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Kinetics of Codon Recognition on the Ribosome by tRNAs and Release Factors

A dissertation submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy

in

Chemistry and Biochemistry

by

Byron Anthony Hetrick

Committee in charge:

   Professor Simpson Joseph, Chair
   Professor Joseph Adams
   Professor Thomas Hermann
   Professor Patricia Jennings
   Professor Wei Wang

2010

The Dissertation of Byron Anthony Hetrick is approved, and is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2010

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LIST OF ABBREVIATIONS

A	adenine
ATP	adenosine triphosphate
A-site	aminoacyl site
ASL	anticodon stem loop
C	cytosine
Cryo-EM	cryo electron microscopy
DNA	deoxyribonucleic acid
D-arm	dihydrouridine arm
E-site	exit site
EF	elongation factor
G	guanine
GTP	guanosine triphosphate
GDP	guanosine diphosphate
GAC	GTPase activation center
IF	initiation factor
IC	initiation complex
LB	Luria Broth
mRNA	messenger ribonucleic acid
P-site	peptidyl site
Psi (Ψ)	pseudouridine
PheRS	phenylalanyl tRNA synthetase

RNA	ribonucleic acid
RRF	ribosome recycling factor
rRNA	ribosomal ribonucleic acid
RF	release factor
RC	release complex
S	svedberg unit
SRL	sarcine ricin loop
T	thymine
tRNA	transfer ribonucleic acid
TC	ternary complex
U	uracil

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ACKNOWLEDGEMENTS

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ABSTRACT OF THE DISSERTATION

Kinetics of Codon Recognition on the Ribosome by tRNAs and Release Factors

by

Byron Anthony Hetrick

Doctor of Philosophy in Chemistry and Biochemistry

University of California, San Diego, 2010

Professor Simpson Joseph, Chair

Ribosomes translate messenger ribonucleic acid (mRNA) into proteins in all domains of life. During the elongation phase of protein synthesis, tRNAs bind to the ribosome in a codon dependent fashion as ternary complexes consisting of a protein elongation factor (EF-Tu), guanosine triphosphate (GTP), and an aminoacylated transfer

ribonucleic acid (tRNA). The first step in the termination of protein synthesis is the recognition of stop codons by release factor 1 or 2 (RF1 or 2) in order to hydrolyze the completely synthesized protein from the tRNA bound in the peptidyl (P) site of the ribosome.

We have developed a fluorescence based method designed to monitor codon recognition by tRNAs and RFs in the aminoacyl (A) site of the 30S subunit of the ribosome. Using the change in fluorescence of a pyrene molecule attached to the 3' end of a short mRNA as a probe, we have investigated the kinetic mechanism of ternary complex and release factor binding to the A-site of the ribosome. Codon recognition by ternary complexes occurs as part of the second order association step between the ribosome and ternary complex. By interacting with the codon during the first encounter, competition between cognate and near or non-cognate ternary complexes is reduced and rapid screening of ternary complexes may occur. We have found that physiological concentrations of the polyamines spermine and spermidine stimulate ternary complex binding to the A-site of the ribosome at least as well as unphysiologically high concentrations of magnesium ions commonly used during in vitro translation experiments. We have also investigated the thermodynamics and kinetics of RF1 binding to the ribosome when a stop codon or a variety of sense codons were positioned in the A-site. The relative affinity of RF1 to different sense codons and the catalysis of peptide release by the RF were not directly related. The observed disparity between binding and catalysis indicates that RF1 employs an induced fit mechanism in the discrimination of stop codons from sense codons.

Chapter 1: Introduction

The flow of information in the cell

According to the central dogma of molecular biology, the flow of information in cells proceeds from deoxyribonucleic acid (DNA) to ribonucleic acid (RNA) to protein (Figure 1.1) (Crick 1970). Replication of DNA, the genetic material, is performed by DNA polymerase and utilizes pre-existing strands of DNA as the template directing the sequence of nucleotides to be polymerized, forming a new strand of DNA (Johnson 1993; Alberts, Johnson et al. 2002). The sequence of nucleotides polymerized is determined by the base pairing interactions observed in Watson and Crick's structure of double stranded DNA (Watson and Crick 1953). In the structure of double stranded DNA, it was observed that adenine (A) and thymine (T) make specific hydrogen bonding interactions and that guanine (G) and cytosine (C) make specific hydrogen bonding interactions, allowing the formation of a uniform double helical structure (Watson and Crick 1953). These interactions have since been known as Watson-Crick base pairs in order to differentiate them from other nucleotide interactions that may occur (Leontis, Stombaugh et al. 2002).

A variety of RNA molecules are synthesized from a DNA template by RNA polymerase in a process called transcription (Alberts, Johnson et al. 2002; Brosnan and Voinnet 2009). The sequence of an RNA being synthesized is determined by similar base paring rules to those observed in the structure of double stranded DNA.
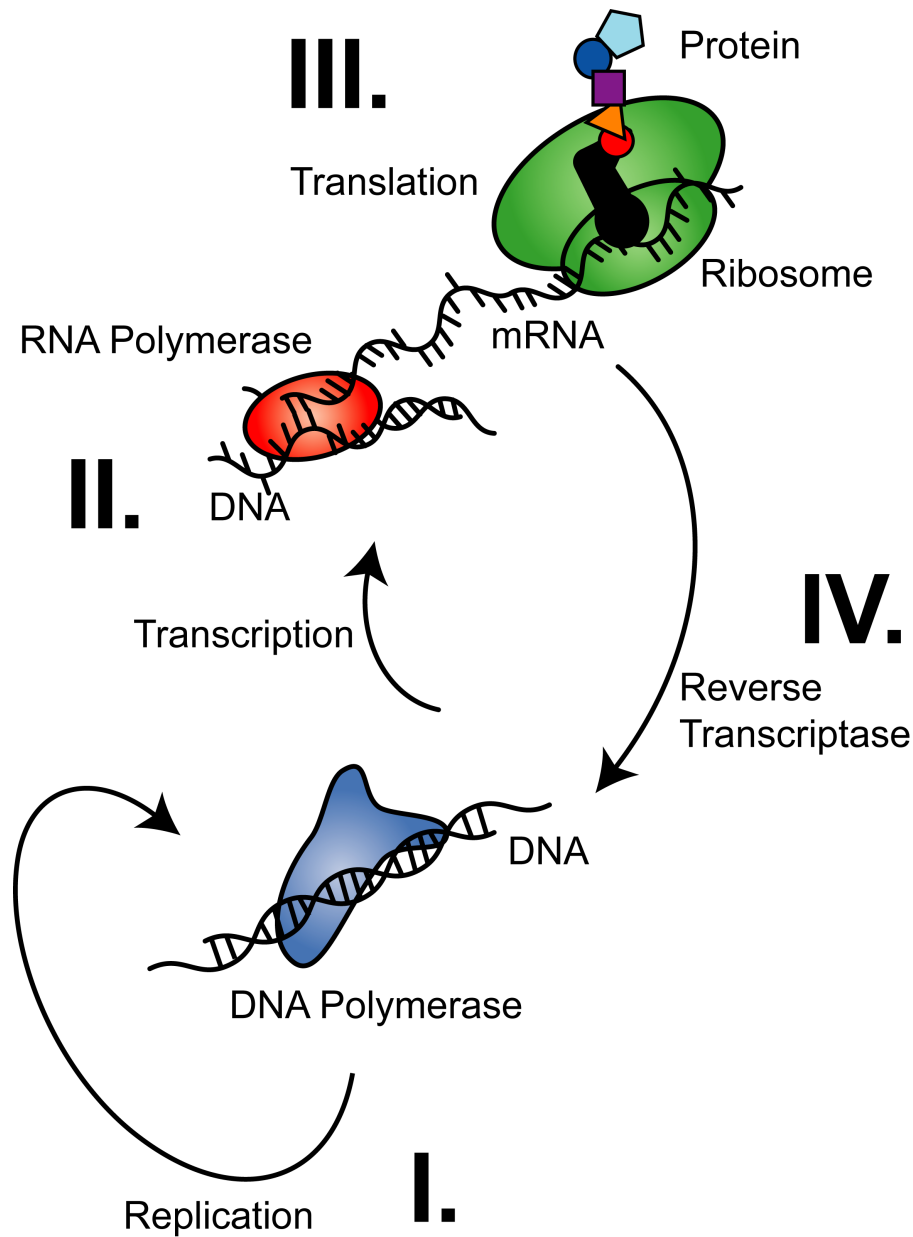
Figure 1.1: The central dogma of molecular biology. Cartoon representation of the central dogma of molecular biology as described by Crick. *I. Replication.* A DNA template is used to synthesize new strands of DNA during replication and is catalyzed by DNA polymerase. *II. Transcription.* DNA may be transcribed into RNA by RNA polymerase. *III. Translation.* mRNA is translated into protein by the ribosome. Translation in prokaryotes may occur co-transcriptionally before the mRNA template has been completely synthesized. *IV. Reverse transcription.* RNA may also be reverse transcribed back into DNA by reverse transcriptase. Reverse transcription commonly occurs in viruses with RNA genomes.

In RNA however, uracil (U) replaces thymine in its interaction with adenine (Saenger 1984; Alberts, Johnson et al. 2002).

RNA products from transcription can be broadly classified into coding and noncoding RNAs (Storz, Altuvia et al. 2005). Coding RNAs, also known as messenger RNA (mRNA), determine the sequence of amino acids in a protein (Brenner, Jacob et al. 1961). All other RNAs are known as noncoding RNA (Brosnan and Voinnet 2009). A specific type of noncoding RNA, known as transfer RNA (tRNA), acts as an "adapter" molecule by interacting with mRNA according to Watson-Crick base pairing rules (Crick 1958; Soll, Jones et al. 1966; Wilcox and Nirenberg 1968). This mRNA-tRNA interaction occurs in sets of three discreet nucleotides (Crick, Barnett et al. 1961; Nirenberg, Leder et al. 1965). Each set of three nucleotides in an mRNA corresponding to a specific tRNA has been termed a codon (Soll, Ohtsuka et al. 1965). The nucleotides in the tRNA that base pair with a codon have been termed the anticodon (Crick 1966). Thus each mRNA codes for a sequence of tRNA molecules. Each tRNA corresponds to a specific amino acid (Berg and Offengand 1958). An amino acid is enzymatically attached to its corresponding tRNA in a process called aminoacyl tRNA synthesis (Ibba and Soll 2000). The codon-anticodon interaction between tRNA and mRNA occurs within a large macromolecular complex called the ribosome. The ribosome is composed of more than 50 proteins (the specific number depends upon the organism) and another type of noncoding RNA called ribosomal RNA (rRNA) (Kurland 1960; Stanley and Bock 1965; Kurland 1972). As a ribosome moves along the mRNA template, codon-anticodon base pairing

specifies which tRNA binds to the ribosome and the ribosome catalyzes the polymerization of amino acids attached to tRNAs into a polypeptide chain (Green and Noller 1997). The process of mRNA directed protein synthesis by the ribosome is called translation. The mechanistic detail of the process of translation is the focus of our lab and of this thesis.

Overview of the three phases of translation and the three site model of protein synthesis

The process of translation can be separated into three phases; initiation, elongation, and termination (Figure 1.2) (Lucas-Lenard 1971). Prior to the initiation of protein synthesis, the ribosome exists as two separate subunits, the 30S, or small, ribosomal subunit and the 50S, or large, ribosomal subunit (Subramanian, Ron et al. 1968). During initiation, the ribosomal subunits are assembled into the 70S ribosome on an mRNA at the correct site to begin protein synthesis (Gold, Pribnow et al. 1981). In prokaryotes, three protein initiation factors, initiation factor 1 (IF1), initiation factor 2 (IF2), and initiation factor 3 (IF3) assemble the ribosome, initiator tRNA, and mRNA into the 70S initiation complex (IC) (Figure 1.2) (Myasnikov, Simonetti et al. 2009). The 70S ribosome-initiator tRNA-mRNA complex formed at the end of initiation is known as the 70S initiation complex (IC). Once the 70S IC has been formed, the elongation phase of protein synthesis begins (Figure 1.2). At the beginning of the elongation phase of protein synthesis, an initiator tRNA is bound in
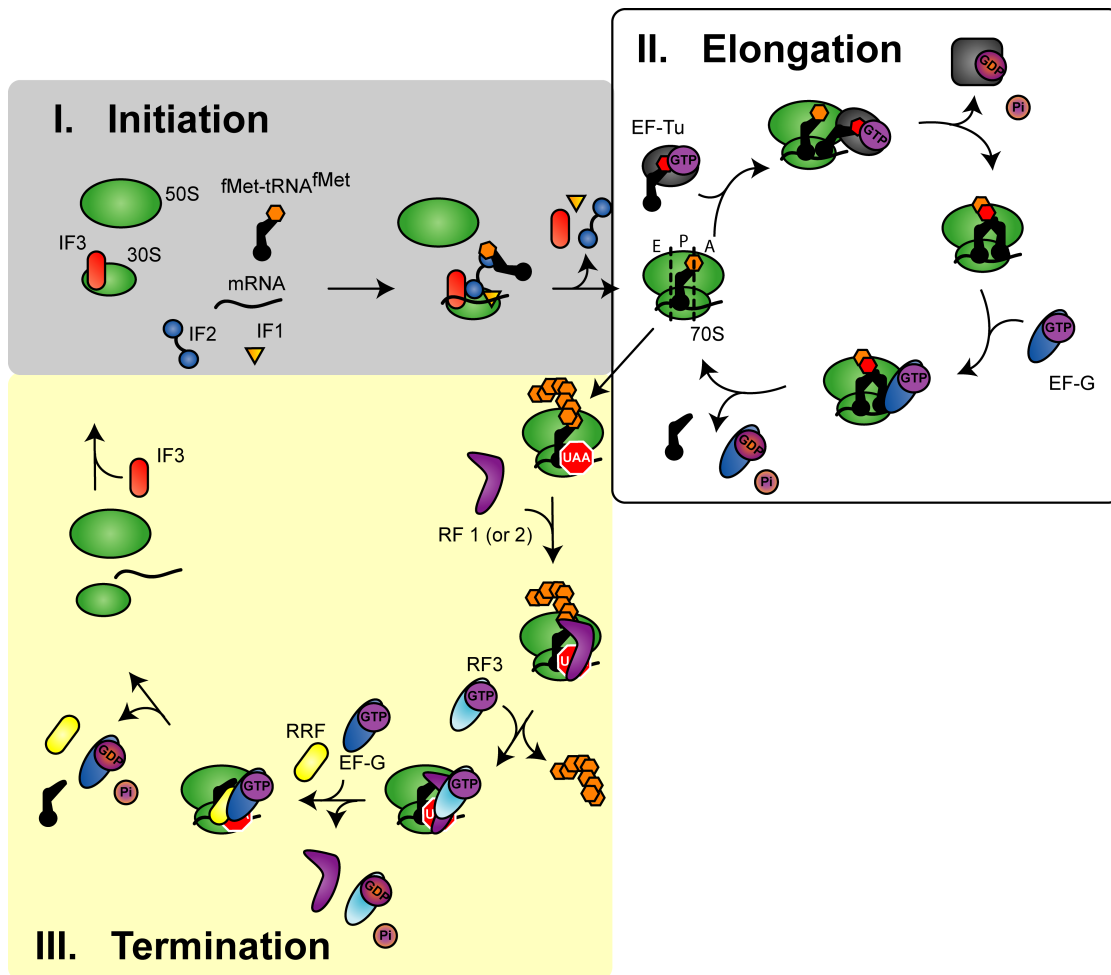
Figure 1.2: The three phases of protein synthesis. *I. Initiation of protein synthesis.* The two ribosome subunits are assembled together with an mRNA and fMet-tRNA^{fMet}. This assembly is catalyzed by three protein initiation factors, IF1, IF2, and IF3. *II. Elongation phase of protein synthesis.* tRNAs bind to the A-site of the ribosome as a ternary complex with EF-Tu and GTP. EF-Tu hydrolyzes GTP, releasing the tRNA into the A-site. Peptide bond formation transfers the peptide being synthesized from the P-site tRNA to the A-site tRNA. EF-G catalyzes the movement of the tRNA-mRNA complex through the ribosome, opening the A-site for a new tRNA to bind. *III. Termination of protein synthesis.* Once a stop codon enters the A-site, RF1 or 2 binds and releases the protein from the P-site tRNA. RF3 removes RF1 or 2 from the A-site. RRF and EF-G together split the subunits. The bound tRNA and mRNA dissociate and IF3 binds to the 30S subunit to prevent premature reassociation of the two subunits.

