**BACTERIAL RIBOSOME CONFORMATIONAL
DYNAMICS DURING
TRANSLATION TERMINATION AND RESCUE**

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by

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TRANSLATION TERMINATION AND RESCUE

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DEDICATION

I dedicate this dissertation to my awesome wife who has supported me throughout this entire process in ways that words cannot describe. From motivating me to wake up early, to supporting my going to the lab for a second shift and working late hours to get the job done. You are an awesome young woman and I look forward to riding the rollercoaster of life with you.

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ABSTRACT

Hydrolysis of polypeptide from the ribosome is a critical step that must occur prior to the ribosome recycling phase of translation. Inability of cells to do so can result in translation inhibition and eventually leading to cell death. In bacteria, class one release factors bind to the ribosome to catalyze the release of the mature polypeptide during translation termination. However, in the event of ribosome stalling as a result of mRNA truncation, ribosome rescue factors bind to the ribosome to catalyze the release of the growing polypeptide from the stalled complex. This rescue process is then followed by ribosome recycling. Here we employ smFRET to study the effects of the class I release factors, RF1 and RF2, and an alternative release factor known as YaeJ on the conformational dynamics of the ribosome following hydrolysis of peptidyl tRNAs. Further, we investigated the role of A-site mRNA on the global conformation of the ribosome. Our results demonstrate that upon binding to their cognate stop codon, the class I release factors stabilize ribosome complexes in the non-rotated state. Similarly, binding of YaeJ to complexes that are assembled on truncated mRNAs resulted in ribosomes that occupy primarily the non-rotated state. We also observe that absence of mRNA in the A-site induces a hyper-rotated conformation between the two subunits. Together, these findings further characterize the interactions between these different ligands and the bacterial ribosome. In addition, these results suggest that stabilization of the ribosome in the non-rotated state is critical for priming the ribosome for the recycling phase of translation.

PRELUDE

In the first chapter we provide an overview of bacterial translation while emphasizing recent significant advances made in the field with the help of single molecule Förster Resonance Energy Transfer (smFRET). We provide a detailed description of bacterial translation termination and ribosome rescue via the YaeJ pathway.

In the second chapter we further characterize the intersubunit rotation of the ribosome using smFRET. We build various ribosome constructs using six different mRNA and tRNA^{fMet} to determine the effects of the class I release factors and an alternative release factor known as YaeJ on the intersubunit rotation of the ribosome. Our results indicate that when these factors bind to the ribosome effectively, they freeze the ribosome in a non-rotated state.

In the third chapter we visualize different ribosome constructs when assembled on varying lengths of truncated mRNA. Further, we determine the effects of mRNA length on the intersubunit rotation of these ribosomes. We show that when the A-site is emptied the ribosome occupies a hyper-rotated state in addition to the rotated and non-rotated states.

In the fourth chapter we provide a brief summary of our major advances in the field regarding translation termination and the ribosome rescue pathway mediated by YaeJ. Lastly, we propose some experiments that can be performed in the future regarding translation termination, ribosome rescue, and improvement in smFRET technique.

CHAPTER 1

INTRODUCTION

1.1 Background

In cells, protein synthesis is carried out by essential macromolecular machines known as ribosomes. In *Escherichia coli*, each ribosome consists of a large 50S subunit and small 30S subunit, which together form the 70S ribosome during translation (**Figure 1.1**). The translation process has been divided into four main phases, which include the initiation, the elongation, the termination, and the ribosome recycling phase. Each of these phases is characterized by a number of factors that interact with the ribosome. These factors are known collectively as the initiation, the elongation, the termination, and the ribosome recycling factors. They bind primarily to three sites within the ribosome which are the acceptor (A-site), the peptidyl (P-site), and the exit site (E-site). While some of these factors alter the structure of their respective binding sites, others induce large scale conformational changes in highly dynamic regions of the ribosome. Hence, these factors are at the center of the major ribosome conformational changes that drive protein synthesis.

1.1.1 Ribosome dynamics

Experimental evidence demonstrated the structure of this macromolecular machinery contains highly dynamic regions. Some of these regions include the L1 stalk, the head of the small subunit, and the intersubunit rotation of the ribosome. Elucidation of these motions is driven primarily by developments and advancements in single

molecule techniques such as X-ray crystallography, and cryo-electron microscopy (cryo-EM). In recent years, however, single molecule Förster resonance energy transfer (smFRET) has been used extensively to further characterize these different regions of the ribosome. Various laboratories have employed smFRET to investigate the dynamics of the ribosome in every aspect of translation ranging from initiation to ribosome recycling. Further, this technique has been very useful at providing precise information about specific regions of the ribosome in real-time. Highlights of the significant advances made in the field of ribosome dynamics after the introduction of the smFRET technique nearly two decades ago are presented here. Also, in chapters two and three we report data that were obtained by using smFRET as the major technique.

1.1.2 SmFRET technique

Since its debut in 1996, the *smFRET* technique has been used to answer a variety of biological questions involving macromolecular complexes¹⁻³. In FRET a donor fluorescent molecule transfers energy to an acceptor fluorescent molecule upon excitation^{1, 4}. The distance between the two fluorescent molecules can be determined based on the ratio of the intensity of the acceptor molecule and the total emission intensity that is observed^{1, 4}. The measurement can be performed by tracking the FRET changes that take place over time. Both confocal microscopy and total internal reflection fluorescence (TIRF) microscopy have been used to detect FRET in smFRET studies¹⁻⁴. As a ratiometric method, smFRET is advantageous in that it enables measurements of internal distance with less noise and drift in a molecular frame as opposed to measurements in a laboratory frame.

1.2 Methodology

Twice, in 1974 and again 2006, the Nobel Prize was awarded for groundbreaking understanding of the ribosome^{5, 6}. Structural and biochemical studies performed by these Nobel laureates and many other researchers generated a massive wealth of knowledge regarding how the translation machinery works⁷⁻¹¹. Their findings elucidated various mechanistic details of the translation process and the structural compositions of the ribosome. Hence, it is worth mentioning that these studies pioneered the usage of smFRET in exploring the mechanisms of translation¹².

X-ray crystallography demonstrated that the L1 stalk of the ribosome can exist in an opened or a closed conformation based on the occupancy of the E-site¹³⁻¹⁹. In later studies, several groups used smFRET to demonstrate that the L1 stalk motion is in fact highly dynamic as it mediates deacylated tRNA release from the E-site^{18, 20, 21}. Further, Bretscher and Spirin independently hypothesized that mRNA-tRNA translocation within the ribosome was coupled with intersubunit rotation^{22, 23}. Later, this hypothesis was tested via chemical footprinting and cryo-EM; the findings from these studies revealed that this translocation/rotation process was coupled with a ratchet-like motion between the two subunits^{10, 11}. Remarkably, in 2008 Cornish et al. used smFRET to visualize the two subunits of the ribosome in real time. This group demonstrated that, indeed, the subunits of the ribosome occupied a rotated and a non-rotated conformation as it interacted with different ligands²⁴. Together, these studies showed that this newly emerging technique, smFRET, can be a practical tool for characterizing various motions that occur within the ribosome during translation.

Investigation of the various large-scale motions that occur within the ribosome throughout translation using smFRET requires a few steps. First, the two sites of interest that are being examined for interactions within the ribosome are tagged with donor and acceptor fluorophores. Second, ribosomes complexes are immobilized on a slide (quartz) surface that had been treated to prevent non-specific binding. Finally, TIRF microscopy and a high-speed camera are used to visualize ribosome complexes following excitation of the donor dye with a laser. Signals from the acceptor dye implicate energy transfer. These major steps are described below from the preparation of the ribosomes to the analysis of the data obtained. In these descriptions, the ribosome subunit rotation as it relates to the elongation phase of translation is the motion of interest.

1.2.1 1- Ribosome preparation for monitoring intersubunit rotation

The rapid increase in knowledge regarding the mechanisms of translation and the ribosome's structural composition has stimulated further related investigations. Driven by the high level of interest in learning more about the ribosome, Ederth et al. developed a novel approach to purify active ribosome complexes using affinity chromatography²⁵. This method is consisted of an *E. coli* strain which was genetically engineered to have a 6(His)-tag fused to the C-terminus of L12 ribosomal protein²⁵. Use of this strain enabled ribosome purification using His-tag affinity purification protocols. This novel approach was demonstrated to be effective at improving the way in which ribosomes can be purified for either structural or biochemical studies²⁶.

Our group further modified the His-tag method to prepare ribosomes that were suitable for investigating subunit rotation via smFRET. Briefly, an *E. coli* strain was engineered to lack the genes that code for S6 (*rpsF*) and L9 (*rplI*) ribosomal proteins in

addition to having the L12-6×His Tag modification. These proteins, S6 and L9, are late assembly proteins and are nonessential for bacterial survival²⁷⁻³¹. Further, the S6 protein is one of many proteins that bind to the 16S rRNA of the small subunit, whereas L9 along with many other proteins bind to the 23S rRNA of the large subunit of the ribosome. Purification of ribosomes from this bacterial strain yielded S6 and L9 depleted ribosomes. Subsequently, S6 D41C and L9 N11C mutant ribosomal proteins that were tagged, respectively, with an acceptor and a donor fluorophore and assembled into the purified S6/L9 depleted ribosomes. Addition of these fluorescently labeled ribosomal proteins to the small and the large subunits resulted in S6(acceptor dye)/L9(donor dye) fluorescently labeled ribosomes. Extensive activity and the structural dynamic testing of these ribosome complexes revealed that they were comparable with previous studies that used long and complicated purification protocols^{25, 26, 32, 33}.

1.2.2 2- Ribosome constructs immobilization

Though various methods have been used to immobilize macromolecules onto a slide surface, a common method for immobilizing ribosome complexes on a quartz slide involves using either the 5' or the 3' end of the mRNA^{4, 24}. As part of the immobilization process, a biotinylated riboprimer is usually annealed to one end of the mRNA. The biotin molecule that is covalently attached to the riboprimer can then be used to tether the complex on passivated quartz slide. Passivation of the quartz slide requires extensive cleaning and preparation of the surfaces to eliminate non-specific binding^{4, 34-36}. Several methods have been used for slide preparation. In the past, passivation of slide surfaces involved usage of polyethylene glycol (PEG)^{34, 37, 38}. This method had been used by a large number of groups that demonstrated it was effective at significantly reducing non-

specific binding of macromolecule to the slide^{24, 26, 34, 39}. Recently a new passivation method using dichlorodimethylsilane–Tween-20 was shown to reduce non-specific binding to an even greater extent³⁵. However, fluorescence intensities derived from impurities are still seen in both methods³⁵. Nonetheless, both approaches are effective for smFRET studies of macromolecules. Once slides are passivated with a mixture of PEG and biotin-PEG, a solution that contains neutravidin can be used to facilitate the tethering of ribosome constructs to the slides. In this process, neutravidin molecules simultaneously form covalent interactions with the biotin molecule on the PEG and the biotin molecule attached to the primer. The K_d for the interaction between the biotin molecules and the neutravidin is about 1×10^{-15} M⁴⁰. Thus, these strong interactions facilitate immobilization of ribosomes to the slide and enable exchange of components in the buffer without disturbing the binding to the surface.

1.2.3 3- Visualization of fluorescently labeled constructs

Following immobilization of the ribosome and exchange of the assembly buffer with the imaging buffer containing ligands of interest, the complexes are visualized using the pre-assembled smFRET instruments. The assembly of this instrument requires various components and can be customized by the builder^{4, 41, 42}. Briefly, a laser source is required to (i.e. Nd:YAG) excite the donor fluorophore. Once excited, the energy from the excited fluorophore is transferred to the acceptor fluorophore^{4, 41, 42}. Then, TIRF microscopy with either oil or a water emersion lens is used to collect the total internal reflection fluorescence signals emitted by the donor and acceptor fluorophores^{41, 42}. The setup, which requires the oil emersion lens, is referred to as an objective-type TIRF, whereas the setup that uses the water emersion lens is referred to as a prism-type TIRF⁴².

In the prism-type TIRF a prism is placed above the slide to perform each experiment⁴¹. In this process, the fluorescence emission from the donor and acceptor fluorophores are guided through the objective lenses and different sets of mirrors which include dichroic mirrors. The dichroic mirrors are used to separate the donor and acceptor fluorescence signals. Once separated, these signals are recorded using either an avalanche photodiode (APD) or an electron multiplying charge coupled device (EMCCD) camera. Subsequently, time traces are extracted from these movies using interactive data language (IDL) scripts, and further analysis is performed with Matlab software. Interpretation of the extracted time traces can be used to explain the motion of interest. Also, additional analysis of the time traces can be performed to obtain information such as the rate of fluctuation between distinctive motions.

1.3 Application of smFRET in translation

1.3.1 Initiation

Extensive structural analysis of 30S and 50S subunits interactions showed that in the 70S ribosome complex the two subunits are connected via twelve bridges⁴³⁻⁴⁸. These bridges consist of a network of RNA-RNA, RNA-protein, and protein-protein interactions which are mediated by hydrogen bonds and electrostatic forces⁴³⁻⁴⁸. Formation of the bridges occurs in the later stages of the initiation phase of translation during the stage of 70S assembly^{43, 44}. This process begins when the anti-Shine-Dalgarno region (near the 3' end of the 16S rRNA in 30S subunit) binds to the Shine-Dalgarno region of the mRNA. This binding is aided by initiation factors (IFs) IF1 and IF3 that guide the mRNA AUG-start codon into the P-site of the ribosome (**Figure 1.1 and 1.2**)⁴⁹⁻⁵⁴. Subsequently, IF2-GTP directs the binding of fMet-tRNA^{fMet} and recruits the 50S

subunit, facilitating docking at pre-initiation to form the complete translation initiation complex⁴⁹⁻⁵⁴. After the hydrolysis of GTP, these initiation factors exit and the ribosome transitions to the elongation phase of translation⁵¹⁻⁵⁴. This sequence of event enables translation to initiate at the correct reading frame, thus contributes to fidelity in translation initiation^{55, 56}. Also, hydrolysis of the GTP by IF2 yields the energy required for overcoming the activation energy for ribosome complexes to enter translation. As the committed step of translation, the 30S pre-initiation complex is an attractive target for antibiotics aiming at inhibiting translation^{57, 58}. Therefore, understanding the mechanistic details of the IFs in 70S initiation complex formation is important for antibiotic design and to understand the mode of action of antibiotics that inhibit this crucial aspect of translation.

The application of smFRET in translation initiation has significantly contributed to our understanding of pre-initiation and initiation complexes. In a recent smFRET study Elvekrog et al. elucidated the various conformational changes that IF3 undergoes during the early stages of translation initiation⁵⁹. This group monitored the FRET changes between the N-terminal domain and the C-terminal domain of IF3 bound to the 30S pre-initiation complex and determined that IF3 exist in a conformational equilibrium among three different states. These conformational changes are characterized as being compact, intermediate, or extended. While in the presence of IF1, the IF3 favors the intermediate conformation; in the presence of IF2 the IF3 favors the compact conformation. However, in a more physiologically relevant manner, (meaning in the presence of both IF1 and IF2 in a preinitiation complex) IF3 appears to favor the extended and intermediate form⁵⁹. In a follow up study, the function of IF3 was further investigated to determine its role in the

formation of 30S and 50S subunit joining to form the mature 70S initiation complex⁵⁰.

The smFRET experiments from the aforementioned study demonstrate that IF3 modulates the strength of the interaction between the 30S and the 50S subunit of the ribosome during translation initiation.

1.3.2 Elongation

Once the initiation complex is formed, the 70S ribosome enters the elongation phase of translation. In this phase, the ribosome interacts with various elongation factors **(Figure 1.1)**⁶⁰⁻⁶². In addition, aminoacylated-tRNA molecules bind the ribosome following recognition of a codon in the A-site. Each tRNA molecule serves as an adapter that carries the amino acid which is to be added to the growing peptide chain. Binding of each aminoacylated-tRNA to the ribosome is mediated by the elongation EF-Tu at the expense of GTP hydrolysis following proper accommodation of the tRNA to the A-site⁶³. Structural and biochemical studies demonstrated that A-site tRNA exists in a classical state upon binding, and then undergoes a change to a hybrid (A/P) conformation once the peptide bond is formed⁶⁴⁻⁶⁹. Subsequently, the tRNA, initially in the P-site of the ribosome, moves to the E-site to exit the ribosome upon translocation. Simultaneously, the hybrid A/P peptidyl tRNA moves to the P-site leaving the A-site vacant for an incoming aminoacylated tRNA.

The elongation factor EF-G catalyzes the translocation of tRNAs by hydrolyzing GTP⁷⁰⁻⁷³. This entire process occurs repeatedly until a stop codon in the mRNA occupies the A-site. This is the signal for termination of translation, which is described below.

Effective interaction between the anticodon of the tRNA and each codon of the mRNA is a coordinated process which is critical for high fidelity during translation elongation^{74, 75}.

While some aspects of the elongation phase are spontaneous processes, energy is required for tRNA-mRNA translocation through the ribosome and for other large scale motions within the ribosome.

Recent developments in probing tRNA molecules and different parts of the ribosome have made it possible to further characterize the conformational dynamics of tRNAs with respect to the ribosome, other tRNAs, and the various elongation factors using smFRET. Employing smFRET, other researchers have visualized the classical and the hybrid conformation of tRNA when bound to the ribosome^{18, 20, 64, 76-79}. Further, these findings supported existing structural and biochemical evidence which relate to ribosome-tRNAs interactions. In addition, the smFRET studies yielded additional information that is only observed when structure investigations are performed in real time⁸⁰⁻⁸⁴. Finally, probing of tRNA molecules with fluorophores has been very useful at describing the mechanistic details of tRNA-tRNA interaction within the ribosome as tRNA translocation occurs^{79, 85, 86}. Consistent with structural and biochemical studies, these different smFRET studies demonstrate the different conformational rearrangements that tRNA molecules undergo while traversing the ribosome^{69, 70, 72, 87}.

