

**Utilizing Single-Molecule Fluorescence Microscopy to Elucidate
Conformational Dynamics of RNA**

by

nico e. conoan nieves

A dissertation accepted and approved in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy
in Chemistry

Dissertation Committee:

James Prell, Chair

Julia R. Widom, Advisor

Scott Hansen, Core Member

Jayson Paulose, Institutional Representative

University of Oregon

Spring 2025

© 2025 nico e. conoan nieves
This work is openly licensed via CC BY-NC-ND 4.0.

DISSERTATION ABSTRACT

nico e. conoan nieves

Doctor of Philosophy in Chemistry

Title: Utilizing Single-Molecule Fluorescence Microscopy to Elucidate Conformational Dynamics of RNA

Single-molecule fluorescence microscopy offers information-rich avenues for investigating the mechanisms of many biological interactions. This dissertation will detail my work building a single-molecule fluorescence microscope and two applications involving different ribonucleic acids (RNAs): a bacterial messenger RNA (mRNA) and a yeast transfer RNA (tRNA). Specifically, the instrument is a prism-type total internal reflection fluorescence (TIRF) microscope that utilizes simultaneous multi-channel detection of fluorescent reporters to determine structural fluctuations of surface immobilized RNA molecules with exceptional sensitivity. By employing smFRET, i demonstrate one of many ways the home-built instrument is capable of examining RNA behavior.

My goal in the first story was to elucidate the binding interaction of a nicotinamide adenine dinucleotide (NAD^+) riboswitch and NAD^+ . A riboswitch is commonly found as part of the 5' untranslated region of certain bacterial mRNA and acts as a switch when bound or unbound to a complementary small molecule. The switching behavior causes either protein translation or nucleic acid transcription to start or stop depending on the conformation adopted by the riboswitch. The Class II NAD^+ riboswitch is a translational “off” switch because, when NAD^+ is bound, the ribosome binding site is sequestered. In the absence of

NAD⁺, the riboswitch frequently samples several distinct conformations represented by five FRET states. The complexity of these structural rearrangements is reduced in the presence of Mg²⁺ and are further repressed to one predominant conformation in the presence of NAD⁺. Thus, our results support the predicted pseudoknot structure adopted by the riboswitch when bound to NAD⁺.

In the second story, my aim was to reveal how a pseudouridine synthase, Pus4, changes the structural dynamics of a tRNA using smFRET. Pseudouridine is a ubiquitous modification of tRNAs and it may be first in line of many other modifications catalyzed by other enzymes. We developed a time-course method to investigate protein binding over 30 minutes. This method allowed for binning of movies into time intervals as the system reaches equilibrium. Our results indicate that Pus4 catalysis is followed by remodeling to achieve the appropriate sampling of conformations suitable for further processing downstream.

This dissertation includes previously published and unpublished, co-authored material.

CURRICULUM VITAE

NAME OF AUTHOR: nico e. conoan nieves

GRADUATE AND UNDERGRADUATE SCHOOLS ATTENDED:

University of Oregon, Eugene

University of Nebraska, Omaha

Metropolitan Community College, Omaha, Nebraska

DEGREES AWARDED:

Doctor of Philosophy, Chemistry, 2025, University of Oregon

Bachelor of Science, Chemistry, 2014, University of Nebraska

Liberal Arts Transfer/Associate of Science, 2011, Metropolitan Community College, Omaha, Nebraska

AREAS OF SPECIAL INTEREST:

RNA structure and dynamics

Fluorescence-detected resonance energy transfer

Single-molecule microscopy: instrument design, build, and applications

PROFESSIONAL EXPERIENCE:

Owner, NCNB & Co. Licensed Residential General Contractor (OR CCB#251132), 2024-present

Graduate Research Assistant, University of Oregon, 2020-present

Chemist, US Army Corps of Engineers, 2014-2019

GRANTS, AWARDS AND HONORS:

Emanuel Fellowship, support for dissertation writing for graduate students in their last term by the Oregon Center for Optical, Molecular, and Quantum Science, University of Oregon, 2025

Director's Fellowship, awarded to three promising first-year graduate students by the Oregon Center for Optical, Molecular, and Quantum Science, University of Oregon, 2019

Dr. Nell Ward Memorial Scholarship, given to the top rising senior by the Department of Chemistry, University of Nebraska, 2013

Undergraduate Award, ACS Division of Inorganic Chemistry, nominated by the Department of Chemistry for excellence in inorganic chemistry, University of Nebraska, 2013

TRiO Pacesetter Award, for completing an associate degree in six quarters, graduating with a superior GPA and transferring to a four-year university, Metropolitan Community College, 2011

PUBLICATIONS:

Conoan Nieves, N. E. and Widom, J., "Ligand binding characteristics of an NAD⁺ riboswitch revealed by FRET and biolayer interferometry", Chem. Comm., **2024**

Conoan Nieves, N. E., Dennis, E., Vaaler, Kadrmas, M., Barry, M., Garcia, D., and Widom, J., "A pseudouridine synthase shapes tRNA structural dynamics through both catalysis and remodeling", *manuscript submitted*

to Ari, Eli, and Jada
you're all my favorites

TABLE OF CONTENTS

Chapter		Page
I.	INTRODUCTION	14
1.	RNA Composition, Structure, and Function	14
2.	Nonradiative Electronic Resonance Energy Transfer	16
3.	Document Structure	20
II.	METHODS	23
1.	RNA Sample Design and Preparation	23
2.	Single-Molecule Fluorescence Microscopy	24
3.	FRET Data Analysis	27
4.	Biolayer Interferometry	28
III.	BUILDING A SINGLE-MOLECULE INSTRUMENT FOR TIRF MICROSCOPY	30
1.	Background	30
2.	Custom Design of an smTIRF Instrument	32
2.1.	Excitation Optics	32
2.2.	Imaging Area	32
2.3.	Fluorescence Optics and Detector	34
3.	Conclusion and Applications	37
IV.	LIGAND-BINDING CHARACTERISTICS OF AND NAD ⁺ RIBOSWITCH REVEALED BY FRET AND BIOLAYER INTERFEROMETRY	39
1.	Prologue	39
2.	Manuscript	39
3.	Supplemental Information	48

Chapter	Page
3.1. Materials and Methods	48
3.2. Supplementary Figures	52
V. IMPACTS OF ADENOSINE ON NAD ⁺ -II RIBOSWITCH CONFORMATIONAL DYNAMICS	57
1. Prologue	57
2. Hidden Markov Modeling of FRET States	57
3. β -NMN concentration series reveals increasing shift towards predominant HF state	61
4. Adenosine Moiety of NAD ⁺ Affects Ligand Recognition	65
5. Future Directions	66
VI. A PSEUDOURIDINE SYNTHASE SHAPES tRNA STRUCTURAL DYNAMICS THROUGH BOTH CATALYSIS AND REMODELING	68
1. Prologue	68
2. Abstract	68
3. Introduction	69
4. Results	72
4.1. Exogenously purified Pus4 pseudouridylates yeast tRNAs <i>in vitro</i>	72
4.2. Synthetic modified tRNAs enable binding and single molecule FRET studies	73
4.3. Wild-type Pus4 binds rapidly to tRNA	73
4.4. Pus4 also binds robustly to pre-modified tRNA and non-substrate RNA	76
4.5. A catalytically-inactive mutant of Pus4 has impaired binding to tRNA	78
4.6. tRNA undergoes dynamic fluctuations between three predominant conformational states	78

Chapter	Page
4.7. Pus4 alters the conformational states and their transition dynamics in tRNA	81
4.8. Catalytically-inactive Pus4 alters the tRNA remodeling pathway	84
5. Discussion	86
6. Methods	90
7. Supplemental Tables and Figures	103
VII. CONCLUDING REMARKS	118
REFERENCES CITED	121

LIST OF FIGURES

Figure	Page
1.1. Example of RNA tertiary structure	15
1.2. Cartoon representation of molecular dipoles	17
1.3. Cy3 and Cy5 spectra excitation and emission spectra	18
1.4. Graphical FRET efficiency representation	19
3.1. Excitation Path	33
3.2. Sample Stage Fitted with Spacers	33
3.3. Prism-Type TIRF Geometry	34
3.4. Aperture Reducing Image Size	35
3.5. Fluorescence Path	36
4.1. Chemical Structures of NAD^+ and the NAD^+ -II Riboswitch	41
4.2. Gaussian-Fitted Histograms and Sample Traces	43
4.3. BLI and Bulk FRET Results	45
4.4. Dimer Study Results	46
4.5. UV/Vis Absorbance Spectrum of b-P1aCy5-Cy3 RNA	52
4.6. smFRET Histograms of b-U14Cy5-Cy3 RNA	52
4.7. BLI Traces for b-U14Cy5-Cy3 RNA	53
4.8. Bulk FRET Measurements on b-P1aCy5-Cy3 RNA	54
4.9. Raw Image of Polyacrylamide Gel	55
4.10. Dimer Study Single-Molecule Scenario Schematics	56
5.1. Selected smFRET traces of RNA only	59
5.2. TODP of NAD^+ -II riboswitch	60

Figure	Page
5.3. Histograms comparing RNA behavior pre- and post-experiment	62
5.4. β -NMN Concentration series histograms and TODPs	63
5.5. Graphical representation of the change in FRET state peak heights . . .	64
5.6. 2D Chemical structure of ε -NAD ⁺	65
5.7. Materials and results of NAD ⁺ and ε -NAD ⁺	66
6.1. Predicted Pus4 structure, tRNA sequences, and overall study approach. .	71
6.2. Association of Pus4 to tRNA using biolayer interferometry	74
6.3. BLI traces and rate	77
6.4. Unmodified and pre-modified tRNAs alone display distinct structural states and dynamics	80
6.5. smFRET histograms and TODPs of U ₅₅ and Ψ_{55} tRNA with Pus4 WT	83
6.6. smFRET histograms and TODPs of U ₅₅ and Ψ_{55} tRNA with Pus4 R286K	85
6.7. Proposed model	89
6.8. Protein purification	103
6.9. Heatmaps of Nanopore direct RNA sequencing	104
6.10. BLI traces	105
6.11. Pus4 has altered affinity to U ₅₅ tRNA compared with Ψ_{55} tRNA	106
6.12. Electrophoretic Mobility Shift Assay	107
6.13. Fits to association phases of BLI curves	108
6.14. Cross-correlation analysis	108
6.15. Representative FRET Traces	109
6.16. Histogram overlays	110
6.17. Predicted secondary structures	111

LIST OF TABLES

Table	Page
6.1. Yeast strains	112
6.2. Plasmids	112
6.3. Oligonucleotides	116
6.4. Direct RNA Sequencing Data Files	117

CHAPTER I

INTRODUCTION

1 RNA Composition, Structure, and Function

RNA is a fascinating, dynamic material with an abundance of roles! While DNA provides the “blueprints” for genes and proteins carry out a plethora of functions, RNAs are biological facilitators responsible for decoding genetic information¹, synthesizing proteins², splicing³, catalysis⁴, and much more⁵. The functions of RNA are directly linked to their structure. Nucleic acids are dynamic polymers capable of adopting different conformations, whether on their own or influenced by nearby materials such as water, ions, and macromolecules like proteins. Double-strandedness arises from a combination of nearest neighbors base-stacking along the length of the chain, like the rungs of a ladder, and base-pairing, which involves hydrogen bonding between nonadjacent bases. While DNA is almost always double-stranded, RNA is often single-stranded, but can form double and triple helices allowing for complex tertiary structures including a pseudoknot or cloverleaf and quaternary structures such as dimers.^{6,7} Figure 1.1 demonstrates the complexity in how the bases of a relatively small RNA molecule can coordinate with each other⁶.

The building blocks of nucleic acids, DNA and RNA, are referred to as nucleotides, each of which contains a base, sugar and phosphate. The bases in DNA include the purines, adenine and guanine, and pyrimidines, cytosine and thymine. RNA consists of the same two purines, but the pyrimidines are cytosine

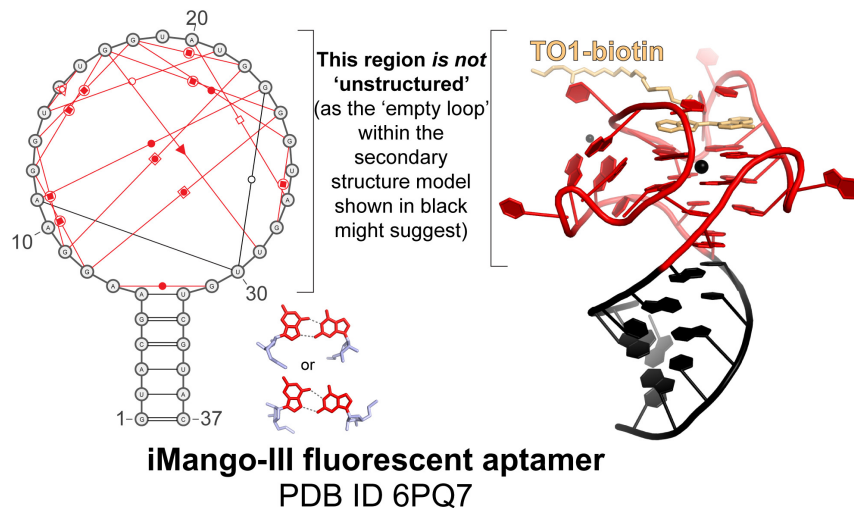


Figure 1.1: A short segment of RNA as an example for how to consider a secondary structure prediction model (left) versus the ball and stick model based on its reported crystal structure (right). The small inset diagram (left-center) highlights the non-canonical G-G pairing this small molecule demonstrates nine times within the "empty loop" of the aptamer.

and uracil. A nucleoside is a molecule consisting of a base and a sugar. while nucleotides can contain a varying number of phosphates attached to the sugar. For example, this naming convention applied to the base adenine gives rise to adenosine, the nucleoside, and adenosine triphosphate, for the nucleotide containing a chain of three phosphates. Nucleic acid bases are connected by a sugar-phosphate backbone to form long chains tasked with the very important functions discussed in this dissertation. The negatively charged phosphate backbone is neutralized by monovalent and divalent cations such as Na^+ and Mg^{2+} . The 2' position of the sugar on RNA contains a hydroxyl (-OH) group, whereas a lone hydrogen is in the same position for a DNA ribose; therefore, the latter is referred to as deoxygenated or "deoxy"-ribonucleic acid.

I studied two types of RNA: a messenger RNA (mRNA) and a transfer RNA (tRNA). In brief, DNA that is transcribed into mRNA contains the series of bases that provide the genetic sequence of codons. A sequence complementary to

each codon, an anticodon, is found in a tRNA that carries amino acids—molecules containing both carboxylic ($-\text{COOH}$) acid and amino ($-\text{NH}_2$) functional groups—to the ribosome as substrates for protein translation. Each codon carries information resulting in the incorporation of one amino acid. Proteins are polymers of amino acids whose actions are responsible for the vast majority of biological functions. In some cases, the 5'-untranslated region (UTR) of a mRNA can switch on or off protein translation or nucleic acid transcription by interacting with a small molecule, or ligand. This segment of an mRNA is called a riboswitch and contains an aptamer domain that binds the ligand and an expression platform downstream. When the aptamer binds a ligand and sequesters the expression platform, it is called an “off” switch. Fluctuations and structural rearrangements of RNA, whether innate or influenced, often occur on the millisecond time scale—a regime for which single-molecule microscopy methods excel. To give the reader an opportunity to understand the foundation of the primary method used for the work in this dissertation, i will provide an overview of the theory that supports it.

2 Nonradiative Electronic Resonance Energy Transfer

The quantum mechanical description of nonradiative electronic resonance energy transfer (RET) was first derived in 1932 by F. Perrin in his report, *Théorie Quantique des Transferts D'Activation*, on why fluorescence anisotropy (polarization) decreased with the concentration of a dye⁸. Applying a two electronic oscillator model system to a dilute, viscous solution of fluorescent molecules, he derived a distance dependence for the probability of electronic energy being transferred nonradiatively to a nearby molecule as a function of the distance,

R_o , such that,

$$R_o = \frac{\lambda}{\pi} \left(\frac{\bar{t}_i}{\tau_o} \right)^{\frac{1}{6}} \quad (1.1)$$

with λ being the wavelength of the excitation energy, $\bar{t}_i$ is the average interaction time for two fluorophores, and τ_o is the lifetime of the excited state. Supposing that for two molecules in solution, $\bar{t}_i$ is on the order of 10^{-13} to 10^{-14} and an average τ_o of 10^{-8} s, we have

$$\frac{\lambda}{30} < R_o < \frac{\lambda}{20} \quad (1.2)$$

meaning two (fluorescein) molecules in solution must be within 150-250 Å for energy transfer to be probable. However, this distance was found to be slightly larger than observed by experimentalists as concentration-dependent depolarization of fluorescence⁹.

In 1948, Förster improved on Perrin's results and connected them to fluorescence anisotropy spectra by utilizing the Coulomb interaction energy for moving electron charges and incorporating Einstein's spontaneous transition probability.⁹ He ascribed the distance dependence of RET as resulting from the alignment of the transition moment for two molecular dipoles as depicted below.

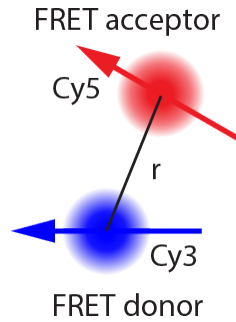


Figure 1.2: Cartoon representation of molecular dipoles oriented close to parallel.

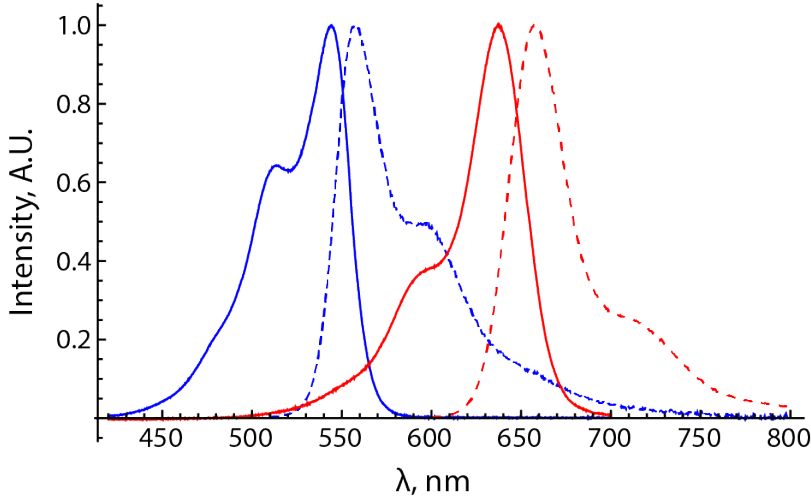


Figure 1.3: Excitation and emission spectra of Cy3 (blue) and Cy5 (red). Solid lines indicate excitation and dashed lines indicate emission.

Förster further demonstrated that spectral overlap of the emission of one molecule (the “donor”) and excitation of a second molecule (the “acceptor”) is required. Figure 1.3 shows the excitation and emission spectra for the FRET pair utilized in my work, with Cy3 as the donor and Cy5 as the acceptor.

Förster referred to R_o as the critical molecular separation at which the probability of donor emission (fluorescence) is equal to that of RET such that, for the transfer of purely electronic excitation energy,

$$R_o = \sqrt[6]{\frac{3\tau_o c^2 J(\nu_o)}{8\pi^4 n^2 (N')^2 \nu_o^2}}, \quad (1.3)$$

where ν_o is the frequency of the purely electronic excitation, the overlap integral, $J(\nu_o) = (\ln 10)^2 \int_o^\infty \epsilon(\nu) \epsilon(2\nu_o - \nu) d\nu$, is written in terms of the absorption, $\epsilon(\nu)$, and fluorescence, $\epsilon(2\nu_o - \nu)$, spectra of the acceptor and donor molecules, respectively, n is the refractive index of the solvent, τ_o is the excited state lifetime of the donor, and N' is the number of molecules per millimole, 6.02×10^{20} .

As an aside, Förster pointed out that values of R_o are estimated from the above equation by approximating the width of the spectral overlap integral, $\Delta\nu$, and excited state lifetime, τ_s as

$$\frac{1}{\Delta\nu} \approx \frac{\int \epsilon(\nu)\epsilon(2\nu_o - \nu)d\nu}{[\int \epsilon(\nu)d\nu]^2} \quad (1.4)$$

and

$$\frac{1}{\tau_s} \approx \frac{8\pi(\ln 10)n^2\nu_o^2}{N'c^2} \int \epsilon(\nu)d\nu. \quad (1.5)$$

Thus we have an equation similar to Perrin's formula in Eq. 1.1,

$$R_o \approx \frac{\lambda_o}{2\pi} \left(\frac{\bar{t}}{\tau_o} \right)^{\frac{1}{6}}. \quad (1.6)$$

Applying equation 1.3 for the molecules used in this work, Cy3 and Cy5, where the overlap integral is centered around 600 nm, yields $R_o = 50 \text{ \AA}$. The distance dependence of RET is summarized in Fig. 1.4.

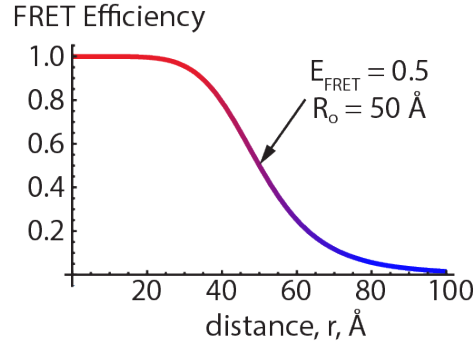


Figure 1.4: Graphical representation of the relationship between molecular distance and FRET efficiency. For the FRET pair used in this work, the critical molecular separation is 50 Å.

The equation for R_o is most commonly presented in a form that incorporates the fluorescence quantum yield, Q_D , and a dipole orientation factor, κ .

$$R_o^6 \frac{9\log(10)\kappa^2 Q_D J}{128\pi^5 N_A n^4} \quad (1.7)$$

N_A is Avogadro's number, 6.022×10^{23} and n is the refractive index of the medium. The overlap integral is defined as $J = \frac{\int f_D(\lambda) \epsilon_A(\lambda) \lambda^4 d\lambda}{\int f_D(\lambda) d\lambda} = \int \overline{f_D}(\lambda) \epsilon_A(\lambda) \lambda^4 d\lambda$, where f_D is the donor emission spectrum, $\overline{f_D}$ is the area of f_D normalized to 1, and ϵ_A is the molar extinction coefficient of the acceptor. The dipole orientation factor is $\kappa = \hat{\mu}_A \cdot \hat{\mu}_D - 3(\hat{\mu}_D \cdot \hat{R})(\hat{\mu}_A \cdot \hat{R})$, where $\hat{\mu}_i$ is the normalized transition dipole moment of each fluorophore and $\hat{R}$ is the normalized vector connecting the fluorophores. κ^2 is equal to $2/3$ if the transition dipoles sample all orientations faster than their fluorescence lifetime, which is often assumed when the dyes are free to rotate.¹⁰ In reality, κ^2 is often not $2/3$, although it can be measured via fluorescence depolarization.¹¹

3 Document Structure

This dissertation provides background information on the techniques and an overview of the mathematics involved in single-molecule fluorescence-detected electronic resonance energy transfer (smFRET) microscopy in Chapter II. I will then describe in detail how to build a custom single-molecule instrument as i built in-house in Chapter III. Our instrument is designed to capture the interactions of ribonucleic acids (RNAs) and other biological materials such as small molecules and proteins. Building an instrument is a non-trivial endeavor that requires extensive time and financial resources, but in the end is an information-rich way to biophysically investigate many fundamental behaviors of biological interactions. However, the cost savings and customizability of an instrument built and maintained in-house compared to a commercial instrument, are significant. Our fluorescence microscope is being used to elucidate the conformational dynamics of RNA immobilized to microscope slides for many types of systems including resolving the secondary structures of long non-coding RNA, exploiting FRET

to observe fluctuations in RNA folding dynamics in real time, and sequencing of RNAs.

After describing the design and construction of our single-molecule microscope, i will discuss how i used this instrument to investigate the structural rearrangements two RNAs. In the first story, i will discuss my investigation of a riboswitch, a motif found in the 5' untranslated region of a bacterial mRNA, that binds the nicotinamide moiety of nicotinamide adenine dinucleotide, NAD^+ , a ubiquitous enzyme cofactor. As a riboswitch binds a metabolite, it adopts a different tertiary structure and acts as a switch to either start or stop protein translation or nucleic acid transcription. For the NAD^+ -II riboswitch, binding NAD^+ results in the ribosome binding site being sequestered. If the RBS continues to be unavailable, protein translation is ceased. Therefore, NAD^+ -II is considered a translational “off” switch. As NAD^+ -II is found in many pathogenic bacteria, this interaction may be a promising therapeutic target for antibiotic medication. Chapter IV, includes recently published work in which the on NAD^+ -II riboswitch was observed to sample many conformations in the absence of ligand, but prefers the pseudoknot structure in the presence of 20 mM Mg^{2+} and 1 mM β -NMN with a 2:1 binding stoichiometry. Chapter V documents my results on NAD^+ -II behavior with increasing concentrations of NAD^+ , which demonstrates nicely the ligand-induced shift in conformations adopted by the riboswitch.

The second story, discussed in Chapter VI, is about a tRNA and its interactions with a pseudouridine synthase, Pus4, an enzyme which catalyses isomerization of the canonical base uracil to pseudo-uracil. tRNAs are heavily chemically modified and our hypothesis is that pseudouridylation and proper tRNA remodeling by Pus4 is required for further modification by other factors. This study

leverages two biophysical methods to elucidate the conformational dynamics of this system during and after catalysis, namely bilayer interferometry and smFRET. Bilayer interferometry is a useful method for reporting on the accumulation rate of protein on immobilized tRNA. In our investigation, we utilized smFRET to observe how the tRNA structure changes over time after protein has bound and catalyzed pseudouridylation. We report that while catalysis is accomplished in the first few minutes, it can take up to 30 minutes for the tRNA to settle into the conformational states sampled by the properly folded tertiary structure.

This dissertation includes previously published and unpublished, co-authored material.

CHAPTER II

METHODS

1 RNA Sample Design and Preparation

For smFRET microscopy, the riboswitch was designed with a 5'-biotin for surface immobilization, a 3'-Cy3, and an in-sequence amino-functionalized uracil (Figs. 4.1 and 6.1) This uracil was labeled with Cy5 via a monoreactive dye pack followed by ethanol precipitation and polyacrylamide gel electrophoresis (PAGE) purification as described in Chapter IV. The tRNA was designed in two pieces, which were ligated together with a DNA splint (Chapter VI). The 5' piece contained a 5'-biotin and Cy3 attached to the 2' ribose of an internal uracil in the acceptor stem close to the 5' end, and the 3' piece contained Cy5 conjugated to a uracil in the anticodon stem. While it is convenient to acquire an RNA in a single segment, as done for the riboswitch, the ligation strategy used for tRNA provides greater flexibility in the constructs one can use in their experiments. For example, we needed two versions of the same tRNA molecule containing a single base modification: uracil versus pseudouracil at position 55 (Fig. 6.1. Ligation consists of annealing the RNA pieces with a DNA splint, then allowing an enzyme (T4 RNA Ligase 2) to stitch them together for 3 hours at room temperature. Following the ligation, DNase was introduced to digest the splint. Ligated RNAs were then purified by PAGE and recovered via electro-elution as described in Chapter VI.

When not in use, RNA is stored at -80°C fully concentrated, usually in the 1-100 μ M range. Storage at much lower concentrations, or over an extended period of time, can result in RNA degradation. Prior to loading samples onto a

microscope slide, they must first be annealed, by heating and then cooling slowly to room temperature. My samples were annealed at 90°C for 2-3 minutes. This denatures the secondary and tertiary structure the RNA had adopted while freezing and allows it to refold into its functional structure. Samples are then diluted to 10 pM in imaging buffer (50 mM Tris-HCl pH 8.0, 100 mM KCl) for loading onto the microscope slide.

As discussed at length in Chapter IV, placement of the FRET pair must not only be at a distance suitable for FRET to occur, but also be able to report on the structural fluctuations of the RNA without interfering with them. Selection of labeling sites can therefore be quite challenging when one does not have a crystal structure available to facilitate their design, as was the case for the NAD⁺-II riboswitch when we initiated our study. Once crystal structures had been reported^{12,13}, it was apparent that Cy5 could interfere with ligand recognition as its original location was very close to one of the ligand binding pockets. With the guidance of crystal structures, we were able to design a functional riboswitch for FRET measurements.

2 Single-Molecule Fluorescence Microscopy

Measuring the intensities of donor and acceptor fluorescence allows for calculating the FRET efficiency as

$$E_{\text{FRET}} = \frac{I_A}{I_D + I_A} = \frac{1}{1 + \left(\frac{r}{R_o}\right)^6} \quad (2.1)$$

where I_A is the intensity of fluorescence from the acceptor molecule and I_D is that of the donor with r as the distance between the two.

Discussions of FRET results frequently refer to high-FRET (HF), mid-FRET (MF), or low-FRET (LF) states with respect to the distance between, for example, two RNA bases. While there is a direct dependence of E_{FRET} on distance, calculating the actual distance between two RNA bases is not straightforward, as mentioned in the previous chapter.¹¹ Dyes are often tethered to RNA molecules by a five-carbon chain to allow the dyes to rotate somewhat freely. On the one hand, this can minimize interference with RNA mobility and function, but this also allows their intermolecular distance to shift. Therefore, we utilize FRET states in our discussions to refer to relative distances (closer or farther) when observing changes over time and generally avoid claims based on their actual separation distance.

When collecting FRET data on our single-molecule instrument, a standard microscope slide with fluorescent beads (FluoSpheres™ Carboxylate-Modified Microspheres, Thermo Fisher Scientific, F8810) immobilized to it is first examined. These beads fluoresce brightly across both donor and acceptor detection channels which allows for calculating a mapping function (using custom MATLAB code) for molecule identification since the spots on the left half of the detector are in the same physical location as those represented on the right half (details in Chapter III).

Microscope slides are prepared for samples following a standard dichlorodimethylsilane (DDS)/Tween-20® protocol¹⁴ and stored at 4°C. DDS degradation occurs readily in air, thus storing slides under vacuum is recommended. In practice, we found that adequate surface immobilization can be achieved for up to two weeks after slides are prepared and assembled (i.e. sealed with double-sided tape, a coverslip, epoxy, and tubing epoxied into micropipette tips cut to fit the inlet and outlet ports of the flow-through sample chamber).

Prior to loading RNA, a series of solutions must be introduced to the sample chamber and allowed to incubate for several minutes followed by flushing with buffer (10 mM Tris-HCl pH 8.0, 50 mM NaCl) between solutions. First, 0.2 mg/mL biotinylated bovine serum albumin (b-BSA) is introduced. b-BSA adsorbs to the DDS-passivated surface and allows for immobilization of biotinylated RNA via the biotin-streptavidin interaction. Next, 0.2% v/v Tween-20[®] is used to prevent non-specific sticking of any sample components by occupying sites directly on the slide surface where no BSA was immobilized. Finally, 0.2 mg/mL streptavidin is introduced to provide a link between the b-BSA and biotinylated RNA. Since streptavidin has four biotin-binding sites, it is important to determine the appropriate incubation times and concentrations for the materials used for slide preparation to achieve a suitable molecular density for the analyte of interest (in our case, riboswitch or tRNA). For our experiments, we determined the optimal incubation times were 5 minutes for 0.2 mg/mL b-BSA and 10 minutes for 0.2% Tween-20[®] and 0.2 mg/mL streptavidin.

Sample preparation consists of diluting RNA to 10 nM in imaging buffer, heating to 90°C for 2-3 minutes, then cooling to room temperature for 10 minutes. Once the slide has been prepared, RNA is further diluted to 10 pM in imaging buffer with an oxygen scavenger system (OSS). The OSS contains 5 mM Trolox[®], 5 mM Protocatechuic acid (PCA), and 5 nM Protocatechuate 3,4-dioxygenase (PCD). Trolox[®] helps to suppress fluorophore blinking and photobleaching and PCD degrades molecular oxygen with the assistance of free radicals generated by PCA.¹⁵

Data must be collected using enough laser power and an appropriate frame rate to obtain a suitable signal to noise ratio (S:N); ideally greater than 5:1. For

the data reported in this dissertation, movies were collected using either 40 ms (riboswitch) or 100 ms (tRNA) integration time—25 Hz or 10 Hz, respectively. For Cy3, a S:N ratio of 8:1 can be achieved using 140 mW green laser (532 nm) and with movies collected at 25 Hz. Cy5, being less photostable but brighter on our instrument than Cy3, provides a S:N of 10:1 using 120 mW red laser (637 nm) at 25 Hz. By increasing the integration time, less excitation power is needed. While shorter integration times allow for better resolution of faster structural rearrangements, it results in a lower signal on the detector unless a higher laser power is used. One must optimize the specific laser power and frame rate for the fluorophores and samples being investigated. Data were collected using Andor[®] software to control the camera and custom-modified Coherent[®] drivers for LabVIEW[®].

3 FRET Data Analysis

Raw data (movies) were imported into FIJI for image rotation, background subtraction and spot identification. Custom MATLAB codes were used for the next several processing steps. First, a mapping function is generated based on the image collected of the fluorescent microspheres. The map provides coordinates for the spots in the left (green) channel based on the spots found in the right (red) channel. Traces, consisting of Cy3 and Cy5 intensities at each frame of the movie, are then extracted for each molecule based on these coordinates. Finally, traces are filtered manually and those that meet the minimum criteria for FRET activity are selected for further processing. These criteria include: donor-acceptor intensity fluctuations (if present) are anticorrelated, single-step photobleaching of fluorescence, and a S:N>4. Histograms were compiled from the E_{FRET} values observed in the first 50 frames of all traces. Mathematica was used

to fit histograms with Gaussian functions, compile all selected traces and export specific examples for figures. Adobe[®] Illustrator was used to format figures for publication.