the peptidyl site (P-site) of the ribosome (Nomura and Lowry 1967; Guthrie and Nomura 1968). The P-site contains the tRNA that is still covalently attached to the protein being synthesized (Moazed, Robertson et al. 1988). A new tRNA is recruited to the ribosome by a protein elongation factor called elongation factor Tu (EF-Tu) (Lucas-Lenard and Lipmann 1966). The next tRNA binds to the aminoacyl site (A-site) of the ribosome based upon codon-anticodon base pairing interactions. The A and P sites of the ribosome were originally defined functionally based upon the reactivity of an aminoacylated tRNA with the antibiotic puromycin (Traut and Monro 1964). An aminoacylated tRNA bound in the P-site reacts well with puromycin, while an aminoacylated tRNA bound in the A-site does not react with puromycin (Traut and Monro 1964). EF-Tu is a guanosine 5'-triphosphatase (GTPase). Upon recruitment of the tRNA to the ribosome, EF-Tu hydrolyzes guanosine 5'-triphosphate (GTP) and releases the tRNA to the ribosome (Lucas-Lenard and Lipmann 1966; Swart and Parmeggiani 1989). The ribosome then catalyzes the transfer of the polypeptide being synthesized from the tRNA bound in the P-site to the tRNA bound in the A-site of the ribosome in the peptidyl transfer reaction (Traut and Monro 1964). Another elongation factor, elongation factor G (EF-G), also a GTPase, catalyzes the movement of the tRNA-mRNA complex through the ribosome (Lucas-Lenard and Lipmann 1966; Bretscher 1968). The movement of the tRNA-mRNA complex through the ribosome is know as translocation (Bretscher 1968). During translocation, the tRNA bound in the P-site is moved into the exit site (E-site), where it binds prior to dissociating from the ribosome, and the tRNA in the A-site is moved to the P-site

(Skogerson and Moldave 1968; Rheinberger, Sternbach et al. 1981; Moazed and Noller 1989). The mRNA, still base paired to the tRNAs, also translocates, presenting a new codon in the A-site (Joseph and Noller 1998). Elongation continues in a cyclic fashion with a new tRNA binding at the A-site, donating its amino acid to the growing polypeptide, then being moved through the ribosome so a new tRNA may bind (Kurland 1972). Owing to the fact that tRNAs may be bound to the ribosome at three different sites, this process is known as the three-site model of protein synthesis (Rheinberger, Sternbach et al. 1981). Three special codons, called nonsense codons, do not code for tRNAs (Brenner, Stretton et al. 1965; Zipser 1967). Nonsense codons signal the end of the elongation phase of protein synthesis when they enter the A-site of the ribosome (Brenner, Stretton et al. 1965). Nonsense codons are more commonly known as stop codons because they signal that the end of the coding portion of the mRNA has been reached (Petry, Weixlbaumer et al. 2008).

The termination phase of protein synthesis begins when a stop codon enters the A-site of the ribosome (Figure 1.2) (Petry, Weixlbaumer et al. 2008). Stop codons code for class I protein release factors (RFs) instead of tRNAs (Scolnick, Tompkins et al. 1968). In prokaryotes, there are two class I release factors, release factor 1 (RF1) and release factor 2 (RF2) that recognize stop codons in the A-site of the ribosome (Scolnick, Tompkins et al. 1968). RFs bind to the A-site and catalyze the release of the completely synthesized protein from the tRNA bound in the P-site of the ribosome (Brown and Tate 1994). Once the protein has been released by the ribosome, the class I RF is removed by a class II RF, release factor 3 (RF3) (Freistroffer, Pavlov et al.

1997). RF3 is a GTPase which induces a structrual change in the ribosome that is incompatible with class I RF binding (Gao, Zhou et al. 2007). After ejection of the class I RF, the ribosome is then disassembled so it can translate a new mRNA (Petry, Weixlbaumer et al. 2008). Ribosome recycling factor (RRF) and EF-G together split the ribosome back into two separate subunits (Karimi, Pavlov et al. 1999; Peske, Rodnina et al. 2005). Once the ribosomal subunits have been separated IF3 binds to the free 30S subunit, preventing premature re-association with 50S subunits (Subramanian, Ron et al. 1968). The ribosome may now re-enter the initiation phase of protein synthesis to begin synthesizing a new protein.

Structure of tRNAs and Ribosomes

The ribosome and tRNAs play a role in all steps of protein synthesis (Figure 1.2). Here, the general structural features of these two major components of the translation pathway will be reviewed. More specific structural details will be described later in the appropriate sections, as necessary.

Ribosome Structure

The 30S and 50S subunits of the ribosome combine to make the 2.6 megadalton 70S ribosome (Figure 1.3) (Kurland 1960). The 50S subunit is composed of two strands of RNA, the 23S and 5S rRNAs, and about 30 proteins (Kurland 1972).

The 30S subunit is composed of the 16S rRNA and about 20 proteins (Kurland 1972). Crystal structures of the individual 50S (Figure 1.3A) and 30S (Figure 1.3B) subunits provided the first high resolution structural information of the ribosome (Ban, Nissen et al. 2000; Wimberly, Brodersen et al. 2000).   Subsequently, a low resolution structure of the complete 70S ribosome with an mRNA and tRNAs bound (Figure 1.3C) was solved, providing the first picture of a functional state of the ribosome (Yusupov, Yusupova et al. 2001). More structures have revealed the molecular details of many intermediate functional states of the ribosome (Schmeing and Ramakrishnan 2009).

The 50S subunit interacts with the 3' ends of the aminoacyl-tRNAs bound to the ribosome (Yusupov, Yusupova et al. 2001). The 3' ends of the tRNAs bound in the A and P sites are located directly adjacent to each other in the peptidyl transferase center of the ribosome (Figure 1.3A, C) (Yusupov, Yusupova et al. 2001).   The peptidyl transferase center carries out the main catalytic activity of the 50S subunit, the transfer of the nascent peptide from the peptidyl-tRNA in the P-site to the aminoacyl-tRNA in the A-site, in the peptidyl transferase reaction (Traut and Monro 1964). The 3' end of the tRNA bound in the E-site is located far from the other ribosome bound tRNAs (Figure 1.3A, C) (Yusupov, Yusupova et al. 2001). The major architectural features of the 50S subunit are the central protuberance (CP), the L1, region and the L7/L12 stalk which stick out from the main body of the subunit (Fig. 1.3A) (Ban, Nissen et al. 2000). Cryo-Electron microscopy (CryoEM) maps as well as crystal structures have verified observations that the sarcine ricin loop (SRL) and the
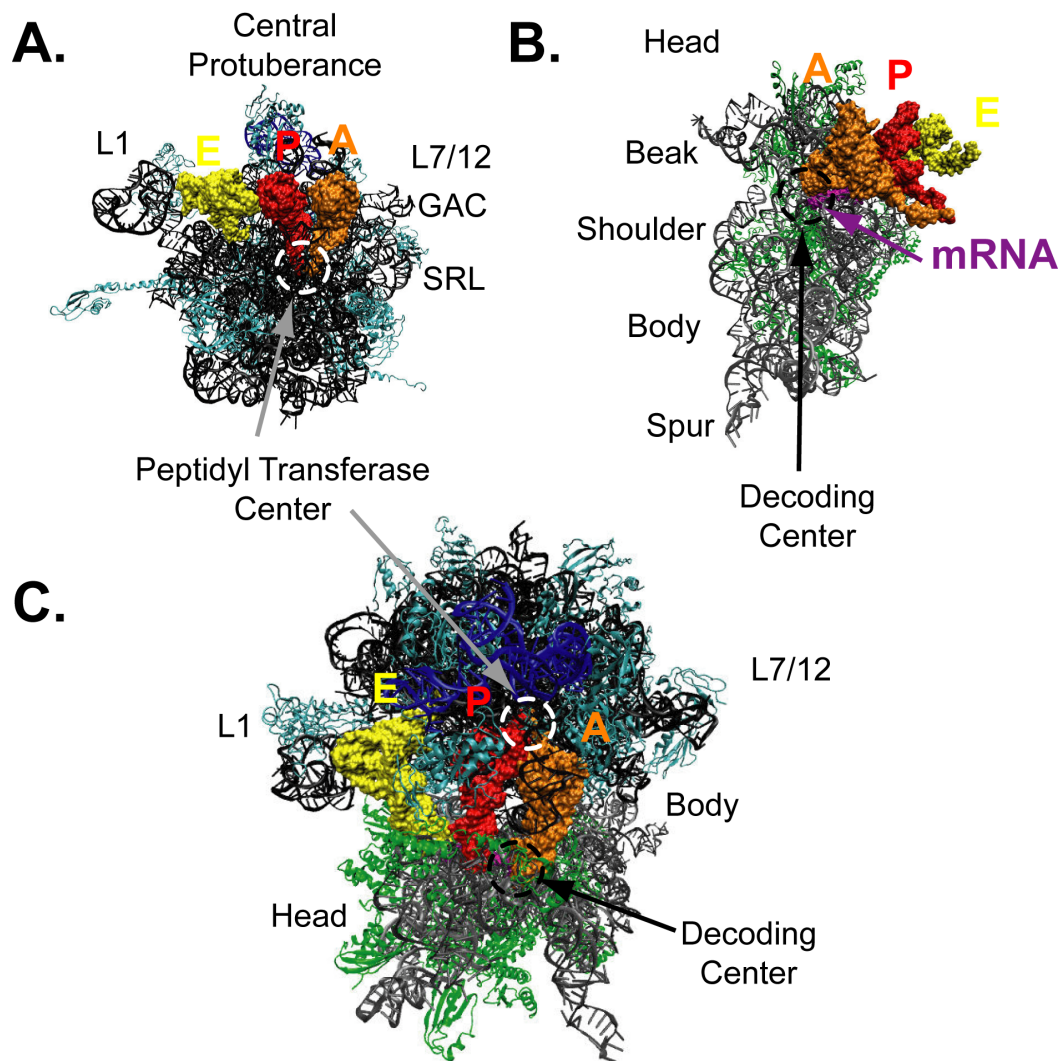
Figure 1.3: Structure of the 70S ribosome with tRNAs and mRNA bound. (A) Structure of the 50S subunit with tRNAs bound viewed from the subunit interface. 23S rRNA (black), 5S rRNA (blue), large subunit proteins (cyan), A-site tRNA (orange), P-site tRNA (red), E-site tRNA (yellow). L1 and L7/12 indicate regions where these large subunit proteins bind. L7/12 are not present in this structure. (B) Structure of the 30S subunit with tRNAs and mRNA bound viewed from the subunit interface. Colors are the same as (A) except 16S rRNA (gray), small subunit proteins (green), and mRNA (purple). (C) Structure of the 70S ribosome from *T. thermophilus* with tRNAs and mRNA bound. Colors are the same as in (A) and (B). Protein Data Bank ID 2WDK and 2WDL (Voorhees, Weixlbaumer et al. 2009).

GTPase activation center (GAC) are regions of the 50S subunit important for the binding and the activation of GTP hydrolysis by the GTPase proteins, EF-Tu, EF-G, RF3, and IF2 (Klaholz, Myasnikov et al. 2004; Allen, Zavialov et al. 2005; Gao, Selmer et al. 2009; Schmeing, Voorhees et al. 2009). The binding of the GTPase protein factors to the ribosome is abolished upon clevage of the SRL by the ribotoxins sarcine or ricin (Wool, Gluck et al. 1992; Garcia-Ortega, Alvarez-Garcia et al. 2010) .

The mRNA being translated by the ribosome is bound in a cleft between the head and body of the 30S subunit (Figure 1.3B) (Yusupova, Jenner et al. 2006). This cleft is also where the codon-anticodon interactions between the mRNA and tRNAs takes place (Yusupov, Yusupova et al. 2001). The region of the ribosome where codon-anticodon base pairing occurs in the 30S subunit A-site is known as the decoding center (Yusupov, Yusupova et al. 2001). The decoding center is where the critical decision is made by the ribosome determining whether a tRNA should be accepted or rejected based upon the codon-anticodon base pairing interaction (Wimberly, Brodersen et al. 2000; Yusupova, Yusupov et al. 2001). The major architectural features of the 30S subunit are the head and body portions of the 30S subunit which are positioned on either side of the mRNA binding channel (Figure 1.3B) (Wimberly, Brodersen et al. 2000). The beak and shoulder regions of the 30S subunit are positioned on the A-site side of the head and body respectively (Wimberly, Brodersen et al. 2000).

The prokaryotic ribosome is composed of approximately 2/3 RNA and 1/3 protein (Kurland 1960). The proteins of the ribosome are primarily scattered around

the surface, with the interior regions and subunit interface being nearly devoid of proteins (Ban, Nissen et al. 2000; Wimberly, Brodersen et al. 2000). While some of the ribosomal proteins are globular in nature, many have long extensions into the core of the ribosome with basic residues that can neutralize charge in the RNA rich interior (Ban, Nissen et al. 2000; Wimberly, Brodersen et al. 2000). The observation of protein extensions into the RNA rich interior of the ribosome has led to the suggestion that the proteins act as a "mortar" holding together the "RNA bricks" of the ribosome (Ban, Nissen et al. 2000). The ribosomal proteins have in fact been shown to be important in the folding of the ribosomal RNA (Adilakshmi, Bellur et al. 2008; Sykes and Williamson 2009). Some ribosomal proteins do perform specific functions however. The L7/L12 proteins, for example, mediate protein-protein interactions which are important for binding and GTP hydrolysis by translation factors (Diaconu, Kothe et al. 2005).

Transfer RNA Structure

tRNAs are involved in nearly every step of protein synthesis (Figure 1.2). Cells must have a great variety of tRNAs in order to read all of the possible sense codons. *Escherichia coli* for example contains 45 different tRNAs (Komine, Adachi et al. 1990). A fundamental understanding of the structure of tRNAs is essential in order to understand translation.

