UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Role of Critical RF1 Residues in Stop Codon Recognition and Peptidyl-tRNA Hydrolysis

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by

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LIST OF ABBREVIATIONS

A (nucleic acid)	Adenine
Arg	Arginine
Asp	Aspartic acid
A Site	Aminoacyl Site
C (nucleic acid)	Cytosine
DNA	Deoxyribose Nucleic Acid
EF	Elongation Factor
E Site	Exit Site
G (nucleic acid)	Guanine
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
GTP	Guanosine triphosphate
H	Histidine
His	Histidine
His-Tag(ged)	C-Terminal 6-Histidine Tag(ged)
IF	Initiation Factor
kDa	Kilodalton
Leu	Leucine
mRNA	Messenger Ribonucleic Acid

P Site	Peptidyl Site
PTC	Peptidyl Transferase Center
Q	Glutamine
R	Arginine
RC	Release Complex
RF1	Release Factor 1
RF2	Release Factor 2
RF3	Release Factor 3
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
Ser	Serine
T (nucleic acid)	Thymine
T (amino acid)	Threonine
Thr	Threonine
tRNA	Transfer Ribonucleic Acid
U	Uracil

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ABSTRACT OF THE THESIS

The Role of Critical RF1 Residues in Stop Codon Recognition and Peptidyl-tRNA Hydrolysis

by

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Master of Science in Biology

University of California, San Diego, 2011

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In the termination phase of protein synthesis, class I release factors, RF1 and RF2, have the ability to recognize stop codons with great affinity and catalyze peptidyl-tRNA hydrolysis; however, the mechanism behind how this occurs is not well known. Recent crystal structures have allowed the investigation of critical residues in these release factors that may be critical to codon recognition as well as

peptide release (Laurberg, et al. 2008). Therefore, the goal of this study is to investigate critical residues of RF1 and determine their role in the recognition process of codon specificity or in the hydrolysis of peptidyl-tRNA.

To investigate the roles of Thr 186, Arg 182, Thr 194, and Gln 181, a recently developed fluorescence based assay was used to study the equilibrium binding of these mutants to the ribosome (Hetrick, et al. 2009). According to the results as seen by the change in fluorescence intensity, these mutants all seemed to have a major defect in binding. The next step was to check if these residues were crucial to peptide release; therefore, to eliminate the binding deficiency, saturated amounts of the factors were added to the ribosome complex, and the rate of catalysis was quantified through the peptide release assay. Surprisingly, all of the mutants were found to have similar rates of peptidyl-tRNA hydrolysis when compared to wild type RF1. Therefore, these mutants seem to be only involved in the recognition of the stop codons in the decoding center.

I. Introduction

The premise of molecular biology states that information throughout the cell is passed from DNA to RNA to protein (Crick 1970). DNA and RNA are linear polymers made of four different types of nucleotide subunits; however, DNA is composed of deoxyribonucleic acids, while the nucleotides in RNA are ribonucleotides. Moreover, DNA contains the bases adenine (A), guanine (G), cytosine (C), and thymine (T), while RNA contains A, G, C, and uracil (U) instead of the thymine. Transcription refers to the process of DNA being converted to RNA, while translation refers to the process of converting RNA to protein, otherwise known as protein synthesis. Protein synthesis is a fundamental process in all living organisms; it consists of three phases, 1) Initiation, 2) Elongation, and 3) Termination. Moreover, the process of protein synthesis is carried out by the ribosome, ribonucleoprotein complexes which contain three tRNA binding sites. The functions and importance of proteins are an invaluable source of information and thus important to study.

In order to understand how genes function, how muscles grow, and how our bodies function, it is vital to understand the mechanism of protein synthesis. In addition, protein synthesis is essential to comprehend due to its contribution and effect to science and medicine. Bacteria are a major cause of infection and disease, which includes bacterial meningitis, Chlamydia pneumonia, diphtheria, meningitis, and scarlet fever among others (Moxon and Siegrist 2011). The study of protein synthesis

and ribosome research is essential for the discovery of novel antibiotics. Several antibiotics function to inhibit these infections and diseases by targeting the bacterial ribosome, resulting in the inactivation of the ribosome. Moreover, antibiotics such as kanamycin, erythromycin, and thiostrepton, specifically inhibit protein synthesis, thus alleviating the ongoing infection. Although regarded as agents used to treat bacterial infections and diseases, antibiotics also have the ability to be used as chemical probes providing information on some of the most complex questions in the field of biology, even dating back to the characterization of penicillin (Falconer, Czarny and Brown 2011). Understanding the mechanism of protein synthesis is vital to developing more effective antibiotics as well as novel inhibitors of bacterial translation. In order to understand the complexity of protein synthesis, it is vital to understand the phases of protein synthesis in detail, comprehend the components involved, and recognize the mechanism of how the process works together.

i. Overview of Protein Synthesis

As previously stated, protein synthesis involves three stages: initiation, elongation, and termination (Figure 1) (Lucas-Lenard 1971). During initiation, the ribosomal subunits are assembled with assistance from initiation factors. Following initiation, elongation takes place. Elongation refers to the assembly of the protein with the assistance of elongation factors and tRNAs. Termination refers to the end of the

process of protein synthesis; this occurs when a stop codon reaches the decoding center in the (aminoacyl site) A site of the small ribosomal subunit. This leads to the release of the nascent polypeptide with the help of release factors. These stages represent the culmination of converting the information present in DNA into proteins.

Initiation

During initiation, the ribosome positions the initiator fMet-tRNA^{fmet} over the start codon of mRNA in the peptidyl site (P site). This binding along with the binding of initiation factors allow the precise positioning of the start codon in the P site (Schmeing and Ramakrishnan, 2009). The overall process of initiation revolves around forming the ribosomal complex in order to carry out protein synthesis (Figure 1). In prokaryotes, the ribosome is composed of two distinct subunits: the 50S and 30S subunits (Subramanian, Ron and Davis 1968). The 30S initiation complex is formed by the binding of the 30S-IF3 complex to mRNA, initiator tRNA, and initiation factors 1 and 2 (IF1 and IF2, respectively). GTP (Guanosine Triphosphate) hydrolysis then takes place, while the initiation factors dissociate from the complex. This leaves the aminoacyl site (A site) open to accept a new aminoacyl-tRNA, while simultaneously preparing the way for elongation.

Elongation

After the 70S initiation complex is formed, the process of elongation can begin. Elongation involves three distinct steps: 1) codon directed binding of aminoacyl tRNA to a ribosomal site, 2) peptidyl transfer between the newly bound aminoacyl tRNA and fmet-tRNA, and 3) translocation (Figure 1) (Lucas-Lenard 1971). These steps contribute to the addition of amino acids to the polypeptide chain.

The first stage involves elongation factor TU (Ef-Tu) recruitment and insertion of aminoacyl-tRNAs to the A site. Through direction of the mRNA codon, the correct aminoacyl-tRNA is selected and inserted in the A site (Rodnina, Fricke and Wintermeyer 1994). The binding of the selected tRNA to the A site results in GTP hydrolysis, the dissociation of Ef-Tu, and the movement of the tRNA into the PTC. Following this, peptide bond formation can take place. In this process, the ribosome catalyzes the transfer of the P site tRNA bound polypeptide to the A site bound aminoacyl-tRNA in the peptidyl transferase center (Traut and Monro 1964). Therefore, the A site has the newly formed peptide chain, while the P site only contains a deacylated tRNA. In order to proceed with elongation, the tRNAs and mRNAs need to move in relation to the ribosome; this process is called translocation. During translocation, elongation factor G (EF-G) catalyzes the mRNA to shift by one codon with respect to the ribosome (Bretscher 1968; Rheinberger, Sternbach and Nierhaus 1981). As more aminoacyl-tRNAs bind to the A site, the ribosome continues

to translate the mRNA codons until a stop codon is recognized (Kurland 1972; Joseph and Noller 1998).

Termination

The termination of protein synthesis occurs when a stop codon enters the A site (Korostelev, et al. 2008). The stop codons, UAA, UAG, and UGA, otherwise known as nonsense codons, signal the termination of translation since they do not code for tRNA (Brenner, Stretton and Kaplan 1965; Zipser 1967; Petry, Weixlbaumer and Ramakrishnan 2008). These stop codons are recognized by class I release factors, known as release factor one (RF1) and release factor two (RF2) (Scolnick, et al. 1968). Although RF1 and RF2 are both categorized as Class I Release Factors, they both differ in their specificity for stop codons. RF1 recognizes the stop codons UAG and UAA, while RF2 recognizes the stop codons UGA and UAA (Korostelev, et al. 2010). These release factors recognize the stop codons and cleave the nascent polypeptide chain from the P site tRNA. This cleavage occurs through the hydrolysis of the bond between the nascent polypeptide and the transfer RNA at the peptidyl-tRNA site, resulting in the release of the newly synthesized protein from the ribosome (Brown and Tate 1994). Following peptide release, release factor three (RF3) binds to the subunit. The binding of RF3, a class II release factor, results in the dissociation of the class I release factors from the ribosome (Freistroffer, et al. 1997). The binding of

RF3 induces conformation changes in the ribosome, thus destabilizing the binding of class I release factors (Schmeing and Ramakrishnan 2009). The last phase of termination is the recycling of ribosomes. Following the hydrolysis on RF3, the release factor dissociates and makes way for the ribosome recycling factor (RRF) and EF-G to recycle the ribosome. In addition, GTP hydrolysis is also required in order to provide separation of the 50S subunit, 30S subunit, mRNA, and deacylated tRNA (Karimi 1999). Moreover, initiation factor three (IF3) is also necessary for the recycling of the 30S subunit to prevent premature subunit association for the next cycle. Therefore, these steps describe the known mechanism of protein synthesis.