In addition to its application in visualizing tRNA movements within the ribosome, smFRET has also been used to monitor other ribosomal large-scale motions which have been shown to be critical for translation. Of these various motions, the highly dynamic L1 stalk of the 50S ribosomal subunit was probed with fluorophores to determine its conformation during translation^{20, 86, 88, 89}. These real-time measurements allowed measurement of transition and fluctuation rates. Specific states were observed as different ligands bound to the ribosome at different stages of translation^{20, 86, 88, 89}. For instance,

aminoacylated tRNA in the P-site gave rise to the open conformation of the L1 stalk. In the open conformation, the L1 stalk was positioned away from the body of the 50S subunit of the ribosome, whereas in the closed conformation the L1 stalk was closer to the body of the 50S subunit of the ribosome. Another large-scale conformation involved the head region of the 30S subunit of the ribosome which had been shown to interact with the mRNA during translation^{45, 72, 90-92}. The head of the bacterial 30S subunit is one of six major regions (head, beak, neck, shoulder, platform, and spur) of the small subunit comprised of 16S rRNA and some small subunit ribosomal proteins^{7, 14, 46, 93, 94}.

A recent study used smFRET to visualize the rotational motion of the head region; the findings described the role of the head region in translocation⁹⁵. SmFRET was also used to identify the rotation between the large and the small subunit of the ribosome, described in the methodology section. This motion was regulated in part by the conformation of the nucleotide residues of helix 69 (H69) of the 23S rRNA and helix 44 (h44) of the 16S rRNA that form the B2 bridge (**Figure 1.3**). These nucleotide residues include A1913 of H69 interactions with A1492 and A1493 of h44⁹⁶. Structural studies demonstrated changes in the conformation of these residues between rotated and non-rotated ribosome complexes⁹⁶⁻¹⁰². The nucleotide residues A1492 and A1493 are stacked within h44 when ribosomes occupy the rotated conformation. However, in ribosomes that occupy the non-rotated conformation, A1493 of h44 stacks on A1913 of H69 while occupying the position of A1492 which flips out of h44 in that process (see **Figure 1.2**).

1.3.3 Termination

In bacteria, translation termination is signaled by a UAA, a UAG, or a UGA stop codon in the A-site of the ribosome. The presence of a stop codon in the A-site is

recognized by one of the class I release factors (RFs) which binds to the complex and catalyzes the hydrolysis of the peptide chain from the peptidyl tRNA (**Figure 1.1**)¹⁰³⁻¹⁰⁶. Although a majority of the translation factors that bind the ribosome at different stages of translation use GTP or other sources of energy for their functions, the release factors do not use an external energy source for their activity. The major requirement is a cognate stop codon in the A-site. In addition to the class I RFs, the peptidyl transferase center (PTC) also plays an important role in the hydrolysis of the peptide chain from the peptidyl-tRNA^{97, 103, 105, 107}. Thus, the mechanisms of peptide release rely on both the activity of the class I RFs and structural rearrangement at the PTC.

The class I RFs includes RF1 and RF2 which share structural similarities but have distinct differences (**Figure 1.4**). Both of these factors consist of 4 domains, domain 1, 2, 3, and 4¹⁰³⁻¹⁰⁶. Domain 1 interacts with both the 50S subunit, in the L11 region, and at the beak region of the 30S subunit of the ribosome¹⁰⁵. Based on a crystal structure of RFs bound to the ribosome, domain 1 can rotate away from domain 2 and 4 and this rotation results in domain 1 anchoring to L11¹⁰⁵.

Domain 2 contains the tripeptide motif that interacts with the stop codon in the A-site of the ribosome^{106, 108}. The amino acid sequence for the tripeptide motif for RF1 is PVT while in RF2 this sequence is SPF. These motifs provide specificity for the different stop codons and allow effective binding to the ribosome^{103-106, 109, 110}. Both RF1 and RF2 recognize the UAA stop codon; however, RF1 is specific for UAG while RF2 is specific for UGA. The specificity of the class I RFs can be compromised following mutations in these motifs^{108, 111}. Likewise, the function of these proteins can be interchanged if the two motifs are swapped^{108, 111}. Although the tripeptide motif is different in RF1 and RF2,

interactions with the stop codon are analogous to that of a tRNA anticodon with a sense codon.

Domain 3 of the class I RFs contains the residues glycine, glycine, and glutamine (GGQ at positions 228-230), that participate in the hydrolysis of the peptide chain from peptidyl tRNA. These residues form the GGQ motif in both RF1 and RF2 (**Figure 1.4**)¹⁰³⁻¹⁰⁶. Domain 3 is flexible, binds near the PTC and interacts with the CCA end of the tRNA. The degree of flexibility can be observed between individually crystallized RFs and RFs crystallized in while bound to the ribosome^{103-106, 112}. Also, this structural rearrangement of the class I RFs has been observed in a recent study using fluorescently labeled RF1¹¹³.

Domain 4 of the class I RFs interacts primarily with the beak of the 30S subunit¹⁰³⁻¹⁰⁶. Domains 2 and 4 form a superdomain that interacts with the A-site of the 30S subunit in the decoding center of the ribosome. Structural studies showed that domains 2, 3, and 4 in both RF1 and RF2 occupy the space where incoming aminoacylated tRNA bind to the ribosome during translation elongation. Thus, the similarities that exist between RF1 and RF2 and their interactions with the ribosome can help explain why the mechanism of peptide hydrolysis by these factors is strikingly similar.

In the crystal structures of RFs bound to ribosomes with a deacylated P-site tRNA, the backbone amide nitrogen of the glutamine residues of the GGQ motif is at hydrogen bonding distance from the 3' hydroxyl of A76 of the tRNA while the side chain of this residue is housed in a pocket at the PTC^{97, 107}. This pocket consists of residues A2451, C2452, and U2506 of the 23S rRNA. The structure of the termination complex suggests that a water molecule serves as a nucleophile that attacks the peptidyl tRNA

ester bond. This reaction in turn creates a transition state with an oxyanion. Then, the amide group of the glutamine residue of the GGQ motif forms a hydrogen bond with the oxyanion of the transition state analogue of the peptidyl-transferase. Formation of this hydrogen bond stabilizes this transition state which enables the peptide to become the leaving group. These steps are thought to be crucial and can drastically enhance the rate of catalysis^{114, 115}. In fact, mutation of this glutamine residue into a proline residue abolished the catalytic activity of these factors due to the absence of an amide group in proline residues^{116, 117}. Further, a proton shuttle mechanism has been proposed to describe the different steps of peptidyl-tRNA hydrolysis. Although the presence of a water molecule is required for this mechanism, the mode of transport for the water molecule into the PTC remains unknown. One speculation is that the GGQ motif brings along a water molecule while positioning itself at the PTC¹⁰⁷. Nevertheless, the PTC decreases the activation energy barrier of these different reactions by excluding additional molecules and only allows presence of one water molecule at the site catalysis. The PTC also provides the platform that brings the peptidyl-tRNA and the GGQ motif of peptide to close proximity^{97, 107}. If in the realm of possibility, a high-resolution structure of translation termination that illustrates a release factors bound to the ribosome prior to the release of the polypeptide would help further explain translation termination.

1.3.4 Recycling of the ribosome

The amount of energy that goes into the synthesis of the 2.5 megadalton bacterial ribosome is astounding; therefore, recycling of this macromolecular machinery at the termination of translation is evolutionarily advantageous. This process is facilitated by the class II RF RF3, ribosome recycling factors, Ef-G, and IF3. First, RF3 binds to the

termination complexes that contain one of the class I RFs and catalyzes the release of the class I RF (**Figure 1.1**). More specifically, the GTP-bound form of RF3 binds at the A-site near domain 1 of the class I RF; GTP hydrolysis by RF3 occurs allowing the release of the class I RF from the termination complex¹¹⁸. However, other studies suggest that RF3 is not required for ribosome recycling. Nevertheless, following dissociation of the class I RF the ribosome recycling factors (RRFs) and Ef-G-GTP bind to the ribosome to catalyze the dissociation of the large subunit from the small subunit at the expense of GTP hydrolysis.

Biochemical and structural studies have significantly advanced our knowledge regarding translation termination and ribosome recycling. Recent smFRET studies including our work in chapter three of this dissertation have shed light on the process of translation termination and ribosome recycling^{119, 120}. While monitoring tRNA dynamics with respect to the L1 stalk motion, two mechanistic models were provided to describe the L1 stalk motion and tRNA dynamics in these processes¹²⁰. Further, in the second chapter of this dissertation we used smFRET to demonstrate that class I RFs shift the equilibrium between non-rotated and rotated states of the ribosome to favor the non-rotated conformation upon effective binding. The non-rotated conformation is only favored when a cognate stop codon is present in the A-site.

Our work also demonstrated that the GGQ motif is not directly involved in influencing conformational dynamics. We also determined the rates and frequencies to which these factors influence the subunit rotation of ribosomes that interconvert between the rotated and the non-rotated conformations. These findings were consistent with previous structures of these factors when bound to the ribosome^{97, 103, 105-107, 109}. In an

independent study, Prabhakar et al were able to visualize the various translation termination and recycling factors to the ribosome while simultaneously monitoring intersubunit rotation of the ribosome¹¹⁹. Together these smFRET studies provided a detailed description of the conformational dynamics of the ribosome and describe a mechanistic detail of translation termination and ribosome recycling.

1.3.5 Ribosome stalling and rescue

A number of check points are in place to ensure efficiency and high fidelity in the translational machinery. Likewise, there are different pathways to assist the ribosome in translation defects that result in stalled ribosome complexes. Events that can result in ribosome stalling and yield nonstop complexes include mRNA damage, synthesis of truncated mRNA as a result of premature termination, or frameshifting, amongst many others¹²¹⁻¹²⁴. The ribosome must be rescued in each of these cases, and this is important for regulation of gene expression and cellular maintenance¹²⁵. In bacteria there are three main mechanisms to rescue ribosomes depending on the nature of the stalling. These mechanisms include the trans-translation system, ArfA, and ArfB (YaeJ)^{121, 122, 126-131}. The trans-translation system consists of transfer mRNA (tmRNA) and small protein B (SmpB). This system rescues ribosomes that are stalled on truncated mRNAs while simultaneously flagging the truncated peptide chain for degradation. ArfA is a small protein that binds to stalled ribosome complexes and recruits RF2 to hydrolyze the peptide chain from the stalled complex. YaeJ, on the other hand, binds to the ribosome and catalyze the release of the polypeptide chain from the peptidyl tRNA without the help of external factors; similarly to the class I release factors. Although these factors act independent of each other, they share structural similarities in addition to their functions

in the bacterial cell. Below we present a brief description of YaeJ and its role on the dynamics of the ribosome.

1.3.6 Structure

The alternative release factor B (ArfB), also known as YaeJ, is a relatively small protein comprised of 140 amino acids. Similar to the class I RFs, YaeJ contains a GGQ motif that it uses to catalyze the release of peptides from the peptidyl tRNA of stalled ribosome complexes (**Figure 1.5**). The crystal structure of the *E. coli* YaeJ bound to *Thermus thermophilus* 70S ribosome in the presence of tRNA^{fMet} and a truncated mRNA was solved recently to 3.2 angstroms by molecular replacement¹⁰⁰. This structure indicates that YaeJ consists of an N-terminal domain that interacts with the large subunit and a C-terminal tail that interacts with the small subunit of the ribosome upon binding. In this process, the GGQ motif located in the N-terminal domain is positioned at the PTC center. The C-terminal tail is positively charged and binds to the 30S subunit near the mRNA entrance tunnel. Also, this C-terminal tail occupies the mRNA the entrance tunnel which is downstream of the A-site of the 30S subunit of the ribosome. In addition to the N-terminal domain, YaeJ contains a helical C-terminal tail that is attached to the N-terminal domain by an unstructured linker. Although YaeJ shares some similarities with the class 1 release factors, it does not, however, have a domain that recognizes stop codons. Its interaction with stalled ribosome complexes depends primarily on the length of the mRNA that is downstream of the P-site codon.

1.3.7 Function/mechanism

Similar to the class I RFs, the GGQ motif of YaeJ has been extensively characterized biochemically^{100, 127, 132}. In YaeJ the GGQ residues stabilize the 3' CCA end of tRNA to facilitate the release of the growing polypeptide from the peptidyl tRNA. Stabilization of the tRNA CCA end reduces the activation energy barrier for the hydrolysis reaction to take place. Further, these residues activate the water molecule to become a nucleophile which is used to hydrolyze the ester bond between the polypeptide chain and the peptidyl tRNA. In addition to the GGQ motif, other residues required for the activity of YaeJ include Arg118, Leu119, Lys122, Lys129, and Arg132 at the C-terminal tail^{127, 133}. Furthermore, an Arg105Ala mutation in the linker region reduces the peptidyl hydrolysis activity of YaeJ. In contrast to the class I RFs, YaeJ uses its C-terminal tail to bind to the ribosome.

1.4 Final remarks and Conclusion

In the last few years, smFRET have gained popularity as it reveals critical dynamic structural information. This technique has also been used across disciplines to determine answers to a large number of biological questions. Furthermore, applications of smFRET allow us to know more about the conformational dynamics of the different interacting components and macromolecular machinery that are involved in each stage of life's central dogma. We have been able to use smFRET in studies that have direct implication in medicine. For instance, this technique has been used to study how different inhibitors might influence the conformational dynamics ribosome, DNA polymerase, and RNA polymerase. Although we have seen these interesting advancements being made in the field there is still room for improvement. Automation of the data analysis, compaction and simplification of the different components of the instrument to render it much more

cost effective and readily commercializable would be significant improvements to this technique.

1.5 References

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Figure 1.1: Simplified version of the translation process

The various phases of translation which include the initiation, the elongation, the termination, and the ribosome recycling phase are highlighted. Also, the different factors that are involved in each phase are pointed out. The different phases and their corresponding factors are color-coded. The 50S and the 30S subunits of the 70S ribosome are depicted in yellow and green respectively. This process begins with the assembly of the 30S (Yellow) and the 50S (Green) subunits of the ribosome on the mRNA along with the various initiation factors and ends with the release of the polypeptide from the ribosome and dissociation of the two subunits of the ribosome.

Figure 1.2: Interactions between the interface of the 50S and 30S to form the 70S ribosome

Surface structure of the 50S (yellow) and the 30S (green) ribosome subunits interfaces. The arrows indicate formation of the 70S as a result of the interactions between the 50S and the 30S interface during translation (PDB: 4V95).

Figure 1.3: Major residues of the B2-Bridge

Interactions between helix 69 of the 23S rRNA of the large subunit and helix 44 of the 16S rRNA of the small subunit to form the B2-Bridge (PDB: 4V95). The residues A1913 of H69 and A1493 along with A1492 of h44 are shown as sticks. These residues have direct implications in the B2-Bridge formation.

Figure 1.4: Structures of the class I release factors

Crystal structures of RF1 and RF2 (PDB: 4V63; PDB: 4V5J). The GGQ motifs for these two proteins are depicted in domain III. The PVT motif and the SPF motif for RF1 and RF2 respectively and are shown in domain II in each of these factors.

Figure 1.5: Structure of the ribosome rescue factor known as YaeJ

An illustration of the 70S bound form of YaeJ. Regions of the ribosome were removed to focus primarily on the different structural components of YaeJ. The GGQ motif is highlighted in the N-terminal domain. Also, both, the C-terminal tail and the linker that connects the C-terminal tail to the N-terminal domain are depicted.

CHAPTER 2

Operative binding of class I release factors and YaeJ stabilizes the ribosome in the non-rotated state

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2.1 Abstract

During translation, the small subunit of the ribosome rotates with respect to the large subunit primarily between two states as mRNA is being translated into a protein. At the termination of bacterial translation, class I release factors (RFs) bind to a stop codon in the A-site and catalyze the release of the peptide chain from the ribosome.

Periodically, mRNA is truncated prematurely and the translating ribosome stalls at the end of the mRNA forming a non-stop complex, requiring one of several ribosome rescue factors to intervene. One factor, YaeJ, shares structural homology with the catalytic region of RFs, but differs by binding to the ribosome directly through its C-terminal tail. Structures of the ribosome show that the ribosome adopts the non-rotated state conformation when these factors are bound. However, these studies do not elucidate the influence of binding on the dynamics of intersubunit rotation, whether to cognate or non-cognate codons. Here, we investigate the effects of wild-type and mutant forms of RF1, RF2 and YaeJ binding on ribosome intersubunit rotation using smFRET. We show that both RF1 and RF2 binding is sufficient to shift the population of post-hydrolysis ribosome complexes from primarily the rotated to the non-rotated state only when a cognate stop codon is present in the A-site. Similarly, YaeJ binding stabilizes non-stop ribosomal complexes in the non-rotated state. Along with previous studies, these results

are consistent with the idea that directed conformational changes and sequential factor binding to the ribosome are requisite for efficient termination and ribosome recycling.

2.2 Introduction

Translation is the process by which the ribosome uses mRNA as a template to synthesize a corresponding polypeptide chain. The mRNA template consists of consecutive sense codons that code for a specific protein. This sequence of sense codons is followed by a nonsense codon (UAA, UAG, or UGA) that signals for translational termination. Then, a release factor binds to the ribosome and catalyzes the release of the full-length peptide chain from the P-site tRNA. Upon binding, the release factor lowers the activation energy barrier needed for the release of the peptide chain from the ribosome³. This process involves conformational changes in both the release factor and the ribosome^{2, 4, 5}. Efficient interactions between the release factor and the nonsense codon are essential for high fidelity in translation termination, hence high efficiency in regulation of gene expression⁶.