For kinetics analyses, raw traces were processed for import into QuB^{16,17} for Hidden Markov Modeling (HMM). Pre- and post-HMM processing was accomplished using custom MATLAB code. We used the results of HMM to populate heat maps called transition occupancy density plots (TODPs). The output of HMM is a list of the time, starting E_{FRET} value and ending E_{FRET} value of every transition identified in each raw trace. Each trace contributes intensity to the TODP at the locations (potentially more than one) corresponding to the initial and final E_{FRET} values it transitions between. Intensity along the diagonal axis of a TODP (lower left to upper right corners) indicates a population of molecules that remain in one state throughout their measurement. For transitions, dwell times spent in the initial FRET state before transitioning to the final FRET state are calculated by selecting a two-dimensional area on the TODP, then fitting the resulting number of instances vs. dwell time curves with a single or double exponential function.

4 Biolayer Interferometry

BLI is well-suited to investigate binding affinities of biomolecular interactions. Experimentally, biotinylated samples are immobilized to a streptavidin-functionalized probe, then dipped into a series of different buffered solutions. As material accumulates near (or dissipates from) the probe, an increasing (or decreasing) “shift” in a diffraction pattern is measured and plotted on the y-axis. Probes labeled with sample are next soaked in a solution of

biotinylated-lysine to “quench” any unoccupied streptavidin binding sites and minimize unwanted adsorption of downstream analytes. A second quenching step may be used for particularly sticky analytes such as proteins. For example, in the tRNA experiment described in Chapter VI, non-specific sticking by the protein under study was inhibited after a second quenching step using a solution of 0.02% Tween-20[®] and including 0.02% Tween-20[®] in the buffer during the association and dissociation phases. Non-specific sticking was not observed in the riboswitch experiments. The quenching and passivation steps are followed by soaking the probes in fresh buffer to establish a new baseline shift. Finally, probes are introduced to the analyte of interest and “accumulation” is reported as the association phase. However it is important to keep in mind that what is being measured is the sum of the association and dissociation rates as the system comes to equilibrium in this solution. This method was used to determine whether the fluorophore-labeled variants of the NAD⁺ riboswitch were capable of binding ligand (See Chapter V) and to determine the time-scales of protein accumulation on tRNA (see Chapter VI). BLI data analysis was performed in Mathematica.

CHAPTER III

BUILDING A SINGLE-MOLECULE INSTRUMENT FOR TIRF MICROSCOPY

1 Background

Single-molecule (sm) microscopy instrumentation involves a light source to excite a sample, a microscope objective and system of lenses and mirrors to enlarge and direct the resulting image, and a detector on which the image lands. By utilizing sm techniques, we are able to observe interactions of biological molecules such as DNA, RNA, and proteins in real time without loss of information due to ensemble averaging.

Several sm methods have been developed over the years for various types of measurements. To name a few, fluorescent imaging allows for co-localization studies or super-resolution imaging in live cells¹⁸, optical tweezers are used to determine forces and displacement that occurs during protein/nucleic acid interactions¹⁹, and smFRET was developed to study structural rearrangements as mentioned above. The first reported smFRET microscopy experiment is attributed to Takjip Ha et. al. who expanded a method called near-field scanning optical microscopy. This measurement was carried out using a probe placed within 10 nm of a sample to measure the emission spectra and excited state lifetimes of single molecules²⁰. In this article, the authors describe two-color imaging and single-molecule spectroscopy of DNA labeled with a donor-acceptor fluorophore pair immobilized on a microscope slide at a concentration of 10 molecules per μm^2 , with some 60 molecules imaged simultaneously. Improvements in instrumentation have

resulted in an imaging capability of >70 molecules per μm^2 with several hundred molecules imaged simultaneously (as observed on our instrument).

Whereas samples in the first smFRET experiments were excited via 514 nm Ar^+ ion laser sent through an optical fiber that carried light directly to a single molecule, our instrument uses an evanescent field propagated into the sample chamber via a laser light incident on a prism at a suitable angle for total internal reflection (TIR) to occur. The prism-type TIR geometry, as opposed to objective-type, facilitates use of a flow-through sample chamber for solution exchange throughout the experiment without affecting the focal plane of the samples.²¹ Another significant benefit of this custom-built smTIRF (single-molecule total internal reflection fluorescence) instrument design is in the simultaneous detection of multiple fluorophores on each molecule; achieved by splitting the fluorescence path into the appropriate number of channels. Design of the fluorescence path was heavily influenced by Selvin and Ha's Single-Molecule Techniques laboratory manual²¹. Our smTIRF instrument is capable of collecting data at temporal resolution on the order of milliseconds and can capture a $2 \times 4 \mu\text{m}^2$ area on the slide and record hundreds of molecules simultaneously when projected onto a $13 \times 13 \mu\text{m}^2$ detector chip. Significant cost savings add up when comparing the direct build cost to a TIRF microscope offered commercially and include routine maintenance, repairs, and further optimization that can all conveniently be performed in-house by mechanically-savvy researchers.

2 Custom Design of an smTIRF Instrument

2.1 Excitation Optics

The excitation path (Fig. 3.1) consists of two solid-state lasers: one is an optically pumped semi-conductor laser at 532 nm and the another is a diode laser at 637 nm (Coherent OBIS 532 LS and Coherent OBIS 637 LX, respectively). Lasers are individually controlled at an interface built in LabVIEW via one external remote for each laser. Emission from the lasers are combined at a 567 nm long-pass dichroic mirror (ThorLabs DMLP567) then navigated vertically along a periscope onto the breadboard that sits atop the microscope. Two mirrors shift the combined beam horizontally to a final mirror angled at 30° , which directs it onto a Pellin-Broca prism (Newport). A biconvex (BCX) lens before the prism focuses the beam to a size comparable to the area imaged onto the camera, enhancing the power within the imaging area. This angle of incidence is needed to create TIR at the slide-water interface via the prism which propagates an evanescent field into the sample chamber for gentle excitation of the fluorophores. An aluminum post was milled at an angle to match the upper surface of the prism, and the prism was mounted to it with standard 5-min epoxy. Several irises (e.g. ThorLabs ID25) are installed along the excitation path to facilitate realignment as needed: two after the first dichroic, two between the second mirror after the periscope and the angled mirror on the breadboard, and one between the lens and the prism.

2.2 Imaging Area

The microscope is a Leica DMI8 inverted microscope fitted with a 63x oil immersion objective. The sample stage was custom fitted with spacers to prevent shifting of the microscope slide during data collection, which can occur if several



Figure 3.1: **Excitation Path.** Two OBIS™ lasers are shown (bottom left) with the green and red excitation paths overlain. The two paths combine at the DMLP567 dichroic mirror (top right) and are aligned with two irises (top center and left).

iterations of sample or buffer injections are needed between recordings of molecules in the same area. Inlet and outlet ports were drilled into quartz microscope slides (GM Quartz, 7525-01) using an upright mounted Dremel® tool with a diamond tip bit. Prepared slides (see Chapter II) are then fitted with Tygon® tubing epoxied together with pipette tips cut to size.

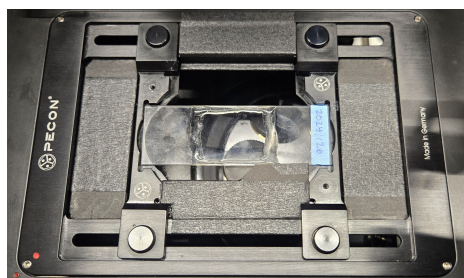


Figure 3.2: **Sample Stage Fitted with Spacers.** The stage was retrofitted with spacers to prevent the slide from shifting as new solutions are introduced to the sample chamber during data collection.

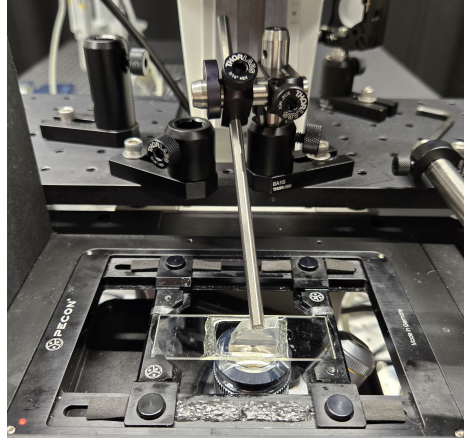


Figure 3.3: **Prism-Type TIRF Geometry.** Laser light enters the prism from the right and exits left to a back-drop to minimize stray light.

2.3 Fluorescence Optics and Detector

The image that exits the microscope port will do so as a wide diverging beam at some angle different from parallel to the table. The final image will need to land on a camera, ideally at the same size as the chip. Therefore, a rectangular aperture must be used to cut the image to the correct size at the microscope port. This was achieved using four standard straight razor blades wrapped in black gaff tape and mounted perpendicularly on two separate small translation stages. The apparatus is placed up against the outlet port of the microscope body to minimize stray light from entering the fluorescence optics chamber.

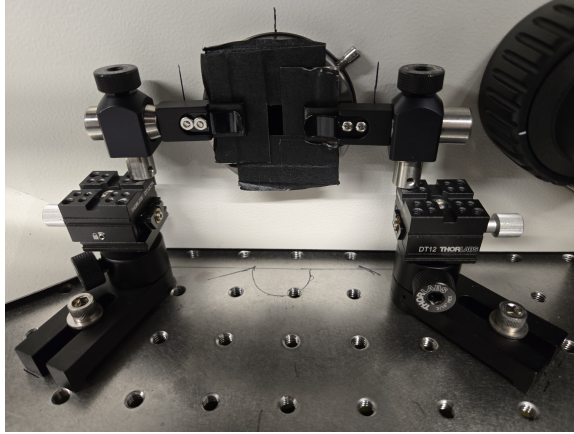


Figure 3.4: **Four-Sided Aperture Reducing Image Size.**

The correctly-sized image passes through a 2" BCX lens (ThorLabs LB1199-AB $f=200$) set 7 inches from the port, then split into red and green channels by a dichroic mirror (Chroma T635lpxr). If the image was generated by a point source, collimation of the beam would occur; however, the result of installing a biconvex lens here is a converging beam. Fortunately, beam convergence allows the false images that propagate along with the real image to be blocked downstream by an iris.

The mirror in the green channel corrects the vertical angle of the light so the image runs parallel to the table. Green light passes through a filter that transmits 585 nm light with a bandwidth of 65 nm then encounters a second dichroic mirror (Chroma T635lpxr) where it is directed to the camera. Light in the red channel passes through the first dichroic mirror and continues through a long-pass filter (Chroma ET655lp) that reflects light of 655 nm and shorter to block laser light and scatter. An iris is installed in both channels immediately upstream from the second dichroic as another aperture blocking the halos that propagate along with the desired beam. These iris apertures are crucial for having a clean, focused image

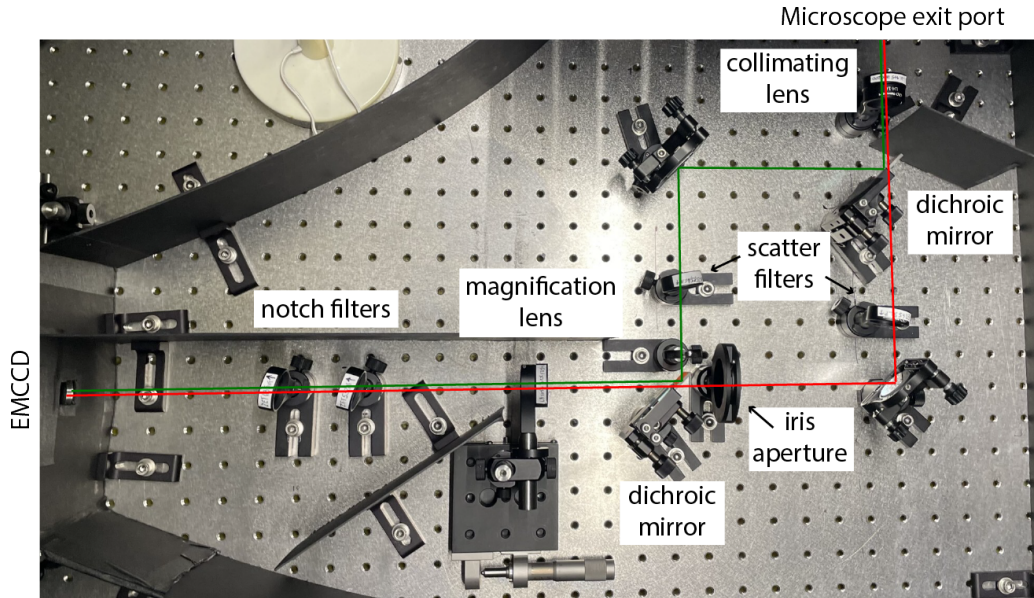


Figure 3.5: **Fluorescence Path.** Light exits the microscope port and is carried along a series of lenses, mirrors, and filters to project a focused image with red light incident on one half of the detector chip and green light on the other half. The various roles of each optical component are labeled. Additional channels may be added by installing different dichroic mirrors and filters to one or both paths.

on the detector. The two channels are combined side by side at the second dichroic mirror in the fluorescence path after which point they encounter another BCX lens (Thor Optics LB 1056 BCX F250) for proper magnification and focus at the detector. Between the second BCX lens and the detector, two notch filters are installed to reflect any remaining laser light (Chroma ZET635nf and ZET532nf).

The camera (Andor iXon 888) has an electron-multiplying charge coupling device (EMCCD) detector chip with 1054×1054 pixels each $13 \times 13 \mu\text{m}^2$ in size and is capable of recording data at a rate of 40 ms per frame without binning the pixels. Faster frame rates are achievable with pixel binning or by restricting the region of interest. Recording movies without binning pixels allows for a spatial resolution that supports simultaneous detection of hundreds of spots in both channels where

each spot, represented by a point-spread function having ~ 3 pixel radius, is either green or red fluorescence from fluorophores such as Cy3 and Cy5, respectively.

Initial configuration entails illuminating a micron-scale reticle with white light to obtain horizontal and vertical alignment for both channels in the fluorescence path as well as the proper depth of focus on the camera. First, the razor aperture must be fitted to the correct image size. Corrections for horizontal and vertical alignment are adjusted by the mirror in each channel, while the lenses are used to adjust the focus on the camera. Following the reticle alignment step, imaging a slide with immobilized fluorescent microspheres (Invitrogen F8810) allows for fine-tuning of the fluorescence path alignment.

Routine maintenance consists of monthly cleaning and realigning the optics in the excitation path using in-place irises and cleaning the breadboard and surrounding areas to prevent dust accumulation. On rare occasions (once to twice annually), the optics in the fluorescence path will need to be cleaned and realigned as needed. The prism will need to be remounted every few years when the epoxy fails.

3 Conclusion and Applications

The versatility of the home-built instrument enables the investigation of a number of research questions. Presently, the types of applications our instrument is accommodating, beyond smFRET, include colocalization and sequencing experiments. For example, the instrument is being utilized to explore the structure and dynamics of a long non-coding RNA by documenting the colocalization of short oligonucleotide probes with the RNA. The colocalization indicates probe binding to a particular segment of the RNA. As such, these events can be correlated with

the structure of the RNA at a specific, targeted sequence, and single-molecule resolution allows the researcher to observe structural changes of that targeted RNA segment. Another application involves developing a high-throughput method that integrates real-time smFRET with single-molecule RNA sequencing to map RNA sequence-dependent structural dynamics. Our instrument enables the simultaneous probing of RNA folding and interactions across vast sequence landscapes in a single experiment.

The following two chapters detail the ways that i have employed our custom single-molecule fluorescence microscope to investigate how a bacterial riboswitch interacts with a ubiquitous enzyme cofactor, NAD^+ . The first part of this story is presented in Chapter IV and continues in Chapter V. Chapter VI continues with an investigation into the interactions of a tRNA and a uracil-modifying enzyme, in which smFRET investigations were carried out with the assistance of my colleague, Madison Kadrmas.

CHAPTER IV

LIGAND-BINDING CHARACTERISTICS OF AND NAD^+ RIBOSWITCH REVEALED BY FRET AND BIOLAYER INTERFEROMETRY

1 Prologue

This chapter contains previously published material.

2 Manuscript

The Class II NAD^+ riboswitch is a bacterial RNA that binds ligands containing nicotinamide. Herein, we report a fluorescence and biolayer interferometry study of riboswitch interactions with β -NMN. The results reveal a shift in the prevalence of a pseudoknot structure in the presence of ligand and Mg^{2+} .

Riboswitches are a topic of increasing interest due to their potential as antibiotic targets²⁰ and molecular sensors.²² A riboswitch is a gene regulatory element, commonly found in the 5' untranslated region of bacterial messenger RNA, that contains an aptamer domain where a ligand binds and an expression platform located just downstream. The Class II NAD^+ riboswitch (NAD^+ -II) is a translational off-switch that has been reported to bind specifically to the nicotinamide moiety of nicotinamide adenine dinucleotide (NAD^+) (Fig. 4.1a), a ubiquitous cellular cofactor involved in many enzymatic reactions.²³ We aimed to elucidate the conformational dynamics and ligand-binding interactions of NAD^+ -II using fluorescence techniques including single-molecule Förster resonance energy transfer (smFRET). This method relies on a donor and acceptor pair of

fluorophores, selected such that an excited donor fluorophore may nonradiatively transfer energy to the acceptor fluorophore with an efficiency that depends on their proximity and relative transition dipole orientations.^{9,24} FRET is sensitive to changes in inter-fluorophore distance on the nanometer length-scale, and modern single-molecule instruments can routinely resolve changes in FRET on the tens-of-milliseconds timescale.²⁵ Appropriate fluorophore labeling sites will change distance as the RNA samples different conformations without interfering with the folding mechanism or binding interaction under investigation.²⁶

A biochemical study of NAD^+ -II reported the potential for the formation of a pseudoknot in which three bases within the ribosome binding site (RBS) form base pairs with three bases within a loop near the 5' end of the aptamer domain (Fig. 4.1b).²³ Titrations based on in-line probing²³ and calorimetry¹² reported a 1:1 binding stoichiometry with K_D values of 100 mM for NAD^+ and 40 mM for β nicotinamide mononucleotide (β -NMN). Two crystallography studies further reported NAD^+ - and β -NMN-bound structures, finding that the primary binding pocket of the riboswitch is specifically sensitive to the nicotinamide moiety.^{12,13} One study observed RNA dimerization with each monomer binding one ligand molecule¹² and the other observed RNA monomers, each of which binds two ligands in distinct domains¹³ (Fig. 4.1c). Both structures demonstrate RBS sequestration through pseudoknot formation, supporting a translational off-switch mechanism.

We designed two riboswitch variants based on the *pnuC* motif from *S. parasanguinis* with the 5' end of the RNA biotinylated for surface immobilization, the donor fluorophore Cy3 on the 3' end, and the acceptor fluorophore Cy5 conjugated internally to a 5-aminohexylacrylamino-uridine modification (Fig. 4.1b). Based on previous studies of the pseudoknot-structured Class I preQ1

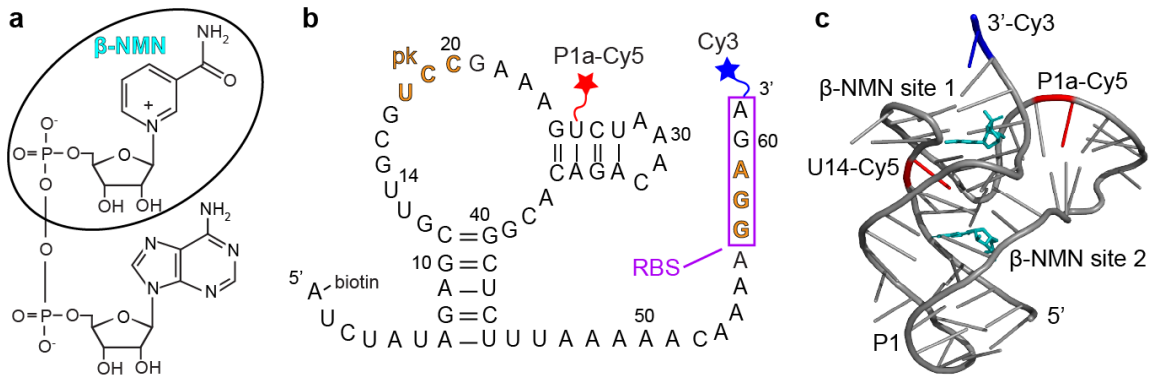


Figure 4.1: (a) Chemical structure of nicotinamide adenine dinucleotide (NAD^+). The circled moiety is β -nicotinamide mononucleotide (β -NMN). (b) Sequence and secondary structure of the NAD^+ -II riboswitch from *S. parasanguinis*. The ribosome binding site (RBS) is indicated by a box and the bases that form the pseudoknot (pk) are indicated in orange. Cy3 (blue star) is placed at the 3' end, and Cy5 (red stars) is placed at either U14 (b-U14Cy5-Cy3 riboswitch construct) or U26 (b-P1aCy5-Cy3 construct). (c) Crystal structure of the riboswitch (PDB ID 8HB8) with the P1 and P1a domains and β -NMN binding sites labeled. Locations of the U14 and P1a Cy5 labeling sites are indicated in red and β -NMN molecules are shown in cyan.

riboswitch,^{27–29} Cy5 was placed at either U14 (in the loop adjacent to the P1 helix) or U26 (in the P1a helix) to ideally result in a high-FRET (HF) state when the riboswitch exists in its compact “docked” pseudoknot structure and a mid-FRET (MF) or low-FRET (LF) state when undocked. We refer to these constructs as b-U14Cy5-Cy3 (abbreviated U14-Cy5) and b-P1aCy5-Cy3 (abbreviated P1a-Cy5) RNAs based on the locations of the 5' biotin (b), internal Cy5, and 3' Cy3 (Fig. 4.1b). The acrylamino-modified U14 construct prior to Cy5 labeling is referred to as b-aminoU14-Cy3 (abbreviated aminoU14).

We first performed smFRET (details in SI) on the U14-Cy5 riboswitch in the absence and presence of 1 mM Mg^{2+} , as divalent cations support RNA folding by coordinating to its 2' hydroxyl groups and negatively charged phosphate backbone. Cy3 and Cy5 fluorescence were recorded in real-time and the first 50 frames of each trace were compiled to populate histograms that display the relative frequencies of the FRET efficiencies accessed by the RNA. Three FRET states are

observed in the histogram for apo-form RNA in the absence of Mg^{2+} with FRET efficiencies of $E_{HF} = 0.92$, $E_{MF} = 0.64$ and $E_{LF} = 0.16$, with the HF state being the most prevalent (Fig. 4.2a). The prevalence of the MF state increases slightly in the presence of 1 mM Mg^{2+} (Fig.4.6). After increasing the divalent cation concentration to 20 mM Mg^{2+} , the concentration used most widely in solution-phase studies,^{3,8} the MF state is substantially suppressed in favor of the HF state (Fig. 4.2a). With the fluorophores placed adjacent to the two arms of the helix forming the pseudoknot, we hypothesized that addition of ligand would further shift population to the HF state. Surprisingly, upon introducing 1 mM β -NMN in combination with either 1 mM or 20 mM Mg^{2+} , no significant difference is observed in histograms or time traces compared to the same Mg^{2+} concentration in the absence of ligand. This observation could be explained by the riboswitch preferring the pseudoknot structure, or a different HF structure, under all conditions tested. Alternatively, fluorophore placement may either not be sensitive to the particular structural changes undertaken by the RNA or may be impairing the ability of the riboswitch to bind ligand altogether.

Inspection of the crystal structures indicated that placement of Cy5 on U14 has the potential to interfere with ligand binding. The nicotinamide moiety of NAD^+ hydrogen-bonds to form a planar “base” triple and intercalates into a four-rung ladder of base triples and quadruples.^{12,13} U14 is immediately adjacent to the base quadruple that forms a “lid” on the nicotinamide binding pocket (Fig. 4.1c). We utilized biolayer interferometry (BLI) to directly investigate whether ligand was binding to the U14-Cy5 riboswitch using the aminoU14 RNA, which lacks Cy5, as a control. The aminoU14 RNA was observed to bind β -NMN, whereas the U14-Cy5 RNA did not respond when exposed to 1 mM β -NMN in the presence

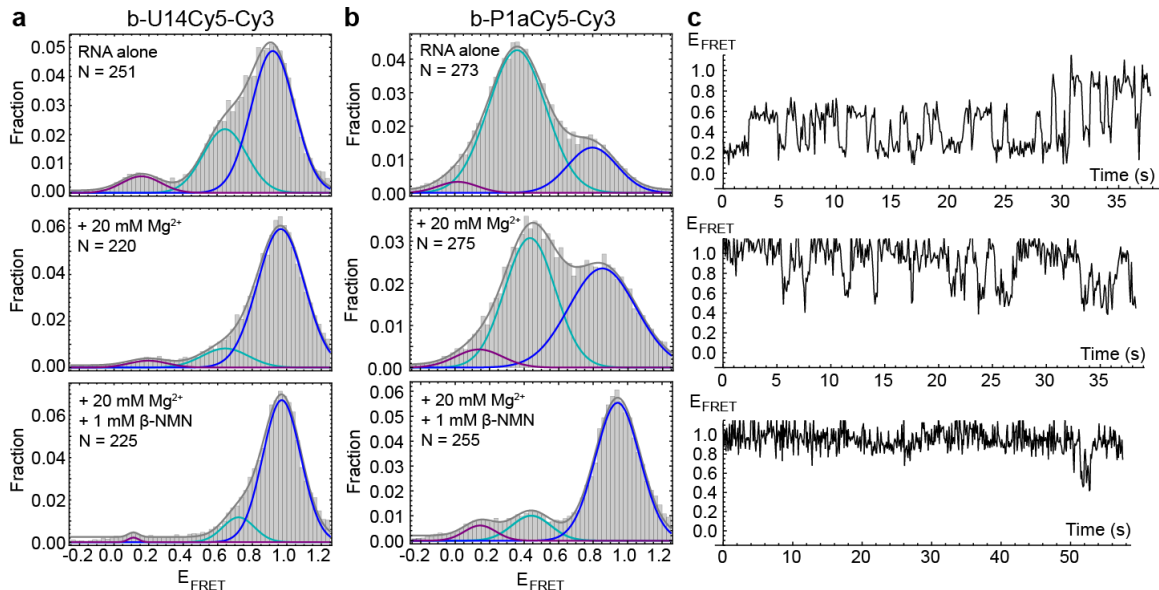


Figure 4.2: (a) smFRET histograms of the U14-Cy5 riboswitch construct recorded in imaging buffer (top), buffer + 20 mM MgCl₂ (middle) or buffer + 20 mM MgCl₂ + 1 mM β-NMN (bottom). 3-Gaussian fits are shown in gray, with underlying components shown in purple, cyan and blue. “N” indicates the number of single-molecule traces that were used to compile the histogram. (b) Corresponding smFRET histograms of the P1a-Cy5 construct. (c) Representative FRET efficiency traces from single molecules of P1a-Cy5 RNA in imaging buffer (top), buffer + 20 mM MgCl₂ (middle) and buffer + 20 mM MgCl₂ + 1 mM β-NMN (bottom).

of 20 mM Mg²⁺ (Fig. 4.3a). These results indicate that the Cy5 modification suppresses ligand binding, either by directly blocking or altering the binding sites, or by promoting misfolding of the RNA. Fitting the BLI trace of aminoU14 RNA revealed fast and slow components to the association phase, which is most readily explained by a 2:1 binding stoichiometry (as seen in one crystal structure¹³) with ligand accumulating more slowly at one binding site than the other (Fig. 4.3b). The slow rate at which a second binding site appears to get populated may explain why solution-phase measurements have typically suggested a 1:1 binding stoichiometry.^{12,23}

We identified an alternative Cy5 labeling site in the P1a helix (U26), which exhibits the highest sequence variability in the aptamer domain and does

not appear to be directly involved in ligand binding.^{12,13,23} This segment of P1a resides near the 3' end of the RNA in crystal structures, suggesting that a HF state would be favored in the presence of ligand. BLI results confirm functionality and again show fast and slow components to the association phase when the P1a-Cy5 riboswitch is exposed to β -NMN (Fig. 4.3c and Fig.4.7). Bulk FRET titrations over the range of 0–10 mM β -NMN indicate that there is a global shift to higher FRET efficiencies with increasing ligand concentration (Fig. 4.3d), which was not seen in a mock titration (only buffer added) or a negative control titration with the related compound nicotinic acid mononucleotide (NaMN; Fig.4.7). Fitting the ratio of Cy5 to Cy3 intensity with the Hill equation after correction for photobleaching (details in methods) yielded a K_D of 32 mM and a Hill coefficient, a reporter on the cooperativity of binding sites, of $n = 0.8$ (Fig. 4.3e). This K_D is comparable to previously reported affinities for β -NMN,^{12,23} confirming that the modifications on the P1a-Cy5 riboswitch do not significantly hinder ligand binding.

smFRET measurements revealed that the P1a-Cy5 riboswitch briefly samples several distinct conformations in the absence of Mg^{2+} and ligand, which are observable as two prominent features in FRET histograms. A broad MF peak centered on $E_{FRET} = 0.36$ is dominant (Fig. 4.2b) and inspection of traces suggests that several different FRET states may be resolvable within this peak (Fig. 4.2c). The riboswitch's dynamics are substantially suppressed upon adding 20 mM Mg^{2+} , with HF states ($E_{FRET} > 0.8$) being stabilized relative to MF states. In the presence of 1 mM β -NMN and 20 mM Mg^{2+} , the complexity of the traces further simplifies to three commonly accessed states with $E_{LF} = 0.14$, $E_{MF} = 0.44$, and $E_{HF} = 0.94$. The prevalence of the HF state increases significantly, and a greater fraction of traces exhibit static rather than dynamic behavior. This response to

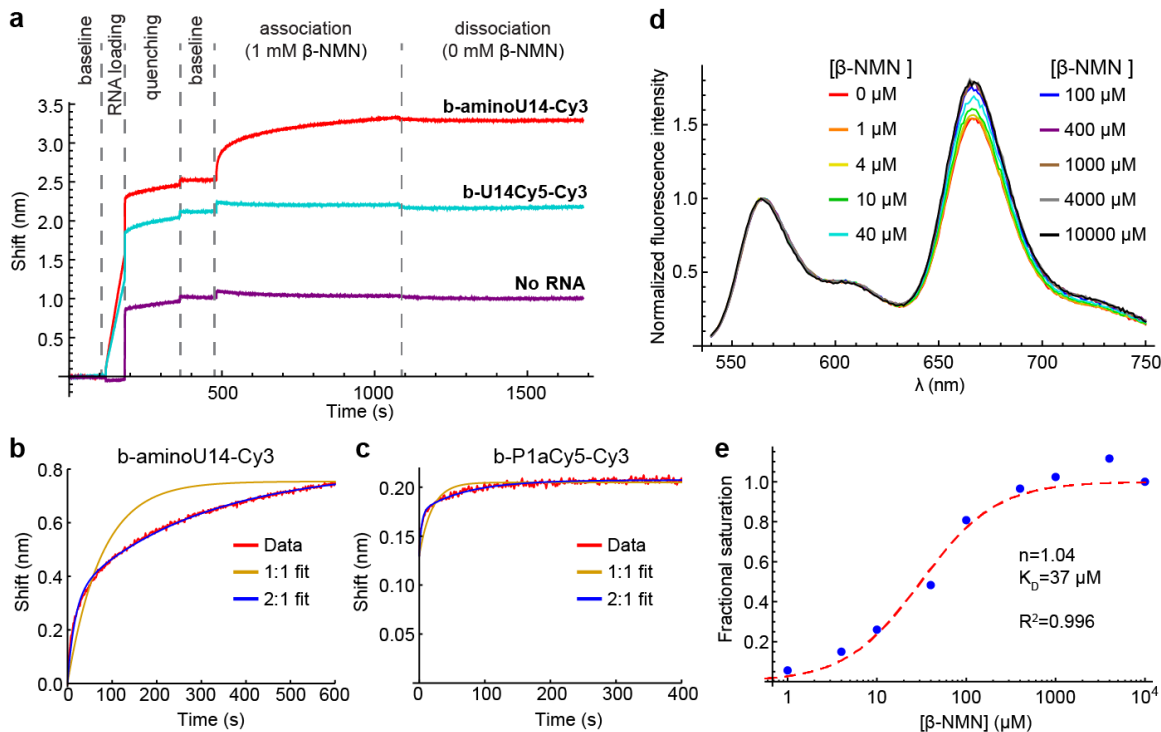


Figure 4.3: (a) Biolayer interferometry (BLI) measurements on aminoU14 (red) and U14-Cy5 RNA (cyan). Shifts are observed due to binding of RNA to the streptavidin-coated probe (“RNA loading”), binding of biocytin to the probe (“quenching”) and binding of β -NMN to the RNA (“association”). A probe was run with no RNA loaded to control for nonspecific binding of β -NMN to the probe (purple). (b) Close-up of the association phase for aminoU14 RNA (red), showing fits for a 1:1 (yellow) and 2:1 (blue) β -NMN:RNA binding stoichiometry. (c) Close-up of the association phase for P1a-Cy5 RNA and fits. (d) Bulk FRET titration of P1a-Cy5 RNA with β -NMN. Emission spectra were recorded under excitation at 532 nm and the Cy3 peak was normalized to an intensity of 1. (e) Blue: titration curve extracted from the ratio of Cy5 to Cy3 emission intensity. Datapoints show the mean and standard deviation across three titrations. The curve was fit with the Hill equation (red dashed line), yielding a cooperativity of 0.8 and a K_D of 32 mM.

ligand is reminiscent of the Class I preQ₁ riboswitch, which like NAD⁺-II has a pseudoknot-structured architecture and adenosine-rich 3' tail.^{27–29} Overall, our smFRET data indicate that the ensemble of structures sampled by the NAD⁺-II riboswitch is more complex than simply undocked or docked (pseudoknot-structured) conformations and that the dynamic sampling of these structures is suppressed in the presence of ligand.