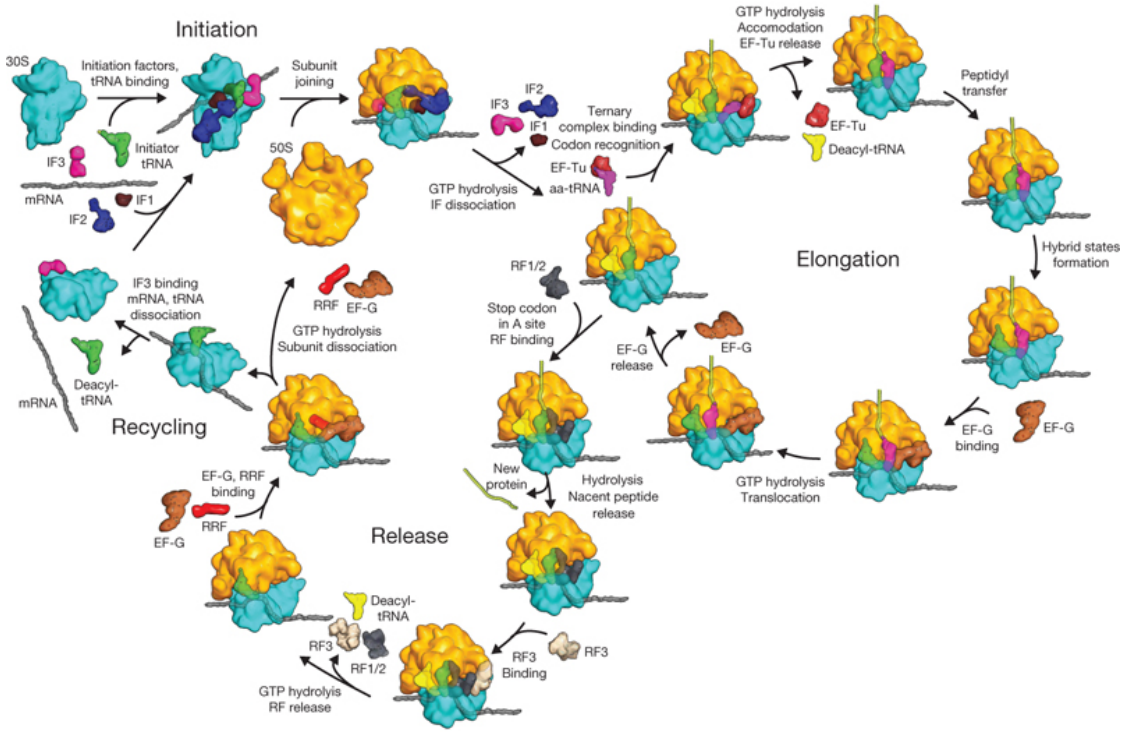

Figure 1: Overview of Prokaryotic Protein Synthesis. This diagram depicts the three stages of protein synthesis: Initiation, Elongation, and Release (Termination). Initiation depicts the joining of the ribosomal subunits with the mRNA and tRNA through the use of initiation factors. Elongation follows initiation; it involves the codon directed binding of aminoacyl tRNA to a ribosomal site, the peptidyl transfer between the newly bound aminoacyl tRNA and fmet-tRNA, and translocation. Immediately following elongation is termination, which is signaled when a stop codon is recognized. During termination, class I release factors recognize the stop codon and catalyze peptide release. Through other factors, the complex is disassembled and recycled for future use. Reprinted by permission from NPG and Macmillan Publishers Ltd: [Nature] (Schmeing and Ramakrishnan, 2009). Nature

ii. Components of Protein Synthesis

Protein synthesis is a complicated process requiring numerous factors in order to synthesize a peptide. The ribosome is the ribonucleoprotein necessary to carry out protein synthesis (Figure 2). In addition, without release factors, among other important features, protein synthesis would not be efficient or even possible. Therefore, in order to fully comprehend the mechanism of protein synthesis, it is crucial to examine the components involved.

Ribosome Structure and Function

The ribosome is a large ribonucleoprotein particle that synthesizes proteins in all cells. A 2.5-megadalton complex, the ribosome is composed of approximately two-thirds ribosomal RNA (rRNA) and one-third protein. Prokaryotic ribosomes are comprised of two subunits: a 50S subunit and a 30S subunit, which join to make a 70S complex; eukaryotic ribosomes, on the other hand, consist of a 60S subunit and a 40S subunit, which join to make an 80S complex. Moreover, the 50S subunit consists of 23S and 5S rRNAs, while the 30S subunit consists 16S rRNA (Schmeing and Ramakrishnan 2009). Due to the availability of high/low resolution crystal structures, it is possible to witness the structures of the 50S subunit and 30S subunit individually, as well as the complete 70S ribosome with bounds tRNAs (Ban, et al. 2000; Yusupov,

et al. 2001; Schmeing and Ramakrishnan 2009).

The ribosome, which consists of mainly RNA, has distinct features. The major distinguishable factor is that the ribosome contains three tRNA binding sites: the aminoacyl site (A site), the peptidyl site (P site), and the exit site (E site) (Figure 2) (Moazed and Noller 1989). The A site, which is adjacent to the P site, is responsible for the incoming aminoacyl-tRNA; all the selected incoming aminoacyl-tRNAs are first inserted into the A site. The P site, which is adjacent to the A site and E site, holds the peptidyl-tRNA which is attached to the polypeptide chain. The E site, which is adjacent to the P site, signifies where the deacylated tRNA is ejected from the ribosome, after completion of the peptide bond formation (Yusupov, et al. 2001). The mRNA binds in a cleft of the 30S subunit, allowing its codons to interact with the tRNA in these binding sites (Figure 2) (Yusupova, et al. 2006).

The ribosome also contains another domain of importance. The peptidyl transferase center (PTC) is a catalytic domain of the ribosome that has a critical role in protein synthesis. Residing in the large ribosomal subunit (50S), the PTC catalyzes two major functions: peptide bond formation and peptide release (Polacek and Mankin 2005). The main function of the PTC is to covalently link amino acids into polypeptides; located between the A site and P site, the PTC is located where the peptide attached to the peptidyl-tRNA in the P site is transferred to the new aminoacyl-tRNA that enters the A site. Another reaction that takes place here is peptidyl-tRNA hydrolysis, which is required for the termination of translation. Hence,

the PTC is crucial to the mechanism of protein synthesis.

Class I Peptide Release Factors

Class I Peptide Release Factors function by recognizing the stop codons in the ribosomal decoding site and catalyzing peptidyl-tRNA hydrolysis. The stop codons, UAA, UAG, and UGA, all signal the termination phase of protein synthesis. The first step for class I release factors is to recognize the stop codons upon entry to the A site. In prokaryotes, there are two class I release factors, RF1 and RF2; RF1 has the ability to recognize UAA and UAG, while RF2 can only recognize UAA and UGA (Scolnick, et al. 1968). UAA is the universal stop codon, allowing both release factors to recognize it. In eukaryotes, there is only a single class I release factor, eRF1, which can recognize all the stop codons (Konecki, et al. 1977). Surprisingly, even without a proofreading mechanism, the release factors have the ability to recognize stop codons with high accuracy (1 x 10-3 to 1 x 10-6); therefore, it is evident that the release factors have a specificity to distinguish the stop codons from the sense codons (Freistroffer, et al. 2000; Jorgensen, et al. 1993).

Genetic experiments and biochemical studies have proposed that tripeptide motifs (PXT in RF1 and SPF in RF2) determine the specificities of the release factors (Ito, Uno and Nakamura 2000). Exposed on the surface, the PXT and SPF motifs, located on domain 2 of RF1 and RF2 respectively, comprise a major part of a

recognition loop sequence as seen in crystal structures of the two proteins bound to the ribosome (Vestergarrd, et al. 2001). Although these motifs are critical for codon specificity, it is insufficient to determine specificity alone thus suggesting that there are other aspects crucial to codon recognition (Young, et al. 2010).

While PXT and SPF are crucial for codon recognition, the universally conserved GGQ motif, located in domain 3 of RF1 and RF2, is vital for peptide-tRNA hydrolysis and peptide release (Frolova, et al. 1999). There are various implicated roles for the GGQ motif such as orienting a water molecule for nucleophilic attack, opening a path for access of a water molecule to the PTC, and excluding other nucleophiles from the esterase reaction (Laurberg 2008). Structures also show that the GGQ motif, located in the peptidyl transferase center, positions its backbone amide group of the conserved Gln to participate in catalysis of the peptidyl-tRNA hydrolysis reaction (Korostelev, et al 2010). Cryoelectron microscopy and crystal structures of RF1 and RF2 indicate that RF1 and RF2 span the ~75Å distance between the decoding center and the PTC, when RF1 and RF2 are bound to the ribosomes. However, crystal structures of unbound RF1 and RF2 indicate that the tripeptide anticodon (motif) and GGQ motif is only ~25Å apart. This strongly suggests that release factors bind to the ribosome in a closed conformation and extend into the PTC after binding, most likely due to a conformation change (Hetrick, et al. 2009). Therefore, the precise positioning of the GGQ motif in the PTC due to conformational changes impact peptidyl-tRNA hydrolysis.