In bacteria, class 1 release factors (RFs), RF1 or RF2, are recruited to the ribosome to catalyze the release of the peptide chain from the P-site tRNA⁷⁻⁹. Both RF1 and RF2 can recognize UAA however RF1 is specific for UAG and RF2 is specific for the UGA stop codon^{2, 10, 11}. Structural and biochemical studies have identified a wide range of similar structural features and functions between the RFs^{2, 5, 12, 13}. RFs bind to the ribosome by interacting first with a stop codon in the A-site of the ribosome via a tripeptide motif in domain 2 that acts as a tRNA-like-anticodon¹⁴. Additionally, the RFs contain a conserved GGQ motif in domain 3 that aides in the hydrolysis of the peptide chain from the P-site tRNA (RF1 shown **Figure 2.1A**)^{3, 15}. Mutations in the GGQ motif have been shown to compromise the catalytic activity of the RFs^{16, 17}. Precise recognition of the stop codon coupled with conformational changes within the RFs leads to

movement of the GGQ motif into the peptidyl transferase center (PTC) and thus results in efficient bacterial translational termination ^{4, 11}.

Recently, biochemical and structural studies have identified an alternative release factor known as YaeJ, which shares structural and functional similarities with the RFs ¹⁸⁻²⁰. In *Escherichia coli*, YaeJ rescues ribosomes that are stalled on truncated mRNA (non-stop complexes) by hydrolyzing the peptide chain from the P-site tRNA. YaeJ contains an N-terminal domain and a C-terminal tail (**Figure 2.1B**). The N-terminal domain contains a GGQ motif that catalyzes the hydrolysis of the peptide chain from the stalled ribosome complex ¹. Unlike the RFs, YaeJ does not interact with mRNA directly, rather, the C-terminal tail inserts into the mRNA entrance channel of the small subunit of the ribosome to facilitate the binding of YaeJ ¹. YaeJ is found in most Gram-negative bacteria and its presence has not been identified in any Gram-positive bacteria ¹⁸. However, its homolog, ICT1, is present in eukaryotic cells and it is known to have similar functions as YaeJ ²⁰. Due to the high degree of similarity between the structures and functions of this class of proteins, it is likely that they have a similar mode of action.

The ribosome undergoes various conformational changes during protein synthesis as it interacts with different ligands. Previous studies have demonstrated that the large subunit and the small subunit of the ribosome rotate with respect to each other as the ribosome translates mRNA ²¹⁻²⁵. The primary rotational states of the ribosome have been classified as either rotated (hybrid tRNA state) or non-rotated (classical tRNA state) (**Figure 2.1C**) ²⁶. These different states are predominantly controlled by the interaction of different ligands (e.g. tRNA and elongation factors) with the ribosome during translation.

Thus, understanding how additional interacting factors influence the dynamics of the ribosome is key to understanding ribosome function.

Previous groups have shown that the RFs remains bound to the ribosome following hydrolysis of the peptidyl tRNA in the P-site. In addition, structural and smFRET studies have shown that binding of either of these factors results in the non-rotated state conformation of the ribosome when bound to a stop codon in the A-site²⁷⁻³⁰. These studies further indicate that the class I RFs forms substantial interactions with bridge B2 of the ribosome composed of nucleotide residues from the 23S and the 16S ribosomal RNA, which is proposed to have a significant influence on intersubunit rotation^{2, 31}. Similarly, a recent X-ray crystal structure of YaeJ bound to the ribosome shows analogous interactions with the 23S and 16S rRNA and that the ribosome is in the non-rotated state¹. Taken together, these elegant studies have largely contributed to our understanding of the interactions of RF1, RF2 and YaeJ to the ribosome. However, a comprehensive understanding of the effects of RF1, RF2, and YaeJ binding on intersubunit rotation of the ribosome at equilibrium remains to be elucidated.

Here, we employ smFRET to characterize the influence of RF binding on the UUU sense codon and the three stop codons in the A-site on intersubunit rotation. Our findings reveal that RF binding influences the conformational dynamics of the ribosome in a codon dependent manner. We show that binding of RFs to their cognate stop codons is necessary for non-rotated state stabilization to occur. In contrast, binding of YaeJ to the ribosome only resulted in a significant shift of ribosome populations to the non-rotated state when the mRNA was truncated near the A-site. Further, through a series of point and truncation mutations of YaeJ, we demonstrate that the linker region between

the N-terminal domain and C-terminal tail is necessary for influencing the conformational shift of intersubunit rotation to the non-rotated state. Together, these results demonstrate that stabilization of the ribosome in the non-rotated state is correlated with the activity of these factors and perhaps priming of the ribosome for the recycling phase of translation ³².

2.3 Materials and Methods

2.3.1 Cloning, mutagenesis, expression, and purification of RF1 and YaeJ

Genomic DNA from MRE600 was used as a template to PCR-amplify the RF1, RF2 and YaeJ genes. These genes were cloned into pET-28a vectors with an N-terminal 6X His-tag on each protein ³³. The vectors were transformed into BL21 (DE3), grown up in 1.5L liter LB, and induced at an OD of 0.6 with 1 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG) for 4 hours. The cells were harvested by centrifugation, resuspended in buffer, and lysed by French press. The RFs lysis buffer was composed of 20mM HEPES-KOH (7.5), 1M KCl, and 6mM β ME with the YaeJ lysis buffer consisting of 20 mM HEPES-KOH (7.5), 500 mM NaCl, and 10% (v,v) glycerol similar to previous methodology ¹⁸. Each lysate was independently loaded on a His 16/10 prep column that was pre-equilibrated with lysis buffer. Following binding, the column was washed with 5 column volumes of lysis buffer and an additional 5 column volumes of buffer consisting of 20mM HEPES-KOH (pH 7.6), 1M KCl, 20 mM imidazole, and 6 mM β ME (for RFs only). The proteins were eluted by addition of an elution buffer containing 20 mM HEPES-KOH, 500 mM KCl, 150 mM imidazole, and 6 mM β ME (for RFs only). Fractions with the highest purity were dialyzed into the storage buffer (20mM HEPES-KOH (pH 7.5), 6mM $\text{Mg}(\text{CH}_3\text{COO})_2$, 150mM NH_4Cl , and 6 mM β ME) and

subsequently concentrated²¹. Site directed mutagenesis was performed to mutate the GGQ motif of RF1 and YaeJ to GAP (**Table 2.2**). The mutants were transformed into BL21 (DE3) and expressed and purified as described above. In the case of truncated YaeJ, inclusion bodies were observed following lysis. The inclusion bodies were dissolved in 6 M urea. Once dissolved, an additional spin was performed to remove the undissolved pellet. The supernatant was dialyzed back in the lysis buffer and purified as described for the wild-type form of YaeJ.

2.3.2 *In vitro* transcription of full-length and truncated mRNAs

The DNA templates for *in vitro* transcription were generated via PCR using forward and reverse primers that were synthesized by Integrated DNA Technologies. The template for the wild-type m291 sequence (5' GTAAAGTGTCATAGCACCAACTGTTAATTAAATTAAATTAAGGAAA TAAAAATGTTTGTATACAAATCTACTGCTGAACTCGCTGCACAAATGGCTAA ACTGAATGGCAATAAAGGTTTTTCTTCTGAAGATAAAG 3') was previously cloned into pUC19³⁴. This was used as a template to generate the full-length and truncated PCR products. Site directed mutagenesis was performed to make three different templates with a TAA, TAG, or TGA stop codon immediately after the ATG start codon. The forward primer was the same for both transcription reactions as indicated (**Table 2.2**). However, the reverse primer sequence for the full-length mRNA differs from the primer of the truncated mRNA (**Table 2.2**). Also, the reverse primer for the truncated mRNA was designed to have a 2' O-methyl on the 5' end to yield homogeneous 3' ends following transcription³⁵. To generate the truncated mRNA with a stop codon in the A-site, we performed site directed mutagenesis on the m291 containing pUC19 vector to

substitute the TTT sequence with a TAA stop codon (**Table 2.2**). Using the TAA mutant sequences above, PCR was performed to truncated versions of the TAA m291 containing sequence. All six of the isolated PCR products were then used as templates for *in vitro* transcription (**Figure 2.2**). *In vitro* transcription was carried out using T7 RNA polymerase and the RNA was purified using urea PAGE ^{36, 37}.

2.3.3 Ribosome preparation for smFRET studies

S6(Cy5)/L9(Cy3) fluorescently labeled ribosomes were prepared as described previously ³⁸. Briefly, ribosomes were purified via nickel affinity chromatography from a strain that lacks the S6 and L9 genes and contains a 6X-His tag on the L7/L12 stalk. S6 and L9 ribosomal proteins were purified via affinity chromatography independently ³⁸. Following purification, S6 was fluorescently labeled with Cy5 and L9 was labeled with Cy3 following previously described protocols ²¹. Finally, the S6(Cy5) and L9(Cy3) proteins were incorporated into purified S6 and L9 KO ribosomes as described ³⁸.

2.3.4 Preparation and immobilization of ribosome complexes for smFRET experiments

For ribosome complexes, we employed tRNA^{fMet} which were purchased from MP Biomedicals. We acquired a biotinylated primer (5' CAGTTGGTGCTATGACACTTTAC-Biotin 3') used to immobilize the ribosome samples on PEG passivated quartz slide surfaces (**Figure 2.1D**) ³⁹. The biotinylated primer was annealed upstream of each mRNA prior to assembling the ribosome constructs for each smFRET experiment. To prepare ribosome samples for smFRET experiments, fluorescently labeled 70S ribosomes (0.25 μ M) were incubated with the

various mRNA/biotin primer duplexes (0.5 μM), and tRNA^{fMet} (1 μM) at 37 °C for 30 minutes. These ribosome complexes were diluted to a final concentration of 1nM for visualization using total internal reflection fluorescence (TIRF) microscopy. In cases where one of the RFs, YaeJ, or mutant versions of these proteins were investigated, the proteins were added at a concentration of 5 μM to the imaging buffer (20mM HEPES-KOH (pH 7.5), 6mM Mg(CH₃COO)₂, 150mM NH₄Cl, 6 mM βME , 0.1% (v/v) Nikkol, 2mM spermidine, and 0.1mM spermine). This was based on binding studies where it was demonstrated that RF1 affinity to sense codons (KD of 1.6 μM to UUU) in the A-site is reduced as compared to the UAA stop codon (KD < 0.003 μM)^{40, 41}. In these cases, immobilized ribosome complexes were incubated with imaging buffer for ~10 mins prior to visualization. Data was recorded on an iXon⁺ EMCCD (Andor) using home-built software. Individual time trajectories were extracted using IDL and analyzed using Matlab as previously described to generate FRET histograms³⁴. The data were fit in Igor to Gaussian distributions with either two or three peaks. Selection of the best fit was determined by comparing the peak widths and chi squared similar to previous reports^{34, 38}. To determine transition frequencies between conformational states, hidden Markov modelling was performed on individual time trajectories using HaMMMy software⁴². Subsequently, the fitted traces were analyzed using TDP software.

2.4 Results

2.4.1 Effects of RF1, RF2, and YaeJ binding on ribosome intersubunit rotation

Optimal RF1 and RF2 activity has been shown to require a stop codon in the A-site of the ribosomes while effective YaeJ binding requires ribosome complexes that are stalled on truncated mRNA^{2, 10, 14, 18, 19, 43}. In this study, a total of six different mRNAs

were designed to test the influence of RF1, RF2 and YaeJ binding on the intersubunit rotation of the ribosome (**Figure 2.2**). These mRNA sequences were derived from a standard m291 mRNA template that has been employed previously by several groups (**Materials and Methods**)^{21, 34, 44, 45}. Of the six different mRNAs, four of them were full-length, meaning that they extend 79 nucleotides beyond the A-site codon, and two were truncated immediately following the A-site codon. One of the full-length mRNAs was the wild type version of the m291 mRNA sequence, which positions an AUG start codon in the P-site and a UUU sense codon in the A-site (sequence in **Materials and Methods**). The three other full-length mRNAs were each built to have a UAA, UAG, or UGA stop codon in place of the UUU codon found in the m291 sequence. Finally, the two shortened mRNAs were truncated immediately following the A-site codon and contained either a UUU or UAA codon as the last codon in the sequence. The 5' end of each of these mRNAs was annealed to a biotinylated DNA oligomer as a means to immobilize ribosome constructs containing these mRNAs on a polyethylene glycol (PEG) passivated microscope slide chamber following assembly (**Materials and Methods**).

To directly visualize intersubunit rotation, we employed a previously characterized fluorescently labeled ribosome construct in which ribosomal proteins S6 from the small subunit and L9 from the large subunit were fluorescently labeled with Cy5 and Cy3 fluorophores, respectively (**Materials and Methods**)^{21, 26, 38}. Previous studies have indicated that these labeling sites do not interfere with either ligand binding to the ribosome or the fluorescence properties of the dyes^{21, 26, 38}. Further, this design enables direct visualization of changes in the rotational state of the ribosome by monitoring FRET changes due to the proximity of these sites across the subunit interface and dye-

dye distance changes between the non-rotated and rotated states as determined by structural studies (2.5Å distance change)²⁶. Several smFRET studies have shown that this construct adopts a low ~0.4 FRET state when in the rotated state and a high ~0.6 FRET state when in the non-rotated state (**Figure 2.1C**)^{21, 26, 38}. We assembled S6(Cy5)/L9(Cy3) fluorescently labeled ribosomes on the various mRNAs mentioned above with deacylated tRNA^{fMet} in the P-site, which yielded six different constructs (**Figure 2.2**). To perform the smFRET measurements, each construct was immobilized on a quartz slide using the 5' end of the mRNA (**Figure 2.1D**)²¹. We employed total internal reflection fluorescence (TIRF) microscopy to visualize these ribosome complexes (**Materials and Methods**). In line with previous findings, each of the six ribosome complexes initiated with tRNA^{fMet} was observed to predominantly occupy the rotated state, as quantified from the normalized FRET histograms (**Figure 2.9; Table 2.1; Materials and Methods**).

We sought to determine the influence of RF1 binding on intersubunit rotation of post-hydrolysis ribosomes when the A-site of the ribosome contains a UUU sense codon or one of the three stop codons (UAA, UAG, UGA). When RF1 was introduced to ribosome complexes assembled on a full-length mRNA with a UUU sense codon in the A-site (**RNA 1**), 40% of these ribosomes occupied the non-rotated state indicating that in the presence or absence of RF1 the ribosome is primarily in the rotated state (**Figure 2.3A versus Figure 2.9A; Table 2.1**). Next, we added RF1 to ribosome complexes assembled on full-length mRNA with a UAA stop codon in the A-site (**RNA 2**). Interestingly, 75% of these ribosome constructs were in the non-rotated state as compared to 33% when RF1 was absent (**Figure 2.3C; Figure 2.9C; Table 2.1**). Similarly, when

RF1 was supplied to constructs that contained an A-site UAG stop codon (**RNA 3**), we observed a majority of these ribosomes in the non-rotated state (**Figure 2.3E; Figure 2.9E; Table 2.1**). Both of these results are consistent with structures of the ribosome in the presence of RF1^{2, 11, 46}. Since RF1 is not specific for the UGA stop codon, we hypothesized that the introduction of RF1 would have minimal influence on the conformational state of the ribosome. Indeed, when RF1 was introduced to ribosome complexes that contained an A-site UGA stop codon (**RNA 4**), a majority of the ribosome population remained in the rotated state with only 35% of the population occupying the non-rotated state (**Figure 2.3F; Table 2.1**). Our findings demonstrate that non-rotated state stabilization induced by RF1 correlates with the recognition of the UAA and UAG stop codons by RF1 and has minimal influence when UUU or the UGA stop codon is present.

As mentioned, RF2 recognizes the UAA and UGA stop codon, but not UAG. We were curious to determine if an analogous pattern of non-rotated state stabilization occurred when RF2 binds to the ribosome in the presence of the same set of RNAs (**Figure 2.2**). First, RF2 was introduced to ribosome complexes that contained a UUU sense codon in the A-site (**RNA 1**). Similar to RF1, we observed that only 36% of these ribosomes occupied the non-rotated state (**Figure 2.4A; Table 2.1**). However, when RF2 was introduced to constructs that contained a UAA stop codon (**RNA 2**), we observed that 68% of these complexes occupied the non-rotate state (**Figure 2.4B; Table 2.1**). This result suggests that, similar to RF1, RF2 also shifts a majority of the population of the ribosome to the non-rotated state. As the specificity of UAG versus UGA is switched for RF2, we expected that the ability of RF2 to induce non-rotated state formation would also

be altered. As anticipated, our data showed that when RF2 was introduced to ribosome complexes containing an A-site UGA stop codon (**RNA 4**) 73% of the population of these ribosomes occupied the non-rotated state (**Figure 2.4C; Table 2.1**). Also, when RF2 was introduced to constructs that have the UAG stop codon in the A-site (**RNA 3**) a majority of the ribosome population remained in the rotated state with only 27% occupying the non-rotated state (**Figure 2.4D; Table 2.1**). These results indicate that RF2 binding to the ribosome in the presence of an A-site UAA or UGA stop codon is sufficient to shift the ribosome population from primarily the rotated to non-rotated state, but not in the case of UAG or UUU. Similar to RF1, this observation correlates with the functional specificity of RF2.

