Published in final edited form as:

Methods Enzymol. 2014 ; 549: 435–450. doi:10.1016/B978-0-12-801122-5.00018-0.

ITC Analysis of Ligand Binding to PreQ₁ Riboswitches

Joseph A. Liberman^{1,2}, Jarrod T. Bogue^{1,2}, Jermaine L. Jenkins^{1,2,3}, Mohammad Salim^{1,2}, and Joseph E. Wedekind^{1,2,3,*}

¹Department of Biochemistry & Biophysics, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642, USA

²Center for RNA Biology, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642, USA

³Structural Biology & Biophysics Facility, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642, USA

Abstract

Riboswitches regulate genes by binding to small-molecule effectors. Isothermal titration calorimetry (ITC) provides a label-free method to quantify the equilibrium association constant, K_A , of a riboswitch interaction with its cognate ligand. In addition to probing affinity and specific chemical contributions that contribute to binding, ITC can be used to measure the thermodynamic parameters of an interaction (G , H , and S), in addition to the binding stoichiometry (N). Here we describe methods developed to measure the binding affinity of various preQ₁ riboswitch classes for the pyrrolopyrimidine effector, preQ₁. Example isotherms are provided along with a review of various preQ₁-II (class 2) riboswitch mutants that were interrogated by ITC to quantify the energetic contributions of specific interactions visualized in the crystal structure. Protocols for ITC are provided in sufficient detail that the reader can reproduce experiments independently, or develop derivative methods suitable for analyzing novel riboswitch-ligand binding interactions.

Keywords

preQ₁; ITC; crystallography; gene regulation; riboswitch; ligand binding; K_D ; thermodynamic analysis

Introduction

Pre-queuosine₁ (7-aminomethyl-7-deazaguanine or preQ₁) is a secondary metabolite synthesized in five steps exclusively in bacteria starting from GTP (McCarty & Bandarian, 2012). The resulting pyrrolopyrimidine is incorporated subsequently by a transglycosylation reaction that replaces guanine within tRNA(GUN) anti-codons (Figure 1). In two additional enzymatic steps, the hypermodified base queuosine (Q) is produced, which enhances the ability of tRNAs(QUN) to read degenerate codons (Meier, Suter, Grosjean, Keith, & Kubli, 1985). The Q modification is widely distributed in bacteria and animals (Yokoyama et al.,

Correspondence to: Prof. Joseph E. Wedekind, Telephone: +1 585 273-4516, Facsimile: +1 585-275-6007, joseph.wedekind@rochester.edu.

1979), although the latter organisms must obtain the Q-base (queuine) from dietary sources or gut flora (Farkas, 1980). PreQ₁ appears to be unique to the bacterial metabolome, and has been found to be the ligand that affects three phylogenetically distinct riboswitches known as preQ₁-I (class 1), preQ₁-II (class 2) and preQ₁-III (class 3) (McCown, Liang, Weinberg, & Breaker, 2014; Meyer, Roth, Chervin, Garcia, & Breaker, 2008; Roth et al., 2007; Weinberg et al., 2007). This discovery provides a rich system to explore how evolutionarily diverse riboswitches recognize a common ligand, as well as the chemical diversity of effector binding that is accessible through the tertiary folding of RNA.

Significant progress has been made on crystallographic structure determinations and biophysical analysis of preQ₁-I and preQ₁-II riboswitches involved in translational regulation (Jenkins, Krucinska, McCarty, Bandarian, & Wedekind, 2011; Liberman, Salim, Krucinska, & Wedekind, 2013; Spitale, Torelli, Krucinska, Bandarian, & Wedekind, 2009; Suddala et al., 2013). ITC has been an integral part of this work since it provides a basis to quantify the preQ₁ binding affinity of various riboswitch constructs generated for crystallization trials. Moreover, once a structure has been solved, ITC can be used to corroborate the observed mode of binding of the riboswitch to preQ₁. To illustrate, ITC was used to measure the equilibrium dissociation constant (K_D) for preQ₁ binding to the wild type preQ₁-II riboswitch from *Lactobacillus rhamnosus*. The resulting K_D of 17.9 ± 0.6 nM was then compared to individual nucleobase mutants that were designed to disrupt hydrogen bond interactions to the ligand based on the wild type structure (Liberman et al., 2013). Figure 2 shows the binding site and the relative changes in affinity (K_{rel}) for the respective mutants. Whereas the C30U mutant produced a K_{rel} of ~46 ($K_D = 0.81 \pm 0.12$ μ M), the U41C mutant produced a K_{rel} of ~90 ($K_D = 1.60 \pm 0.02$ μ M). The ΔG values for C30U and U41C were 2.2 and 2.6 kcal mol⁻¹, respectively, which is consistent energetically with the loss of two or three hydrogen bonds to preQ₁. The results support the structural observations but also suggest that each mutant causes the loss of one additional hydrogen bond between the mutated base and the ligand compared to what might be expected if the respective nucleobase mutations were isostructural with the wild type binding pocket. Overall, this work demonstrates the general utility of ITC to interrogate the binding of ligands to riboswitches, which can be analyzed in terms of K_D and relative energetic differences. Moreover, ITC can provide insight into the cellular concentration of the metabolite required to elicit a biological response. For a broader description of ITC theory, methodology, and a general analysis of ITC-based RNA-ligand interactions, the reader is referred to several recent reviews that include analyses of RNA binding to small ligands, ions, sugars, and large molecules such as tRNA (Gilbert & Batey, 2009; Salim & Feig, 2009; Sokoloski & Bevilacqua, 2012; Zhang, Jones, & Ferre-D'Amare, 2014).