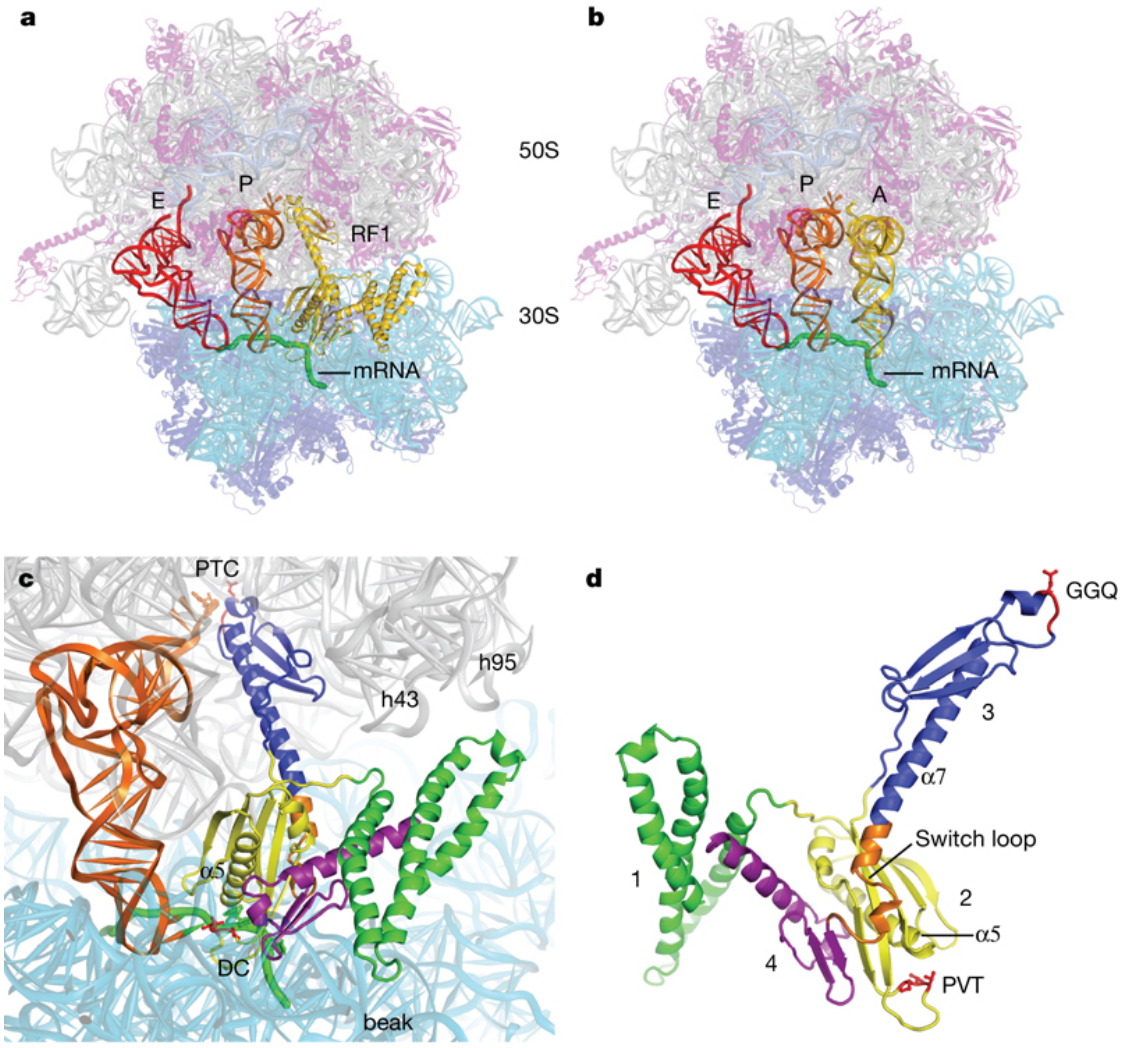

Figure 2: Overview of the Ribosome Structure. This figure depicts the structure of the ribosome. There are the two ribosomal subunits, the 50S and the 30S. In addition, the three tRNA binding sites are visible: the aminoacyl site (A site), the peptidyl site (P site), and the exit site (E site). a) Depicts the three tRNA binding sites with RF1 and mRNA attached; b) Depicts the ribosome structure without the RF1 attached; c) Displays GGQ motif as well as the PTC motif; d) The domains of RF1 are shown. Reprinted by permission from NPG and Macmillan Publishers Ltd: [Nature] (Laurberg, et al., 2008). Nature

iii. Recognition of the Stop Codon

Due to high resolution crystal structures, recently it has been possible to investigate more specifically the mechanism of how the stop codons are recognized (Wiexlbaumer, et al. 2008; Laurberg, et al. 2008; Korostelev, et al. 2008; Korostelev, et al. 2010). As stated earlier, Class I Release Factors, RF1 and RF2, are capable of identifying the stop codons UAA, UAG, and UGA; however, RF1 recognizes UAA and UAG, while RF2 recognizes UAA and UGA. Therefore, there is variability in the second and third nucleotide on the stop codon in regards to how it is read by the release factors. Although we know the 'anticodon tripeptide' motif in RF1 and RF2 interact with the stop codons in the decoding center, crystal structures indicate that other residues in RF1 and RF2 are also major contributors. Therefore, in order to understand how these residues contribute, it is necessary to examine the interactions between the stop codons and the release factors.

RF1 Recognition of the Stop Codons UAA or UAG

Recently, crystal structures of a translational termination complex containing *T. thermophilus* 70S ribosome, an mRNA, a $tRNA^{fmet}$ bound to an AUG codon in the P site, non-cognate $tRNA^{fmet}$ in the E site, and release factor bound in response to a UAA stop codon in the A site was solved (Laurberg, et al. 2008). These structures

have made it possible to examine the various crucial elements regarding the termination of protein synthesis.

First Nucleotide of the Stop Codon: U

In all three stop codons, the first nucleotide of the stop codons is U. As evident by the crystal structures, U interacts with a conserved glycine in domain 2 of RF1 and RF2. Moreover, U1 forms hydrogen bonds with conserved residues in the tripeptide motif of RF1 and RF2. These interactions strongly discriminate against a purine, only allowing a uridine in the first position (Hetrick, Lee and Joseph 2009).

In addition, the first position of the stop codon is a U1 due to three major interactions: 1) the packing of the tip of helix α5 of RF1 at Gly 116 (all numbering in *T. thermophilus*) against the edge of uridine discriminates against a purine, 2) the hydrogen bond formed between U and Gly 116 is only possible with a uridine, and 3) the hydrogen bonds formed between Glu 119 backbone amide and the hydroxyl of Thr 186 in the PxT motif are only allowed with a uridine (Laurberg, et al. 2008). All these interactions make the discrimination the strongest for the first nucleotide.

Second Nucleotide of the Stop Codon: A or G

For the second nucleotide, RF1 only recognizes an A2 in the second position,

while RF2 can recognize both an A and a G. For RF1, the A stacks against conserved residues in the release factor and forms hydrogen bonds with a threonine in the tripeptide motif; in RF2, the A and G stacks against conserved residues in the release factors, but forms hydrogen bonds with a serine in the tripeptide motif.

The second nucleotide, A2, also is stacked between the U1 and a histidine. The A2 forms hydrogen bonds with the hydroxyl of Thr 186; G is discriminated against here due to its inability to form hydrogen bonds with Thr 186. Although it is not clear why pyrimidines are excluded here, a possible explanation is due to weak propensity for stacking with U1 (Laurberg, et al. 2008).

Third Nucleotide of the Stop Codon: A or G

For the third nucleotide of the stop codon, RF1 recognizes A and G, while RF2 only recognizes A. The third nucleotide is not only unstacked from the second nucleotide of the stop codon, but it also forms several hydrogen bonds with various residues of the release factors, which explains the selection of an A or G by RF1 and A by RF2.

Specifically, the third nucleotide, A3, is unstacked from the first two bases of the stop codon. The amino group (N6) of A3 donates hydrogen bonds to the side chains of Glu 181 and Thr 194, while its N7 can accept a hydrogen bond from the side chain of Thr 194; this permits RF1 to recognize both A and G and discriminate against

pyrimidines (Laurberg, et al. 2008).

iv. Investigate the Binding Kinetics/Peptide Release of Critical Residues in RF1

Recently, higher-resolution crystal structures have allowed the specific insight into the inner-workings of the mechanism of translation. These structures have revealed that it is not only the tripeptide motifs alone that make interactions with the stop codons, but there are other important residues contributing to the function and specificity of translation (Young, et al. 2010). Although the recent crystal structures have benefitted the study of translation, there are still fundamental questions regarding the mechanism that have yet to be answered; the major question being, how are the stop codons recognized?

The objective of this thesis is to investigate the importance of these various residues via fluorescent-based transient kinetic analysis as well as test the catalytic functionality by examining the rate of peptide release (Hetrick, Lee and Joseph 2009). By using the assays mentioned above to examine various residues, the data collected will convey whether these residues contribute to the mechanism of stop codon recognition.

The residues below were selected based on their interactions with the stop codons and mutated to alanine by site-directed mutagenesis (all numbering in *T. thermophilus*).

RF1 T186

Threonine 186 (T186) is vital to the selection of the first and second positions of the stop codon (Figure 3). The hydrogen bonds of the hydroxyl of T186 in the PxT motif to the O4 position of U1 require this position to only contain a uridine (Laurberg, et al. 2008). Moreover, energy diagrams from simulations, depicting the interactions of RF1 and the stop codons, display that the interactions of U1 with Gly 116 and Glu 119, as well as the side chains of Thr 186, require a U in the first position (Sund, et al. 2010). In addition to contributing to the first position, T186 also affects the second position recognition. Not only does the N6 of A2 hydrogen bond with the hydroxyl of T186 allowing only an A in the second position, but the backbone carbonyl of T186 in RF1 also discriminates against a G2 due to a repulsive interaction with surrounding residues (Sund, et al. 2010).

RF1 R182

Arginine 182 (R182) is crucial for the selection of an adenine in the second position of the stop codon for RF1. For RF2, the specificity in the second position lies is a recognition switch, comprising of Glu 128, Asp 131, Arg 191, and Ser 193; by rotation of the Glu 128 carboxylate group, it is able to read both A and G. However, in RF1 the Asp 131 is replaced by Leu 122 and the Ser 193 is replaced by

Pro 184. These replacements not only causes this recognition switch to be lost, but also causes Glu 119 to form a more stable ion pair with Arg 182, inhibiting a guanine to be present (Sund, et al. 2010).

RF1 T194

Threonine 194 (T194) contributes to the selection of the adenine/guanine in the third position (Figure 3). The amino group (N6) of A3 donates hydrogen bonds to the side chains of Glu 181 and Thr 194, while its N7 can accept a hydrogen bond from the side chain of Thr 194; this permits RF1 to recognize both A and G and discriminate against pyrimidines (Laurberg, et al. 2008). The release factors recognize an A3 through bifurcated hydrogen bonding to the Thr 194 side chain. In RF1, the simulations predict that a water molecule bridges U531 and the third stop-codon base, allowing the water molecule to change orientation so it can hydrogen bond to A or G (Sund, et al. 2010).

RF1 Q181

Glutamine 181 (G181), which hydrogen bonds with the third codon base, is important for the selection of guanine in the third position (Figure 3) (Korostelev, et al. 2010). By rotating its amide side chain, Gln 181 can either donate a hydrogen bond

to the O6-keto group of guanine, or accept a hydrogen bond from the N6 amino group of adenine, most likely explaining why an A or G can be in the third position (Korostelev, et al. 2010). In addition, the reading of G is possible due to glutamine's NH_2 group replacing the hydrogen bond lost by T194 when A is substituted for G (Sund, et al. 2010). Therefore, the dual specificity of the different nucleotides in the third position can be explained by the Gln 181 and a water molecule (mentioned above), conveying that the tripeptide motif has little to do with the third position reading. Another key point to recognize is also that in RF2, which requires strictly an A, a hydrophobic side chain replaces the Gln 181.

