MODELING THE IMPACTS OF 2-AMINOPURINE ON RNA BINDING AFFINITY AND STRUCTURAL DISRUPTIONS VIA FLUORESCENCE SPECTROSCOPY

by

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A THESIS

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Title: Modeling the Impacts of 2-aminopurine on RNA Binding Affinity and Structural Disruptions via Fluorescence Spectroscopy

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Within bacteria, Riboswitches function as regulatory RNA that components that control the gene expression of an mRNA by responding to a ligand and causing conformational changes to the riboswitch. The growing knowledge of riboswitches and their implications on mRNA regulation of gene expression shows promises in future therapeutic targets. A current issue with antibiotic therapies is the dangers associated with increasing antibiotic resistance in certain bacteria so the field of study of non-antibacterial therapeutic targets is ever growing. Non-antibacterial therapeutic targets are potential mechanisms for executing the same antibacterial activity as traditional antibiotics, but the bacteria does not develop resistance as it is not a traditional antibiotic. It has been hypothesized that bacterial death can be induced when enough non-essential genes are turned off by controlling the conformation of the riboswitch that regulates the different mRNAs. Although there is a lot of promise in developing riboswitches as therapeutic target without increasing antibacterial resistance, there is still a lot to be understood and learned regarding the fundamentals of riboswitches and their folding dynamics.

In this study, we aim to resolve discrepancies in existing literature for the folding dynamics of a riboswitch that senses the ligand $preQ_1$ where one source demonstrates the riboswitch maintaining as single conformation regardless of the ligand's presence while another source shows dynamic conformational responses to the ligand. A clearer understanding of these fundamental elements of riboswitches is required to further the field of its efficacy as a non-antibacterial therapeutic target. We show in this study that the fluorescent base analogue 2-aminopurine can be used as a probe to study the local structural changes of the $preQ_1$ riboswitch in response to a ligand. Future optimization is required to resolve the discrepancy found in existing literature, but current methods discussed here shows promise in resolving these issues.

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Table of Contents

List of Figures

List of Tables

Introduction

Riboswitches are RNA molecules that plays a crucial role in gene regulation in bacterial species[1]. A riboswitch contains two regions that will interact with a metabolite-like ligand. The aptamer domain provides a very selective binding site for a specific ligand and the expression platform responds to this signal by folding which directly affects the regulation of mRNA the riboswitch resides in[2]. Amongst other bacterial species, the aptamer domain for each class is highly conserved allowing different riboswitches in different organisms to bind the same type of ligand[1]. Furthermore, roughly 4% of bacterial genes are regulated by riboswitches and disruptions to these regulatory steps could be sufficient to have a lethal effect on bacteria[3].

The $preQ_1$ riboswitch

The $preQ_1$ riboswitch used in this study is derived from *Bacillus subtilis* which regulates the gene expression in this bacterium for producing queuosine[4]. The $preQ_1$ riboswitch is found in several bacterial species, but this study utilizes the one derived from *Bacillus subtilis*. The figure below shows a cocrystal structure of the $preQ_1$ riboswitch from *Bacillus subtilis* bound to its ligand[5].

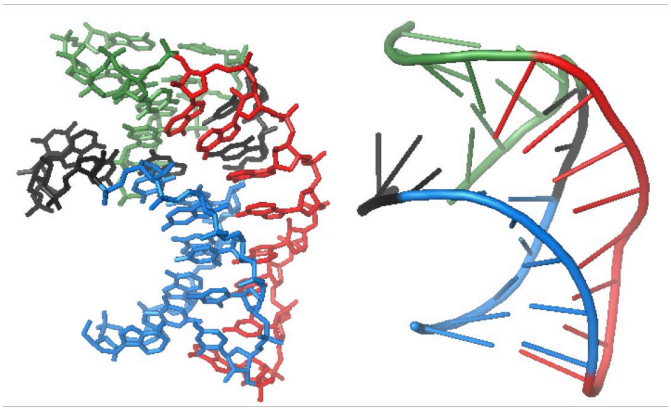

Figure 1. Cocrystal structure of the preQ$_1$ riboswitch from *Bacillus subtilis*

This figure depicts a cocrystal structure of the preQ$_1$ riboswitch primarily used in this study. PDB ID: 3FU2

The preQ$_1$ riboswitch has an aptamer domain only 34 nucleotides long making it the smallest known riboswitch. This riboswitch can be found in *Bacillus subtilis* where it regulates the synthesis of queuosine nucleobases by binding a preQ$_1$ metabolic intermediate that induces folding and a drop in queuosine production[1].

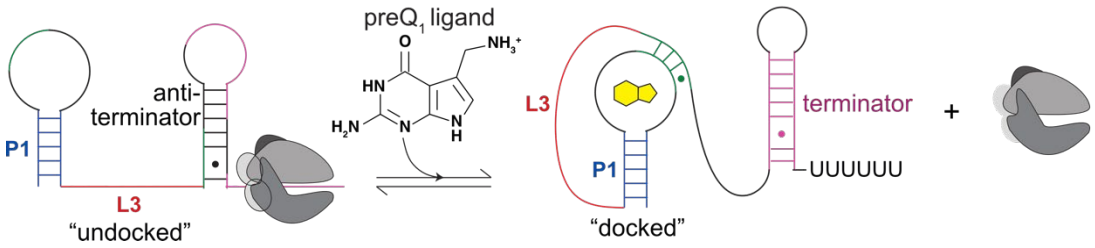

Figure 2. Riboswitch regulation of bacterial gene expression

This reaction schematic shows how recognition of the preQ$_1$ ligand causes a conformational change to the riboswitch. Before binding the ligand, RNA polymerase synthesizes the mRNA regulated by the riboswitch and binding the ligand forms a pseudoknot structure forming a terminator loop. This causes RNA polymerase to dissociate from the mRNA thus terminating transcription. The P1 region will interact with the L3 region, where base analogue substitution is performed, in response to its ligand. Figure created by Dr. Julia Widom.

The preQ$_1$ riboswitch is an interest of study because there is a large discrepancy in the riboswitch behavior when the FRET (Förster resonance energy transfer) data are

analyzed against cocrystal structures in both conformations. FRET measurements have revealed that the riboswitch will fold tightly into a pseudoknot structure in the presence of $preQ_1$ ligand while it remains in the unfolded state in the ligand's absence[1]. However, cocrystal structures in alternative literature has indicated that the riboswitch remains in the tightly folded conformation in both the presence and absence of $preQ_1$ revealing a large discrepancy[5]. We would like to better understand the origins of this discrepancy and the potential interactions between the P1 and L3 regions through fluorescence spectroscopy.