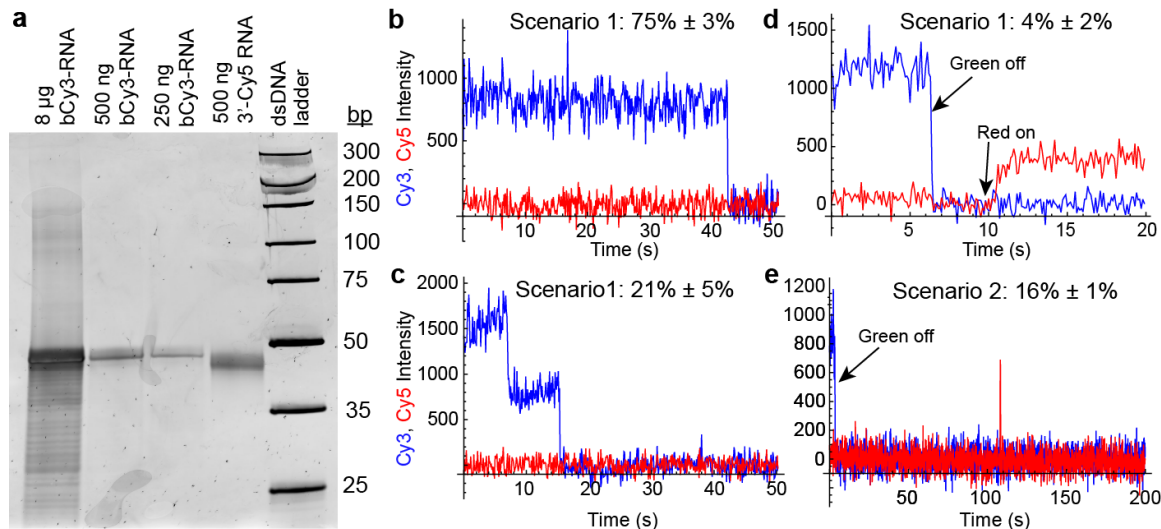


Figure 4.4: (a) Native polyacrylamide gel showing the presence only of a monomer band (61 nucleotides) when varying quantities of b-aminoU14-Cy3 RNA (lanes 1–3) or 3'-Cy5 RNA (lane 4) are loaded. Weak slower-migrating bands are observed only in an overloaded lane. (b) Example of a trace showing single-step photobleaching of Cy3 on a slide prepared under dimerization scenario 1 (see Fig. 4.10a). (c) Example of a trace showing two-step photobleaching of Cy3 and no Cy5 in scenario 1. (d) Example of a trace showing a stable heterodimer in scenario 1. The green laser was turned off at 6 s and the red laser was turned on at 10 s. (e) Example of a trace showing Cy3 and transient presence of Cy5 in scenario 2 (see Fig. 4.10b). The green laser was turned off at 4 s and the red laser was on continuously.

We next tested the potential of the riboswitch to form dimers (as observed in one crystal structure¹²) in solution. Nondenaturing polyacrylamide gel electrophoresis did not indicate the presence of stable dimers, even at high RNA concentrations (Fig. 4.4a and Fig. 4.9). To further probe the potential for dimer formation, we performed a single-molecule colocalization study using two single fluorophore-labeled riboswitches: b-aminoU14-Cy3 RNA and a 3' Cy5-labeled RNA. Different annealing and imaging conditions were tested to provide opportunities for homo- and heterodimers to form and be observed (Fig. 4.4b–e and Fig. 4.10). Homodimers were observed as multi-step photobleaching of Cy3 (Fig. 4.4c). Transient or extended observations of Cy5 revealed the presence of heterodimers (Fig. 4.4d and e).

When b-aminoU14-Cy3 and 3' Cy5 RNAs were annealed in a 1:10 ratio before immobilization (scenario 1, Fig. 4.10a), a small fraction of traces exhibited Cy5 signal under direct excitation, $4\% \pm 2\%$ (mean $\pm$ standard deviation, σ , across 4 movies). When b-aminoU14-Cy3 RNA was annealed alone, followed by imaging with freely diffusing 3' Cy5 RNA present in the sample chamber (scenario 2, Fig. 4.10b), transient heterodimer observations occurred in $16\% \pm 1\%$ (mean $\pm \sigma$ across 2 movies) of traces (Fig. 4.4e). Multi-step photobleaching of Cy3 (Fig. 4.4c), observed in $21\% \pm 5\%$ (mean $\pm \sigma$ across 3 movies) of traces in scenario 1, can result from dimerization or from independent immobilization of two molecules within the diffraction-limited width of the point-spread function.³⁰ The annealing conditions in scenario 1 were designed to favor the formation of heterodimers over homodimers, so it is likely that many of these Cy3 colocalizations are coincidental. Therefore, our data do not support the extensive formation of stable RNA dimers in solution in the absence of Mg^{2+} and ligand.

In summary, our results indicate that the NAD^+ -II riboswitch dynamically samples a complex landscape of conformations with compact structures being favored in the presence of Mg^{2+} and static occupation of a compact conformation being favored in the presence of β -NMN. We found that stable RNA dimers are not prevalent in solution in the absence of Mg^{2+} and ligand, and our BLI measurements suggest a 2:1 binding stoichiometry of ligand to RNA. Our findings also emphasize the potential impacts of fluorophore labeling on riboswitch function, with ligand binding being inhibited when Cy5 is conjugated to U14. Ongoing work includes a more extensive smFRET study of the NAD^+ -II riboswitch in which we further investigate its dynamics as well as its interactions with NAD^+ and other ligand variants.

N. E. C. N.: conceptualization, investigation, visualization, writing - original draft. J. R. W.: conceptualization, supervision, funding acquisition, visualization, writing – revision and editing.

The authors thank the National Institutes of Health (R35-GM147229) for supporting this research. We also thank the labs of Marian Hettiaratchi and Parisa Hosseinzadeh at the University of Oregon for the use of their biolayer interferometry instrument and Justin Svendsen for training and guidance.

3 Supplemental Information

3.1 Materials and Methods

Materials: RNA was obtained from Horizon Discovery and HPLC-purified and deprotected by the manufacturer. RNA concentrations were determined via A260 using the extinction coefficient provided by the manufacturer. Cy5-labeled constructs were labeled using Cy5 mono-reactive dye packs (Cytiva PA25001). One tube of dye was dissolved in 20 μ L of anhydrous DMSO and combined with 30 μ L of 167 mM Na_2CO_3 containing 3-4 nanomoles of RNA. The reaction was incubated at RT for 2 hours and excess dye was removed via ethanol precipitation. The RNA was then purified via denaturing polyacrylamide gel electrophoresis and recovered via electrocution and ethanol precipitation. RNA was annealed before each experiment by heating to 90°C for 2 minutes in imaging buffer (50 mM Tris-HCl pH 8.0, 100 mM KCl) and then air-cooling over 10 minutes. MgCl_2 , β -NMN (Millipore Sigma N3501) and/or NaMN (Millipore Sigma N7764) were added to the desired concentrations after annealing. For single-molecule measurements, the buffer additionally contained an oxygen scavenging system consisting of 50 nM protocatechuate 3,4-dioxygenase (PCD, Millipore

Sigma P8279), 5 mM protocatechuic acid (PCA, Millipore Sigma 08992) and 5 mM Trolox[®] (Millipore Sigma 238813). Microscope slides were prepared by the DDS Tween-20[®] method¹, in which slides are passivated with dichlorodimethylsilane (Millipore Sigma 440272), followed by incubation with biotinylated bovine serum albumin (Thermo Scientific PI29130), streptavidin (Molecular Probes S888) and Tween-20[®] (Millipore Sigma P9416).

smFRET measurements: Data were collected on a home-built prism-type total internal reflection fluorescence (TIRF) microscope (Leica DMI8 inverted microscope, 63x oil immersion objective). Cy3 and Cy5 were excited by a 532 nm laser (Coherent OBIS 532 LS) at 100-120 mW and a 637 nm laser (Coherent OBIS 637 LX) at 60-75 mW, respectively. The slide was illuminated at 532 nm continuously and at 637 nm at the beginning and end of each movie to confirm that Cy5 was active. For U14-Cy5 RNA and dimer study movies, fluorescence was recorded on a Hamamatsu C13440 Orca Flash 4.0 sCMOS with an exposure time of 100 ms. P1a-Cy5 RNA movies were recorded on an Andor iXon 888 emCCD with an exposure time of 40 ms. Traces exhibiting single-step photobleaching of Cy3 and Cy5 were selected manually and analyzed using custom MATLAB codes. Histograms were compiled from the first 50 frames of each selected trace. Gaussian fitting of histograms was performed in Mathematica.

Bulk FRET measurements: Fluorescence spectra for bulk FRET measurements were collected using an Edinburgh Instruments FS5 fluorometer by exciting Cy3 at 532 nm and recording emission from 540 nm to 750 nm. β -NMN or NaMN was titrated over a range of 0-10 mM into 150 nM RNA in imaging buffer containing 20 mM Mg²⁺. Decreasing fluorescence intensity for both dyes was observed over time due to photobleaching of molecules during each 210-second

scan (Fig. 4.8). To correct for this, we performed a “mock” titration in which aliquots of buffer without ligand were added, keeping all other measurement parameters and timing the same. The ratio of the peak intensity of the Cy5 peak to the Cy3 peak was calculated for each spectrum in the β -NMN and mock titrations, and the ratio observed in the mock titration’s spectrum was subtracted from the ratio observed in the corresponding β -NMN titration’s spectrum (Fig. 4.7g). This quantity was then converted to fractional saturation (FS) using the following equation:

$$FS(c) = \frac{R_c - R_o}{R_f - R_o}$$

where R_c is the corrected Cy5/Cy3 peak ratio at β -NMN concentration c , R_o is the peak ratio at 0 μ M β -NMN, and R_f is the peak ratio at the final β -NMN concentration measured, 10 mM. The fractional saturation curve was then fit with the Hill equation:

$$FS(c) = \frac{c^n}{c^n + K_D}$$

where n is the Hill coefficient and K_D is the dissociation constant.

Native polyacrylamide gel electrophoresis: After annealing, samples were mixed 1:1 with 2x TBE (Tris-Borate-EDTA) buffer containing 40% glycerol, then run out on a 12% non-denaturing polyacrylamide gel in TBE buffer at 4°C. The gel was stained in Sybr Gold and imaged on a Typhoon scanner.

Dimer study: In one experimental design (“Scenario 1”), a mixture of b-aminoU14-Cy3 RNA and 3’ Cy5-RNA was annealed at a 1:10 ratio in imaging buffer. The mixture was flowed onto the microscope slide, which was then flushed with buffer prior to imaging. Annealing at this ratio would allow for preferential formation of heterodimers over homodimers. To observe monomers

and homodimers, the samples were excited at 532 nm until photobleaching of Cy3 occurred. Traces were categorized by whether they exhibited single- or double-step blinking and photobleaching. Spots that exhibited two-step blinking or photobleaching indicate either homodimers or independent colocalized molecules. Spots exhibiting Cy5 signal under excitation with a red laser were classified as heterodimers. In another design (“Scenario 2”), the RNAs were annealed separately, b-aminoU14-Cy3 RNA was immobilized onto the slide, then 100 pM 3’ Cy5 RNA was introduced and allowed to freely diffuse while movies were collected. The slide was illuminated briefly at 532 nm to localize the b-aminoU14-Cy3 molecules, then illuminated at 637 nm to view Cy5 colocalization under direct excitation.

Biolayer interferometry: BLI measurements were performed on a GatorPlus Biolayer Interferometer (GatorBio, Paolo Alto, CA) utilizing proprietary GatorOne software for data collection, reference subtraction, and calculation of binding stoichiometry. GatorBio streptavidin-coated glass probes were presoaked for 3 min in imaging buffer containing 1 or 20 mM MgCl₂ before collecting a baseline signal for 180 s. The probes were immersed in 100 nM biotinylated RNA until an adequate shift ($\sim$ 1 nm) in signal was observed (1-1.5 min). Probes were then submerged into a 1 ppt biocytin solution to saturate all remaining streptavidin sites. Loaded probes were submerged into imaging buffer to ensure the baseline wavelength reading had stabilized (180 s). To measure association, loaded probes were submerged into 1 mM β -NMN in imaging buffer for 400-600 s. Traces from reference probes with no RNA loaded were subtracted from sample results to correct for non-specific background signal prior to calculating association stoichiometry.

3.2 Supplementary Figures

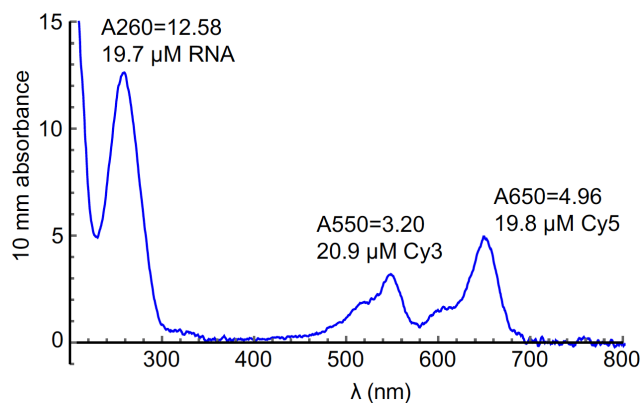


Figure 4.5: Example UV/visible absorbance spectrum of b-P1aCy5-Cy3 RNA recorded on a Nanodrop spectrophotometer. A260, A550 and A650 (values indicated on the plot) reflect the expected 1:1:1 RNA:Cy3:Cy5 labeling stoichiometry.

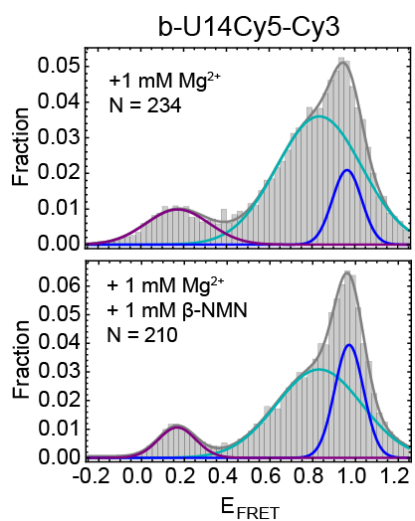


Figure 4.6: smFRET histograms and 3-Gaussian fits of b-U14Cy5-Cy3 RNA in the presence of 1 mM MgCl_2 (top) and 1 mM MgCl_2 + 1 mM β -NMN (bottom). “N” indicates the number of single-molecule traces that were used to compile the histogram.

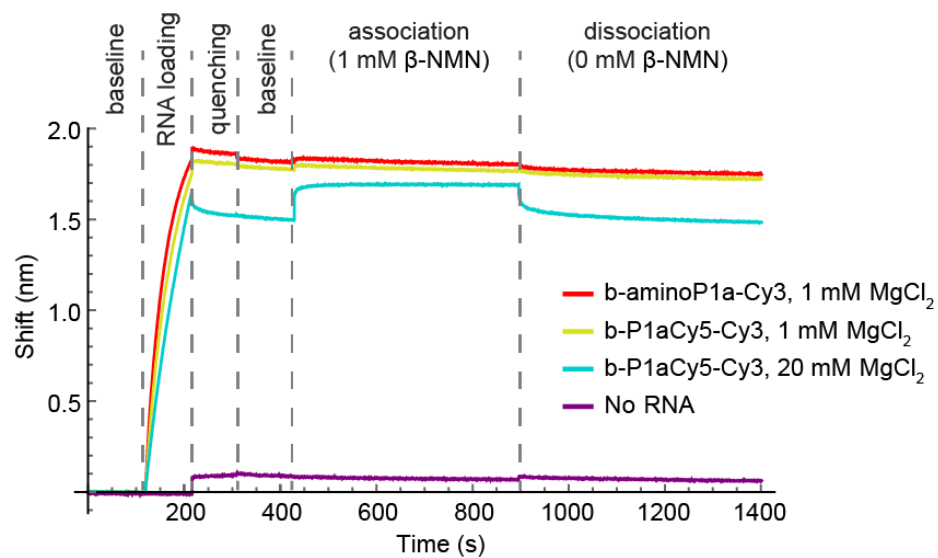


Figure 4.7: Complete BLI traces for b-P1aCy5-Cy3 RNA. Red: b-aminoP1a-Cy3 in 1 mM MgCl_2 . Yellow: b-P1aCy5-Cy3 in 1 mM MgCl_2 . Cyan: b-P1aCy5-Cy3 in 20 mM MgCl_2 . Purple: no RNA.

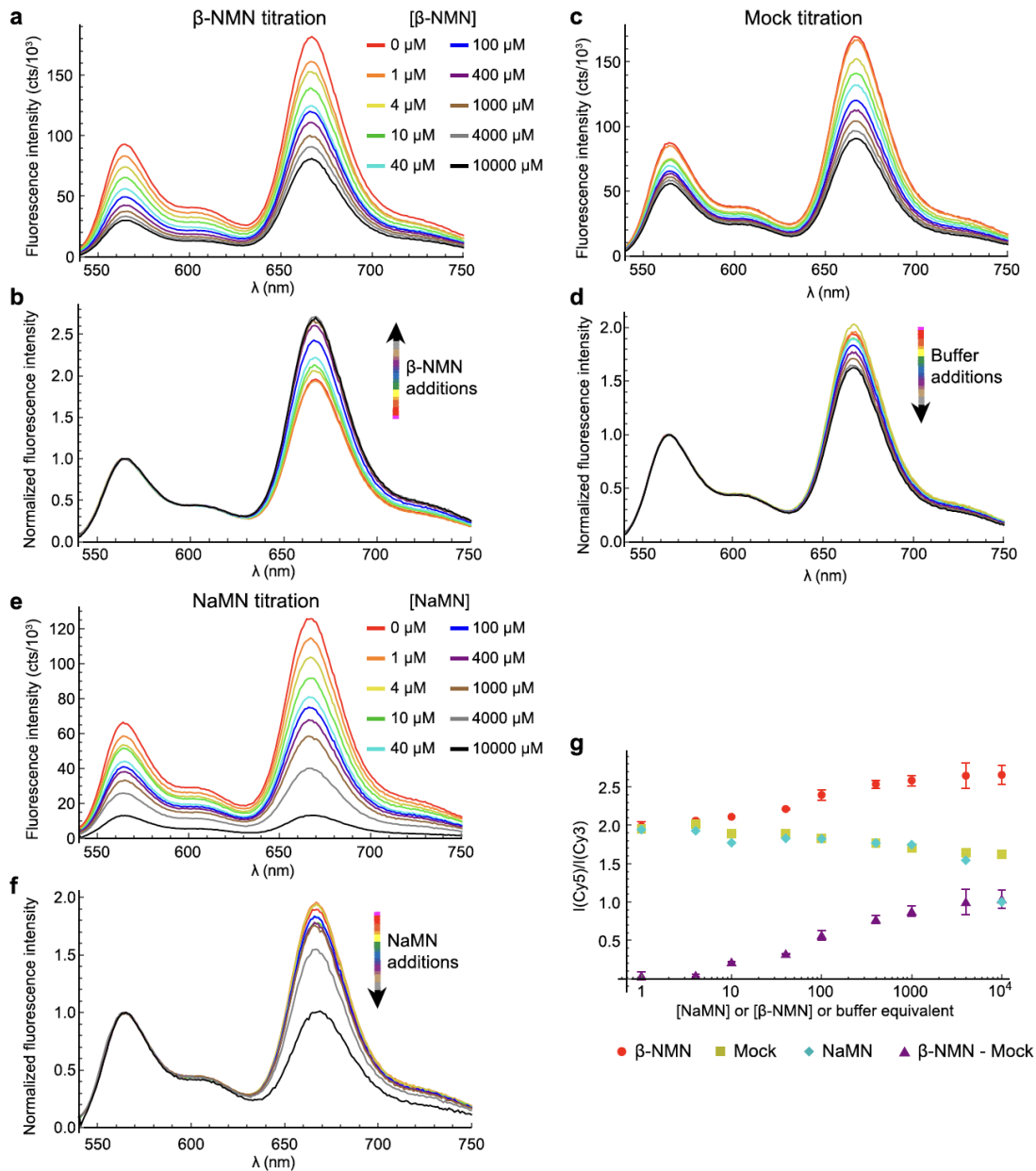


Figure 4.8: Bulk FRET measurements on b-P1aCy5-Cy3 RNA. (a) Raw fluorescence emission spectra following sequential additions of β -NMN to the indicated concentrations. (b) Spectra normalized to the peak of the Cy3 emission feature (reproduced from Fig. 4.3 for ease of comparison). (c-d) Raw (c) and normalized (d) spectra from a mock titration in which buffer was added in the same series of volume increments as β -NMN. Photobleaching causes a decrease in Cy5 emission relative to Cy3. (e-f) Raw (e) and normalized (f) spectra following sequential additions of nicotinic acid mononucleotide (NaMN) to the indicated concentrations. The trend closely tracks the mock titration until 4 mM NaMN. (g) Cy5 to Cy3 peak intensity ratio extracted from the β -NMN (red), mock (yellow) and NaMN (cyan) titration spectra. The photobleaching-corrected peak ratio used for fitting is plotted in purple.

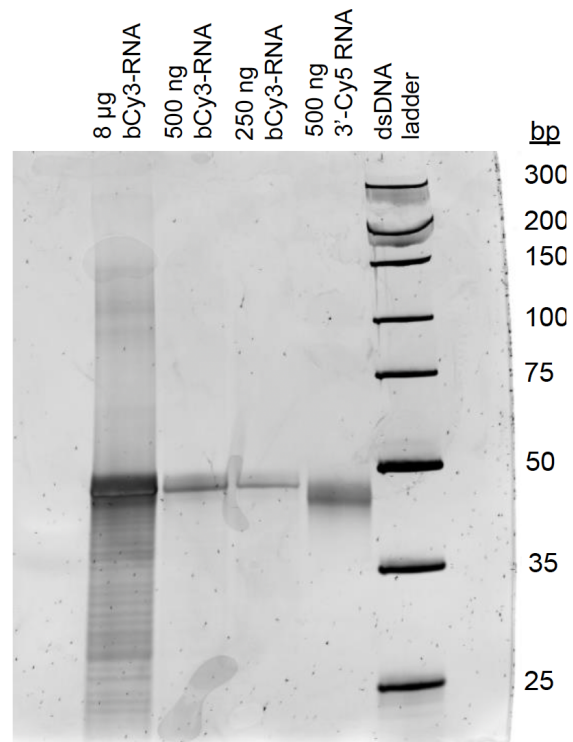


Figure 4.9: Raw image of gel in Fig. 4.4a

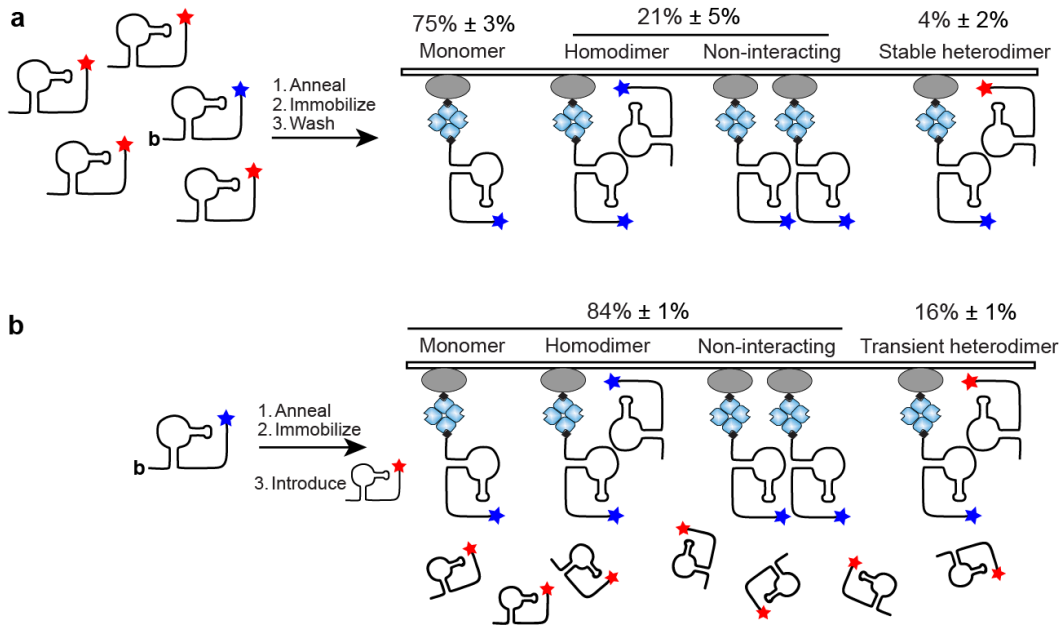


Figure 4.10: (a) Schematic of experimental design and results for “dimerization scenario 1”. 3' Cy5 and b-aminoU14-Cy3 RNAs were annealed in a 10:1 ratio and immobilized on a microscope slide that was then thoroughly flushed with buffer. Imaging allowed the observation of monomers (75% of analyzed spots), stable homodimers and non-interacting colocalized molecules (21%) and stable heterodimers (4%). (b) Schematic of experimental design for “dimerization scenario 2”. b-aminoU14-Cy3 RNA was annealed and immobilized, then imaging was performed with 100 pM 3' Cy5 RNA in the sample chamber. 16% of analyzed traces showed evidence of heterodimer formation with all colocalizations being transient.

CHAPTER V

IMPACTS OF ADENOSINE ON NAD⁺-II RIBOSWITCH CONFORMATIONAL DYNAMICS

This section includes unpublished material.

1 Prologue

As an extension of the investigation discussed in Chapter IV, the results presented in this Chapter offer more insight into the behavior of the NAD⁺-II riboswitch. A discussion of Hidden Markov Modeling (HMM) for this system where several FRET states are sampled with very short dwell times is included. Results of single-molecule data are presented including the concentration dependence on the shift toward the static pseudoknot structure the riboswitch adopts in the presence of 1 mM β -NMN and the effect that the adenosine moiety of NAD⁺ has on ligand recognition; a crucial component of the potential of NAD⁺-II to be a drug target. That NAD⁺-II recognizes NAD⁺ and binds predominantly via the nicotinamide moiety has been established.^{12,13,23} However, I show here that the adenosine moiety does impact ligand recognition, observable in single-molecule data as a decrease in the prevalence of RNA that occupies the HF state. Interestingly, there is no significant difference in the distribution of states sampled by the riboswitch in the presence of NAD⁺ versus ϵ -NAD⁺, which contains a modified adenine.

2 Hidden Markov Modeling of FRET States

Selecting the appropriate number of states for Hidden Markov Modeling is inherently subjective because the inputs to specific FRET states and transition

rates are derived by observation. This subjectivity is especially true for cases where more than three clearly distinguishable states are sampled and the time-scales of dynamics are comparable to the frame rate of data collection. This can result in overfitting in which noise is mis-identified as transitions between FRET states, which introduces error in calculating their respective dwell times. Three or fewer FRET states can potentially be resolved in a histogram by eye, assuming there is adequate separation of the peak centers. For cases with more than three states, a more rigorous analysis is warranted. My approach for fitting these data was to first take note of FRET states observed during trace analysis and then to fit the histogram with a sum of up to five Gaussian functions. Two HMM inputs were taken from the fitted histograms: the FRET efficiencies directly and their relative occurrence frequencies, which were used to estimate relative rates of the forward and backward transitions between them. The magnitudes of the transition rates, $k_{a \rightarrow b}$ in s^{-1} were estimated as observed in the raw traces. HMM calculations were performed using QuB¹⁶, an established online modeling tool.

In the absence of divalent cations or ligand, the NAD^+ -II riboswitch rapidly samples several conformational states, observable in the vast majority of traces where transitions occur. A collection of smFRET traces is presented in Fig. 5.1 to demonstrate 1) the number of potential FRET states sampled by single molecules, 2) that determining the actual number of states and their transition rates by eye can be challenging, and 3) that rapid sampling of different conformations is common for apo form NAD^+ -II.

The histogram for this experimental condition shows two broad features centered around $E_{\text{FRET}} = 0.4$ and 0.8 , referred to as mid-FRET (MF) and high-FRET (HF) states, respectively (Fig. 4.2). Transitions between the MF and HF

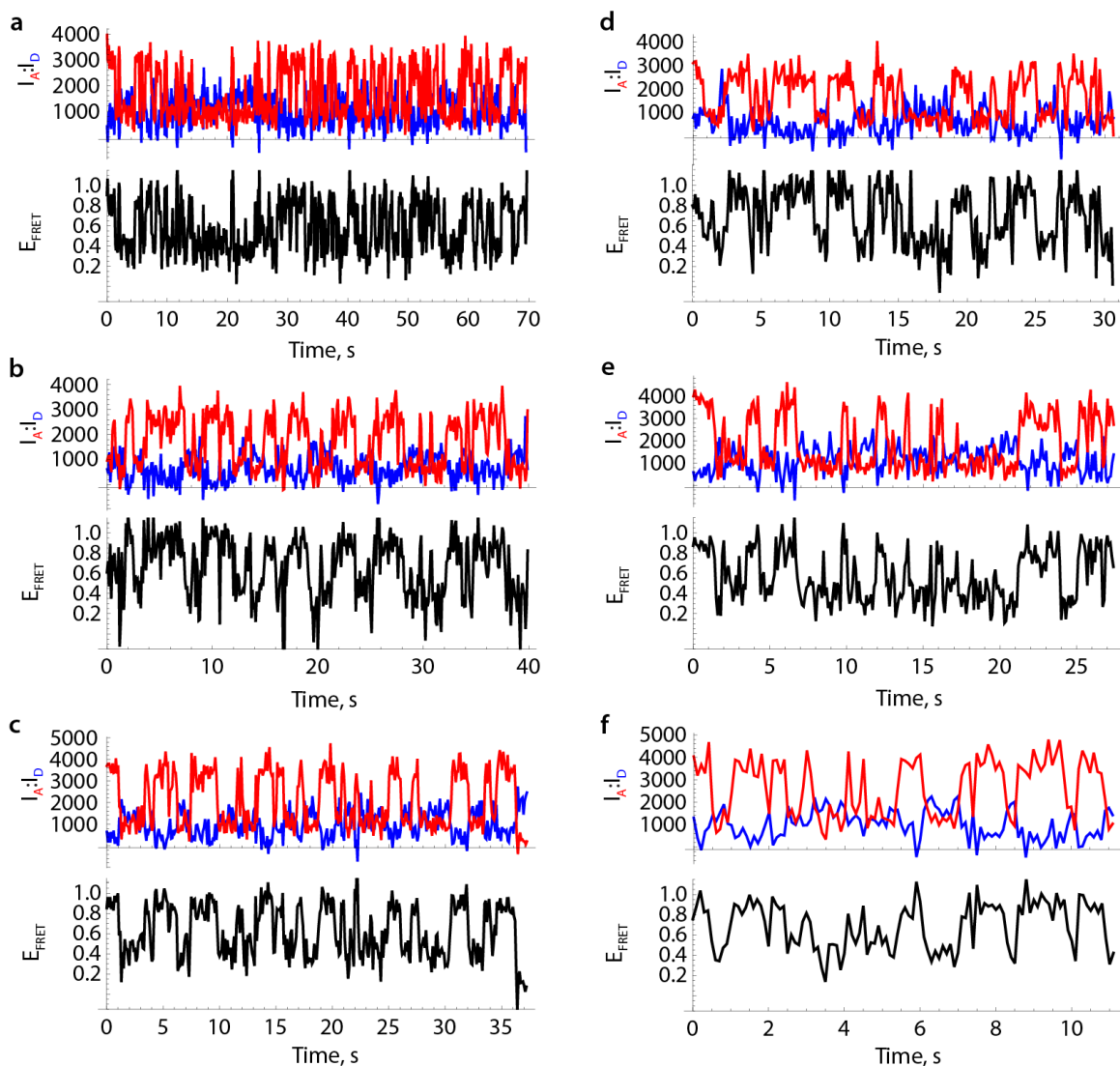


Figure 5.1: **Selected smFRET traces of NAD⁺-II riboswitch.** Each panel portrays the dynamics of a different molecule. Transitions occur so rapidly that determining the number of FRET states sampled by observation is challenging. Each trace provides a robust example of FRET donor-acceptor intensity anti-correlation on a different time scale. Traces are presented in order of decreasing duration, starting at 70 s in panel **a** to 11 s in panel **f**, demonstrating that rapid transitions can be identified by inspection.

states occur most frequently as indicated by the two brightest spots in the TODP (Fig. 5.2a). Several other transitions are observed with high prevalence, including frequent sampling of LF states. Almost all of the possible transition pathways are accessed to some degree, with the least likely transition occurring between the highest- and lowest- FRET states.