The FRET histograms are useful in describing the overall conformational distribution of the ribosome between the rotated and non-rotated state. However, a quantitative understanding of the frequency with which the ribosome moves between the rotated and non-rotated states requires additional analysis. One metric that is useful in describing the flexibility of the system is the percentage of traces with observed FRET transitions between the two states. This is a useful indication of how prevalent transitions are between states and is most useful when there is insufficient data to perform an extensive analysis with more rigorous modeling techniques. The percentage of traces that contain at least one transition between the two states among all data sets range from 10.3% to 34.5% whether in the presence or absence of RF1 or RF2 on full-length mRNA (**Table 2.1**). This shows that binding of RFs to the ribosome alters the frequency with which FRET transitions are observed by at most ~3 fold with no correlation to the presence of bound ligand. This observation is contrary to the case when EF-G and

viomycin bind the ribosome²¹. In those instances, the absence of bound ligand exhibited as much as a 30- to 40-fold increase in the observation of FRET transitions. Next, we employed hidden Markov modeling (HaMMy) to determine transition rates between the two global conformations of the ribosome for constructs that displayed a sufficient number of transitions ($> \sim 90$)⁴². For the constructs containing full-length mRNA, the forward transition rate (non-rotated to rotated state) ranged from 0.31 to 0.67 s⁻¹ while the reverse transition rate (rotated to non-rotated state) ranged from 0.09 to 1.06 s⁻¹ in the presence or absence of RFs (**Table 2.1**). These values reveal that there is at most a ~10-fold change in transition rates with no clear correlation between the presence or absence of RFs. However, binding of EF-G and viomycin showed a significant difference with at least a ~10-fold reduction in the rotated to non-rotated state transition, which resulted in a very strong stabilization of the ribosome in the rotated state²¹

2.4.2 A-site downstream mRNA is not required for RFs function

Occasionally during translation, the ribosome stalls at the end of a truncated mRNA prior to encountering a stop codon, which requires one of several rescue pathways to intervene. Each of these rescue pathways employs a rescue factor that binds in part to the empty mRNA entry tunnel^{1, 47-51}. We designed two mRNA molecules (**Figure 2.2**) that are truncated following the A-site codon, which position either UUU or UAA in the A-site. Prior to employing these mRNAs with the YaeJ rescue factor, we wanted to investigate whether the length of the mRNA downstream of the A-site has any influence on the ability of class I RFs to influence the intersubunit rotation of the ribosome. Thus, we introduced RF1 to ribosome complexes that were assembled with tRNA^{fMet} and mRNA containing either UUU (**RNA 5**) or UAA (**RNA 6**) in the A-site. When RF1 was

added to ribosome complexes containing UUU in the A-site (**RNA 5**), only 27% of these complexes occupied the non-rotated state as compared to 29% in the absence of RF1 (**Figure 2.3B; Figure 2.9B; Table 2.1**). When RF1 was introduced to ribosome complexes containing a UAA stop codon in the A-site (**RNA 6**), 85% of these complexes were in the non-rotated state as compared to 39% without RF1 (**Figure 2.3D; Figure 2.9D; Table 2.1**). These results demonstrate that the length of the mRNA did not affect the ability of RF1 to stabilize a majority of the ribosome population in the non-rotated state when a UAA stop codon was present in the A-site and thus it appears that the presence of mRNA in the mRNA entry channel is not required for the function of RFs (**Figure 2.3C, 2.3D; Table 2.1**).

2.4.3 YaeJ stabilizes post-hydrolysis ribosomes in the non-rotated state

We investigated the influence of truncated and full-length mRNAs on the ability of YaeJ to modulate intersubunit rotation of post-hydrolysis ribosome complexes (**Figure 2.2**). When we introduced YaeJ to ribosomes assembled with tRNA^{fMet} and the full-length UUU m291 mRNA (**RNA 1**) we observed that 24% of the ribosome population was in the non-rotated state (**Figure 2.4A; Table 2.1**). This result is similar to when no factors were introduced in that a majority of the ribosome population remained in the rotated state (**Figure 2.9A; Table 2.1**). Interestingly, when YaeJ was provided to ribosomes assembled with tRNA^{fMet} and the truncated UUU m291 mRNA (**RNA 5**), a majority (61%) of these ribosomes were in the non-rotated state as compared to only 29% without YaeJ (**Figure 2.5B; Figure 2.9B**). To determine the influence of the A-site codon, YaeJ was introduced to ribosome complexes with tRNA^{fMet} and full-length mRNA with an A-site UAA stop codon (**RNA 2**). Similar to the full-length UUU m291

mRNA, these ribosomes were observed predominantly in the rotated state (31%) (**Figure 2.5C; Figure 2.9C**). However, when YaeJ was supplied to ribosome complexes with tRNA^{fMet} and a truncated mRNA (**RNA 5**) with a UAA A-site stop codon 50% of these constructs occupied the non-rotated state (**Figure 2.5D; Figure 2.9D; Table 2.1**). These results demonstrate that YaeJ binding modulates intersubunit rotation of the ribosome independent of the identity of the A-site codon.

2.4.4 The YaeJ linker region is required to stabilize the ribosome in the non-rotated state

Previously, it was shown that removal of the C-terminal 40 amino acids (Δ 101-140) of YaeJ compromised binding to the ribosome¹⁸. We were interested in performing a similar set of experiments using smFRET to investigate whether the presence of the N-terminal domain of YaeJ could exert an influence on intersubunit rotation. We introduced truncated YaeJ to ribosomes containing tRNA^{fMet} with either full-length (**RNA 1**) or truncated UUU m291 mRNA (**RNA 5**). In both cases, we observed that there was no significant influence on the equilibrium population whether or not truncated YaeJ was present with ~30% of these ribosomes occupying the non-rotated state (**Figure 2.6A, 2.6B and Figure 2.9A, 2.9B; Table 2.1**). Our analysis also indicates that the truncated form of YaeJ does not significantly influence the transition rate for these ribosomes between the rotated and non-rotated state as compared to the corresponding ribosome construct in the absence of truncated YaeJ (**Table 2.1**). As expected, our results are consistent with the notion that the C-terminal tail of YaeJ is necessary for efficient binding to the ribosome^{1, 18}.

Next, we asked whether the C-terminal tail of YaeJ alone could influence the conformational dynamics of the ribosome. We synthesized a short peptide (TRASKERRLASKAQKSSVKAMRGKVRSGRE) that corresponds to the same sequence as residues 101-140 of the C-terminal tail of YaeJ. Previous studies have shown that the C-terminal tail of YaeJ is essential for the activity of YaeJ^{3, 18}. Also, structural studies have shown that the C-terminal tail of YaeJ interacts the mRNA entry channel of the ribosome^{1, 2, 31}. We introduced this peptide to ribosomes containing tRNA^{fMet} and truncated UUU m291 mRNA (**RNA 5**). As shown, the short peptide was not able to significantly alter the conformation of the ribosome in this context with only 32% of ribosomes in the non-rotated state as compared to 29% without the peptide (**Figure 2.6C; Figure 2.9B; Table 2.1**). We cannot eliminate the possibility that the peptide does not bind independently to the ribosome, which would also result in no change in intersubunit motion. Subsequently, we introduced both the truncated form of YaeJ and the short C-terminal peptide to ribosome complexes simultaneously to determine if supplying the two components of YaeJ *in trans* would have similar results as the full-length protein. This experiment was performed with ribosomes that are assembled with tRNA^{fMet} and truncated mRNA (**RNA 5**) with an A-site UUU codon. We did not observe a substantial change in non-rotated state formation with only 26% of the ribosome population occupying the non-rotated state (**Figure 2.6D; Table 2.1**). Thus neither the C-terminal tail nor the truncated forms of YaeJ separately or in combination are able to stabilize the ribosome in the non-rotated state.

2.4.5 GAP RF1 and GAP YaeJ mutant are able to influence ribosome intersubunit rotation

Previous groups have shown that mutations in the GGQ motif of RF1 and YaeJ compromised the catalytic activity of both RF1 and YaeJ by up to 3 orders of magnitude with minimal influence on the structure of RF1 ^{5, 16, 18, 52, 53}. To investigate what influence mutations to the GGQ motif may have on intersubunit rotation, we changed the GGQ motif of both RF1 and YaeJ to GAP. These specific mutations were selected since they showed the largest reduction in activity. We introduced the mutant form of RF1 (GAP) to ribosomes that were assembled on a full-length mRNA with a UAA stop codon (**RNA 2**) and tRNA^{fMet} in the P-site. We observed that 60% of these ribosomes occupied the non-rotated state (**Figure 2.7A; Table 2.1**). The non-rotated state population that we observed in the presence of RF1(GAP) is ~0.8 fold lower than what we observed in the presence of wild-type RF1 (**Figure 2.1C; Table 2.1**). While we observed a reduction in non-rotated state formation, this result suggests that mutation of the GGQ motif is still sufficient to shift the ribosome population to the non-rotated state. The mutant and wild type forms of RF1 have a similar influence on the transition rate of these constructs between the rotated and non-rotated state (**Table 2.1**). Next, we introduced the GAP mutant form of YaeJ to ribosomes that are assembled on truncated m291 UUU with tRNA^{fMet} in the P-site. In this case, 45% of these ribosomes occupied the non-rotated state (**Figure 2.7B; Table 2.1**). This is reduced from 61%, which is ~ 0.7 fold lower than what we observed in the presence of the wild type form of YaeJ (**2.5B; Table 2.1**). However, there is still an increase in non-rotated state formation as compared to ribosome complexes in the absence of YaeJ (29%) (**Figure 2.6B, 2.6C, and 2.6D**). Together, these results suggest that mutating the GGQ motif of either RF1 or YaeJ influences intersubunit rotation to a lesser extent than the wild-type forms of each protein.

2.5 Discussion

2.5.1 Subunit rotation is altered by Class I RFs binding

By employing smFRET, we showed that RF1, RF2, and YaeJ strongly influence the conformational dynamics of the ribosome following peptidyl tRNA hydrolysis. These measurements were obtained by directly monitoring intersubunit rotation of the ribosome prepared under different conditions. Our results demonstrate that binding of RF1 and RF2 to post-hydrolysis ribosomes shifts the equilibrium of the ribosome to the non-rotated state when bound to the A-site containing a UAA stop codon. Also, binding of RF1 or RF2 to ribosomes that contained an A-site UAG or UGA stop codon, respectively, resulted in an equilibrium shift that favored the non-rotated state (**Figure 2.8**). These observations are consistent with structural studies of both RF1 and RF2 bound to the ribosome^{2, 5, 28, 54-56}. Also, we observed that these release factors exhibited specificity towards their cognate stop codons and the RFs were unable to influence the subunit rotation of ribosomes with a sense codon in the A-site. In an smFRET study, Sternberg et al. monitored L1 stalk motion with respect to P-site tRNA in the ribosome. They observed that RF1 prevented P-site tRNA to go from the classical to the hybrid state, and RF1 restricted the L1 stalk to stay in the open rather than the closed conformation²⁹. While conceptually similar to what we observe here, a strict correlation between L1 stalk motion and intersubunit rotation has not been directly observed. In a separate smFRET study, researchers monitored subunit rotation during translational termination and also noted that class I RFs stabilized the ribosome in the non-rotated state following release of the peptide chain³². However, these experiments were performed in the presence of all translational termination factors and thus information regarding the equilibrium

distribution between the non-rotated and rotated state and relevant transition frequencies when RFs are bound could not be determined³². Our work is distinguished from that report since we conducted our experiments at equilibrium. Thus, we obtained quantitative information for many different ribosome complexes, which allowed us to specifically focus on how subtle differences in mRNA sequence contributes to the effectiveness of RF1, RF2, and YaeJ binding in modulating ribosome conformational changes.

In *E. coli*, RF2 is present at a 5-7 times higher concentration than RF1, which is proportional to the abundance of the UGA and UAG stop codons in the genome⁵⁷. Interestingly, the presence of the UAA stop codon is much more prevalent than the other two stop codons⁵⁷. Since both RFs recognize UAA, translational termination of the genes that contain a UAA stop codon can occur more rapidly. It has been shown that RF2 is more efficient at terminating translation than RF1 *in vivo*⁵⁷. Indeed, peptidyl-tRNA hydrolysis by RF1 can take as long as 2.0s, whereas RF2 requires less than half that time at 0.7s⁵⁸. In looking for a connection between non-rotated state stabilization by both RFs and activity, it is reasonable to speculate that the efficiency of RF1 termination for UAG is reduced as compared to other cases due to the reduced ability of RF1 to stabilize the ribosome in the non-rotated state (**Figure 2.8; Table 2.1**). However, it is important to note that another study suggests that RF2 is less efficient in decoding UGA stop codons than RF1 in decoding UAG stop codons⁵⁹. Therefore, further experiments are necessary to address this controversy and establish any connection between the extent of non-rotated state stabilization and activity. Further, the abundance of UAG versus UGA stop

codons varies from species to species⁵⁷. How this relates to the ability of RF1 and RF2 to stabilize the non-rotated state in the ribosome across species is unclear.

2.5.2 YaeJ stabilizes the ribosome in the non-rotated state

The effects of YaeJ on the conformational dynamics of the ribosome have not been previously characterized. Our results demonstrate that, upon binding, YaeJ stabilizes the ribosome in the non-rotated state similar to what we observed for RF1 and RF2. As mentioned the X-ray crystal structure of YaeJ bound to *Thermus thermophiles* ribosomes has been solved¹. While the authors mention that binding of YaeJ does not induce major changes in intersubunit rotation, this is likely stated as a comparison between the YaeJ bound structure and the pre-hydrolysis state, which would also be in the non-rotated state⁶⁰. Thus, this structure would be fully consistent with our observation of non-rotated state stabilization by YaeJ. Additionally, we show that non-rotated state stabilization does not occur as the mRNA is lengthened beyond the mRNA entrance channel implicating the importance of the C-terminal tail in YaeJ binding in this location. Indeed, the X-ray crystal structure does reveal a substantial difference between the structures of YaeJ when free in solution compared to the structure of YaeJ bound to the ribosome^{1, 20}. In solution, the C-terminal tail of free YaeJ is unstructured while the linker region (101-108) that connects the C-terminal tail with the N-terminal domain remains relatively ordered. Upon binding to the ribosome, the C-terminal tail adopts an alpha helical structure and forms extensive interactions with the mRNA entry channel suggesting that it serves as an anchor for YaeJ on the ribosome¹. While other short, positively charged peptides could bind to the ribosome in a similar fashion, the full-length version of YaeJ is required to bind, stabilize the ribosome in the non-rotated state

and hydrolyze the peptidyl-tRNA in the P-site. An example of another positively charged peptide that binds to the ribosome is alternative release factor A (ArfA), which is involved in a separate non-stop complex rescue pathway^{47, 61-63}. However, ArfA requires an additional protein, RF2, to exert similar functions as full-length YaeJ.

Previously, it was shown that removal of the C-terminal tail reduced binding of YaeJ to the ribosome^{18, 20}. Similarly, we removed the C-terminal tail domain from YaeJ starting at residue 101 to determine if the N-terminal region was sufficient to induce changes in intersubunit conformational dynamics. We observed that the truncated form of YaeJ was not able to alter intersubunit rotation, and we interpreted this to indicate that truncated YaeJ does not bind, which is consistent with previous binding studies^{18, 20}. Separately, we introduced the C-terminal tail of YaeJ to determine if binding of this short peptide would influence the conformational dynamics of the ribosome. We prepared ribosomes with truncated mRNA that contained tRNA^{fMet} in the P-site with a UUU codon in the A-site. We did not observe any significant differences between ribosome complexes in the presence or absence of the C-terminal tail peptide (**Figure 2.6B and Figure 2.9B**). These results demonstrate that the C-terminal tail of YaeJ alone does noticeably alter the conformational dynamics of the ribosomes. Although, a more systematic study of the binding of this C-terminal tail of YaeJ would be necessary for further characterization of its role on ribosome dynamics.

When we introduced the C-terminal peptide along with the truncated form of YaeJ *in trans*, non-rotated state stabilization was not observed. This suggests that the linker connecting the C-terminal tail with the N-terminal domain of YaeJ may play a critical role in ribosome rescue. Indeed, shortening the linker by deletion of residues 100-

101 was shown to decrease activity, but did not prevent binding²⁰. Thus, these results suggest that one main role of the linker may be to optimally position the N-terminal region in the PTC. However, further studies varying the length and or the nature of the amino acids in the linker region are necessary to establish this. Nevertheless, it is clear from our studies that full-length YaeJ is necessary to induce non-rotated state stabilization of the ribosome.

In trans-translation, another non-stop complex rescue pathway, transfer messenger RNA (tmRNA) and SmpB bind to the ribosome to restart the process of translation and tag the stalled peptide for degradation⁶⁴. Interestingly, SmpB contains a C-terminal tail analogous to the C-terminal tail found in YaeJ and has been shown structurally to bind to the same location^{1, 19, 65, 66}. However, the absence of mRNA in the A-site is required for full activity of the tmRNA·SmpB pathway^{67, 68}. Deletion of the C-terminal tail of SmpB prevents the accommodation of the tmRNA portion of the complex to the A-site of the ribosome^{69, 70}. The C-terminal tail of SmpB alone cannot promote hydrolysis of GTP by EF-Tu during the rescue process. When the C-terminal tail was introduced *in trans* along with tmRNA and full-length SmpB, binding did occur resulting in GTP hydrolysis, but did not result in accommodation of tmRNA. As a backup mechanism to trans-translation, ArfA recruits RF2 rescuing non-stop ribosomal complexes. Recent structural studies and previous hydroxyl radical probing have shown that the C-terminal tail of ArfA also binds to the ribosome mRNA entry channel^{61, 63, 71}. These findings in addition to the results we present here highlight the importance of the C-terminal tail in the mechanism of ribosome rescue in bacteria.