Information Content in an ITC Experiment

Since the purpose of this chapter is to provide a practical context for ITC, as well as pedagogical instruction for those new to the field, it is worth mentioning some of the significant properties of ITC. Some knowledge is assumed with respect to the instrument design and the reader's understanding of free energy relationships in the context of ligand binding by biological macromolecules. Importantly, ITC is a label-free method to characterize the binding thermodynamics for any (usually two) interacting molecules – such

as a riboswitch and its small molecule effector. The approach allows characterization of the thermodynamic parameters including: enthalpy (ΔH), entropy (ΔS), and free energy (ΔG) changes that result from binding interactions in solution. In addition, the heat capacity change (ΔC_p) can be measured by acquiring ΔH values at various temperatures. Such information can be useful to evaluate buried surface area, as described (Salter, Lippa, Belashov, & Wedekind, 2012). Herein we are interested in the integrated heats of injection that directly report on the ΔH of a binding event, and allow us to extract K_A from a curve fit of the integrated heat as a function of the ligand-to-receptor molar ratio; notably, the reciprocal of K_A equals K_D . We can extract other parameters by the knowledge that $\Delta G = -RT \ln K_A$ and $\Delta G = \Delta H - T \Delta S$, where R is the gas constant and T is the absolute temperature. Although the approach can require relatively large amounts of material compared to other methods – usually mg to μ g – ITC is operative over a wide range of affinities between 1 mM and 1 nM. In practice this range is governed by the solubility of the respective ligand and receptor. Low-affinity interactions may require very high concentrations, whereas very high-affinity interactions can have large heat changes that preclude efforts aimed at monitoring the true titration. In practice, the amount of material consumed by a single ITC experiment on a wild type preQ₁ riboswitch is more than that required for a single, 96-condition crystallization screen, assuming in the latter case that one uses a liquid-handling robot at an RNA concentration of 5 mg per mL. Although the inexperienced crystallographer may be conflicted about experimental priorities, the ability to quantitatively evaluate an RNA crystallization construct for ligand binding is key to a successful crystallization outcome, since the information garnered will be necessary for designing screens that ensure a ligand concentration ~10-fold greater than the K_D measured for binding the target RNA.

Experimental Procedures for ITC

Assessing the Feasibility of ITC Experiments

Before initiating any ITC experiment, it is important to evaluate the feasibility of the proposed experiment. Aside from having sufficient amounts of pure receptor and ligand, it is helpful to know approximately how tightly the molecules will interact based on an educated guess. It is not unusual for riboswitches to bind their ligands with apparent K_D values ranging from 2 nM to 2 μ M (Jenkins et al., 2011; Rieder, Kreutz, & Micura, 2010). There are examples of exceptionally tight binding riboswitches that bind with affinities of 100 pM (Nelson et al., 2013) to 10 pM (Smith et al., 2009). Such high affinity can create difficulties for ITC experiments. Problems arise during data analysis because the isotherms resulting from these experiments will produce binding curves that are not well populated with measurements at the sigmoidal inflection point, thus yielding a curve that conforms to a step function. The resulting K_A values from fitting such curves are not reliable and should only be considered as an estimate of affinity, although the ΔH and stoichiometry values can still be reliable. When the affinity is too high, it is possible to weaken the interaction by changing the temperature, lowering the Mg²⁺ concentration, increasing monovalent ion concentration, or by the addition of a mild denaturant. Isotherms with an ideal curve will appear sigmoidal with an initial flat slope, an obvious inflection point within a gradually sloping region, and a clear point of saturation with a zero slope. Populating the curve with data points is especially

important near the inflection region since these observations are needed to accurately fit the K_A .