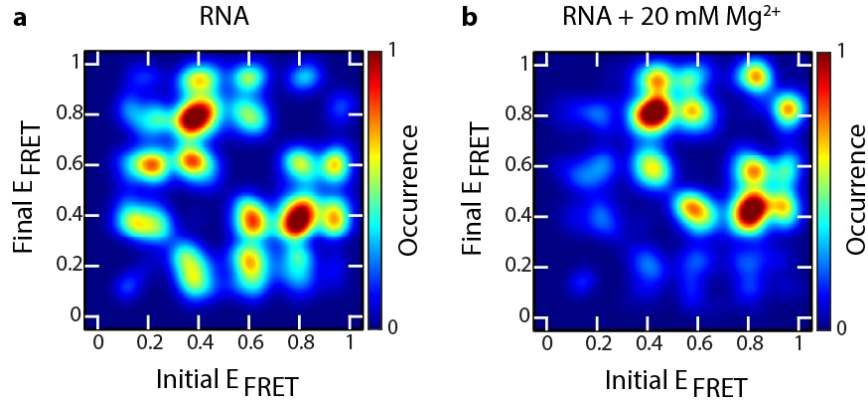


Figure 5.2: Transition occupancy density plots of NAD⁺-II riboswitch in the a) absence and b) presence of 20 mM Mg²⁺ generated using a 5-state Hidden Markov Model with $E_{\text{FRET}} = 0.2, 0.4, 0.6, 0.8$, and 0.9 .

Upon addition of 20 mM Mg²⁺, the RNA rarely samples conformations with $E_{\text{FRET}} < 0.4$ (Fig. 5.2b). The transitions between $E_{\text{FRET}} = 0.8$ or 0.6 and 0.2 are dramatically reduced. There is also an increase in sampling of transitions between the highest FRET states, with $E_{\text{FRET}} \sim 0.95$ and 0.8 , although the most frequent transition sampled remains between the MF and HF states. Aside from a small number that occupied the LF state statically, almost no traces were observed where RNA adopted a particular conformation without transitioning to another for the duration of data collection, up to ~ 1 minute.

Given that the number of states with well-resolved transitions is suppressed with 20 mM Mg²⁺ and collapses with 1 mM β -NMN (Fig. 5.4), I elected to model

RNA behavior with three FRET states with E_{LF} , E_{MF} , and E_{HF} of 0.15, 0.45, and 0.90, respectively. Details lost due to the reduction from the 5-state to the 3-state model for RNA $\pm$ Mg²⁺ in the absence of ligand include any transitions to/from $E_{FRET} = 0.6$. There is also a loss in the distinction between the $E_{FRET} = 0.8$ and $E_{FRET} = 0.9$ states.

3 β -NMN concentration series reveals increasing shift towards predominant HF state

As demonstrated in Chapter IV, the riboswitch adopts the HF state in the presence of 20 mM Mg²⁺ and 1 mM β -NMN with a 2:1 ligand:RNA binding stoichiometry. To determine whether cooperativity between the two binding sites could be observed in histograms or TODPs, I tested the riboswitch with a range of concentrations of β -NMN between 10 and 1000 μ M. Experimentally, smFRET data were first collected on the RNA alone, then with 20 mM Mg²⁺. Next, the first aliquot of 20 mM Mg²⁺ + β -NMN was introduced. For subsequent iterations of β -NMN, the sample chamber was flushed thoroughly with imaging buffer containing 20 mM Mg²⁺ between introducing the various concentrations of β -NMN. Ligand concentrations were introduced in a random (not increasing) order to reduce bias. After all concentrations were tested, RNA with 20 mM Mg²⁺ was tested again to show that there were no residual effects of introducing ligand throughout the experiment and the efficacy of flushing the sample chamber between tests on the same RNA (Fig. 5.3).

Fitting the histograms with the sum of three Gaussians given the above inputs (where $E_{HF} = 0.15, 0.45$, and 0.90) reveals a shift in the MF state from $E_{MF} = 0.37$ in the absence of Mg²⁺ to 0.42 ± 0.03 for all other conditions (mean

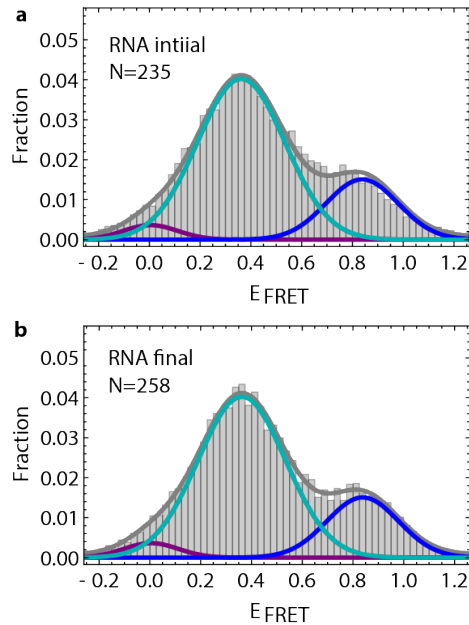


Figure 5.3: **Histograms comparing RNA behavior pre- and post-experiment.** Between the RNA initial and final movies of RNA were collected, several ligands were tested including β -NMN, NAD^+ , and $\epsilon\text{-NAD}^+$. The two histograms being identical support the efficacy of the flushing method described above sufficiently purged the sample chamber and that RNA behavior is reversible after interacting with all ligands.

$\pm$ standard deviation, σ , across histograms recorded in the presence of 20 mM Mg^{2+} at six ligand concentrations from 0 to 1000 μM). The HF state also shifts higher, albeit more gradually. For RNA without Mg^{2+} , the HF state sampled was $E_{\text{HF}} = 0.79$. With 20 mM Mg^{2+} , $E_{\text{HF}} = 0.85$. Adding 10 μM β -NMN results in a further shift to $E_{\text{HF}} = 0.89$. For the remaining concentration series, $E_{\text{HF}} = 0.93 \pm 0.02$ (mean $\pm \sigma$ of four histograms). These results suggest that NAD^+ -II does not significantly alter its tertiary structure to bind ligand, rather that the pseudoknot architecture it prefers becomes stabilized by Mg^{2+} and more compact when ligand-bound. In other words, in the presence of external factors such as Mg^{2+} and ligand, the riboswitch demonstrates a strong preference to only a subset of the states it can access when unbound.

The following figure panel shows the collection of histograms and TODPs for the β -NMN concentration series (Fig. 5.4) with the 3-Gaussian fits overlain. All samples contained 20 mM Mg^{2+} . The RNA + 20 mM Mg^{2+} histogram (reproduced from Fig. 4.2b) and TODP demonstrate that there is no significant population of RNA occupying the HF state statically. Sampling of the HF $\leftrightarrow$ MF transition dominated all other transitions observed. Compared with the histograms of RNA only (Fig. 5.3), sampling of the MF state is reduced in favor of sampling the HF state.

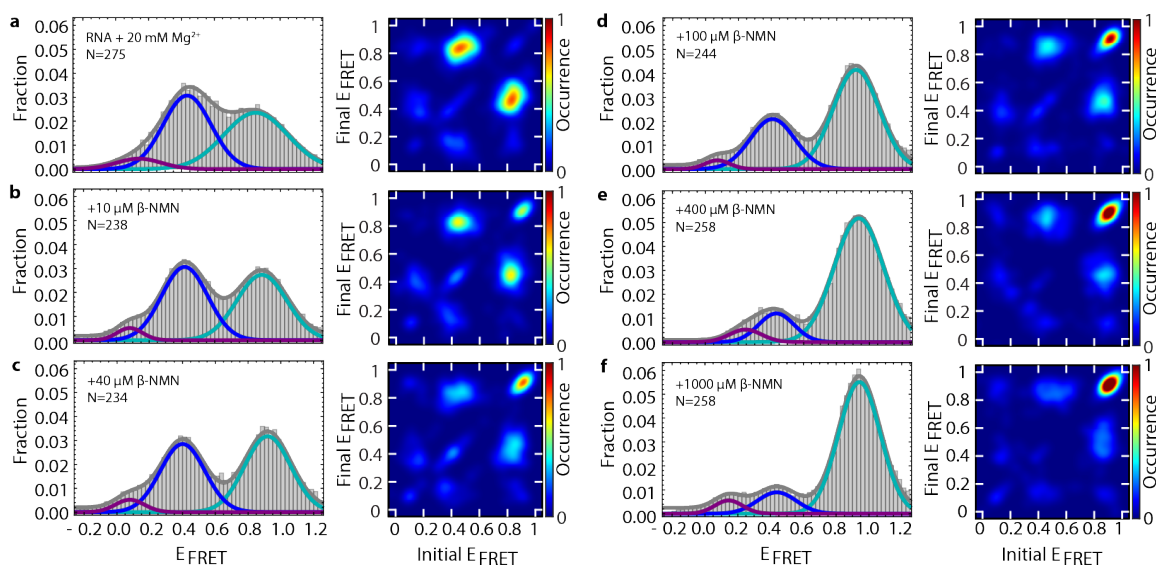


Figure 5.4: **smFRET histograms of NAD^+ -II in imaging buffer.** All data are of RNA with 20 mM MgCl_2 , but with different concentrations of β -NMN. **a)** 0 μM β -NMN, **b)** 10 μM β -NMN, **c)** 40 μM β -NMN, **d)** 100 μM β -NMN, **e)** 400 μM β -NMN, **f)** 1000 μM β -NMN. “N” indicates the number of single-molecule traces that were used to compile the histograms (left columns). TODPs (right columns) generated using a 3-state Hidden Markov Model. Prominence of the high-FRET peak along the diagonal increases with increasing concentration of β -NMN, even at concentrations higher than the reported $K_D^{12,23?}$.

After introducing 10 μM β -NMN (Fig. 5.4b), more of the population begins to sample the HF state without transitioning (statically) for the duration of the experiment, ~ 1 minute. There is now close to a 1:1 ratio of the peak heights for the MF to HF states. Sampling of the HF $\leftrightarrow$ MF transitions is reduced in favor of

sampling the HF state statically. As the concentrations of β -NMN is increased (Fig. 5.4c-f), the prevalence of the HF to MF state continues to increase to the final concentration of 1 mM with the majority of RNA adopting the HF state statically. A graph of the peak heights for LF, MF, and HF states (Fig. 5.5) demonstrates the trend of increasing HF population, decreasing MF population, and low, unchanging LF population.

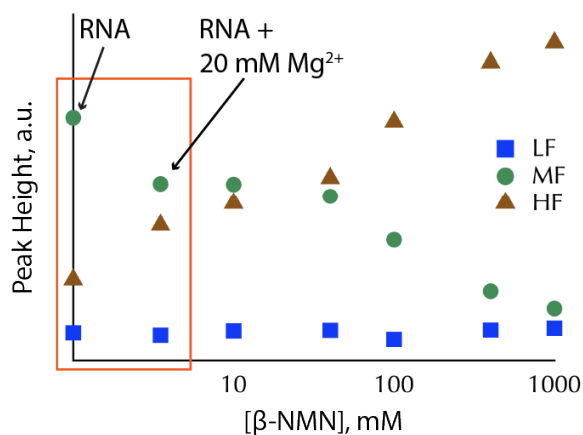


Figure 5.5: **Graphical representation of FRET state peak heights.** RNA only (far left) and RNA + 20 mM Mg^{2+} (second from left) with increasing concentration of β -NMN over the concentration range depicted in Fig. 5.4 and plotted on a logarithmic scale.

With respect to whether the results support noncooperativity of the two binding sites as observed by the bulk FRET measurements presented in Chapter IV, these data neither support nor refute that conclusion. There was no glaringly obvious difference in the trend of increasing HF and decreasing MF maxima with concentration of ligand, although the rate at which the change occurs does appear slower between 10 and 40 μM β -NMN and faster at higher concentrations. However, the lack of observable difference in the histograms for the initial and final RNA conditions (Fig. 5.3), demonstrates the reversibility of the conformations the RNA adopts before binding of β -NMN and after dissociation.

4 Adenosine Moiety of NAD^+ Affects Ligand Recognition

Much focus has been placed on how NAD^+ binds the riboswitch primarily via the nicotinamide moiety, yet there is precedent to consider the potential impact of the other half of the dinucleotide ligand. Studies have shown that the Class I NAD^+ riboswitch specifically senses adenine and its derivatives.³¹ However, the crystal structure of NAD^+ -II (PDB 8HBA) demonstrating two NAD^+ binding sites shows the nicotinamide and adenosine moieties both interacting with RNA bases at site 2.¹³ Specifically, adenine demonstrates hydrogen bonding with A24 and base-stacking with C36 at the base of the P1a helix (Fig. 5.7). We sought to determine how significantly adenine affects riboswitch structure by testing both NAD^+ and a ligand candidate with a modified adenine, nicotinamide 1,N(6)-ethenoadenine dinucleotide (Fig 5.7b).

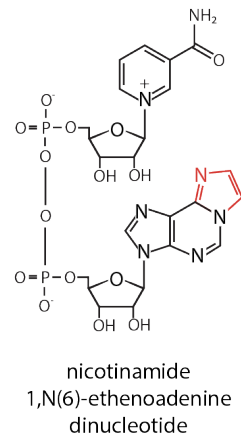


Figure 5.6: **2D Chemical structure of $\epsilon\text{-NAD}^+$**

I tested the behavior of the riboswitch in the presence of NAD^+ and $\epsilon\text{-NAD}^+$ using smFRET. Histograms were fitted with the same 3-state model as with $\beta\text{-NMN}$. Dwell times were calculated for the transitions between $E_{\text{FRET}} = 0.4$ and 0.8. While the static HF feature is still present in the TODP, the histogram for the riboswitch in the presence of 1 mM NAD^+ indicates a sustained dynamic sampling of the MF state not observed with $\beta\text{-NMN}$, which almost exclusively adopted the pseudoknot structure. Dwell times for the $\text{HF} \leftrightarrow \text{MF}$ transition are on the order of 1 s. As evidenced by the similarities in the histograms and TODPs for both NAD^+ and $\epsilon\text{-NAD}^+$, this modification to adenine does not appear to impact ligand recognition any more than an unmodified adenine.

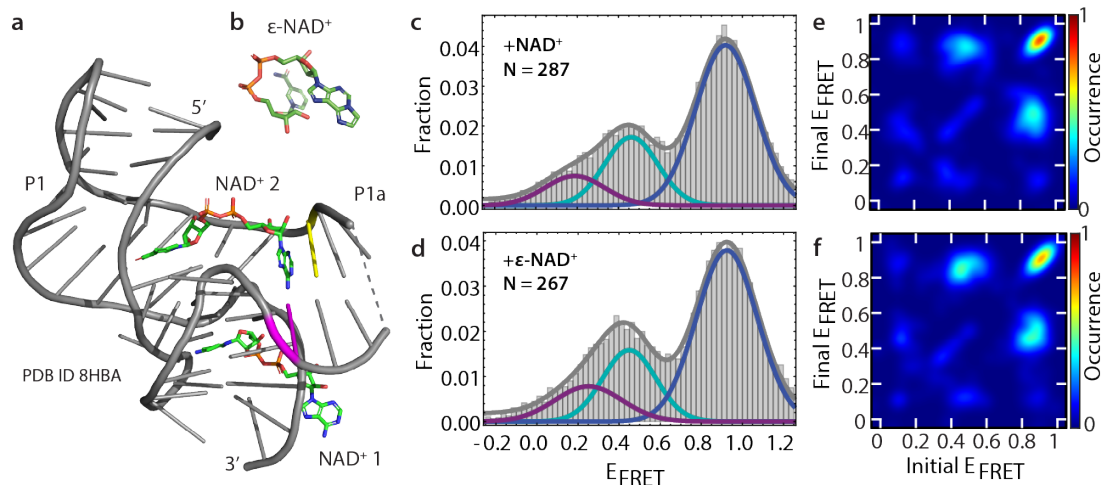


Figure 5.7: **Materials and results of NAD⁺ and ε-NAD⁺.** **a)** Crystal structure of the riboswitch with NAD⁺ (PDB ID 8HBA) and **b)** chemical structure of ε-NAD⁺. Only the nicotinamide moiety interacts with the RNA at site 1, but the adenosine moiety of NAD⁺ at site 2 appears to be intercalating into the core-end of the P1a helix via non-canonical hydrogen bonding with A24 (magenta) and base stacking under C36 (yellow) within the P1a loop. While the presence of an adenosine moiety clearly alters the behavior of the riboswitch, it does not distinguish between NAD⁺ **c)** and ε-NAD⁺ **d)**. Differences in the TODPs for the NAD⁺ **e)** and ε-NAD⁺ **f)** are subtle.

These results reveal behavior indicative of a riboswitch that is a good candidate for drug targeting in that a drug could modify one part of a ligand without interfering with the ability of the riboswitch to sense it. Inhibition of a riboswitch responsible for critical protein production in a pathogenic bacterial cell could shut down such replication and halt bacterial propagation.

5 Future Directions

The investigation described herein for the NAD⁺-II riboswitch could be easily adapted to test the ligand-binding dynamics of another riboswitch that is a potential candidate as an antibacterial target. Pursuit of this type of investigation could lead to collaboration with computational chemists to determine a series of

potential riboswitch candidates as well as synthetic chemists to fabricate the drug candidates themselves.

CHAPTER VI

A PSEUDOURIDINE SYNTHASE SHAPES tRNA STRUCTURAL DYNAMICS THROUGH BOTH CATALYSIS AND REMODELING

1 Prologue

This section includes unpublished, co-authored material. A copy of the manuscript as submitted can be found on BioRxiv. I, co-first author, designed the smFRET and BLI experiments and processed smFRET data, including Gaussian fits of histograms, developing the HMM model, and generating TODPs. Emily Dennis, co-first author, prepared/purified proteins and collected/analyzed BLI data. Abigail Vaaler designed the tRNA FRET constructs and performed their ligations. Maddison Kadrmas collected and analyzed smFRET data. Margaret Barry performed the tRNA sequencing analysis. This manuscript was produced with the direction and writing contributions of Julia Widom and David Garcia.

2 Abstract

Transfer RNA has long served as an exemplar of a thermodynamically stable, structured RNA. Yet it undergoes significant structural changes upon binding and catalysis by diverse modification enzymes. We leveraged optical binding assays and single-molecule FRET to observe tRNA structural dynamics before and upon interaction with the conserved pseudouridine synthase Pus4/TruB. We show that unmodified and pseudouridylated tRNA similarly sample one open and two closed conformations, dynamically. Binding by Pus4 to unmodified tRNA populates additional conformational states, gradually approaching an ensemble

that is adopted sooner by tRNA that was pseudouridylated prior to engaging Pus4. A catalytically incompetent mutant of Pus4 binds more slowly and remodels unmodified and pre-modified tRNAs into different conformational ensembles than wild-type enzyme. Thus, Pus4 both catalyzes a lasting chemical change on tRNA and remodels it over time, perhaps to advance subsequent steps of tRNA maturation.

3 Introduction

Pseudouridine (Ψ) is the most abundant RNA chemical modification in nature. Position 55 in the T-loop is pseudouridylated in nearly all tRNAs, which is catalyzed by Pus4/TruB/TRUB1+TRUB2 (yeast/bacteria/human). This change from U₅₅ to Ψ_{55} provides an additional hydrogen bond donor, increases base-stacking and helix rigidity, and promotes tertiary interactions between the T-arm and D-arm in the “elbow” region of a folded tRNA, contributing to the characteristic three-dimensional structure of tRNA^{32–36}. In the budding yeast *Saccharomyces cerevisiae*, Pus4 (Fig. 6.1a) pseudouridylates 41 out of 42 cytosolic tRNA isoacceptors³⁷, and 23 out of 24 mitochondrial tRNA isoacceptors (Julia Reinsch, unpublished), and it does so with a high conversion efficiency of U to Ψ . In *E. coli*, all tRNAs are known to be pseudouridylated by TruB³⁸. Therefore, the majority of tRNA molecules examined thus far are bound and modified by Pus4/TruB during at least one point of their biogenesis/maturation, implicating this enzyme as a benchmark for understanding how tRNA structure and dynamics are affected by an enzyme that chemically modifies it.

Previous studies have identified various RNA sequences and/or structures important for binding and modification by various pseudouridine synthase

enzymes^{39–55}, including Pus4/TruB^{56–58}. Yet binding by these synthase enzymes can remodel RNAs in ways distinct from the lasting chemical change that they catalyze. An early study on bacterial TruB noted that growth phenotypes associated with its absence could not be explained by its catalytic activity alone⁵⁹. Subsequently, a second role was more clearly established: that it aids tRNA folding and functions as a tRNA chaperone⁶⁰. Other studies have also demonstrated RNA-chaperone-like roles for other tRNA modifying enzymes^{61–64}. Thus, to exert their full function, tRNA modifying enzymes should, 1) bind tRNA, and 2) catalyze chemical modification of tRNA; while some may also, 3) remodel tRNA structure. These three components could each independently affect the dynamics of tRNA structure, or do so interdependently. We sought to clarify these models by addressing the following questions: 1) Are there differences between the structural ensembles of unmodified tRNA and pseudouridylated tRNA? 2) How does binding by Pus4 affect the distribution of tRNA structures over time? 3) Does the outcome depend on whether the tRNA is modified prior to engagement, modified by Pus4, or not modified at all?

To address these questions, we leveraged single-molecule Förster resonance energy transfer (smFRET) to track the conformational fluctuations of tRNA and biolayer interferometry (BLI) to monitor association of Pus4 to tRNA (Fig. 6.1c). We show that Pus4 binds rapidly to both unmodified (U_{55}) and pseudouridylated (Ψ_{55} , hereafter also referred to as “pre-modified”) tRNAs, and subsequently navigates unmodified tRNA through a pathway of catalysis and remodeling lasting tens of minutes, that is bypassed by pre-modified tRNA. This pathway involves a temporary population of conformations not prevalent for either U_{55} or Ψ_{55} tRNA in the absence of enzyme. We also show that a mutant with impaired binding

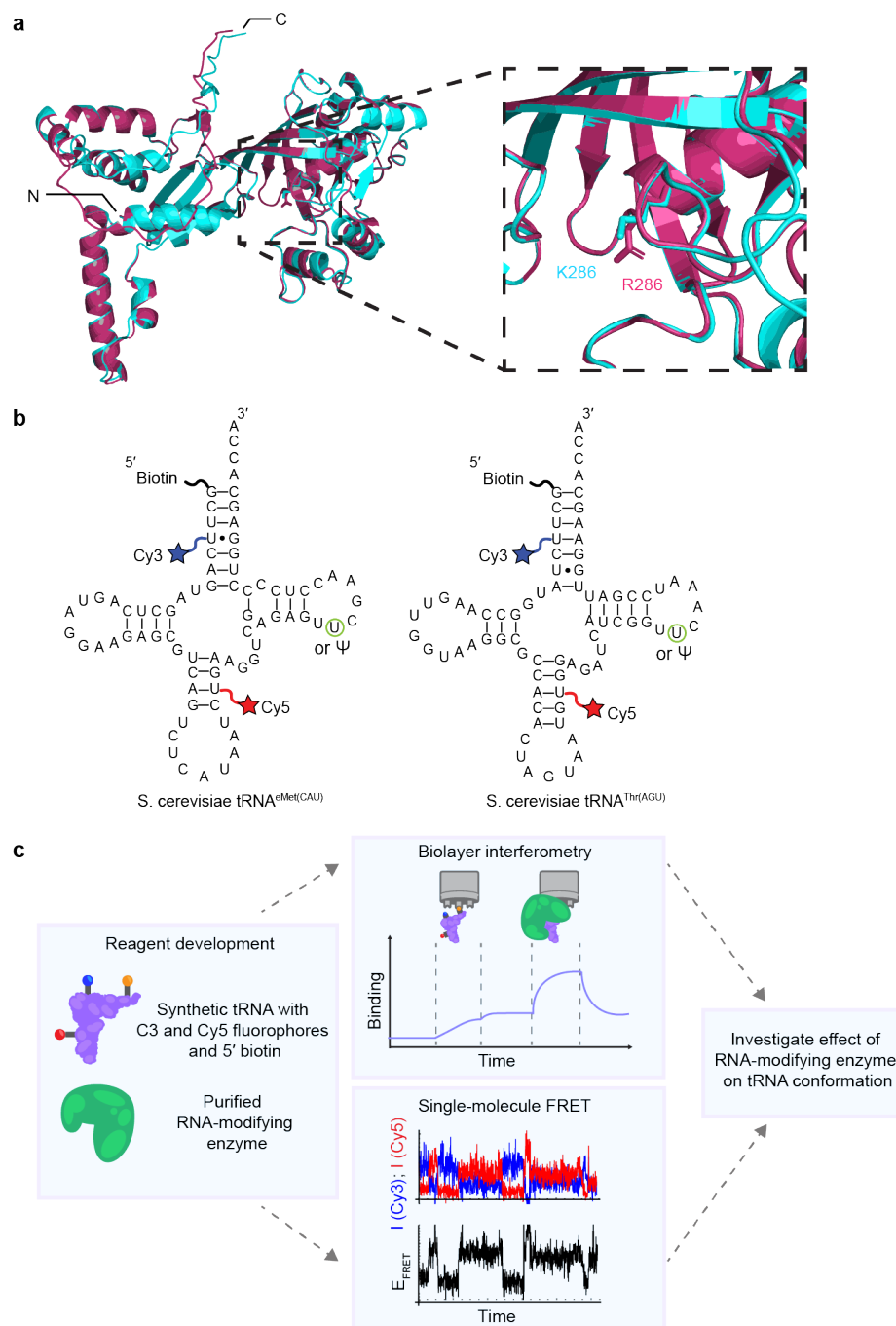


Figure 6.1: Predicted Pus4 structure, tRNA sequences, and overall study approach.
a, Structures of Pus4 WT (magenta) and Pus4 R286K (cyan) predicted with AlphaFold 3 and aligned using PyMol, with inset showing residue 286 in the active site. **b**, Predicted secondary structures of the tRNA sequences used in this study, including the positions of biotin, Cy3, Cy5 and pseudouridine modifications. **c**, Summary of experimental approach and workflow undertaken in this study.

and catalytic activity (Pus4 R286K) still remodels U₅₅ and Ψ_{55} tRNAs, but into different conformational ensembles than those promoted by Pus4 wild-type (WT). Thus our results show that a prototypical RNA-modifying enzyme shapes the chronology of tRNA structural dynamics through both its catalysis and remodeling.

4 Results

4.1 Exogenously purified Pus4 pseudouridylates yeast tRNAs *in vitro*

After establishing a protocol to exogenously express and purify Pus4 protein (Supp. Fig. 6.8a), we tested its catalytic activity *in vitro* (Supp. Fig. 6.9). Using direct RNA sequencing (DRS), a method we previously established to investigate this modification *in vivo*³⁷, Ψ_{55} was newly detected on nearly all tRNAs isolated from a *pus4* Δ yeast strain (Supp. Table 1) following incubation with an excess of purified Pus4 (Supp. Fig. 6.9a). Modification levels were qualitatively comparable to those previously observed in tRNA isolated from WT strains³⁷. While some differences in pseudouridine levels were observed between the *in vivo* and *in vitro* data—most notably a lack of modification of tRNAs Arg^{ACG} and Leu^{CAA} *in vitro*—the data supported near-full catalytic activity of purified Pus4 on these cell-derived yeast tRNAs.

We previously identified a single point mutation to Pus4, converting arginine to lysine at position 286 (R286K; AlphaFold 3 structure prediction⁶⁵ in Fig. 6.1a), that impairs its pseudouridylation activity *in vivo*³⁷, and was based on a homologous mutant of TruB that also impairs catalysis without reducing tRNA binding *in vitro*⁶⁶. Compared to Pus4 WT, incubation of R286K with yeast tRNAs *in vitro* produced almost no modification signal at U₅₅ across isoacceptors (Supp. Fig. 6.9a). Low levels of modification were observed on certain isoacceptors,

however, the overall pattern resembled that of untreated tRNA isolated from *pus4* Δ strains. Thus Pus4 R286K is catalytically impaired *in vitro*.

4.2 Synthetic modified tRNAs enable binding and single molecule FRET studies

Having established recombinant Pus4 WT and R286K with desired enzymatic activities, we prepared synthetic tRNA for our biophysical studies of RNA binding by Pus4 (using BLI) and tRNA conformational fluctuations (using smFRET). We prepared synthetic tRNA^{eMet(CAU)} and tRNA^{Thr(AGU)} containing either U or Ψ at position 55, with modifications required for BLI and smFRET (Fig. 6.1b). Specifically, the 5 ends were biotinylated for surface immobilization on a BLI probe/microscope slide, the FRET donor fluorophore Cy3 was placed in the acceptor stem, and the acceptor fluorophore Cy5 was placed in the anticodon stem, based on a previous design for human mt-tRNA^{Lys} (ref)^{67–70}.

4.3 Wild-type Pus4 binds rapidly to tRNA

We utilized BLI to assess the binding specificity of Pus4 and the timescales of its association to and dissociation from both substrate and non-substrate RNAs. RNAs were immobilized on a streptavidin-coated probe that was then passivated to prevent non-specific adsorption of protein, exposed to buffers containing Pus4 protein (the “association phase”), and finally exposed to buffer lacking protein (the “dissociation phase”) (Fig. 6.2a, Supp. Fig. 6.10a). A BLI “shift” is observed when material accumulates on the probe, such as protein binding to immobilized tRNA during the association phase. At a concentration of 200 nM protein, the association curves show rapid binding of Pus4 to tRNA^{eMet(CAU)} and tRNA^{Thr(AGU)}, with the accumulation rate slowing after ~ 100 seconds (Fig. 6.2b-d; replicate data in Supp. Fig. 6.11a-c). This is followed by a period of slow continued accumulation of

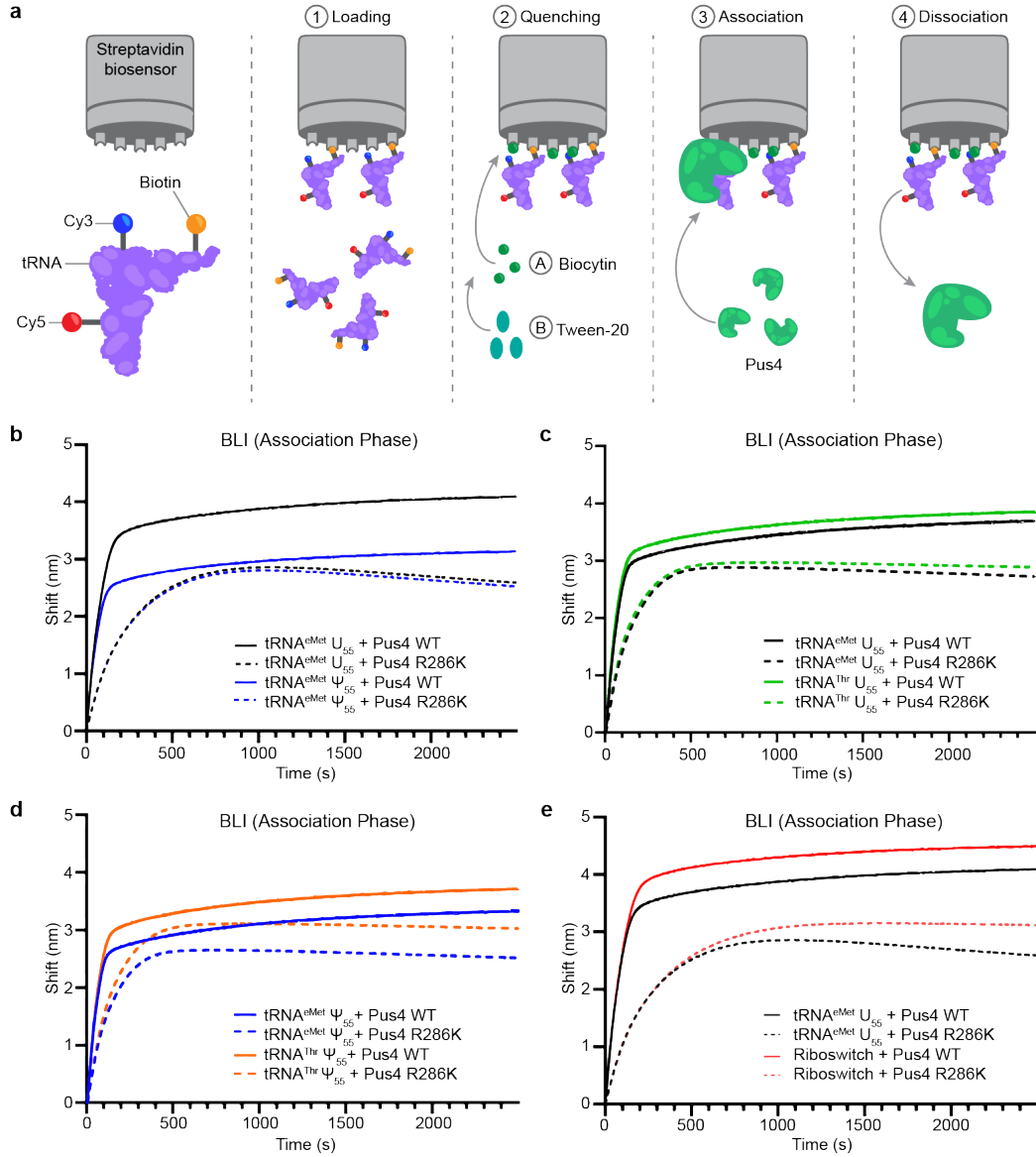


Figure 6.2: Association of Pus4 to tRNA using biolayer interferometry.

a, Summary of BLI experimental design. The synthetic tRNAs used contain Cy3, Cy5, biotin and pseudouridine modifications. The Cy3 and Cy5 are not needed for BLI measurements, but were used here to keep the data comparable with smFRET measurements. These tRNAs are immobilized onto streptavidin probes (1) that then proceed through two quenching steps and a baselining (2). The probe is then transferred into buffer with purified Pus4, where association of Pus4 to the immobilized tRNAs can occur (3). The final step transfers the probe to a buffer without protein, so that dissociation of the protein from the tRNA can be measured (4). **b**, BLI traces showing the association phase of 200 nM wild-type Pus4 (solid) and R286K Pus4 (dotted) to both 60 nM unmodified (black) and pre-modified tRNA^{eMet(CAU)} (blue). Traces are aligned to start at zero. **c**, Same as **b**, but with unmodified tRNA^{eMet(CAU)} (black) and tRNA^{Thr(AGU)} (green). **d**, Same as **b**, but with pre-modified tRNA^{eMet(CAU)} (blue) and tRNA^{Thr(AGU)} (orange). Data collected in the same experiment as **c**. **e**, Same as **b**, but with unmodified tRNA^{eMet(CAU)} (black) and NAD⁺ riboswitch (red). tRNA^{eMet(CAU)} data are the same as is shown in panel **b**.