Broad research goals

Learning about riboswitches and the mechanisms that dictate their folding allows for control over the production of proteins at key regulatory steps. Although a certain riboswitch is not guaranteed to control the regulation of an essential gene for survival, targeting and disrupting multiple nonessential genes can be sufficient to produce a lethal effect. Additionally, understanding the binding mechanisms of a riboswitch allows researchers to design a metabolite analogue as a ligand that could selectively repress a targeted expression pathway and achieve the desired lethal effect[3].

Background: principles of fluorescence spectroscopy

Fluorescence spectroscopy uses the principle of excitation and emission where a molecule can be excited before emitting a specific wavelength of light as it relaxes back to the ground state[6]. Fluorescence spectroscopy can be used to study the $preQ_1$ riboswitch by replacing adenine with its fluorescent analogue 2-aminopurine (2-AP) within the L3 region. 2-AP can replace adenine without disrupting the structure too

much since it differs from adenine only by the position of the NH_2 group on the purine ring[7]. We can use 2-AP to study the L3 region it is placed in because its fluorescence is quenched by bases in the immediate vicinity. Location-specific data regarding structure can be obtained from fluorescence measurements since a change in its fluorescence between measurements indicates a change in the local structure due to bases quenching 2-AP fluorescence. Using this data, we can learn about how 2-AP substitution in the L3 region perturbs the riboswitch's binding abilities and how the fluorescent probe can be used to selectively disrupt the structure furthering efforts in new antibacterial targets.

A discrepancy in existing literature

Current literature reveals that 2-AP is a functional base analogue that can be used to study RNA and DNA molecules due to its quenching characteristics, but it is also understood that 2-AP perturbs the structure in some way. The binding affinities of the unmodified riboswitch have also been recorded allowing for a comparison of the modified samples' data[8]. However, there is no research yet that provides a way for selective disruption of the riboswitch by 2-AP substitution. Learning how to alter a gene regulatory step in bacteria through selective 2-AP substitution allows for medical applications where antibacterial drugs can be made without concern of raising antibiotic resistance.

A crucial discrepancy between research on the $preQ_1$ riboswitch's folding response to the ligand also reveals a need for further study on the local changes that occur during the folding process. The crystal structures reported in previous literature has shown that the riboswitch will remain tightly folded in a pseudoknot structure in both the presence and absence of the ligand[5]. However, FRET measurements performed

on the riboswitch in both the presence and absence of ligand that the riboswitch does not remain tightly folded in the ligand's absence[1]. My research aims to understand the root of these through studying the local changes that occur during folding and the possibility of different 2-AP positions perturbing the structure in a different way. In addition, studying the interactions that might occur within the less-folded structure might afford new information to explain these discrepancies. This demand for study of the local riboswitch structure coupled with 2-AP and its ability to perturb a structure makes fluorescence spectroscopy a convenient way of studying the two phenomena.

My research looks to study the structural disruptions of 2-AP in the L3 region of the $preQ_1$ riboswitch to learn how selective probing and structural disruptions might be induced. Previously, it has been recognized that designing a small-metabolite analogue that functions as the ligand opens an avenue for designing an antibacterial drug therapy by directly affecting gene regulatory steps on a molecular level[3]. However, it has not been investigated how 2-AP might be used as a selective probe for perturbing the riboswitch structure therefore affecting its ability to bind its ligand. Both ways accomplish new antibacterial targets, however in cases where designing a functional metabolite analogue proves to be challenging, altering the riboswitch aptamer domain with selective 2-AP substitution might prove to be more functional.

Specific research question

Time permitting, we would like to study the local structure and effects of 2-AP substitution at six different positions within the L3 region. It is understood that 2-AP slightly disrupts the riboswitch's structure, but we are looking at how the local structure is disrupted depending on the position of the 2-AP substitution. Comparing the binding

constants between the unmodified and modified riboswitch provides a convenient way to understand how 2-AP could be used to selectively perturb the structure since the ligand's dissociation constant (K_D) will only change when there is a difference in local interactions. Depending on the base sequence, base stacking quenches 2-AP fluorescence to different extents, revealing the local folding at different concentrations of the ligand rather than a global perspective. Due to the high sensitivity of fluorescence spectroscopy, we are able conduct experiments on samples with low concentrations of the riboswitch.

With the advantage of fluorescence spectroscopy's high sensitivity, we are currently studying samples where the concentration of the riboswitch is lower than the riboswitch's K_D. The dissociation constant gives a direct measure for the tightness of the ligand binding to the riboswitch because it measures the concentration of $preQ_1$ required so that 50% of the riboswitch is bound to it. This is convenient in insuring that the extrapolated K_D is the true 50% bound state of the riboswitch and ligand and not from a lack of ligand when the riboswitch concentration is too high. However, initial data collected with this protocol has led to issues in baseline fluorescence, thus we are adjusting the protocol so that the riboswitch concentration is higher than what we are currently working with in hopes that this corrects the baseline issues.

Smaller dissociation constants indicate more stable binding, and the unmodified riboswitch has a K_D in the low nanomolar range[8]. Investigation of the different K_D values of the different 2-AP positions on the riboswitch allows for a comparison with the dissociation constant of the unmodified riboswitch. The sample whose K_D is very similar to the unmodified sample reveals that placing 2-AP at this location within the L3

region will allow the modified sample to bind ligand like the unmodified riboswitch. Further comparison will also reveal the positions in the L3 region that might be probed with 2-AP for intentional, selective disruption of the structure so that the riboswitch's binding mechanism is altered from the unmodified riboswitch. The K_D is calculated using equation 1 through a ligand titration where fluorescence is measured with fluorescence spectroscopy.

Project design

To study the impacts of base substitution in the L3 region of the riboswitch, adenines in various positions within this region can be substituted with a fluorescent base analogue. Adenines in positions 27 and 29 are substituted with 2-AP, and fluorescence emission scans are taken to create a titration curve from which we can extrapolate binding coefficients. Substitution in different positions within the L3 region allows for studies of local conformational changes and how slight perturbations to the riboswitch structure can affect its binding kinetics. This project design hopes to further illuminate the reason behind the discrepancy found in existing literature.