2.5.3 Importance of the GGQ motif in the function of YaeJ and the class I RFs

Previous studies have demonstrated that mutation of the glutamine residue of the GGQ motif completely abolishes the hydrolytic activity of RFs and YaeJ ^{3, 5, 16, 18, 72}. Structural studies have shown that the GGQ motif of RF1 (PDB: 4V63), RF2 (PDB: 4V5E), or YaeJ (PDB: 4V95) interacts with the CCA end of P-site tRNA and ribosomal RNA at the PTC ^{1, 2, 12, 73}. We investigated whether the GGQ motif is required for YaeJ to stabilize the ribosome in the non-rotated state. Our results show that mutations in the GGQ motif of YaeJ to GAP did not prevent YaeJ from shifting the ribosome population towards the non-rotated state (**Figure 2.7; Table 2.1**). However, we did observe a reduction of the non-rotated state population as compared to the wild-type protein (**Figure 2.7; Table 2.1**). Also, we demonstrated that the mutant RF1 (GAP) shifted ribosome complexes containing a P-site deacylated tRNA towards the non-rotated state, but not to the extent of wild-type RF1 (**Figure 2.7; Figure 2.8; Table 2.1**). This suggests that the GGQ motif has a role in stabilization of the ribosome in the non-rotated state, but this is secondary to codon recognition. More importantly, however, it is apparent that mutations to the GGQ motif of RF1 and YaeJ eliminate the stabilizing hydrogen bonding interactions at the PTC affecting the ability of these proteins to hydrolyze peptidyl-tRNA in the P-site ^{5, 16, 18, 52, 53}. It is important to note, following class I RF binding and hydrolysis of peptidyl-tRNA in the P-site, that RF3 binds and shifts the ribosome to the rotated state ^{29, 32}. This shift to the rotated state requires that both the P-site tRNA is deacylated and that RF1 or RF2 have effectively stabilized the ribosome in the non-rotated state ³². It was shown recently that a mutation in the GGQ motif of RF2 (GAQ) resulted in a significant reduction of catalytic activity ³². Subsequent binding of RF3 was not able to shift the ribosome to the rotated state thus reducing the efficiency of

termination. Whether this was the result of the presence of a peptidyl-tRNA in the P-site or the inability of RF3 to effectively remove the mutant form of RF2 from the ribosome, is not clear.

2.5.4 Mechanism of YaeJ and Class 1 RFs in peptide release and ribosome recycling

Diverse groups of ribosome binding molecules dictate the different conformational changes that are observed during translation. It was established by smFRET that the aminoacylation state of the P-site tRNA is a strong determinant for the conformational state of the ribosome and substantially influences the subunit transition frequencies by up to two orders of magnitude²¹. When an aminoacylated tRNA occupies the P-site, the ribosome is predominantly in the non-rotated state with very infrequent transitions to the rotated state^{21, 74}. However, in the presence of a deacylated tRNA in the P-site following peptide release, the ribosome is mainly in the rotated state with frequent transitions to the non-rotated state²¹. Until recently, however, the significance of the A-site in altering the conformational dynamics of the ribosome has not been as well scrutinized^{21, 31, 75}. Several structures of the ribosome have been determined with various factors bound to the A-site including RF1, RF2, tmRNA·SmpB, and ArfA·RF2^{2, 5, 12, 28, 46, 47, 63, 76-78}. In previous smFRET studies, we investigated binding of both peptidyl-tRNA and EF-G with several GTP analogues to the A-site²¹. While slight alterations in intersubunit rotation were observed, there was no significant shift in the population of ribosomes towards the non-rotated state²¹. In contrast to these studies, when we introduced either RF or YaeJ to stop and non-stop complexes, respectively, that have a deacylated tRNA in the P-site, these ribosomes primarily occupied the non-rotated state

consistent with the structural studies and frequent transitions to the rotated state were still observed²⁸. It should be noted that previous single molecule experiments from another research group showed no difference in L1-tRNA conformational dynamics whether or not the tRNA was aminoacylated prior to addition of RF1²⁹. Thus, the primary role for both RF1 and YaeJ is to hydrolyze P-site peptidyl-tRNA independent of GTP hydrolysis while remaining bound following peptide release²⁹. This differs from the role of both EF-Tu and EF-G, which show reduced affinity following GTP hydrolysis⁷⁹. As mentioned above, stabilization of the ribosome in the non-rotated state following peptide release is critical and prepares the ribosome to recruit additional factors as necessary for ribosome recycling by maintaining the required conformation of the ribosome^{29, 32}. In the case of RF1/RF2, RF3 is recruited, which displaces RF1/RF2 and subsequently recruits ribosome recycling factor (RRF) to separate the 30S and 50S subunits^{29, 32}. It has been shown that YaeJ is able to recycle the ribosome following peptide release, however, whether additional factors are necessary is not clear, but is likely similar⁶².

A critical feature that stabilizes the ribosome in the non-rotated state appears to be bridge B2, which is an interaction between H69 of the 23S rRNA and h44 of the 16S rRNA. A recent study combining smFRET and X-ray crystallography, showed that the aminoglycoside paromomycin was sufficient to stabilize the ribosome in the non-rotated state by interacting closely with residue 1913 in H69 and 1493 in h44³¹. Like the RFs, YaeJ (residues 102-116) interacts quite extensively with bridge B2 of the ribosome. This likely provides an additional explanation as to why the truncated form of YaeJ and the C-terminal tail supplied *in trans* was not sufficient to stabilize the ribosome in the non-rotated state. A longer linker length for example may still support positioning of the N-

terminal domain in the PTC, but may be incompatible with maintaining contacts with bridge B2 necessary for stabilization of the ribosome in the non-rotated state. Additional experiments that involve lengthening the linker region and the composition would be necessary to establish this. It should be noted that bridge B2 is not maintained when EF-G is bound to the ribosome presumably allowing the ribosome to adopt the rotated state

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2.6 Conclusion

In this study, we determined the effects of class I RFs and YaeJ binding on the intersubunit rotation of post-hydrolysis ribosomes using several different ribosome complexes. We showed that stabilization of the ribosome by RFs and YaeJ in the non-rotated state is dictated by the nature of the mRNA and tRNA present in the ribosome. These investigations contribute to the large body of evidence detailing how translation factors control large-scale conformational changes in the ribosome and thus determine its fate during translation. While these studies have advanced our understanding of the mechanism of RFs and YaeJ, future studies are necessary to enhance our knowledge of the role of YaeJ in ribosome recycling and also to determine how the ribosome selects among the non-stop complex rescue pathways.

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2.9 References

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Figure 2.1: Experimental Design

(A and B) Illustration of the crystal structure of RF1 (orange, PDB: 4V63) and YaeJ (orange, PDB: 4V95) bound to the ribosome (gray) with tRNA^{fMet} (blue) and a short mRNA (red)^{1, 2}. (C) An illustration showing the transition between the rotated and non-rotated conformational state of the ribosome. The upper portion of the block diagram represents the 50S subunit with the lower portion representing the 30S subunit. Each subunit is divided into three regions, which represent the E-, P-, and A-sites. The vertical black line denotes tRNA. The forward transition frequency (k_1) and reverse transition frequency (k_{-1}) are indicated. (D) Diagram indicating how the constructs were assembled and immobilized onto quartz slide surfaces for TIRF microscopy using the 5' end of the mRNA. The 50S subunit is shown in yellow, the 30S subunit is shown in blue, and tRNA in black. Approximate dye locations to investigate intersubunit rotation are indicated. The green star represents Cy3 attached to ribosomal protein L9 and the red star represents Cy5 attached to ribosomal protein S6.

Figure 2.2: Illustration of the different mRNAs that were used in this study (Materials and Methods).

For each mRNA an AUG start codon was followed by a UUU sense codon or one of the three stop codons. **RNA 1** is a full-length mRNA with a UUU sense codon, **RNA 2** is a full-length mRNA with a UAA stop codon, **RNA 3** is a full-length mRNA with a UAG stop codon, **RNA 4** is a full-length mRNA with a UGA stop codon, **RNA 5** is a truncated mRNA with a UUU sense codon, and **RNA 6** is a truncated mRNA with a UAA stop codon.

Figure 2.3: RF1 binding to full-length and truncated m291 mRNA · ribosome complexes

Normalized FRET histograms of S6(Cy5)/L9(Cy3) ribosomes with 5 μ M RF1, tRNA^{fMet} and (A) full-length UUU m291 mRNA, (B) truncated UUU m291 mRNA, (C) full-length UAA m291 mRNA, (D) truncated UAA m291 mRNA, (E) full-length UAG m291 mRNA, or (F) full-length UGA m291 mRNA. The diagram in each panel represents a schematic of the ribosome similar to those shown in Figure 2.1C. In this case, only the predominant conformation is indicated whether rotated (~ 0.4 ; offset diagram) or non-rotated (~ 0.6 ; aligned diagram). RF1 is shown in red and tRNA^{fMet} is shown as a vertical black line. The number of traces and percentage in each conformational state for each complex are indicated (**Table 1**).

Figure 2.4: RF2 binding to full-length m291 mRNA-ribosome complexes

Normalized FRET histograms of S6(Cy5)/L9(Cy3) ribosomes with 5 μ M RF2, tRNA^{fMet} and (A) full-length UUU m291 mRNA, (B) full-length UAA m291, (C) full-length UGA m291 mRNA, or (D) full-length UAG m291 mRNA. The diagram in each panel represents a schematic of the ribosome similar to those shown in Figure 2.1C. In this case, only the predominant conformation is indicated whether rotated (~ 0.4 ; offset diagram) or non-rotated (~ 0.6 ; aligned diagram). RF2 is shown in green and tRNA^{fMet} is shown as a vertical black line. The number of traces and percentage in each conformational state for each complex are indicated (Table 1).

Figure 2.5: YaeJ binding to full-length truncated m291 mRNA·ribosome complexes

Normalized FRET histograms of S6(Cy5)/L9(Cy3) ribosomes with 5 μ M YaeJ, tRNA^{fMet} and (A) full-length UUU m291 mRNA, (B) truncated UUU m291mRNA, (C) full-length UAA m291 mRNA, or (D) truncated UAA m291 mRNA. The diagram in each panel represents a schematic of the ribosome similar to those shown in Figure 2.1C. In this case, only the predominant conformation is indicated whether rotated (~ 0.4 ; offset diagram) or non-rotated (~ 0.6 ; aligned diagram). YaeJ is shown in yellow, and tRNA^{fMet} is shown as a vertical black line. The number of traces and percentage in each conformational state for each complex are indicated (**Table 1**).

Figure 2.6: Truncated YaeJ and or YaeJ C-terminal tail peptide binding complexes

Normalized FRET histograms of S6(Cy5)/L9(Cy3) ribosomes with 5 μ M truncated YaeJ RF1, tRNA^{fMet} and (A) full-length UUU m291 mRNA, or (B) truncated UUU m291mRNA. (C) Normalized FRET histograms of S6(Cy5)/L9(Cy3) ribosomes with 5 μ M YaeJ C-terminal tail peptide, tRNA^{fMet} and truncated UUU m291 mRNA. (D) Normalized FRET histograms of S6(Cy5)/L9(Cy3) ribosomes with 5 μ M truncated YaeJ, 5 μ M YaeJ C-terminal tail peptide, tRNA^{fMet} and truncated UAA m291 mRNA. The diagram in each panel represents a schematic of the ribosome similar to those shown in Figure 2.1C. In this case, only the predominant conformation is indicated whether rotated (~ 0.4 ; offset diagram) or non-rotated (~ 0.6 ; aligned diagram). The number of traces and percentage in each conformational state for each complex are indicated (**Table 1**).

Figure 2.7: Binding of GAP mutants RF1 and YaeJ to cognate binding sites in ribosome complexes

Normalized FRET histograms of S6(Cy5)/L9(Cy3) ribosomes with (A) 5 μ M RF1(GAP), tRNA^{fMet} and full-length UAA m291 mRNA, or (B) 5 μ M GAP-YaeJ, tRNA^{fMet} and truncated UUU m291 mRNA. The diagram in each panel represents a schematic of the ribosome similar to those shown in Figure 1C. In this case, only the predominant conformation is indicated whether rotated ($\sim$ 0.4; offset diagram) or non-rotated ($\sim$ 0.6; aligned diagram). GAP-RF1 is shown in red, GAP-YaeJ is shown in yellow, and tRNA^{fMet} is shown as a vertical black line. The number of traces and percentage in each conformational state for this complex are indicated (**Table 1**).

Figure 2.8: Summary of class 1 RF smFRET experiments

A bar graph summarizing the smFRET experiments that were conducted in the current study in the presence or absence of RFs (**Table 1**). The blue bar represents the percentage of the ribosome complex in the non-rotated state and the red bar represents the percentage of the ribosome complex in the rotated state.

Table 2.1: Statistical analysis for all the complexes

	Fig	% NR A-	% R B-	K _{eq} C-	ΔG (kJ/mo l) D-	% Fluctuation E-	# of traces F-	# of Transitions G-		k _i (S ⁻¹) H-	k _i (S ⁻¹) I-	K _{eq} ^{rate} J-
Full Length UUU m291 mRNA												
tRNA ^{fMet}	S1A	34	66	0.51	1.6	23.2 (70)	302	370	374	0.46 ±0.07	0.88 ±0.22	0.52
tRNA ^{fMet} with RF1	3A	40	60	0.66	1.0	10.3 (33)	321	266	269	0.63±0. 14	0.79±0.12	0.8
tRNA ^{fMet} with RF2	4A	36	64	0.56	1.43	30.3 (103)	340	270	256	0.51±0. 21	0.78±0.13	0.65
tRNA ^{fMet} with YaeJ	5A	24	76	0.31	2.9	3.2 (7)	217	18	12	κ	κ	
tRNA ^{fMet} with Truncate d-YaeJ	6A	33	67	0.49	1.7	25.7 (87)	339	220	216	0.38±0. 28	0.77±0.41	0.49
Truncated UUU m291 mRNA												
tRNA ^{fMet}	S1B	29	71	0.4	2.2	56.7 (204)	360	1889	188 3	0.63±0. 06	0.88±0.05	0.72
tRNA ^{fMet} with RF1	3B	27	73	0.36	2.5	21.9 (53)	242	121	120	0.36±0. 08	0.83±0.33	0.43
tRNA ^{fMet} with YaeJ	5B	61	39	1.56	-1.1	18.7 (40)	213	36	35	κ	κ	
tRNA ^{fMet} with Truncate d-YaeJ	6B	30	70	0.42	2.1	5.3 (20)	375	285	282	0.4±0.0 5	1.04±0.12	0.39
tRNA ^{fMet} with Peptide	6C	32	68	0.47	1.8	8.6 (21)	243	86	72	κ	κ	
tRNA ^{fMet} with Peptide & Truncate d YaeJ	6D	26	74	0.35	2.6	7.6 (18)	238	115	105	0.52±0. 1	0.94±0.42	0.55
tRNA ^{fMet} with GAP- YaeJ mutant	7B	45	55	0.81	0.5	16.6 (54)	326	121	120	0.36±0. 16	0.83±0.36	0.43
Full Length UAA m291 mRNA												
tRNA ^{fMet}	S1C	33	67	0.49	1.7	34.5 (78)	226	109	109	0.45±0. 19	0.72±0.37	0.63
tRNA ^{fMet} with RF1	3C	75	25	3	-2.7	22 (72)	328	121	109	0.51±0. 07	0.46±0.32	1.11
tRNA ^{fMet} with RF2	4B	68	32	2.1	-1.83	20.7 (68)	329	100	88	0.38±0. 11	0.73±0.09	0.52
tRNA ^{fMet} with YaeJ	5C	31	69	0.44	2.0	22.4 (66)	294	36	35	κ	κ	
tRNA ^{fMet} with GAP-RF1 mutant	7A	60	40	1.5	-1.0	22.8 (98)	430	100	75	0.41±0. 13	0.47±0.25	0.87
Truncated UAA m291 mRNA												
tRNA ^{fMet}	S1D	39	61	0.63	1.1	30 (90)	300	189	182	0.66±0. 15	1.06±0.2	0.62
tRNA ^{fMet} with RF1	3D	85	15	5.6	-4.2	47.7 (142)	298	544	557	0.4±0.2 5	0.45±0.17	0.88
tRNA ^{fMet} with YaeJ	5D	50	50	1	0	20 (82)	411	159	147	0.43±0. 29	0.37±0.2	1.16
Full length UAG m291 mRNA												
tRNA ^{fMet}	S1E	29	71	0.4	2.27	16.8 (55)	327	154	144	0.31±0.	0.22±0.04	1.41

										14		
tRNA^{fMet} with RF1	3E	54	46	1.17	-0.38	12.5 (40)	321	106	94	0.37±0. 12	0.17±0.11	2.17
tRNA^{fMet} with RF2	4D	27	73	0.36	2.5	12.1 (39)	321	115	106	0.64±0. 36	0.52±0.27	1.23
Full length UGA m291 mRNA												
tRNA^{fMet}	S1F	35	65	0.53	1.57	20.2 (70)	347	108	100	0.42±0. 30	0.09±0.04	4.67
tRNA^{fMet} with RF1	3F	27	73	0.36	2.5	24.9 (82)	329	90	89	0.65±0. 15	1.0±0.39	0.65
tRNA^{fMet} with RF2	4C	73	27	2.7	-2.4	11.6 (45)	388	94	90	0.67±0. 22	0.71±0.27	0.94

A- Percent non-rotated; **B**- Percent rotated; **C**- Equilibrium constant (percent non-rotated/percent rotated); **D**- Gibbs Free energy ($\Delta G = -RT \cdot \ln(K_{eq})$); **E**- Percent Fluctuation; **F**- Number of traces; **G**- Number of transitions; **H**- Forward transition rate; **I**- Reverse transition rate; **J**- Equilibrium rate constant (forward transition rate/reverse transition rate); **K**- Due to the low number of transitions for these complexes transition rates are not reported.