Perhaps the best way to establish whether an ITC experiment will yield a suitable isotherm is by calculation of the unitless parameter c , which dictates the shape of the curve. The c value is the product of the equilibrium association constant, K_A , the receptor concentration in the cell at the start of the experiment, $[M]$, and the stoichiometry of ligand binding to the receptor, N :

$$c = K_A \cdot [M] \cdot N$$

Large c values approaching 500 will result in a very steep inflection point, which precludes a proper fit of K_A . Here, values for N and ΔH can still be measured reliably, which is an important consideration if one is assessing heat capacity (C_p) changes (Salter et al., 2012; Sokoloski, Dombrowski, & Bevilacqua, 2012). By contrast, c values less than 10 are associated with flat, featureless curves that do not possess the information necessary to extract K_A , N or ΔH accurately. Such isotherms are characteristic of weak binding interactions. In practice, one should strive for the range $20 < c < 100$ (Myszka et al., 2003). Because we typically have an estimate of the K_A (i.e., $1/K_D$) for the interaction in question, we can calculate the $[M]$ value for our desired receptor concentration in the sample cell at the onset of ITC analysis. A good starting point for the ligand concentration in the syringe is ~10 times the value of $[M]$, which is necessary to ensure saturation. For example, the experimental preparation for titration of preQ₁ into a new class 1 type III preQ₁ riboswitch from *Enterobacter cloacae* (McCown et al., 2014) was based on a knowledge of the K_D (7.3 ± 2.3 nM and N value of 0.98) measured previously by ITC for a related class 1 type I preQ₁ riboswitch (Suddala et al., 2013). As such, the $[M]$ value was estimated to be 0.75 μ M assuming a c value of 100. In practice, the type III riboswitch was observed to possess 10-fold poorer affinity ($K_D = 72$ nM) than the type I molecule. Following the initial ITC experiment, the $[M]$ value was adjusted accordingly to a value of 8.7 μ M with an appropriate increase in the starting ligand concentration in the syringe. This resulted in a c value of 112 (Figure 3). If such estimations are not possible based on known K_D values, the reader is encouraged – as with all aspects of the ITC experiment – to read the manufacturer's guidelines for the appropriate starting concentration of the receptor and ligand, which is described in the operation manual.

Instrumentation, Materials and Solutions for ITC

The following instruments or equivalents are required for ITC analysis: a ThermoVac (GE Life Sciences) system for solution degassing, a Nanodrop UV Spectrophotometer (Thermo Scientific), Pipettes (Gilson Inc), an aluminum heating block with a thermostatic controller, and a VP-ITC Calorimeter (GE Life Sciences). Other ITC calorimeters in common use include the iTC-200 (GE Life Sciences), and the Nano ITC (TA Instruments).

The following stock solutions and supplies are required: RNA resuspension buffer: 0.10 M NaCl, and 0.050 M Na•HEPES pH 7.0; dialysis buffer: 0.10 M NaCl, 0.050 M Na•HEPES pH 7.0 and 0.0060 M MgCl₂; a stock solution of 1.0 M MgCl₂; 3.5K MWCO Slide-A-Lyzer

(Thermo Scientific) for dialysis. All stocks should be sterile filtered through 0.2 μm cellulose acetate filters (Millipore) and prepared under ribonuclease free conditions. UV-irradiated water with a resistivity of 18 M Ω should be used from a Barnstead NanoPure™ UV/UF system or equivalent.

RNA and Ligand Preparation

The RNA sample must be of the highest possible purity, such as that used for crystallization. Effective purification methods include HPLC (Spitale et al., 2009; Spitale & Wedekind, 2009; Wedekind & McKay, 2000) or denaturing polyacrylamide gel electrophoresis (Liberman et al., 2013; Lippa et al., 2012). At all stages of the experiment, the user is advised to avoid ribonuclease contamination by using approaches described in the aforementioned methods manuscripts. Riboswitches can be folded for ITC using any protocol that has been found to be effective. For the preQ₁-I and preQ₁-II riboswitches, we developed a procedure that entails heating the RNA in a neutral pH buffer containing sodium chloride, followed by addition of magnesium chloride while the RNA is still hot with subsequent slow cooling to room temperature (details below). The volumes described herein are for the VP-ITC instrument, and should be revised accordingly if a different instrument will be used that possesses a smaller cell.