The nucleotide sequence of an RNA defines its primary structure (Cantor and

Schimmel 1980). The first primary structure of a tRNA was discovered in 1965 (Holley, Apgar et al. 1965). From the primary structure, the secondary structure could be deduced (Levitt 1969; Soll and RajBhandary 1995) . The secondary structure of an RNA is defined primarily by local base pairing interactions between nucleotides resulting in the formation of double helices (Cantor and Schimmel 1980). Upon the determination of the sequences of more tRNAs, it was discovered that the canonical cloverleaf secondary structure that was initially observed describes the general secondary structure for nearly all tRNAs (Soll and RajBhandary 1995). The general cloverleaf secondary structure of tRNAs consists of four stems (double stranded, base-paired regions) radiating out from a central core (Figure 1.4A). These four regions are known as the aminoacyl stem, the anticodon arm, the TΨC arm, and the D arm (Soll and RajBhandary 1995). The aminoacyl stem is named due to the fact that the 3' end of the tRNA located here is where an amino acid to be brought to the ribosome is attached through an aminoacyl linkage (Skogerson and Moldave 1968; Ibba and Soll 2000). The anticodon arm contains the anticodon of the tRNA which base pairs with mRNA in the ribosome (Soll and RajBhandary 1995). The D arm and TΨC arm are named for conserved, post transcriptionally modified bases found in these regions (Soll and RajBhandary 1995). D stands for dihydro-uracil, T stands for 5-methyl uracil, and Ψ (psi) stands for pseudouridine (Soll and RajBhandary 1995). Additionally, there is a region of the tRNA known as the variable loop (Figure 1.4A) (Soll and RajBhandary 1995). As the name suggests, the variable loop varies in size depending upon the tRNA while the length of the other regions remains relatively

A.

B.
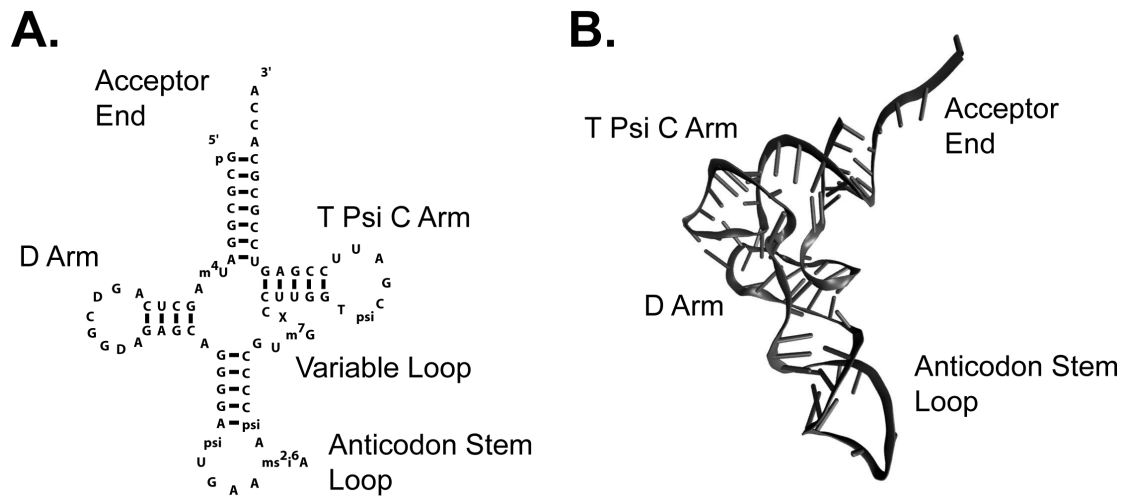
Figure 1.4: tRNA structure. (A.) The secondary structure of tRNA[phe]. (B.) Tertiary structure of tRNA[phe]. Protein Data Bank ID 1EHZ (Shi and Moore 2000).

constant (Soll and RajBhandary 1995).

The crystal structures of many tRNAs have been solved. While there may be a high degree of variability in the primary structure and some variability in the secondary structure of tRNAs, all tRNAs appear to fold into a similar "L-shaped" tertiary structure (Figure 1.4B) (Soll and RajBhandary 1995). Two crystal forms of tRNA[phe] (notation for phenylalanine accepting tRNA) have been shown to be indistinguishable, helping to solidify this tRNA as a prototypical tRNA (Sussman and Kim 1976). The tertiary structure of an RNA is defined by the long range interactions which generally consist of interactions in the major or minor grooves of base paired regions of the RNA (Cantor and Schimmel 1980). In the tertiary structure of tRNAs, the TΨC arm stacks on the acceptor arm to form the acceptor stem and the D arm

stacks onto the anticodon arm to form the anticodon stem (Soll and RajBhandary 1995). Interactions between the loop of the TΨC arm and the loop of the D arm "lock" the corner of the L-shaped tRNA structure into position (Soll and RajBhandary 1995). Crystal structures of tRNAs have provided a "treasury of stereochemical information" (Saenger 1984). Nearly all early known RNA structural motifs came from tRNA structures (Saenger 1984; Soll and RajBhandary 1995).

Due to the highly negative charge of RNA in solution, cations are extremely important in stabilizing the structure of RNAs (Soll and RajBhandary 1995). Consistent with this fact, magnesium ions and polyamines have been found in deep grooves of tRNAs making nonspecific contacts (Quigley, Teeter et al. 1978). The polyamine spermine has also been shown to be an important additive for tRNA crystallization (Kim, Quigley et al. 1971; Ichikawa and Sundaralingam 1972; Ladner, Finch et al. 1972).

The Initiation Phase of Protein Synthesis

During the initiation phase of protein synthesis, the translational machinery is assembled on the message in order to begin protein synthesis (Figure 1.2). Experiments observing the accumulation of polysomes (multiple ribosomes attached to one mRNA template) suggested that ribosomes attach to an mRNA at the beginning of the message and detach when the message has been completely translated (Knopf and Lamfrom 1965; Williamson and Schweet 1965). The idea that ribosomes are

dynamically bound to the message and are recycled was extended when subunit exchange experiments indicated that ribosomes dissociate into separate 50S and 30S subunits between rounds of translation (Eisenstadt and Brawerman 1967; Kaempfer, Meselson et al. 1968). The fact that ribosomes begin the initiation phase of protein synthesis as separate subunits was confirmed when it was shown that crosslinked subunits could not initiate protein synthesis but, could elongate on a template once attached (Hawley, Miller et al. 1974; van Duin, Kurland et al. 1975). A factor stimulating the dissociation activity of the subunits was found to co-purify with 30S subunits and was sensitive to degradation by trypsin, suggesting a protein factor is important in the subunit separation (Subramanian, Ron et al. 1968). Three protein initiation factors (IF1, IF2, and IF3) were eventually identified to be important for efficient initiation of protein synthesis (Iwasaki, Sabol et al. 1968). Each of these factors could be found with the 30S subunit fractions (Iwasaki, Sabol et al. 1968).

IF3 was initially assigned the function of the subunit dissociation factor but has been shown to trap the free 30S subunit, preventing docking with the 50S subunit rather than actively inducing the separation of subunits (Slobin 1972; Grunberg-Manago, Dessen et al. 1975). There remains some evidence however that IF3 may actively promote the dissociation of subunits (Laursen, Sorensen et al. 2005). IF3 was also shown to improve the binding of mRNA to the 30S subunit (van Duin, Kurland et al. 1975; Kurland 1977). This stimulation of mRNA binding likely occurs by facilitating interaction of the Shine-Dalgarno (SD) sequence of an mRNA with the 16S rRNA (van Duin, Kurland et al. 1975). The Shine-Dalgarno sequence is a sequence

found in the 5' untranslated region of many mRNAs (Shine and Dalgarno 1974). The Shine-Dalgarno region of the mRNA base pairs with a region of the 16S rRNA called the anti-Shine-Dalgarno (anti-SD) sequence, helping to fix the starting position of translation (Yusupova, Yusupov et al. 2001). IF1 was found to improve the activities of IF3 and binds at the A-site of the 30S subunit, effectively blocking a tRNA from binding at this position prematurely (Gualerzi and Pon 1990; Dahlquist and Puglisi 2000; Carter, Clemons et al. 2001). Blocking the A-site is important as initiation requires a specific initiator tRNA (tRNA^{fMet}) bound to the 30S subunit P-site to begin protein synthesis (Salas, Miller et al. 1967).

N-formylmethionine was implicated as the N-terminal amino acid in all *E. coli* proteins, however, in most cases the N-terminal formylmethionine is removed (Adams and Capecchi 1966; Schweet and Heintz 1966; Webster, Engelhardt et al. 1966). Formylmethionyl-tRNA^{fMet} (fMet-tRNA^{fMet}) was found to bind at AUG codons (Clark and Marcker 1965; Uhlenbeck, Baller et al. 1970). AUG codons also act as part of the signal to initiate protein synthesis (Kurland 1972). IF2 was shown to recruit fMet-tRNA^{fMet} to the initiation complex at AUG codons (Iwasaki, Sabol et al. 1968; Lelong, Grunberg-Manago et al. 1970). These findings show that the initiatior tRNA^{fMet} is essential for the formation of the 70S initiation complex, as other tRNAs are unable to substitute for it during initiation (Salas, Miller et al. 1967). fMet-tRNA^{fMet} is also excluded from binding at internal AUG codons by the fact that it is the only tRNA that EF-Tu is unable to interact with (Ono, Skoultchi et al. 1968). Finally, it was shown that fMet-tRNA^{fMet} is recruited into the P-site during initiation complex formation as it

is sensitive to the puromycin reaction after the 50S subunit has joined with the 30S subunit (Hille, Miller et al. 1967). Once the initiation factors, mRNA, and initiator tRNA are bound to the 30S subunit, the 50S subunit may join with the 30S subunit (Guthrie and Nomura 1968). IF2 interacts with the GAC of the 50S subunit, and catalyzes the hydrolysis of a bound GTP (Lelong, Grunberg-Manago et al. 1970; Tomsic, Vitali et al. 2000; Allen, Zavialov et al. 2005). The three initiation factors may then dissociate from the 70S ribosome and the initiation of protein synthesis ends.

While many of the mechanistic details of the initiation phase of protein synthesis remain elusive, a general model for the series of events of initiation can be described (Laursen, Sorensen et al. 2005). Much of the evidence for the following model has been presented in primary research articles and reviews previously referenced therefore, while describing the series of events during initiation of protein synthesis, work from references will only be cited if it has not previously been mentioned. At the first step of initiation, the 30S ribosomal subunit, which was separated from the 50S subunit during the termination phase of protein synthesis (to be described later) most likely has IF3 bound in order to prevent the premature association of the 30S and 50S subunits. IF1 probably also binds at this step as it has been reported to aid in the function of both IF3 and IF2. An mRNA to be translated then binds to the 30S subunit and the mRNA binding, including SD-Anti-SD interactions is facilitated by IF3 (and IF1). The location of the SD sequence of the mRNA helps to place the initiaton codon, AUG, in the P-site of the 30S subunit where fMet-tRNA^{fMet} is recruited to bind by IF2. The 50S subunit is now able to bind to the

30S subunit, IF2 hydrolyzes GTP upon interaction with the 50S GAC and the initiation factors leave. Structured mRNAs are also able to bind to the 30S subunit and unfold to expose the SD sequence and initiator codon (de Smit and van Duin 2003). This unfolding appears to be facilitated by IF2 and fMet-tRNA[fMet] (Studer and Joseph 2006). The final product of the initiation phase of protein synthesis is the 70S ribosome with an mRNA bound and fMet-tRNA[fMet] in the P-site (Laursen, Sorensen et al. 2005). Unfortunately, crystallography has not provided much structural information on the initiation phase of protein synthesis, other than a structure of the 30S subunit with IF1 bound near the A-site (Carter, Clemons et al. 2001; Schmeing and Ramakrishnan 2009). Cryo-EM structures however have provided useful structural information, clarifying many of the experimental results described (Allen, Zavialov et al. 2005; Simonetti, Marzi et al. 2008).

The Elongation Phase of Protein Synthesis

Once the 70S initiation complex has been assembled during the initiation phase of protein synthesis, the elongation phase begins (Figure 1.2). The elongation phase of protein synthesis can be broadly separated into two different functions. First, the selection of aminoacyl-tRNAs by the ribosome based upon codon-anticodon base pairing is known as decoding (Rodnina and Wintermeyer 2001). Second, the movement of the tRNA-mRNA complex through the ribosome is known as translocation (Green and Noller 1997). The process of decoding is a major subject of

this thesis and will be reviewed in the most detail.

Aminoacyl-tRNA selection by the ribosome (Decoding)

During translation, it is essential that proteins be assembled with the correct sequence of amino acids in order to ensure proper function. Once it was observed that the genetic information contained in DNA passes through a transient RNA intermediate, it was still unknown how this information could be translated into a protein sequence (Brenner, Jacob et al. 1961). Crick proposed the existence of a special "adapter" molecule (likely a small nucleic acid) that would interact specifically with the mRNA and carry the correct amino acid to be incorporated into the protein (Crick 1958). This adapter hypothesis proved correct in theory when specific small RNAs were found to be enzymatically linked to amino acids (Berg and Offengand 1958). These RNAs would then interact with ribosomes, bringing their attached amino acids to the site of protein synthesis (Hoagland and Comly 1960). Finally, it was shown that the amino acids attached to these small RNAs were transferred into proteins on the ribosome (Nathans and Lipmann 1961). Initially, these small RNAs were called soluble RNAs (sRNA) because they could be found in supernatant fractions of cell lysate (Hoagland, Zamecnik et al. 1957). Eventually, these sRNAs would be recognized as tRNAs, named for their transfer activity of amino acids into proteins (Hoagland and Comly 1960).

Specific binding of tRNAs to the ribosome based upon the mRNA codon

present in the ribosome provided further evidence that tRNAs act as the adapter molecule, translating between an mRNA and protein sequence (Nirenberg, Leder et al. 1965; Wilcox and Nirenberg 1968). Direct binding experiments revealing the specificity of each amino acid (and the attached tRNA) for an mRNA sequence was the basis for the elucidation of the genetic code (Nirenberg, Leder et al. 1965). However, synonyms in the code were found. Multiple codons could specifiy the same amino acid (Kellogg, Doctor et al. 1966). The presence of synonymous codons and the observation that there are an insufficient number of tRNAs to bind every possible codon led to a thematic problem in how tRNAs could specify the sequence of all amino acids on all codons (Soll and RajBhandary 1995). Crick's wobble hypothesis describes how an individual tRNA is capable of reading multiple codons by exhibiting reduced specificity in the third position of the codon-anticodon base pairs (Crick 1966).

Additional protein factors were identified to be important in the association of tRNAs to the ribosome (Nishizuka and Lipmann 1966). Supernatant fractions important for *in vitro* protein synthesis were eventually separated into a G fraction (corresponding to the GTPase activity of EF-G), and T fraction (corresponding to the transfer of tRNAs to the ribosome) (Lucas-Lenard and Lipmann 1966). The T fraction was further separated into stable (corresponding to EF-Ts) and unstable (corresponding to EF-Tu) fractions (Lucas-Lenard and Lipmann 1966). It was also shown that these factors are general for the transfer of all tRNAs to the ribosome (Nathans and Lipmann 1961).

EF-Tu was found to be the factor that transfers aminoacyl-tRNAs to the ribosome in a GTP dependent process (Ravel 1967). EF-Tu forms a ternary complex with GTP and an aminoacyl-tRNA, which is the active intermediate in the transfer of tRNAs to the ribosome (Ravel, Shorey et al. 1968; Shorey, Ravel et al. 1969). During the delivery of aminoacyl-tRNAs to the ribosome, one GTP is hydrolyzed for every peptide bond formed (Nishizuka and Lipmann 1966; Gordon 1969). Interestingly, it was shown that the hydrolysis of GTP was not required for the association of the ternary complex to the ribosome, however GTP hydrolysis was required for peptide bond formation to occur (Skoultchi, Ono et al. 1970). Upon hydrolysis of GTP, the aminoacyl-tRNA was found to be bound in the A-site of the ribosome, indicated by the insensitivity of the aminoacyl-tRNA to the puromycin reaction (Skoultchi, Ono et al. 1970). These early experiments on the EF-Tu dependent binding of aminoacyl-tRNAs to the ribosome eventually led to an early formulation of the reaction mechanism of ternary complex binding to the ribosome (Hachmann, Miller et al. 1971). First, EF-Tu•GTP binds an aminoacyl-tRNA to form a ternary complex. The ternary complex then binds to the ribosome, EF-Tu hydrolyzes GTP, releasing the aminoacyl-tRNA and the aminoacyl-tRNA participates in peptide bond formation.