Figure 2.9: Conformational state of full-length and truncated m291 mRNA·ribosome complexes

Normalized FRET histograms of S6(Cy5)/L9(Cy3) ribosomes with tRNA^{fMet} and (A) full-length UUU m291 mRNA, (B) truncated UUU m291 mRNA, (C) full-length UAA m291 mRNA, (D) truncated UAA m291 mRNA, (E) full-length UAG m291 mRNA, or (F) full-length UGA m291 mRNA. The diagram in each panel represents a schematic of the ribosome similar to those shown in

Figure 1C. In this case, only the predominant conformation is indicated whether rotated (~ 0.4 ; offset diagram) or non-rotated (~ 0.6 ; aligned diagram). The number of traces and percentage in each conformational state for each complex are indicated (**Table 1**).

Table 2.2: Indicating the different primers that were used in this study

Name	Primer
pET-28a-RF1 Wild-type GGQ motif	Using sequence ligation independent cloning (SLIC) pET-28a Forward primer: CAAAGCCCGAAAGGAAGC pET-28a Reverse primer: GTGATGATGATGATGATGGC RF1 Forward primer: gccatcatcatcatcacAAGCCTTCTATCGTTGCC RF1 Reverse primer: cagcttcctttcgggctttgTTATTCCTGCTCGGACAAC
pET-28a-RF2 Wild-type GGQ motif	RF2 Forward primer: gccatcatcatcatcacTTTGAAATTAATCCGGTAAATAATC RF2 Reverse primers: aggcgcgctccattcgccaTCATAACCCTGCTTTCAAAC
pET-28a-RF1 GAP mutant at the GGQ motif	Forward: CGT CAG GGG CGG GTG CTC CGC ACG TTA ACA CCA C Reverse: GTG GTG TTA ACG TGC GGA GCA CCC GCC CCT GAC G
pET-28a-YaeJ Wild-type GGQ motif	Using BamHI and NdeI restriction sites YaeJ Forward primer: GCCCCGCATATGATGATTGTGATTTCCCGAC YaeJ Reverse primer: GAATAAGGATCCTTATTCCCGACCGCTGCGC
pET-28a-YaeJ GAP mutant at the GGQ motif	Forward primer GAP mutant YaeJ: 5' CGCAGGGCGCGGGCGCGCCGCATGTTAATAAGACC 3' Reverse primer GAP mutant YaeJ: 5' GGTCTTATTAACATGCGGCGCGCCGCGCCCTGCG 3'
Truncated-YaeJ: Amber stop codon introduced at Q103	Forward: GATTTAACAACAGAATAAAAAGCCCGACGACCCAC Reverse: GTGGGTCGTCGGGCTTTTTATTCTGTTGTAAATC
m291 mRNA mutation to have a stop codon after the AUG start codon	Forward: GGA AAT AAA AAT GTA AGT ATA CAA ATC TAC Reverse: GTA GAT TTG TAT ACT TAC ATT TTT ATT TCC
Reverse primer for generating UUU full length m291	5' CTTTATCTTCAGAAGAAAAACC 3'
Reverse primer for generating UAA full length m291	5' CTTTATCTTCAGAAGAAAAACC 3'
Forward primers for TAA	GGAAATAAAAAATGTAAGTATACAAATCTAC
Forward primers for TGA	GGAAATAAAAAATGTGAGTATACAAATCTAC
Forward primers for TAG	GGAAATAAAAAATGTAGGTATACAAATCTAC
Reverse primers for TAA	GTAGATTTGTATACTTACATTTTATTTC
Reverse primers for TGA	GTAGATTTGTATACTCACATTTTATTTC
Reverse primers for TAG	GTAGATTTGTATACCTACATTTTATTTC
Forward primer for generating each of the PCR templates	5' GCCGGTAATACGACTCACTATAGGG 3'
Reverse primer for generating UAA truncated m291	5' mUmUACATTTTATTTC 3'
Reverse primer for generating UUU truncated m291	5' mAmAACATTTTATTTC 3'

CHAPTER 3

Conformational dynamics of ribosomes with a vacant A-site and the influences of rescue factor YaeJ

Widler Casy and Peter V. Cornish. This chapter is a preliminary version of a manuscript which is being prepared for potential publication.

3.1 Introduction

At the termination of bacterial translation, a class I release factor (RF) recognizes a stop codon in the A-site and binds to the ribosome to catalyze the release of the full-length protein. Under certain circumstances, however, translation is forced to stop permanently as the ribosome stalls on a truncated mRNA and forms a non-stop complex. These stalled ribosome complexes occur as result of events such as readthrough of the stop codon, mRNA damage, and premature transcription termination¹⁻⁹. In bacteria, various pathways are in place to rescue non-stop complexes^{4, 10-14}. In these different pathways a ribosome rescue factor binds to the non-stop complex to catalyze the release of the growing peptide chain from the peptidyl tRNA yielding a truncated protein^{12, 15}. Although the mechanisms that rescue factors employ when rescuing non-stop ribosome complexes vary, the resulting products are similar in that ribosomes are ultimately recycled post hydrolysis of the peptidyl tRNA¹⁶.

In *Escherichia coli*, there are three different ribosome rescue pathways that have been extensively studied^{4, 10, 12, 17, 18}. One of these pathways uses the alternative release factor B (ArfB), also known as YaeJ, consisting of an N-terminal domain that catalyzes peptide chain release and a C-terminal tail which interacts with the mRNA entry channel^{17, 19}. This C-terminal tail acts intrinsically as a sensor to identify ribosomes that are

stalled on a truncated mRNA^{19, 20}. In another pathway, the alternative release factor A (ArfA) works with RF2 to help recycle stalled ribosomes^{16, 21, 22}. In this pathway, ArfA binds near the neck region of the small subunit of ribosomes that are stalled on a truncated mRNA and recruits RF2 to catalyze the release of the polypeptide chain **(Figure 3.1)**^{16, 21, 23, 24}. In addition, ArfA spans the distance between the decoding center and the mRNA entrance channel^{17, 21}. The other well-characterized pathway has two major components, the small protein B (SmpB) and a transfer mRNA (tmRNA)^{6, 17, 22, 25-28}. In this system, SmpB binds to the 30S subunit near the vacant mRNA entrance channel while the tmRNA region participates in the release of the polypeptide allowing translation to resume^{6, 17, 25-31}. Following this process, the peptide is transferred to the alanine tRNA-like region of the tmRNA while an mRNA-like portion of the tmRNA serves as a template which encodes a degradation signal for the defected polypeptide^{6, 8, 17, 25-29, 32}. Interestingly, the C-terminal tails of YaeJ, ArfA, and SmpB are highly similar in their rich composition of basic amino acid residues and how they specifically interact with ribosomes that are stalled on truncated mRNAs. Aside from these similarities and their requirement for truncated mRNA, the structural influence in the selection process for stalled ribosome complexes has remained elusive.

A wide variety of translation factors that bind to the ribosome during translation require specific interaction with the A-site codon. Upon effective binding, some of these factors force the subunits of the ribosomes to exist in a rotated conformation, while others induce a non-rotated conformation³³. Further, these different binding events to the A-site and sequential modulation of ribosomal subunit rotation are some of the major driving forces for the different phases of translation. Therefore, the A-site, more specifically the

A-site mRNA codon, plays a critical role in providing the correct platform for these factors to bind to the ribosome. Unlike these translation factors, however, the aforementioned ribosome rescue factors bind preferentially to ribosomes that are stalled on a truncated mRNA. In fact, the optimal binding condition for these rescue factors is a vacant A-site. Additionally, different studies have demonstrated that the length of the mRNA downstream of the P-site help determines the binding effectiveness of these rescue factors to the stalled complexes^{4, 10, 17, 21, 31, 34}. These various findings suggest that perhaps the number of mRNA nucleotide (one or two nucleotides) or lack thereof downstream of the P-site provide the structural conformations required for the binding of these rescue factors to the ribosome. Thus, structural investigation of ribosome complexes with zero, one, or two nucleotides downstream of would help elucidate the importance of A-site mRNA.

Intersubunit rotation between the large and the small subunit of the ribosome is critical for mRNA translation. In the current study, we designed ribosome constructs with varying length of mRNA downstream of the P-site and employed single molecule Förster Resonance Energy Transfer (smFRET) to determine the effects of mRNA length on ribosome intersubunit rotation. Further, we investigated the effects of a short peptide rich in basic amino acid residues on the intersubunit rotation of the ribosome when the A-site is unoccupied by mRNA. The sequence of that peptide was designed to be the same as the amino acid sequence for the C-terminal tail of YaeJ, which is analogous to the C-terminal tails of ArfA and SmpB. Our major observations suggest that A-site mRNA truncations resulted in substantial change in ribosome subunit rotation. Our result showed that the ribosome occupied the non-rotated (~0.6 FRET), the rotated (~0.4 FRET), and to

our surprise a hyper-rotated (~0.2 FRET) states in the absence of an mRNA codon in the A-site. Interestingly, we observed that the hyper-rotated state disappeared when an mRNA codon was present in the A-site. Further, we observed that the frequency of the hyper-rotated state diminished when the short peptide was supplied to the complexes in the hyper-rotated state. Together, our findings suggest potential structural roles of a codon in the A-site and the mRNA entrance channel on intersubunit rotation of the ribosome.

3.2 Materials and method

3.2.1 Truncated and full-length mRNA synthesis

A total of eight different mRNAs were used in the current study, which were generated via *in vitro* transcription (**Figure 3.2-A**). DNA templates for *in vitro* transcription were generated via PCR. To perform these reactions, the m291 DNA sequence

(GTAAAGTGTTCATAGCACCAACTGTTAATTAAATTAAATTAAAAAGGAAATAAAAATGTTTGTATACAAATCTACTGCTGAACTCGCTGCACAAATGGCTAAACTGAATGGCAATAAAGGTTTTTCTTCTGAAGATAAAG), which was previously cloned in a pUC19 plasmid under a T7 promoter was used as a template³⁵. **Table 3.1** displays the sequences for each of the different primers. While the forward primer (5' GCCGGGTAATACGACTCACTATAGGG 3') was the same in all of these different reactions, the reverse primers were different. Each of the 5' ends of the reverse primers contained a 2' O-methyl to ensure that the 3' end of the corresponding transcripts was homogeneous (**Table 3.1**). Following the first round of PCR, each of the different reactions underwent DpnI digestion to remove the pUC19-m291 vector. Following the

plasmid digestion, each of the different PCR products was purified via gel extraction. Then, these PCR products were used as templates in a larger scale PCR using the corresponding forward and reverse primers to generate the templates needed to perform the *in vitro* transcription (**Table 3.1**). After the purification of these DNA templates, *in vitro* transcription was performed using these new templates. Following urea polyacrylamide gel electrophoresis (PAGE) of the mRNA products from these *in vitro* transcription reactions, an Elutrap was used to isolate the mRNAs. Afterwards, the isolated mRNA products were verified via urea PAGE. Each 5' end of the purified mRNAs was annealed to a biotinylated primer (5' CAGTTGGTGCTATGACACTTTAC-Biotin 3') for the smFRET experiments. **Table 3.1** contains the mRNAs sequences.

3.2.2 Preparation and immobilization of ribosome complexes for smFRET experiments

Recently, our group developed a protocol for preparing fluorescently labeled ribosomes to visualize subunit rotation via smFRET and this method employed here to generate our fluorescently labeled ribosomes³⁶. Briefly, a mutant (D41C) form of the S6 (small subunit) ribosomal protein was independently expressed, purified and conjugated with Cy5 dye using maleimide chemistry. This same procedure was performed for a mutant (N11C) version of the L9 (large subunit) ribosomal protein and it was tagged with Cy3 dye. Next, these proteins were incorporated into ribosomes using partial reconstitution. These ribosomes were isolated from a bacterial strain (BW2113) that was engineered to lack the S6 and the L9 genes³⁶. Following the partial reconstitution step, the S6(Cy5)/L9(Cy3) fluorescently labeled ribosomes were aliquoted and stored in the at -80°C for smFRET experiments as done previously³⁶.

The ribosome complexes were prepared and immobilized on a quartz slide similar to previously, with some slight modifications^{33, 36}. Here, tRNA^{fMet} or tRNA^{Phe}, which were purchased from MP Biomedicals, was used to assemble the S6(Cy5)/L9(Cy3) fluorescently labeled ribosomes on the different mRNAs. The first three constructs were generated following assembly of these fluorescently labeled ribosomes with tRNA^{fMet} and **mRNA 1**, **mRNA 2**, or **mRNA 3** (**Figure 3.2-A; Table 3.1**). Five other constructs were generated with the fluorescently labeled ribosomes using tRNA^{Phe} and **mRNA 4**, **mRNA 5**, **mRNA 6**, **mRNA 7**, or **mRNA 8** (**Figure 3.2-A; Table 3.1**). Following assembly of these different complexes, they were each diluted to 1nM and immobilized on passivated quartz slides as was done previously^{33, 36, 37}. The imaging buffer that was used to visualize these different constructs consisted of 20 mM HEPES-KOH (pH 7.5), 150 mM NH₄Cl, 6 mM Mg(CH₃COO)₂, 6 mM βME, 0.1% (v/v) Nikkol, 0.1 mM spermine, and 2 mM spermidine. Further, a short peptide rich in basic amino acid residue was added to this imaging buffer to determine its influence on the intersubunit rotation of ribosomes with a vacant A-site (**mRNA1** and tRNA^{fMet}). The amino acid sequence (TRASKERRLASKAQKSSVKAMRGKVRSGRE) for this short peptide was the same as YaeJ C-terminal tail sequence and it was purchased from the University of Missouri Molecular Interactions Core. The data from these experiments were recorded using a home-built software and iXon⁺ EMCCD (Andor). Also, IDL software was used to extract individual time trajectories as a function of donor and acceptor fluorophores intensities with respect to time. Further, this software also generated individual FRET traces which are related to the donor and the acceptor fluorophores intensities over time^{35, 36, 38}. The individual FRET traces for a given construct were isolated via Matlab in order to build

the FRET histogram for that specific construct, as was done previously^{35, 36}. The FRET histogram for every construct was built by binning individual FRET traces accordingly using Igor software.

3.2.3 Data analysis

The histograms generated here were fitted with Gaussian curves to further analyze the major conformational states that were recorded per construct. The median of each Gaussian curve represented the FRET state for the ribosomes in that subpopulation. Whereas, the area calculated under the curve is a fraction of the ribosomes in the entire population that exists in that particular FRET state. The values for both the FRET state and the area under the curve were computed using Igor. Each fraction value for the area under the Gaussian curve was converted and expressed in terms of percent. The entire ribosome population that were monitored per construct was randomly split into three clusters. This enabled calculation of the standard error for the different FRET states and the standard error for the percent of ribosomes in each state for every construct that were monitored.

Additional calculations were performed for further characterization of these different ribosome constructs. These calculations were based on the time traces which were recorded and the different FRET states that we observed. The FRET states in some of these time traces showed back and forth fluctuations between the rotated and the non-rotated states. In populations where such fluctuations were observed, the percent of ribosomes that underwent these frequent conformational changes were obtained as a fraction of the number of fluctuated traces over the total number of traces. The standard errors for the percent of ribosome with intersubunit rotation fluctuations were also

determined. To do so, the total ribosomes which were monitored for a specific construct were divided into three clusters and the percent fluctuation for each individual cluster was calculated. The values that were obtained in each of the three different sub-groups were in turn used for computing the standard errors.

3.3 Results

3.3.1 Construct preparation

A majority of translation factors that interact with the ribosome require the presence of a codon in the A-site for their function. However, the absence of a codon in the A-site due to truncation in the mRNA results in non-stop stalled ribosome complexes. These stalled ribosome complexes must be rescued to reduce cellular toxicity and to prevent impairment of the translation machineries ^{4, 9, 18, 39, 40}. In bacteria, ribosome rescue pathways that involve YaeJ, ArfA-RF2, or tmRNA-SmpB are in place to relieve such stalled complexes. YaeJ binding has been shown to be tolerated when a codon is present in the A-site. However, the binding of this factor to the ribosome is abolished in the presence of an mRNA that extends downstream of the A-site ^{17, 19}. In contrast, the rescue factors ArfA-RF2 and tmRNA-SmpB bind preferentially to the ribosomes when mRNA is absent from the A-site and the mRNA entrance channel ^{4, 23, 41}. Thus, these different findings highlight the important role that mRNA lengths have on the binding of these different factors to the ribosome. The need for mRNA to be absent in the A-sites and the mRNA entrance channel suggests that mRNA truncation might induce ribosome configurations that are different in comparison to when there is a codon in the A-site. Here, we designed eight different mRNAs of various lengths to determine whether the number of mRNA nucleotides following the P-site codon influences ribosomal subunit

rotations (**Figure 3.2**). These mRNAs were derived from the full-length m291 mRNA that has been used in previous smFRET studies^{33, 35, 36}. The first mRNA, **mRNA 1**, was designed as a truncated version of the full-length m291 mRNA with the truncation positioned immediately after the AUG start-codon, which is positioned in the P-site. Whereas, **mRNA 2** through **mRNA 7** consisted of a sequential addition of one nucleotide to the **mRNA 1** sequence as indicated in **Figure 3.2A**. The last mRNA however, **mRNA 8**, was the same sequence as the full-length form of the m291 mRNA. These mRNAs were synthesized via *in vitro* transcription and a biotinylated DNA oligomer was annealed to the 5' end of each these mRNAs for immobilization purposes (**Materials and Methods**).