A key consideration is that the buffer used to suspend the sample in the reference cell (i.e. the receptor) is matched perfectly to that of the titrant (i.e. ligand) in the syringe. This is achieved for the small-molecule ligands that typically interact with riboswitches by dissolving the ligand, or diluting a concentrated stock, in the same buffer used for dialysis of the riboswitch. It is essential that the ligand is of the highest purity possible; in our investigations preQ₁ was produced by chemical synthesis as described (Liberman et al., 2013).

The step-by-step protocol for sample preparation is as follows. First dissolve the lyophilized RNA stored at -80 °C in 2.2 mL of RNA 'resuspension buffer' described in the prior section. Use the *c*-value calculation above to determine the starting receptor concentration, [M]. Ensure that a sufficient amount of RNA is resuspended to perform all the required titrations; at this point it is more desirable to have a slightly higher concentration than one that is too low since it is easier to dilute the sample than to concentrate it. Heat the RNA solution to 65 °C for 3 min in an aluminum heating block, then add sufficient volume from the MgCl₂ stock to give a final concentration of 0.0060 M. Return the sample to the heating block at 65 °C for 5 min. Remove the aluminum block from the heater, and cool the hot RNA inside the block until it reaches room temperature. Load the RNA into 3.5K molecular weight cutoff Slide-A-Lyzer for dialysis. Dialyze the RNA overnight against 4 L of dialysis buffer; 0.2 μm sterile filter and degas the used dialysis buffer using a vacuum-based filter bottle (Nalgene) and save it for use later. This step will help preserve the buffer (see below), and will facilitate the removal of undesirable trapped gas that can evolve during the ITC experiment, leading to measurement artifacts. The concentration of the RNA recovered from the Slide-A-Lyzer should be determined by OD₂₆₀ based on the calculated extinction coefficient; use the Beer-Lambert relationship. Dilute the RNA to the desired concentration using the 0.2 μm , vacuum filtered dialysis buffer above; reuse of the dialysis buffer ensures a

precise solvent match, which is necessary to avoid heat-of-dilution artifacts during ITC. Although the minimum sample volume required to fill the ITC cell is 1.8 mL, a volume of 2.2 mL of RNA per experiment is recommended to ensure proper loading with no air bubbles. Dissolve the ligand in the same dialysis buffer used for the RNA dialysis (above) to ensure a proper buffer match. The ligand concentration should be ~10-fold higher than the RNA concentration. A ligand volume of 600 μ L will allow facile loading of the VP-ITC syringe. Degas both the RNA and ligand solutions, respectively, for 10 min using the ThermoVac set at a temperature 1–2 $^{\circ}$ C lower than the temperature at which ITC will be conducted. Take care to avoid boiling the sample; reduce the vacuum if bubbling is observed.

The Isothermal Titration Calorimetry Experiment

Prior to conducting ITC experiments, the water in the reference cell should be changed according to the manufacturer's instructions. Additionally, after each ITC experiment the sample cell and syringe should be flushed thoroughly with at least 150 mL of degassed dialysis buffer. At the end of each day of ITC use, the sample cell and syringe should be cleaned according to the manufacturer's instructions. During all procedures, it is especially important to avoid placing mechanical stress on the tip of the loading syringe. A bent tip can cause measurement artifacts and usually necessitates a costly replacement. Review the manufacturer's instructions for proper handling and storage.

The following protocol describes how to initiate ITC measurements to analyze ligand interactions with a riboswitch, and can be adapted readily to the analysis of other RNA-small molecule complexes. First, flush the sample cell with 300 mL of 0.2 μ m filtered, degassed dialysis buffer retrieved from the RNA dialysis experiment. Set the temperature of the sample cell; values of 25 or 30 $^{\circ}$ C work well for initial experiments. Notably, the VP-ITC has a temperature range of 2 to 80 $^{\circ}$ C but we have not been able to collect useful data higher than 60 $^{\circ}$ C due to baseline fluctuations (Suddala et al., 2013). Next, adjust the instrument settings for the VP-ITC. The reference power is set to 15 μ cal per sec, the mixing speed should be 300 rpm, and the feedback mode/gain is set to 'high'. The time allocated between injections is a critical parameter. If an injection begins before the prior injection has returned fully to baseline the measured peak magnitudes will be inaccurate. A spacing of 300 sec is a safe starting point that can be increased or decreased as necessary in subsequent experimental runs. The injection volume and duration rarely need to be changed from defaults of 10 μ L and 20 sec. The first injection is rarely accurate, so the initial injection volume is usually 3 μ L to avoid wasting sample; this data point is discarded later in fitting of the binding model. In the computer interface, enter the concentration of RNA [M] (receptor) in the cell, and ligand in the syringe; accurate values are essential for successful analysis. Importantly, the concentration of [M] or ligand can be revised *a posteriori* if an erroneous value was entered at the onset of the experiment. Load the RNA into the cell and the ligand into the syringe following the manufacturer's instructions. Perform the purge/refill procedure three times to remove air bubbles that may be in the syringe. Carefully place the syringe into the cell and start the run. As a heuristic, one should strive for an average integrated heat of 5 μ cal evolved (or absorbed) per injection for the first two-thirds of the injections. The sensitivity of the VP-ITC is about 0.1 μ cal.