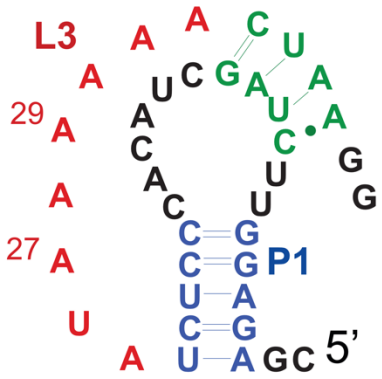

Figure 3. The preQ$_1$ riboswitch sequence map for selective base analogue substitution

The L3 region are colored in red. For the purposes of this study, probing the modified riboswitch in positions 27 and 29 with a fluorescent-base analogue allows for studying the changes in local dynamics. This figure maps out the entire modified riboswitch used in the study along with the positions where adenine in the L3 region is substituted for 2-AP. An understanding of how structural disruptions can perturb the binding kinetics of the riboswitch is conducted this way via selective substitution.

How 2-AP can be used in this study

The molecule 2-aminopurine (2-AP) is a fluorescent analogue of adenine where this can be substituted for an adenine in the L3 region while only causing slight perturbations to the structure. The figure below depicts how 2-AP is only slightly different in its binding motifs with uracil, allowing for the modified riboswitch to have preserved folding dynamics since Watson-Crick base-pairs can still form.

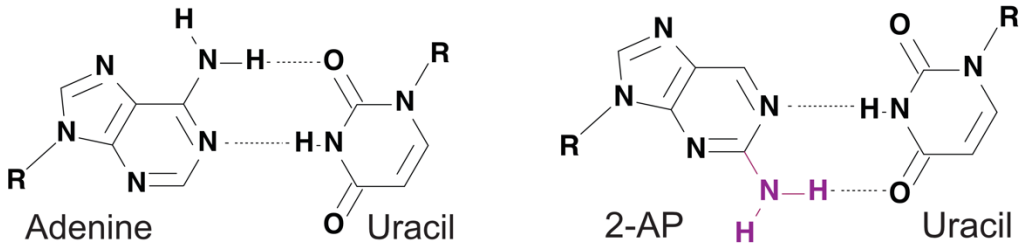

Figure 4. The functionality of adenine base substitution with 2-aminopurine preserves Watson-Crick base pairing

2-aminopurine is a fluorescent base analogue of adenine where it is still able to base pair with uracil in an RNA such as the riboswitch under study. Rather than forming a hydrogen bond with the oxygen adjacent to the amine on uracil, 2-AP will form a hydrogen bond with the other oxygen atom on uracil.

In addition to the usefulness of 2-AP as a fluorescent analogue of adenine, it can preserve the base pairing motifs while exhibiting fluorescence that can be quenched via base stacking, allowing for fluorescence studies as a way of understanding the folding dynamics of this riboswitch. In theory, the fluorescence emission should no longer change once the riboswitch is saturated with ligand since no more folding occurs thus no more fluorescence quenching. Furthermore, 2-AP is also helpful in conducting local structural studies because it can be selectively excited independent of the absorbances of the native bases (Figure 5). This project design allows for the study of local structural changes while minimally modifying the structure of the riboswitch.

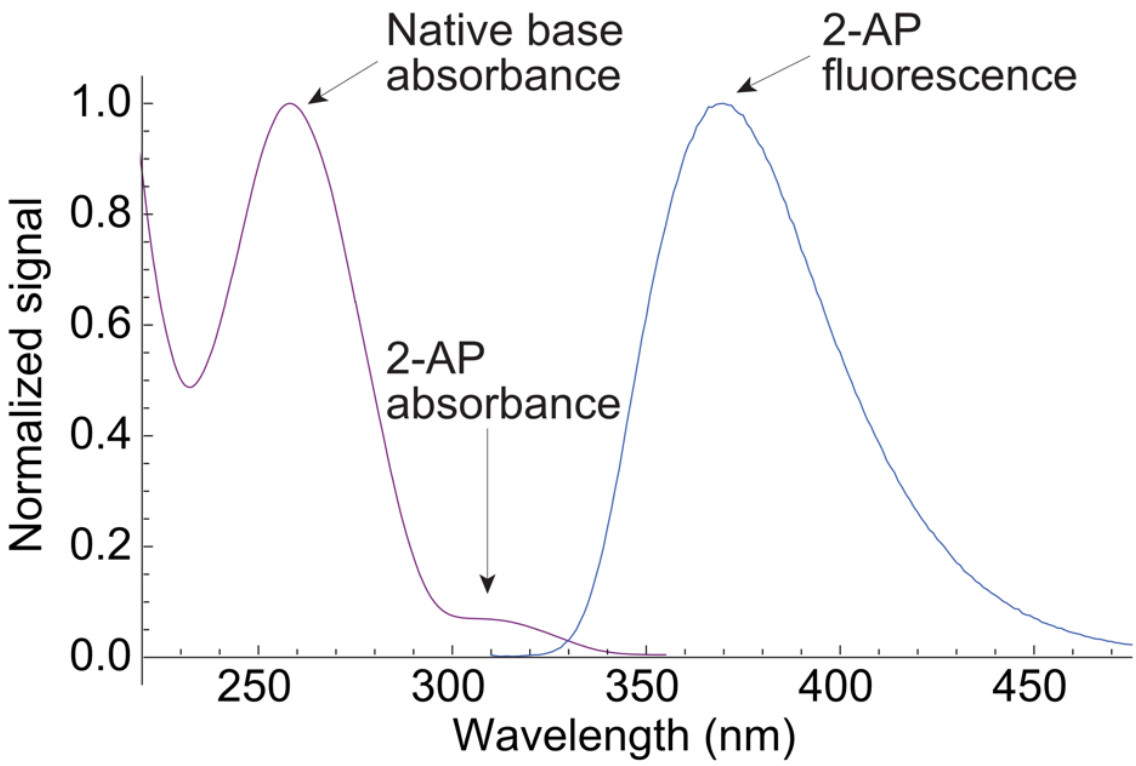

Figure 5. Comparison of native RNA bases to 2-AP absorbance and fluorescence

2-AP absorbance is red shifted from the absorbance of the native bases allowing for selective excitation of the fluorescent probe while minimally perturbing the native bases. The fluorescent 2-AP can be excited at 295 nm so that only the probe is excited.

The figure above depicts how 2-AP can be used for studying the local changes in structure of the riboswitch due to both base stacking quenching fluorescence and its ability for selective excitation of the probe since it is red shifted from the absorbance of the native bases.

Results

Protocol I fluorescence emission titration

The data presented in figure 6 was collected using "protocol I". In this protocol, the riboswitch is at a 100 nM concentration. Upon each ligand addition, the sample was pipette mixed with an autoclaved multiflex tip that inadvertently introduced a contaminant present in the negative fluorescence at higher wavelengths and major peaks shifts.

