IDENTIFICATION AND BIOPHYSICAL CHARACTERIZATION OF FACTORS INFLUENCING THE THERMOSTABILITY OF BIOLOGICAL RNAs

A Thesis in
Chemistry
by
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ABSTRACT

The popular model of RNA folding is that its highly stable and organized secondary structure allows for pre-organization of the tertiary structure, which drives RNA to fold on a biological timescale. The crowded conditions of the cell may also play a role in facilitating RNA folding, reducing the stability of any misfolded intermediates. In addition, metal ions (and in particular divalent metal ions) stabilize RNA folding by neutralizing the inherent clash of negatively charged phosphate backbones. Studies in this thesis focus on the effects of RNA folding under macromolecular crowding conditions, as well as the role of divalent metals on RNA secondary structural elements.

Although the folding of functional RNAs under idealized conditions of dilute solution and high ionic strength has been well studied, much less is known about RNA folding under the more in vivo conditions of low Mg\(^{2+}\) concentrations and high macromolecular crowding. It is believed that while RNA may fold hierarchically, a certain cooperativity of folding transitions might be necessary to fold the RNA on a biological timescale. We propose that a functional RNA (transfer RNA) can fold cooperatively in the presence of a molecular crowder and physiological Mg\(^{2+}\) concentrations (Chapter 2). In addition, through a study of secondary structure strengthening mutants, we established that under cooperative (two-state-like) conditions, thermostability of a functional RNA can be increased through base pairing.

In Chapter 3, the divalent metal ion interactions with several GAAA tetraloops were studied using thermal denaturation by UV absorbance (referred to as “melting” throughout the thesis) to compare thermodynamic parameters, in particular, Gibbs free energy values (\(\Delta G_{37}\)). GAAA tetraloops with both cg and gc closing base pairs were studied in the presence of divalent alkaline earth metals to determine if there is preference for a particular
divalent metal ion to stabilize the GAAA tetraloop with a gc closing base pair. Preliminary results suggest that, surprisingly, both closing base pairs prefer larger ions for stabilization. An inner-sphere coordination site for the metal ion in the tetraloops may explain these results.

Further studies for both Chapters 2 and 3 are discussed in Chapter 4. After studying the thermodynamics of RNA folding under crowding conditions, folding kinetics of a functional RNA can be studied using stopped-flow spectroscopy as well as fluorescence resonance energy transfer (FRET) single molecule studies. We hypothesize that macromolecular crowding conditions should cause slower unfolding of tertiary structure under cooperative conditions. In addition, Raman spectroscopy and phosphorothioate substitutions can be used to further probe GAAA tetraloops to determine the nature of a metal-coordination site.
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List of Abbreviations

**CoHex**: Cobalt (III) Hexammine

**FRET**: Fluorescence Resonance Energy Transfer

**NLPB**: Non-Linear Poisson Boltzmann
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“I have no special talent. I am only passionately curious.” -Albert Einstein
Chapter 1  
Introduction  

1.1 Hierarchy of RNA Folding  
At the heart of understanding the structure-function relationship in RNA is the “RNA folding problem” (1). Similar to the “Levinthal Paradox” for protein folding (2,3), the question arises as to the ability of the complex nucleic acid to fold in minutes or less in spite of the vast length of time required to sample all possible conformational states before finding the most stable native structure. The popular consensus is that the highly stable and organized secondary structure in RNA allows for pre-organization of the tertiary structure, allowing RNA to fold much faster (4). Nearest neighbor interactions lead to stable secondary structures which form weaker tertiary and finally global structures along the folding pathway.  

1.2 Primary Structure  
At the most basic level, RNA is composed of four Watson-Crick nucleobases: adenine, guanine, cytosine, and uracil, attached to a phosphate group and ribose sugar to create a nucleotide. Phosphodiester bonds between the 5’ phosphate and the 3’ hydroxyl group of the ribose sugar connect each nucleotide base. The nucleotide bases are pseudoaromatic and largely planar, and classified into purines or pyrimidines. Pyrimidines (cytosine and uracil) are composed of one six-member ring with two nitrogen atoms, while purines (adenosine and guanine) contain two joined rings with two nitrogen atoms on each ring. Figure 1.1 shows the structures of the four Watson-Crick nucleobases. Stacking of the aromatic rings stabilizes structure, despite the repulsion of the negatively charged phosphate backbones. The primary sequence contains the information required to fold into the native structure.
1.3 Secondary Structure

While DNA is typically double-stranded, RNA is often single-stranded (i.e. a single strand folds up rather than two strands) which allows it to assume diverse secondary structures such as hairpins, junctions, and other common motifs (5). The conventional Watson Crick base pairing pairs adenosine with uracil (AU base pairs) and cytosine with guanine (CG base pairs). The base pairs are joined by hydrogen bonding: an AU base pair contains two hydrogen bonds, and a CG base pair contains three hydrogen bonds (making it more thermodynamically stable). In addition, the RNA bases are sometimes heavily modified in vivo (ie. tRNA) (6,7), which strengthens or weakens secondary or tertiary structure.

Hairpins or stem-loops are very common secondary motifs in RNA. Hairpins are local regions where the RNA folds back on itself defined by a region of base pairing followed by a loop (of varying sizes) of unpaired nucleotides. The last base pair before the loop is called the closing base pair and often plays a large role in the stabilization of the hairpin (8). The size of the loop is also a factor in determining stability. Of particular importance are the very stable and phylogenetically conserved UUCG and GAAA tetraloops (9). Both tetraloops have a defined structure. UUCG tetraloops are stabilized by a U1-G4 reverse wobble with a syn G, and the C3 base stacking on the U1 base (10,11). In addition, there is a hydrogen bond between the C3 amine and U2 phosphate oxygen (10,11). GAAA tetraloops are stabilized by a sheared base pair between G1 and A4, and stacking between A2 and A3 (12). For both families of tetraloops, the second nucleotide in the loop does not make specific loop contacts – but is required to relieve steric clashes. These tetraloops are normally closed by a cg or gc closing base pair. In particular, cg closing base pairs are more stabilizing for GAAA tetraloops because of the G1 –
g5 cross strand stack (12) and favorable electrostatic interactions (13). Hairpin structures regularly participate in tertiary interactions, such as the well-characterized GAAA tetraloop – receptor (14).

1.4 Tertiary Structure

Conventionally, stable Watson-Crick interactions between complementary RNA strands provide the foundation for tertiary interactions to form and assemble the correct native structure. These tertiary interactions are typically not Watson-Crick, although they are possible. Some of the most stable tertiary interactions are kissing loops (tertiary contacts formed between two loops), with $\Delta G_{37}$ ranging from -6 to -15 kcal/mol (15,16), depending on the number of base pairs between the loops, and the docking of GAAA tetraloops to helical receptors is also a stable tertiary interaction with a $\Delta G_{37}$ of -2 to -4 kcal/mol (17,18). Most other tertiary interactions have not been studied thermodynamically in any detail. Depending on solution conditions such as Mg$^{2+}$ concentrations and macromolecular crowding, tertiary structure formation can be more stable than secondary structure formation, which is especially important for Chapter 2 of this thesis. These effects arise because tertiary structures cause closer contact of backbone phosphates whose interaction is electrostatically and entropically unfavorable (19).

Typically, multivalent cations such as Mg$^{2+}$ are required for proper folding of RNA tertiary structures, since those structures are often more compact, requiring smaller cations to stabilize the smaller pockets between the negatively charged phosphates. To form a functional structure many RNAs must fold into a native tertiary conformation, sometimes through several intermediate states. A lack of populated intermediate states in the folding pathway has been defined as cooperativity (20). The idea of cooperativity of folding in vivo may speak to the
RNA folding problem – the suppression of intermediates along the folding pathway – allowing RNA to find its most stable native structure without needing to sample all the possibilities.

1.5 Crowding and RNA Folding

Although RNA forms its native structure in a relatively crowded, nonhomogeneous aqueous environment inside of cells, most biophysical studies have characterized RNA under dilute homogeneous aqueous conditions. The cell contains many macromolecules, which can occupy almost 40% of the cellular volume (21). Therefore, to more closely approximate cellular conditions, nucleic acids can be studied and characterized in a “crowded” environment. This crowded environment is shown herein to enhance the cooperativity of RNA folding.

Polyethylene glycol (PEG) has been used to simulate the crowding felt by RNA in the cell (22). PEG is a polymer available in varying lengths that acts as a hydrophilic cosolute. PEG appears to alter their stability by lowering the activity of water in a solution (22), although the excluded volume effect may play a role as well (23). In a study on the hammerhead ribozyme, it was shown that cosolutes (including PEG) decreased the stability of the secondary structure but increased the thermal inactivation temperature of the ribozyme (22). In addition, it was determined that PEG lowers the melting temperature of duplexes (24) and other motifs containing Watson-Crick base pairing, while increasing thermostability of compact states such as G-quadruplexes (21). When secondary structure forms, water is “taken up” to interact with the minor and major grooves which stabilizes the secondary structure. However, when tertiary structure forms, water is released so the strands can come closer together (22). The cosolutes lower the concentration of water by up to 40% and so stabilize the water-release reaction of the RNA tertiary folding and destabilize the water-uptake reaction of Watson-Crick base pairing
These observations imply that the depopulation of less stable intermediates disfavored with crowding leads to an increase in the folding cooperativity of RNA.

PEG is just one of the more studied cosolutes – others include ficoll and various sizes of dextran (shown in Figure 1.2). All are long repetitive polymers that dissolve in water and create a heterogeneous environment, more akin to cellular conditions than the idealized dilute conditions. The specific interactions between the cosolutes and water or RNA that cause an increase in cooperativity of RNA folding have been speculated upon, but are inconclusive (22,23). In moving towards more in vivo conditions, we plan to add proteins and other cellular components to the system, although their addition further complicates the study of RNA behavior.