Pus4 onto tRNAs, observed at all protein concentrations tested. While this slowed accumulation plateaued after ~ 15 minutes when Pus4 was present at our highest tested concentration (800 nM), it failed to plateau even after 45 minutes for Pus4 at 50 or 200 nM concentrations (Fig. 6.3a,b). The BLI shift at 200 nM Pus4 eventually converged with the data for 800 nM, suggesting that complete binding occupancy was achieved in this concentration range. We confirmed this using non-denaturing gel electrophoresis as an orthogonal tRNA binding assay, which showed that in free solution, all tRNA^{eMet(CAU)} molecules were bound after 30-minute incubation with Pus4 at concentrations of 200 nM or higher (Supp. Fig. 6.12). Combined with the prior observation that both tRNA^{eMet(CAU)} and tRNA^{Thr(AUG)} were robustly modified within two minutes during the *in vitro* modification experiment (in which Pus4 was present at 1 μ M) (Supp. Fig. 6.9), we presumed that the majority of the immobilized tRNAs were pseudouridylated in the early period of this association phase once engaged with Pus4. The slow, steadily increasing second phase of the association curve may represent 1) gradual “trapping” of tRNA or Pus4 molecules that began the association phase in conformations not amenable to binding, or 2) a modest level of Pus4 multimerization such that the stoichiometry of Pus4:tRNA exceeded 1:1 on some of the immobilized tRNA molecules (the latter is supported by the shift of Pus4:tRNA bands to the wells for the highest Pus4 concentrations in Supp. Fig. 6.12).

We analyzed the association rates quantitatively by modeling the initial binding of Pus4 as a reversible process occurring under pseudo-first order conditions (details in Methods). Bi-exponential fits of the complete association curves and mono-exponential fits of the rapid phase (Supp. Fig. 6.13) both revealed that the on-rate (k_{on}) was slightly faster for tRNA^{Thr(AUG)} (1.36×10^5

$\text{M}^{-1}\text{s}^{-1}$ from the bi-exponential fit) than for $\text{tRNA}^{\text{eMet(CAU)}}$ ($1.00 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$) (Fig. 6.3c,d). These on-rates are comparable to ones previously observed for yeast Pus1 binding to tRNA^{Phe} and tRNA^{Val} ($4 - 6 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$)⁷¹. These fits could in principle have been used to obtain k_{off} , however the off-rate during the initial phase of Pus4 accumulation was indistinguishable from zero. When the BLI probe was transferred into a solution lacking protein, an initial fast phase of dissociation was observed, but even after 45 minutes, less than 10% of the shift observed in the association phase was reversed (Supp. Fig. 6.10b-d). This suggests that Pus4 WT binds all RNAs investigated with high affinity and dissociates very slowly.

4.4 Pus4 also binds robustly to pre-modified tRNA and non-substrate RNA

To test whether Ψ_{55} altered the binding of Pus4 to tRNA, we tested synthetic tRNAs that had pseudouridine present at position 55 using BLI. Although the BLI curve of $\Psi_{55} \text{tRNA}^{\text{eMet(CAU)}}$ exhibited a smaller maximum shift than that of U_{55} (Fig. 6.3a), non-denaturing gel electrophoresis indicated that this still corresponded to complete binding occupancy at Pus4 concentrations at or above 200 nM (Supp. Fig. 6.12). Furthermore, the overall association and dissociation profile was similar between the unmodified and pre-modified variants of both tRNAs, and k_{on} values were unchanged or higher with Ψ_{55} tRNA ($1.12 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ for $\Psi_{55} \text{tRNA}^{\text{eMet(CAU)}}$ and $1.36 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ for $\Psi_{55} \text{tRNA}^{\text{Thr(AUG)}}$), indicating weak discrimination between binding substrate and product. It is worth noting that previous studies have shown pseudouridine synthases can be promiscuous binders of RNA^{39,41-43}, consistent with their roles of modifying multiple sequences and structures in various classes and sizes of RNA. For example, Pus1 has been found to have broad affinity for RNA, even those lacking defining tRNA structural features, as well as tight binding to catalytic products (e.g., K_D

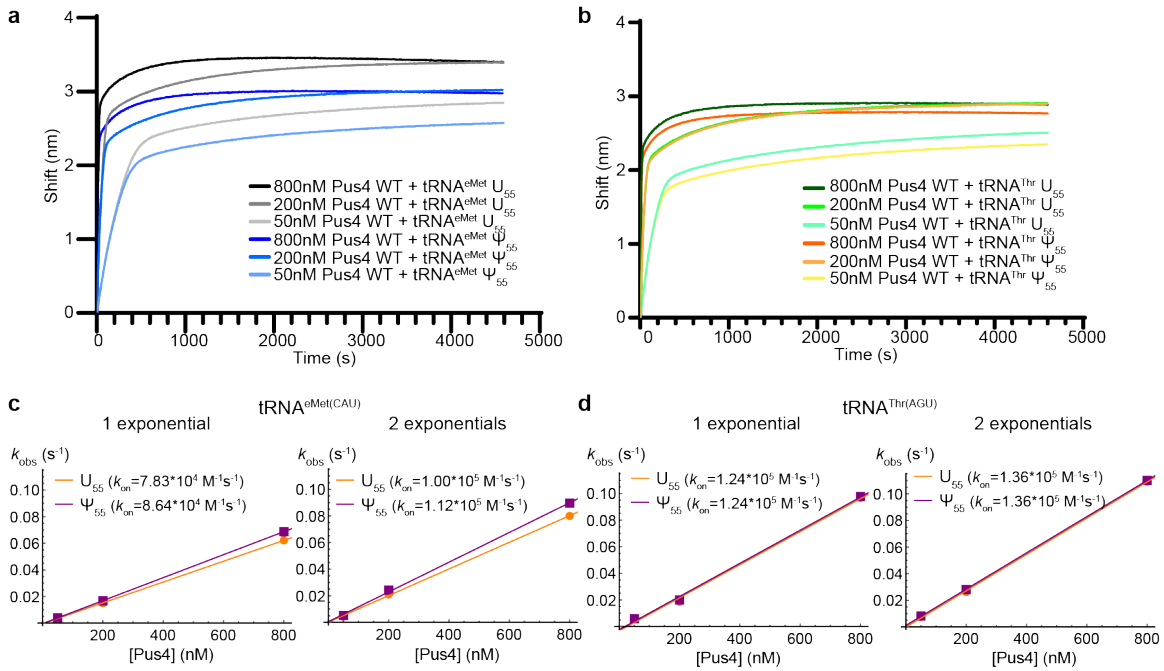


Figure 6.3: Pus4 binds tRNAs with a high k_{on} , but a low k_{off} , with similar affinities for unmodified and pre-modified tRNAs.

a, BLI traces showing the association phase of 50 nM, 200 nM, and 800 nM Pus4 WT to U₅₅ (black) and Ψ₅₅ (blue) tRNA^{eMet(CAU)}. **b**, BLI traces showing the association phase of 50 nM, 200 nM, and 800 nM Pus4 WT to U₅₅ (green) and Ψ₅₅ (orange) tRNA^{Thr(AGU)}. **c,d**, Linear fits to the observed association rate at three Pus4 concentrations. Left columns: k_{obs} determined from a single exponential fit to the time range of the association phase during which ~75% of the BLI shift occurs. Right columns: k_{obs} determined from a double exponential fit to the entire association phase. **c**, U₅₅ (orange) and Ψ₅₅ (purple) tRNA^{eMet(CAU)}. **d**, U₅₅ (orange) and Ψ₅₅ (purple) tRNA^{Thr(AGU)}.

= 14 nM for pseudouridylated tRNA^{Val})⁷¹. In addition to tRNA, Pus4 modifies mRNA^{51,72,73} and rRNA⁷⁴, *in vivo*. To better contextualize our BLI results, we also performed experiments with an RNA that is not a natural substrate of Pus4 but is similarly sized to tRNA: the bacterial class II NAD⁺ riboswitch, a 61-nt RNA that adopts a pseudoknot structural motif to regulate translation initiation^{23,75}. As we observed for tRNA, Pus4 rapidly associated with the riboswitch, followed by slow continued accumulation and slow dissociation (Fig. 6.2e, Supp. Fig. 6.10e). We note the possibility that *in vivo*, re-binding of tRNA by Pus4 may be repressed by m¹A₅₈, that depends on Ψ₅₅ (ref)^{37,76}. *In vivo*, most tRNA modifications are

capable of being added to tRNAs lacking Ψ_{55} (ref)³⁷; however, our *in vitro* data also demonstrated that these modifications did not generally inhibit exogenous Pus4's ability to pseudouridylate them (Supp. Fig. 6.9).

4.5 A catalytically-inactive mutant of Pus4 has impaired binding to tRNA

To determine how Pus4's catalytic function influences its binding to tRNA, we performed BLI experiments with Pus4 R286K. This mutant showed a reduced rate of initial association to tRNA as well as a lower maximum occupancy (Fig. 6.2b-d). Pus4 R286K had impaired binding to the riboswitch as well, suggesting broad impairment of its RNA binding capability (Fig. 6.2e). After around 800 seconds (~ 13 minutes) of the association phase at 200 nM Pus4, the catalytically inactive mutant began a slow but steady dissociation from substrate (Fig. 6.2b). This behavior was identical for unmodified and pre-modified tRNA sequences of both isoacceptors, though it was not observed for the riboswitch (Fig. 6.2b-e). We ruled out RNase contamination as a possible explanation for the decreasing association signal in these samples (Supp. Fig. 6.8b). It is possible this decrease in signal results from a population of tRNA molecules that acquire a structure bound more weakly by this mutant, although this is difficult to disentangle in a bulk measurement. Together, these data suggest that the active site residue R286 is required for catalytic activity and rapid association with tRNA, as well as promoting continued engagement or re-engagement with tRNA molecules.

4.6 tRNA undergoes dynamic fluctuations between three predominant conformational states

We utilized smFRET to investigate how tRNA conformations are affected by Pus4 binding and pseudouridylation. The biotinylated- and fluorophore-labeled

tRNA^{eMet(CAU)} constructs used for BLI were immobilized on microscope slides, and fluorescence from single tRNA molecules was recorded using prism-based total internal reflection fluorescence (TIRF) microscopy. We recorded short movies (~ 2 minutes) sequentially across different regions of the slide of tRNA alone, as well as tRNA during up to 30 minutes of incubation with 200 nM Pus4 protein. Cy3 and Cy5 fluorescence intensity traces were extracted from individual molecules, and data from at least two separate experiments were combined to achieve 200 or more individual molecules for each condition. In order to observe evolution of the conformational landscape over tens of minutes, for samples incubated with protein, the data were combined into discrete time bins of 2–6 minutes, 7–9 minutes, 10–15 minutes and 30–34 minutes after introduction of Pus4 (see Methods for an explanation of bins).

In the absence of protein, both U₅₅ and Ψ_{55} tRNAs dynamically sample several different conformational states. Three are visible upon inspection of individual traces (Fig. ??a,b) and in histograms compiled from the first 50 frames of $N \geq 200$ traces (Fig. 6.4c,d). A low-FRET efficiency feature (LF)—representing a conformation in which the fluorophores are most separated from each other relative to other states—is centered around $E_{\text{FRET}} = 0.30$ for U₅₅, and 0.22 for Ψ_{55} tRNA. An LF feature could arise from molecules that are either less structured or that adopt a structure such as an extended helix that enforces separation of the fluorophores. Two high-FRET features (HF1 and HF2)—representing conformations in which the fluorophores are closer to each other relative to LF—were observed for both tRNAs and are centered around $E_{\text{FRET}} = 0.77$ and 0.98 for HF1 and HF2, respectively (Fig. 6.4c,d). The LF and HF1/HF2 states we observed may approximate the U (unfolded) and C (cloverleaf-based-L-shape)

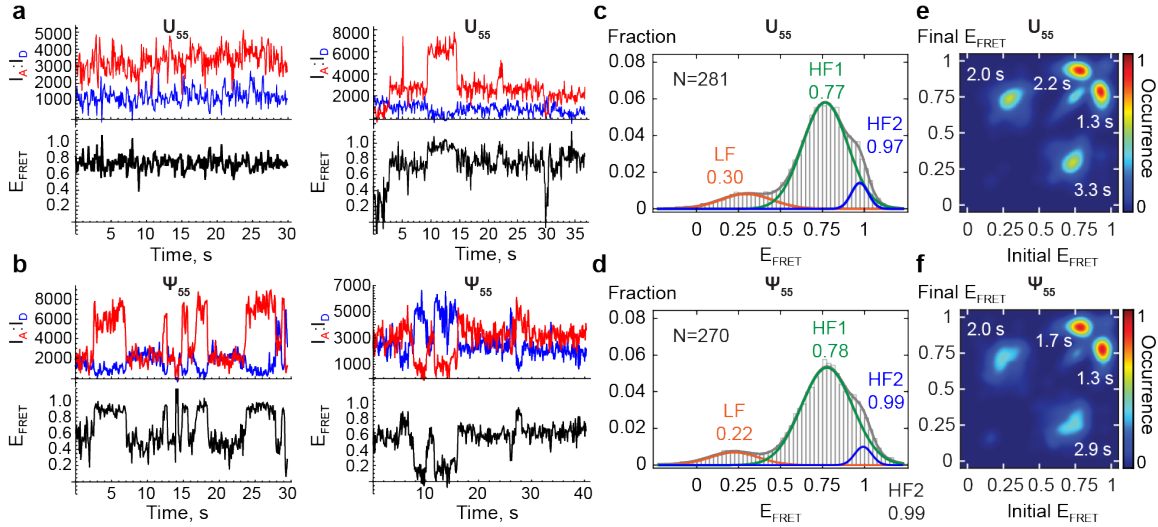


Figure 6.4: Unmodified and pre-modified tRNAs alone display distinct structural states and dynamics.

a, Representative donor (blue) and acceptor (red) E_{FRET} (black) traces of U_{55} tRNA^{eMet(CAU)} in the absence of Pus4. **b**, Same as **a**, but for Ψ_{55} tRNA. **c**, Histogram of the first 50 frames of $N = 281$ traces for U_{55} tRNA. A 3-Gaussian fit is overlaid with the peak centers labeled. **d**, Same as **c**, but for Ψ_{55} tRNA ($N = 270$). **e**, TODP for U_{55} tRNA, where the intensity of the heatmap at position (X,Y) quantifies the fraction of molecules that transition from $E_{\text{FRET}} = X$ to $E_{\text{FRET}} = Y$ at least once during the measurement. **f**, Same as **e**, but for Ψ_{55} tRNA.

conformations, respectively, that were previously identified through smFRET analysis of the human mt-tRNA^{Lys} (ref)⁶⁷. We hypothesize that the HF1 and HF2 states we have defined here share the overall C secondary structure, yet differ in certain tertiary contacts. In summary, we observed three predominant conformational states in the absence of protein, though this behavior was highly similar for U_{55} and Ψ_{55} tRNAs.

We analyzed the dynamics of U_{55} and Ψ_{55} tRNA^{eMet(CAU)} by performing Hidden Markov Modeling (HMM)⁷⁷ using a 3-state model that included the LF, HF1, and HF2 states. The pathways of transitions among these three states were visualized using transition occupancy density plots (TODPs), which show the prevalence of dynamic or static behavior across hundreds of molecules within each condition (Fig. 6.4e,f). A “feature” on the diagonal axis of a TODP (i.e. lower left

corner to upper right corner) indicates a population of molecules that remain in one state throughout their measurement, while an off-diagonal feature at position (X,Y) indicates a population of molecules that transition from states with $E_{\text{FRET}} = X$ (initial) to $E_{\text{FRET}} = Y$ (final) at some point during the measurement. This analysis showed that a minority of molecules ($\sim 5\%$ for both U_{55} and Ψ_{55}) remained in the HF1 or HF2 state for longer than 30 seconds, while the majority of molecules exhibited dynamic behavior involving fluctuations between conformations. This is consistent with previous bulk^{78,79} and smFRET⁷⁰ studies that showed that tRNAs can interconvert between multiple secondary and tertiary structures at physiological salt and temperature. In both U_{55} and Ψ_{55} tRNAs, bright off-diagonal features in the TODPs indicate that tRNAs readily and reversibly interconverted between the LF and HF1 states and between the HF1 and HF2 states (Fig. 6.4e,f). In contrast, almost no intensity was observed at the locations corresponding to transitions between the LF and HF2 states. We analyzed the rates of these transitions using cross-correlation analysis (details in Methods), which identifies how rapidly Cy3 and Cy5 intensities become uncorrelated with one another within a collection of smFRET traces. The cross-correlation curves of U_{55} and Ψ_{55} tRNAs were both well-fit with a mono-exponential decay with a time constant of ~ 1 s (Supp. Fig. 6.14), suggesting that interconversion between LF and HF1 and between HF1 and HF2 both occur on this time-scale. In summary, our analysis revealed that U_{55} and Ψ_{55} tRNAs can undergo frequent conformational changes involving substantial changes in their 3-dimensional architecture.

4.7 Pus4 alters the conformational states and their transition dynamics in tRNA

After Pus4 was added to U_{55} tRNA, a rapid increase in conformational complexity was observed, indicating that the protein changed the free energy

landscape of tRNA molecules (Fig. 6.5a). The earliest time window probed by smFRET, 2–6 minutes, begins when the initial fast phase of Pus4 association observed by BLI is nearly complete, and continues into the slow accumulation phase (Fig. 6.2b-d). Inspection of individual traces recorded during this time window revealed a new population of mid-FRET (MF) states with E_{FRET} between 0.4–0.6 (Supp. Fig. 6.15), which were not among the predominant states in U_{55} tRNA alone. Consistent with this observation, transitions between the LF and HF1 states were less clearly resolved in the TODPs, with a cluster of off-diagonal features representing discrete “microstates” with E_{FRET} between 0.5–0.8 interconverting with microstates with E_{FRET} between 0.2–0.5. As observed in tRNA alone, transitions between HF1 and HF2 remained well resolved after addition of Pus4 (Fig. 6.5a-d), although a subtle shift in E_{HF1} from 0.77 in U_{55} alone, to 0.80 after 2–6 minutes of incubation with Pus4, may indicate changes to specific tertiary contacts previously held (Supp. Fig. 6.16a). After 7–9 minutes of incubation, less sampling of MF states was observed, and the peak E_{FRET} value for HF1 returned to 0.77. By 10–15 minutes after addition of Pus4, U_{55} tRNA mostly ceased sampling MF states in lieu of well-resolved HF1–LF or HF1–HF2 transitions, resembling behavior observed in tRNA-only data. After 30–34 minutes (at which point all tRNA molecules are bound by Pus4, Fig. 6.12), a significant increase in the occupancy of HF2 was observed relative to earlier time points (Supp. Fig. 6.16a). Therefore, within the first 15 minutes after addition of Pus4, it altered tRNA conformation by promoting temporary sampling of MF states that were nearly absent without protein. We hypothesize that these MF states involve remodeling of the T-loop in order to facilitate pseudouridylation, as observed in crystallography data of the bacterial homolog TruB with tRNA^{80,81}. While our approach precluded real-time, simultaneous measurement of pseudouridylation

of tRNA immobilized on microscope slides, our *in vitro* pseudouridylation assay showed that the majority of tRNAs were pseudouridylated by Pus4 WT within 2 minutes (Supp. Fig. 6.9a). Therefore, results over the course of these smFRET experiments reflect the enduring influence of catalysis on the trajectory of the tRNA conformations sampled.

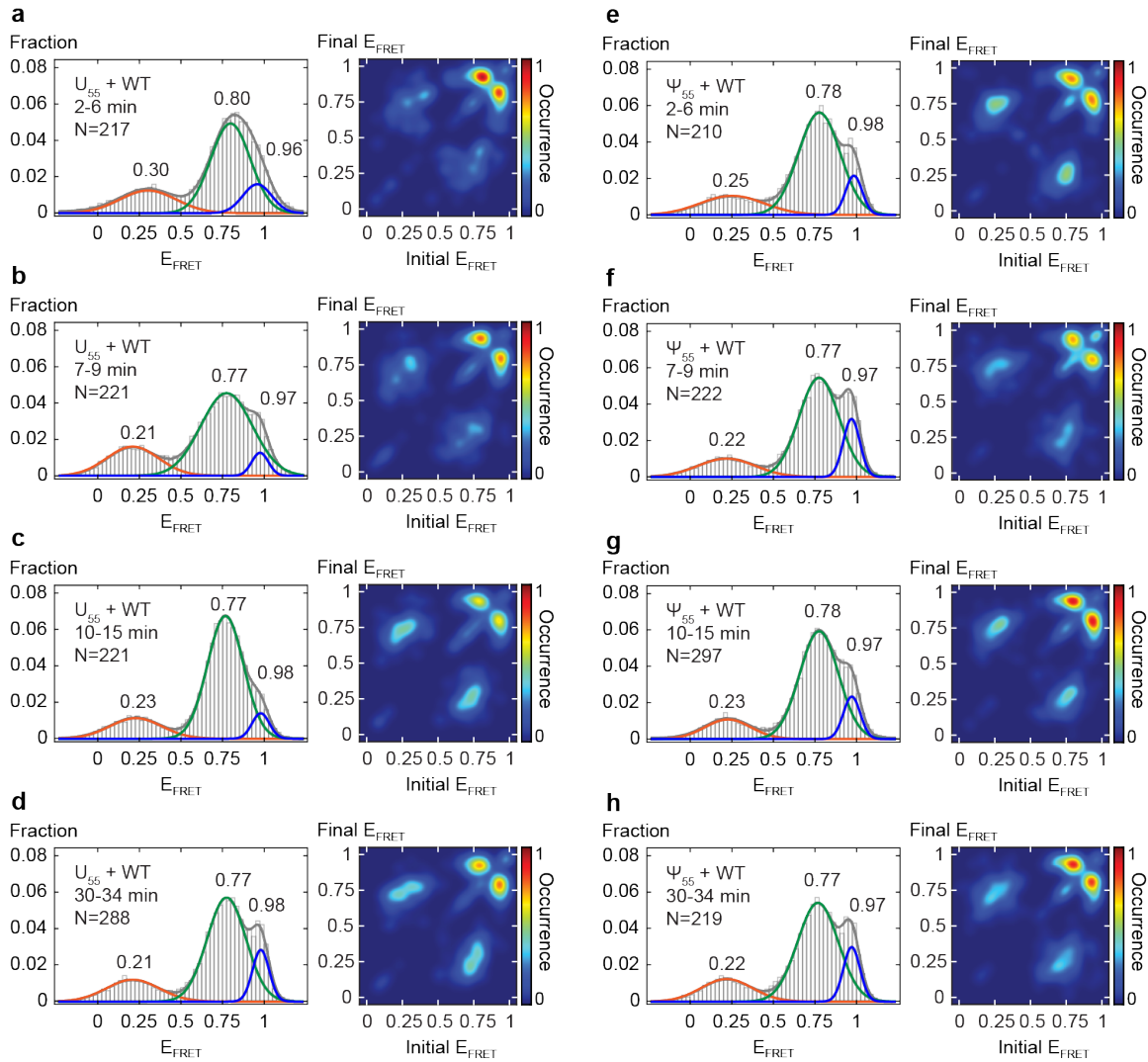


Figure 6.5: **smFRET histograms and TODPs of U_{55} and Ψ_{55} tRNA with Pus4 WT.** Histograms (left column) of the first 50 frames of $N > 200$ traces of U_{55} and TODPs (right column) at a timepoint following addition of Pus4 WT **a**, 2-6 minutes **b**, 7-9 minutes **c**, 10-15 minutes **d**, 30-34 minutes **e-h**, Same as **a-d**, but with Ψ_{55} .

To help isolate the influence of Pus4 binding and remodeling of tRNA from its pseudouridylation activity, we performed smFRET experiments with the pre-modified Ψ_{55} tRNA^{eMet(CAU)} in the presence of Pus4. In contrast to the dramatic changes observed in U_{55} tRNA shortly after addition of Pus4, Ψ_{55} tRNA's smFRET traces resolved into sharp yet dynamic LF, HF1, and HF2 states as early as the 2–6 minute window (Fig. 6.5e-h). Pus4 slightly increased the population of the LF state of Ψ_{55} tRNA rapidly after binding (also seen with U_{55}) (Supp. Fig. 6.16b), but it did not promote as much sampling of MF states, which we propose are repressed by the presence of Ψ_{55} . Furthermore, the initial shift of HF1 to higher E_{FRET} exhibited by U_{55} tRNA was not observed in Ψ_{55} tRNA. We hypothesize that Ψ_{55} tRNA thus bypassed some conformational changes that U_{55} tRNA transitioned through during its catalysis and subsequent remodeling by Pus4. In agreement with this model, the conformational landscape of U_{55} tRNA evolved toward the more steady conformational landscape of Ψ_{55} , but required 30 minutes to attain the distribution of structures that Ψ_{55} attained in only a few minutes (Fig. 6.5, Supp. Fig. 6.16e,f).

4.8 Catalytically-inactive Pus4 alters the tRNA remodeling pathway

While the Pus4 R286K mutant had impaired binding and catalysis, we found that it still promoted structural remodeling. It initially influenced U_{55} and Ψ_{55} tRNA similarly (Fig. 6.6a,e), unlike Pus4 WT which remodeled the two differently at early time points (Fig. 6.5a,e). During the 2–6 minute time period with Pus4 R286K, the population of the LF state increased and tRNA sampled a diverse array of MF conformations (Fig. 6.6a,e), despite only being bound at – of its peak protein occupancy (Fig. 6.2b). The distribution of MF states sampled was less broad than observed for U_{55} + Pus4 WT at early time points (Fig. 6.5a),

as indicated by the persistence of a prominent feature (rather than a cluster of features) at the locations corresponding to LF $\rightarrow$ HF1 transitions in their TODPs (Fig. 6.6a,e). This suggests that the mutant is less effective at facilitating the exploration of different structures. In contrast to their similarity at early times, the conformational

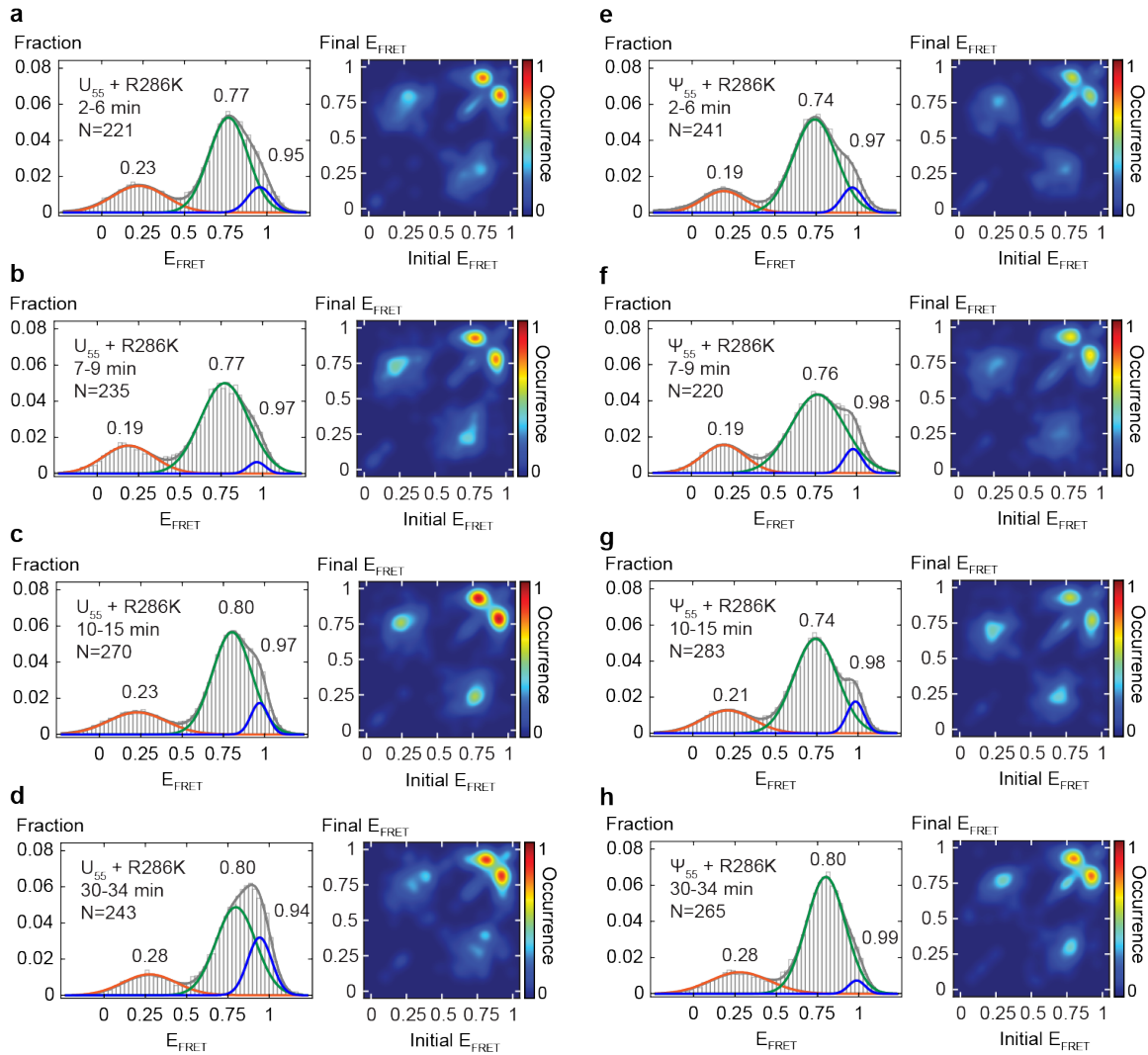


Figure 6.6: smFRET histograms and TODPs of U_{55} and Ψ_{55} tRNA with Pus4 R286K.

a-d, Histograms of the first 50 frames of $N > 200$ traces of U_{55} and TODPs at a timepoint following addition of Pus4 R286K **a**, 2-6 minutes **b**, 7-9 minutes **c**, 10-15 minutes **d**, 30-34 minutes **e-h**, Same as **a-d**, but with Ψ_{55} .

landscapes of U_{55} and Ψ_{55} tRNA diverged significantly by the 30-34 minute window (Fig. 6.6d,h, Supp. Fig. S9e–h). With protein binding occupancy still close to its peak (Fig. 6.2b), U_{55} tRNA maintained transitions between HF1 and both LF and MF states, as was observed at early time points for Pus4 WT. In contrast, Ψ_{55} tRNA exhibited much less sampling of MF states and a smaller HF2 population, compared to U_{55} tRNA (Fig. 6.6d,h). Thus, despite the deficiency of Pus4 R286K in discriminating between U_{55} and Ψ_{55} tRNAs immediately after binding, it ultimately remodels them into different structural ensembles. These results suggest that Pus4 R286K can still remodel tRNA, with the presence or absence of Ψ_{55} impacting the outcome.

5 Discussion

For many decades since its three-dimensional structure was first elucidated⁸², tRNA has been presented as an archetype of structured non-coding RNA. Yet every tRNA undergoes many processing steps during biogenesis—including trimming, tailing and addition of multiple chemical modifications—necessitating many conformational changes. In this study, we combined biophysical and biochemical approaches to document the dynamics of these conformational changes using a yeast tRNA and a ubiquitous pseudouridine synthase as a model. Our data demonstrate how Pus4 impacts tRNA conformation through its binding, promotion of conformations compatible with catalysis, and resolution toward a more sharply defined ensemble of conformations. The data show this final step occurring over a period of minutes if catalysis is bypassed (i.e. Pus4 WT+pre-modified tRNA) or *tens* of minutes if catalysis occurs or is precluded through interaction with catalytically-incompetent enzyme. Pus4 R286K influenced the conformational states explored by the tRNA differently than Pus4

WT did, thus demonstrating the impact of a single active site residue on the ability of Pus4 to bind to, chemically modify *and* remodel tRNA.