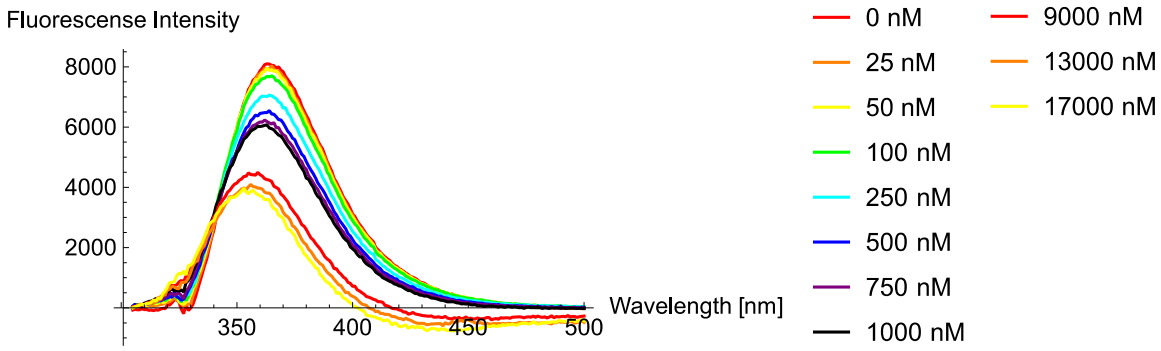

Figure 6. Emission spectra of riboswitch modified with 2-AP at position 29

This fluorescence emission titration was taken on February 25, 2021, where the sample was pipette mixed with the autoclaved multiflex tip upon each ligand addition. Several of the emission intensities towards higher wavelengths are not baseline corrected to reflect negative fluorescence. Baseline correction at higher wavelengths refers to averaging and setting the intensities to zero when their raw values are close enough to zero.

In the titration curve above, the major peaks shift around different wavelengths where they are expected to remain along the same wavelength. This is evidence of contamination of the sample, and we hypothesized that it could be coming from the multiflex tip used to pipette mix the sample upon each ligand addition. The major peak shift and negative fluorescence seen at higher wavelengths and ligand concentrations

further reflects the need for more optimization. The data presented in figure 6 has no baseline correction for the last data points towards higher wavelengths to further show the accuracy of the data collection. Negative fluorescence should not be observed indicating a possible error and need for adjusting the protocol. We repeated this experiment on a separate day, testing the hypothesis of the multiflex tip contaminant by replacing it with a 200 μL pipette tip for mixing.

In the figure below, a binding isotherm equation fits the data where a curve of is presented as fluorescence absorbed changes with ligand absorption as a function ligand concentration at fixed temperatures. Figure 7 shows the equation fitting of the data with a binding isotherm equation where the data fits better than the quadratic equation 1. This occurs when the riboswitch is at a much lower concentration than the K_D of the riboswitch, so the data taken in figure 6 is fit better with equation 2 (binding isotherm equation) than equation 1.

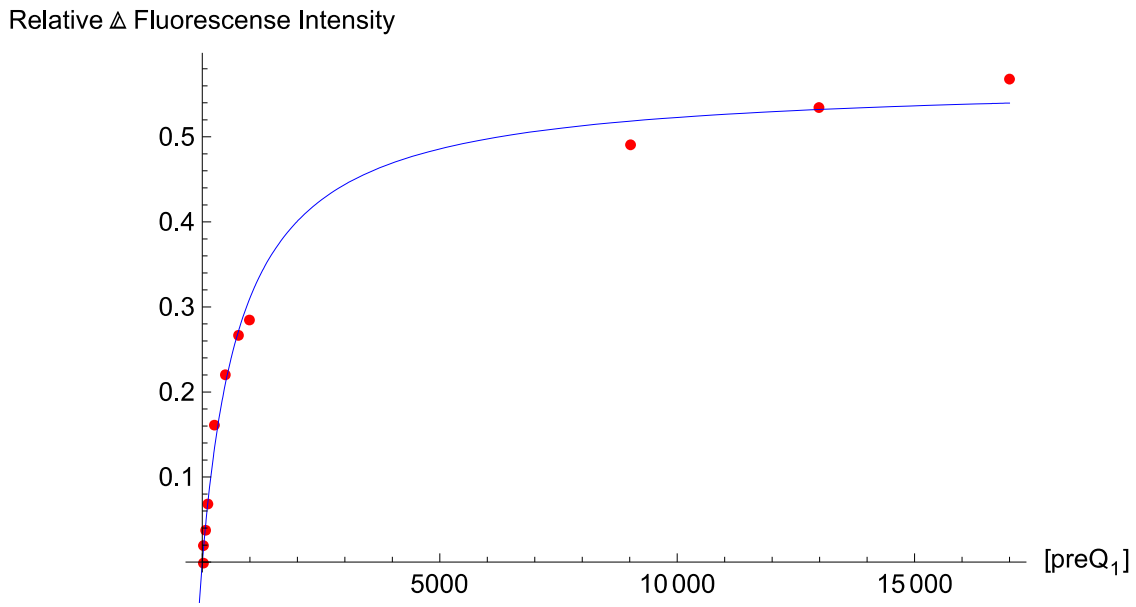

Figure 7. Position 29 modified riboswitch titration fitted to a binding isotherm equation

The K_D of the data fitted in this equation returns a value of 824 nM. The data is fitted into equation 2 where this is a binding isotherm equation rather than the quadratic equation in equation 1.

The binding isotherm fits the data points from the titration performed in figure 6 much better than the quadratic equation because the riboswitch is at a much lower concentration of 100 nM compared to the K_D in protocol I.

Protocol I titration replicate: exclusion of pipette tip contaminant

The data presented in figure 6 and 7 uses protocol I where the pipette tip used to mix the sample upon each ligand addition was switched from the multiflex tip to standard 200 µL pipette tip. Here, the data comes much closer to an ideal dataset where there is less negative fluorescence at higher wavelengths and ligand concentrations and all major peaks do not shift. The improvement of this dataset using protocol I over the data collected in figure 6 represents successful elimination of the pipette tip contaminant, however still not an ideal dataset.