1.6 Role of Metals in RNA Folding

Because of the inherent electrostatic clash between the negatively charged phosphate backbones of nucleic acids, metal cations are essential in stabilizing RNA folding. Some of the most commonly studied biological metal ions – with regards to RNA folding – are sodium (Na⁺), potassium (K⁺), magnesium (Mg²⁺), and calcium (Ca²⁺). Approximate mammalian cellular concentrations of these metals are 10 mM Na⁺, 140 mM K⁺, 30 mM Mg²⁺, and 1 mM Ca²⁺ (25) although concentrations of free Mg²⁺ and Ca²⁺ are 0.5 mM (26) and 0.1 uM (27), respectively. The physical properties of these metals; size, electronic character, and degree of hydration, play a large role in the structure and stabilization of RNA. Table 1.1 lists the approximate ionic radii and enthalpy of hydration of alkaline earth metals. Note that on going down the periodic table, size increases, and the magnitude of enthalpy of hydration decreases.
While monovalent cations such as sodium and potassium can stabilize some secondary structures, divalent cations (particularly Mg\(^{2+}\)) seem to be necessary for most three-dimensional folding in RNA. The effect of Mg\(^{2+}\) ions on RNA folding and stability has been extensively studied (4,5,28,29). The higher the charge density of the RNA, the higher the local concentration of ions. Multivalent ions are more entropically favorable in order to relieve the electrostatic stress than monovalent ions, as they are both smaller and have much higher charge density (2.54 e\(^-\) for Mg\(^{2+}\) compared to 1.14 e\(^-\) for Na\(^+\) (30). In essence, one divalent ion can do the job of two monovalent ions in terms of neutralization, with only half the entropy loss for binding.

Draper classifies the different metal interactions with RNA as either “diffuse ions” or “chelated ions” (31). Figure 1.3 shows a model for both diffuse and chelated Mg\(^{2+}\) ions interacting with RNA. Diffuse ions interact with the RNA electrostatic field and keep their shells of hydration. Interaction of RNA with diffuse ions is driven by long-range electrostatic forces, and the local concentration is determined by the amount of electrostatic potential in the particular region. Chelated ions, on the other hand, coordinate directly to a specific location on the RNA. Some of the water molecules coordinated to both the RNA and the metal are displaced, accruing an energetic penalty that must be overcome in the new electrostatic interaction; in addition, there are repulsive electrostatic forces between the diffuse ions and the partially dehydrated cation. However, these large unfavorable free energies for the chelated cation are compensated by the strong interaction between the RNA and the cation.

While RNA folding might involve a few chelated Mg\(^{2+}\) ions, diffuse ions are a major part of the stabilization of tertiary structures (31). Cobalt (III) hexammine (CoHex) is a trivalent metal cation commonly used to simulate diffuse ions (32), as it is tightly coordinated to six ammine groups and is exchange-inert, thus it cannot participate in chelated interactions with
RNA. CoHex is similar in size to a fully hydrated Mg\(^{2+}\) ion (hexahydrated), therefore it is a good probe for diffuse Mg\(^{2+}\) ions that stabilize RNA structure.

### 1.7 Thesis Format

Chapter 2 discusses approximating the folding of a functional RNA (tRNA) under *in vivo* conditions, using low Mg\(^{2+}\) concentrations and the addition of cosolutes such as PEG and dextran to simulate macromolecular crowding in the cell. While RNA stability and folding has been heavily studied under idealized dilute conditions, its behavior in the crowded environment of the cell is largely unknown and may be quite different. Cosolutes can act as macromolecular crowders, increasing the cooperativity of the folding transition. We show that PEG destabilizes secondary structure, while strengthening tertiary structure to make the transition more cooperative. Similar results are found using dextran 10 (Avg MW of 10kDa). In addition, under crowded conditions less Mg\(^{2+}\) is required (~0.5 mM) to allow for native folding of transfer RNA phenylalanine (tRNA\(^{Phe}\)), more akin to cellular concentrations of free Mg\(^{2+}\) (26). Destabilization of secondary structure elements by PEG is observed, as supported by thermal denaturation experiments on tRNA\(^{Phe}\) fragments. The study of tRNA\(^{Phe}\) mutants revealed that increasing the stability of secondary structure not directly involved in tertiary contacts can increase the thermostability of the RNA, but only if RNA folding from primary to tertiary structure is cooperative.

Chapter 3 focuses on the study of the effects of metals on RNA secondary structure, specifically hairpins. Previously in our lab, nonlinear Poisson-Boltzmann (NLPB) calculations revealed that GAAA loops with a gc closing base pair have higher surface charge density than with a cg closing base pair (13). It was speculated that a ring of negative potential observed in the loop could serve as a site for partially chelated divalent metal ions (13). Therefore we
studied GAAA tetraloops with both cg and gc closing base pairs in the presence of various
divalent alkaline earth metals to determine if there is a metal preference for the gGAAAc
tetraloop. Preliminary results suggest that the larger cation is preferred by both closing base
pairs, and that stability increases with increasing ionic radius. This surprising trend was
confirmed to be peculiar to the GAAA tetraloop, as it was not observed with a control UUUU
tetraloop.

Finally, Chapter 4 focuses on future experiments that stem from the work in Chapters 2
and 3. As mentioned, in Chapter 2, the thermostability of RNA folding in the presence of
cosolutes was examined. Building upon the idea that cosolutes increase the cooperativity of the
folding transition in RNA, thermodynamic and pre-steady state kinetic experiments can be
performed using tRNA$_{\text{Phe}}$ and other biologically relevant RNAs to study the effect of crowding.
In Chapter 3, the influence of divalent ion size on GAAA tetraloop stability was examined.
Future experiments include Raman (33) and phosphorothioate (34,35) spectroscopy to analyze
for direct interaction of the ions with the phosphates.
<table>
<thead>
<tr>
<th>Metal Cation (group II of PT)</th>
<th>Hydration Number</th>
<th>Ionic Radii(^1) (Dehydrated) (Å)</th>
<th>Approx. Ionic Radii(^2) (Hydrated) (Å)</th>
<th>Enthalpy of Hydration(^1) ((\Delta H_{\text{hyd}})) (kJ/mol)</th>
</tr>
</thead>
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<td>5.5</td>
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</tr>
<tr>
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<td>1.35</td>
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</table>

Table 1.1. Ionic radii and enthalpy of hydration of alkaline earth metals.\(^1\) From *The RNA World, Second Edition* (23). \(^2\)Estimated from Mg\(^{2+}\)-O and Ca\(^{2+}\)-O distances in water molecules, 2 Å (30) and 2.64 Å (31), respectively and the hard sphere diameter of a water molecule 2.8 Å (32). For example, Approx. Ionic Radius Mg\(^{2+}\) = 2 Å + 0.72 Å + 2.8 Å = 5.5 Å
Figures:

**Adenine**

**Uracil**

**Guanine**

**Cytosine**

**Figure 1.1.** Structure of the four Watson-Crick nucleobases.
Figure 1.2. Structures of monomers of (A) PEG, (B) dextran, and (C) ficoll.
Figure 1.3. Potential ion environments in RNA. Red circles are nonbridging phosphate oxygens and the oxygen of water molecules. Blue circles are hydrogen atoms. Yellow circles are Mg$^{2+}$ ions. At the left, a fully hydrated diffuse interaction is shown. In the middle, a partially dehydrated interaction is shown. At the right, a chelated Mg$^{2+}$ ion interaction is shown. The Mg$^{2+}$ ion is hexacoordinated in all panels, although not all interactions are depicted. Adapted from (26).
1.8 References


Chapter 2

Molecular Crowding Enhances the Folding Cooperativity and Evolvability of a Functional RNA

[Will be submitted as a paper titled, “Molecular Crowding Enhances the Folding Cooperativity and Evolvability of a Functional RNA” by Elisabeth E. Whitman, Joshua M. Blose, Subba Rao Nallagatla, and Philip C. Bevilacqua.]

2.1 Abstract

While folding of functional RNAs under dilute solution and high ionic strength has been well studied, far less is known about RNA folding under the more in vivo conditions of low Mg$^{2+}$ concentrations and high macromolecular crowding. It is generally thought that while RNA may fold hierarchically, a certain cooperativity of folding transitions may be required to fold the RNA on a biological timescale. In previous research from our lab, it has been shown that a DNA triplex could be tuned to unfold cooperatively from tertiary to primary structure (without population of the secondary intermediate) using low pH of 5.5 (1). Additionally, strengthening Watson-Crick base pairing (by increasing GC base pairs not directly involved in tertiary structure) in the DNA triplex increased the thermostability of the native structure when folding was cooperative (1). However, more biologically relevant parameters than low pH, such as macromolecular crowding and a low 0.5 mM magnesium concentration, would be more useful in studying the cooperativity of RNA under in vivo conditions. Therefore, a well-characterized RNA, transfer RNA phenylalanine (tRNA$^{\text{Phe}}$, secondary and tertiary structures shown in Figure 2.1) was used as a model system to study the effects of macromolecular crowding on the folding of biological RNAs. Thermal denaturation and SHAPE chemistry were employed which showed that tRNA folds cooperatively in physiological conditions of low Mg$^{2+}$ (0.5 mM (2)) and high concentrations of a molecular crowder. This cooperativity is shown in sharp, two-state like
folding transitions. Similar to the earlier triplex studies, strengthening secondary structure of the tRNA not directly involved in tertiary structure can increase thermostability of the tRNA if the molecule folds under cooperative conditions.

2.2 Introduction

In order to form a functional structure many RNAs must fold into a tertiary native conformation, sometimes through several intermediate states. A lack of populated intermediate states in the folding pathway has been defined as cooperativity by Professor Ken Dill (3). In previous research from our lab, it was shown that a DNA triplex could be tuned to unfold cooperatively from tertiary to primary structure (without population of the secondary intermediate) using a low pH of 5.5 (1). Additionally, strengthening Watson-Crick base pairing (by increasing GC content in base pairs not directly involved in tertiary structure) in the DNA triplex increased the thermostability of the native structure when folding was cooperative (1).