EF-Tu does not bind aminoacyl-tRNAs in the GDP bound state and preferentially binds GDP over GTP (Ravel, Shorey et al. 1968; Cooper and Gordon 1969) . Therefore, a GDP/GTP exchange factor is necessary in order for EF-Tu to form a new ternary complex, after GTP hydrolysis. In the presence of aminoacyl-tRNAs, EF-Ts, and GTP, EF-Tu efficiently forms a ternary complex with GTP and an

aminoacyl-tRNA (Shorey, Ravel et al. 1969). The second elongation factor found in the original, T fraction, EF-Ts was found to improve the exchange of nucleotides on EF-Tu (Weissbach, Miller et al. 1970). When EF-Ts is present, EF-Tu exchanges nucleotides more rapidly, allowing EF-Tu to bind GTP. The GTP bound state of EF-Tu is then stabilized by interaction with aminoacyl-tRNAs (Cooper and Gordon 1969). This interaction effectively "pulls" the equilibrium bound state of EF-Tu almost entirely into the GTP bound conformation in spite of the factor's greater affinity for GDP. This idea is supported by the observation that EF-Tu and tRNAs are present in cells in approximately a 1:1 ratio, and that nearly all EF-Tu and tRNAs are present in ternary complex (Furano 1975).

The decoding problem and kinetic proofreading

While early work on the selection of tRNAs by the ribosome focused on identification of the components of the process (tRNAs, EF-Tu, EF-Ts, etc...) and their roles in tRNA binding to the ribosome, most work on tRNA selection has been done under the framework of "the decoding problem" (Ogle and Ramakrishnan 2005). Early experiments measuring the frequency of errors in protein synthesis showed that the ribosome incorporates an incorrect amino acid no more than once out of every 3,000 amino acids polymerized (Loftfield 1963). This error frequency agrees well with the currently accepted range of the error frequency of protein synthesis (one error in every 1,000-10,000 amino acids) (Zaher and Green 2009). In order to obtain this

low error rate, a difference in binding stability of tRNAs for the mRNA template would be expected to be about 5 kcal/mol (Loftfield 1963). Non-cannonical base pairing interactions between the tRNA and mRNA could result in the loss of only one hydrogen bond when compared to Watson-Crick base pairs (Loftfield 1963). The loss in stability of these non-cannonical interactions cannot account for the low error rate of protein synthesis (Loftfield 1963). The observation that the difference in stability between correct and incorrect codon-anticodon base pairing interactions is not sufficient to account for the accuracy of protein synthesis is known as the decoding problem (Ogle and Ramakrishnan 2005).

Two lines of reasoning have explained how the decoding problem can be overcome. First, the ribosome may provide a special environment that increases the difference in stability between correct and incorrect codon-anticodon interactions. Antibiotics, such as streptomycin, and mutations in the ribosome that affect the error frequency of protein synthesis supported this view (Davies, Gilbert et al. 1964; Gorini and Kataja 1964; Rosset and Gorini 1969). These experiments suggested that the ribosome directly interacts with the codon-anticodon base pairs however, the details of this interaction would not be elucidated until the crystal structure of the 30S subunit was solved (Ogle, Brodersen et al. 2001). The details of how the ribosome stabilizes the codon-anticodon interaction will be discussed further in a later section describing the structural aspects of decoding. The second line of reasoning describing how the ribosome may overcome the decoding problem deals with specific mechanisms that can be used to improve accuracy.

The idea of a kinetic proofreading (or kinetic amplification) reaction mechanism allowing an increase in accuracy beyond the thermodynamically available energy difference among different substrates was published nearly simultaneously by two groups (Hopfield 1974; Ninio 1975). In Hopfield's application of kinetic proofreading to the ribosome, two selection phases are required, initial selection, and proofreading (Figure 1.5) (Hopfield 1974). These two stages of selection must be separated by a high energy (effectively irreversible) reaction (Hopfield 1974). In the case of ternary complex mediated tRNA binding to the ribosome, this intermediate is GTP hydrolysis by EF-Tu. According to Hopfield's initial hypothesis, discrimination would occur primarily through differences in the dissociation rates of correct versus
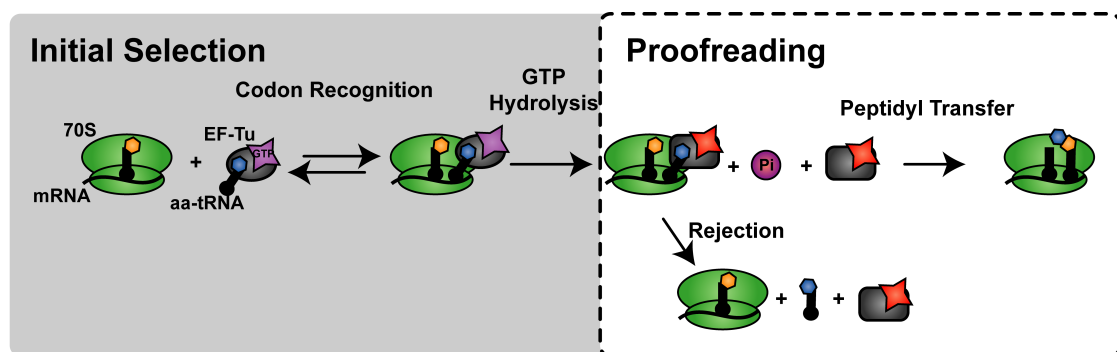
Figure 1.5: Hopfield's kinetic proofreading mechanism. In Hopfield's kinetic proofreading mechanism, there are two selection stages. First, during initial selection (gray background), the ternary complex binds to the ribosome and EF-Tu hydrolyzes GTP. During the second stage, proofreading (dashed outline), the tRNA is accepted into the ribosome and undergoes peptide bond formation. Dissociation of incorrect tRNAs may occur through the dissociation of the codon recognition complex or by rejection of the tRNA after GTP hydrolysis. In this scheme, an irreversible process, such as GTP hydrolysis, is essential to prevent association through the reverse of the rejection step.

incorrect substrates (Hopfield 1974). Thus, correct (cognate) or incorrect (non-cognate) ternary complexes could associate with the ribosome but, non-cognate tRNAs would dissociate much faster from the ribosome during either the initial selection or proofreading step (Hopfield 1974). By describing discrimination as being exclusively dissociation rate driven, the assumption is made that forward reaction rates are the same for correct and incorrect substrates (Hopfield 1974). This assumption is not required for Hopfield's kinetic proofreading mechanism but, it simplifies the results (Hopfield 1974; Thompson and Karim 1982). By having a selection process driven by differences in dissociation rates, the minimum error fraction attainable is described by the ratio of the equilibrium dissociation constants (K_D's) of two substrates, or equivalently, the ratio of the dissociation rates (k_{off}) of two substrates (Hopfield 1974). Using a kinetic proofreading mechanism, the overall error rate could be as low as the square of the error rate for a single selection step (Hopfield 1974). Thus, if one incorrect amino acids is incorporated in one hundred codons, a single selection step would give you an error frequency of 0.01. A kinetic proofreading mechanism could decrease this error frequency to as low as 0.0001 or one incorrect amino acid in 10,000 codons.

The efficiency of proofreading depends critically upon the rate of the irreversible step separating the two selection phases relative to the dissociation rate of an incorrect substrate (Hopfield 1974). If the rate of GTP hydrolysis (and peptidyl transfer) is slow relative to the dissociation rate of an incorrect substrate, the codon recognition step (Figure 1.5) will be allowed to come to equilibrium before the

hydrolysis of GTP by EF-Tu. This allows initial selection to achieve the error fraction expected by a single selection mechanism (Hopfield 1974). In proofreading, if the rate of peptidyl transfer is slow relative to the rejection of an incorrect tRNA then an equivalent discrimination step will occur on a pool that has already passed through one selection step. The resulting error fraction will be as low as the ratio of the K_D of an correct and incorrect tRNA squared. It is not guaranteed however that the rate of GTP hydrolysis and peptidyl transfer are slow relative to the dissociation of an incorrect tRNA. If GTP hydrolysis and peptidyl transfer are too rapid, the complexes will not be able to reach equilibrium prior to each step and the error fraction will increase (Hopfield 1974). This observation has led to the idea of an accuracy versus efficiency tradeoff (Thompson and Karim 1982).   If reactions occur too quickly, incorrect substrates will be shuttled through and incorporated before having the opportunity to dissociate.

An equivalent "kinetic amplification" scheme was also proposed by Ninio (Ninio 1975). While the mathematical outcome of the two selection mechanisms (kinetic proofreading and kinetic amplification) are identical, Ninio's description of kinetic amplification scheme was based upon "sticking times" of correct and incorrect substrates (Ninio 1975; Ninio 2006) . In this mechanism, intermediate steps act as delay functions, giving an incorrect substrate the opportunity to dissociate before being committed forward (Ninio 1975). Irreversible steps also prevent binding of substrates at a point in the binding process that would allow the kinetic amplification to be shortcut (Ninio 2006). In this mechanism, again, if the steps of GTP hydrolysis

or peptidyl transfer, which act as the delay functions, occur too rapidly, the improvement in selectivity is reduced as the incorrect substrates do not have an opportunity to dissociate. Even if the intermediate steps are rapid however, some improvement in the selectivity is to be expected. When transit times (rather than rate constants) are considered, it is clear that even steps that are not rate limiting take some amount of time to complete and would allow a slightly greater time period for an incorrect substrate to dissociate (Pape, Wintermeyer et al. 1998; Johansson, Bouakaz et al. 2008). While the results of Ninio and Hopfield's models are equivalent, slight differences make Hopfield's kinetic proofreading mechanism (Figure 1.5) more directly applicable to tRNA selection by the ribosome and it has been been the model conventionally used in past studies (Thompson, Dix et al. 1980). For example, in Ninio's scheme, the enzyme becomes "activated" and must have a path to return to its ground state (Ninio 2006). This may be more directly applicable to a reaction where the irreversible step is phosphorylation or some other modification of the enzyme rather than tRNA selection where it is the ternary complex that undergoes the activation or deactivation through GTP hydrolysis and guanine nucleotide exchange.

Experimental criteria to determine if a kinetic proofreading mechanism is being employed was established while studying tRNA aminoacylation by aminoacyl-tRNA synthase enzymes (Hopfield, Yamane et al. 1976; Yamane and Hopfield 1977). Aminoacyl tRNA synthase enzymes catalyze the attachment of an amino acid to its corresponding tRNA in a process called aminoacylation (Ibba and Soll 2000). Aminoacyl tRNA synthases first activate an amino acid to be attached to a tRNA by

adenylation, with AMP-amino acid and pyrophosphate as the products of this step (Nismann, Bergmann et al. 1957; Berg and Offengand 1958). The second step of the reaction is the attachment of the amino acid to the 3' end of the tRNA. Mischarging reactions where tRNA^{ile} was aminoacylated with valine showed that the hydrolysis of ATP and the aminoacylation of the tRNA became uncoupled (Hopfield, Yamane et al. 1976). This indicated that multiple attempts at aminoacylation were made at the initial selection step that were filtered out by proofreading (Hopfield, Yamane et al. 1976). Decoupling of the intermediate high-energy reaction step from final product formation in the case of reactions with incorrect substrates has become the standard method to diagnose a kinetic proofreading mechanism (Thompson and Stone 1977; Yamane and Hopfield 1977; Ruusala, Ehrenberg et al. 1982).

Kinetic proofreading and the mechanism of tRNA selection

Subsequent to the proposal of the kinetic proofreading mechanism of ternary complex binding to the ribosome, several lines of research verified many of the predictions and assumptions that were made. The amount of GTP hydrolyzed compared to the amount of peptide bonds formed were compared when a variety of incorrect tRNAs were mixed with ribosomes (Thompson and Stone 1977). When incorrect ternary complexes attempt to bind the ribosome, the intermediate step of GTP hydrolysis, and the final step of peptide bond formation become uncoupled (Thompson and Stone 1977). Many more GTPs must be hydrolyzed in order for an

incorrect tRNA to be incorporated, indicating that there is a proofreading step that rejects incorrect tRNAs after GTP hydrolysis by EF-Tu (Thompson and Stone 1977). These experiments also provided a functional differentiation between near-cognate and non-cognate tRNAs. Ternary complexes with near-cognate tRNAs exhibited a stimulation of GTP hydrolysis, indicating that the proofreading step is essential to prevent their misincorporation (Thompson and Stone 1977). Ternary complexes with non-cognate tRNAs did not appreciably cause GTP hydrolysis by EF-Tu when mixed with ribosomes (Thompson and Stone 1977). The finding that the ribosome does proofread was verified in a more complete elongation system incorporating EF-G to allow ribosomes to elongate, rather than observing single binding attempts (Ruusala, Ehrenberg et al. 1982). As an experimental note, most work on the mechanism of tRNA selection by the ribosome has made use of the codon UUU in the A-site (or poly-U mRNA). The cognate tRNA for this codon is tRNA^{phe} which has the anticodon GAA. This incorporates a GU wobble base pair in the third position of the codon-anticodon interaction as predicted by Crick (Crick 1966). tRNA$_2^{Leu}$ (with a GAG anticodon) was the most likely tRNA found to be misincorporated on a UUU template (Thompson and Stone 1977).

Experimental evidence for the sequence of events in Hopfield's model was strongly supported by studies measuring the single turnover kinetics of GTP hydrolysis and peptide bond formation during the reaction of ternary complex with ribosomes (Thompson, Dix et al. 1980). GTP hydrolysis displayed apparent second order kinetics, suggesting it occurs rapidly and that experiments were performed under

conditions where binding is the rate limiting step (Thompson, Dix et al. 1980). The significantly slower kinetics of peptidyl transfer (containing a small lag phase) and the fact the GTP hydrolysis was required to observe peptidyl transfer proved that peptidyl transfer occurs after GTP hydrolysis by EF-Tu (Thompson, Dix et al. 1980; Thompson and Karim 1982). The kinetics of peptide bond formation measured could support an elongation rate of 5-10 amino acids per second, agreeing reasonably well with the accepted overall rate of protein synthesis (Thompson, Dix et al. 1980).

The rate of the irreversible processes in the kinetic proofreading mechanism are important in allowing efficient two step selection rather than channeling incorrect substrates into the final reaction step (Hopfield 1974). Single turnover kinetics of GTP hydrolysis by EF-Tu in the reaction of ternary complex with ribosomes suggested that the rate of this step is not slow and may not allow proofreading to occur with maximum efficiency (Thompson, Dix et al. 1980; Eccleston, Dix et al. 1985). GTP[γS] is an analog of GTP that efficiently participates in the formation of ternary complex and the binding of ternary complex to the ribosome but, is hydrolyzed more than 2,500 times more slowly than GTP (Thompson and Karim 1982). The reaction of ternary complex containing GTP[γS] rather than GTP exhibited greater selectivity in the two step selection mechanism of ternary complex binding to the ribosome (Thompson and Karim 1982). Improving selectivity by slowing the intermediate reaction of GTP hydrolysis in ternary complex binding to the ribosome highlights the fact that while tRNAs do bind through a two step selection, kinetic proofreading mechanism, the rate of GTP hydrolysis is too rapid to allow maximum utilization of

the kinetic proofreading scheme (Thompson and Karim 1982). The ribosome is unable to use the kinetic proofreading mechanism to its maximum efficiency due to the tradeoff between accuracy and speed. If GTP hydrolysis were slow enough to allow more efficient tRNA selection, then protein synthesis as a whole would be too slow to support the high rate of protein synthesis required by cells (Thompson 1988).