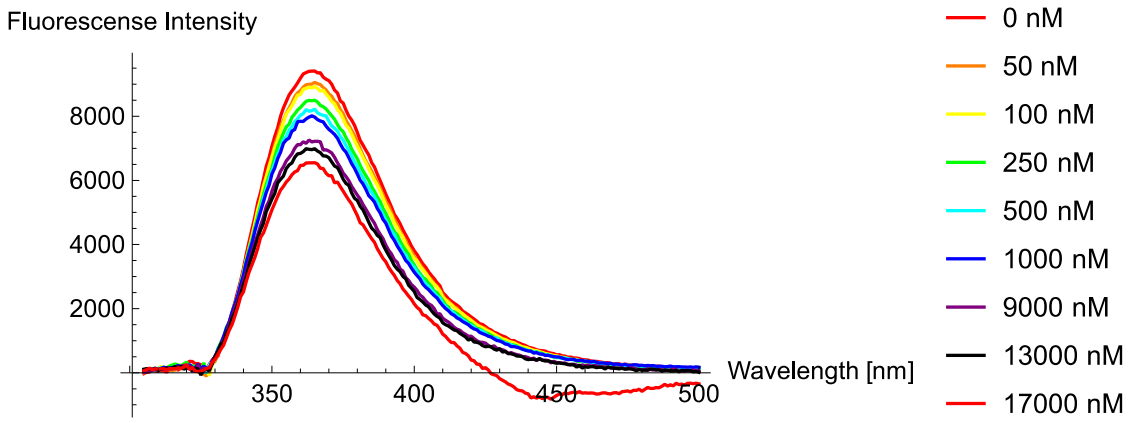

Figure 8. Emission spectra of riboswitch modified with 2-AP at position 29 replicate with new pipette tip

This data was taken on March 3, 2021, where the sample was pipette mixed with a new 200 µL tip rather than the multiflex tip. Major peaks do not shift. Replicate data taken on different days are still not ideal with the present negative fluorescence prompting change in protocol. Several of the emission intensities towards higher wavelengths are not baseline corrected to reflect negative fluorescence.

Data in figure 8 still has the riboswitch at a 100 nM concentration and apparent negative fluorescence is still observed at higher wavelengths and ligand concentrations, although the major peaks are now stable at the same wavelength. The last few data points towards higher wavelengths were not baseline corrected to further reflect accurate data. Since this data set is still not ideal and the K_D values from each were inconsistent, a greater adjustment to the protocol was made.

The figure below shows the fitting of the titration data with equation 2. The binding isotherm equation provides a much better fit than equation 1 since the riboswitch is still at 100 nM.

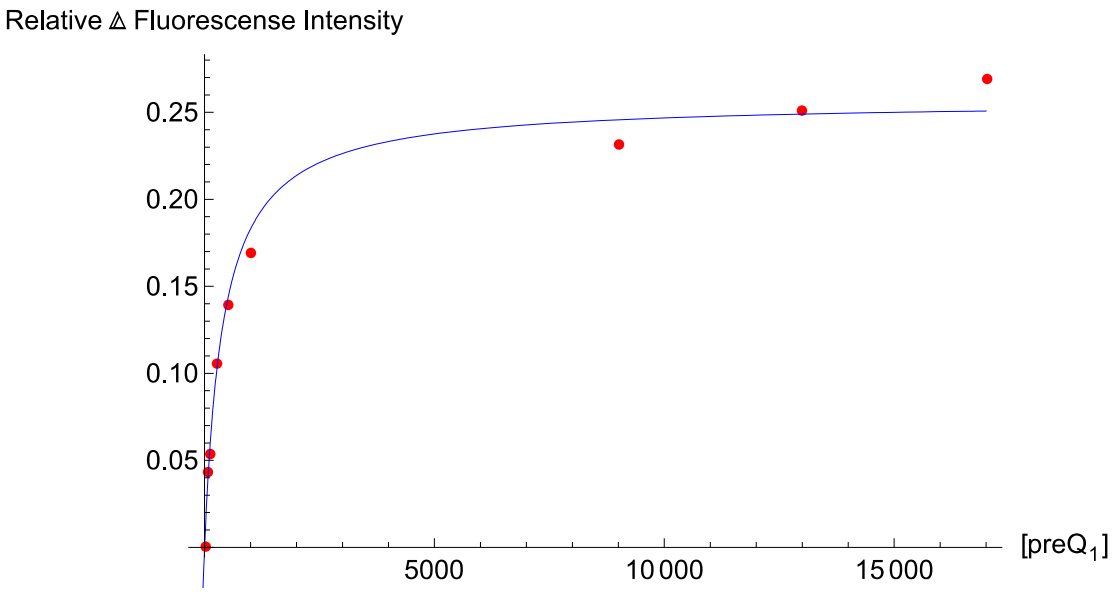

Figure 9. Position 29 modified riboswitch replicate data titration fitted with binding isotherm equation

The K_D from this replicate dataset is 402 nM. The K_D from this replicate data is almost half the K_D of the equation fitting presented in figure 7. The riboswitch is at 100 nM again, however the K_D is at a much higher concentration than the riboswitch representing accurate data.

The data presented in figures 6 and 8 both utilize protocol I with a slight adjustment to the pipette tip used to mix the sample. However, the K_D extrapolated from each dataset are vastly different from one another suggesting further optimization of the protocol being used to study the $preQ_1$ riboswitch. The K_D in presented in figure 9 is much more accurate due to a better fitting into the binding isotherm equation along with a K_D that is much higher than the riboswitch concentration, which is expected.

Protocol II fluorescence emission titration

In the data presented in figure 10 and 11, the riboswitch is now at a 500 nM concentration similar to the riboswitch concentration used in Frener and Micura's reported results, which reflects an ideal titration curve with this method[9]. The ligand

concentrations were also adjusted to reflect the ligand concentrations used in their paper. The modified riboswitch was probed in position 27 rather than position 29. Instead of pipette mixing, an overall improvement was seen in switching from the previous mixing method to magnetic stirring in the stacked spectra.