RNA forms its native structure in a crowded environment, and cosolutes such as PEG have been used to simulate macromolecular crowding (4-7). Folding transitions in tRNA have been well characterized (8-10); thus tRNA is a good model system to test ideas on cooperativity and thermostability gained from the very simple DNA triplex model. It was shown previously in our lab (11) that macromolecular crowding could be used to increase the cooperativity of transfer RNA phenylalanine (structure shown in Figure 2.2), both as a T7 transcript and a naturally modified wild type. Adding 2 mM MgCl₂ and 40% PEG (wt./vol.) increases the melting temperature (T_M) of the tertiary structure by ~23°C for the T7 tRNA transcript, and ~28°C for the WT tRNA (11). As in the triplex model, creating T7 transcribed tRNA^{Phe} mutants that stabilize secondary structure base pairing without directly affecting tertiary structure should
therefore increase the thermostability ($T_M$ of the functional tertiary structure) of the tRNA when the complex is tuned to fold cooperatively.

2.3 Materials and Methods

**RNA Preparation**

Wild type yeast tRNA$^{Phe}$ was purchased from Sigma Aldrich, and purity confirmed by denaturing 7 M Urea 10% PAGE. The RNA was dialyzed as described below at 4 °C for 8 h in 10 mM NaCl and 0.1 mM EDTA and for another 8 h in 10 mM sodium cacodylate (pH 7.0) prior to usage. WT and mutant transcripts were made using T7 RNA polymerase in 40 mM Tris-HCl (pH 8.0), 25 mM MgCl$_2$, 2 mM DTT, 1 mM spermidine, and 3 mM NTPs, and purified using 10% PAGE. DNA oligonucleotide templates to make WT and mutants transcripts were purchased from Integrated DNA Technologies (IDT, Coralville, IA) and used without further purification. The TS T7-23 oligonucleotide was annealed to the template prior to transcription. Sequences of DNA oligonucleotides for T7 transcription of tRNAs were as follows (modifications are underlined):

**TS T7-23:** 5’ TAA TAC GAC TCA CTA TAG

**WT tRNA template:** 5’ TGGTGCGAATTCTGTGGATCGAACACAGGACCTCCAGATCTTCAGTCTGGCGCTCTCCCAACTGAGCTAATCCGCTATAGGTGA GTCGTATTA

**M1 tRNA template:** 5’TGGTGCGAGATTCTGTGGATCGAACACAGGACCTCCAGATCTTCAGTCTGGCGCTCTCCCAACTGAGCTAATCCGCTATAGGTGA GTCGTATTA

**M2 tRNA template:** 5’ TGGTGCGAGTTCTGTGGATCGAACACAGGACCTCCAGATCTTCAGTCTGGCGCTCTCCCAACTGAGCTAAAACCGCTATAGGTGA GTCGTATTA
M3 tRNA template:  
5’-TGGTGCGGGTTCTGTGGATCGAACACAGGACCTCCAGATCTCTCAGTCTGGCGCTCTCCCCAAGCTAAAAACCGCTATAAGTGAGTCGATATT

Sequences of T7 tRNAs were as follows:

WT tRNA: 5’ GCGGAUUUAGCUCAGUUGGGAGAGCGCCAGACUGAAGAUCUGGAGGUCCUGUGUUCGAUCCACAGAAUUCGCACCA

M1 tRNA: 5’ GCGGAUUUAGCUCAGUUGGGAGAGCGCCAGACUGAAGAUCUGGAGGUCCUGUGUUCGAUCCACAGAAUUCGCACCA

M2 tRNA: 5’ GCGGGAUUUAGCUCAGUUGGGAGAGCGCCAGACUGAAGAUCUGGAGGUCCUGUGUUCGAUCCACAGAAUUCGCACCA

M3 tRNA: 5’ GCGGGAUUUAGCUCAGUUGGGAGAGCGCCAGACUGAAGAUCUGGAGGUCCUGUGUUCGAUCCACAGAAUUCGCACCA

Hairpin fragments for tRNA were purchased from DharmacoM, Inc. (Chicago, IL) and dialyzed into 10 mM sodium cacodylate (pH 7.0) and purity confirmed by denaturing PAGE.

Sequences of tRNA hairpin fragments are as follows:

Acceptor Stem: 5’-GCGGAUUUUUUUUUUAAUUCGC

D Loop Stem: 5’-GCUCAGUUGGGAGAGC

AC Loop Stem 5’-CCAGACUGAAGAUCUGG

TΨC Loop Stem: 5’-CUGUGUUCGAUCCACAG

All RNA was dialyzed into 10 mM sodium cacodylate (pH 7.0) buffer using a microdialysis system (Gibco-BRL Life Technologies). Secondary structures of fragments are provided in Figure 2.2 and folds were confirmed with mFold: at 25% suboptimal free energy and a window size of 0, these sequences gave only the expected hairpin fold. The acceptor stem was
designed based on a compromise: the size of its loop during melting will depend on the sequence of the stem. For a very stable stem, like in M3, the loop would be the remainder of the sequence (or ~ 58 nt) because the acceptor stem would be the last species to melt, while in other cases, it is more likely that there is co-melting of other helical species, as per data in Figures 2.3, 2.4 and 2.5. As such, we made a loop of 8 U’s as a compromise. Given the approximation in the loop we did not make a literal 3’-overhang of the ACCA for all RNAs. We did prepare this overhang sequence for the WT, and in this case saw a $T_M$ change of 6.5 °C under standard melt conditions of 0.5 mM Mg$^{2+}$/140 mM KCl/0% PEG, which would serve to make the melts in Figure 2.6A only more non-cooperative. In the presence of 0.5 mM Mg$^{2+}$/140 mM KCl/40% PEG-200, the $T_M$ change for the overhang sequence was only 1.7°C, which keeps the melts in Figure 2.6B cooperative. Overall, these effects suggest that the acceptor stem models used herein are reasonable.

Determination of Thermodynamic Parameters by UV Melting

All RNA, including full-length tRNA and helical fragments, was first renatured by heating to 90°C for 3 min (in buffer and KCl only) and cooled to room temperature over 15 min. The MgCl$_2$ was then added to RNA and the samples were heated to 55°C for 3 min and cooled to room temperature over 15 min. Melting experiments were performed on a Gilford Response II spectrophotometer with a data point acquired every 0.5°C and a heating rate of ~0.6°/min at 260 nm. Melts of WT and mutant transcripts were normalized by dividing absorbances by the high-temperature absorbance value, while tRNA$^\text{Phe}$ helical fragments were normalized by dividing absorbances to give a final concentration (5 μM). These differences in normalization methods caused the dA$_{260}$/dT to take on different values between plots of WT and mutant tRNA$^\text{Phe}$, and a
plot of tRNA\textsuperscript{Phe} and stem loop fragments (notably between panels A and B-E and panels F and G-J in Figures 2.3, 2.4, and 2.5). Monophasic melt data were fit to a two-state model using sloping baselines and analyzed using a Marquadt algorithm for nonlinear curve fitting in KaleidaGraph v. 3.5 (Synergy software). Derivative plots were of normalized data and were smoothed using an 11 point window prior to taking the derivative. Samples for yeast tRNA\textsuperscript{Phe}, WT transcript, and mutants were 0.5 μM RNA, and in the standard melt buffer of 10 mM sodium cacodylate (pH 7.0) and 140 mM KCl, along with 0 – 2.0 mM MgCl\textsubscript{2} and 0 – 40% (w/v) PEG200 depending on the melt.

*Chemicals*

Polyethylene glycol (PEG) 200 was obtained from Sigma. PEG8000 was obtained from Sigma (Fluka) and no RNases or DNases were detected. MgCl\textsubscript{2} was obtained from J.T. Baker, KCl was obtained from EMD Chemicals, and sodium cacodylate was obtained from Sigma.

**2.4 Results and Discussion**

**2.4.1 Mg\textsuperscript{2+} and PEG can tune cooperativity of tRNA**

Thermal denaturation studies of both the natural tRNA\textsuperscript{Phe} from yeast and the T7 transcript of tRNA\textsuperscript{Phe} revealed that both Mg\textsuperscript{2+} and PEG can independently tune cooperativity of the RNA folding. Figure 2.7C shows that increasing Mg\textsuperscript{2+} concentration in the range of 0 – 2 mM can increase the cooperativity of the transition (the steepness of the melt derivative plot) of the T7 transcript tRNA\textsuperscript{Phe} as well as the thermostability of the tRNA (tertiary melting temperature) by approximately 10°C. Figure 2.7D shows that increasing PEG200 percentage (w./v.) also
increases the cooperativity of the T7 transcript tRNA\textsuperscript{Phe}. However, thermostability is slightly decreased in this case, speculated to be because of the actual formation of tertiary structure – under conditions of only 10 mM Na\textsuperscript{+} and no PEG200, it is very likely that tertiary structure of the T7 transcript is not fully formed. Similar results are seen for the natural yeast tRNA\textsuperscript{Phe} – Figure 2.1A-C shows an increase in the thermostability and cooperativity upon addition of increasing amounts of Mg\textsuperscript{2+}, and Figure 2.1D-F shows an increase in cooperativity upon addition of increasing PEG200 percentage. Unlike in the T7 transcript (Figure 2.7D), thermostability is not decreased by PEG200, most likely due to increased tertiary strength in the naturally chemically modified tRNA\textsuperscript{Phe}. Thermodynamic parameters for the T7 transcript tRNA\textsuperscript{Phe} are shown in Table 2.1. In addition, the reversibility of melts in 40% PEG200 and the absence of Mg\textsuperscript{2+} was confirmed (shown in Figure 2.8). Thus, both Mg\textsuperscript{2+} and PEG200 increase thermostability and cooperativity of the folding transition in tRNA\textsuperscript{Phe}.