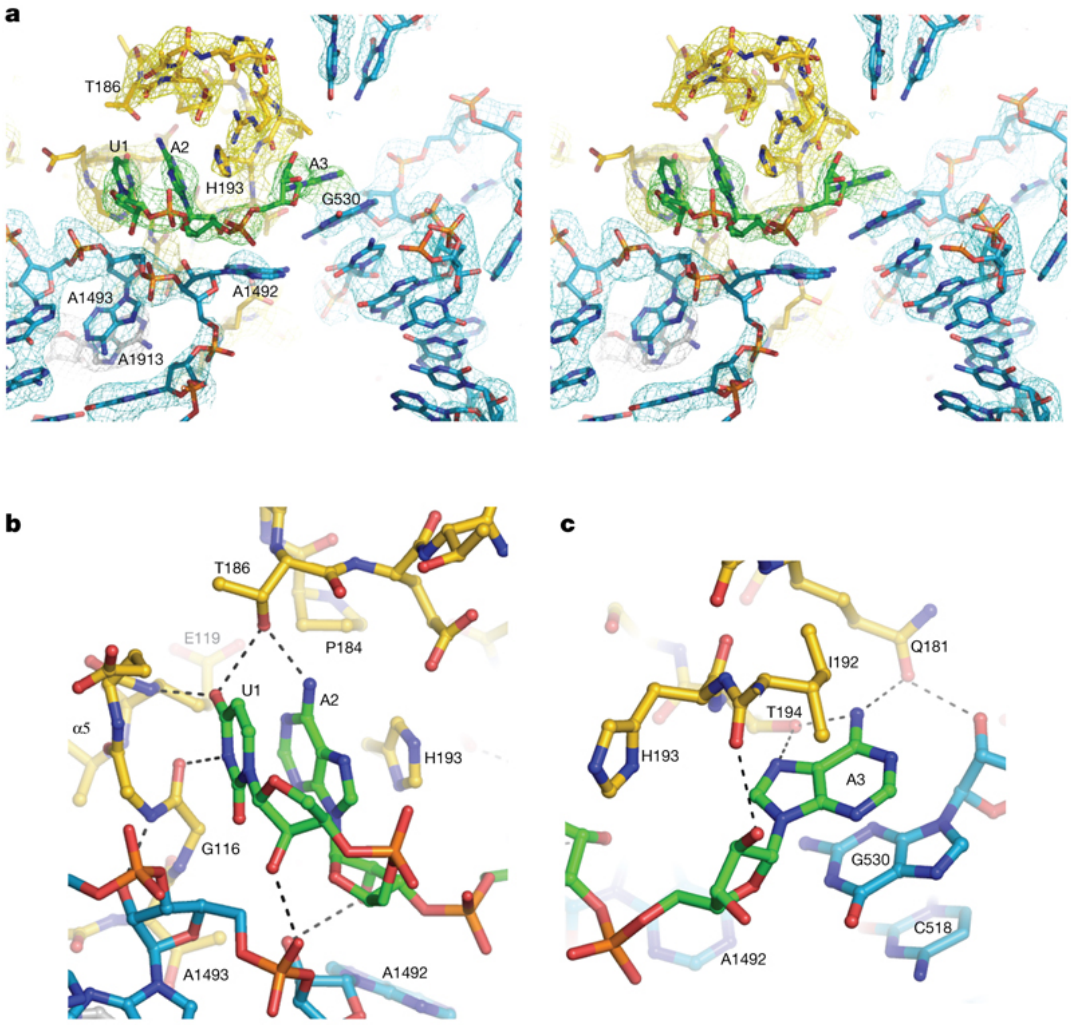

Figure 3: Critical RF1 Residues. These figures depict the critical residues of RF1 that play a crucial role to RF1 binding and stop codon recognition. Threonine 186 is vital to the selection of the first and second nucleotides (U1 and A2) of the stop codon through its interactions with hydrogen bonds. Arginine 182 is also involved in the selection of an adenine in the second position of the stop codon; Arg182 forms a stable ion pair with Glu119, thus inhibiting a guanine to be present. Threonine 194 is important for the selection of an adenine/guanine for the third position. The A3 of the stop codon can either donate or accept hydrogen bonds from Thr194, allowing A or G to be recognized. Glutamine 181 is also involved in the selection of guanine in the third position. By rotating its side amide side chain, Gln181 can also either donate or accept a hydrogen bond, allowing an A or G to be present; in RF2, a hydrophobic side chain replaced Gln181, only allowing an A. Reprinted by permission from NPG and Macmillan Publishers Ltd: [Nature] (Laurberg, et al., 2008). Nature

II. Materials and Methods

i. RF1, Ribosomes, mRNA, tRNA, and Buffer Preparations

His-tagged *E. coli* RF1 (also referred to as Wild Type) was purified using the techniques described in the QIAexpessionist manual. The protein was purified through a column and the fractions containing RF1 were pooled and concentrated in an Amicon 10kDA cutoff filter. The next step performed was buffer exchange to greater than 3000-fold dilution of the unretained buffer. The RF1 was then quantified by the Bradford Assay, flash-frozen in liquid nitrogen, and stored at -80ºC (Hetrick, Lee and Joseph 2009).

Escherichia coli MRE600 were used to produce tightly-coupled 70S ribosomes, as previously described (Powers and Noller 1991). The mRNA, with a UAA stop codon attached, was purchased through Dharmacon; pyrene was covalently attached to the mRNA as described previously (Studer, Feinberg and Joseph 2003). Native $tRNA^{fmet}$ was purchased through Sigma. All of the experiments were performed in 20mM Hepes-KOH with a pH of 7.6, 6mM $MgCl_2$, 150mM NH_4Cl, 4mM β–mercaptoethanol, .05 mM spermine, and 2mM spermidine (Bartetzko and Nierhaus 1988).

ii. Mutants T186A, R182A, T194A, and Q181A

In order to create these mutants, Quickchange (Stratagene) site-directed mutagenesis was performed. This technique was utilized in order to produce the

various mutants by using the wild type RF1 plasmid. The DNA primers were designed and subsequently ordered and purchased from ValueGene. The mutants were then sequenced and transformed into BL21 (DE3) cells. The mutants were then purified in the same manner as the Wild Type RF1, as described earlier.

iii. K_D Titrations using Fluorescent Measurements of RF1 Binding

In order to test the K_D, release complexes had to be formed. Release complexes were formed by heat activating .25uM of tightly coupled 70S ribosomes for 10 minutes at 42 ºC. The ribosomes were then cooled to 37 ºC for 10 minutes. After cooling, pyrene-labeled mRNA was added to the mixture and incubated for 10 minutes at 37 ºC; tRNA was then added and incubated at 37ºC for an additional 30 minutes (Hetrick, Lee and Joseph 2009). The final concentration of the release complex was 50 nM, while the final concentration for the mutants was 5 nM. Specific amounts of RF1 were added to the ribosome mixtures; this mixture was then incubated for at least 20 minutes prior to fluorescent measurement.

The fluorescence of the ribosome complexes were measured on a Fluoromax-P instrument (J.Y. Horiba, Inc.). The samples were read with an excitation wavelength of 343 nm and an emission wavelength of 376 nm. The data was analyzed using the equilibrium K_D equation below using Graphpad Prism as described previously (Studer and Joseph 2007). The experiments were performed at a minimum of three times.

$$Y = m \{K + R + X - [(K + R + X)^2 - 4RX]^{1/2}\} / 2R$$

where Y is the fluorescence, m is the maximum fluorescence signal, K is the dissociation constant (K_D), R is the 70S ribosome concentration, and X is the concentration of RF1.

iv. Peptide Release Assay

In order to test the peptide release, release complexes had to be formed. Release complexes were formed by heat activating the 70S ribosome at 42ºC for 10 minutes. The complex was then slow cooled to 37ºC for an additional 10 minutes; then mRNA was added to the mixture for an addition 10 minutes at 37ºC. Simultaneously, $tRNA^{fMet}$ was aminoacylated as previously described (Hetrick, Lee, and Joseph 2009), added to the complex, and the complex was incubated at 37ºC for 30 minutes. Following incubation, the complex was then passed and washed through an Amicon Ultra 100K Ultracel centrifugal filter for a final dilution greater than 200,000 fold, in order to remove excess [^{35}S] and other unbound agents. The final concentrations for the experiment as well as the time courses were 70S, mRNA, 70S release complex, and RF1 were .5μM, 1.0μM, .25μM and 50μM, respectively. The time courses were performed using 25% formic acid and then run on an eTLC plate. The data was analyzed as described previously (Feinberg and Joseph 2006). This experiment was carried out at least two times.

III. Results

The termination phase of protein synthesis is activated when a stop codon enters the ribosomal A site. Class I Release factors are responsible for the recognition of the stop codons, UAA, UAG, and UGA (Caskey et al. 1968). In prokaryotes, there are two release factors; RF1 recognizes UAA and UAG, while RF2 recognizes UAA and UGA. In eukaryotes, there is only one release factor, eRF1, which has the ability to recognize all three stop codons. In order to prevent premature termination, the accurate recognition of the stop codons is crucial. The error frequency of the recognition is so minimal (1×10^{-3} to 1×10^{-6}), demonstrating that the mechanism of codon recognition is highly sophisticated (Hetrick, et al. 2009). Moreover, the discrimination between the stop codons and the sense codons occur without the use of a proofreading mechanism that is used in tRNA selection (Petry, et al. 2005). After recognition of the stop codon, the release factors are responsible for catalyzing the peptidyl-tRNA hydrolysis, followed by the release of the newly synthesized protein from the ribosome.

Although the recent crystal structures have provided a foundation for the mechanism of how stop codons are recognized by release factors, the dynamics of how release factor binding is influenced by stop and sense codons remains a mystery. Previous experiments managed to study the kinetics of RF1 and RF2 discrimination of stop and sense codons under steady-state conditions; these studies showed that a sense codon in the decoding center increased the K_M between the release factors and the

ribosome by 400-3000-fold and reduced the catalytic rate of peptide release (k_{cat}) by 2-180 fold (Friestroffer, et al. 2000). Therefore, it is evident that the binding step is the principal component of how release factors discriminate between codons; however, this experiment did not directly measure binding. A more recent experiment tested the binding of RF1 to ribosomes with stop or sense codons in the decoding center using a fluorescence-based, pre-steady-state kinetic assay (Hetrick, Lee and Joseph 2009). This study showed that 1) RF1's association rate constant is similar with both stop codons and sense codons, while the dissociation rate constant increased by ~4000-fold when a sense codon was inserted into the decoding center and 2) conformation changes in the ribosome-release factor complex may occur before catalysis, since the rate of peptide release is inhibited by the H197A mutation even under conditions where RF1 binding to the ribosome is saturated (Fields, et al. 2010).