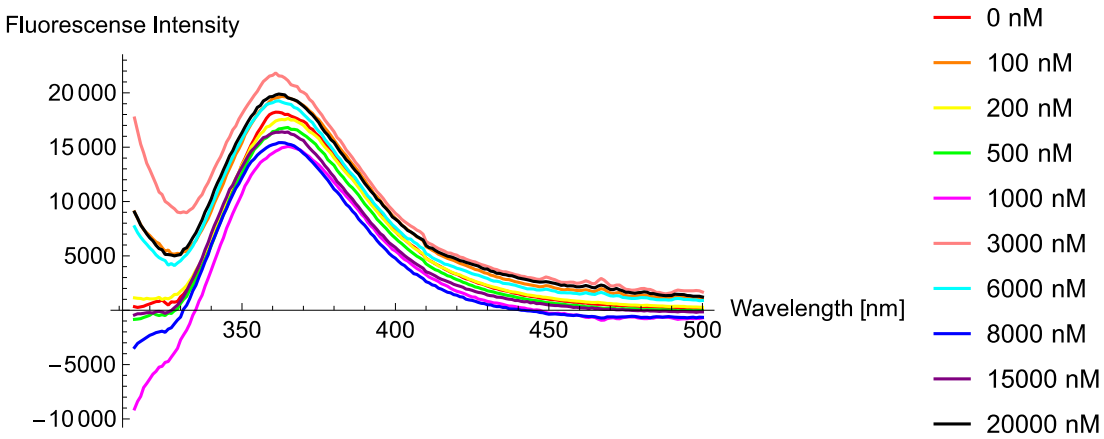

Figure 10. Emission spectra of riboswitch modified with 2-AP at position 27 with protocol II

This data was taken on February 8, 2022, where protocol II implementation is reflected. Major peaks stay within the same relative wavelength and negative fluorescence is greatly reduced or mostly negligible at higher wavelengths. Emission intensities towards higher wavelengths are not baseline corrected to reflect more accurate data.

The improvements of magnetic stirring over pipette mixing can be seen in this dataset where there is no negative fluorescence at higher wavelengths and the major peaks do not shift compared to the previous datasets using protocol I. This is closer to the ideal titration dataset presented in Frener and Micura's supplemental information[9]. However, there is still negative fluorescence and peaks seen at lower wavelengths that further work and optimization is required with "protocol II". In this protocol, the riboswitch is at a 500 nM concentration.

The data is expected to be fit much better into the quadratic equation than the binding isotherm equation because the riboswitch is now at 500 nM, an order of magnitude larger than its K_D. However, the figure below that data taken with protocol II yielded non-monotonic behavior, making it impossible to obtain a K_D, possibly due to experimental errors.

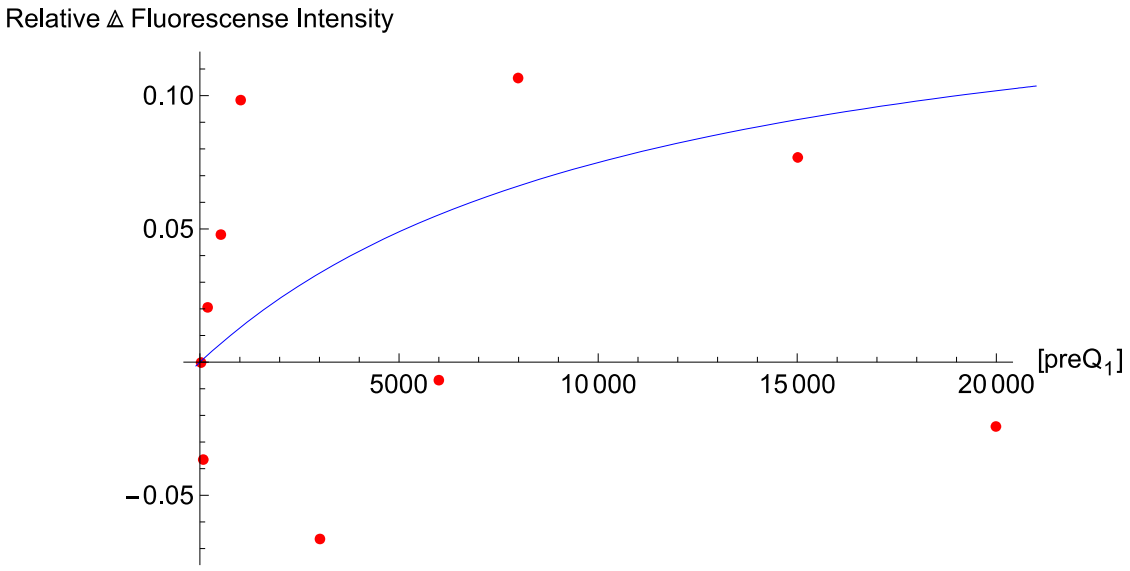

Figure 11. Non-monotonic titration of Position 27 modified riboswitch

The titration curve above attempts to fit the data using equation 1, but the experimental data behaves non-monotonically making it impossible to extrapolate a K_D. Since the experimental data behaves non-monotonically, the data cannot be fitted into either the quadratic or binding isotherm equation.

The K_D extrapolated from this dataset is not meaningful because the fluorescence intensity changes non-monotonically as ligand is added. When fitting the fluorescence spectra using an equation to construct a titration curve, we expect either no change due to no response of the riboswitch to the ligand or monotonic responses to the ligand. Further optimization of protocol II and additional data collection shows future promise and efficacy in studying local structural changes that can help settle the discrepancy

found in literature via analysis of K_D. However, the data presented in figure 9 represents a good titration equation fitting into the binding isotherm equation indicating that future work with protocol I with magnetic stirring could show promise in resolving the literature discrepancy as well.

Discussion

Initial results and a need for further optimization

The initial result of this experiment shows that this method of 2-AP substitution and construction of a titration curve can allow for the study of local structural changes and their impacts on riboswitch folding dynamics. Clearly, protocol II shows promise in addition to refining protocol I since the dataset is more accurate such that monotonic behavior is observed as a response to the ligand and principally behaves better. Furthermore, refinement of Protocol II will hopefully correct issues in negative fluorescence and major peak shifts occurring across the datasets. Data with protocol II should be recollected to see whether monotonic behavior can be observed so that a K_D can be extrapolated. However, further work and optimization with protocol II are required to complete this study since consistent replicate data that is monotonic has not been collected. Furthermore, the last few intensities at higher wavelengths should all be close enough to zero that they can be baseline corrected without affecting the K_D upon equation fitting to the quadratic equation. The data collected using protocol I was fitted with a binding isotherm equation since the concentration of the riboswitch at 100 nM is much lower than the K_D, however data collected in protocol II is fit poorly with the quadratic equation (equation 1) due to unexpected non-monotonic behavior. Overall, the titration curves for protocol I return better equation fittings, but the spectra from protocol II appears to be more ideal. Thus, further optimization is required.

In the future, we hope to collect data along with replicates for different probed positions along the riboswitch so that we can further study the local structural changes that occur. At this time, there is not enough data to make a definitive statement on how

2-AP substitution can impact the riboswitch and create structural disruptions. An ideal example dataset using this method of fluorescence emission titration is shown below to depict the end goal of what the data should look like.

Example data of a titration with $MgCl_2$

A titration of the $preQ_1$ riboswitch with $MgCl_2$ rather than ligand is shown in figure 9 where all ideal characteristics were met. The example data below shows the efficacy of using 2-AP to probe riboswitch folding where major peaks do not shift wavelengths and no negative fluorescence is observed.