2.4.2 Use of helical fragments to analyze cooperativity

To further investigate the role of PEG in increasing the folding cooperativity of tRNA\textsuperscript{Phe}, helical fragments (structures shown in Figure 2.2) of the T7 transcript were studied under similar cooperative (low Mg\textsuperscript{2+}, high crowding) and noncooperative (low Mg\textsuperscript{2+}, no crowding) conditions. (See Materials and Methods section for fragment sequences and design considerations.) If tertiary structure is less stable than the independent helical fragments, then folding would be noncooperative. However, if tertiary structure is more stable than the independent helical fragments, folding would be cooperative. Thermal denaturation experiments were performed on the helical fragments in various combinations of 0, 0.5, and 2 mM Mg\textsuperscript{2+}, and in 0%, 20%, and
40% PEG200. Thermodynamic parameters for the full tRNA and helical fragments are shown in Tables 2.2 and 2.3.

In the absence of Mg\textsuperscript{2+}, RNA folding transitions are mostly noncooperative, although the addition of 40% PEG200 did stabilize tertiary structure (see Figure 2.3A-B and 2.3F-G). Without Mg\textsuperscript{2+} and PEG200, there is a hint of tertiary structure around 45°C, with the bulk of the secondary structure melting out around 54°C (although the transitions are quite broad). The helical fragments melt out at temperatures that correspond to transitions in the full tRNA (Figure 2.3B), further indicative of a noncooperative transition. Upon addition of 40% PEG200, tertiary structure appears more defined, with an approximate T\textsubscript{M} of 40°C (Figure 2.3F). The bulk of the secondary structure of the T7 transcript tRNA\textsuperscript{Phe} melts out around 49°C. In addition, the acceptor stem, D Loop, and AC Loop melt out nearer to the full length tRNA\textsuperscript{Phe} (49.1°C), with T\textsubscript{M}’s of 46.8°C, 44.9°C, and 48.9°C, respectively (Table 2.2). However, the TΨC Loop melts out after the full length tRNA (54°C), indicating that the tRNA folding transition is at most semi-cooperative.

In physiological Mg\textsuperscript{2+} conditions (0.5 mM Mg\textsuperscript{2+}) and no PEG, the folding of the full tRNA\textsuperscript{Phe} appears to be noncooperative, as most of the helical pieces have higher T\textsubscript{M}’s (57.9°C, 65.7°C, 54.9°C, and 60.2°C for the A stem, D Loop, AC Loop, and TΨC Loop fragments, respectively) than the full length RNA (57.6°C, Table 2.2). As shown in Figures 2.6B and 2.9A, in 0.5 mM Mg\textsuperscript{2+} and no PEG, the helical fragments melted out before or with the full length tRNA\textsuperscript{Phe}. However, when physiological-like crowding is added (40% PEG200) in the background of 0.5 mM Mg\textsuperscript{2+}, the T\textsubscript{M}’s of most of the independent helical fragments decreased (48.5°C, 52.9°C, 56.9°C, and 48.4°C for the A stem, D Loop, AC Loop, and TΨC Loop fragments, respectively), melted out well before the full length tRNA\textsuperscript{Phe} (57.7°C) (Figures 2.6
and 2.9B). Upon addition of 40% PEG (in 0.5 mM Mg\(^{2+}\)), the T\(_{M}\) of the full length tRNA\(^{\text{Phe}}\) is unchanged, only cooperativity is increased – in effect, tertiary structure is strengthened, while secondary structure is destabilized (comparing panel A and B in Figure 2.9). The net effect is a sharper transition. This is further demonstrated in Figure 2.4A, where the T\(_{M}\) of the sum of the fragments decreases as PEG200 is added (cooperativity is unaffected), while the full length tRNA\(^{\text{Phe}}\) increases in cooperativity upon addition of PEG200. Thus, just the presence of physiological Mg\(^{2+}\) will not allow the tRNA\(^{\text{Phe}}\) to fold cooperatively; molecular crowding is also required for a cooperative folding transition.

In 2 mM Mg\(^{2+}\), results are slightly different. The increase in Mg\(^{2+}\) concentration above 0.5 mM allows the folding transition to be semi-cooperative in the absence of PEG200: both the acceptor stem and the T\(\Psi\)C Loop melt out before the full tRNA\(^{\text{Phe}}\) (64.7°C), with T\(_{M}\)’s of 58.0°C and 60.5°C, respectively. However, the D Loop and the AC Loop melt out after the full tRNA, with T\(_{M}\)’s of 65.7°C and 68.3°C. These transitions are shown in Figure 2.5B. The full length T7 transcript tRNA\(^{\text{Phe}}\) shows a single, relatively cooperative transition (Figure 2.5A). Upon the addition of 40% PEG200, the folding transition becomes even more cooperative, with the melting transition taking place over a smaller temperature range (Figure 2.5F) and the T\(_{M}\) unaffected. The helical fragments are destabilized with the addition of PEG200, all melting out before the full length tRNA\(^{\text{Phe}}\) (63.9°C), with T\(_{M}\)’s of 49.9°C, 55.5°C, 52.7°C, and 58.7°C for the acceptor stem, D Loop, AC Loop, and T\(\Psi\)C Loop, respectively. In sum, cooperative folding transitions occur for the T7 transcript tRNA\(^{\text{Phe}}\) in 0.5 mM and 2 mM Mg\(^{2+}\) in 40% PEG200.
2.4.3 Effect of strengthening base pairing in tRNA

Increased GC content is often found in the functional RNA of thermophiles (12,13). To test the effects of strengthening secondary structure on the thermostability of RNA, mutants were designed that increased the GC base pair content of the acceptor stem. These mutations were made as they did not directly involve any nucleotides involved in tertiary structure formation (Figure 2.7A) and allowing us to look only at stability gained by increasing secondary structure strength. Mutant 1 (M1) changed base pair 4 from an GU wobble to a GC pair, M2 changed base pair 5 from a AU to a GC pair, and M3 made both mutations (secondary structures shown in Figure 2.7). As with the helical fragments, the full length tRNA\textsuperscript{Phe} mutants were studied in various Mg\textsuperscript{2+} concentrations (0-2 mM) and PEG200 percentages (0-40% w/v). All thermodynamic parameters for the T7 transcript tRNA\textsuperscript{Phe} and mutants are shown in Table 2.1. Thermodynamic parameters for the mutant acceptor stems are shown in Table 2.3.

In the absence of Mg\textsuperscript{2+} and PEG, all mutants showed broad transitions, as expected under such low salt conditions (and lack of divalent metals). Shown in Figure 2.3A, there was perhaps a hint of tertiary structure formation around 40°C for the mutants. As expected, all four full length tRNAs had different high temperature T\textsubscript{M}’s, which depended on the extent of secondary structure stabilization made to the acceptor stem. Comparison of the helical fragments (with acceptor stem for each mutant) showed a noncooperative transition, with most of the helical pieces melting out with the full length tRNA (Figure 2.3C-E).

Upon addition of 40% PEG200, the above destabilization of the secondary structure of the mutants was seen as expected from studies on the WT (Figure 2.3F-J). All the tRNAs had a tertiary transition at approximately 42°C and a secondary transition corresponding to the strength of the secondary structure of each mutant (49.1°C, 53.9°C, 53.7°C, 58.8°C for the WT, M1, M2,
and M3, respectively). Similar results were seen for conditions of no Mg\(^{2+}\) and 20\% PEG, with a tertiary T\(_M\) of approximately 41°C (data not shown). Thus, in the absence of Mg\(^{2+}\), the folding transition of tRNA\(^{\text{Phe}}\) and mutants are noncooperative, so strengthening base pairing only strengthens secondary structure, it does not affect the thermostability of the RNA.

In physiological 0.5 mM Mg\(^{2+}\), the folding was noncooperative without the addition of a molecular crowder. The helical pieces with mutant acceptor stems melted out with the full length tRNA (Figure 2.6C-E). The WT and mutants have a tertiary T\(_M\) of approximately 50°C, with secondary structure T\(_M\)’s of 57.6°C for the WT, 63.6°C for M1, 64.0°C for M2, and 58°C and 71°C for M3 (Figures 2.6A, 2.9C). Upon addition of 20\% PEG, the folding transition became more cooperative, becoming more two-state with T\(_M\)’s of 56.9°C, 60.7°C, 61.8°C, and 62.5°C for the WT, M1, M2, and M3, respectively. While the T\(_M\) of the RNAs was not greatly affected by the further addition of 40\% PEG200, cooperativity of the mutants was increased by the added PEG200 (Table 2.1). Indeed, this was further illustrated in Figure 2.4, where increasing PEG200 percent increased the cooperativity of the full length tRNA while decreasing the stability of the helical fragments. The addition of a GC base pair added approximately 3-4°C to the thermostability of the tRNA (Table 2.1, Figure 2.6F) in 0.5 mM Mg\(^{2+}\) and 40\% PEG200. Therefore, while strengthening secondary structure in physiological Mg\(^{2+}\) alone does not affect thermostability, adding a molecular crowder in the presence of Mg\(^{2+}\) allows the folding transition to become cooperative and thermostability of the RNA is increased by strengthening secondary structure.