The original postulation of the kinetic proofreading mechanism also predicted that discrimination is entirely driven by dissociation rates (Hopfield 1974). Faster dissociation of incorrect substrates during the initial binding and proofreading steps eliminate mistakes before being irreversibly incorporated (Hopfield 1974). Dissociation rate driven selection is not a requirement of kinetic proofreading but, early work on the kinetics of tRNA selection indicated that the faster dissociation of incorrect tRNAs through the reverse of the initial selection step and through rejection after GTP hydrolysis are the major pathways that incorrect tRNAs are selected against (Thompson, Dix et al. 1981; Thompson 1988). The forward rates of GTP hydrolysis and peptidyl transfer were not significantly slower when the near cognate ternary complex containing $tRNA_2^{leu}$ reacted with ribosomes compared to the cognate reaction (Thompson and Karim 1982; Thompson 1988). Ternary complexes containing $tRNA_2^{leu}$ were found to dissociate much more rapidly in the initial selection and rejection steps (Thompson and Karim 1982; Thompson 1988). Additionally, reactions of the binary complex, EF-Tu•GTP, with ribosomes showed similar reaction kinetics of GTP hydrolysis even without a tRNA bound (Thompson, Dix et al. 1986). The observation that forward reactions of tRNA selection are independent of the tRNA but,

the dissociation steps change depending upon the tRNA indicate that tRNA selection is in fact driven by dissociation of incorrect substrates (Thompson, Dix et al. 1986).

Intermediates and induced fit in tRNA selection

It is clear that GTP hydrolysis and peptide bond formation are important checkpoints in the kinetic proofreading mechanism of tRNA selection. There are a variety of intermediates that had not been identified by measuring only the chemical steps in tRNA selection. Fluorescent probes have made it possible to identify intermediate steps in the process of tRNA selection by the ribosome (Figure 1.6). Yeast tRNA[phe] labeled with proflavin at positions 16 and 17 has been used extensively to identify conformational transitions of the tRNA upon ribosome binding (Figure 1.6A) (Rodnina, Fricke et al. 1994). Wybutine, a natural post transcriptionally modified nucleotide in the anticodon loop of tRNA[phe] has been used to measure the codon recognition step of ternary complex binding to the ribosome (Figure 1.6A) (Rodnina, Fricke et al. 1995). 3'-O-(N-methylanthraniloyl)-2-deoxyguanosine triphosphate (mant-dGTP) is a fluorescent analog of GTP that has been used to observe conformational changes of EF-Tu during ribosome binding (Figure 1.6B) (Rodnina, Fricke et al. 1995). Single molecule fluorescence resonance energy transfer (smFRET) experiments have also provided insights into the mechanism of ternary complex binding to the ribosome by labeling the P-site tRNA and incoming A-site tRNA with FRET donor/acceptor dyes (Figure 1.6C)(Blanchard, Gonzalez et al. 2004)
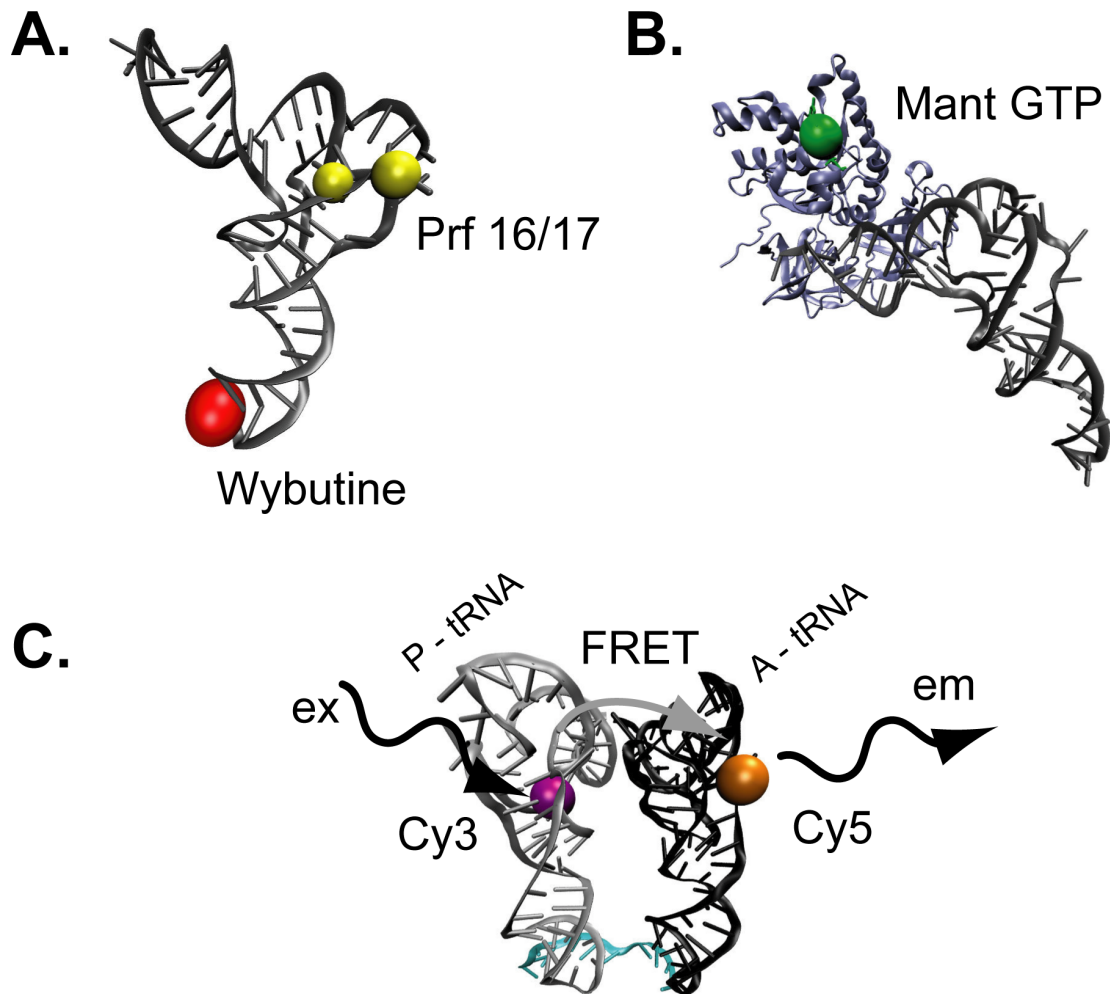
Figure 1.6: Fluorescent probes used to monitor tRNA binding to the ribosome. (A) Yeast tRNA[phe] (gray) has been labeled with proflavin at positions 16 and 17 (dihydrouracil nucleotides) (yellow). A naturally fluorescent post transcriptionally modified nuceotide, wybutine (red) has also been used as a probe for tRNA binding. Protein Data Bank ID 1EHZ (Shi and Moore 2000). (B) The crystal structure of a ternary complex shows the position of GTP (green). Mant-dGTP, a fluorescent analog of GTP has been used to measure conformational changes in EF-Tu (violet). Protein Data Bank ID 1B23 (Nissen, Thirup et al. 1999) (C) Labeling the tRNA in the P-site (silver) and the tRNA in the A-site (black) with Cy3 and Cy5, and monitoring FRET between the flurophores has allowed single molecule studies of ternary complex binding to the ribosome. Protein Data Bank ID 2WDK and (Voorhees, Weixlbaumer et al. 2009).

(Blanchard, Gonzalez et al. 2004).

Using some of the fluorescent probes described above in addition to single-turnover measurements of GTP hydrolysis and peptide transfer, the so called complete kinetic mechanism of tRNA selection has been established (Figure 1.7) (Pape, Wintermeyer et al. 1998). In some cases, these studies have reinforced previous knowledge on how the ribosome selects the correct tRNA but overall they have led to a major paradigm shift in our understanding of tRNA selection.

The complete kinetic mechanism of tRNA selection still posses the characteristic features of a kinetic proofreading mechanism. An initial selection phase, followed by a proofreading phase, separated by a high energy process (GTP hydrolysis) (Figure 1.7). The first step in initial selection is though to be a nonspecific second order binding step called initial binding (Pape, Wintermeyer et al. 1998). During initial binding, the ternary complex associates with the ribosome but, no codon-anticodon interactions takes place thus, any cognate, near-cognate, or non-cognate ternary complex may interact with the ribosome equivalently at this step (Rodnina, Fricke et al. 1994; Rodnina, Pape et al. 1996). After the initial binding step, the codon-anticodon base pairing interaction takes place, stabilizing the binding of cognate ternary complex, preventing their dissociation (Rodnina, Pape et al. 1996; Pape, Wintermeyer et al. 1998). A poorly understood conformational change during ternary complex binding to the ribosome then takes place, inducing GTP hydrolysis by the elongation factor (GTPase activation) (Rodnina, Fricke et al. 1995; Vorstenbosch, Pape et al. 1996; Pape, Wintermeyer et al. 1998). EF-Tu then immediately hydrolyzes

GTP and must release the hydrolyzed phosphate before the elongation factor changes conformation to release the bound tRNA (Rodnina, Fricke et al. 1995; Pape, Wintermeyer et al. 1998; Kothe and Rodnina 2006). No longer held in place by EF-Tu, the tRNA may either accommodate fully into the A-site of the ribosome and participate in the peptidyl transfer reaction or dissociate from the ribosome in the rejection step (Rodnina, Fricke et al. 1994; Pape, Wintermeyer et al. 1998).

Initial binding of ternary complex to the ribosome

The first step in the binding of ternary complex to the ribosome is thought to be the second order initial binding step (Rodnina, Fricke et al. 1994; Rodnina, Pape et al. 1996; Pape, Wintermeyer et al. 1998). The initial binding step of ternary complex binding to the ribosome was identified using proflavin labeled tRNAs (Figure 1.6A) (Rodnina, Fricke et al. 1994). When ternary complex containing proflavin labeled tRNAs binds to the ribosome, a flourescence change occurs that takes place in three apparent phases (Rodnina, Fricke et al. 1994). The first phase of the fluorescence change was found to occur independently of any codon-anticodon interaction (Rodnina, Fricke et al. 1994). When ternary complexes were mixed with ribosomes programmed with an AAA codon in the A-site (making a noncognate codon-anticodon interaction) an equivalent change in fluorescence was observed (Rodnina, Fricke et al. 1994). Additionally, if the A-site was blocked by addition of an unlabeled tRNA so
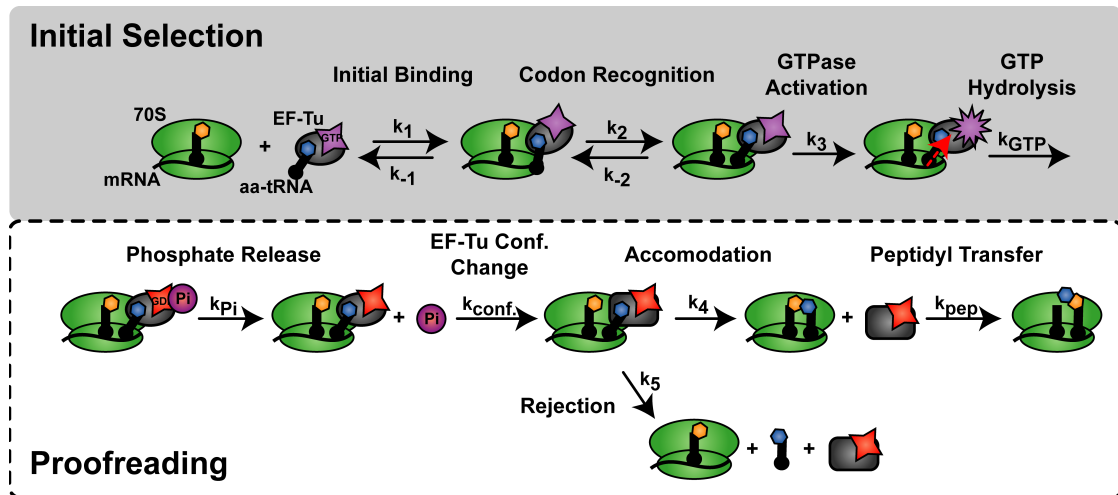
Figure 1.7: The complete kinetic mechanism of tRNA selection by the ribosome. The mechanism of tRNA selection consists of two phases, initial selection and proofreading, separated by the high energy process of GTP hydrolysis. During initial selection, the ternary complex binds in a nonspecific, initial binding complex. Codon-anticodon base pairing then occurs during codon recognition. A conformational change, GTPase activation, stimulates GTP hydrolysis by EF-Tu. EF-Tu releases the hydrolyzed phosphate then undergoes a conformational change, loosing its affinity for the aminoacylated tRNA. The tRNA may then either accommodate into the A-site of the ribosome and participate in the peptidyl transfer reaction or dissociate from the ribosome in the rejection step.

another ternary complex could not bind to the A-site, the fluorescence change ascribed to initial binding could still be observed (Rodnina, Pape et al. 1996). While the evidence is convincing that a codon independent interaction between the ternary complex and ribosome is possible, no other techniques, including single molecule FRET studies have been able to identify this step (Blanchard, Gonzalez et al. 2004).

Two different models have been suggested to describe how the initial binding interaction may take place. In a recent paper describing the crystal structure of ternary complex bound to the ribosome in an intermediate state thought to resemble the codon

recognition complex prior to GTP hydrolysis, it was proposed that the ternary complex binds in a position identical to the codon recognition complex except that the position of the anticodon arm of the tRNA renders it unable to interact with the codon in the A-site (Schmeing, Voorhees et al. 2009). This proposal seems to most closely follow the original data identifying the initial binding step where the initial binding complex was treated as a single binding site and non-cognate ternary complexes would be expected to competitively inhibit cognate ternary complex binding (Rodnina, Pape et al. 1996). The ribosome must always operate with a large excess of incorrect substrates. A nonspecific binding step such as this would be expected to severely inhibit protein synthesis (Lovmar and Ehrenberg 2006; Johansson, Lovmar et al. 2008). Simulations predicting the rate of protein synthesis with an excess of non-cognate ternary complex at first indicated that the initial binding step would cause at most a 5-fold decrease in the rate of protein synthesis (Rodnina, Pape et al. 1996). These predictions relied on the assumption that at physiological concentrations of magnesium ions (Mg^{2+}) the equilibrium dissociation constant (K_D) of the initial binding complex would be fairly large (30 μM) (Rodnina, Pape et al. 1996). Direct measurement of the initial binding step at low Mg^{2+} concentrations (3.5 mM) have indicated a significantly lower K_D of 0.6 μM (Gromadski and Rodnina 2004). Simulations taking into account the greater initial binding affinity have indicated that a much greater inhibition of protein synthesis is expected to occur (Johansson, Lovmar et al. 2008). Experiments directly observing the inhibition of protein synthesis by near-cognate ternary complexes have experimentally shown that delivery of cognate tRNAs to the ribosome is not inhibited

by a large excess of near-cognate ternary complex (Bilgin, Ehrenberg et al. 1988).