ITC Data Analysis

Here we present a rudimentary protocol for data analysis using the VP-ITC associated software Origin® 7. Treatment of isotherms to arrive at high precision measurements is described elsewhere (Keller et al., 2012). First, load the ITC data by clicking the “Read Data ...” box under “ITC Main Control” on left side of screen, and select the appropriate .itc file (in this protocol, the data file will be referred to as “YourFile.itc”). Under the “Window” pull-down menu, select the item that has the same name as your .itc file. Under the column labeled “NDH” select the last three cells. These are the heats of injection of the ligand into buffer in units of kcal mol^{-1} , provided that saturation was achieved. As such, these heats must be subtracted from all injections. Right click on the highlighted cells and select “statistics on column” in the menu that opens, then copy the value from the cell labeled “Mean(Y)”. Return to the original window that was opened after selecting your ITC data. Ensure that no cells are selected. Open the “Math” pull down menu and select “Simple Math...”. In the “Available Data” column select “YourFile_NDH” and click the top double arrow. The box next to Y1 will now show “YourFile_NDH”. In the “operator” box enter a minus sign (–). In box Y2, paste the value copied above for the Mean(Y) value, representing the last three heats of injection; press “OK”. The heat of dilution of the ligand has now been removed from all data points. Open the “Window” pull down menu and select “DeltaH”. The first data point from the 3 μL injection must be removed. Select the “Remove Bad Data...” box from under “Data Control,” click on the first data point, and press the “return” key.

Now we will consider the binding model for the interaction between the ligand and riboswitch. There are several possibilities, but for preQ₁ riboswitches the most likely model will be the “One Set of Sites...” model, which assumes all sites are equivalent. To fit this binding model, click “One Set of Sites...” under “Model Fitting” on the left side of the screen. In the window that opens, click the box that says “100 Iter.”; the chi squared value denoted “chi^2” should appear in a new dialogue box, and is an indicator of agreement between the experimental data and the binding-model curve. After the first 100 iterations of least-squares fitting, there should be an updated display that shows improvement in the agreement between the data and the trend curve, in addition to a reduced “chi^2” value. Since our goal here is to reach convergence, continue to click the “100 Iter.” button until no reduction is observed in chi^2, then select the “Done” box. As a result of fitting, a new box will appear next to the isotherm. This ‘DeltaH’ box will contain the associated binding including: the stoichiometry of binding “N”, the equilibrium K_A in units of M^{-1} , designated “K”, “H” in calories per mole, and “S” in units of calories per mole per degree, which is calculated from H and K after fitting. A publication-quality figure, such as Figure 3, can be generated by selecting “Final Figure” in the “ITC” pull down menu. Be sure to adjust the spacing between units so that the values on the ordinate and abscissa do not overlap.

In our experiments thus far, we have observed 1:1 binding stoichiometry between preQ₁ riboswitches and their ligands. As stated, the choice of binding model is “One Set of Sites” but other stoichiometries and binding models are feasible. These should be considered if the visual fit between the binding model and the data is poor. Importantly, the best choice model

should improve visibly relative to other choices (also χ^2 should be reduced significantly) and the binding model should be biologically sensible.