Finally, in 2 mM Mg\(^{2+}\), stabilizing secondary structure in the absence of a molecular crowder added some thermostability to the tRNA, as the transition appears two-state. M3 shows the effect of over-stabilization of secondary structure (Figure 2.5A), and puts a limit on tertiary
structure stabilization. $T_M$’s for the folding transition were 64.7°C, 69.6°C, 68.5°C, and 72.1°C for the WT, M1, M2, and M3, respectively. Transitions with the mutant helical fragments also showed a partial to full cooperative transition (Figure 2.5C-E). The addition of 20% or 40% PEG200 did not affect the $T_M$ of the full length mutant tRNAs, as expected (Figure 2.5, compare upper and lower rows), but it does increase the cooperativity of the folding transition for all mutants (Table 2.1). The addition of a GC base pair added approximately 3-4°C to the thermostability of the tRNA (similar to conditions of 0.5 mM Mg$^{2+}$ and 40% PEG). Thus, the cooperativity of the folding transition allows for increased thermostability upon the strengthening of secondary structure.

### 2.4.4 Crowding effect is general

To see if the results described were unique to PEG200, thermal denaturation studies were performed with a larger PEG polymer (PEG8000) and dextran 10 in 0.5 mM Mg$^{2+}$, comparing wild-type and M3. As shown in Figure 2.10A and 2.10B, the addition of PEG8000 increases the cooperativity of the tRNA folding transition for the WT and M3. For M3 especially, increasing percent of PEG8000 from 20 to 40% increased the cooperativity of the folding transition. Figure 2.10C further shows the similarity of the PEG200 and PEG8000 results. In addition, the tRNA behaved similarly in dextran 10. Increasing the percentages of dextran 10 increased the folding cooperativity of both the WT and M3 (Figure 2.10D, 2.10E). Likewise, dextran 10 sharpened the melts in a manner similar to PEG200 (Figure 2.10F). Therefore, crowding effects appear to not be unique to one crowder or polymer size.
2.4.5 Assigning melt transitions

Several controls were carried out to confirm the nature of the melting transition. A tRNA\textsubscript{Phe} lacking tertiary structure was designed ("No Tert") to compare to the WT folding transitions. We changed the D, T\psiC, and variable loops to U’s, which removes the bulk of the tertiary interactions (Figure 2.11A). Melts of the No Tert RNA are shown in Figure 2.11B. In 0.5 mM Mg\textsuperscript{2+} and no PEG, the No Tert and WT tRNA looked similar, consistent with the lack of cooperativity. Upon the addition of 40% PEG200, the No Tert tRNA melted approximately 8°C lower than the WT (and lacked an increase in cooperativity), while the WT retained the same T\textsubscript{M} and increased cooperativity. Thus, the tertiary structure in the WT prevents the secondary structure from melting at its “normal” T\textsubscript{M}, whether this secondary structure comes from helical fragments or a physically connected secondary structure in No Tert.

2.5 Conclusions

Through the study of a tRNA, we established that a functional RNA can fold cooperatively in the presence of physiological Mg\textsuperscript{2+} concentrations when there is a molecular crowder. This observation suggests that RNA secondary structure may be less stable \textit{in vivo} than what is generally assumed and that tertiary structure may be more stable than predicted. Future studies will be needed to determine if this trend can be applied to other functional RNAs.

The study of secondary structure-strengthening mutants established that, under conditions of a cooperative, two-state system, thermostability of a functional RNA can be increased through secondary structure alone. By increasing GC base pair content in the acceptor stem of tRNA\textsubscript{Phe} (uninvolved in tertiary structure formation) under cooperative conditions, the thermostability of the RNA was increased by approximately 3-4°C per GC base pair. This speaks to the
evolvability of RNA – instead of creating new tertiary interactions, RNA might be able to increase thermostability by strengthening base pairing alone. Thus, using crowding to more accurately mimic RNA under cellular conditions may provide insight into RNA adaptation to extreme environments *in vivo*, as well as routes to engineer stability of RNA for in vitro uses such as medicinal tools. Further, as described in Chapter 4, kinetics of folding may be faster and more monophasic under crowded conditions.

### 2.6 Acknowledgements

EEW conducted experiments and co-wrote the manuscript with PCB. JMB performed thermal denaturation experiments on Yeast tRNA$^{\text{Phe}}$. This work was supported by NSF Grant MCB-0527102 to P.C.B.
### TABLES:

#### Table 2.1. Thermodynamic parameters for unfolding of T7 transcript tRNA<sup>Phe</sup> and mutants.

<table>
<thead>
<tr>
<th>Mg (mM),</th>
<th>T&lt;sub&gt;M&lt;/sub&gt;(°C)</th>
<th>ΔH (kcal/mol)</th>
<th>max dA/dT</th>
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<tbody>
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<td>PEG200(%)</td>
<td>WT M1 M2 M3 WT M1 M2 M3 WT M1 M2 M3</td>
<td>WT M1 M2 M3</td>
<td></td>
</tr>
<tr>
<td>0, 0</td>
<td>Broad 56.9 50, 63.2 50, 63.0 50, 69.5 42.7 33.3 33.2 23.0 0.0090 0.0094 0.0098</td>
<td>0.0084 0.0095 0.0093</td>
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<tr>
<td>0.33, 0</td>
<td>Broad, 57.6 50, 63.6 50, 64. 50, 58, 71 50.5 34.4 31.5 27.1 0.0092 0.0084</td>
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<tr>
<td>0.5, 0</td>
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<tr>
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<tr>
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<tr>
<td>2, 40</td>
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<td>0.0237 0.0247 0.0200</td>
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All melts were performed in a background of 10 mM sodium cacodylate (pH 7.0) and 140 mM KCl as described in Materials and Methods. Melts of tRNA<sup>Phe</sup> and mutants were normalized by dividing absorbances collected as a function of temperature by the high-temperature absorbance value. In order to extract enthalpy and T<sub>m</sub> parameters, data were fit to a two-state model as described above.
Table 2.2. Thermodynamic parameters of T7 transcript tRNA<sup>Phe</sup> and tRNA fragments.

<table>
<thead>
<tr>
<th>Mg (mM), PEG200 (%)</th>
<th>Melt transition of WT T7 tRNA</th>
<th>T7 WT tRNA</th>
<th>T&lt;sub&gt;M&lt;/sub&gt; (°C)</th>
<th>A stem</th>
<th>D Loop</th>
<th>AC Loop</th>
<th>TΨC Loop</th>
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<td>Non-cooperative</td>
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<td>55.3</td>
<td>61.6</td>
<td>55.9</td>
<td>64.3</td>
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<tr>
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<td>Non-cooperative</td>
<td>57.6</td>
<td>57.9</td>
<td>65.7</td>
<td>54.9</td>
<td>60.2</td>
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<tr>
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<td>Semi-cooperative</td>
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<td>Non-cooperative</td>
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<td>52.9</td>
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<td>0.5, 40</td>
<td>Cooperative</td>
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<td>2, 40</td>
<td>Cooperative</td>
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<td>49.9</td>
<td>55.5</td>
<td>52.7</td>
<td>58.7</td>
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</tbody>
</table>

All melts were performed in a background of 10 mM sodium cacodylate (pH 7.0) and 140 mM KCl as described in Materials and Methods. Full length WT T7 tRNA<sup>Phe</sup> and helical fragments were normalized for concentration (5 μM). These values are all for the WT acceptor stem; values for mutant acceptor stems can be found in Table 2.3.

Table 2.3. Thermodynamic parameters of mutant acceptor stems (Astems).

<table>
<thead>
<tr>
<th>Mg (mM), PEG200 (%)</th>
<th>Astem T&lt;sub&gt;M&lt;/sub&gt; (°C)</th>
</tr>
</thead>
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<tr>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>0, 0</td>
<td>55.3</td>
</tr>
<tr>
<td>0.5, 0</td>
<td>57.9</td>
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<td>2, 0</td>
<td>58.0</td>
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<tr>
<td>0, 40</td>
<td>46.8</td>
</tr>
<tr>
<td>0.5, 40</td>
<td>48.5</td>
</tr>
<tr>
<td>2, 40</td>
<td>49.9</td>
</tr>
</tbody>
</table>