It has also been suggested that the initial binding interaction could occur through the L7/L12 stalk of the 50S subunit (Kothe, Wieden et al. 2004; Diaconu, Kothe et al. 2005). The L7/L12 stalk consists of the large subunit proteins L10, L7 and L12 (Diaconu, Kothe et al. 2005). L10 binds to the 23S rRNA and mediates the binding of several copies of the L7/L12 proteins (Diaconu, Kothe et al. 2005). Most of the L7/L12 stalk is absent from crystal and Cryo-EM structures of the ribosome (Valle, Zavialov et al. 2003; Selmer, Dunham et al. 2006). Crystal structures of L7/L12 proteins in isolation and fitting into Cryo-EM maps have enabled a model of the complete L7/L12 stalk to be created (Diaconu, Kothe et al. 2005). EF-Tu appears to interact directly with the L7/L12 proteins in the stalk and disruption of this interaction has been shown to decrease the rate of formation of the initial binding complex (Kothe, Wieden et al. 2004). The presence of multiple copies of the L7/L12 proteins in the highly mobile stalk and the interaction of these proteins with EF-Tu suggests that the stalk may act to loosely bind multiple ternary complexes in an initial binding complex that would likely not block the A-site (Diaconu, Kothe et al. 2005). Interaction of multiple ternary complexes with the stalk could explain how a nonspecific initial binding complex can be formed without inhibiting protein synthesis in the presence of an excess of non-cognate ternary complex.

Codon recognition

After the nonspecific initial binding step, the codon-anticodon base pairing interaction occurs in the codon recognition step (Figure 1.7) (Rodnina, Fricke et al. 1994). A crystal structure of the ribosome with ternary complex frozen in a state though to resemble codon recognition has recently been solved (Figure 1.8A) (Schmeing, Voorhees et al. 2009). Crystal structures of the 30S subunit with and without an anticodon stem loop (ASL) analog of a tRNA showed how the ribosome stabilizes the codon-anticodon interaction to improve the fidelity of translation (Wimberly, Brodersen et al. 2000; Ogle, Brodersen et al. 2001). Three 16S rRNA nucleotides, A1492, A1493, and G530 change conformations when a tRNA binds (Figure 1.8B) (Ogle, Brodersen et al. 2001). A1492, A1493, and G530 interact primarily with the 2'-OH groups of the codon-anticodon minihelix (Figure 1.8C) (Ogle, Brodersen et al. 2001). These structural motifs occur commonly in structured RNAs and are known as type I and type II A-minor interactions (Ogle, Brodersen et al. 2001; Battle and Doudna 2002). Type I and II A-minor interactions selectively stabilize Watson-Crick base pairs, increasing the energy difference between a cognate and near-cognate codon-anticodon interaction to ~5 kcal/mol (Battle and Doudna 2002; Ogle, Murphy et al. 2002). This energy difference is sufficient to account for the fidelity of protein synthesis however, as previously described, the high rate of translation makes it impossible for the ribosome to completely utilize the difference in binding energy between a cognate and near-cognate codon interaction in selectivity

(Loftfield 1963; Thompson and Karim 1982).

Near-cognate codon-anticodon interactions could not be observed by crystallography without the use of the miscoding antibiotic paromomycin due to the transient nature of the interaction (Ogle, Murphy et al. 2002). Molecular Dynamics (MD) simulations have helped to understand how the interactions in the decoding center select against near-cognate codon-anticodon interactions (Sanbonmatsu and Joseph 2003). In MD simulations, a GA mismatch resulted in a shallow minor groove that would sterically prevent interaction of the rRNA nucleotides with the minor groove of the codon-anticodon helix (Sanbonmatsu and Joseph 2003). A shallow minor groove precluding A-minor interactions has been experimentally observed in other systems as well (Battle and Doudna 2002). A GG mismatch was found to skew the base pairs to prevent interactions in the minor groove and to affect the geometry of neighboring Watson-Crick base pairs, preventing interactions with the 16S rRNA (Sanbonmatsu and Joseph 2003). Unexpectedly, a kink in the mRNA between nucleotides in the A- and P-sites appears to play a significant role in decoding (Yusupova, Yusupov et al. 2001; Sanbonmatsu and Joseph 2003). The kink in the mRNA prevents stacking interactions between the first nucleotide in the A-site codon and its neighboring nucleotide in the P-site (Yusupova, Yusupov et al. 2001). Due to loss of this stacking interaction, mismatches in the first position of the codon-anticodon interaction allows greater fluctuations of the first nucleotide in the codon, disrupting interactions with A1493 (Sanbonmatsu and Joseph 2003). In summary, mismatches in the second position are most deleterious due to disruption of the
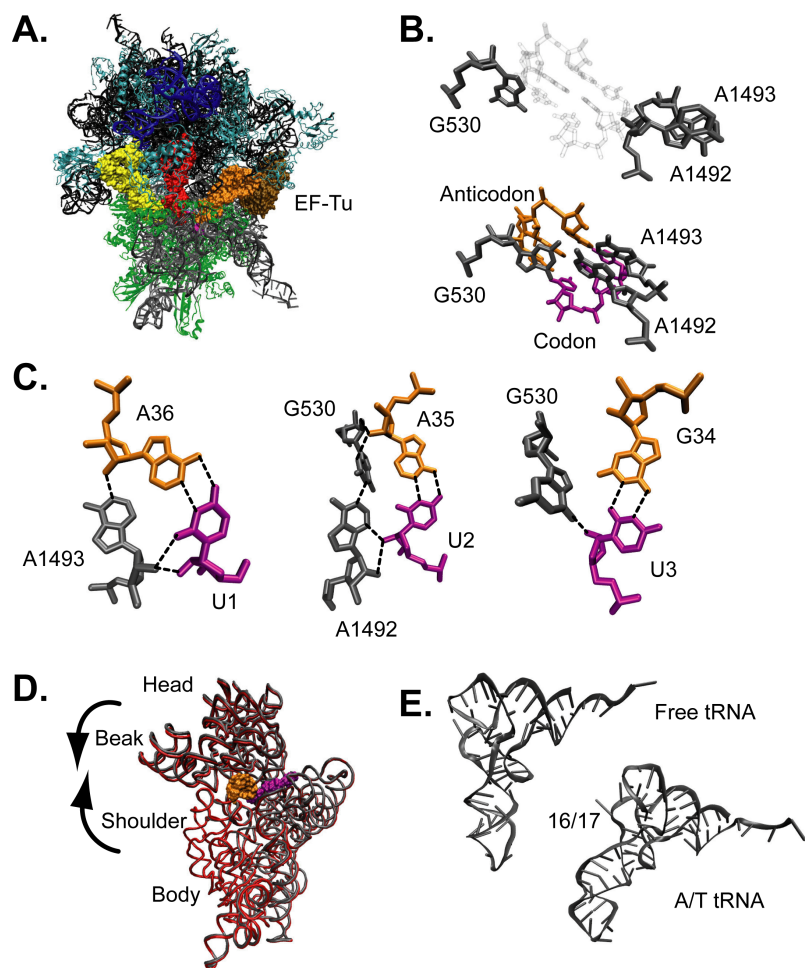
Figure 1.8: Structural transitions in codon recognition. (A) The crystal structure of the ribosome with ternary complex bound. Ternary complex is frozen in the A/T state by the nonhydrolyzable analog GDPNP. 23S rRNA (black), 5S rRNA (blue), 16S rRNA (gray), large subunit proteins (cyan), small subunit proteins (green), E-tRNA (yellow), P-tRNA (red), A-tRNA (orange), mRNA (purple), EF-Tu (ocher). Protein Data Bank ID 2WRN, 2WRO (Schmeing, Voorhees et al. 2009). (B) Conformational change of 16S rRNA nucleotides A1492, A1493, G530 (gray) when a tRNA binds the A-site. Conformation shown in a vacant A-site (top) (location of the codon-anticodon interaction shown as transparent) and filled A-site (bottom) Protein Data Bank ID 1FJF, 1IBL (Wimberly, Brodersen et al. 2000; Ogle, Brodersen et al. 2001). (C) Interactions between the mRNA (purple), tRNA (orange), and 16S rRNA (gray) at each position of the the codon-anticodon duplex. (D) Global conformational change of the 30S subunit known as domain closure. Backbone of the 16S rRNA in 30S subunit with a vacant A-site (gray) and with an ASL and paromomycin bound (red). (E) Comparison of the conformation of a free tRNA and a tRNA bound in the A/T state.

geometry of the entire codon-anticodon helix (Sanbonmatsu and Joseph 2003).

Mismatches in the first position are the next most deleterious due to extra structural

fluctuations allowed through the absence of stacking interactions with a neighboring

nucleotide (Sanbonmatsu and Joseph 2003). Mismatches in the third position are least

deleterious because the mRNA nucleotide is still partially restrained by stacking

interactions with neighboring nucleotides.

Biochemical experiments have shown that the observed decoding center

interactions are important in tRNA selection. Replacement of either the anticodon or

codon nucleotides with deoxynucleotides resulted in the destabilization of the tRNA in

the A-site due to faster dissociation rates, while P-site binding was unaffected

(Potapov, Triana-Alonso et al. 1995; Phelps, Jerinic et al. 2002; Fahlman, Olejniczak

et al. 2006). While the 2'-OH groups of the codon-anticodon base pairs have been

shown to be important in binding of tRNAs, their role in ensuring the fidelity of

protein synthesis has not been directly tested.

Mutagenesis of nucleotides A1492, A1493, and G530 have been shown to be

lethal (Powers and Noller 1990). Experiments studying the decoding of tRNAs by

ribosomes with A1492G, A1493G, or G530A mutations showed that these nucleotides

are important in tRNA selection (Cochella, Brunelle et al. 2007). While these

experiments, suggested that mutagenesis of nucleotides A1492, A1493, and G530

reduces the stability of the tRNA in the A-site, the stability of the codon recognition

complex was not directly measured (Cochella, Brunelle et al. 2007). The role of

A1492, A1493, and G530 in later steps of tRNA selection will be discussed further in

later sections.

Upon binding of a cognate ASL to the 30S subunit in the presence of the miscoding antibiotic paromomycin, a global conformational change of the 30S subunit called domain closure was observed (Ogle, Murphy et al. 2002). During domain closure, the head and shoulder of the 30S subunit rotate inward towards the decoding center (Figure 1.8D) (Ogle, Murphy et al. 2002). It was hypothesized that domain closure occurs when a full length tRNA binds to the ribosome and that the antibiotic paromomycin was required to induce domain closure because the interaction of an ASL with the 30S subunit does not have enough binding energy to induce the domain closed state (Ogle, Murphy et al. 2002). This hypothesis seems to have been verified as the domain closed state of the 30S subunit has been observed in 70S ribosomes with a full length tRNA bound in the A-site and in the crystal structure of a ternary complex bound to the ribosome (Selmer, Dunham et al. 2006; Schmeing, Voorhees et al. 2009). Binding of paromomycin the the 30S decoding center induces the same structural changes in nucleotides A1492 and A1493 as observed when a cognate ASL is bound (Ogle, Murphy et al. 2002). The fact that when an ASL is bound to the 30S subunit, paromomycin is required to induce the domain closed state and that paromomycin induces the same changes in A1492 and A1493 as observed with a cognate tRNA bound in the A-site has led to the suggestion that the conformational switch of A1492, A1493, and G530 is important for domain closure (Ogle, Murphy et al. 2002). So far, domain closure has not been observed in solution experiments and its role in the mechanism of tRNA selection is unclear.

In the codon recognition complex, the tRNA must be bound simultaneously to the 30S subunit A-site and EF-Tu (Figure 1.8A) (Schmeing, Voorhees et al. 2009). This state of tRNA binding, termed the A/T state, was initially identified by chemical protection experiments (Moazed and Noller 1989). Cryo-EM and crystallography have shown that while bound in the A/T state, the tRNA undergoes a significant structural distortion compared to a free tRNA (Figure 1.8E) (Valle, Sengupta et al. 2002; Valle, Zavialov et al. 2003; Schmeing, Voorhees et al. 2009). Cryo-EM structures first showed that the tRNA must adopt a kinked conformation and defined nucleotides 44, 45, and 26 as the hinge region of the tRNA that must be flexible enough to adopt this kinked intermediate (Valle, Zavialov et al. 2003). The crystal structure of the ternary complex bound to the ribosome clarified this kink as an untwisting of the tRNA anticodon helix from base pair 30:40 to base pair 25:45 (Schmeing, Voorhees et al. 2009).

Biochemically, the hinge region of the tRNA has been shown to be very important in tRNA selection. Mutagenesis of G24 to A in the hinge region of tRNA[trp] has been shown to allow this tRNA to decode UGA stop codons (Hirsh 1971). G24A tRNA[trp] has been called the Hirsch suppressor due to the ability of the tRNA to suppress UGA stop codons (Cochella and Green 2005). The elevated miscoding ability of this tRNA has been linked to later steps in the decoding mechanism (the stability of the codon recognition complex was not affected) and will be discussed further in the appropriate sections (Cochella and Green 2005). Disruption of the 9:12:23 base triple also yields a miscoding phenotype, emphasizing the importance of

the hinge region on the selection tRNA by the ribosome (Smith and Yarus 1989; Schmeing, Voorhees et al. 2009).

Kinetically, the codon recognition step of tRNA selection has been characterized primarily through the use of proflavin labeled tRNAs (Figure 1.6A). Upon interaction of ternary complexes carrying fluorescently labeled tRNA, a fluorescence change with three apparent phases occurs (Rodnina, Fricke et al. 1994). The first phase of this fluorescence change was identified as codon independent initial binding (Figure 1.7) (Rodnina, Pape et al. 1996). The second phase was identified as the codon recognition step because a much greater change in fluorescence intensity was observed when ternary complex bound to ribosomes programmed with a cognate codon (Rodnina, Pape et al. 1996). Quenching studies indicated that the fluorescence change attributed to codon recognition is due to the partial unfolding of the D-loop, where the fluorescent probes are located (Rodnina, Pape et al. 1996). The crystal structure of the ternary complex bound to the ribosome verified that this region of the tRNA does undergo a conformational change at this step (Figure 1.8E) (Gao, Selmer et al. 2009; Schmeing, Voorhees et al. 2009).

The interpretation of the fluorescence change of proflavin labeled tRNAs has been controversial. First, the greater extent of fluorescence change observed when the ribosome is programmed with a cognate mRNA is possibly due simply to the higher affinity of tRNAs for the cognate complex (Pape, Wintermeyer et al. 1998). The K_D reported for the initial binding complex indicates that under the conditions tested, only a fraction of the ternary complexes would be bound to the ribosome (Rodnina, Pape et

al. 1996). In the presence of a cognate codon, the ternary complex has a much higher affinity for the ribosome and would be expected to be completely bound (Cochella and Green 2005). Because the fluorescence change associated with the initial binding complex was not tested under saturating concentrations of ternary complex, it is not known if the lower fluorescence change is due to a decrease in the fraction bound or due to a difference in the final bound complex (Rodnina, Pape et al. 1996). Additionally, when the fluorescence change attributed to the codon recognition step is analyzed, the smaller amplitude phase attributed to initial binding is ignored (Rodnina, Fricke et al. 1994). The concentration dependence of the rates of each phase indicates similar association kinetics but faster dissociation kinetics, again, consistent with the only difference being the stability of the complexes rather than the mechanistic step being monitored (Rodnina, Pape et al. 1996; Pape, Wintermeyer et al. 1998).

While the cause of the fluorescence change observed when ternary complexes containing proflavin labeled tRNAs binds to the ribosome may not be well determined, the analysis of the kinetics of the fluorescence change has led to results that have become entrenched in the ribosome field. Discrimination of correct and incorrect tRNAs in the codon recognition step occurs through the faster dissociation of incorrect ternary complexes (Pape, Wintermeyer et al. 1999; Gromadski and Rodnina 2004; Gromadski, Daviter et al. 2006). The dissociation of near-cognate tRNAs from the codon recognition complex has been shown to be roughly uniform regardless of the identity of the mismatch between the codon-anticodon base pairs (Gromadski, Daviter et al. 2006). The dissociation rate of incorrect ternary complexes also has

been suggested to be rate-limited by the dissociation of the initial binding complex (Rodnina, Pape et al. 1996; Gromadski and Rodnina 2004; Gromadski, Daviter et al. 2006). It is likely that the reverse of the codon recognition step is not uniform regardless of the mismatch between the codon-anticodon interaction but simply appears to be because the codon independent initial binding step is rate limiting for dissociation (Gromadski, Daviter et al. 2006).