Although it is understood that the anticodon motif is involved during the recognition process of the stop codon, recent x-ray crystal structures have shown that additional residues may also be involved in stop codon recognition (Laurberg, et al. 2008). Therefore, the following residues have been analyzed using equilibrium binding studies and peptide release assays in order to study their functional importance.

i. Equilibrium Binding of RF1 Mutants to the Ribosome

In order to study the functional roles of the conserved residues below, site-directed mutagenesis was performed; the RF1 residues T186, R182, T194, and Q181 were all changed to an alanine. Alanine was used because it generally does not perturb protein structure. These RF1 residues are known to make critical interactions with the stop codon, as discussed previously. By mutating these residues to alanine, we investigated if the binding affinity would change through the disruption of the hydrogen bonds as well as the various interactions between the residues and the stop codon. The following mutants of RF1, T186A, R182A, T194A, and Q181A, were then purified; using a fluorescence-based assay, these mutants were then tested for their ability to bind to the ribosomes (Hetrick, et al. 2009). In order to conduct this assay, release complexes were formed; the release complexes were formed by the addition of 70S ribosomes, followed by the addition of pyrene-labeled mRNA, and then the addition of $tRNA^{fmet}$. The binding of the $tRNA^{fmet}$ to the P site allows the UAA stop codon to position in the A site of the ribosome. After the release complexes were formed, it was necessary to add increasing amount of the RF1 mutants to the complexes. With the release complex concentration fixed, the increase in fluorescence emission intensity was measured for each concentration of RF1. As the concentration of RF1 increased, the fluorescence increased due to RF1 binding to the ribosome. "For sufficient signal to noise, the minimum concentration of RC required for the titration experiment is 5nM, which is close to the K_D of wild-type RF1 binding to the

ribosome, and hence, the K_D could not be accurately determined for wild-type RF1; our best estimate is that is it below 3nM" (Fields, et al. 2010). However, the mutants T186A, R182A, T194A, and Q181A, had values of 791 nM, 833 nM, 1194 nM, and 635 nM, respectively (Figures 4-7). Therefore, mutants T186A and R182A show approximately a 250-fold increase in the K_D compared to the wild type RF1, while T194A shows an even greater difference with a 400-fold increase. Q181A also demonstrated a ~200 fold increase compared to wild type. Therefore, it is clearly evident that Threonine 186, Arginine 182, Threonine 194, and Glutamine 181 are all crucial elements that play a role in RF1 binding to the ribosome (Figures 4-7).

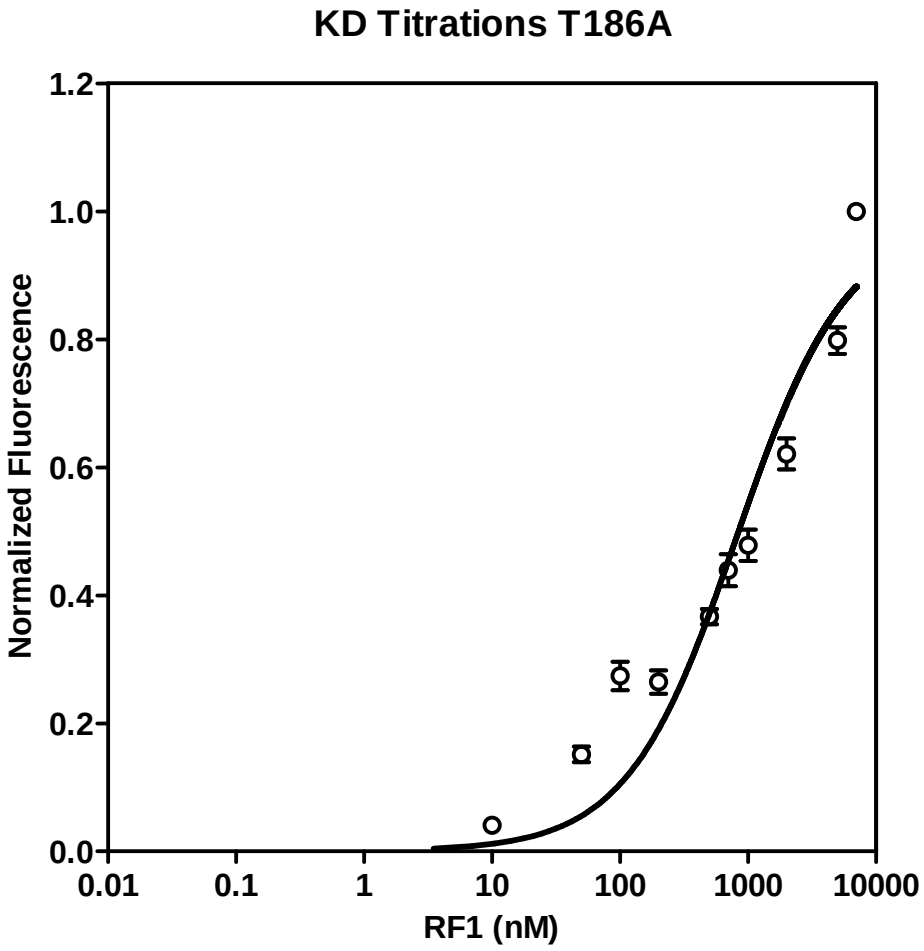

Figure 4: K_D Titration of T186A. This graph displays the normalized changes in fluorescence intensity with increasing concentrations of RF1. Using the fluorescence assay, the K_D of T186A could be determined. This graph also depicts the standard deviations for three independent experiments.

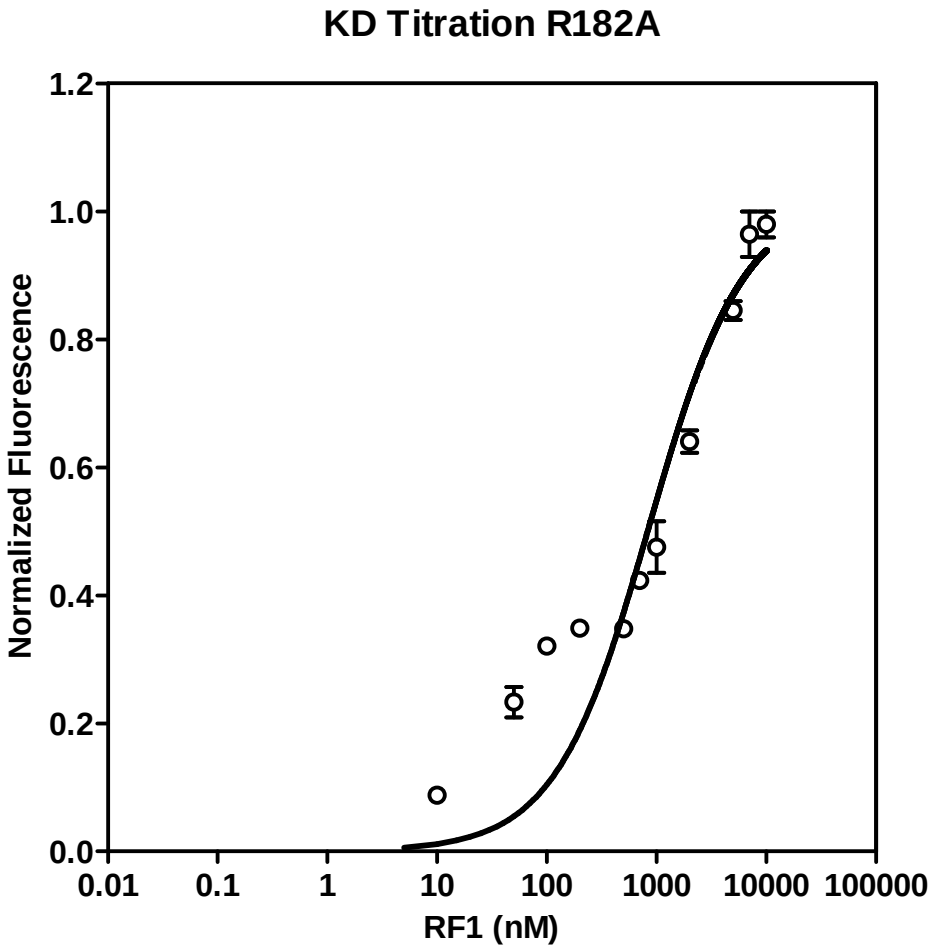

Figure 5: K_D Titration of R182A. This graph displays the normalized changes in fluorescence intensity with increasing concentrations of RF1. Using the fluorescence assay, the K_D of R182A could be determined. This graph also depicts the standard deviations for three independent experiments.

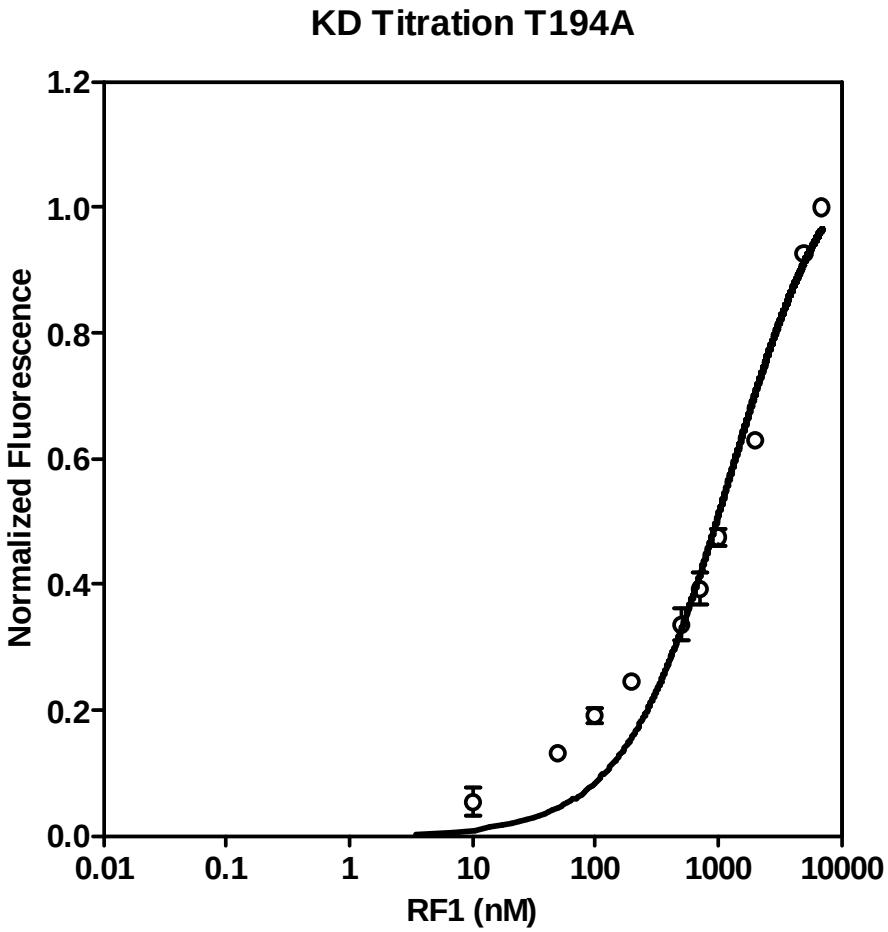

Figure 6: K_D Titration of T194A. This graph displays the normalized changes in fluorescence intensity with increasing concentrations of RF1. Using the fluorescence assay, the K_D of T194A could be determined. This graph also depicts the standard deviations for three independent experiments.