We find that tRNA maintains the fundamental conformational landscape of LF, HF1 and HF2 states throughout its interactions with Pus4 and independently of its modification status, with MF states being populated only under certain circumstances. We consistently observe a higher HF population in the yeast tRNA^{eMet(CAU)} profiled in this study than previously observed by smFRET in human mt-tRNA^{Lys} (ref)⁶⁷⁻⁷⁰, which we propose can be reconciled by predicted free energies. The predicted cloverleaf secondary structure of the tRNA that we investigated has a predicted $\Delta G_{\text{folding}} = -18.9$ kcal/mol, versus -16.1 kcal/mol for human mt-tRNA^{Lys} studied in Voigts-Hoffman et al., 2007 (details of free energy calculations in Methods) (Supp. Fig. 6.17). mt-tRNA^{Lys} populated a mid-FRET state with $E_{\text{FRET}} \sim 0.4$ that interconverted with C on a time-scale of hundreds of milliseconds, which the authors assigned to an extended hairpin secondary structure (E)⁷⁰. Interconversion between C- and E-type structures was also observed in a different tRNA, mt-tRNA^{Leu} (ref)⁷⁹, using biochemical methods. We identified a plausible secondary structure of tRNA^{eMet(CAU)} that mimics the E structure but, at $\Delta G = -12.9$ kcal/mol, it is 6 kcal/mol less stable than this tRNA's predicted C structure (Supp. Fig. 6.17a,b), potentially explaining its low prevalence. In contrast, the E structure proposed in Voigts-Hoffman et al., 2007 is predicted to have a free energy of -15.3 kcal/mol, within 1 kcal/mol to that of the C structure (Supp. Fig. 6.17c,d). Differences between the relative stabilities of E (non-native) and C structures have been previously shown to influence tRNA biogenesis and neurodegeneration⁸³. Once Pus4 was introduced, we observed a rapid increase in MF conformations, though our smFRET approach does not distinguish whether

these resemble the aforementioned E structure, or originate from altered tertiary contacts. Future studies of tRNA models with alternate placement of FRET pairs may enable delineation of these structures.

The resolution of the C structure of tRNA^{eMet(CAU)} into two distinct states (HF1 and HF2) is another noteworthy observation in our study, and is consistent with prior steady-state and kinetic measurements that identified multiple interconvertible tertiary structures under conditions generally favoring the C secondary structure^{78,84}. Splitting of the HF feature into two distinct states was not observed for human mt-tRNA^{Lys} under any condition reported; instead its C conformation consistently peaked around $E_{\text{FRET}} \sim 0.8$ over a variety of ionic conditions and chemical modification states^{67–70}. It is possible that the low thermodynamic stability of the C structure of mt-tRNA^{Lys} precludes multiple stable tertiary structures from being resolved within the time resolution of the smFRET measurements reported for it.

We summarize our results with a model for the conformational dynamics of yeast tRNA^{eMet(CAU)}, and how they are influenced by pseudouridylation and interaction with Pus4 (Fig. 6.7). We propose that each instance of Pus4 engaging with tRNA follows a three-to-four-step process: 1) Initial binding of Pus4 occurs. 2) Pus4 “tests” whether Ψ_{55} is already present. 3) If Ψ_{55} is not present, Pus4 can convert U_{55} to Ψ_{55} . 4) Pus4 remodels tRNA into its final distribution of structures. Pus4 WT effectively discriminates U from Ψ , bypassing catalysis for pre-modified tRNA and remodeling within minutes, as indicated by the rapid convergence of the structural ensemble when Pus4 has bound Ψ_{55} tRNA. In checking for Ψ_{55} prior to catalysis, Pus4 induces an additional population of intermediate (MF) states, including states that may promote catalysis, or an indiscriminate sampling of states

similar to the catalytically active one (e.g. both tRNAs+Pus4 R286K). Overall our results demonstrate two important layers of influence on tRNA conformational dynamics: chemical catalysis and remodeling resulting from extended interactions with modification enzymes. Why Pus4 should exert such malleability toward tRNA conformational dynamics, even on already pseudouridylated tRNAs, remains an important question. Since Pus4 catalysis can strongly influence the addition of subsequent modifications⁶, some added over many hours after pseudouridylation⁷, we propose that part of the role of these enzymes is to make tRNA structure amenable to binding and modification by subsequent modification enzymes critical for tRNA function.

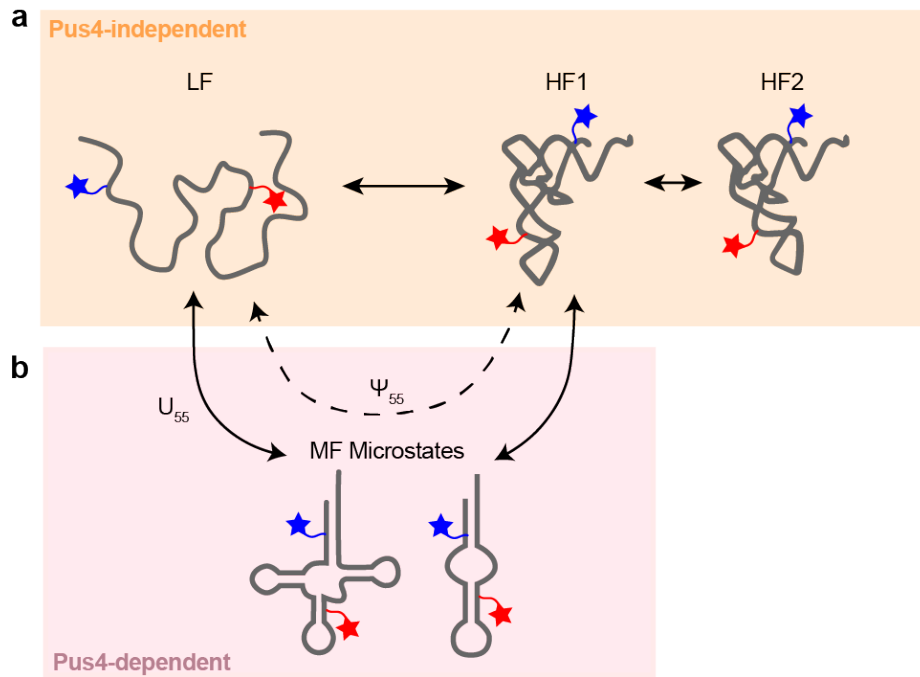


Figure 6.7: Proposed model for the conformational dynamics of tRNA as influenced by Pus4.

a, tRNAs interconvert between multiple cloverleaf-based conformations (HF1, HF2) and an extended (LF) conformation, independent of pseudouridine modification, independent of pseudouridine modification. **b**, Addition of Pus4 adds a new population of intermediate (MF) states that resolve into various HF states after 30 or more minutes. This step is bypassed by Ψ_{55} +Pus4 WT, which more quickly resolves to HF1 and HF2 states. Pus4 may enable additional secondary structures to be accessed in the MF states, such as an extended hairpin.

6 Methods

Synthetic tRNA sequence design and preparation

Fluorophore placement was modeled after that used in Voigts-Hoffmann et. al., 2007⁷⁰. The yeast tRNA^{eMet(CAU)} and tRNA^{Thr(AGU)} were chosen for several reasons: 1) both contain uridines at positions 4 and 41, which were the positions labeled with fluorophores and match those labeled in the prior work⁷⁰, 2) both have a minimum free energy structure as predicted by RNAFold^{85,86}, which closely resembles the tRNA cloverleaf secondary structure, theoretically reducing the propensity for the formation of non-canonical structures *in vitro*, and 3) prior evidence suggested both tRNAs are pseudouridylated by Pus4 efficiently *in vivo*⁷⁰ and our work herein demonstrated their pseudouridylation by Pus4 *in vitro*. The tRNAs were designed to contain a Cy3 attached to the carbon-5 position of U4 and Cy5 attached to the carbon-5 position of U41, as well as biotinylation of the 5'-phosphate to enable attachment of the molecules to the microscope slide or probe for FRET and BLI, respectively.

Full-length tRNA constructs were made by ligation of two RNA oligos using a DNA splint (Supp. Table 2). Ligation was performed using 2 nmol of each of three oligos in a final volume of 67.5 μ L containing 7.5 μ L hybridization buffer (150 mM HEPES pH 7.5 and 500 mM KOAc). This solution was heated for 2 minutes at 90°C and allowed to cool at room temperature for 30 seconds before 10x T4 RNA ligase 2 buffer (NEB) was added to a final concentration of 1X. The mixture was then cooled at room temperature for an additional 10 minutes, at which time additional ligase buffer, ATP, water, and T4 RNA ligase 2 (MC Labs) were added to achieve a reaction volume of 100 μ L containing 0.4 mM ATP, 1X RNA ligase 2

buffer, and 10 μ L (100 units) T4 RNA ligase 2. The ligation mixture was incubated at room temperature for 3 hours. Following this, 8 μ L (16 units) DNase I (NEB) was added along with 10X DNase I buffer to bring the final concentration of that buffer to 1X. This mixture was incubated at 37°C for 15 minutes to digest the DNA splint.

The ligation mixture was then heated for 2 minutes at 90°C to inactivate the enzyme and unfold the tRNA, and then it was run on a 12% polyacrylamide, 8 M urea denaturing gel. Gels were imaged using an Amersham Typhoon (GE) and further visualized using a Dark Reader transilluminator (Clare Chemical Research). The 76nt band corresponding to the full length tRNA was extracted by electro-elution, and the product was isolated by ethanol precipitation.

Protein expression and purification

Starting with a pGEX-5X-3-ScPus4 backbone plasmid expressing GST-Pus4 with a Factor Xa cleavage site between the GST tag and Pus4 WT protein sequence (PDG344), the Pus4 R286K was generated using QuikChange Site-Directed Mutagenesis (Agilent Technologies) (Supp. Table 1). GST-tagged Pus4 WT and R286K vectors were transformed into *E. coli* BL21(DE3) competent cells (ThermoFisher Scientific). A single transformant of each was inoculated to make a 50 mL overnight starter culture, which was then diluted into a 4 L culture grown until reaching $OD_{600}=0.80$. Cultures were grown at 37°C in TB medium containing 100 μ g/mL ampicillin and shaken at 225rpm. Upon reaching the desired OD_{600} , the 4 L culture was shifted to 16°C for 30 minutes. Protein expression was induced by adding IPTG (Gold Bio) to a final concentration of 1 mM, and cells were grown overnight for ~16 hours at 16°C with continuous shaking at 225rpm. Cells were

harvested by centrifugation, and the cell pellets were resuspended in 15 mL of Buffer A (50 mM Tris-HCl pH 7.5, 400 mM NaCl, 10 mM MgCl₂, 10% Glycerol, 0.5 mM EDTA, 5 mM BME, 1 tablet of Protease Inhibitor (ThermoFisher Scientific)), divided into two 50 mL conical tubes, and then flash-frozen in liquid nitrogen before storage at -80°C. Each conical contained enough material for a single protein purification. All subsequent procedures were performed at 4°C unless otherwise stated.

Conical tubes were thawed on ice. The following reagents were added to achieve their final concentrations, respectively: lysozyme (Sigma) to 1 mg/mL, DNase (GoldBio) to 10 µg/mL, MgCl₂ (Fisher) to 0.5 mM, and RNase A (Fisher) to 30 µg/mL. This cell suspension was incubated for 30 minutes. Cells were lysed via sonication using a sonicator (Branson Sonifier 450) with 30-seconds-on/30-seconds-off cycles (50% duty cycle, power level of 4), repeated three times. Insoluble material was removed by centrifugation for 30 minutes at 30,000xg in a JA-20 rotor. After clarification, the remaining soluble material was incubated with 2 mL of a 50% slurry of Glutathione-Sepharose beads (Cytiva) that had been pre-equilibrated with Buffer A for 1 hour with end-over-end rotation at room temperature. After incubation, the protein-bound beads were isolated using centrifugation (5 minutes at 500xg) and then washed with 20 mL of Buffer A. This wash step was repeated for a total of five times. Protein was then eluted from the beads with 1 mL of freshly prepared Elution buffer (50 mM Tris-HCl pH 7.6, 10 mM reduced glutathione) after incubating for 5 minutes at room temperature with end-over-end rotation. This elution step was repeated five times and the elutions were pooled together (~5 mL).

The eluate was concentrated to ~1mL final volume using a 30 kDa Vivaspin concentrator (GE Healthcare). Using the same concentrator, the sample underwent buffer exchange into Imaging buffer (50 mM Tris-HCl pH 7.5, 100 mM KCl) by adding 10 mL of Imaging buffer, and performing centrifugation to concentrate to ~1 mL final volume. This was repeated two more times. These protein samples were aliquoted into Eppendorf tubes and flash frozen in liquid nitrogen before storage at -80°C. The yield from each of the two vectors was comparable. Protein purity was determined using SDS-PAGE using samples collected across steps of the protein purification process (Supp. Fig. 6.8).

Yeast growth conditions

*pus4*Δ (YDG127) cells were streaked out onto YPD agar media plates from a frozen -80°C stock and grown at 30°C for 2 days (Supp. Table 1). Colonies were inoculated into 5 mL YPD media in culture tubes and grown at 30°C in a rolling drum overnight. In the morning, 400 μL of each culture was transferred to culture tubes with 5.2 mL liquid YPD and grown at 30°C in a rolling drum to an OD600 of ~0.8. Cultures were transferred to 15 mL conical tubes and centrifuged at 4°C, 3000xg for 2 minutes. Media was decanted, and the cell pellets were resuspended in 1 mL cold 1X PBS and transferred to microcentrifuge tubes. Tubes were centrifuged at 4°C, 9500rpm for 1 minute to pellet. Supernatant was decanted and cell pellets frozen by placing microcentrifuge tubes in liquid nitrogen. Cell pellets were stored at -80°C until used for total RNA isolation.

RNA isolation from yeast

*pus4*Δ (YDG127) yeast pellets were thawed on ice (Supp. Table 1). For cell lysis and RNA preservation, pellets were resuspended in 400 μL TES buffer

(10 mM Tris, 10 mM EDTA, 20% SDS). 400 μ L acidic phenol was added to each tube. Tubes were vortexed for 10 seconds and incubated in a 65°C water bath for one hour, with 10 seconds of vortexing every 15 minutes. Tubes were put on ice for 5 minutes then centrifuged at 4°C, 13000rpm for 10 minutes. The top aqueous layer of each sample was transferred to a new tube. 400 μ L of chloroform was added to each of the new tubes. Tubes were vortexed for 10 seconds then centrifuged at 4°C, 13000rpm for 10 minutes. The top aqueous layer of each sample was transferred to a new tube and the addition of chloroform, vortexing, and centrifuging repeated. The top aqueous layer of each sample was transferred to a new tube. To precipitate RNA, 1/10 volume of 3M sodium acetate pH 5.5 and 2.5 volumes of ice cold 100% EtOH was added to each sample and mixed. Tubes were stored at -80°C for one hour. Tubes were centrifuged at 4°C, 13000rpm for 10 minutes. The supernatant was removed and 500 μ L cold 70% EtOH added to each tube to wash pellets of salt. Tubes were centrifuged at 4°C, 13000rpm for 10 minutes. Ethanol was removed and tubes left open for 5-10 minutes to allow residual ethanol to evaporate. Dried pellets were resuspended in 25 μ L water, and their concentrations were quantified using a Nanodrop.

An 8% urea acrylamide gel was made using the National Diagnostics UreaGel 19:1 Denaturing Gel System. Gel was pre-run in 1X TBE running buffer at 45 mA for 30 minutes. Total RNA samples were aliquoted to 10 μ L volumes and 10 μ L 2X RNA loading dye (NEB) added to each sample. 3 μ L Low Range ssRNA Ladder (NEB) was mixed with 7 μ L water and 10 μ L 2X RNA loading dye. Samples were incubated at 70°C for 8 minutes. Samples were loaded on the gel and empty wells loaded with 10 μ L water + 10 μ L 2X RNA loading dye. Gel was run at 20 mA for 15 minutes and then at 60 mA for ~1 hour. Gel was stained

with SYBR gold (Invitrogen) in 1X TBE running buffer for 10 minutes. Gel was imaged with an Amersham Typhoon (GE). Using UV shadowing, tRNA bands were cut from the gel. Gel fragments were placed in tubes with 450 μ L of 0.3 M NaCl. Tubes were put on a tube inverter overnight at 4°C. Liquid was transferred to new tubes and 1.05 mL 100% EtOH was added to each sample and mixed. Samples were stored at -80°C for one hour. Samples were centrifuged at 4°C, 13000rpm for 35 minutes. The supernatant was removed and tubes left open for 5-10 minutes to allow residual ethanol to evaporate. Dried pellets were resuspended in 10-20 μ L water and stored at -80°C.

***in vitro* modification**

To perform the *in vitro* modification, 1 μ M Pus4 protein was mixed with 300 nM total tRNA harvested from *pus4* Δ cells and incubated at 30°C for 2 minutes in a 200 μ L reaction volume in reaction buffer (50 mM Tris-HCl pH 7.5, 100 mM KCl, 4 mM MgCl₂). The reaction was halted with the addition of 200 μ L of saturated phenol (pH 6.6) to each reaction. Samples were mixed and then stored at -80°C.

To purify *in vitro* modified tRNA, samples were thawed on ice, then vortexed for 10 seconds and centrifuged at 4°C, 13000rpm for 10 minutes. tRNA was re-purified as done in the total RNA isolation, beginning with the addition of 400 μ L chloroform. Samples were resuspended in 10 μ L water and concentrations quantified using a Nanodrop. Samples were stored at -80°C.

Direct sequencing of *in vitro* modified tRNA using Oxford Nanopore Technology (ONT)

To perform library preparation for sequencing, samples were first thawed on ice. Samples were incubated at 95°C for 2 minutes, room temperature for 2 minutes, then on ice for 2 minutes. In a new tube, 7.25 μ L tRNA sample was mixed with 2 μ L 10X RNA ligase 2 buffer (NEB), 0.8 μ L each of: 10 μ M ACCA, 10 μ M GCCA, 10 μ M UCCA, 5 μ M CCCA, and 5 μ M CCA specific adapters², 2 μ L 50% PEG, 2 μ L 250 mM MgCl₂, 1.25 μ L 100 mM DTT, 2 μ L 20 mM ATP, and 1 μ L RNA ligase 2 (NEB), for a total volume of 20 μ L. The reaction was incubated at room temperature for 45 minutes. 20 μ L water was added to the reaction. tRNA was purified with 1.8X volume RNAClean XP Beads (Beckman Coulter). Samples were washed twice with 500 μ L 80% EtOH. Samples were eluted with 23 μ L water. Samples were mixed with 8 μ L 5X quick ligase buffer (NEB), 6 μ L RMX adapter (ONT), and 3 μ L T4 DNA ligase (2,000,000 U/mL; NEB) for a total volume of 40 μ L. Reaction was incubated at room temperature for 30 minutes. tRNA was purified with 1.5X volume RNAClean XP Beads. Samples were washed twice with 150 μ L WSB (ONT). Samples were eluted with 15 μ L ELB (ONT). Water was added for a total volume of 37.5 μ L. Samples were sequenced with MinION devices following the ONT SQK-RNA002 protocol.

Pseudouridylation of U₅₅ in tRNA leads to a base miscall, due to an alteration in the ionic current when a pseudouridine transitions through the nanopore, as compared to the different disruption in ionic current caused by the unmodified uridine base. This causes a “mismatch” between the sequenced base and the unmodified reference base. The “reference match probability” refers to the probability that a given base will match the reference base. Therefore, a low reference match probability at position 55 is indicative of a pseudouridylated base. For more details see reference².

Sequencing results were basecalled using Guppy v3.0.3, aligned with BWA-MEM (-W 13 -k 6 -xont2d), and quality controlled with SAMtools (Q1). marginAlign was used to create alignment and error models, and marginCaller was used to calculate mismatch probabilities. Python was used to calculate the reference match probability—equal to a value of 1.0 minus the mismatch probability value—and generate heatmaps (Supp. Fig. 6.9). DRS data files are listed in Supp. Table 3.

Testing purified protein for RNase contamination

Approximately 1 μg of tRNA isolated from yeast was incubated with 0.4 μM of Pus4 (WT or R286K) in a 10 μL total volume for one hour at room temperature. Control samples of tRNA without protein and tRNA plus protein without incubation were also performed. Samples were combined with 10 μL of RNA loading dye, heated at 70°C for 8 minutes on a heat block, and then loaded onto a fresh pre-run PAGE gel, along with RNA ladder, using an Apogee V16 gel system. After running the gel at 60 mA for about 1.5 hours, the gel was stained with SYBR Gold for 10 minutes at room temperature, and imaged using an Amersham Typhoon (GE) to assess tRNA degradation (Supp. Fig. 6.14).

RNA preparation for BLI and smFRET

The RNA was diluted in Imaging buffer (50 mM Tris-HCl pH 7.5, 100 mM KCl) to a concentration of 10 nM for smFRET and 60 nM for BLI measurements. The solutions were heated to 90°C on a heat block for 2 minutes, then allowed to cool for 10 minutes at room temperature before placing on ice. The same RNA substrates were used for both BLI and smFRET measurements. For smFRET measurements, the RNA was further diluted to 10 pM with the addition of 5 mM

D-glucose, 165 U/mL glucose oxidase from *Aspergillus niger*, 2150 U/mL catalase from *Corynebacterium glutamicum* and 5 mM Trolox[®] as an oxygen scavenger. This oxygen scavenger was used for RNA slide injection due to its fast acting capability, allowing for a higher level of control over RNA concentration during slide immobilization.

Biolayer Interferometry (BLI)

BLI measurements were performed on a GatorPlus Biolayer Interferometer (GatorBio, Palo Alto, CA) utilizing proprietary GatorOne software for data collection. All BLI measurements were obtained at 30°C. GatorBio streptavidin-coated glass probes were presoaked in the same Imaging buffer used during smFRET measurements to allow for comparisons between the two datasets. Probes were presoaked in Imaging buffer for at least 20 minutes before collecting baseline signal for 300 seconds. The probes were immersed in 60 nM biotinylated tRNA until an adequate shift (~ 1.2 nm) in signal was observed (~ 60 seconds). The probes were then submerged in 1:1000 biocytin in Imaging buffer to quench the remaining streptavidin sites. This was followed by a second quenching in 0.2% of Tween-20[®] in 50 mM Tris-HCl pH 7.5, 100 mM KCl, which also aided in reducing non-specific protein binding. Loaded probes were then incubated in 0.02% Tween-20[®] in 50 mM Tris-HCl pH 7.5, 100 mM KCl until baseline was achieved (~ 300 seconds). To measure association of the protein to the tRNA, the loaded probes were then incubated in 200 nM of GST-Pus4 (WT or R286K) in 0.02% Tween-20[®] in 50 mM Tris-HCl pH 7.5, 100 mM KCl for ~ 3000 seconds. To measure dissociation, the probes were then submerged in 0.02% Tween-20[®] in 50 mM Tris-HCl pH 7.5, 100 mM KCl for the same amount of time as the association step. 0.02% Tween-20[®] was included in all buffer steps following quenching to reduce

non-specific binding of GST-Pus4 to the probe. Reference probes were included in some runs to allow for baseline subtraction of non-specific interactions and signal resulting from probe background.

Analysis of BLI data

To estimate the kinetics of Pus4 WT binding to tRNA, we attempted to isolate the initial rate of association before pseudouridylation (in the case of tRNA that was not premodified) or refolding occurred. In isolation, this early phase of binding represents a reversible second order reaction ($\text{RNA} + \text{Pus4} \rightleftharpoons \text{RNA} \cdot \text{Pus4}$). We approximated its kinetics as pseudo-first order in Pus4, as the protein was present in bulk solution while the quantity of tRNA was limited by the number of binding sites on the probe. This is expected to yield an exponential shape to the association curve (Eq. 6.1):

$$[\text{RNA} \cdot \text{Pus4}]_t = [\text{RNA}]_0(1 - e^{-k_{obs}t}), \quad (6.1)$$

where the observed rate of the reaction is: $k_{obs} = k_{on}[\text{Pus4}] - k_{off}$.

Considering that at each timepoint t , $[\text{RNA}]_0 = [\text{RNA}] \cdot \text{Pus4}]_t + [\text{RNA}]_t$, and that at the final timepoint $[\text{RNA}]_{final} = [\text{RNA}]_0 - [\text{RNA} \cdot \text{Pus4}]_{final}$, Eq. 6.1 becomes:

$$\frac{[\text{RNA} \cdot \text{Pus4}]_{final} - [\text{RNA}] \cdot \text{Pus4}]_t}{[\text{RNA} \cdot \text{Pus4}]_{final}} = e^{-k_{obs}t}. \quad (6.2)$$

To estimate k_{on} and k_{off} , the association phases of the BLI curves were converted to fractional saturation (FS) using Eq. 6.3, where $s(t)$ is the shift at time t and s_{final} is the shift at the final timepoint of the association phase:

$$FS(t) = 1 - \frac{s_{final} - s_t}{s_{final}}. \quad (6.3)$$

Two methods were then used to fit the fractional saturation curve: 1) The early segment of the association phase over which $\sim 75\%$ of the overall shift occurred was fitted with a single exponential (Eq. 6.4), or 2) the entire association phase was fitted with the sum of 2 exponentials (Eq. 6.5). In Eq. 6.5, the faster of the two rates was taken to be k_{obs} .

$$FS(t) = y - a \cdot e^{-k_{obs}t} \quad (6.4)$$

$$FS(t) = y - a_1 \cdot e^{-k_1t} - a_2 \cdot e^{-k_2t} \quad (6.5)$$

These fits were performed for BLI curves obtained at 50 nM, 200 nM and 800 nM Pus4. k_{obs} was then plotted against [Pus4] and the slope of a linear fit yielded an estimate for k_{on} . In principle, the y-intercept of the fit yields k_{off} . However, determining it accurately would require measurements at lower [Pus4], conditions that reduce both the accuracy of the pseudo-first order kinetics approximation and the separation of the timescales on which Pus4 accumulates on the probe and on which it modifies and/or remodels the tRNA it has already bound to. Because k_{obs} was less than 0.01 s^{-1} at 50 nM Pus4 (the lowest concentration tested) for all RNAs and fitting approaches, we can reasonably assume that k_{off} is less than that value. Given the values obtained for k_{on} (Supp. Fig. 6.13), this constrains the dissociation constant $K_D = k_{off}/k_{on}$ to a value less than $\sim 130 \text{ nM}$.

Single molecule FRET (smFRET)

The RNA of interest, prepared as described above, was flowed onto a slide that had been passivated with DDS, incubated with 0.2 mg/mL of bBSA for 5 minutes, 0.2% v/v Tween-20[®] for 10 minutes, and 0.2 mg/mL streptavidin for 5

minutes. Imaging was performed with Imaging buffer (50 mM Tris-HCl pH 7.5, 100 mM KCl) containing 5 mM 3,4-dihydroxybenzoic acid (PCA), 5 mM Trolox[®], and 50 nM Protocatechuate 3,4-dioxygenase (PCD) as an oxygen scavenging system. 200 nM Pus4 WT or R286K in imaging buffer containing the above oxygen scavenger as well as 0.02% Tween-20[®] as a surfactant was prepared and allowed to tube incubate with minimal headspace for 10 minutes before injection into the system. This pre-incubation allowed for movie capture as early as two minutes after injection. Data were collected on a home-built prism-type total internal reflection fluorescence (TIRF) microscope (Leica DMi8 inverted microscope, 63x oil immersion objective). Cy3 and Cy5 were excited by a 532 nm laser (Coherent OBIS 532 LS) at 140 mW and a 637 nm laser (Coherent OBIS 637 LX) at 60 mW, respectively. The slide was illuminated at 532 nm continuously and at 637 nm at the beginning and end of each movie to confirm that Cy5 was active. tRNA movies were recorded on an Andor iXon 888 EMCCD with an exposure time of 100 ms. Two minute movies were collected back to back for the first 15 minutes following protein injection. Additional movies were collected 30 minutes after protein injection.

Coordinates and fluorophore intensity traces for each molecule were extracted from raw video files using FIJI. Traces that exhibited single-step photobleaching of Cy3 and Cy5 and anticorrelation between donor and acceptor signals when transitions occurred were selected manually and analyzed using custom MATLAB codes. The FRET efficiency was calculated as $I_A/(I_A+I_D)$, where I_A and I_D are the background corrected fluorescence intensities of Cy5 and Cy3 respectively. FRET histograms were compiled from the first 50 frames of each selected trace for a large number of traces (>200). Gaussian fitting of histograms

was performed in Mathematica. Hidden Markov Modeling was performed in QuB (Quantifying unknown Biophysics) online^{16,17,87}. TODPs were generated using custom MATLAB codes.

Free energy calculations

The free energies of different secondary structures were predicted using the RNAfold web server⁸⁸. The sequence used for mt tRNA^{Lys} was the one utilized in Voigts-Hoffman et al., 2007 with U instead of dT at its fluorophore labeling sites, and the sequence used for tRNA^{eMet(CAU)} was the one utilized in this work. Mild base-pairing constraints were applied to achieve the C and E secondary structures of mt tRNA^{Lys} as depicted in Fig. 1 of Voigts-Hoffman et al., 2007 (1 and 2 enforced BP, respectively), the canonical tRNA^{eMet(CAU)} structure as depicted in Fig. 6.1 of this work (2 enforced BP), and a plausible E-like structure of tRNA^{eMet(CAU)} (4 enforced BP). The secondary structures were optimized under these constraints with energy parameters re-scaled to 25°C and 0.1 M salt and isolated BP allowed. The resulting minimum free energy structures were re-rendered using R2DT⁸⁹ to generate the visualizations shown in Supp. Fig. ??.

7 Supplemental Tables and Figures

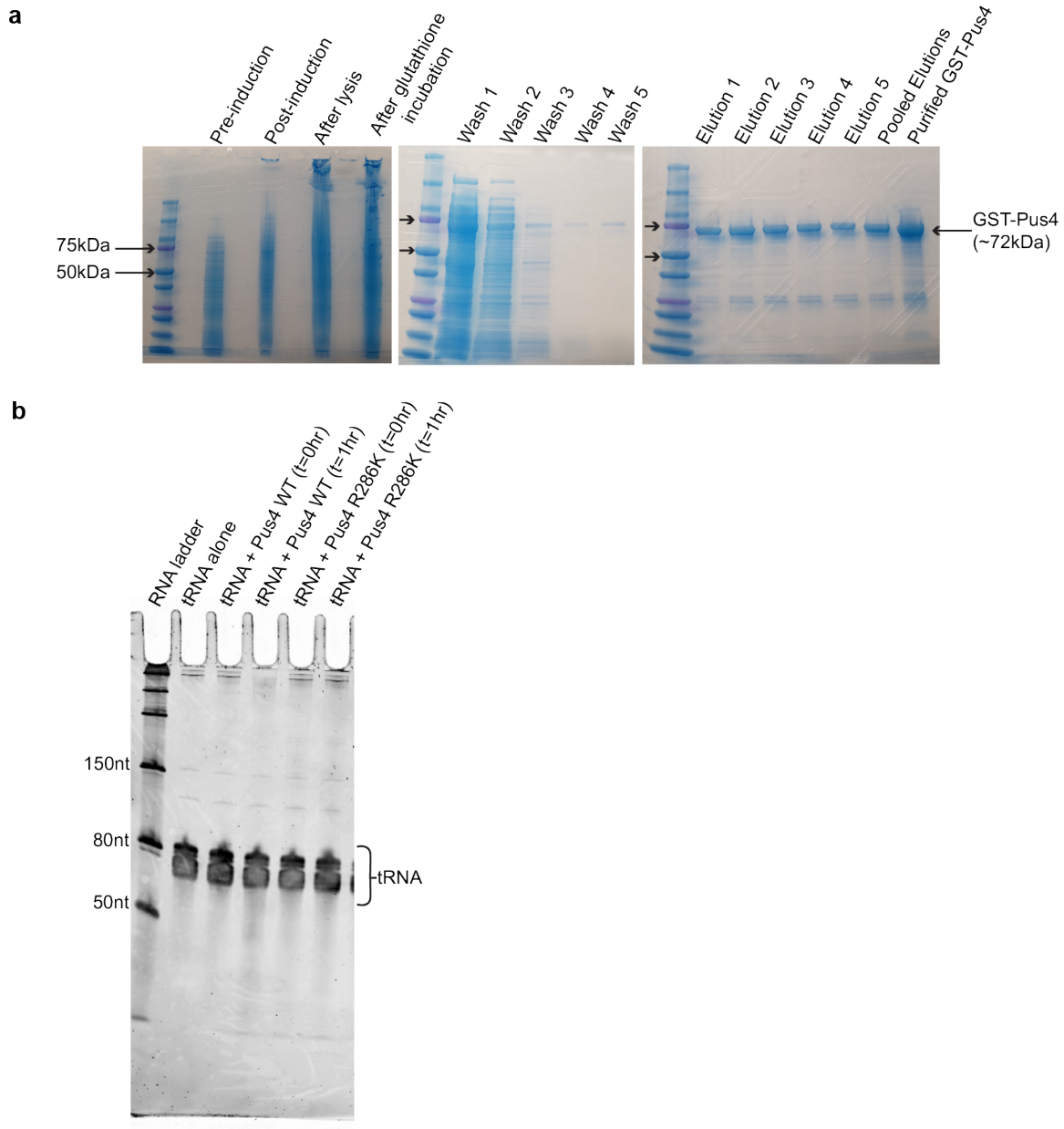


Figure 6.8: Protein purification

a, Denaturing PAGE gels stained with GelCode Blue showing protein present throughout purification, shown here for Pus4 WT (results similar for R286K, data not shown). Upper and lower arrows indicate ladder standards of 75 and 50 kDa, respectively. **b**, Denaturing PAGE gel showing total yeast tRNA remains without any appreciable degradation after incubation with purified Pus4 protein for 1 h at room temperature.