Manual Adjustment of the Baseline

Origin® 7 will fit automatically the baseline of ITC data. As stated above, a sufficiently long spacing is required between injections to ensure that the baseline does not overlap with neighboring injections, which is a prerequisite to proper fitting. However, there are occasionally instances where the automatic baseline does not fit the data despite adequate separation. Here manual intervention is recommended to obtain the best results. To conduct manual fitting, enter the “Window” pull-down, and select “RawITC”. Click “Adjust Integrations...” under ITC Main Control on the left side of the screen. Click the first peak. The “<<” and “>>” arrows will advance to the previous or next peak. Here, only one peak is considered at a time. The area between the two blue lines is integrated to determine the heat of injection (Figure 4a). The blue lines can be dragged to alter the window considered for integration (for example, to remove a bubble spike in the baseline region); the red line is the baseline. To adjust the baseline, click the “Baseline” button. A series of black boxes appear on the red baseline that can be dragged up or down to account for irregularities. After adjustments have been made to the baseline, click the “Integrate” button before advancing to the next peak. The integrated area will be filled with vertical black lines (Figure 4b). Following manual adjustment and integration of each individual peak, click “Quit”. The results should show a better fit of the data to the curve (Figure 4c versus Figure 4d), which can have a subtle effect on the K_A , N , and H , as well as other parameters. Notably, both the flatness of the baseline and the apparent separation between injections have improved in Figure 4d. To gauge the productiveness of one’s baseline adjustment efforts, the reader is encouraged to experiment with a subset of injections to ascertain whether the newly integrated data fits better to the model based on the criteria described above.

Publishing ITC Results and Representative Analysis

As in any experimental approach, it is preferable to conduct multiple measurements to improve accuracy and generate error estimates. In this respect ITC is no exception, although the quantity of sample required often precludes numerous measurements due to the costly nature of producing the RNA and the associated ligand, such as preQ₁. At a minimum, at least two ITC experiments should be conducted per sample (preferably more), and the results should be presented as the arithmetic mean in textual, or tabular form, depending on the number of samples. The reported values should include: K_A or K_D , N , H , $-T \Delta S$ and G along with the associated standard deviation of each. It is recommended to always include a representative isotherm and the associated curve fit to demonstrate the quality of the data. A plot of kcal per mole of injectant versus molar ratio should be sigmoid with a visually discernable fit of the binding model to the experimental data points (Figure 3). The c value for each experiment should also be provided, or sufficient information to calculate the c value and to reproduce the experiments. In cases where the c value is high (approaching 500), the K_A or K_D results should be described with a disclaimer that the values cannot be accurately determined, which may be the only alternative if the use of a lower $[M]$ results in poor heats. Alternatively, a high c may be acceptable if the goal is to measure H as part of a heat capacity measurement (Salter et al 2012). The reader is also

encouraged to explore various temperatures to improve the quality of isotherms and values of *c*. For a practical example of how systematic changes to the ITC conditions can influence binding isotherms, see (Sokoloski et al., 2012). Altering a riboswitch's ligand can provide information on the chemical groups involved in aptamer binding (Gilbert, Mediatore, & Batey, 2006). Conversely, mutations of a specific aptamer can provide insight into conserved groups required for ligand recognition (Gilbert, Love, Edwards, & Batey, 2007; Gilbert, Stoddard, Wise, & Batey, 2006). For a summary of thermodynamic parameters obtained by ITC for various riboswitches see (Zhang et al., 2014).

Acknowledgments

We thank Clara Kielkopf for helpful discussions, and Philip Bevilacqua for critical comments on this manuscript. This research was funded by NIH grants RR026501 and GM063162 to J.E.W. J.A.L. was funded in part by NIH T32 training grant GM068411, and an E.H. Hooker graduate fellowship. J.T.B was funded in part by a fellowship from NIH/NCATS CTSA award TL1 TR000096 to the University of Rochester. Portions of this research were carried out at the Stanford Synchrotron Radiation Lightsource (SSRL, Menlo Park CA), a Directorate of SLAC National Accelerator Laboratory and an Office of Science User Facility operated for the U.S. DOE by Stanford University. The SSRL Structural Molecular Biology Program is supported by the DOE and NIH grants GM103393 and RR001209.