We employed smFRET to directly visualize intersubunit rotation between the two ribosomal subunits of S6(Cy5)/L9(Cy3) fluorescently-labeled ribosomes (**Materials and Methods**). These experiments yielded quantitative information that was used to determine how various length mRNA molecules change ribosome intersubunit rotation. This specific ribosome labeling scheme has been used previously for the characterization of the rotated (~0.4 FRET) and the non-rotated (~0.6 FRET) states of the ribosome^{33, 35, 36}. The rotated and non-rotated conformations are the two major structural rearrangements that the subunits of the ribosome undergo during translation of mRNAs and these motions are required for the activity of the translation machinery. In fact, previous studies have demonstrated that inhibition of ribosomal intersubunit rotation inhibited translation by preventing mRNA-tRNA translocation^{42, 43}. Here, our primary focus was to determine whether the length of mRNA downstream of the P-site has any influence on the intersubunit rotation of the ribosome. In order to conduct these

investigations, the S6(Cy5)/L9(Cy3) fluorescently-labeled ribosomes were assembled with tRNA^{fMet} or tRNA^{Phe} and with one of the eight different aforementioned mRNAs (**Figure 3.2A**). The deacylated versions of these tRNAs were used because the forward and the reverse rates of rotated and non-rotated conformations for the ribosome is faster in the presence of deacylated tRNA^{33, 44, 45}. Also, the effects of deacylated tRNA on the intersubunit rotation of the ribosome is well-characterized^{33, 36, 46}. In previous smFRET studies, the presence of deacylated tRNA in the P-site was shown to shift the equilibrium between the rotated and the non-rotated states to favor predominantly the rotated state^{33, 37}. Therefore, deacylated tRNA can be used to study the effects of these different mRNAs on the intersubunit rotation of the ribosome. The various generated constructs allow us to systematically visualize changes in subunit rotation with respect to the occupancy of the A-site with mRNA ranging from no nucleotide to a full codon (**Figure 3.2A**).

3.3.2 Absence of mRNA codon in the A-site induces ~0.2 FRET state

We investigated ribosome subunit rotation when mRNA is absent in the A-site. In order to do so, ribosome constructs assembled with tRNA^{fMet} and **mRNA 1** were immobilized on a passivated quartz slide and visualized using total internal reflection fluorescence (TIRF) microscopy (**Figure 3.2B; Materials and Methods**). Afterwards, individual FRET traces for a given construct were acquired and combined into normalized FRET histograms as described in the **Materials and Methods** section. Examples for the time traces that were compiled to generate the histogram are illustrated in **Figure 3.3**. These FRET histograms showed the presence of the rotated (at ~0.4 FRET) and non-rotated (at ~0.6 FRET) states. In addition to these two states, there was another significant population which was observed at ~0.2 FRET. The assignments of

these different conformational states are based on previous findings and represent the conformational states of the ribosomes in that population^{33, 35}. Analysis of the different FRET states revealed that 21±4% of the ribosome in that population occupied the ~0.2 FRET state, while a majority (57±12%) of these traces occupied the ~0.4 FRET, and the remainder (22±6%) in the ~0.6 FRET state (**Figure 3.4A; Table 3.2**). We did not observe the hyper-rotated state formation in previous studies when the A-site was occupied with an mRNA codon^{33, 35, 36}. Thus, this surprising observation suggests that absence of mRNA in the A-site induced the ~0.2 FRET state. Interestingly, the hyper-rotated state was reported previously, although, these ribosomes were assembled with a structured mRNA at the mRNA entrance channel³⁵. Additional structural experiments will be needed to determine whether these two phenomena involving structured mRNA and truncated mRNA are related.

The hyper-rotated conformation was not observed in previous smFRET experiments when ribosomes were assembled with unstructured full-length mRNAs^{33, 35, 36}. Therefore, it was of interest to determine the minimal number of mRNA nucleotides required in the A-site for these constructs to behave similarly to ribosomes that have a full-length unstructured mRNA. To answer this question, we investigated the intersubunit rotation of different ribosome constructs by increasing the length of mRNA downstream of the P-site one nucleotide at a time (**Figure 3.2A**). Hence, the intersubunit rotation of ribosomes that are assembled with tRNA^{fMet} and **mRNA 2** were monitored to obtain information about the conformational state of these ribosomes when only one mRNA nucleotide is present in the A-site (**Figure 3.2A**). In these ribosome complexes, the P-site contained an AUG start codon followed by one nucleotide (uridine), which is positioned

in the A-site of the ribosome. The results from these smFRET experiments indicated that a majority ($57\pm 9\%$) of these ribosomes occupied the ~ 0.4 FRET state, while $24\pm 5\%$ of these ribosomes were in the ~ 0.4 FRET state (**Figure 3.4B; Table 3.2**). Interestingly, the hyper-rotated state was observed in $19\pm 2\%$ of the ribosome population. Next, we sought to find out whether the presence of two mRNA nucleotides in the A-site of the ribosome that were assembled with **mRNA 3** and tRNA^{Met} could reduce if not remove the ~ 0.2 FRET state. The smFRET data from this construct indicated that a large number ($56\pm 11\%$) of the ribosomes in that population exhibited the ~ 0.4 FRET state, while $36\pm 6\%$ of these ribosomes existed in the ~ 0.6 FRET state and $8\pm 1\%$ in the ~ 0.2 FRET state (**Figure 3.4C; Table 3.2**). These results showed a decrease in the ~ 0.2 FRET state in this ribosomes population when compared to ribosomes constructs assembled with **mRNA 1** or **mRNA 2**. Another interesting observation was the increase in ribosome population exhibiting the ~ 0.6 FRET state. This was different when compared to ribosomes that were assembled with **mRNA 1** and **mRNA 2** in that a lesser percentage of the ribosomes population for these constructs was seen in the ~ 0.6 FRET state. Together, these findings demonstrate that increasing number of mRNA nucleotides in the A-site lessens the hyper-rotated state conformation.

3.3.3 Ribosome ~ 0.2 FRET state is independent of deacylated tRNA

In order to validate our findings which showed that the number of mRNA nucleotides in the A-site influenced the presence of the hyper-rotated state, we employed a different approach to generate the constructs in which this state was observed. These constructs were generated by assembling the fluorescently labeled ribosomes with tRNA^{Phe} and **mRNA 4**, **mRNA 5**, **mRNA 6**, or **mRNA 7** (**Figure 3.2a**). The ribosomes

that were assembled with **mRNA 4** contained no mRNA in the A-site. When we monitored these ribosome complexes, the data revealed that $65\pm 13\%$ of these ribosomes occupied the rotated state, $16\pm 3\%$ of these constructs were in the non-rotated state, and the remaining $19\pm 4\%$ existed in the hyper-rotated state (**Figure 3.5A; Table 3.2**). In the ribosomes that were assembled with **mRNA 5**, this design resulted with one mRNA nucleotide being present in the A-site. Our smFRET studies of this particular constructs showed that $73\pm 8\%$ of these ribosome were in the rotated state, $22\pm 3\%$ were in the non-rotated state, while the remaining $5\pm 1\%$ occupied the hyper-rotated state (**Figure 3.5B; Table 3.2**). Next, the complexes that were built with **mRNA 6** where the A-site contained two mRNA nucleotides also presented the three different conformations. Analysis of the data obtained from these constructs showed that $70\pm 15\%$ of the ribosome in that population occupied the rotated state, $15\pm 3\%$ were in the non-rotated state, and $15\pm 3\%$ in the hyper-rotated state (**Figure 3.5C; Table 3.2**). In the case of the ribosomes which were assembled with mRNA 7 where the A-site contained an mRNA codon, we saw that these ribosomes occupied the rotated ($80\pm 12\%$) the non-rotated ($9\pm 1\%$), and the hyper-rotated ($11\pm 2\%$) states (**Figure 3.5D; Table 3.2**). Interestingly, in previous studies where the ribosomes were assembled with tRNA^{fMet} and a truncated mRNA with the last codon (UUU) in the A-site, the hyper-rotated state was not observed (see Chapter two). This suggests that the nature of the tRNA in the P-site and the sequence of mRNA codon in the A-site might also have implications in the observation of that state. Overall, these findings further support the claim that absence of mRNA in the A-site induces the hyper-rotated state.

In a previous study it was demonstrated that when ribosome are assembled with the full-length m291 mRNA (**mRNA 8**) and tRNA^{Phe}, these ribosome were predominantly in rotated state and the hyper-rotated state was not observed³³. Although, the way in which the fluorescently labeled ribosomes in the aforementioned study were prepared differed from the procedure that we used here (see **Materials and Methods**). We decided to perform the same smFRET experiment with our fluorescently labeled ribosome using mRNA 8 and tRNA^{Phe} (**Figure 3.2A**). The goal was to determine whether lengthening the mRNA could influence the intersubunit rotation of this specific ribosome construct³³. The results from this experiment indicated that there were only two states, with the majority (97±22%) of the ribosomes occupying the ~0.4 FRET state and the remaining 3±1% occupying the ~0.6 FRET state (**Figure 3.6A; Table 3.2**). This finding was similar to the smFRET study which showed that when ribosomes are assembled on a full-length mRNA, these ribosomes occupied only the rotated and the non-rotated states. Together these results demonstrate that the length of the mRNA downstream of the P-site has substantial effects on the intersubunit rotation of the ribosome.

3.3.4 Small peptide rich in basic amino acid residues influences subunit rotation

Recent studies have demonstrated that YaeJ binding to the ribosome is mediated by its C-terminal tail^{17, 19}. In chapter two we demonstrated that the C-terminal tail of YaeJ is required for YaeJ to influence ribosome subunit rotation. This C-terminal tail, which is rich in basic amino acid residues, interacts with the vacant mRNA entrance channel in a fashion similar to the way that the ArfA and the C-terminal tail of SmpB interact with this channel (**Figure 3.7A; Figure 3.7B**). In essence, the C-terminal tail of each of these proteins serves as a sensor that detects the degree of occupancy of the A-

site and the mRNA entrance channel^{16, 17, 22, 34, 47-52}. Structural analysis of the mRNA entrance channel with these factors bound revealed that the C-terminal tail interacts with the mRNA entrance channel similarly to the interactions of mRNA with this channel. Hence, we asked whether the binding of a short peptide to the mRNA entrance channel influence ribosome subunit rotation. In order to test this hypothesis, a small synthetic peptide which is analogous in sequence to the C-terminal tail of YaeJ was used to determine its effect on ribosomes that contain a vacant A-site. We have used this short peptide in previous investigations with the A-site occupied with an mRNA codon (see chapter two for more details). We introduced this peptide to ribosomes that were assembled with tRNA^{fMet} and **mRNA 1 (Figure 3.2A)**. In this construct the A-site deprived of mRNA nucleotides completely vacant. The data indicated that 72±10% of the ribosomes were in the ~0.4 FRET state, while 18±2% were in the ~0.6 FRET state, and with the rest 10±3% in the ~0.2 FRET state (**Figure 3.6B; Table 3.2**). Interestingly, we observed that the ~0.2 FRET state decreased in the presence of this peptide in comparison to when this peptide was not introduced to that similar ribosome constructs. Altogether, these results indicate the effects the hyper-rotated conformation of on the ribosome.

3.3.5 Presence of nucleotide in the A-site mRNA changes ribosome subunit rotation

The histograms in **Figure 3.4, 3.5, and 3.6** provided a clear representation of the distribution for the ~0.2 FRET, the ~0.4 FRET, and the ~0.6 FRET states of the ribosome population in each of the 9 unique constructs that were tested. However, some ribosomes remained strictly in the one of the three states, others transitioned back and forth between

the rotated and the non-rotated conformations (**Figure 3.3A and Figure 3.8A; Table 3.2**). The fraction of ribosomes that underwent these transitions differs amongst the eight different constructs and the values are listed in **Table 3.2** and illustrated in **Figure 3.8B**. These changes are likely due to the presence or absence of mRNA nucleotide in the A-site of the ribosome. Interestingly, when the peptide was introduced to ribosomes that are assembled with tRNA^{fMet} and **mRNA 1** we observed a one-and-a-half fold increased in the percent of ribosomes that had their conformations changing overtime between the ~0.4 and the ~0.6 FRET states. This observation suggests that the peptide increased the ability of these ribosomes to interconvert between the rotated and the non-rotated states. Also, as the number of nucleotide present in the A-site increased, a slight increase in the percent fluctuation was observed (**Figure 3.8B; Table 3.2**). This trend was primarily observed when ribosomes were assembled with the tRNA^{fMet} and to a lesser extent when the ribosomes were assembled with tRNA^{Phe} (**Figure 3.8B; Table 3.2**). The difference in ribosome fluctuation that is observed between ribosomes that are assembled with tRNA^{fMet} and tRNA^{Phe} could be a result of the nature of these tRNAs. Our observations based on these different conditions suggest that the number of mRNA nucleotides downstream of the P-site could influence fluctuation between the rotated and the non-rotated states of the ribosome.

3.4 Discussion

The ability to use smFRET to measure structural rearrangements in single molecules on a nanoscale and in real time has enabled visualization of changes in the conformational dynamics of ribosome constructs in the current study. These ribosome complexes differed primarily by mRNA lengths and the number of nucleotides that are

found in the A-site. Using smFRET, we observed that the ribosome subunits adopt a hyper-rotated conformation when the A-site and the mRNA entrance channel have no mRNA. Further, our findings show that as the number of nucleotides in the A-site increases the percentage of ribosomes that transition between the rotated and the non-rotated states. In addition, we used a synthetic peptide rich in basic amino acid residues analogous to the C-terminal tail of YaeJ and we saw that this peptide reduced the hyper-rotated state in the construct that contains a vacant A-site. The hyper-rotation also disappeared in ribosome construct that were assembled with an mRNA that extend beyond the mRNA entrance channel. Hence, our observations here suggest that the occupancy of the A-site and the mRNA entrance channel have direct effects on the conformational dynamics of the ribosome.

3.4.1 Small basic-amino-acids-rich peptide lessens hyper-rotated conformation

Structural and biochemical studies have been instrumental at depicting the A-site of the ribosome^{46, 53-57}. This site houses the decoding center of the ribosome which provides the correct platform for the accommodation of tRNA anticodon and mRNA codon interactions during translation elongation^{55, 58-62}. In addition, other factors ranging from the initiation, elongation, release, and recycling factors require binding to that site for their activity. In fact, majority of these factors bind to the A-site of the ribosome and modulate the conformational dynamics of the ribosome^{33, 43, 56, 63, 64}. These critical interactions highlight the importance of the A-site in the different phases of translation. Here, we used a short peptide that is analogous to the C-terminal tail of YaeJ and recorded a slight decrease in the hyper-rotated state in ribosome constructs that in the absence of the peptide exhibited this state. This result suggests that binding of this

peptide to the ribosome shifted the conformational dynamics of the ribosome to favor mainly the rotated and the non-rotated states. We speculate that this short peptide is able to transiently interact with the ribosome similarly to A-site codons. Though, additional investigations that further characterize the binding of the peptide to the ribosome both structurally and biochemically are required to confirm this observation.

3.4.2 Absence of A-site mRNA induces hyper-rotated state

Observations of the hyper-rotated conformation when ribosomes are assembled on a truncated mRNA with the final codon in the P-site were similar to a recent finding that showed that structured mRNA in the mRNA entrance channel induced a hyper-rotated state ³⁵. In that study, the mRNA sequence contained a hairpin derived from the dnaX gene. This hairpin has been shown to induce -1 frameshifting ⁶⁵. When the ribosomes were assembled with that structured-mRNA and a P-site tRNA^{fMet}, these constructs occupied mainly the hyper-rotated conformation ³⁵. However, here when ribosomes are assembled with truncated mRNAs these constructs remained primarily in the rotated conformation with a portion of the population distributed between the non-rotated and the hyper-rotated states. Observation of the hyper-rotated state suggests that absence of codon in the A-site alters mRNA-rRNA interactions at that site which are critical for ribosome conformation. In an independent study, analysis of a wide range of mutations in regions (A1400 to A1500) of the 16S rRNA that form the 30S A-site were shown to significantly compromise translation fidelity ⁶⁶. Some of these mutations resulted in lethal phenotypes. Also, expression and isolation of β -galactosidase from a one of these mutants bacterial strain, C1407U mutant, was shown to result in -1 frameshifting and readthrough of the three different stop codons. This group suggested

that these mutations altered tRNA-mRNA-rRNA interactions and resulted in these defects. Hence, we speculate that absence of mRNA codon in the A-site induces the hyper-rotated state following destabilization of mRNA-rRNA interaction at A-site. Whether these structural rearrangements are similar to those observed in mutants that results in -1 frameshifting or structured mRNA that result in -1 frameshifting are unclear. Structures of ribosomes that are assembled on a truncated mRNA with a completely vacant A-site or an A-site with one or two nucleotides are needed to further explain this conformation.

3.4.3 A-site mRNA influences ribosome subunit rotation

Interactions between residues A1492 of the 16S rRNA in helix 44 and A1913 of 23S rRNA in helix 69 mediate the formation of bridge B2 between the large and the small ribosomal subunits. This bridge occurs at the center of the ribosome and it is one of three major bridges that connect the large subunit and the small subunit of the ribosome. X-ray crystal structures and smFRET studies have revealed that the arrangements of the residues in that bridge have effects on ribosome intersubunit rotation. In fact, ligands such as the class I RFs, YaeJ, and even the antibiotic paromomycin stabilize the ribosome in the non-rotated state by inducing rearrangement of the A1492 and A1913⁶⁶⁻⁷¹. In one study, Abdi et al. performed a wide range of mutations of 16S rRNA residues (G530, A1492, and A1493) that make up the A-site and they observed that mutant variations of the residues A1492 and G530, and A1493C resulted in annihilation of translation in vivo⁷². This was likely due to compromises in key actions such as mRNA translocation as a result of interrupting the interaction between the A1492 and A1493. It was shown previously that A1493 participates in mRNA translocation throughout translation⁷³.

Also, mutations that result in reorientation of A1492 could have altered or compromised interactions with the second nucleotide in the A-site. Perhaps, the current findings with the truncated mRNA are linked with the roles of residue A1492 as it relates to interactions with A-site mRNA. These interactions are likely to have an indirect effects on mRNA dynamics, while directly influence the dynamics of the ribosome.