Many of the interactions in the codon recognition step of tRNA selection have been found to be important for subsequent steps in the decoding pathway. The roles of these interactions will be discussed further when describing the steps they have been found to play an important mechanistic role in.

GTPase activation and GTP hydrolysis

After codon recognition, a conformational change of the ribosome and ternary complex results in the stimulation of GTP hydrolysis activity by EF-Tu (Rodnina, Fricke et al. 1995; Pape, Wintermeyer et al. 1998). This conformational change has been proposed to play a role in an induced fit mechanism that compliments kinetic proofreading to improve the fidelity of protein synthesis (Pape, Wintermeyer et al. 1999). The proposed induced fit mechanism of tRNA selection relies on the acceleration of forward reaction steps for correct substrates, while incorrect substrates react more slowly and have a greater opportunity to dissociate (Johnson 2008).

The GTPase activation step in the binding of ternary complex to the ribosome

was identified through fluorescence changes observed when ternary complex containing the fluorescent GTP analog mant-dGTP bound to the ribosome (Figure 1.6B) (Rodnina, Fricke et al. 1995). When ternary complexes formed with mant-dGTP bound to the ribosome, a biphasic fluorescence change was observed where the fluorescence first increased then decreased (Rodnina, Fricke et al. 1995). The increase in fluorescence was associated with a conformational change in the ribosome and ternary complex (GTPase activation) (Pape, Wintermeyer et al. 1998). The decrease in fluorescence was associated with the dissociation of EF-Tu-mant-dGDP from the ribosome after GTP hydrolysis by EF-Tu (Pape, Wintermeyer et al. 1998).

Kirromycin inhibition of EF-Tu function was essential in the identification of the cause of the fluorescence changes of mant-dGTP (Rodnina, Fricke et al. 1995). Kirromycin prevents the conformational change of EF-Tu that occurs after GTP hydrolysis (Figure 1.9) (Vogeley, Palm et al. 2001). With kirromycin bound, EF-Tu•GDP is capable of binding aminoacyl-tRNAs and the resulting complex binds to the ribosome with the tRNA in the A/T state (Wolf, Chinali et al. 1977; Abrahams, van Raaij et al. 1991; Valle, Sengupta et al. 2002). Additionally, kirromycin stimulates the intrinsic GTPase activity of EF-Tu (Parmeggiani and Swart 1985).

The fluorescence of EF-Tu•mant-dGTP was unaffected by kirromycin, indicating that the binding of the antibiotic or the hydrolysis of GTP is not a cause of the observed fluorescence change (Rodnina, Fricke et al. 1995). In the presence of kirromycin, only an increase in the fluorescence of mant-dGTP was observed when ternary complexes bound to the ribosome, indicating that the dissociation of EF-Tu or

a conformational change subsequent to GTP hydrolysis caused the observed decrease in fluorescence of mant-dGTP (Rodnina, Fricke et al. 1995). In subsequent publications, this interpretation was simplified (apparently, without further experimentation), claiming that the decrease in fluorescence of mant-dGTP was due to the dissociation of EF-Tu from the ribosome (Pape, Wintermeyer et al. 1998; Pape, Wintermeyer et al. 1999). The conformational change of EF-Tu after GTP hydrolysis, which is inhibited by kirromycin, could potentially be the cause of the observed fluorescence decrease in mant-dGTP (Figure 1.9) (Hogg, Mesters et al. 2002). The increase in fluorescence upon interaction of mant-dGTP bound ternary complex to the ribosome was unaffected by the presence of kirromycin (Rodnina, Fricke et al. 1995). Additionally, lack of protection of mant-dGTP from acrylamide quenching upon binding to the ribosome led to the conclusion that binding to the ribosome is not a cause of the observed increase in fluorescence of mant-dGTP (Rodnina, Fricke et al. 1995). This is clearly not a sufficient control however as solvent protection is not the only cause of fluorescence changes due to bimolecular interactions (Lakowicz and Masters 2008). By excluding other steps, it was concluded when ternary complex with mant-dGTP binds to the ribosome, the fluorescence increase observed is due to a conformational change in the complex after codon recognition that leads to GTP hydrolysis by the factor (GTPase activation) (Rodnina, Fricke et al. 1995; Vorstenbosch, Pape et al. 1996; Pape, Wintermeyer et al. 1998).

The observation that the kinetics of GTPase activation and GTP hydrolysis are indistinguishable under most experimental conditions led to the conclusion that

GTPase activation is rate limiting for GTP hydrolysis (Pape, Wintermeyer et al. 1998). Global fitting has led to the rate constant of GTP hydrolysis being reported as >500 s^{-1} however, it is unclear how this number is obtained as the observed rates of GTP hydrolysis at the highest concentrations of ribosomes measured did not exceed 60 s^{-1} (Pape, Wintermeyer et al. 1998). To my knowledge, no other labs have reported such a high rate constant for the hydrolysis of GTP by EF-Tu during ternary complex binding to the ribosome. The rate constant of GTP hydrolysis may be significantly overestimated. Accurate knowledge of the rate of GTP hydrolysis is important because "defects" in GTP hydrolysis are often compared to the very high reported value and may lead to the incorrect understanding of the role of GTP hydrolysis in ternary complex binding (Pape, Wintermeyer et al. 1999).

Measurement of GTP hydrolysis when near cognate ternary complexes bind to the ribosome have indicated that the ribosome employs an induced fit mechanism to discriminate cognate tRNAs from near or non-cognate tRNAs (Pape, Wintermeyer et al. 1999). GTP was hydrolyzed more slowly when a near-cognate ternary complex containing leu-tRNA$_2$^{leu} bound to ribosomes programmed with a UUU codon in the A-site (Pape, Wintermeyer et al. 1999). This conclusion is in direct opposition with the previously proposed model of tRNA selection, where the forward rate constant of GTP hydrolysis was proposed to be constant regardless of the codon-anticodon interaction (Thompson 1988). Comparison of the GTP hydrolysis rates when a cognate ternary complex (containing phe-tRNA^{phe}) or a near-cognate ternary complex (containing leu-tRNA$_2$^{leu}) highlights the point that accurately knowing the rate of GTP hydrolysis is

essential to understanding its role in tRNA selection. The rate of GTP hydrolysis was reported to be ten fold slower in the case of the near-cognate ternary complex (Pape, Wintermeyer et al. 1999). The ten fold difference is due to the fact that global fitting allowed the assignment of the rate constant of GTP hydrolysis by a cognate ternary complex to be >500 s^{-1} (Pape, Wintermeyer et al. 1999). Direct comparison of GTP hydrolysis measurements with a cognate or near-cognate ternary complex binding to the ribosome showed a significantly smaller difference. At the highest concentration of ribosomes measured, GTP hydrolysis occurred with an observed rate of 55 s^{-1} when a cognate ternary complex bound to the ribosome (Pape, Wintermeyer et al. 1999). When the near-cognate complex bound to the ribosome, GTP was hydrolyzed with an observed rate of 35 s^{-1} at the highest concentration of ribosomes measured (Pape, Wintermeyer et al. 1999). The less than two fold difference in the actual measurements is in good agreement with Thompson's model of tRNA selection (Thompson 1988). More recent studies have been able to demonstrate a greater difference between the GTPase activity of ternary complex on near or non-cognate codons and cognate codons (Gromadski and Rodnina 2004; Cochella and Green 2005; Gromadski, Daviter et al. 2006). When near cognate ternary complexes bind to the ribosome, an increase in the dissociation rate accompanies the observed decrease in the rate of GTP hydrolysis. It has been argued that the lower rates of GTP hydrolysis observed do not take into account multiple binding and dissociation events (Ninio 2006). The observed reduction in GTP hydrolysis activity could be due to the faster dissociation rates of near cognate ternary complexes for the ribosome, requiring

multiple binding and dissociation events, on average, before a ternary complex hydrolyzes GTP, rather than a decrease in the rate of the actual catalytic step.

It is not entirely clear that the ribosome employs an induced fit mechanism in the selection of tRNAs during initial selection but, the idea has been very tenacious among ribosome enzymologists. This is because an induced fit mechanism can allow protein synthesis to occur at a rapid rate with improved fidelity. In order for an induced fit mechanism to increase selectivity, binding of correct or incorrect substrates must induce conformations of the enzyme that react differently (Koshland 1958; Post and Ray 1995). In the case of tRNA selection, it has been proposed that binding of cognate ternary complex induces a conformation in the ribosome and EF-Tu that rapidly hydrolyzes GTP (Daviter, Gromadski et al. 2006). When a near or non-cognate ternary complex binds to the ribosome, the highly reactive conformation is not induced, GTP hydrolysis is relatively slow, and the incorrect ternary may dissociate (Daviter, Gromadski et al. 2006). An induced fit mechanism allows kinetic proofreading to occur with high efficiency and with fast intermediate reactions because in the case of incorrect substrates, the intermediate reactions are slow, providing an opportunity for dissociation prior to reacting.

In order for an induced fit mechanism to increase selectivity, the ribosome must interact differently with cognate and near or non-cognate tRNAs (Pape, Wintermeyer et al. 1999; Johnson 2008). Nucleotides A1492, A1493, and G530 interact with the minor groove in a manner specific for Watson-Crick base pairing geometry (Ogle, Brodersen et al. 2001). The minor groove interactions for a cognate

codon-anticodon interaction are not expected to be the same for a near or non-cognate interaction (Sanbonmatsu and Joseph 2003). The conformational change that A1492, A1493, and G530 must undergo in order to interact with the minor groove of the codon-anticodon base pairs has been suggested to be important in inducing the conformation of the ribosome and ternary complex that is more active in GTP hydrolysis (Cochella, Brunelle et al. 2007).

Phosphate release

Upon hydrolysis of GTP, EF-Tu must release the hydrolyzed phosphate (Pi) before it can undergo the conformational change, releasing the tRNA (Figure 1.7) (Kothe and Rodnina 2006). Pi release by EF-Tu during ternary complex binding to the ribosome was measured using phosphate binding protein (PBP) labeled with N-[2-(1-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide (MDCC) (Kothe and Rodnina 2006). MDCC labeled PBP exhibits an increase in fluorescence emission of the probe upon binding of Pi (Brune, Hunter et al. 1994). Pi release was unaffected by the presence of kirromycin, indicating that Pi release occurs prior to the conformational change of EF-Tu that releases the tRNA (Vogeley, Palm et al. 2001; Kothe and Rodnina 2006). Pi release was initially assumed to occur very rapidly after GTP hydrolysis due to charge repulsion (Kothe and Rodnina 2006). However, it was shown that the release of Pi is delayed by a conformational change of the switch 2 region of EF-Tu (Kothe and Rodnina 2006). It is assumed that Pi release does not

play a significant role in the discrimination of tRNAs because the conformational change of EF-Tu after GTP hydrolysis (which Pi release is rate limiting for) is the same in the case of cognate or near-cognate ternary complex binding (Pape, Wintermeyer et al. 1998).

EF-Tu conformational change

Upon GTP hydrolysis and Pi release by EF-Tu, EF-Tu undergoes a conformational change into its GDP bound conformation (Kaziro 1978; Song, Parsons et al. 1999). EF-Tu•GDP does not exhibit any affinity for aminoacylated tRNAs therefore, this conformational change of EF-Tu allows it to dissociate from the ribosome (Weissbach, Miller et al. 1970; Kaziro 1978). Crystal structures of EF-Tu bound to GDP, GDPPNP (a nonhydrolyzable GTP analog), GDP with kirromycin, and in ternary complex show the endpoints of this conformational change (Kjeldgaard, Nissen et al. 1993; Song, Parsons et al. 1999) (Figure 1.9). Comparing EF-Tu in the GTP and GDP bound conformations, an internal rearrangement in EF-Tu is observed (Kjeldgaard, Nissen et al. 1993). This may be part of the EF-Tu conformational change, releasing the tRNA, or this may be related to the conformational change limiting phosphate release. Domain 1 moves relative to domains 2 and 3, destroying the tRNA binding site (Song, Parsons et al. 1999). In the presence of kirromycin, domain 1 does not move relative to domains 2 and 3 upon GTP hydrolysis and the aminoacyl-tRNA binding site is preserved (Vogeley, Palm et al. 2001).

The conformational change of EF-Tu was kinetically identified by the lag phase observed in peptide bond formation kinetics (Pape, Wintermeyer et al. 1998). The lag phase observed in peptide bond formation kinetics is longer than expected based upon the measurement of all preceding steps (Pape, Wintermeyer et al. 1998). Based upon the difference between the expected and observed lag phase in peptide bond formation, the conformational change of EF-Tu was calculated to occur at a rate of 60 s^{-1} (Pape, Wintermeyer et al. 1998). The rate of this conformational change was essentially the same when a cognate or near-cognate ternary complex reacts with ribosomes (Pape, Wintermeyer et al. 1999). The calculation of the rate of the conformational change of EF-Tu after GTP hydrolysis based upon the lag phase observed in peptide bond formation time courses relies on the assumption that all possible intermediates have been characterized and the only remaining step is the conformational change of EF-Tu.