All melts were performed in a background of 10 mM sodium cacodylate (pH 7.0) and 140 mM KCl as described in Materials and Methods. Full length WT T7 tRNA<sup>Phe</sup> and helical fragments were normalized for concentration (5 μM).
Figure 2.1. Derivative melt curves of yeast tRNA$^\text{Phe}$ with varying Mg$^{2+}$ and PEG200 concentrations (w/v). Panels A, B, and C show derivative curves with varying Mg$^{2+}$ in (A) 0%, (B) 20%, and (C) 40% PEG200. Panels D, E, and F show derivative curves with varying PEG200 in (D) 0 mM Mg$^{2+}$, (E) 0.5 mM Mg$^{2+}$, and (F) 2 mM Mg$^{2+}$. All RNA is melted in the background of 10 mM sodium cacodylate (pH 7.0) and 140 mM KCl. These melts were performed by Joshua Blose.
Figure 2.2. Secondary structures of T7 tRNA$^{Phe}$ fragments.
**Figure 2.3.** First-derivative melt curves of WT and mutant T7 transcript tRNA\(^{\text{Phe}}\) and stem-loop fragments. (A) Melt curve of tRNA\(^{\text{Phe}}\) and mutants M1, M2, and M3 in no Mg\(^{2+}\) or PEG. (B-E) Curve of T7 transcript tRNA\(^{\text{Phe}}\) and M1, M2, and M3 with stem loop fragments of tRNA\(^{\text{Phe}}\) in no Mg\(^{2+}\) and PEG. (F) and (G-J) are identical to panels (A) and (B-E), respectively, except in the presence of 40% PEG200. All RNA is melted in a background of 10 mM sodium cacodylate (pH 7.0) and 140 mM KCl.
Figure 2.4. Comparison of increasing PEG200 percent of (A) T7 transcript tRNA\textsuperscript{Phe}, (B) M1, (C) M2, and (D) M3, and the sum of the absorbances of the 4 tRNA\textsuperscript{Phe} fragments for the respective RNA. In this figure, a single y-axis is used for full length and fragments. All RNA is melted in the background of 10 mM sodium cacodylate (pH 7.0), 140 mM KCl, and 0.5 mM Mg\textsuperscript{2+}. 
Figure 2.5. First-derivative melt curves of WT and mutant T7 transcript tRNA\textsubscript{Phe} and stem-loop fragments. (A) Melt curve of tRNA\textsubscript{Phe} and mutants M1, M2, and M3 in 2 mM Mg\textsuperscript{2+}. (B-E) Curve of T7 transcript tRNA\textsubscript{Phe} and M1, M2, and M3 with stem loop fragments of tRNA\textsubscript{Phe} in 2 mM Mg\textsuperscript{2+}. (F) and (G-J) are identical to panels (A) and (B-E), respectively, except in the presence of 40% PEG200. Data between 10 and 30\degree C were flat (not shown). All RNA is melted in a background of 10 mM sodium cacodylate (pH 7.0) and 140 mM KCl.
Figure 2.6. First-derivative melt curves of WT and mutant T7 transcript tRNA$^{\text{Phe}}$ and stem-loop fragments. (A) Melt curve of tRNA$^{\text{Phe}}$ and mutants M1, M2, and M3 in 0.5 mM Mg$^{2+}$. (B-E) Curve of T7 transcript tRNA$^{\text{Phe}}$ and M1, M2, and M3 with stem loop fragments of tRNA$^{\text{Phe}}$ in 0.5 mM Mg$^{2+}$. (F) and (G-J) are identical to panels (A) and (B-E), respectively, except in the presence of 40% PEG200. Data between 10 and 30°C were flat (not shown). All RNA is melted in a background of 10 mM sodium cacodylate (pH 7.0) and 140 mM KCl.
Figure 2.7. Cooperative folding of tRNA is driven by Mg$^{2+}$ and crowding. The (A) secondary and (B) tertiary structure of tRNA$^{\text{Phe}}$. Rendered from a 1.93 Å structure of tRNA$^{\text{Phe}}$ (PDB 1EHZ) (38). Tertiary interactions are depicted in gold. Two mutant T7 transcripts, M1 and M2, that strengthen base pairing without affecting tertiary interactions are depicted; M3 contains both base pair mutations. (C) First-derivative melt curves of WT T7 tRNA$^{\text{Phe}}$ in increasing Mg$^{2+}$ concentration in the absence of PEG. (D) First derivative melt curves in increasing PEG200 percent (w/v) in the absence of Mg$^{2+}$. All RNA is melted in a background of 10 mM sodium cacodylate (pH 7.0) and 140 mM KCl.
Figure 2.8: (A-D) Reversibility of melts in the presence of 40% PEG200 and 10 mM sodium cacodylate (pH 7.0) and 140 mM KCl. Reverse melts were done first, followed a forward melt. We omitted Mg$^{2+}$ so that the RNA did not degrade during the high temperature portion of the forward melt.
Figure 2.9. Cooperative folding of tRNA is driven by crowding in the presence of physiological Mg\(^{2+}\). First-derivative melt curves of WT and mutant T7 tRNAs\(^{\text{Phe}}\) and helical fragments. (A) Melt curve of WT T7 tRNA\(^{\text{Phe}}\) and helical fragments in 0.5 mM Mg\(^{2+}\). Note the double y-axis. (B) Identical to panel (A) except in the presence of 40% PEG w/v. Additional considerations for acceptor stem design and melts are provided in Materials and Methods. (C) Melt curve of tRNA\(^{\text{Phe}}\) and mutants M1, M2, and M3 in physiological 0.5 mM Mg\(^{2+}\). (D) Identical to panel (C) except in the presence of 40% PEG w/v. Data between 10 and 30°C were flat (not shown). All RNA is melted in a background of 10 mM sodium cacodylate (pH 7.0) and 140 mM KCl.
Figure 2.10. Comparison of first derivative absorbance curves of T7 transcript tRNA\textsuperscript{Phe} and M3 in 0.5 mM Mg\textsuperscript{2+} and PEG200, PEG8000, or dextran10K. (A-B) Effect of increasing PEG8000 percent on (A) T7 WT and (B) M3. (C) Comparison of PEG200 and PEG8000 of tRNA\textsuperscript{Phe} T7 transcript and M3 in 40% PEG percent. All RNA is melted in the background of 10 mM sodium cacodylate (pH 7.0) and 140 mM KCl. (D-E) Effect of increasing dextran10K percent on (D) T7 WT and (E) M3. (F) Comparison of PEG200 of tRNA\textsuperscript{Phe} T7 transcript and M3 in 40% PEG and 10% dextran 10. Lines were added to these plots for clarity.
Figure 2.11. (A) Secondary structure of No Tert tRNA where the nucleotides in the loop of the D loop, variable loop, and TΨC loop have been changed to U and boxed. (B) Comparison of derivative melt curves of T7 transcript tRNA\textsuperscript{Phe} and tRNA\textsuperscript{Phe} without tertiary structure (‘No Tert’) in 0.5 mM Mg\textsuperscript{2+} and 0% and 40% PEG200. All RNA is melted in the background of 10 mM sodium cacodylate (pH 7.0) and 140 mM KCl.
2.7 References


3.1 Abstract

Hairpins are common in RNA structures and play important roles in nature. GAAA and UUCG tetraloops are part of phylogenetically conserved hairpin families (1). Previously in the lab, we observed a linear relationship between $\Delta G_{37}^o$ and $\log [\text{Na}^+]$ for both families, and loops with a cg closing base pair had a reduced dependency of stability on salt (2). Through nonlinear Poisson-Boltzmann (NLPB) calculations, it was determined that GAAA loops with a gc closing base pair had higher surface charge density which gave rise to the higher salt dependency, and it was postulated that a ring of negative potential in the loop could be a coordination site for divalent metal ions (2). GAAA tetraloops with both cg and gc closing base pairs were studied in the presence of divalent alkaline earth metals to analyze salt dependence and determine if there is a preference for a particular divalent metal ion to stabilize the GAAA tetraloop with a gc closing base pair. Preliminary results suggest that both closing base pairs prefer larger ions for stabilization, and stability increases with increasing ionic radius – the opposite trend than what was expected, this trend was confirmed with a UUUU tetraloop. These results may be explained if the site is inner-sphere, given the increased energetic penalty for dehyrdration of the smaller metal ions. In addition, the decrease in stability of the gc closing base pair with the exchange-inert cobalt hexamine compared to $\text{Sr}^{2+}$ and $\text{Ba}^{2+}$ further suggests a potential direct (i.e. inner-sphere) coordination site for a divalent metal ion. This trend does not exist for a $\text{U}_4$ loop with identical stem, so it is possibly unique to the structured GAAA tetraloops.
3.2 Introduction

Nearly 50% of hairpin structures in rRNA are tetraloops (3). UNCG and GNRA tetraloops, where N is any nucleotide and R is a purine, are quite thermodynamically stable, and even more so when combined with a cg closing base pair (1,4). Most metal binding sites in RNA are in relatively large RNAs, however we have initial data that the simple GAAA tetraloop has a metal binding site, and that it has unusual preference for inner-sphere coordination. To study the salt dependence of UUCG and GAAA tetraloops with cg and gc closing base pairs, thermal denaturation was previously performed in our lab to measure the thermodynamic parameters including the free energy (ΔG) of the tetraloops (2,5). Plots of ΔG37 versus the log of sodium ion concentration show a greater slope for both UUCG and GAAA loops with a gc closing base pair than a cg closing base pair (2). Out of the four tetraloop combinations, the GAAA tetraloop with a gc closing base pair had the greatest salt dependence (2). In addition, surface potential maps were calculated using non linear Poisson-Boltzmann calculations (6) to look at potential differences in electrostatic potentials between GAAA and UUCG tetraloops with cg and gc closing base pairs. The GAAA tetraloop with a gc closing base pair displayed a ring of negative potential that is absent in the other tested tetraloops, even GAAA loops with a cg closing base pair (shown in Figure 3.1) (2). That ring of negative potential could be a binding pocket for a metal ion, particularly a divalent metal ion which is smaller and could easily fit into a small pocket. Thermal denaturation experiments were performed on GAAA tetraloops with cg and gc closing base pairs (structures shown in Figure 3.2) using different concentrations of alkaline earth metal ions – magnesium, calcium, strontium, and barium – to determine the difference in free energy (ΔΔG) between cg and gc closing base pairs. Preferential stabilization by a particular
divalent metal cation was elucidated by comparison of the tetraloop free energy in the presence of each of the metals. From these studies, a possible trend between salt dependence of the tetraloops and ionic size was determined.

3.3 Materials and Methods

3.3.1 RNA Preparation

All RNA hairpins were chemically synthesized by Dharmacon, Inc. and deprotected as per manufacturer’s suggestions, and dialyzed for 8 h into 10 mM NaCl and 0.1 mM Na₂EDTA (pH 7.0) to remove any heavy metals, and dialyzed for 8 h into 10 mM sodium cacodylate buffer (pH 7.0). The sequence of all hairpins are as follows (where stem nucleotides are underlined):

BW5  5’ GAUCGAAAGAUC
BW6  5’ GAUGGAAACACUC
BW5U  5’ GAUCUUUUGAUC
BW6U  5’ GAUGUUUUCAUC

MgCl₂ and CaCl₂ were obtained from J.T. Baker, and sodium cacodylate, SrCl₂, and BaCl₂ were obtained from Sigma.

3.3.2 UV Melting Experiments

All RNA was first renatured by heating to 90°C for 3 min in 10 mM sodium cacodylate buffer (pH 7.0) and cooled to room temperature over 15 min. All melts were performed in the background of 10 mM sodium cacodylate buffer (pH 7.0). Next divalent metal salt was added to the RNA (MgCl₂, CaCl₂, SrCl₂, or BaCl₂) and the sample was heated to 55°C for 3 min and cooled to room temperature over 15 min. UV melting experiments were performed on a Gilford Response II spectrophotometer, with a data point acquired every 0.5°C and a heating rate of ~0.6°/min at 260 and 280 nm. The UV melts were performed over temperature ranges of 5 to
95°C and 95 to 5°C. Forward and reverse UV melts in the absence of divalent metal salts were similar, which provided data consistent with reversibility of the folding transition.