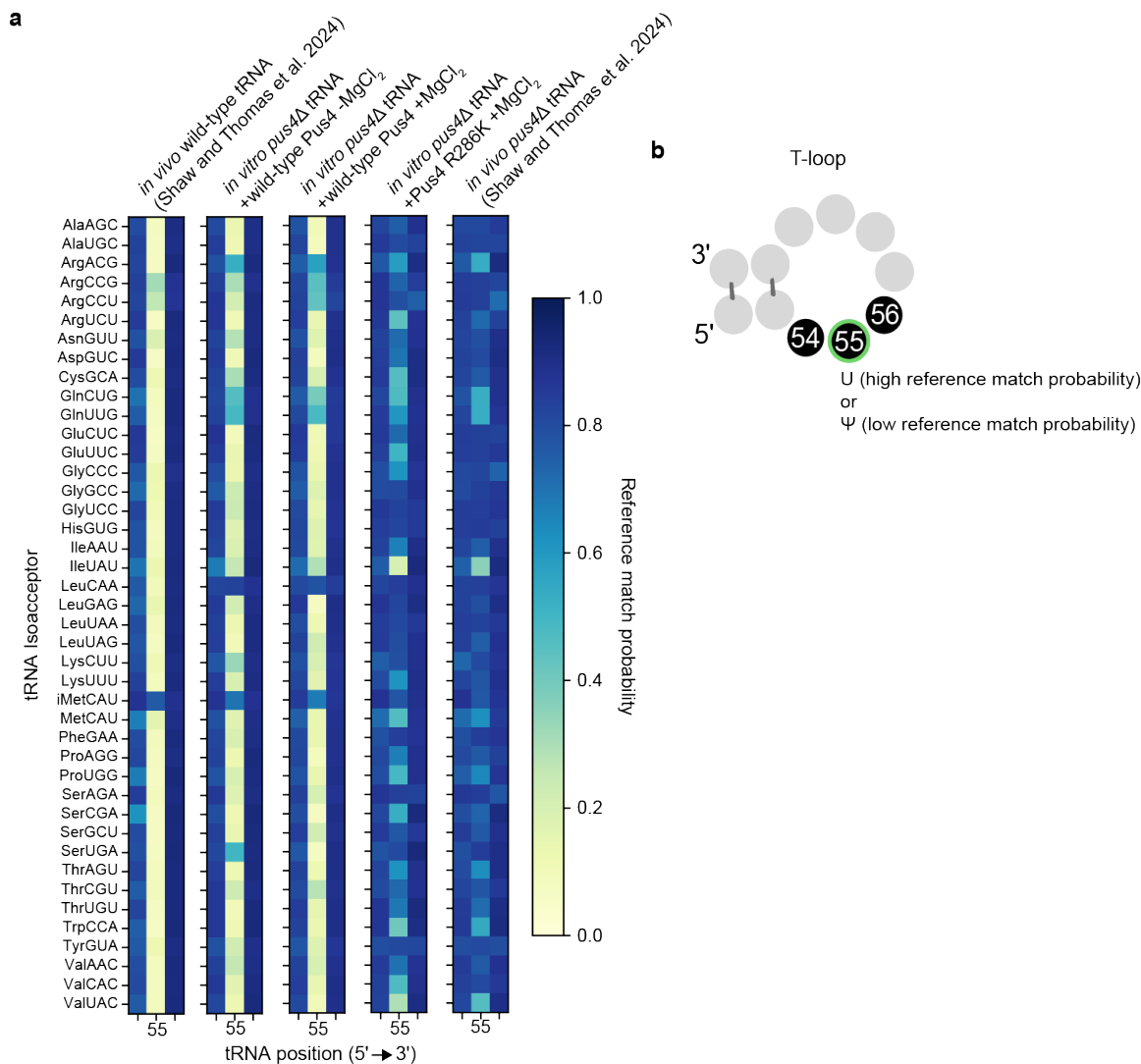


Figure 6.9: Heatmaps of Nanopore direct RNA sequencing base miscalls show *in vitro* modification activity of purified Pus4 WT and R286K. **a**, Heatmaps representing sequenced nucleotide positions 54-56 of the 42 yeast cytosolic tRNA isoacceptors for tRNA harvested from *pus4*Δ cells and reacted *in vitro* with purified Pus4 WT or Pus4 R286K, with or without 4 mM MgCl₂. Results from *in vivo* tRNA sequencing are from Shaw and Thomas et al., 2024. Reference match probability corresponds to the frequency with which a basecalled nucleotide agrees with the reference sequence, where more frequently miscalled positions (indicative of pseudouridine at that position) are light yellow. **b**, Diagram of a generic yeast tRNA T-loop. Black circles denote bases shown in **a**.

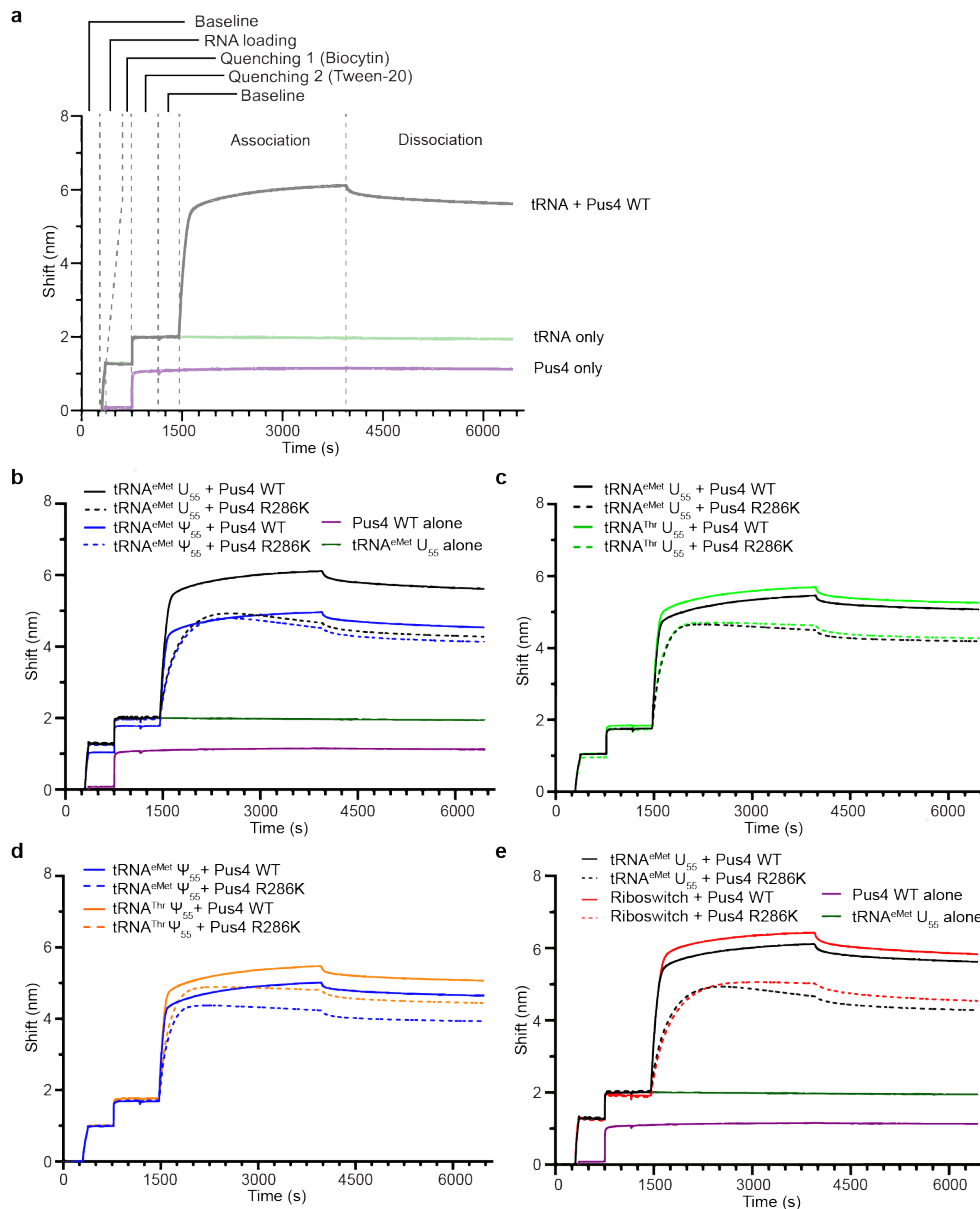


Figure 6.10: **Complete BLI traces of data from Figure 2.**

a, Full BLI traces with each experimental step overlaid and labeled. Step (length of time): Baseline (300 s), RNA loading (~60 s), Quenching 1 (400 s), Quenching 2 (400 s), Baseline (300 s), Association (2500 s), Dissociation (2500 s). **b**, Full BLI traces of 200 nM Pus4 WT (solid) and Pus4 R286K (dotted) showing association with U_{55} (black) and Ψ_{55} tRNA^{eMet}(CAU) (blue). The traces from the reference probes of Pus4 WT alone (purple) and U_{55} tRNA^{eMet}(CAU) alone (green) are also shown. **c**, Same as **b** but with U_{55} tRNA^{eMet}(CAU) (black) and tRNA^{Thr}(AGU) (green). **d**, Same as **b** but with Ψ_{55} tRNA^{eMet}(CAU) (blue) and tRNA^{Thr}(AGU) (orange). Data collected in the same experiment as those shown in panel **c**. **e**, Same as **b** but with Pus4 WT (solid) and Pus4 R286K (dotted) with both U_{55} tRNA^{eMet}(CAU) (black, reproduced from panel **b**) and NAD⁺ riboswitch (red). Data collected in the same experiment as those shown in panel **b**.

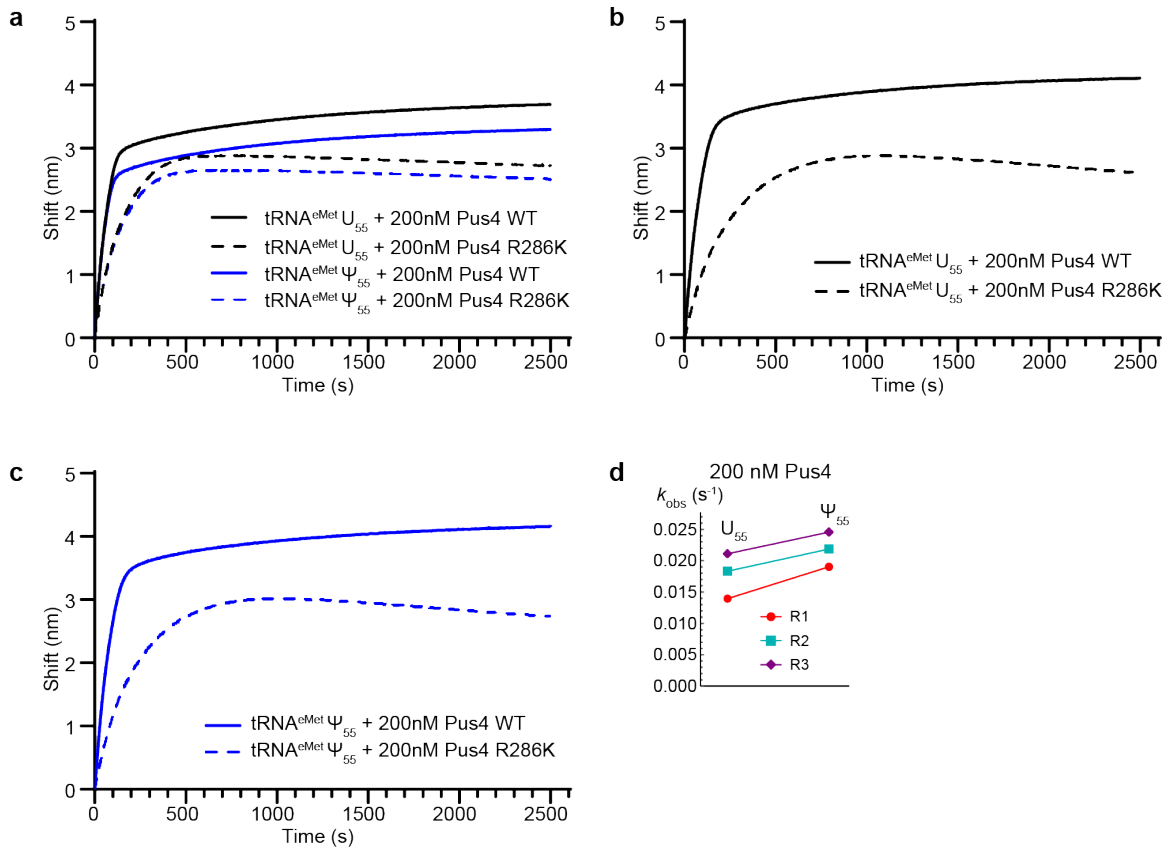


Figure 6.11: **BLI replicate data and resulting rates of fits to association phases of tRNA + Pus4 WT BLI curves.**

Each panel is from a different BLI run. **a**, Association phase of 200 nM Pus4 WT (solid) and Pus4 R286K (dotted) showing association with U_{55} (black) and Ψ_{55} (blue) tRNA^{eMet(CAU)}. Data shown are the same as in Fig. 6.2b, Supp. Fig. 6.10b. **b**, Association phase of 200 nM Pus4 WT (solid) and Pus4 R286K (dotted) showing association with U_{55} (black). **c**, Association phase of 200 nM Pus4 WT (solid) and Pus4 R286K (dotted) showing association with Ψ_{55} tRNA^{eMet(CAU)} (blue). **d**, k_{obs} determined from a bi-exponential fit to the entire association phases of three runs (R1, R2 and R3) of tRNA^{eMet(CAU)} + 200 nM Pus4 on different days. While the rates themselves are variable between runs, Ψ_{55} tRNA consistently exhibits a faster k_{obs} than U_{55} tRNA.

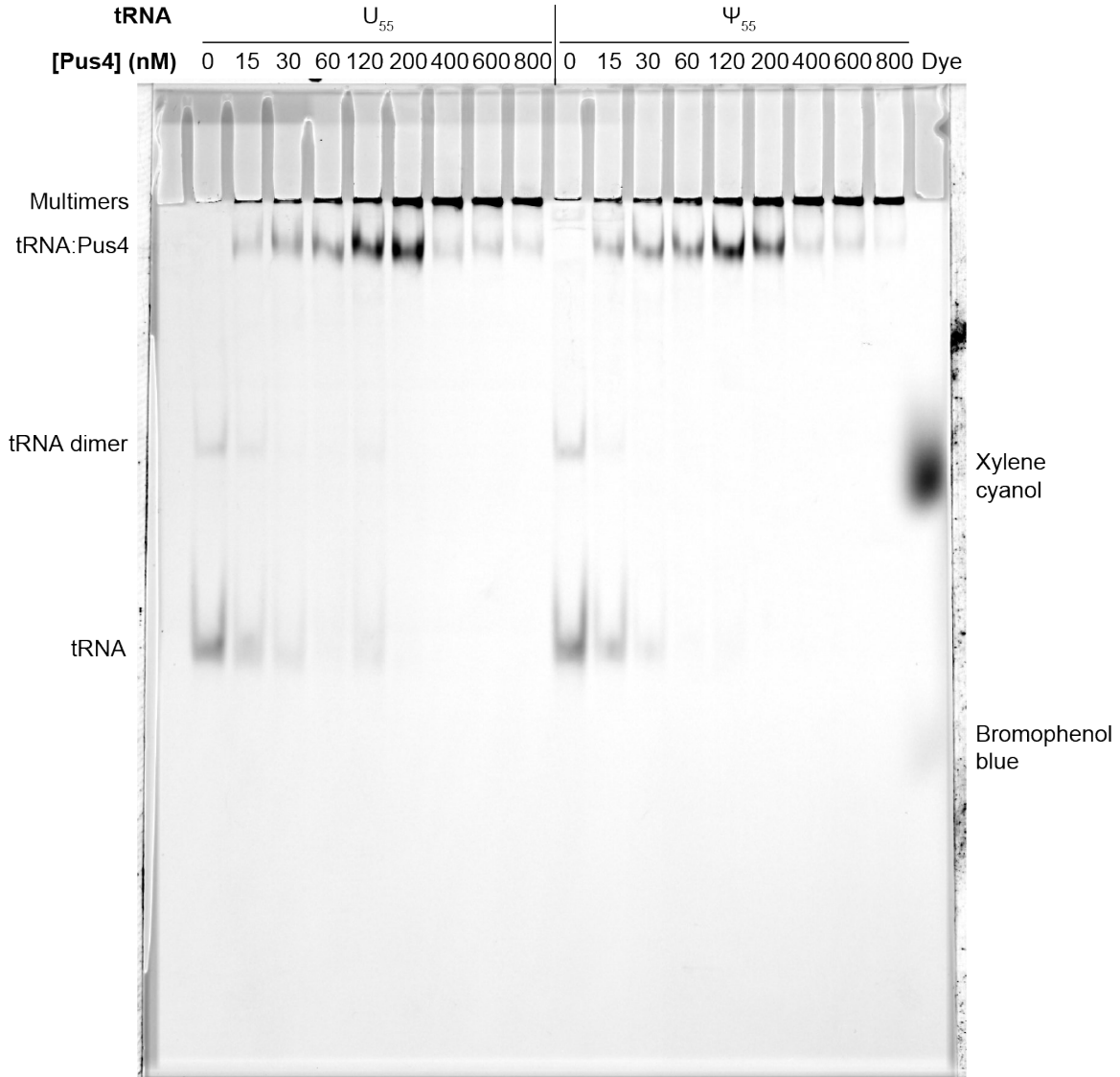


Figure 6.12: Electrophoretic Mobility Shift Assay
 U₅₅ or Ψ₅₅ tRNA^{eMet(CAU)} was incubated for 30 minutes in the presence of 0 to 800 nM Pus4 WT and the resulting protein-RNA assemblies were resolved on an 8% non-denaturing polyacrylamide gel. tRNA was visualized by detecting Cy5 fluorescence. The image was subjected to a 500-pixel rolling ball background subtraction. The slower-migrating band in the no-protein lanes may represent tRNA dimers. The far-right lane contains xylene cyanol and bromophenol blue as a reference.

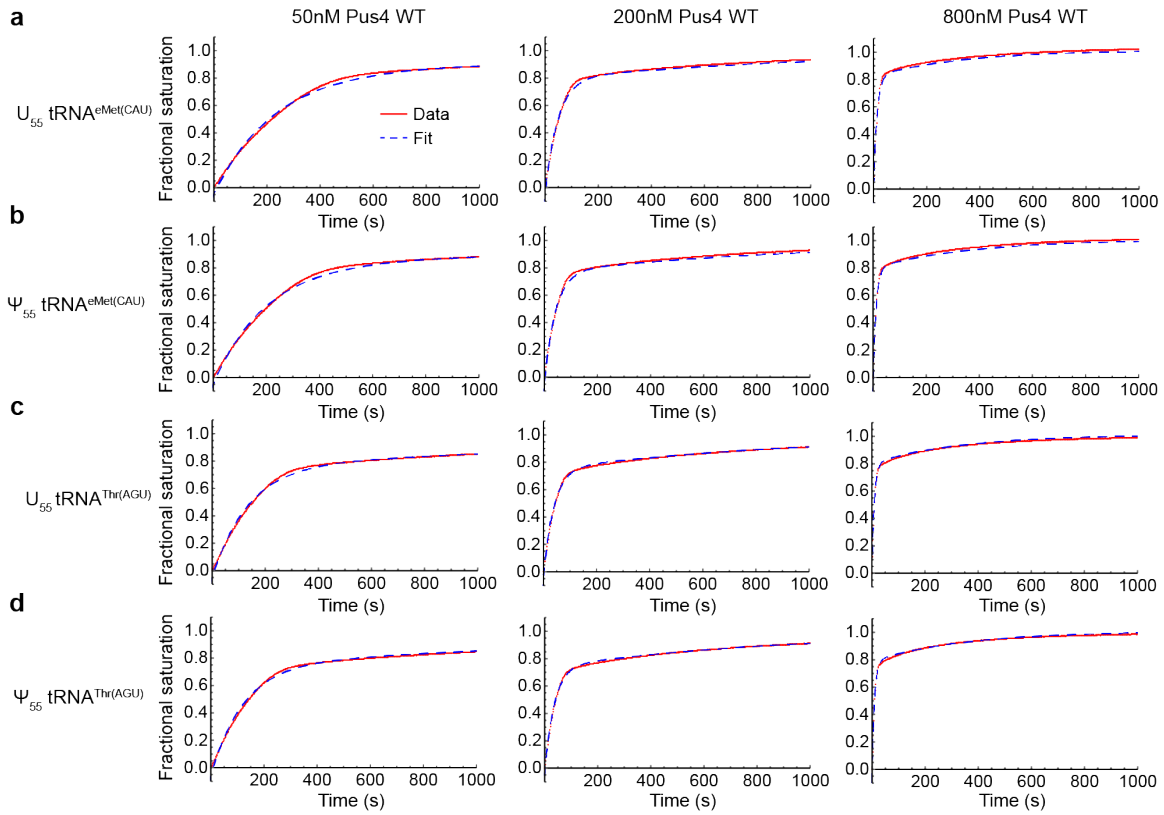


Figure 6.13: Fits to association phases of BLI curves. Bi-exponential fits (blue dashed lines) to the association phases of BLI curves (red) at varying concentrations of Pus4 WT. Left column: 50 nM; Middle: 200 nM; Right: 800 nM. **a**, U_{55} tRNA^{eMet(CAU)}. **b**, Ψ_{55} tRNA^{eMet(CAU)}. **c**, U_{55} tRNA^{Thr(AGU)}. **d**, Ψ_{55} tRNA^{Thr(AGU)}. The entire association phase was fitted, but the first 1000 s are displayed for ease of comparison.

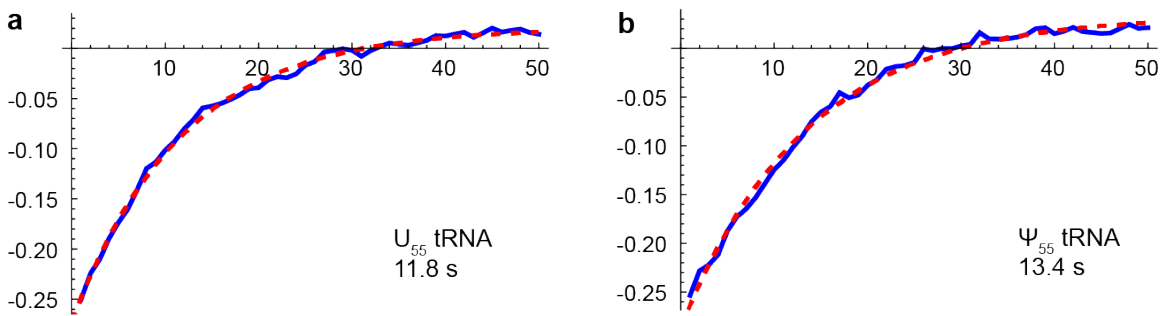


Figure 6.14: Cross-correlation analysis. Cross-correlation curves (blue) and mono-exponential fits (dashed red) for **a**, U_{55} tRNA^{eMet(CAU)}. **b**, Ψ_{55} tRNA^{eMet(CAU)}. See Methods for equation.

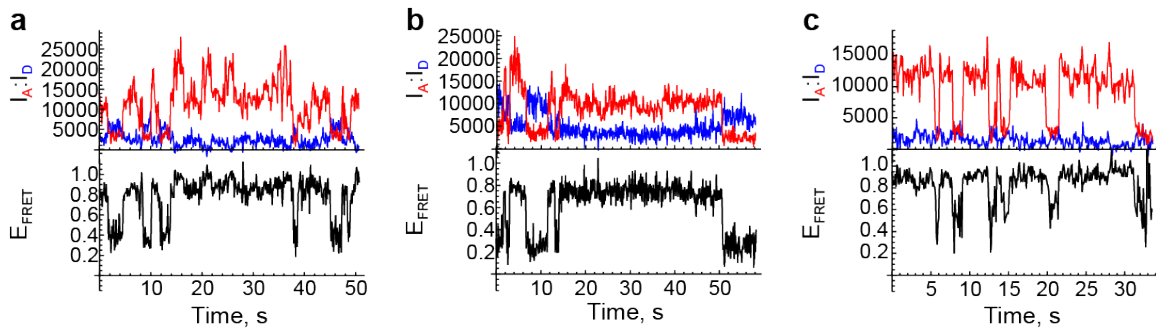


Figure 6.15: **Representative smFRET traces for U_{55} tRNA^{eMet}(CAU) 2–6 minutes following introduction of Pus4 WT**

a, E_{FRET} transitions between 0.75 and 0.4 with brief sampling of the HF2 state from HF1 between 14 and 26 s. **b**, E_{FRET} transitions between 0.75 and 0.25. **c**, Intermediate MF states where $E_{FRET} = 0.5$.

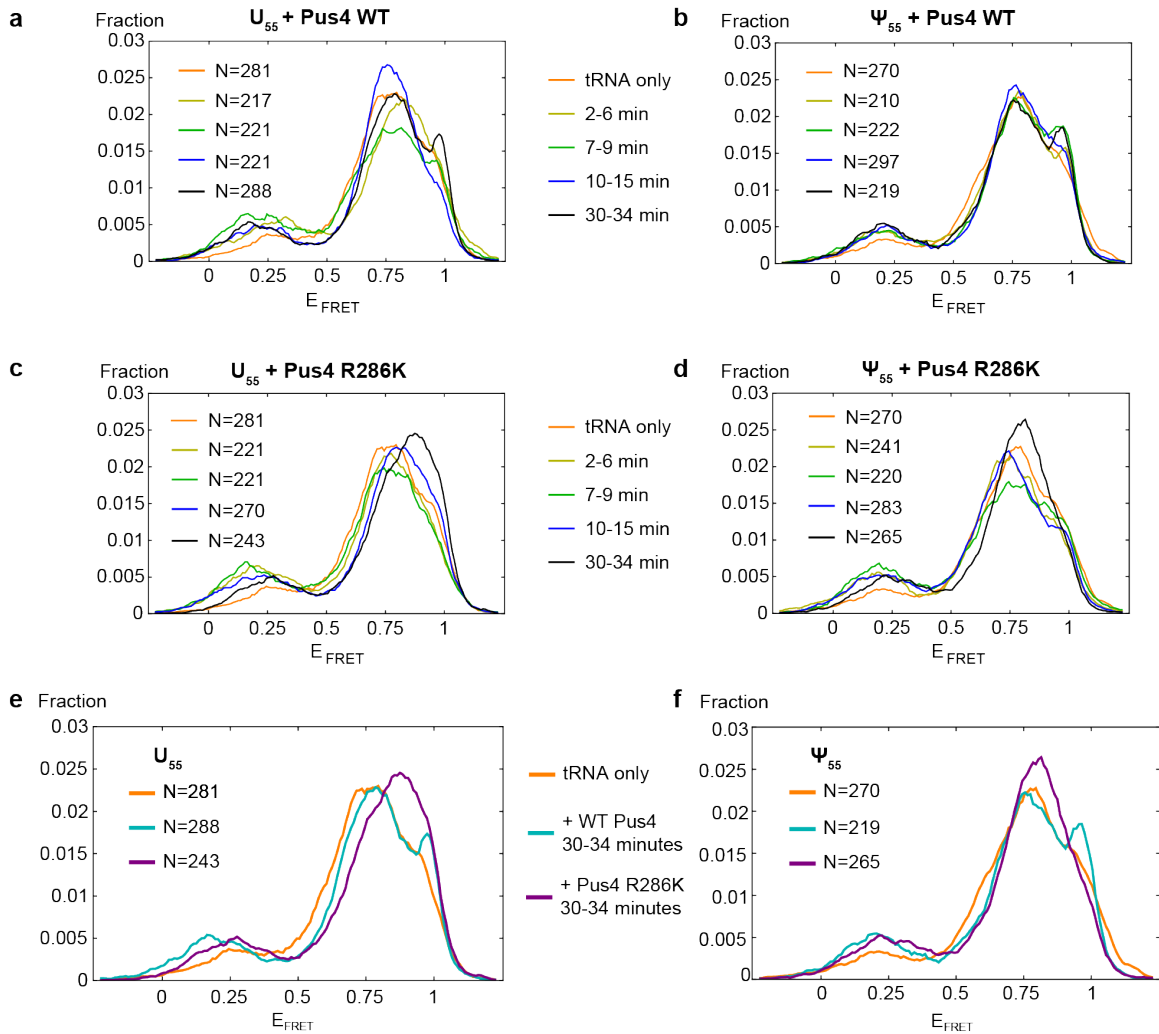


Figure 6.16: Histogram overlays

Overlaid smFRET histograms of tRNA prior to introduction of Pus4 (orange) and tRNA at 2–6 minutes (yellow) 7–9 minutes (green), 10–15 minutes (blue) and 30–34 minutes (black) following introduction of Pus4. a, $U_{55} + \text{Pus4 WT}$. b, $\Psi_{55} + \text{Pus4 WT}$. c, $U_{55} + \text{Pus4 R286K}$. d, $\Psi_{55} + \text{Pus4 R286K}$. e, 30–34 minutes with U_{55} tRNA alone (orange), with Pus4 WT (teal), with Pus4 R286K (purple). f, 30–34 minutes with Ψ_{55} tRNA alone (orange), with Pus4 WT (teal), with Pus4 R286K (purple).

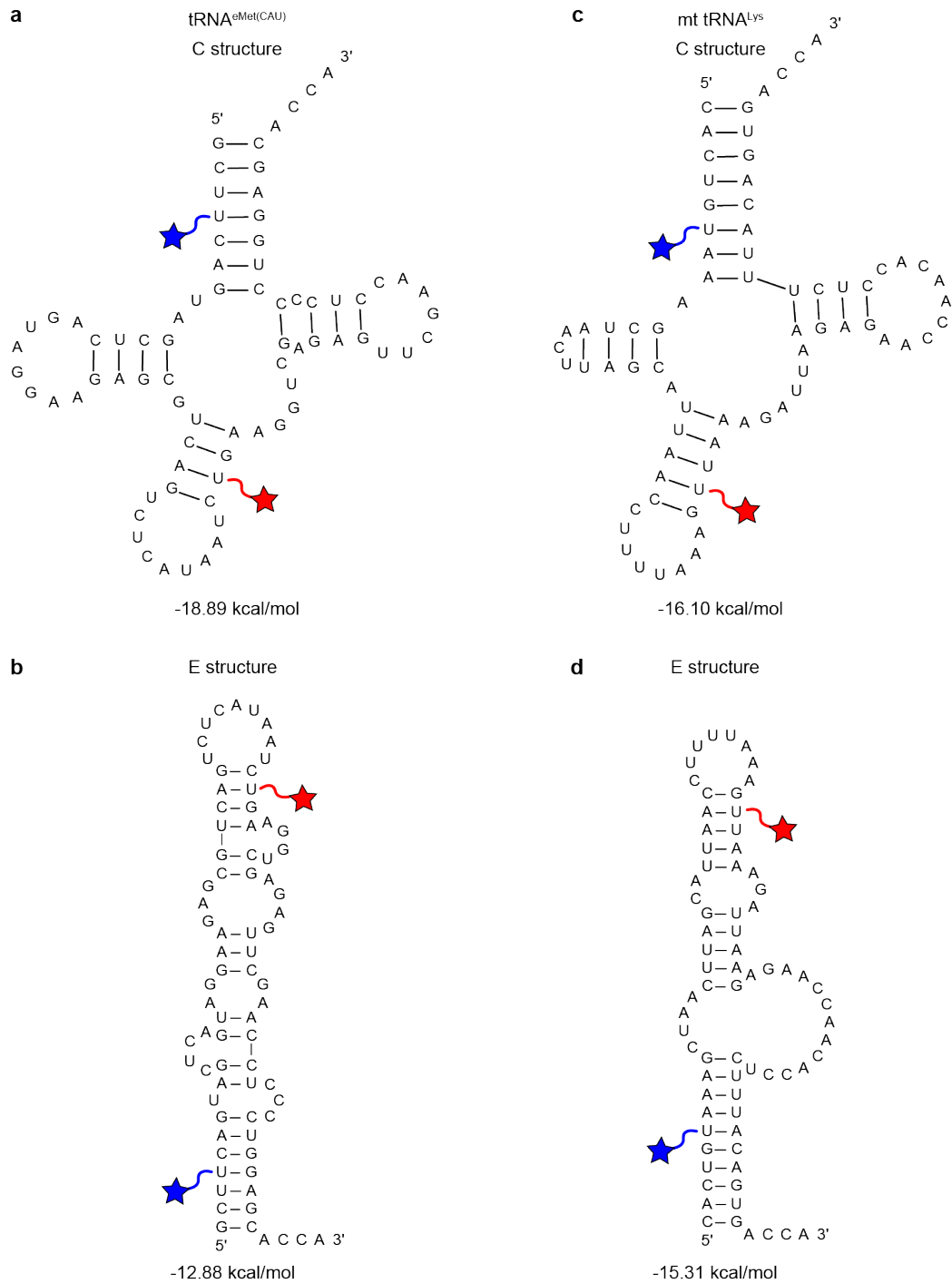


Figure 6.17: **Predicted secondary structures**

Secondary structures and associated free energies at 25°C and 0.1 M salt predicted using the RNAfold web server (see Methods for details). **a**, Predicted C structure of tRNA^{eMet}(CAU) and its predicted free energy. **b**, Plausible E structure of tRNA^{eMet}(CAU). **c**, Predicted C structure of human mitochondrial tRNA^{Lys} from Voigts-Hoffman et al., 2007. **d**, Predicted E structure of human mitochondrial tRNA^{Lys} from Voigts-Hoffman et al., 2007.