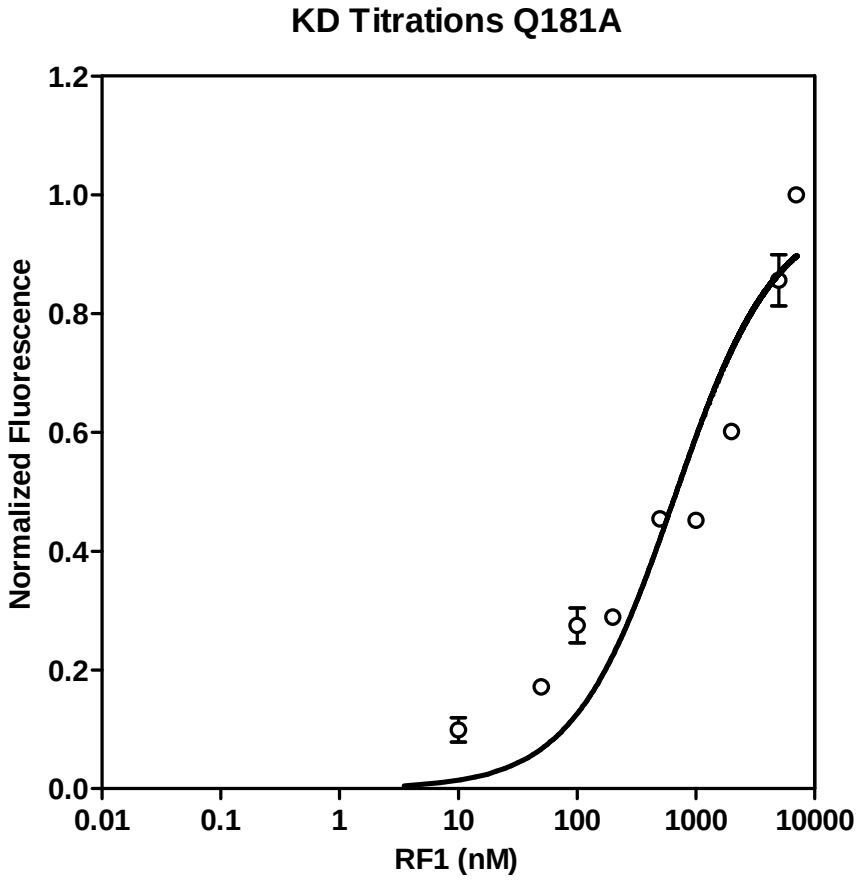

Figure 7: K_D Titration of Q181A. This graph displays the normalized changes in fluorescence intensity with increasing concentrations of RF1. Using the fluorescence assay, the K_D of Q181A could be determined. This graph also depicts the standard deviations for three independent experiments.

ii. Kinetics of Peptide Hydrolysis by RF1 Mutants

We measured the catalytic activity of the RF1 mutants to determine if these mutations affected peptidyl-tRNA hydrolysis. In order to conduct this assay, release complexes were formed; the release complexes were formed by the addition of 70S ribosomes, followed by the addition of mRNA with the UAA stop codon, and then the addition of [^{35}S]fMet-tRNA^{fmet}, which binds to the P site. Peptide release time courses were performed by the addition of saturating amounts of RF1. The RF1-catalyzed release of [^{35}S]fMet was then analyzed by eletrophoretic TLC and quantitated with a phosphorimager (Figure 8) (Fields, et al. 2010).

According to the results, wild type RF1 catalyzed peptidyl-tRNA release with a rate of ~0.14 s^{-1}, which concurs with data previously published (Figure 9) (Hetrick, et al. 2009; Fields, et al. 2010). The mutants T186A, R182A, T194A, and Q181A catalyzed peptide release with rates of 0.14 s^{-1}, 0.19 s^{-1}, 0.15 s^{-1}, and 0.16 s^{-1}, respectively (Figure 10-13). In comparison to the wild type RF1, there seems to be little to no disparity between catalytic rates, suggesting that these residues have a minimal role in peptide release.

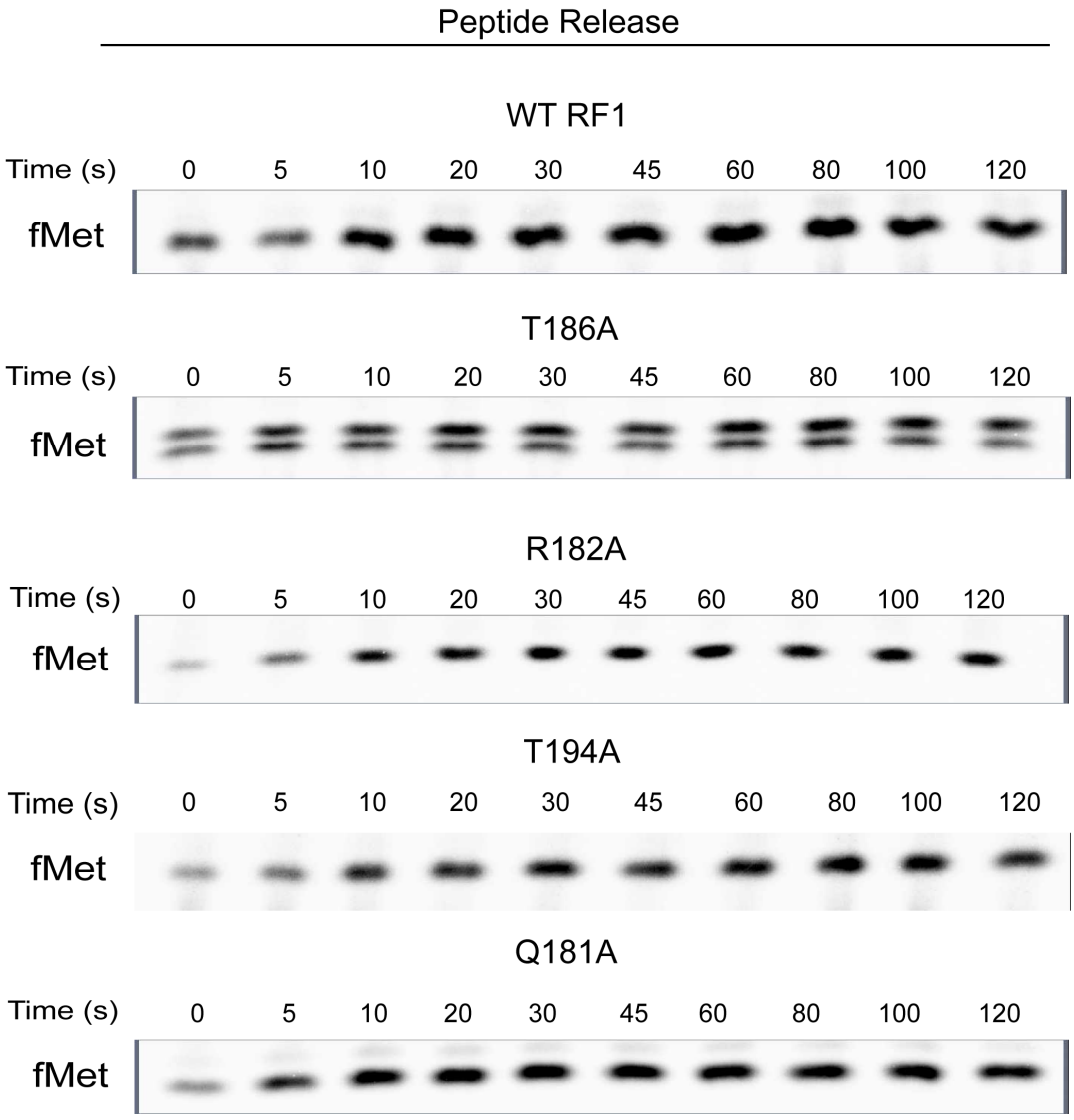

Figure 8: Quantification of Peptide Release. This figure displays the peptide release time courses that were performed with saturating amounts of RF1. This displays the RF1-catalyzed release of [35S]fMet that was analyzed by electrophoretic TLC and quantitated with a phosphoimager.

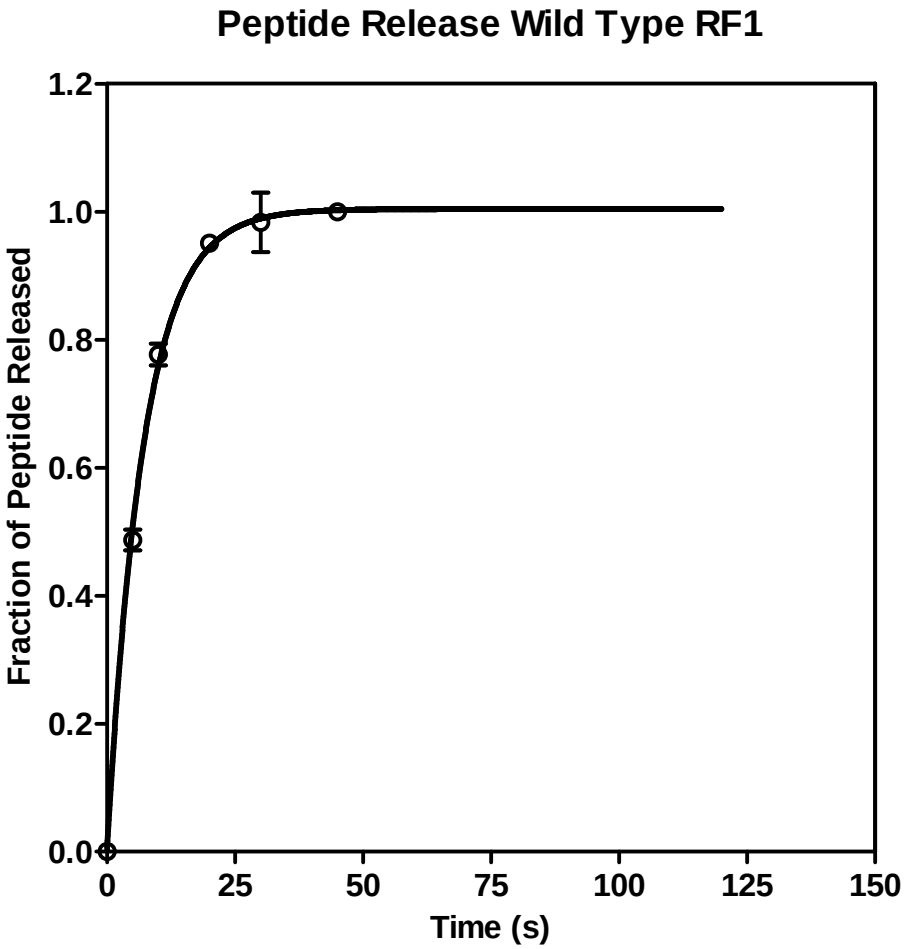

Figure 9: Peptide Release of Wild Type RF1. Using the peptide release assay, time courses were selected at saturating concentration of RF1. The data was quantitated and analyzed in order to determine the catalytic rate of peptide release. Standard deviations are included for two independent experiments.