Thermodynamic parameters are the average of at least two independently prepared samples. Melt data were fit to a two-state model using sloping baselines and analyzed using a Marquardt algorithm for nonlinear curve fitting in KaleidaGraph v. 3.5 (Synergy software), as described previously (7).

### 3.4 Results and Discussion

#### 3.4.1 Divalent metal preference

As Mg$^{2+}$ is the smallest alkaline earth metal (see Table 1.1), we expected that it would be the most stabilizing, at least for the GAAA tetraloop with a gc closing base pair, because it could fit easily into the small ring of negative potential as seen in NLPB calculations (2). Contrary to this hypothesis, thermodynamic parameters derived from optical melting experiments show that Mg$^{2+}$ is the least stabilizing metal for both tetraloops, with free energy values at 37°C ($\Delta G_{37}$) of -2.58 kcal/mol and -1.01 kcal/mol (at 2 mM Mg$^{2+}$) for cg and gc closing base pairs, respectively.

For both tetraloops, stabilization increased with increasing ionic radius (shown in Figure 3.3): free energy values for 2 mM of Ca$^{2+}$, Sr$^{2+}$, and Ba$^{2+}$ are -3.05, -3.55, and -3.76 kcal/mol for the cg closing base pair tetraloop and -2.20, -2.64, and -3.08 kcal/mol for the gc closing base pair tetraloop (all thermodynamic parameters for tetraloops in various metals are shown in Table 3.1).

Enthalpy and entropy for both of the tetraloops in the presence of 2 mM divalent followed a similar trend (as shown in Figures 3.4 and 3.5, and Table 3.1). The difference in free energy for the cg closing base pair is monotonic in salt radius (Figure 3.6), but there is a significantly larger difference in going from magnesium and calcium in the gc closing base pair.
While larger alkaline earth divalent metals lead to greater stability, smaller divalent ions lead to greater CG preference (Figure 3.3, RH set of columns). The GAAA tetraloop with a cg closing base pair is more stable than with a gc closing base pair as expected (8), and a trend for dependence on ionic radius can be extracted from ΔΔG values (the absolute value of the difference between cg and gc closing base pairs). As seen in Figure 3.3, as ionic size increased the ΔΔG decreased. In addition, a plot of ΔG and ionic radius showed a linear dependence of free energy on ionic radius, with the gc closing base pair twice as dependent on size as the cg closing base pair (Figure 3.6).

Although Mg\(^{2+}\) appeared to stabilize the cg closing base pair tetraloop much better than the gc closing base pair (with a difference of 1.56 kcal/mol), the free energy difference between the two tetraloops in 2 mM Ba\(^{2+}\) is smaller at just 0.48 kcal/mol. As the free energy values for the cg closing base pair tetraloop increased fairly regularly with ionic radius, the decreased ΔΔG values appeared to stem from the greater increase in stability of the gc closing base pair tetraloop in the presence of the larger divalent metal ions, especially in going from Mg\(^{2+}\) to Ca\(^{2+}\). It is therefore speculated that if there is an inner-sphere metal ion coordination site in gGAAAc tetraloops, Ba\(^{2+}\) may more easily dehydrate in the dilute homogenous conditions of the experiment. One possibility is that the site on the GAAA tetraloops is at least partially inner-sphere given that larger ions coordinate better because they are easier to dehydrate. In fact, the enthalpy of hydration decreases going from Mg\(^{2+}\) to Ba\(^{2+}\) (9, 10), requiring less of an energetic penalty to form an inner-sphere coordination (see Table 1.1 in Chapter 1).

To further investigate the potential for inner-sphere metal coordination, thermal denaturation studies were performed in the exchange-inert cobalt (III) hexammine (CoHex). While 2 mM CoHex increased the stability of the cg closing base pair tetraloop more than Mg\(^{2+}\)
(Figure 3.3), it was less stabilizing than Sr$^{2+}$ and Ba$^{2+}$ for the gc closing base pair tetraloop (shown in Figure 3.3), because CoHex cannot form inner-sphere contacts. These observations support the inner-sphere nature of the ion binding site in the gc closing base pair GAAA loop. In addition, CoHex confers the highest cg preference of all the metals tested at ~2 kcal/mol in $\Delta G^0$. Therefore, we speculate that it may be too energetically costly for Mg$^{2+}$ to dehydrate and form an inner-sphere coordination under the conditions studied.

To further characterize the uniqueness of this trend in stabilization energies, less structured U$_4$ tetraloops (11) with stems identical to the GAAA loops were studied in the presence of 2 mM of the divalent metals and CoHex. In contrast to the GAAA tetraloops, there seems to be no significant trends for the cg and gc closing base pairs in UUUU tetraloops: there appears to be no preference for a particular metal cation, nor do the $\Delta \Delta G$ values show any trend in stabilization (Figure 3.7).

### 3.4.2 Tetraloop Stability in PEG

Additional studies were performed on the GAAA tetraloops in 2 mM Mg$^{2+}$ and 20, 30, and 40% PEG200. If PEG lowers the activity of water (12), Mg$^{2+}$ may be able to more easily partially dehydrate and participate in inner-sphere metal coordination, although it has also been shown that PEG decreases the stability of secondary structure in nucleic acids (13,14). Preliminary results suggested that increasing PEG percentage decreased stability of the tetraloops, as expected, but there appears to be an interesting effect at 20% PEG200 (shown in Figure 3.8 and in Table 3.2). Although the GAAA tetraloop with a cg closing base pair is more stable than its gc closing base pair under homogenous aqueous conditions, the addition of 20% PEG200 in 2 mM Mg$^{2+}$ slightly better stabilizes the gc closing base pair. This increased stabilization is a
possible indication of some inner-sphere metal coordination by Mg$^{2+}$, which would extend the Ba$^{2+}$ result to biological conditions, although more experiments are necessary. These results suggest that in vivo, where crowding is 20-40%, the preference for the closing base pair might be lowered or even lost (15,16).

### 3.5 Conclusions

Previously performed NLPB calculations revealed a small ring of negative charge present in GAAA tetraloops with gc closing base pair that is absent in GAAA tetraloops with cg closing base pairs (2). Thermal denaturation studies herein showed that the larger metals were more stabilizing for both cg and gc closing base pairs, while the smaller metals had a higher preference for the cg closing base pair GAAA tetraloop. Although it was unexpected that Mg$^{2+}$ was the least stabilizing alkaline earth metal, the larger enthalpy of hydration values for the smaller alkaline earth metals under dilute conditions may have prevented inner-sphere coordination of Mg$^{2+}$ and Ca$^{2+}$ to the gGAAAc tetraloop. Studies with the exchange-inert trivalent metal cobalt (III) hexammine further revealed the potential for inner-sphere coordination, as cobalt (III) hexammine was less stabilizing than Sr$^{2+}$ and Ba$^{2+}$ for the gGAAAc tetraloop. In addition, the lack of any trend in stabilization for the less-structured UUUU tetraloops indicates that the particular inner-sphere coordination seen in gGAAAc may be unique to those loops.

### 3.6 Acknowledgements

Joshua Blose performed NLPB calculations shown in Figure 3.1. This work was supported by NSF Grant MCB-0527102 to P.C.B.
### Table 3.1. Thermodynamic parameters for folding of RNA hairpins. All melts were performed as described in Materials and Methods.

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<th>sequence</th>
<th>Divalent Salt (2mM)</th>
<th>$\Delta H^\circ$ (kcal/mol)</th>
<th>$\Delta S^\circ$ (eu)</th>
<th>$\Delta G^\circ_{37}$ (kcal/mol)</th>
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Table 3.2. Thermodynamic parameters for folding of RNA hairpins in the presence of various cosolutes. All melts were performed as described in Materials and Methods.

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<th>2 mM Mg(^{2+}), Crowding</th>
<th>(\Delta H^\circ) (kcal/mol)</th>
<th>(\Delta S^\circ) (eu)</th>
<th>(\Delta G^\circ_{37}) (kcal/mol)</th>
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FIGURES:

**Figure 3.1.** Electrostatic potential map derived from NLPB calculations (performed by Joshua Blose).
Figure 3.2. Structure of GAAA tetraloops with (A) CG and (B) GC closing base pairs, and structure of UUUU tetraloops with (C) CG and (D) GC closing base pairs.
Figure 3.3. Comparison of the free energy of the GAAA tetraloop with cg and gc closing base pairs in 2 mM divalent ion concentration or 2 mM CoHex. Melts were performed in the background of 10 mM sodium cacodylate buffer (pH 7.0). Error bars for each metal series represent the average of the standard deviation of multiple melt experiments.
Figure 3.4. Comparison of the enthalpy of the GAAA tetraloop with cg and gc closing base pairs in 2 mM divalent ion concentration. Melts were performed in the background of 10 mM sodium cacodylate buffer (pH 7.0). Error bars for each metal series represent the average of the standard deviation of multiple melt experiments.
Figure 3.5. Comparison of the entropy of the GAAA tetraloop with cg and gc closing base pairs in 2 mM divalent ion concentration. Melts were performed in the background of 10 mM sodium cacodylate buffer (pH 7.0). Error bars for each metal series represent the average of the standard deviation of multiple melt experiments.
Figure 3.6. Line plot showing the relationship between free energy and ionic radii (dehydrated) of Mg$^{2+}$, Ca$^{2+}$, Sr$^{2+}$, and Ba$^{2+}$ for GAAA tetraloops with cg and gc closing base pairs.
Figure 3.7. Comparison of the free energy of the UUUU tetraloop with cg and gc closing base pairs in 2 mM divalent ion concentration. Melts were performed in the background of 10 mM sodium cacodylate buffer (pH 7.0).
Figure 3.8. Comparison of free energy values of GAAA tetraloops with cg and gc closing base pairs in 2 mM Mg$^{2+}$ and various PEG200 percent (w./v.). Melts were performed in the background of 10 mM sodium cacodylate buffer (pH 7.0). Error bars for each %PEG series represent the average of the standard deviation of multiple melt experiments.
3.7 References


Chapter 4

Future Directions

[The following chapter presents proposed directions for future research from the thesis work discussed in Chapters 2 and 3.]