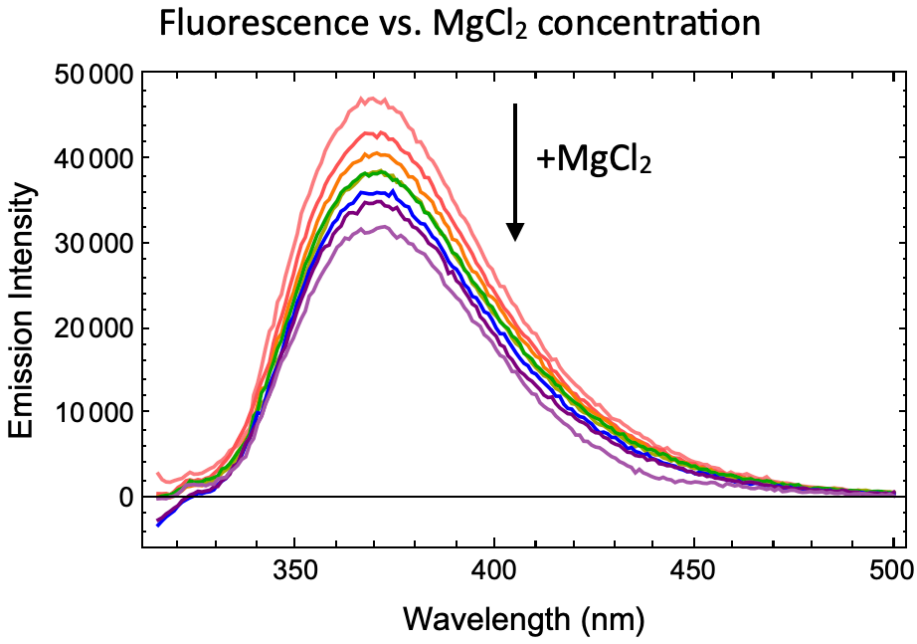

Figure 12. An example titration with the $preQ_1$ riboswitch modified at position 31 responding to $MgCl_2$

This data was collected by Dr. Julia Widom. The purpose of depicting this dataset is to show that the successful studying of local structural changes with 2-AP substitution can be performed.

Further work with the current modified $preQ_1$ riboswitch must be conducted so that a titration curve and replicate data can be obtained such that the curves reflect the

example dataset in figure 9. The data collected with the $preQ_1$ riboswitch needs further optimization rather than being a method that does not work because this method has been proven to be successful for different riboswitches. The error in our data lies in the need for further optimizing the protocol rather than the methods themselves. Additionally, the $preQ_1$ riboswitch will respond differently to its ligand based on the buffer conditions that governs its environment.

Conclusions

The use of 2-AP substitution to study local structural changes can be successfully done due to the ability of 2-AP to quench its fluorescence due to base stacking that occurs as a riboswitch folds in the presence of a ligand. Probing the riboswitch in the immediate vicinity of the L3 region will allow for these structural changes that hopes to resolve the discrepancy found in existing literature. Our data supports the interaction between the L3 and P1 regions, as the decrease in fluorescence as the sample becomes more saturated with ligand indicates base-stacking behavior. The efficacy of this method of using 2-AP probing and fluorescence emission titrations have been proven with other riboswitches that respond to different ligands, thus this method can be applied to the $preQ_1$ riboswitch as well[9]. In conclusion, further work and optimization of protocol II used to study the $preQ_1$ riboswitch is required to obtain consistent dissociation constants and an accurate study of local conformational changes that could be leading to the discrepancy in existing literature. In the future, we hope to refine the protocol enough so that titration curves and replicate data for different positions along the riboswitch can be collected such that the graphs look like the one presented in figure 12.

Methods

RNA oligonucleotides were purchased from Dharmacon and were HPLC-purified by the manufacturer (RNA sequences in table 1). The 3' segments of RNA oligonucleotides were purchased containing a 5' phosphate to enable ligation to another RNA piece. The riboswitch samples probed with fluorescent 2-AP were prepared via DNA splint ligation with T4 RNA ligase 2 (New England Biolabs M0239S). 5 nanomoles of each 5' and 3' segments of the riboswitch were combined with the DNA splint followed by an annealing of 90 °C for two minutes and then an additional cooling at room temperature for ten minutes. 30 seconds into cooling at room temperature, RNA ligase 2 reaction buffer provided by the manufacturer was added. After the cooling cycle, the ligation reaction was diluted to a final volume of 150 µL with reaction buffer and an addition of 60 units of T4 RNA ligase 2. The ligation reactions were then incubated at room temperature for three hours followed by purification on a 15% polyacrylamide-urea gel. The products of the ligation reaction were excised from a gel imaged with a blue-light transilluminator. Product bands were recovered from the gel via electroelution with a BioRad model 422 electro-eluter and then precipitated with ethanol.

Riboswitch	RNA sequence
Unmodified	GCA GAG GUU CUA GCU ACA | CCC UCU AUA AAA AAC UAA GG
Position 27 modified riboswitch	GCA GAG GUU CUA GCU ACA | CCC UCU AU(2-AP) AAA AAC UAA GG

Position 28 modified riboswitch	GCA GAG GUU CUA GCU ACA | CCC UCU AUA (2-AP)AA AAC UAA GG
Position 29 modified riboswitch	GCA GAG GUU CUA GCU ACA | CCC UCU AUA A(2-AP)A AAC UAA GG
Position 30 modified riboswitch	GCA GAG GUU CUA GCU ACA | CCC UCU AUA AA(2-AP) AAC UAA GG
Position 31 modified riboswitch	GCA GAG GUU CUA GCU ACA | CCC UCU AUA AAA (2-AP)AC UAA GG
Position 32 modified riboswitch	GCA GAG GUU CUA GCU ACA | CCC UCU AUA AAA A(2-AP)C UAA GG
$preQ_1$ FFS Rnl2 splint	GTT TTT TAT AGA GGG TGT AGC TAG AAC C

Table 1. Table of samples synthesized with ligation reaction accompanied by corresponding RNA sequences

For the purposes of this research project, data was taken with 2-AP at position 27 and 29 of the riboswitch. In future research, fluorescence data should be taken on the other ligation samples as well.