References

- Farkas WR. Effect of diet on the queuosine family of tRNAs of germ-free mice. *J Biol Chem.* 1980; 255:6832–6835. [PubMed: 6771278]
- Gilbert SD, Batey RT. Monitoring RNA-ligand interactions using isothermal titration calorimetry. *Methods Mol Biol.* 2009; 540:97–114. [PubMed: 19381555]
- Gilbert SD, Love CE, Edwards AL, Batey RT. Mutational analysis of the purine riboswitch aptamer domain. *Biochemistry.* 2007; 46:13297–13309. [PubMed: 17960911]
- Gilbert SD, Mediatore SJ, Batey RT. Modified pyrimidines specifically bind the purine riboswitch. *J Am Chem Soc.* 2006; 128:14214–14215. [PubMed: 17076468]
- Gilbert SD, Stoddard CD, Wise SJ, Batey RT. Thermodynamic and kinetic characterization of ligand binding to the purine riboswitch aptamer domain. *J Mol Biol.* 2006; 359:754–768. [PubMed: 16650860]
- Iwata-Reuyl D. Biosynthesis of the 7-deazaguanosine hypermodified nucleosides of transfer RNA. *Bioorg Chem.* 2003; 31:24–43. [PubMed: 12697167]
- Jenkins JL, Krucinska J, McCarty RM, Bandarian V, Wedekind JE. Comparison of a preQ₁ riboswitch aptamer in metabolite-bound and free states with implications for gene regulation. *J Biol Chem.* 2011; 286:24626–24637. [PubMed: 21592962]
- Keller S, Vargas C, Zhao H, Piszczek G, Brautigam CA, Schuck P. High-precision isothermal titration calorimetry with automated peak-shape analysis. *Anal Chem.* 2012; 84:5066–5073. [PubMed: 22530732]
- Liberman JA, Salim M, Krucinska J, Wedekind JE. Structure of a class II preQ₁ riboswitch reveals ligand recognition by a new fold. *Nat Chem Biol.* 2013; 9:353–355. [PubMed: 23584677]
- Lippa GM, Liberman JA, Jenkins JL, Krucinska J, Salim M, Wedekind JE. Crystallographic analysis of small ribozymes and riboswitches. *Methods Mol Biol.* 2012; 848:159–184. [PubMed: 22315069]
- McCarty RM, Bandarian V. Biosynthesis of pyrrolopyrimidines. *Bioorg Chem.* 2012; 43:15–25. [PubMed: 22382038]
- McCown PJ, Liang JJ, Weinberg Z, Breaker RR. Structural, Functional, and Taxonomic Diversity of Three PreQ₁ Riboswitch Classes. *Chem Biol.* 2014; 21:880–889. [PubMed: 25036777]
- Meier F, Suter B, Grosjean H, Keith G, Kubli E. Queuosine modification of the wobble base in tRNAHis influences 'in vivo' decoding properties. *EMBO J.* 1985; 4:823–827. [PubMed: 2988936]

- Meyer MM, Roth A, Chervin SM, Garcia GA, Breaker RR. Confirmation of a second natural preQ₁ aptamer class in Streptococcaceae bacteria. *RNA*. 2008; 14:685–695. [PubMed: 18305186]
- Myszka DG, Abdiche YN, Arisaka F, Byron O, Eisenstein E, Hensley P, et al. The ABRF-MIRG'02 study: assembly state, thermodynamic, and kinetic analysis of an enzyme/inhibitor interaction. *J Biomol Tech*. 2003; 14:247–269. [PubMed: 14715884]
- Nelson JW, Sudarsan N, Furukawa K, Weinberg Z, Wang JX, Breaker RR. Riboswitches in eubacteria sense the second messenger c-di-AMP. *Nat Chem Biol*. 2013; 9:834–839. [PubMed: 24141192]
- Rieder U, Kreutz C, Micura R. Folding of a transcriptionally acting preQ₁ riboswitch. *Proc Natl Acad Sci U S A*. 2010; 107:10804–10809. [PubMed: 20534493]
- Roth A, Winkler WC, Regulski EE, Lee BW, Lim J, Jona I, et al. A riboswitch selective for the queuosine precursor preQ₁ contains an unusually small aptamer domain. *Nat Struct Mol Biol*. 2007; 14:308–317. [PubMed: 17384645]
- Salim NN, Feig AL. Isothermal titration calorimetry of RNA. *Methods*. 2009; 47:198–205. [PubMed: 18835447]
- Salter JD, Lippa GM, Belashov IA, Wedekind JE. Core-binding factor beta increases the affinity between human Cullin 5 and HIV-1 Vif within an E3 ligase complex. *Biochemistry*. 2012; 51:8702–8704. [PubMed: 23098073]
- Smith KD, Lipchock SV, Ames TD, Wang J, Breaker RR, Strobel SA. Structural basis of ligand binding by a c-di-GMP riboswitch. *Nat Struct Mol Biol*. 2009; 16:1218–1223. [PubMed: 19898477]
- Sokoloski JE, Bevilacqua PC. Analysis of RNA folding and ligand binding by conventional and high-throughput calorimetry. *Methods Mol Biol*. 2012; 905:145–174. [PubMed: 22736003]
- Sokoloski JE, Dombrowski SE, Bevilacqua PC. Thermodynamics of ligand binding to a heterogeneous RNA population in the malachite green aptamer. *Biochemistry*. 2012; 51:565–572. [PubMed: 22192051]
- Spitale RC, Torelli AT, Krucinska J, Bandarian V, Wedekind JE. The structural basis for recognition of the PreQ₀ metabolite by an unusually small riboswitch aptamer domain. *J Biol Chem*. 2009; 284:11012–11016. [PubMed: 19261617]
- Spitale RC, Wedekind JE. Exploring ribozyme conformational changes with X-ray crystallography. *Methods*. 2009; 49:87–100. [PubMed: 19559088]
- Suddala KC, Rinaldi AJ, Feng J, Mustoe AM, Eichhorn CD, Liberman JA, et al. Single transcriptional and translational preQ₁ riboswitches adopt similar pre-folded ensembles that follow distinct folding pathways into the same ligand-bound structure. *Nucleic Acids Res*. 2013; 41:10462–10475. [PubMed: 24003028]
- Wedekind JE, McKay DB. Purification, crystallization, and X-ray diffraction analysis of small ribozymes. *Methods Enzymol*. 2000; 317:149–168. [PubMed: 10829279]
- Weinberg Z, Barrick JE, Yao Z, Roth A, Kim JN, Gore J, et al. Identification of 22 candidate structured RNAs in bacteria using the CMfinder comparative genomics pipeline. *Nucleic Acids Res*. 2007; 35:4809–4819. [PubMed: 17621584]
- Yokoyama S, Miyazawa T, Iitaka Y, Yamaizumi Z, Kasai H, Nishimura S. Three-dimensional structure of hyper-modified nucleoside Q located in the wobbling position of tRNA. *Nature*. 1979; 282:107–109. [PubMed: 388227]
- Zhang J, Jones CP, Ferre-D'Amare AR. Global analysis of riboswitches by small-angle X-ray scattering and calorimetry. *Biochim Biophys Acta*. 2014