Supplementary Tables

Table 6.1: Yeast strains

Strain	Description	From
YDG127	<i>pus4</i> Δ::KanMX	Yeast Deletion Collection ⁹⁰

Table 6.2: Plasmids

Construct	Description	From
PDG312	To express Pus4 R286K mutant in <i>E. coli</i> ; pGEX-5X-3-ScPus4 R286K	This study
PDG322	To express Pus4 WT in <i>E. coli</i> ; pGEX-5X-3-ScPus4	This study, modified from plasmid gifted by Dr. Ute Kothe
PDG344	Transformation of PDG322 into BL21(DE3) cells	This study
PDG374	Transformation of PDG312 into BL21(DE3) cells	This study

Insert sequences

Sequence of expressed wild-type Pus4 protein (including GST tag, Factor Xa cleavage site, and Pus4 protein sequence):

ATGTCCCCTATACTAGGTTATTGGAAAATTAAGGGCCTTGTGCAACCCACTC
GACTTCTTTTGAATATCTTGAAGAAAAATATGAAGAGCATTTGTATGAGCG
CGATGAAGGTGATAAATGGCGAAACAAAAAGTTTGAATTGGGTTTGGAGTTT
CCCAATCTTCCTTATTATATTGATGGTGATGTTAAATTAACACAGTCTATGG
CCATCATACGTTATATAGCTGACAAGCACAACATGTTGGGTGGTTGTCCAAA
AGAGCGTGCAGAGATTTCAATGCTTGAAGGAGCGGTTTTGGATATTAGATAC
GGTGTTTTCGAGAATTGCATATAGTAAAGACTTTGAAACTCTCAAAGTTGATT
TTCTTAGCAAGCTACCTGAAATGCTGAAAATGTTCTGAAGATCGTTTATGTCA
TAAAACATATTTAAATGGTGATCATGTAACCCATCCTGACTTCATGTTGTAT

GACGCTCTTGATGTTGTTTTATACATGGACCCAATGTGCCTGGATGCGTTCC
CAAAATTAGTTTGTTTTAAAAACGTATTGAAGCTATCCCACAAATTGATAA
GTACTTGAAATCCAGCAAGTATATAGATGGCCTTTGCAGGGCTGGCAAGCCA
CGTTTGGTGGTGGCGACCATCCTCCAAAATCGGATCTGATCGAAGGTCGTGG
GATCCCCAGGAATTCGGGGAACATGAATGGAATATTTGCTATTGAGAAGCCT
AGCGGTATTACATCCAATCAGTTTATGCTAAAGTTGCAACATGCTTTAACTA
AAAGTCAAGTATTCTCGAAGGAAATTCAAAGGGCAACAGCGGAAAGAAAGCA
GCAATATGAAAAACAGACAGGTAAGAAGGCCAGCAAGAGGAACTTCGTAAA
GTCTCAAAGGTAAAGATGGGACACGGAGGCACTTTGGATCCGTTGGCCTCTG
GTGTCCTGGTAATTGGGATTGGAGCGGGCACCAAAAAAATTGCAAATTATCT
TTCAGGCACCGTTAAAGTCTATGAAAGTGAAGCCCTGTTTGGTGTTCCTACT
ACTTCAGGAGATGTAGAAGGTGAGATTTTGTCCCAGAACTCCGTTAAACATT
TAAATTTGATGATTTAAAGACTGTGGAGGAAAAATTTGTTGGACAATTAAA
GCAGACGCCGCCAATATACGCAGCTTTGAAGATGGATGGCAAACCCCTGCAT
GAATATGCTCGAGAAGGAAAGCCTTTACCTAGAGCCATTGAGCCCAGACAAG
TTACTATTTATGACTTGAAGGTGTTTTTCAGACTCTCTTAAACGTGATCATGA
CTATCCCTTATTGAGGCCCACTACAGAAGAAGCTGTTGATACCGTGAAAAAT
TTGAATGCAAACATGCTAAACGATGTTTTATACTTTTCAAAGAATATACAG
AGAAGCACGGCCTTGATTCTGAGGTGGCAAAGGTAGAAGAACCATTTCCGCT
GAGTGAACAAGAAGAGCAAGAAATCCAAAAAGAAGGAGATTCTTATAGGGCT
CCAAAATTGCACTTCAAAGCTAATGTGTCTTCTGGAACATACATAAGGTCCT
TAGTGAGCGACATCGGTAAATCTATGAGGAGCTCATGTTATATGGTGAAATT
GATACGTTTGC AACAGCAGGACTGGTCTCTTGAAAAAATAATGTGTTCCAA
TTAACGGATTTTACTGAAAGAGACGAGAAGGTGTGGAGTAAGGTACTGGAGA
AGGTTCTAGACGAGGGAGCAACTGTTGATGTTATAGAAGAGCTAAAGAAAGC
AGAAAAGGAAATACCAGCGGACGTGAAGGAATGTATAGTTTCGAGTGATCAA

CCTGGTGATGAGGCTACGGCTGAAACAATTGAAACTGCTAACGCCGAAGAAC
ATTCTAATACACTAAAAAGAAAAATCGAACAGGTGTAA

Sequence of expressed R286K Pus4 protein (including GST tag, Factor Xa cleavage site, and Pus4 protein sequence):

ATGTCCCCTATACTAGGTTATTGGAAAATTAAGGGCCTTGTGCAACCCACTC
GACTTCTTTTGAATATCTTGAAGAAAAATATGAAGAGCATTTGTATGAGCG
CGATGAAGGTGATAAATGGCGAAACAAAAAGTTTGAATTGGGTTTGGAGTTT
CCCAATCTTCCTTATTATATTGATGGTGATGTTAAATTAACACAGTCTATGG
CCATCATACGTTATATAGCTGACAAGCACACATGTTGGGTGGTTGTCCAAA
AGAGCGTGCAGAGATTTCAATGCTTGAAGGAGCGGTTTTGGATATTAGATAC
GGTGTTTTCGAGAATTGCATATAGTAAAGACTTTGAAACTCTCAAAGTTGATT
TTCTTAGCAAGCTACCTGAAATGCTGAAAATGTTCTGAAGATCGTTTATGTCA
TAAACATATTTAAATGGTGATCATGTAACCCATCCTGACTTCATGTTGTAT
GACGCTCTTGATGTTGTTTTATACATGGACCCAATGTGCCTGGATGCGTTCC
CAAAATTAGTTTGTTTTAAAAAACGTATTGAAGCTATCCCACAAATTGATAA
GTACTTGAAATCCAGCAAGTATATAGCATGGCCTTTGCAGGGCTGGCAAGCC
ACGTTTGGTGGTGGCGACCATCCTCCAAAATCGGATCTGATCGAAGGTCGTG
GGATCCCCAGGAATTCGGGGAACATGAATGGAATATTTGCTATTGAGAAGCC
TAGCGGTATTACATCCAATCAGTTTATGCTAAAGTTGCAACATGCTTTAACT
AAAAGTCAAGTATTCTCGAAGGAAATTCAAAGGGCAACAGCGGAAAGAAAGC
AGCAATATGAAAAACAGACAGGTAAGAAGGCCAGCAAGAGGAAACTTCGTAA
AGTCTCAAAGGTAAAGATGGGACACGGAGGCACTTTGGATCCGTTGGCCTCT
GGTGTCCTGGTAATTGGGATTGGAGCGGGCACCAAAAAAATTGCAAATTATC
TTTCAGGCACCGTTAAAGTCTATGAAAGTGAAGCCCTGTTTGGTGTTTCCAC
TACTTCAGGAGATGTAGAAGGTGAGATTTTGTCCCAGAACTCCGTAAACAT

TTAAATTTTCGATGATTTAAAGACTGTGGAGGAAAAATTTGTTGGACAATTAA
AGCAGACGCCGCCAATATACGCAGCTTTGAAGATGGATGGCAAACCCCTGCA
TGAATATGCTCGAGAAGGAAAGCCTTTACCTAGAGCCATTGAGCCCAGACAA
GTTACTATTTATGACTTGAAGGTGTTTTTCAGACTCTCTTAAACGTGATCATG
ACTATCCCTTATTGAGGCCCACTACAGAAGAAGCTGTTGATACCGTGAAAAA
TTTGAATGCAAACATGCTAAACGATGTTTTTATACTTTTCAAAAGAATATACA
GAGAAGCACGGCCTTGATTCTGAGGTGGCAAAGGTAGAAGAACCATTTCCGC
TGAGTGAACAAGAAGAGCAAGAAATCCAAAAAGAAGGAGATTCTTATAGGGC
TCCAAAATTGCACTTCAAAGCTAATGTGTCTTCTGGAACATACATAAAATCC
TTAGTGAGCGACATCGGTAAATCTATGAGGAGCTCATGTTATATGGTGAAAT
TGATACGTTTGCAACAGCAGGACTGGTCTCTTGAAAAAAATAATGTGTTCCA
ATTAACGGATTTTACTGAAAGAGACGAGAAGGTGTGGAGTAAGGTACTGGAG
AAGGTTCTAGACGAGGGAGCAACTGTTGATGTTATAGAAGAGCTAAAGAAAG
CAGAAAAGGAAATACCAGCGGACGTGAAGGAATGTATAGTTTTCGAGTGATCA
ACCTGGTGATGAGGCTACGGCTGAAACAATTGAAACTGCTAACGCCGAAGAA
CATTCTAATACTACTAAAAAGAAAAATCGAACAGGTGTAA

Table 6.3: Oligonucleotides

Description	Sequence (5' to 3')	Supplier and Purification	Nucleic Acid
eMet CAU 5' oligo	biotin-GCUU[Cy3]CAGUAGCUCAGUAG GAAGAGCGUCAGUCUCAUAAU	Dharmacon, HPLC	RNA
eMet CAU 3' oligo	phosphate-CU[Cy5]GAAGGUCGAGAGU UCCAACCUCCCCUGGAGCACCA	Dharmacon, HPLC	RNA
eMet CAU 3' with Ψ	phosphate-CU[Cy5]GAAGGUCGAGAGU Ψ CGAACCUCCCCUGGAGCACCA	Dharmacon, HPLC	RNA
eMet CAU 3' with 5FU	phosphate-CU[Cy5]GAAGGUCGAGAGU U[5F]CGAACCUCCCCUGGAGCACCA	Dharmacon, HPLC	RNA
Thr AGU 5' oligo	biotin-GCUU[Cy3]CUAUGGCCAAGUUG GUAAGGCGCCACACUAGUAAU	Dharmacon, HPLC	RNA
Thr AGU 3' oligo	phosphate-GU[Cy5]GGAGAUCAUCGGU UCAAAUCCGAUUGGAAGCACCA	Dharmacon, HPLC	RNA
Thr AGU 3' with Ψ	phosphate-GU[Cy5]GGAGAUCAUCGGU Ψ CAAAUCCGAUUGGAAGCACCA	Dharmacon, HPLC	RNA
Splint for 5' and 3' portions of tRNA eMet ^(CAU)	GGTGGGTCTGCTTTGGTGCTCCAGG GGAGGTTTCGAACCTCTCGACCTTCAG ATTATGAGACTGACGCTCTTCCTAC TGAGCTACTGAAGCATTTTGGGTAC	IDT, PAGE	DNA
Splint for 5' and 3' portions of tRNA Thr ^(AGU)	GGTGGGTCTGCTTTGGTGCTTCCAA TCGGATTTGAACCGATGATCTCCAC ATTACTAGTGTGGCGCCTTACCAAC TTGGCCATAGAAGCATTTTGGGTAC	IDT, PAGE	DNA
NAD ⁺ riboswitch	biotin-AUCUAUAGAGCGUU(Cy5)GCG UCCGAAAGUCUAAACAGACACGGCU CUUUAAAAACAAAAGGAGA(Cy3)	Dharmacon, HPLC	RNA
QuikChange F primer for Pus4 R286K	GTCTTCTGGAACATACATAAAATCC TTAGTGAGCGACATCG	IDT, Standard desalting	DNA
QuikChange R primer for Pus4 R286K	CGATGTCGCTCACTAAGGATTTTA TGTATGTTCCAGAAGAC	IDT, Standard desalting	DNA

Accession	Sample	Fast5 file	FastQ file
PRJEB85651	Pus4 WT <i>in vitro</i>	20240718_RNA002_UO _sCer_tRNA_pus4deletion_in_vitro_WT_pus4. tar.gz	20240718_RNA002_UO _sCer_tRNA _pus4deletion_in_vitro _WT_pus4_noU.fastq.gz
PRJEB85651	Pus4 R286K <i>in vitro</i>	20240724_RNA002_UO _sCer_tRNA _pus4deletion_in_vitro _R286K_pus4.tar.gz	20240724_RNA002_UO _sCer_tRNA _pus4deletion_in_vitro _R286K_pus4_noU .fastq.gz
PRJEB85651	Pus4 WT no MgCl ₂ <i>in vitro</i>	20240813_RNA002_UO _sCer_tRNA _pus4deletion_in_vitro _WT_pus4_noMgCl .tar.gz	20240813_RNA002_UO _sCer_tRNA _pus4deletion_in_vitro _WT_pus4_noMgCl _noU.fastq.gz
PRJEB68201	<i>in vivo</i> WT replicate 1	WT_tRNAs_rep1_7_22 _21_fast5s.tar.gz	WT_tRNAs_rep1_7_22 _noU.fastq.gz
PRJEB68201	<i>in vivo</i> WT replicate 2	WT_tRNAs_rep2_8_6 _21_fast5s.tar.gz	WT_tRNAs_rep2_8_6 _21_noU.fastq.gz
PRJEB68201	<i>in vivo</i> WT replicate 3	WT_tRNAs_rep3_9_17 _21_fast5s.tar.gz	WT_tRNAs_rep3_9_17 _21_noU.fastq.gz
PRJEB68201	<i>in vivo</i> <i>pus4</i> Δ replicate 1	pus4_tRNAs_rep1_06_30 _21_fast5s.tar.gz	pus4_tRNAs_rep1_06_30 _21_noU.fastq.gz
PRJEB68201	<i>in vivo</i> <i>pus4</i> Δ replicate 2	pus4_tRNAs_rep2_07_19 _21_fast5s.tar.gz	pus4_tRNAs_rep2_07_19 _21_noU.fastq.gz
PRJEB68201	<i>in vivo</i> <i>pus4</i> Δ replicate 3	pus4_tRNAs_rep3_10_26 _21_fast5s.tar.gz	pus4_tRNAs_rep3_10_26 _21_noU.fastq.gz

Table 6.4: **Direct RNA Sequencing Data Files.** **Sample** is the name of the sample as referred to in Dennis and Conoan Nieves et. al. 2025. **Fast5** and **FastQ** file are the names of the files containing the basecalled sequencing data for the specified sample. Files from this study are available at the European Nucleotide Archive (<https://www.ebi.ac.uk/ena>) with study accession number PRJEB85651. Files from Shaw and Thomas et. al., 2024 reanalyzed in this study are available with study accession number PRJEB68201

CHAPTER VII

CONCLUDING REMARKS

RNA structure and function are essential to life. Structure determines function and single-molecule microscopy provides information-rich techniques to elucidate the behavior of RNAs and their interactions with other biological materials on the millisecond time scale. In my work, i have demonstrated several ways of accessing information regarding the structure and dynamics employing the single-molecule instrument i custom-built in-house. I studied two non-coding RNAs: an NAD^+ riboswitch and its interaction with the small molecule NAD^+ as well as a tRNA and its interactions with the protein Pus4. My findings are summarized in this chapter.

The Class II NAD^+ -riboswitch rapidly samples many conformations in the absence of Mg^{2+} or ligand. The number of states being actively sampled, particularly those with low FRET efficiencies, is reduced in the presence of Mg^{2+} and collapses to three with 1 mM β -NMN. Our data supports the 2:1 ligand:RNA binding stoichiometry observed in one published crystal structure,¹³ whereas stable RNA dimers were not found in solution in the absence of divalent cation or ligand. I further demonstrated the potential impact that fluorophore labeling may have on RNA function in that one labeled construct was rendered incapable of binding its ligand. As an extension of my published work, my smFRET β -NMN concentration course study neither supports nor refutes the noncooperativity of the two ligand-binding sites on the RNA. However, my results suggest that NAD^+ -II behavior before and after interacting with nicotinamide-containing compounds is

reversible. Finally, i have shown that the adenosine moiety, previously assumed to be ignored by the riboswitch, does impact ligand recognition. However, there are not significant differences in the histogram and TODP results for the ligand with the modified adenine, ϵ -NAD⁺, compared to NAD⁺.

This riboswitch has potentially two useful capabilities for future projects: one regarding instrumentation/method development and another as a potential antibiotic therapeutic target. For the first topic, the rapid interconversion of conformational states of apo form RNA suggests that it is a good model system to use to improve the temporal resolution of our single-molecule instrument and data analysis pipeline. Instrument parameter optimization would include reducing the region of interest to enable an increased frame rate. The appropriate laser powers to achieve a desirable S:N would also need to be determined. Regarding data analysis, improving the HMM process could entail establishing a protocol or flow-chart for selecting appropriate (ideally unbiased) HMM inputs for the number of states for a system that samples more than three. Secondly, the results of my investigation of the NAD⁺ riboswitch reveals behavior indicate that it may be a good candidate for drug targeting in that a drug could modify one part of NAD⁺ without interfering with the ability of the riboswitch to sense it. Inhibition of a riboswitch responsible for critical protein production in a pathogenic bacterial cell could shut down such replication and halt bacterial propagation.

In the study of yeast tRNA^{eMet(CAU)}, we demonstrated the structural fluctuations between high- and low-FRET states, including two similar cloverleaf structures independent of pseudouridylation –showcasing the sensitivity of our single-molecule instrument. Upon addition of and following catalysis by Pus4 WT, the tRNAs are rearranged structurally while the protein stays bound for more

than 30 minutes. The catalytically inactive mutant, Pus4 R286K, also affects the conformational landscape of the tRNA, albeit differently compared to Pus4 WT. We hypothesized that pseudouridylation by Pus4 WT followed by remodeling makes the tRNA amenable to further modification by subsequent modification enzymes.

REFERENCES CITED

- (1) O'Donoghue, P.; Ling, J.; ; Söll, D. *RNA Biology* **2018**, *15*, 423–426.
- (2) Joseph, S. In *Encyclopedia of Cell Biology*; Bradshaw, R. A., Stahl, P. D., Eds.; 2016; pp 384–395.
- (3) Neil, C. R.; Fairbrother, W. G. *RNA structure and splicing regulation* **2019**, *1862*, 194439.
- (4) Pitchiaya, S.; Heinicke, L. A.; Custer, T. C.; Walter, N. G. *Chemical Reviews* **2014**, *114*, 3224–3265.
- (5) Graham, S. *The Biochemist* **2016**, *38*, 4–7.
- (6) Vicens, Q.; Kieft, J. S. *Proceedings of the National Academy of Sciences* **2022**, *119*, e2112677119.
- (7) Butcher, S. E.; Pyle, A. M. *Accounts of Chemical Research* **2011**, *44*, 1302–1311.
- (8) Perrin, F. *Ann. Phys.* **1932**, *10*, 283–314.
- (9) Förster, T. *Annalen der Physik* **1948**, *2*, 55–75.
- (10) Dale, R.; Eisinger, J.; Blumberg, W. *Biophysical Journal* **1979**, *26*, 161–193.
- (11) VanDerMeer, B. W. *Methods and Applications in Fluorescence* **2020**, *8*.
- (12) Xu, X.; Egger, M.; Li, C.; Chen, H.; Micura, R.; Ren, A. *Nucleic Acids Research* **2023**, *51*, 54–67.
- (13) Peng, X.; Liao, W.; Lin, X.; Lilley, D. M. J.; Huang, L. *Nucleic Acids Research* **2023**, *51*, 2904–2914.
- (14) Hua, B.; Han, K. Y.; Zhou, R.; Kim, H.; Shi, X.; AbeySirigunawardena, S. C.; Jain, A.; Singh, D.; Aggarwal, V.; Woodson, S. A.; Ha, T. *Nature Methods* **2014**, *11*, 1233–1236.
- (15) Aitken, C. E.; Marshall, R. A.; Puglisi, J. D. *Biophysical Journal* **2008**, *94*, 1826–1835.
- (16) Milescu, L.; Nicolai, C.; Bannen, J. QuB Software. 2000.

- (17) Nicolai, C.; Sachs, F. *Biophysical Reviews and Letters* **2013**, *08*, 191–211.
- (18) Sahl, S. J.; Moerner, W. *Protein-carbohydrate interactions / Biophysical methods* **2013**, *23*, 778–787.
- (19) Bustamante, C. J.; Chemla, Y. R.; Liu, S.; Wang, M. D. *Nature Reviews Methods Primers* **2021**, *1*, 25.
- (20) Ha, T.; Enderle, T.; Ogletree, D. F.; Chemla, D. S.; Selvin, P. R.; Weiss, S. *Proceedings of the National Academy of Sciences of the United States of America* **1996**, *93*, 6264–6268.
- (21) Selvin, P.; Ha, T. *Single Molecule Techniques: A Laboratory Manual*; Cold Spring Harbor, 2008.
- (22) Harbaugh, S.; Goodson, M.; Chushak, Y.; Chávez, J.; Kelley-Loughnane, N. In *Handbook of Cell Biosensors*; Thouand, G., Ed.; 2022; pp 111–140.
- (23) Panchapakesan, S. S. S.; Corey, L.; Malkowski, S. N.; Higgs, G.; Breaker, R. R. *RNA* **2021**, *27*, 99–105.
- (24) Roy, R.; Hohng, S.; Ha, T. *Nature Methods* **2008**, *5*, 507–516.
- (25) Holden, S. J.; Uphoff, S.; Hohlbein, J.; Yadin, D.; Le Reste, L.; Britton, O. J.; Kapanidis, A. N. *Biophysical Journal* **2010**, *99*, 3102–3111.
- (26) Hoehner, J. E.; Sande, N. E.; Widom, J. R. *Photochemistry and Photobiology* **2024**, *100*, 419–433.
- (27) Suddala, K. C.; Rinaldi, A. J.; Feng, J.; Mustoe, A. M.; Eichhorn, C. D.; Liberman, J. A.; Wedekind, J. E.; Al-Hashimi, H. M.; Brooks, I., Charles L.; Walter, N. G. *Nucleic Acids Research* **2013**, *41*, 10462–10475.
- (28) Suddala, K. C.; Wang, J.; Hou, Q.; Walter, N. G. *Journal of the American Chemical Society* **2015**, *137*, 14075–14083.
- (29) Widom, J. R.; Nedialkov, Y. A.; Rai, V.; Hayes, R. L.; Brooks, C. L., III; Artsimovitch, I.; Walter, N. G. *Molecular Cell* **2018**, *72*, 541–552.e6.
- (30) Stallings, S.; Rieger, B. *Optics Express* **2010**, *18*, 24461–24476.
- (31) Huang, L.; Wang, J.; Lilley, D. M. *RNA* **2020**, *26*, 878–887.
- (32) Lorenz, C.; Lünse, C. E.; Mörl, M. *Biomolecules* **2017**, *7*, 35.

- (33) Zhang, J.; Ferré-D'Amaré, A. R. *Life* **2016**, *6*, 3.
- (34) Biela, A.; Hammermeister, A.; Kaczmarczyk, I.; Walczak, M.; Koziej, L.; Lin, T.-Y.; Glatt, S. *Journal of Biological Chemistry* **2023**, 299.
- (35) Robertus, J. D.; Ladner, J. E.; Finch, J. T.; Rhodes, D.; Brown, R. S.; Clark, B. F. C.; Klug, A. *Nature* **1974**, *250*, 546–551.
- (36) Shi, H.; Moore, P. B. *RNA* **2000**, *6*, 1091–1105.
- (37) Shaw, E. A.; Thomas, N. K.; Jones, J. D.; Abu-Shumays, R. L.; Vaaler, A. L.; Akeson, M.; Koutmou, K. S.; Jain, M.; Garcia, D. M. *Nucleic Acids Research* **2024**, *52*, 12074–12092.
- (38) Cappannini, A.; Ray, A.; Purta, E.; Mukherjee, S.; Boccaletto, P.; Moafinejad, S. N.; Lechner, A.; Barchet, C.; Klaholz, B. P.; Stefaniak, F.; Bujnicki, J. M. *Nucleic Acids Research* **2024**, *52*, D239–D244.
- (39) Carlile, T. M.; Martinez, N. M.; Schaening, C.; Su, A.; Bell, T. A.; Zinshteyn, B.; Gilbert, W. V. *Nature Chemical Biology* **2019**, *15*, 966–974.
- (40) Lin, T.-Y. et al. *Molecular Cell* **2024**, *84*, 2472–2489.e8.
- (41) Purchal, M. K.; Eyler, D. E.; Tardu, M.; Franco, M. K.; Korn, M. M.; Khan, T.; McNassor, R.; Giles, R.; Lev, K.; Sharma, H.; Monroe, J.; Mallik, L.; Koutmos, M.; Koutmou, K. S. *Proceedings of the National Academy of Sciences* **2022**, *119*, e2109708119.
- (42) Behm-Ansmant, I.; Urban, A.; Ma, X.; Yu, Y.-T.; Motorin, Y.; Branlant, C. *RNA* **2003**, *9*, 1371–1382.
- (43) Grünberg, S.; Doyle, L. A.; Wolf, E. J.; Dai, N.; Jr, I. R. C.; Yigit, E.; Stoddard, B. L. *PLOS ONE* **2023**, *18*, e0291267.
- (44) Roovers, M.; Hale, C.; Tricot, C.; Terns, M. P.; Terns, R. M.; Grosjean, H.; Droogmans, L. *Nucleic Acids Research* **2006**, *34*, 4293–4301.
- (45) Gurha, P.; Joardar, A.; Chaurasia, P.; Gupta, R. *RNA Biology* **2007**, *4*, 101–109.
- (46) Ansmant, I.; Massenet, S.; Grosjean, H.; Motorin, Y.; Branlant, C. *Nucleic Acids Research* **2000**, *28*, 1941–1946.
- (47) Ansmant, I.; Motorin, Y.; Massenet, S.; Grosjean, H.; Branlant, C. *Journal of Biological Chemistry* **2001**, *276*, 34934–34940.

- (48) Zhou, J.; Liang, B.; Li, H. *RNA* **2011**, *17*, 244–250.
- (49) Tillault, A.-S.; Schultz, S. K.; Wieden, H.-J.; Kothe, U. *Journal of Molecular Biology* **2018**, *430*, 1284–1294.
- (50) Schaening-Burgos, C.; LeBlanc, H.; Fagre, C.; Li, G.-W.; Gilbert, W. V. *PLOS Genetics* **2024**, *20*, e1011100.
- (51) Carlile, T. M.; Rojas-Duran, M. F.; Zinshteyn, B.; Shin, H.; Bartoli, K. M.; Gilbert, W. V. *Nature* **2014**, *515*, 143–146.
- (52) Urban, A.; Behm-Ansmant, I.; Branlant, C.; Motorin, Y. *Journal of Biological Chemistry* **2009**, *284*, 5845–5858.
- (53) Lecointe, F.; Simos, G.; Sauer, A.; Hurt, E. C.; Motorin, Y.; Grosjean, H. *Journal of Biological Chemistry* **1998**, *273*, 1316–1323.
- (54) Motorin, Y.; Keith, G.; Simon, C.; Foiret, D.; Simos, G.; Hurt, E.; Grosjean, H. *RNA* **1998**, *4*, 856–869.
- (55) Gurha, P.; Gupta, R. *RNA* **2008**, *14*, 2521–2527.
- (56) Mukhopadhyay, S.; Deogharia, M.; Gupta, R. *RNA* **2021**, *27*, 66–79.
- (57) Phannachet, K.; Huang, R. H. *Nucleic Acids Research* **2004**, *32*, 1422–1429.
- (58) Gu, X.; Yu, M.; Ivanetich, K. M.; Santi, D. V. *Biochemistry* **1998**, *37*, 339–343.
- (59) Gutgsell, N.; Englund, N.; Niu, L.; Kaya, Y.; Lane, B. G.; Ofengand, J. *RNA* **2000**, *6*, 1870–1881.
- (60) Keffer-Wilkes, L. C.; Veerareddygar, G. R.; Kothe, U. *Proceedings of the National Academy of Sciences* **2016**, *113*, 14306–14311, Publisher: Proceedings of the National Academy of Sciences.
- (61) Gc, K.; Gyawali, P.; Balci, H.; Abeysirigunawardena, S. *ChemBioChem* **2020**, *21*, 1885–1892.
- (62) Johansson, M. J. O.; Byström, A. S. *RNA* **2002**, *8*, 324–335.
- (63) Copela, L. A.; Chakshumathi, G.; Sherrer, R. L.; Wolin, S. L. *RNA* **2006**, *12*, 644–654.
- (64) Keffer-Wilkes, L. C.; Soon, E. F.; Kothe, U. *Nucleic Acids Research* **2020**, *48*, 7981–7990.

- (65) Abramson, J. et al. *Nature* **2024**, *630*, 493–500.
- (66) Friedt, J.; Leavens, F. M. V.; Mercier, E.; Wieden, H.-J.; Kothe, U. *Nucleic Acids Research* **2014**, *42*, 3857–3870.
- (67) Dammertz, K.; Hengesbach, M.; Helm, M.; Nienhaus, G. U.; Kobitski, A. Y. *Biochemistry* **2011**, *50*, 3107–3115.
- (68) Kobitski, A. Y.; Hengesbach, M.; Helm, M.; Nienhaus, G. U. *Angewandte Chemie International Edition* **2008**, *47*, 4326–4330.
- (69) Kobitski, A. Y.; Hengesbach, M.; Seidu-Larry, S.; Dammertz, K.; Chow, C. S.; van Aerschot, A.; Nienhaus, G. U.; Helm, M. *Chemistry & Biology* **2011**, *18*, 928–936.
- (70) Voigts-Hoffmann, F.; Hengesbach, M.; Kobitski, A. Y.; van Aerschot, A.; Herdewijn, P.; Nienhaus, G. U.; Helm, M. *Journal of the American Chemical Society* **2007**, *129*, 13382–13383.
- (71) Arluison, V.; Buckle, M.; Grosjean, H. *Journal of Molecular Biology* **1999**, *289*, 491–502.
- (72) Lovejoy, A. F.; Riordan, D. P.; Brown, P. O. *PLOS ONE* **2014**, *9*, e110799.
- (73) Schwartz, S.; Bernstein, D. A.; Mumbach, M. R.; Jovanovic, M.; Herbst, R. H.; León-Ricardo, B. X.; Engreitz, J. M.; Guttman, M.; Satija, R.; Lander, E. S.; Fink, G.; Regev, A. *Cell* **2014**, *159*, 148–162.
- (74) Begik, O.; Lucas, M. C.; Pryszcz, L. P.; Ramirez, J. M.; Medina, R.; Milenkovic, I.; Cruciani, S.; Liu, H.; Vieira, H. G. S.; Sas-Chen, A.; Mattick, J. S.; Schwartz, S.; Novoa, E. M. *Nature Biotechnology* **2021**, *39*, 1278–1291.
- (75) Conoan Nieves, N. E.; Widom, J. R. *Chemical Communications* **2024**,
- (76) Barraud, P.; Tisné, C. *IUBMB Life* **2019**, *71*, 1126–1140.
- (77) Blanco, M.; Walter, N. G. In *Methods in Enzymology*; Walter, N. G., Ed.; Single Molecule Tools: Fluorescence Based Approaches, Part A; Academic Press, 2010; Vol. 472; pp 153–178.
- (78) Cole, P. E.; Crothers, D. M. *Biochemistry* **1972**, *11*, 4368–4374.
- (79) Bhaskaran, H.; Taniguchi, T.; Suzuki, T.; Suzuki, T.; Perona, J. J. *Biochemistry* **2014**, *53*, 1456–1465.

- (80) Pan, H.; Agarwalla, S.; Moustakas, D. T.; Finer-Moore, J.; Stroud, R. M. *Proceedings of the National Academy of Sciences* **2003**, *100*, 12648–12653.
- (81) Hoang, C.; Ferré-D’Amaré, A. R. *Cell* **2001**, *107*, 929–939.
- (82) Kim, S. H.; Sussman, J. L.; Suddath, F. L.; Quigley, G. J.; McPherson, A.; Wang, A. H. J.; Seeman, N. C.; Rich, A. *Proceedings of the National Academy of Sciences* **1974**, *71*, 4970–4974.
- (83) Lai, L. B.; Lai, S. M.; Szymanski, E. S.; Kapur, M.; Choi, E. K.; Al-Hashimi, H. M.; Ackerman, S. L.; Gopalan, V. *Proceedings of the National Academy of Sciences* **2022**, *119*, e2119529119.
- (84) Cole, P. E.; Yang, S. K.; Crothers, D. M. *Biochemistry* **1972**, *11*, 4358–4368.
- (85) Chan, P. P.; Lowe, T. M. *Nucleic Acids Research* **2009**, *37*, D93–D97.
- (86) Chan, P. P.; Lowe, T. M. *Nucleic Acids Research* **2016**, *44*, D184–D189.
- (87) Qin, F. *Biophysical Journal* **2004**, *86*, 1488–1501.
- (88) Gruber, A. R.; Lorenz, R.; Bernhart, S. H.; Neuböck, R.; Hofacker, I. L. *Nucleic Acids Research* **2008**, *36*, W70–W74.
- (89) McCann, H. et al. *Nucleic Acids Research* **2025**, *53*, gkaf032.
- (90) Giaever, G. et al. *Nature* **2002**, *418*, 387–391.