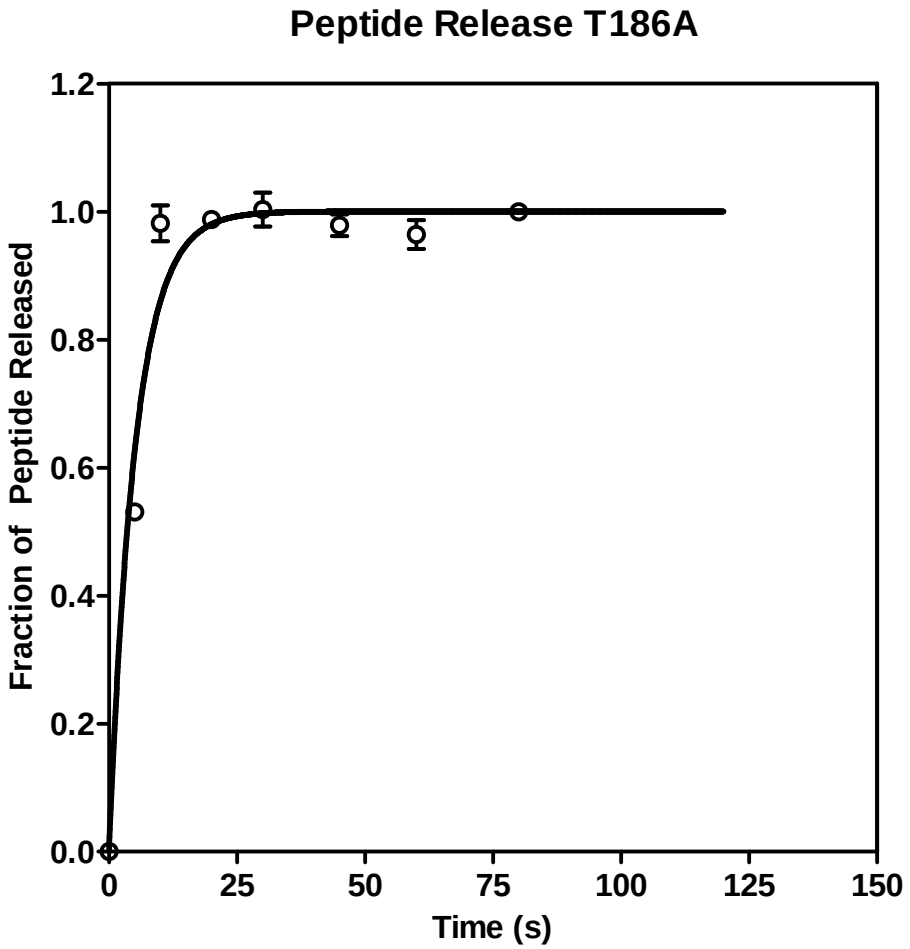

Figure 10: Peptide Release of T186A. Using the peptide release assay, time courses were selected at saturating concentration of RF1. The data was quantitated and analyzed in order to determine the catalytic rate of peptide release. Standard deviations are included for two independent experiments.

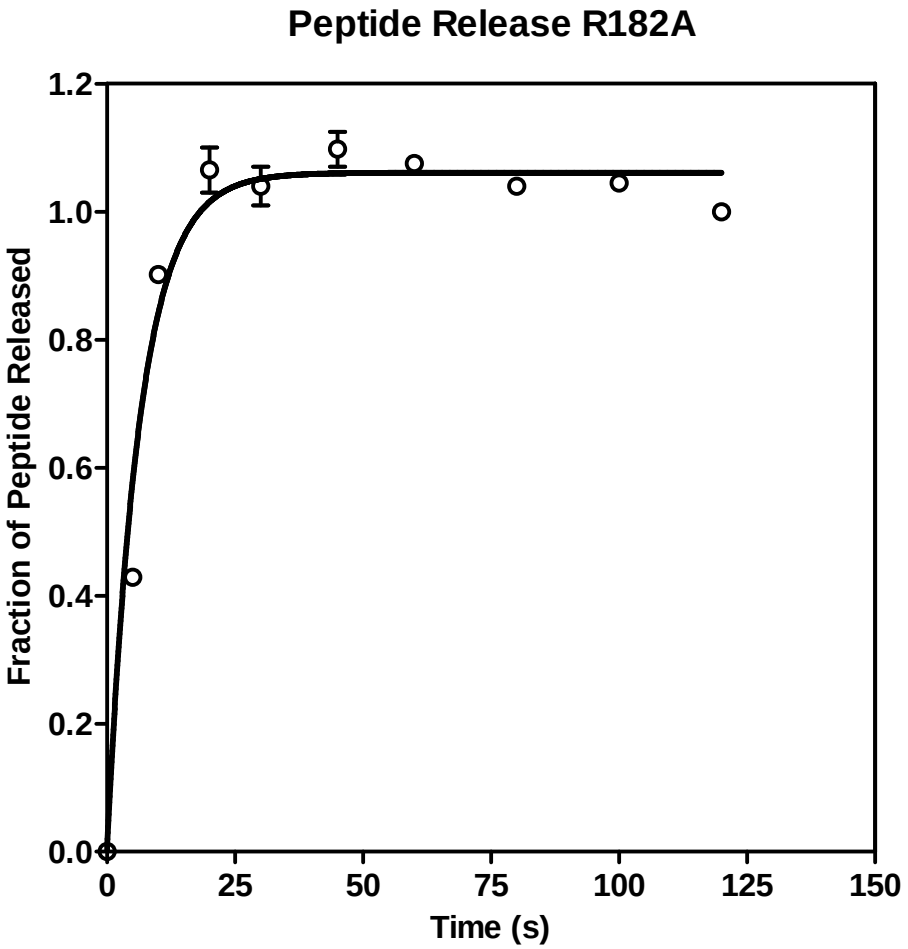

Figure 11: Peptide Release of R182A. Using the peptide release assay, time courses were selected at saturating concentration of RF1. The data was quantitated and analyzed in order to determine the catalytic rate of peptide release. Standard deviations are included for two independent experiments.

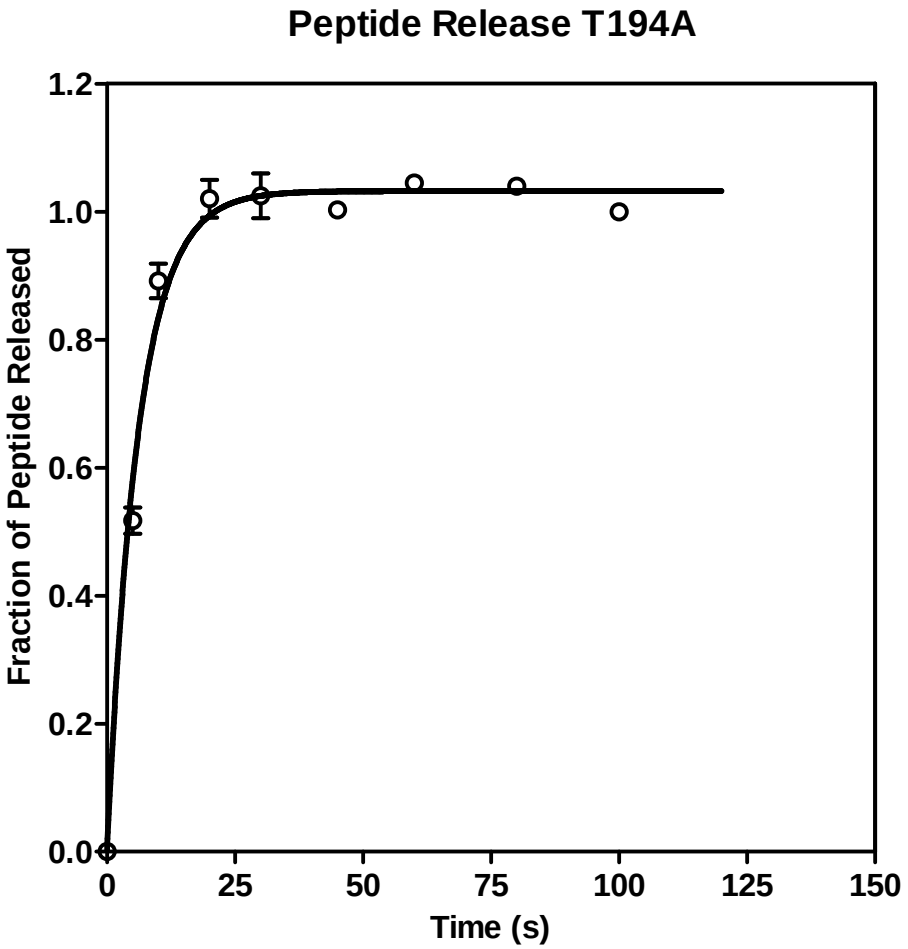

Figure 12: Peptide Release of T194A. Using the peptide release assay, time courses were selected at saturating concentration of RF1. The data was quantitated and analyzed in order to determine the catalytic rate of peptide release. Standard deviations are included for two independent experiments.