Figure 1.

Schematic diagram of prequeuosine₁ (preQ₁) as an intermediate on the queuosine (Q) biosynthetic pathway. For a review of known enzymes see (McCarty & Bandarian, 2012). Although animals must obtain Q from dietary sources or gut flora, bacteria can produce Q by *de novo* synthesis, which proceeds via preQ₁ formation [reviewed in (Iwata-Reuyl, 2003)]. The procedures herein were developed to probe the binding affinity of various bacterial preQ₁ riboswitches for the preQ₁ molecule.

Figure 2.

Mode of chemical recognition of preQ₁ by the preQ₁-II riboswitch as described in (Liberman et al., 2013). Putative hydrogen bonds are shown as broken lines. The “floor” of the binding pocket is formed by a Hoogsteen base pair between A71•U31. The affinity of the C30U and U41C mutants for preQ₁ were probed by ITC and compared to wild type. K_{rel} is defined as the mutant K_D divided by the wild type K_D . In this manner, ITC was used to quantify changes in free energy resulting from binding site mutations, which appear to corroborate the mode of ligand binding based on crystallographic observations.

Figure 3.

Representative isotherm and binding-model fit for a preQ₁-I type III riboswitch. ITC was performed at 25 °C in 0.0060 M MgCl₂, 0.10 M NaCl, and 0.050 M Na-HEPES pH 7.0. The wild type riboswitch was in the cell at 8.74 μM; preQ₁ was in the syringe at a concentration 10-fold higher than the RNA. The c value is 112. The parameters obtained from a “One Set of Sites” binding model are shown as text in the inset DeltaH window. The values are: the binding stoichiometry, $N = 0.93$; $K_A = 1.39 \times 10^7 \text{ M}^{-1}$; $H = -25.39 \times 10^3 \text{ cal/mol}$; and $S = -52.4 \text{ cal/(mol K)}$.

Figure 4.

Diagrams comparing automated and manual baseline corrections of ITC data. (a) Example of a baseline that was determined automatically. The baseline shown in red (horizontal line) is too high but can be adjusted by dragging the rectangular boxes (e.g. see arrow). The area between the two blue lines is integrated to determine the heat of a specific injection; these lines can be maneuvered to change the amount of the peak that is integrated and the associated baseline. (b) Baseline from data in panel (a) after manual adjustment and integration (red). The area filled by the vertical black lines has been integrated; note, the units of the abscissa in panels (a) and (b) are time (s). (c) ITC experiment from panel (a) in which the baseline was derived by automated selection in Origin® 7. (d) Isotherm resulting from manual baseline adjustment, shown in part as panel (b). Although the thermodynamic parameters and stoichiometry of binding have not significantly changed, the fit of the binding model is improved based on visual inspection, and the improved chi-squared value.