I. Experiments to extend Chapter 2 studies

4.1. Investigation of thermodynamics of RNA folding under crowded conditions

While thermodynamic parameters for folding of RNAs have been well characterized under idealized conditions of dilute solution, 1 M NaCl (1), and no divalent ions, and popularized in dynamic programming algorithms such as mFold, little is known about RNA folding under \textit{in vivo}-like crowded conditions and there is no general way to predict RNA structure under such conditions. My studies in Chapter 2 show that crowded conditions and low Mg\textsuperscript{2+} destabilize secondary structures in general. Thus, at the very least there will be different free energies under these conditions. However, given that each secondary structural motif will have a different degree of hydration and that crowding reduces water concentration, there is likely to be a re-ordering of the relative stabilities of various secondary structures. For example, Watson-Crick base pairs may suffer the greatest destabilization because of the lesser availability of water for their well-ordered hydration spines (2), while non-Watson-Crick base pairs may be less destabilized because of less-ordered waters. Measurement of parameters for RNA folding under \textit{in vivo}-like conditions and implementation into the mFold and related programs is likely to have a large impact on the RNA community, and may result in new biological insights both for our research group and many others.

The experimental plan for this area would involve the melting of an array of sequencing containing various Watson-Crick base pairs and non-Watson-Crick motifs. Data would then be
interpreted by single-value decomposition methods and used to predict RNA structure. First, we
would see whether the dataset is self-consistent and can predict stabilities of other RNAs under
these conditions. The challenge to these experiments will be identifying reasonable means of
mimicking crowding. Initial plans are to include polymers and physiological KCl and Mg\(^{2+}\) (135
and 0.5 mM free, respectively). We may also include proteins, other RNA/DNA, and lipids,
although UV-detected melts and calorimetry would no longer be possible; perhaps we could use
helical hairpin constructs to extract thermodynamic parameters under these conditions.

### 4.2 Investigation of pre-steady state kinetics of RNA folding under crowded conditions

The kinetics of RNA folding have been significantly less investigated than thermodynamics (3).
Even less studied are the effects of macromolecular crowding agents on kinetics. RNA folding is
often slowed by formation of stable misfolds; however, as described in Chapter 2, crowding
destabilizes these folds, suggestive of the possibility for faster RNA folding under crowded
biological conditions and that folding may be more cooperative. Figure 4.1 shows possible
changes in the folding landscape owing to crowding agents. A more crowded environment may
affect the energy landscape, destabilizing kinetic traps (i.e. RNA secondary structure as
mentioned), thus allowing RNA to fold faster and more homogeneously (i.e. single rather than
multiple kinetic phases). The conclusion that RNA folds faster under crowded conditions would
be important to the idea of both present biology as well as the RNA World, showing much faster
achievement of the native state. Such folding has implications for engineering RNAs with
improved folding kinetics as well.

Phenylalanine transfer RNA (tRNA\(^{\text{Phe}}\)) is a good model for preliminary studies of folding
kinetics in crowding, as it has been studied extensively through thermal denaturation studies and
kinetics using fluorescence (4), but not in the presence of macromolecular crowding agents. Transfer RNA$^{\text{Phe}}$ has an L-shaped tertiary structure and clover-leaf secondary structure, and has a detectable change in absorbance signal upon transition between tertiary and secondary structures. Stopped-flow spectroscopy experiments to monitor pre-steady state folding kinetics can be performed on tRNA$^{\text{Phe}}$ using the signal from the naturally fluorescent Y base, although other fluorophores will be introduced, similar to Peter Qin et al (5). In these experiments tRNA folding could be induced by addition of Mg$^{2+}$. We can also perform RNA unfolding experiments: in a manner similar to Maglott et al (6), the increase in absorbance upon mixing folded RNA (containing Mg$^{2+}$ and crowding agents) with ethylenediaminetetraacetic acid (EDTA) to chelate the Mg$^{2+}$ and unfold the RNA can be observed.

From both the folding and unfolding studies, the rates can be determined by fitting the kinetic traces to an exponential fit. Measuring the rates over a temperature range (limited by the conditions needed to maintain the folded state) can allow for Arrhenius plots and activation parameters to be determined. Furthermore, rates of folding and unfolding in the presence of macromolecular crowding agents can be compared. If macromolecular crowding promotes tertiary structure stabilization by the excluded volume effect, I would expect rates of tertiary structure unfolding in the presence of crowding agents to be significantly slower, as a more compact structure would be favored.

After characterization of the model tRNA$^{\text{Phe}}$, other biological RNAs can be studied, such as ribozymes and riboswitches, beginning with the guanine riboswitch (structure shown in Figure 4.2)(7). Requirements for this particular experiment are RNAs with well-characterized secondary and tertiary structures whose secondary structure forms in the absence of Mg$^{2+}$ and whose tertiary structure will form upon addition of Mg$^{2+}$. Increasing secondary structure base
pairing strength should cause slower unfolding of tertiary structure until secondary structure becomes so strong that overall folding becomes non-cooperative. At that point, other intermediates will be detectable in the unfolding kinetics. We also intend to study model helices from section 4.1 to uncover the kinetic basis for destabilization. Given that detection of folding can be absorbance hypochromicity, unlabeled oligonucleotides should be completely suitable. We might anticipate that destabilization of Watson-Crick containing oligonucleotides by crowders will be due to faster unfolding rather than slower folding because of weaker water interaction in the folded state.

4.3 Characterization of RNA folding by fluorescence resonance energy transfer (FRET) single molecule studies
To further probe the effects of macromolecular crowding on RNA folding and expand my knowledge of other biophysical techniques, single molecule FRET can be used to compare RNA folding under both crowded and dilute conditions. RNA has a very rugged energy landscape (possibly because of relatively stable secondary structure) and can fold through multiple pathways with various transition states (8) (Figure 4.1). Averaged ensemble measurements (i.e. standard biophysical/biochemical experiments) can only detect accumulative or appreciably populated intermediates. Single molecule studies, on the other hand, are able to probe transient low-populated states and characterize complex folding pathways and dynamics. This would be important for cooperative conditions in which secondary structures are less stable than the native tertiary structure state. In order to assign a FRET signal to a specific change in conformation, mutation studies to disrupt conformational changes will be performed.
The small hairpin ribozyme has been characterized by single molecule FRET, and highly complex folding dynamics have been detected (8). An entropic barrier to the hairpin ribozyme activity was proposed, as two loops in the ribozyme are believed to be in close proximity in the transition state (8). If crowding agents produce an excluded volume effect, the addition of a crowder should accelerate the rate of reactions dependent on entropically unfavorable transition states and show shorter times for population of intermediates. Indeed, Nakano et al. demonstrated higher reaction rates for the hammerhead ribozyme upon addition of a 20 wt % cosolute (9).

Single molecule FRET studies can be performed on ribozymes and other biologically relevant RNAs using cosolutes and could elucidate additional pathways not observed in ensemble averaged studies. In addition, the specific effect of cosolutes on RNA folding could be probed. The folding of tRNA\textsuperscript{Phe} in the presence of cosolutes will be studied and characterized, followed by more complex riboswitches and ribozymes all containing tertiary structures that have been solved. After characterization of folding with cosolutes, it would be interesting to continue to move towards observation of RNA folding in the cellular environment, using engineered cytoplasms. These studies could provide deep insights into the functional role of RNA in the cell.
II. Experiments to extend Chapter 3 studies

4.4 Raman spectroscopy and phosphorothioate substitutions to further examine GAAA tetraloops

As mentioned in Chapter 3, we favor a model for metal binding to GAAA tetraloops in which the metal makes inner-sphere contacts to the tetraloop (Figure 1.3). We need to do additional experiments of a more structural and spectroscopic nature to test this model.

Possible collaborations with the Harris Laboratory at Case Western Reserve may further elucidate the type of metal binding through Raman spectroscopic methods. Raman spectroscopy can be used to assess changes in vibrational energies caused by metal interactions (11). Inner-sphere, hydrogen bonding, and electrostatic interactions cause a decrease of the Raman signal for the symmetric vibration of the non-bridging phosphate oxygens, but only chelated coordination causes a shift of the signal to higher wavenumbers (11). In addition, phosphorothioate substitutions similar to Maderia et al (12) can be used to probe the environment in GAAA loops with both cg and gc closing base pairs. These types of substitutions are commercially available from Dharmacon, and our lab is capable of separating the diastereomers using HPLC. They conducted both UV melts and NMR spectroscopy on variants in different metals. Applying this technique to my GAAA tetraloops can help me determine if there is a different Mg$^{2+}$ coordination environment between GAAA tetraloops with a cg and gc closing base pair.
**Figure 4.1.** Effects of macromolecular crowding on RNA folding landscape. (A) Folding landscape in dilute or non-cooperative conditions, with metastable kinetic traps $M_1$ and $M_2$. (B) Folding landscape in the presence of macromolecular crowding agents, showing an absence of metastable states owing to destabilization of secondary structures by macromolecular crowding.
Figure 4.2. Secondary structure of guanine riboswitch construct with long-range contacts drawn in solid lines (Watson-Crick base pairs) and dashed lines (non-canonical base pairs) (7).
4.5 References