A comparison between when there is only one or no nucleotide in the A-site and when there are two nucleotides in the A-site and tRNA^{fMet} showed a large increase in the non-rotated conformation of the ribosome. Further, when there were two nucleotides in the A-site as opposed to one or no nucleotides, we observed a slight increase in the percent fluctuations between the rotated and the non-rotated state. Although similar increased in non-rotated conformation was not observed when tRNA^{Phe} was used, this was perhaps due to the structure of that tRNA. Our findings further highlight the importance of mRNA-rRNA interactions and some of the interactions that govern mRNA translocation and ribosome dynamics.

In summary, we visualized nine different constructs to determine the importance of A-site mRNA on ribosome dynamics. We observed that in the absence of mRNA codon in the A-site the ribosome occupied hyper-rotated state. Second, when only two nucleotides are present in the A-site, the population of ribosome in the non-rotated state conformation increased. Lastly, we saw that there is a trend between the number of A-site nucleotides. and the percent fluctuation between the rotated and the non-rotated states. Interestingly, as the number of nucleotide in the A-site of the ribosomes increased, the number of ribosomes that fluctuated between the rotated and the non-rotated conformation also increased. Overall, these different observations further highlights the

role of the A-site, the mRNA entrance channel, and the B2-bridge of the ribosome on mRNA dynamics and ribosome intersubunit rotation.

3.5 References

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Figure 3.1: Regions of the 30S subunit of the ribosome

A surface view of the interface of an X-ray crystal structure of the 30S subunit of the ribosome in the absence of the 50S subunit, mRNA and additional translation factors (PDB: 4V95). Regions of this subunit which include the head, the platform and the body are shown.

Figure 3.2: Experimental design and set up

A- Representation of the different mRNA sequences, $\text{tRNA}^{\text{fMet}}$ and tRNA^{Phe} .

The AUG codon is positioned in the A-site and one nucleotide was added to lengthen the mRNA. These constructs were assembled with either $\text{tRNA}^{\text{fMet}}$ or tRNA^{Phe} as illustrated. The last diagram on the bottom right corner illustrates the addition of the peptide to that specific construct. B- Illustration of the ribosome when assembled on a passivated quartz slide using the 5' end of the mRNA.

Figure 3.3: Illustration of FRET traces

Time traces that show (A) ~0.2 FRET state, (B) ~0.4 FRET state, and (C) ~0.6 FRET state. The donor and acceptor intensity over time are shown in green and red respectively, the FRET efficiency overtime is depicted in blue.

Figure 3.4: Truncated mRNAs with tRNA^{fMet}

Normalized FRET histograms of S6(Cy5)/L9(Cy3) fluorescently labeled ribosomes bound to mRNA species that were truncated (A) immediately after the AUG start codon, (B) one nucleotide after the AUG start codon, and (C) two nucleotides after the AUG start codon. The diagrams in the top row of **Figure 2-A (mRNA 1-3)** illustrate these different constructs.

Figure 3.5: Truncated m291 mRNAs with tRNA^{Phe}

Normalized FRET histograms of S6(Cy5)/L9(Cy3) fluorescently labeled ribosomes where the truncation in the mRNA was placed (A) immediately after the P-site UUU sense codon, (B) one nucleotide after the P-site UUU sense codon, (C) two nucleotides after the UUU sense codon, and (D) one codon after the P-site UUU sense codon. The diagrams in the second row and the diagram in the bottom left corner for of **Figure 2-A (mRNA 4-7)** illustrate these different constructs.

Figure 3.6: Full-length m291 mRNA and truncated m291 mRNA with tRNA^{Phe}

Normalized FRET histograms of S6(Cy5)/L9(Cy3) fluorescently labeled ribosomes assembled with (A) the full-length m291 mRNA (**mRNA 8**) and tRNA^{Phe}, and (B) a truncated m291 mRNA (**mRNA 1**) with tRNA^{fMet} and a short peptide.

A

YaeJ (111-140): TRASKERRLASKAQKSSVKAMRGKVRSGRE
SmpB (133-160): KKQHDKRSDIKEREWQVDKARIMKNAHR
ArfA (43-72): GKHGNGNWEASGKKVNHFFTTGLLLSGAC

B

Figure 3.7: YaeJ C-terminal tail interaction with the ribosome mRNA entrance channel

A) Comparison between amino acid residues that form the C-terminal tail of YaeJ, SmpB, and ArfA. The letters that are colored in red are the basic amino acid residues that are in each of the sequences. B) The C-terminal tail of YaeJ and how it interacts with the mRNA entrance channel PDB (4v95).

Figure 3.8: Fluctuation between ~0.6 to ~0.4 FRET

(A) Time trace in which fluctuation occurs between ~0.6 and the ~0.4 FRET state over time until photobleaching of the donor and the acceptor fluorophore occur. (B) The percent fluctuation with respect to each of the different constructs. The following list describes which construct each letter represents as A: mRNA 1 with tRNA^{fMet}; B: mRNA 2 with tRNA^{fMet}; C: mRNA 3 with tRNA^{fMet}; D: mRNA 4 with tRNA^{fMet}; E: mRNA with tRNA^{Phe}; F: mRNA 5 with tRNA^{Phe}; G: mRNA 6 with tRNA^{Phe}; H: mRNA 7 with tRNA^{Phe}; I: mRNA 1 with tRNA^{Phe} and the peptide; J: mRNA 8 with tRNA^{Phe}; K: mRNA 8 with tRNA^{fMet}.

Table 3.1: List of mRNAs and primers that were used in the current study

	<i>mRNA</i>	<i>Description</i>	<i>Reverse Primer</i>
<i>Truncated</i>	mRNA 1	This mRNA contains no nucleotide after the AUG start codon.	5' mCmATTTTTATTTCCTTTT AA 3'
	mRNA 2	This mRNA contains one nucleotide after the AUG start codon.	5' mAmCATTTTTATTTCCTTT TA 3'
	mRNA 3	This mRNA contains two nucleotides after the AUG start codon.	5' mAmACATTTTTATTTCCTTT TT 3'
	mRNA 4	This mRNA codon contains a P-site UUU codon.	5' mAmAACATTTTTATTTC 3'
	mRNA 5	This mRNA contains one nucleotide after the P-site codon.	5' mCmAAACATTTTTATTTCCT TT 3'
	mRNA 6	This mRNA contains two nucleotides after the P-site codon.	5' mAmCAAACATTTTTATTTCCT TT 3'
	mRNA 7	This mRNA contains another codon after the P-site codon.	5' mTmACAAACATTTTTATTTC CT 3'
<i>Full-length</i>	mRNA 8	This mRNA is the full length of the m291 mRNA.	5' CTTTATCTTCAGAAGAAAAA CC 3'

Table 3.2: Statistical analysis for all the complexes

	Fig	% R A-	% NR B-	% HpR C-	% Fluctuation D-	# of traces E-	Peak 1 Area F-	Peak 2 Area G-	Peak 3 Area H-	Peak 1 Location I-	Peak 2 Location J-	Peak 3 Location K-
Full length m291 mRNA												
tRNA ^{Met}	Ref	60±8	40±8		23.2 (70)	302	n/a	1.8 ±0.18	1.2±0.21	n/a	0.4±0.01	0.5±0.01
tRNA ^{Phe}	4a	97±22	3±1		6.3 (22)	352	n/a	3.0±0.48	0.1±0.03	n/a	0.4±0.01	0.6±0.07
Truncated m291 mRNA: AUG												
tRNA ^{Met}	2a	57±12	22±6	21±4	13.3 (36)	271	0.6±0.08	1.6±0.28	0.61±0.16	0.2±0.01	0.4±0.01	0.6±0.01
tRNA ^{Met} with peptide	4b	72±10	18±2	10±3	34.0 (134)	394	0.4±0.1	2.8±0.3	0.7±0.07	0.2±0.02	0.4±0.02	0.6±0.03
Truncated m291 mRNA: AUGU												
tRNA ^{Met}	2b	57±9	24±5	19±2	23.6 (113)	477	0.9±0.08	2.7±0.36	1.1±0.2	0.2±0.03	0.4±0.01	0.6±0.03
Truncated m291 mRNA: AUGUU												
tRNA ^{Met}	2c	56±11	36±6	8±1	27.8 (136)	489	0.4±0.03	2.7±0.47	1.8±0.21	0.2±0.05	0.4±0.03	0.6±0.03
Truncated m291 mRNA: AUGUUU												
tRNA ^{Phe}	3a	65±13	16±3	19±4	17.5 (47)	268	0.5±0.08	1.7±0.27	0.4±0.06	0.2±0.04	0.4±0.02	0.6±0.03
Truncated m291 mRNA: AUGUUUG												
tRNA ^{Phe}	3b	73±8	22±3	5±1	18.5 (106)	573	0.3±0.07	4.1±0.37	1.2±0.12	0.2±0.02	0.4±0.01	0.6±0.01
Truncated m291 mRNA: AUGUUUGU												
tRNA ^{Phe}	3c	70±15	15±3	15±3	23.8 (48)	202	0.3±0.05	1.4±0.25	0.3±0.036	0.2±0.01	0.4±0.01	0.6±0.01
Truncated m291 mRNA: AUGUUUGUA												
tRNA ^{Phe}	3d	80±12	9±1	11±2	21.7 (77)	355	0.4±0.05	2.8±0.32	0.3±0.03	0.2±0.01	0.4±0.00 ₅	0.6±0.01

A- Percent non-rotated; **B-** Percent rotated; **C-** Percent hyper-rotated; **D-** Percent of traces that fluctuates between ~0.6 and ~0.4 FRET states; **E-** Total number of traces; **F-** Fraction of peak 1 area; **G-** Fraction of peak 2 area; **H-** Fraction of peak 3 area; **I-** FRET state for peak 1; **J-** FRET state for peak 2; **K-** FRET state for peak 3.

CHAPTER 4

SUMMARY

Substantial developments in biophysical and biochemical techniques have contributed vastly to our current understanding of bacterial translation termination and ribosome rescue. Recent structures of the class I release factors (RFs) and the ribosome rescue factor, YaeJ, bound to the bacterial ribosomes have mapped the interactions between each of these factors and the A-site¹⁻⁶. In addition to these structural insights, biochemical studies have further characterized these factors in terms of their activities and the mechanisms that they employ to catalyze the release of peptide chain from peptidyl-tRNA⁷⁻⁹. While the class I RFs require a cognate stop codon in the A-site to bind to the ribosome and to become active, YaeJ binding and its activity is optimal when the mRNA is truncated. Nonetheless, the catalytic mechanisms of these three factors are strikingly similar in that they use a water molecule to hydrolyze the polypeptide chain from peptidyl-tRNA. Though various studies have explained the functions of these different factors, other fundamental questions about their interactions with the ribosome remained unanswered. In the work presented in this dissertation we addressed the following questions: how do these factors influence the intersubunit rotation of the ribosome upon effective binding? Is the catalytic activity of these proteins linked to their effects on ribosome dynamics and what effects truncated mRNAs have on ribosome structure? These answers increased the understanding of the large scale structural rearrangements that occur within the ribosome in each of these contexts.

The ribosome field has benefited significantly from the emergence of single molecule Förster resonance energy transfer (smFRET) of nearly two decades ago. This can be attested in scientific literatures that have used this technique to settle debates in the field regarding the intersubunit rotation of the ribosome. Further, this technique has been used to provide answers to insightful questions about other highly dynamic region of the ribosome. Also, several groups have since utilized smFRET to investigate the effects of antibiotics on the structure of the ribosome. Hence, the ability of these different groups to answer groundbreaking questions regarding the ribosome structure using smFRET highlights the major strengths of this technique. In this dissertation, this technique was employed to determine the effects of class I RFs, YaeJ, and mRNA truncation on subunit rotation of the ribosome. These investigations reveal the following novel details about the dynamics of the ribosome under these situations:

- 1- Class I RFs, RF1 or RF2 freezes the ribosome in the non-rotated conformation only when a cognate stop codon is present in the A-site.
- 2- Alternative release factor, YaeJ, stabilizes the non-rotated state of the ribosome only when the ribosome was assembled on a truncated mRNA.
- 3- The GGQ motif of the class I RFs and YaeJ which is required to catalyze peptide chain release is not essential for influencing the dynamics of post-hydrolysis ribosomes.
- 4- The N-terminal domain of YaeJ alone has no influence on ribosome intersubunit rotation. Similar to the N-terminal domain of YaeJ, the C-terminal tail alone is not able to influence the dynamics of the ribosome.

- 5- Absence of mRNA in the A-site causes the subunits of the ribosome to adopt a hyper-rotated conformation.

4.1 Future directions

Though the findings that are presented in this dissertation have contributed significantly to the ribosome literature, other related areas can be explored for further characterization of ribosome dynamics. These avenues are highlighted in the following subsections.

4.1.1 Role of the B-2 Bridge on the conformational dynamics of the ribosome

The B2-bridge is one of twelve bridges that connect the large and the small subunits of the 70S ribosome and it has been shown to be essential for bacterial survival¹⁰⁻¹³. This bridge is mediated by helix 44 (h44) of the 16S rRNA and helix 69 of the 23S rRNA of the small and large subunits of the ribosome, respectively¹⁴. The nucleotide residues A1492 of h44 and A1913 of H69 are the two major residues in each of these helices that are at the center of the interactions in this bridge. Interestingly ligands that bind to the ribosome and that rearrange the structure of these residues also influences the conformational dynamics of the ribosome¹⁻⁶. Thus, experiments that involve mutations at these sites (i.e. A1492C) which compromise interactions between h44 and H69 could potentially result in changes in the conformational dynamics of the ribosome. Also, crystal structures and/or smFRET studies of these different mutants could provide further insights in the structural role of the B2-bridge. Further, a study that examines the binding activities of these different mutants with various translation factors would show why the maintenance of this bridge is crucial for translation. Overall, the experiments that are

suggested here would further explain the role of the B2-bridge in the structure of the bacterial translation machinery.

4.1.2 Effects of tmRNA-SmpB and ArfA-RF2 complexes on the intersubunit rotation of the ribosome

Structural studies have mapped the interactions between the ribosome and the rescue factors tmRNA-SmpB and ArfA-RF2. These structures show similarities and differences that exist between these two different systems when bound to the ribosome. While the tmRNA-SmpB complex interaction with the ribosome is mediated by SmpB, in the case of ArfA-RF2, ArfA mediates binding to the ribosome. Nonetheless, these factors recognize ribosomes that are stalled on a truncated mRNA and bind near the mRNA entrance channel. The crystal structures of ArfA-RF2 bound to the ribosome indicate that this complex stabilizes the ribosome in a non-rotated conformation. Though, the effect of ArfA or SmpB alone on the conformational dynamics of the ribosome has remained elusive. Perhaps, smFRET studies that focus on the intersubunit rotation of the ribosome in the presence of either ArfA or SmpB would help answer that question. Also, in this dissertation we showed that in the absence of mRNA in the A-site there is population of ribosomes that occupy a hyper-rotated conformation. It would be interesting to find out if these factors could alter this conformation upon binding to these specific ribosome constructs. Also, our findings here suggest that the number of nucleotides in the A-site of the ribosome influences the degree to which the hyper-rotation is observed. We speculated that this difference might exist to provide different ribosome template to favor the binding of one rescue factor over another. Therefore, we propose a binding study that explores the affinity of these factors with ribosomes that contains varying number (0-3)

of mRNA nucleotides in the A-site of the ribosome. Together, these investigations would be beneficial in determining the reason why one pathway might be favored over another to rescue stalled ribosomes especially when the functions of these pathways are exceedingly similar.

4.1.3 Influences of ribosome rescue factors on the L1 stalk motion of the ribosome

Aside from the rotation between the two subunits, the L1 stalk motion of the bacterial ribosome has also been very well scrutinized both structurally and biochemically. In fact, translation factors that influence the intersubunit rotation of the ribosome have also been shown to alter the motion of the L1 stalk¹⁵⁻¹⁸. Hence, we propose that YaeJ, ArfA-RF2, or tmRNA-SmpB is likely to influence the dynamics of this stalk upon binding. To test this hypothesis, direct monitoring of the L1 stalk following using smFRET can be performed following the binding of each of these different factors. These different experiments would be divided into three categories. First, the L1 stalk can be visualized in ribosome nascent chain complexes that contain a truncated mRNAs and in the presence of the GAP YaeJ mutant. A similar experiment could be performed for these ribosome constructs in the presence of ArfA-RF2 GAP mutant. In both of these experiment the nascent chain ought to remain bound to the ribosome peptidyl tRNA due to the inability of these mutants to catalyze peptide chain release. The second set of experiments would involve post-hydrolysis ribosomes and the wild-type form of YaeJ or ArfA-RF2 rescue factors. Lastly, these experiments would be performed with either SmpB alone or a mutant form of the tmRNA-SmpB complex that prevent transfer of the peptide to the alanine tRNA of the tmRNA. In addition, structures of the mutant forms of these rescue factors bound to ribosome nascent chain complexes

with truncated mRNA would complement these smFRET experiments. Altogether, these different investigations would provide a wide variety of snapshots of the L1 stalk.

The structural complexity of the ribosome alone and interactions with the various translation factors continue to make this complex a very interesting macromolecular machinery to study. A lot more is known about the ribosome now compared to several decades ago and this wealth of knowledge is a largely a product of advancements in various cutting edge single molecule techniques. With that, we project an increase in learning and discovery of fine details regarding features of the ribosome which are currently beyond our technical capabilities.

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VITA

My name is Widler Casy and I was born and raised in Haiti. In 2007, my family and I immigrated to Florida due to political turmoil. While in Florida I finished high school, and then decided to move to Saint Charles Missouri where I earned a bachelor of science in chemistry with an emphasis in biochemistry from Lindenwood University. Shortly thereafter, I moved to Columbia Missouri to pursue a Ph.D. in biochemistry under the supervision of Dr. Peter V. Cornish. I am married to Kara Jane Casy and we share habitat with a dog name Phoebe.