All titration curves were assembled using fluorescence spectral data recorded on a FS5 spectrofluorometer (Edinburgh Instruments SC-25 FS5 spectrofluorometer) with our ligated sample in buffer consisting of 20 mM Na_iPO_4 at pH7.5 and 100mM NaCl. Sample preparation prior to taking fluorescence scans requires heating to 90 °C for two minutes followed by crash cooling in an ice water bath for ten minutes. This annealing and cooling process is essential so that the RNA can fold in the presence of a ligand without dimerizing. The fluorescence spectra were recorded in a 1 cm path-length cuvette (Starna 29-9F-Q-10) with a riboswitch concentration of 100 nM or 500 nM (see sub-sections for different protocols). The excitation wavelength was kept constant at 295 nm with a bandwidth of 5 nm. The emission wavelength scans were taken from 305

nm to 500 nm in 1 nm steps with a dwell time of 1 s and a bandwidth of 2 nm. The assembled titration curve from the fluorescence data were assembled so that there would be 10 or 12 titration points. A buffer scan at each ligand concentration was recorded so that it can be subtracted from the sample scan at each ligand concentration to afford proper background correction. Prior to loading the cuvette with the sample, the cuvettes were soaked in a 1x Hellmanex solution at 35 °C for 30 minutes followed with a MilliQ water rinse and inert nitrogen gas drying.

The binding constants K_D were determined by following the increase in fluorescence after each titration step with integration of the wavelengths from 395 nm to 500 nm. The changes in fluorescence ($F-F_0$) were normalized to the maximum fluorescence observed in the buffer scan containing no $preQ_1$ riboswitch. The normalized fluorescence intensity of each titration step was plotted against ligand concentration. A stacked plot of each fluorescence spectrum is plotted against wavelength versus intensity at each concentration of ligand added to the sample. A fitting procedure performed in Mathematica (Wolfram Research) was used to extract the binding constants K_D from the analyzed spectral data. Tabulated data of each titration point was imported into the Mathematica notebook to create a fitting using the following quadratic equation:

$$\frac{(F - F_0)}{(F_f - F_0)} = \frac{K_D + [preQ_1] + [RNA] - \sqrt{(K_D + [preQ_1] + [RNA])^2 - 4 \bullet [preQ_1] \bullet [RNA]}}{2 \bullet [RNA]}$$

Equation 1: Quadratic equation for data fitting and K_D extrapolation (Protocol II)

F_0 corresponds to the initial fluorescence and F_f is the final fluorescence[9]. $[preQ_1]$ is a measurement of the total concentration of $preQ_1$ ligand added in each titration step while [RNA] corresponds to the total RNA concentration in each sample.

From here, we can use the fluorescence spectra from each titration step to determine the K_D binding constant. At each ligand titration point, the ligand was added directly to the cuvette followed by pipette mixing. Initial scans were performed at a riboswitch concentration lower than the conditions the quadratic equation above calls for where varying volumes and concentrations of the ligand were added after each titration step rather than a steady volume. The protocol used with this quadratic equation suggests taking measurements where the riboswitch is at a much higher concentration than what we have been currently working with. Equation 2 below was used to fit data taken with protocol I where the high riboswitch concentration condition is not met for a good fit with equation 1. This protocol also calls for using a steady volume after each titration step rather than varying volumes followed by stirring rather than pipette mixing. Transitioning to the protocol recommended in the supplemental information section that accompanies the quadratic equation used to model data may fix current issues with our samples' baseline fluorescence issues that can affect our extrapolated binding constants reflected in protocol II.

Protocol I

The initial protocol used to collect the titration curves has the riboswitch at a 100 nM concentration with 12 titration points. This protocol also used pipette mixing

upon ligand addition prior to taking emission scans of the sample. The data set presented in figure 6 contains a mixing protocol using an autoclaved multiflex 0.5-200 µL round 0.6mm tip provided by FisherScientific. Unexpectedly, these pipette tips had a contaminant that was causing issues with the emission scans for that dataset. For the next dataset using this same protocol I, the current pipette tip was replaced with a 200 µL pipette tip that was used to mix the sample upon ligand addition. This dataset using the new 200 µL pipette tip is shown in figure 8 where the data appears to be better, however still contaminated. The contaminant from the first dataset was eliminated by using a different pipette tip as shown in the second dataset, however the results between the two data sets are consistently inaccurate with negative fluorescence appearing at higher wavelengths and higher ligand concentrations. The only difference between the two datasets is that the major peaks for figure 6 shift indicating contamination from the multiflex tip pipette tip, meanwhile the data presented in figure 8 shows major peaks at a consistent wavelength. Due to inaccurate data collection and dissociation constants extrapolated being different, a new protocol for collecting the titration scans was implemented.

Unexpectedly, the data taken with protocol I was more accurately fit when a binding isotherm equation is used over equation 1. This is since the riboswitch in protocol I is at a much lower concentration than the K_D, whereas the quadratic equation fits the data well when using protocol II since the conditions for higher riboswitch concentration are met. The equation below depicts the equation used to fit titration data with protocol I.

$$\text{Fluorescence}_{\text{intensity}} = \frac{C \bullet [\text{preQ}_1]}{(K_D + [\text{preQ}_1])}$$

Equation 2. Binding isotherm equation for fitting data taken with protocol I

$\text{Fluorescence}_{\text{intensity}}$ is the intensity of the major fluorescence peak at the different ligand concentrations. C is a fitting parameter used to extrapolate the K_D. The K_D represents the dissociation constant while $[\text{preQ}_1]$ reflects the concentration of the ligand per titration point with nanomolar units. Titration curves assembled using protocol I are better fit using the binding isotherm equation over the quadratic equation due to the riboswitch being at 100 nM, much lower than its K_D.

Protocol II

The quadratic equation used to extrapolate the dissociation constants can be found in the supplemental information from work done by Marina Frener and Ronald Micura[9]. Within this supplemental information, the authors present titration data with the same quadratic equation using a preQ_1 riboswitch from a thermophilic organism. The titration data that they show is representative of the datasets that I am currently working on collecting where all major peaks stay at a single wavelength, there is no negative fluorescence, and fluorescence stops changing upon riboswitch saturation with the ligand. From this information, a new protocol was constructed to better represent what the data the authors were able to collect. Adjustments that were made to create Protocol II include using a riboswitch concentration of 500 nM, different ligand concentrations, and ten titration steps instead of 12. This resulted in better data as shown in figure 10 where the new protocol helped eliminate a lot of the issues with the previous emission scans. With this new protocol, pipette mixing was replaced entirely with magnetic stirring to further avoid unnecessary contamination. The adjusted ligand concentrations used in this new protocol match the ligand concentrations used in the

paper by Frener and Micura. Although further work is needed to successfully create replicates of accurate data with correct dissociation constants, this new protocol shows large improvement over the Protocol I and will be used in future work.

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