequence of TiMo to test if a longer, more flexible strand of DNA could help induce the formation of an intramolecular secondary structure.
5) Bibliography