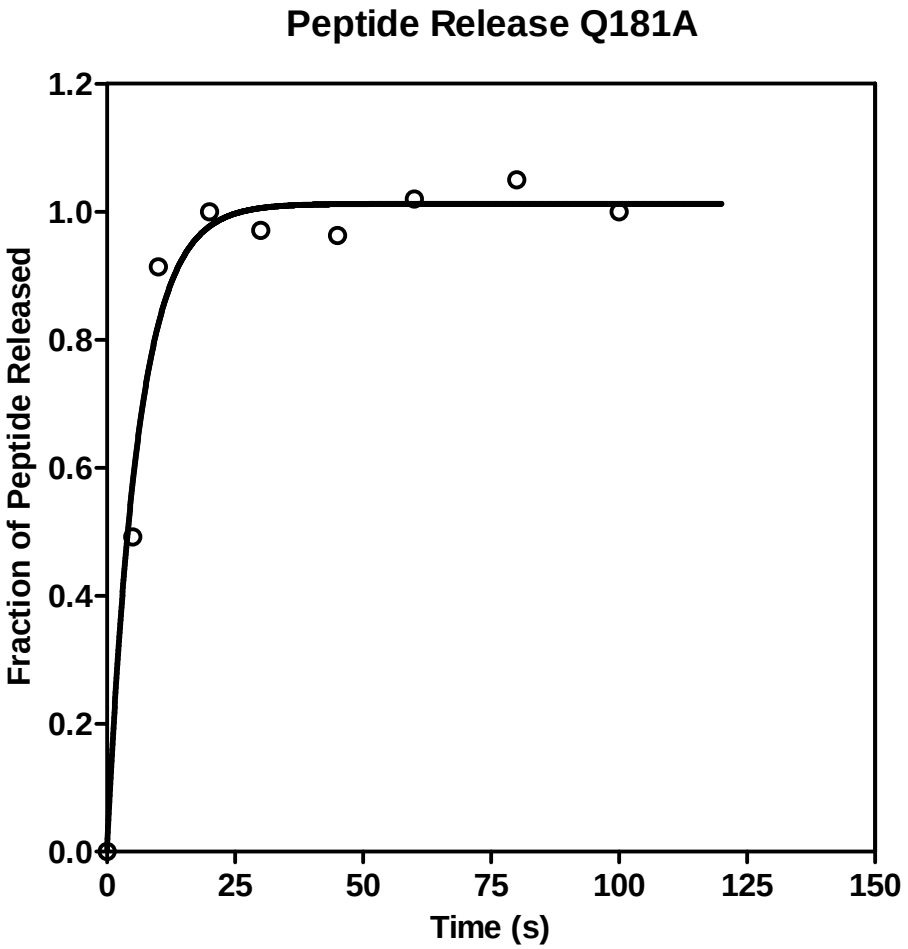

Figure 13: Peptide Release of Q181A. Using the peptide release assay, time courses were selected at saturating concentration of RF1. The data was quantitated and analyzed in order to determine the catalytic rate of peptide release.

IV. Discussion

Protein synthesis is an essential process to all living organisms. The three main stages of protein synthesis are 1) Initiation, 2) Elongation, and 3) Termination (Figure 1). The goal of this thesis is to focus on the stage of termination, with an emphasis on how class I release factors recognize stop codons and catalyze peptidyl-tRNA hydrolysis. Class I release factors, RF1 and RF2, recognize stop codons UAA, UAG, and UGA. Recent crystal structures have demonstrated that not only is the tripeptide motif involved in the process of codon recognition, but there are other vital residues of great importance (Laurberg, et al. 2008; Korostelev, et al. 2010). Moreover, computational simulations have also predicted various critical residues in the process of stop codon recognition (Sund, et al. 2010). "Nevertheless, the contribution of critical residues in the RF to binding, conformational changes, and catalysis has to be determined experimentally to fully understand the mechanism of stop codon recognition" (Fields, et al. 2010).

This study focused on the conserved RF1 residues Thr 186, Arg 182, Thr 194, and Gln 181 (Figure 3). Through the use a fluorescence-based assay, as well as peptide release assays, the binding of the mutants to the ribosome could be investigated as well as the rate of peptidyl-tRNA hydrolysis, respectively. The fluorescence-based assay was used to analyze the equilibrium binding of critical residues in release factor 1; the equilibrium studies demonstrated that the mutants T186A, R182A, T194A, and Q181A have K_D values of 791 nM, 833 nM,

1194 nM and 635 nM, respectively (Figures 4-7). These values indicate that there is a large increase in the K_D values of the mutants when compared to the wild type, which has a value of ~3nM. Threonine 186 and Arginine 182 have values ~25- fold greater than wild type, while Threonine 194 has a value ~400-fold greater and Glutamine 181 showed a value ~200-fold higher. Therefore, undoubtedly, these residues play a crucial role in RF1 binding to the ribosome.

In addition to understanding how these residues play a role in binding, the rate of peptidyl-tRNA hydrolysis was measured. In order to overcome the binding defect, saturating amounts of RF1 were added to the release complex described earlier. Wild Type RF1 showed a rate of ~0.14 s^{-1}; the mutants, T186A, R182A, T194A, and Q181A demonstrated similar values with rates of 0.14 s^{-1}, 0.19 s^{-1}, 0.15 s^{-1}, and 0.16 s^{-1}, respectively (Figures 9-13). Therefore, there seems to be no major difference in the catalytic rate between the wild type and the mutants, strongly suggesting that these mutants do not play a major role in catalysis.

These assays were used to quantitatively investigate the roles of critical residues in RF1, specifically the equilibrium binding of RF1 to the ribosome as well as the rate of peptidyl-tRNA hydrolysis. According to the results, the residues T186, R182, T194, and Q181 are all critical for binding, but not for peptidyl-tRNA hydrolysis. Due to the fact that these residues are not essential for binding, it is evident that they are active in stop codon recognition; by changing the residues to alanine, the mutants showed a significant binding defect. This defect is most likely due to the disruption of the molecular interactions; by changing the residues to

alanine, the mutants most likely do not bind with high affinity, since the proper bonds cannot be made with high efficiency.

Threonine 186 is vital to the selection of the first and second stop codon. Thr 186 hydrogen bonds to the O4 position of U1 of the stop codon, ensuring the selection of a uridine for the first base. Thr 186 also hydrogen bonds with A2, the second base of the stop codon. This interaction limits the selection to an A, while also discriminates against a G. By mutating threonine to an alanine, these hydrogen bonds were clearly disrupted. The binding defect of this mutant suggests that the Thr186 is crucial to the selection of the first two bases of the stop codon.

Arginine 182 is crucial for the selection of an adenine in the second position of the stop codon for RF1. In RF2, there is a recognition switch that causes A and G to both be read. However, in RF1, the differences in residues cause this recognition switch to be lost. These residues change, as described earlier, causes Glu 119 to form a more stable ion pair with Arg 182, inhibiting a guanine and leading to the selection of an A. Mutating this residue to an alanine demonstrated a major binding defect. Although Arg 182 does not hydrogen bond with the stop codon, the binding defect indicates that the interactions between arginine and the surrounding residues were clearly disrupted. Therefore, Arg 182 must contribute to the recognition of the stop codon.

Threonine 194 contributes to the selection of the adenine or guanine in the third position. Thr 194 can accept hydrogen bonds from the N6 of A3, or can donate hydrogen bonds to the N7 of of A3, allowing both the recognition of A or G, while

discriminating against pyrimidines. The mutation of threonine to alanine clearly demonstrates a disruption of hydrogen bonds, demonstrated by T194A having the largest binding defect of all the mutants discussed. This suggests that Thr 194 is crucial to the recognition of the stop codon.

Glutamine 181 is important for the selection of guanine/adenine in the third position. By rotating its amide side chain, Gln 181 can either donate a hydrogen bond to the O6-keto group of guanine, or accept a hydrogen bond from the N6 amino group of adenine, most likely explaining why an A or G can be in the third position. In addition, the reading of G is possible due to glutamine's NH_2 group replacing the hydrogen bond lost by T194 when A is substituted for G. Q181A showed a binding defect of ~200-fold greater than wild type. This indicates that these hydrogen bonds must have been disrupted. The disruption of these hydrogen bonds demonstrates that Glu 181 is necessary for the precise recognition of the stop codon.

Therefore, this study identified that residues Thr 186, Arg 182, Thr 194, and Gln 181, are essential for high-affinity binding of RF1 to the ribosome, but not critical for peptidyl-tRNA release. Moreover, this study contributes to the question of how stop codons are recognized; by recognizing quantitatively that these residues are crucial for binding, details of how these residues contribute to the overall recognition of stop codons can now be investigated. The results from this study are beneficial to understanding how release factors function during the termination phase of translation.

V. Future Directions

Undoubtedly, it is evident that termination is a crucial process for protein synthesis, and essentially vital to all living organisms. Due to the recent crystal structures of ribosomal subunits, as well as crystal structures of RF1 and RF2 bound to the ribosomes, the mechanism of translation, specifically how stop codons are fully recognized and what triggers the catalysis of peptidyl-tRNA hydrolysis can be investigated further. To further understand this unsolved mechanism and to further analyze the data discussed earlier, we can measure the kinetics of RF1 binding to the ribosome as well as investigate how binding, kinetics, and catalysis function with sense codons.

i. Kinetics of RF1 Binding to the Ribosome

In order to complete the entire story behind the RF1 mutants Thr 186, Arg 182, Thr 194, and Gln 181, it would be beneficial to study the transient-state kinetics to determine the rates of stop codon recognition by RF1. By using a stopped-flow instrument, it is possible to determine the rates by taking various time courses of RF1 bound to the ribosome complex. Here, we studied the equilibrium binding of the mutants. By investigating the kinetics of the mutants, it will allow us to compare the equilibrium binding to the transient-state kinetic data. For instance, Histidine 193 demonstrated it had a K_D of 350 nM after the completed reaction, showing one phase.

However, this only indicated the equilibrium binding. After investigating the transient-state kinetics, it became evident that there was not one phase, but there was a two-step binding process (biphasic). The data indicated that the association rate of H193A was unaffected by the mutation (phase one), while H193 was required for the second phase due to the binding defect. Therefore, investigating the transient-state kinetic data for the other mutants would allow us to determine the activity of the mutants during real time.

ii. Use of Sense Codons Rather than the UAA Stop Codon

This study focused on RF1 recognizing the UAA stop codon. A future study could focus on how these mutants interact with a different stop codon or even a sense codon. The first step could be using a different stop codon; since RF1 recognized only UAA and UAG, by using the UGA stop codon, it would be possible to investigate how RF1 reacts to that particular stop codon. Will the mutants perhaps bind to that stop codon or will RF1 have no affinity to that stop codon? Another avenue is using sense codons rather than stop codons. By changing the stop codon UAA to a sense codon such as CAA or UAC, one can investigate the equilibrium binding and rate of peptide release of these mutants interacting with sense codons. It may be possible that the mutations cause RF1 to still recognize these codons or even catalyze peptide release with a higher rate; at the same time, it may be possible that these mutations cause no binding to occur at all.

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