The dissociation of EF-Tu•GDP after GTP hydrolysis was identified through a decrease in fluorescence of mant-dGTP that could be inhibited by kirromycin (Rodnina, Fricke et al. 1995). The dissociation of EF-Tu was observed to occur at a rate of 2-3 s^{-1} regardless of whether the tRNA was cognate or near-cognate (Pape, Wintermeyer et al. 1999). The relatively slow rate of this reaction also suggests that it may be rate limiting for the overall process of ternary complex binding to the ribosome (Pape, Wintermeyer et al. 1998).
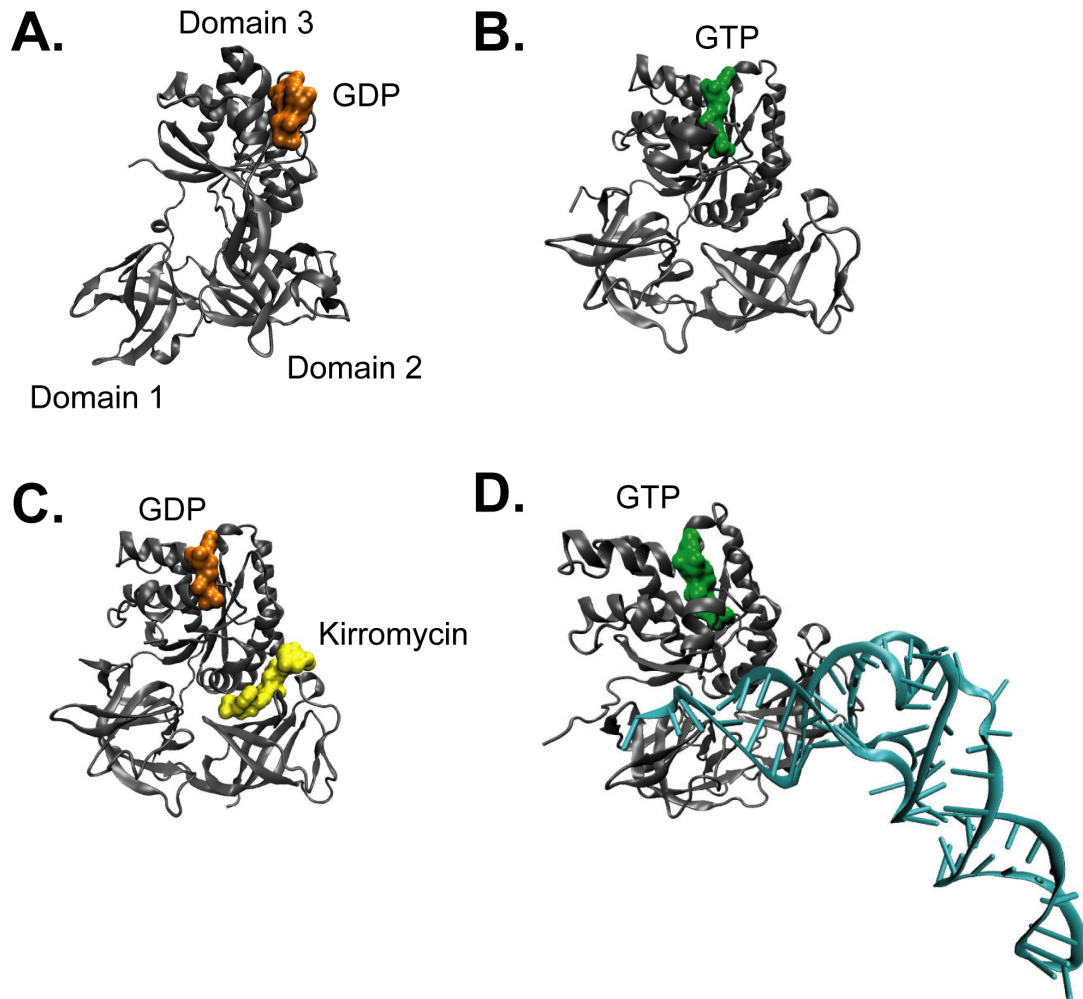
Figure 1.9: Conformational changes of EF-Tu. (A) Crystal structure of EF-Tu (gray) in the GDP (orange) bound conformation. Protein Data Bank ID 1TUI (Polekhina, Thirup et al. 1996) (B) Crystal structure of EF-Tu in the GTP (green) bound conformation. Protein Data Bank ID 1EFT (Kjeldgaard, Nissen et al. 1993). (C) EF-Tu GDP with kirromycin (yellow) bound resembles the conformation of EF-Tu GTP. Protein Data Bank ID 1HA3 (Vogeley, Palm et al. 2001). (D) Crystal structure of EF-Tu GTP aminoacyl-tRNA (cyan) ternary complex. Protein Data Bank ID 1B23 (Nissen, Thirup et al. 1999).

Accommodation and peptidyl transfer

The movement of the acceptor end of the aminoacyl-tRNA into the A-site of the 50S subunit is known as accommodation (Rodnina, Fricke et al. 1994). Prior to GTP hydrolysis by EF-Tu, the aminoacylated tRNA is strained while bound in the A/T state (Valle, Sengupta et al. 2002; Valle, Zavialov et al. 2003; Schmeing, Voorhees et al. 2009; Schuette, Murphy et al. 2009). Directed MD simulations have revealed potential pathways describing how the tRNA may relax from the strained A/T state into the fully accommodated, A/A, state (Sanbonmatsu, Joseph et al. 2005). During accommodation, the tRNA was found to rotate about 45 degrees around the axis of the anticodon stem loop (Sanbonmatsu, Joseph et al. 2005). This observation agrees well with the subsequent crystal structure showing that in the A/T state, the anticodon stem of the tRNA is underwound by a similar amount (Schmeing, Voorhees et al. 2009). During accommodation, the tRNA appears to be simply relaxing back into the unstrained conformation while being held in place at the anticodon end by interactions in the decoding center (Sanbonmatsu, Joseph et al. 2005). While the acceptor end of the tRNA accommodates into the 50S subunit A-site, it encounters a portion of the 23S rRNA impeding the path of the tRNA (Sanbonmatsu, Joseph et al. 2005). The acceptor end of the tRNA must maneuver around this rRNA just before entering the peptidyl transferase center (Sanbonmatsu, Joseph et al. 2005). This final readjustment of the rRNA and the tRNA in the peptidyl transferase center agrees well with the induced fit model proposed by Steitz (Schmeing, Huang et al. 2005). The interactions

of the acceptor end of the tRNA with the 23S rRNA are required because the 23S rRNA must protect the aminoacyl linkage between the peptide chain being synthesized and the P-site tRNA from spontaneous hydrolysis by water (Schmeing, Huang et al. 2005). The tRNA must "move" the rRNA out of the way so it may enter the peptidyl transferase center in order to participate in peptide bond formation.

The accommodation step of ternary complex binding to the ribosome was kinetically identified by the fluorescence change observed when ternary complex containing proflavin 16/17 labeled tRNA bound to the ribosome (Rodnina, Fricke et al. 1994). Three apparent phases were observed when the fluorescently labeled ternary complex bound to the ribosome (Rodnina, Fricke et al. 1994). The third phase was correlated with the accommodation step because it required GTP hydrolysis by EF-Tu in order to be observed and was not affected by peptide bond formation (Rodnina, Fricke et al. 1994). The kinetics of accommodation and peptide bond formation indicate that accommodation is rate limiting for peptidyl transfer (Pape, Wintermeyer et al. 1998). One group has measured very fast rates of peptidyl transfer and suggested that accommodation of the tRNA into the A-site is not rate limiting for peptide bond formation (Johansson, Bouakaz et al. 2008). The possibility of the dissociation of EF-Tu from the ribosome influencing the fluorescence change of the fluorescently labeled tRNA was not addressed (Pape, Wintermeyer et al. 1998).

Upon complete accommodation of the tRNA into the A-site of the ribosome, the peptidyl transferase reaction occurs, transferring the polypeptide being synthesized from the P-site tRNA to the A-site tRNA (Skoultchi, Ono et al. 1970). A major

challenge to the study of the mechanism of the peptidyl transfer reaction is that the actual chemical step must be rate limiting in order to obtain information about the reaction itself (Beringer and Rodnina 2007). Peptidyl transfer is not the rate limiting step in the binding of ternary complex to the ribosome (Pape, Wintermeyer et al. 1998). In order to observe the chemical step of peptidyl transfer directly, the small molecule puromycin has often been employed (Traut and Monro 1964; Maden, Traut et al. 1968; Nissen, Hansen et al. 2000). Puromycin is a small molecule that binds to the peptidyl transferase center in the 50S subunit and participates in the peptidyl transfer reaction as an analog of the A-site tRNA (Nissen, Hansen et al. 2000). Puromycin does not require the conformational changes in the 50S subunit observed when a tRNA binds in order to react therefore there may be significant differences in the puromycin reaction and authentic peptidyl tranfer (Bashan, Agmon et al. 2003). In an effort to make a more authentic peptidyl transfer analog, puromycin was attached to a cytosine or to a CCA, mimicking the CCA-amino acid end of the A-site tRNA and used to study the peptidyl transferase reaction (Wohlgemuth, Beringer et al. 2006).

The rate of the peptidyl transfer reaction appears to be accelerated by the ribosome primarily through a decrease in the enthalpy of activation (Sievers, Beringer et al. 2004). The ribosome catalyzes the peptidyl transfer reaction by precisely positioning the tRNAs and a water molecule (Schmeing, Huang et al. 2005). The mechanism of the reaction proceeds through a concerted proton shuttle involving the hydroxyl groups of A76 of the P-site tRNA and a water molecule (Weinger, Parnell et al. 2004; Beringer and Rodnina 2007). This results in the breakage of the aminoacyl

linkage between the P-site tRNA and the polypeptide by attack of the amino group of the amino acid attached to the A-site tRNA (Beringer and Rodnina 2007). Based upon pH titrations, there do not appear to be any ionizable groups involved in the reaction (Bieling, Beringer et al. 2006).

The accommodation and peptidyl transfer steps are often grouped together in studies on the fidelity of protein synthesis due to the observation that the accommodation step is rate limiting for peptidyl transfer (Cochella and Green 2005). Typically, the rate of peptidyl transfer is measured in a quench flow and is used to report the rate of accommodation (Cochella and Green 2005). The accommodation/peptidyl transfer step of the reaction of ternary complex with the ribosome also appears to follow an induced fit mechanism (Pape, Wintermeyer et al. 1999). Studies comparing the rate of peptide bond formation using cognate and near or non-cognate ternary complexes have shown that peptide bond formation occurs significantly more rapidly with cognate ternary complex (Gromadski and Rodnina 2004; Gromadski, Daviter et al. 2006). An early study using $tRNA_2^{leu}$ (GAG anticodon) ternary complex binding to a UUU codon showed, consistent with Thompson's model, a nearly identical observed rate of peptide bond formation when compared to the reaction with a cognate $tRNA^{phe}$ ternary complex (Pape, Wintermeyer et al. 1999). As expected however, there was a large decrease in the extent of peptide bond formation when near cognate tRNA was used, consistent with the rejection of many incorrect tRNAs in the proofreading step (Pape, Wintermeyer et al. 1999). Decoding studies performed with lower concentrations of magnesium ions and using

codon-anticodon mismatches expected to be more severe showed a much greater difference in the kinetics of peptide bond formation when cognate and near cognate ternary complex reactions were analyzed (Gromadski and Rodnina 2004; Gromadski, Daviter et al. 2006). These studies do not appear to take into account so-called shortcut events (Ninio 2006). Shortcut events involve the binding of free tRNAs to the ribosome, effectively bypassing the initial selection phase of the kinetic proofreading mechanism (Ninio 2006). After GTP hydrolysis and tRNA release by EF-Tu, free aminoacylated tRNAs would be free to bind to the ribosome (Moazed and Noller 1989). *In vivo*, free tRNA binding is not thought to be a problem because EF-Ts regenerates EF-Tu•GTP and ternary complex binds much more rapidly to ribosomes, effectively out competing free tRNA binding (Skoultchi, Ono et al. 1970). In the *in vitro* experiments characterizing induced fit in peptidyl transfer, there is no control for the possibility of free tRNA binding after GTP hydrolysis by EF-Tu (Pape, Wintermeyer et al. 1999; Gromadski and Rodnina 2004; Gromadski, Daviter et al. 2006). Additionally the ternary complex was purified from the other reaction components (aminoacyl-tRNA synthase, GTP, ATP, PEP, EF-Ts, pyruvate kinase) by size exclusion chromatography (Gromadski, Daviter et al. 2006). Purification of ternary complex, removing EF-Ts and GTP would prevent the regeneration of the ternary complex after GTP hydrolysis by EF-Tu, leaving previously rejected aminoacyl-tRNAs free to bind, bypassing initial selection.

Interactions between the rRNA and codon-anticodon base pairs and the structure of the tRNA anticodon stem have been shown to be extremely important in

the accomodation/peptidyl transfer steps of tRNA selection by the ribosome (Youngman, Brunelle et al. 2004). Mutation of 16S rRNA bases A1492, A1493, and G530, which interact with the minor groove of the codon-anticodon base pairs to stabilize Watson-Crick base pairing interactions, results in 5-10 fold decrease in the rate of peptide bond formation (Youngman, Brunelle et al. 2004). A comparatively modest decrease in the extent of the reaction was also observed, suggesting that free tRNA binding in shortcut events was not the cause of the low rates of peptide bond formation in these experiments (Youngman, Brunelle et al. 2004).

Hirsh suppressor tRNA (G24A tRNA[trp]) was also shown to increase the rate of peptidyl transfer on near cognate codons (Cochella and Green 2005). The G24A mutation is in the anticodon stem of the tRNA, in the hinge region where the tRNA must undergo a significant conformational change during the codon recognition and accommodation steps of tRNA selection (Schmeing, Voorhees et al. 2009). The flexibility of the anticodon stem of the tRNA appears to be particularly important in the selection of tRNAs by the ribosome (Sanbonmatsu, Joseph et al. 2005; Schmeing and Ramakrishnan 2009) . Studies with the Hirsh suppressor tRNA provide some of the best evidence for the utilization of an induced fit mechanism in the selection of tRNAs. All other studies have shown that decreases in the rates of the forward steps (GTP hydrolysis and peptidyl transfer) are accompanied with increases in the rates of the rejection steps (dissociation of the codon recognition complex and rejection) (Ninio 2006). In the case of the Hirsh suppressor tRNA binding to near-cognate UGA codons, GTP hyrolysis and petidyl transfer occur more rapidly when compared to a

native tRNA without a significant change in the dissociation rates of the tRNA from the ribosome (Cochella and Green 2005).

The Alignment/Misalignment hypothesis has been proposed to explain the role of the tRNA and decoding center interactions on the induced fit mechanism of tRNA selection by the ribosome (Sanbonmatsu 2006). The tRNA must be recognized as correct or incorrect in the decoding center and reactions must be catalyzed >75 Å away by EF-Tu or the peptidyl transferase center based upon these interactions (Selmer, Dunham et al. 2006; Schmeing, Voorhees et al. 2009). tRNAs are fairly rigid molecules and except for the distortion of the hinge region of the anticodon stem, do not undergo large conformational changes in the decoding process (Sanbonmatsu, Joseph et al. 2005). Slight misalignment of the tRNA in the decoding center by incorrect base pairing interactions or by disrupting the rRNA-codon-anticodon interactions could be translated to greater differences far from the disruption (Sanbonmatsu 2006). Thus, the misalignment of the tRNA results in slower rates of GTP hydrolysis and peptidyl transfer because important catalytic groups would be improperly aligned (Sanbonmatsu 2006). Increasing the flexibility of the anticodon stem in the Hirsh suppressor tRNA by disrupting native interactions could allow the tRNA to adopt a more reactive conformation even when the decoding center interactions are disrupted (Sanbonmatsu 2006).

Rejection

After the hydrolysis of GTP by EF-Tu, EF-Tu undergoes a conformational change, releasing the aminoacyl-tRNA (Kaziro 1978). If the aminoacyl-tRNA is held tightly by the decoding center, it will accommodate into the A-site of the ribosome and undergo the peptidyl transfer reaction (Thompson and Stone 1977). If the tRNA is not held tightly by the decoding center due to incorrect codon-anticodon base pairing interactions, it may dissociate from the ribosome in the rejection step (Thompson and Stone 1977). The rejection of tRNAs from the ribosome was identified through the decoupling of GTP hydrolysis and peptidyl transfer in the case of near-cognate ternary complex binding to the ribosome (Thompson and Stone 1977). If GTP is hydrolyzed by EF-Tu but the tRNA does not undergo peptidyl transfer, it indicates that the tRNA is able to dissociate from the ribosome without EF-Tu (Thompson and Stone 1977). The dissociation of tRNA from the ribosome after GTP hydrolysis by EF-Tu is known as rejection (Pape, Wintermeyer et al. 1998).

The rejection step of tRNA selection has not been directly identified kinetically. The kinetics of rejection are generally calculated based on the observed rate of peptide transfer and the extent of the peptidyl transfer reaction when compared to a cognate reaction (Cochella and Green 2005). One study has characterized the rejection rate of tRNAs with a variety of codon-anticodon mismatches (Gromadski, Daviter et al. 2006). The rejection of near-cognate tRNAs was found to occur in the range of 1-3 s^{-1} without any clear dependence on the type of mismatches in the codon-

anticodon interaction (Gromadski, Daviter et al. 2006). The uniform rates observed in the rejection indicates the possibility of a conformational change of the ribosome limiting the release of the tRNA (Gromadski, Daviter et al. 2006). Domain closure of the 30S subunit is a possible conformational change locking the tRNA into the A-site that could limit dissociation (Ninio 2006). It is not known if domain closure occurs when near-cognate tRNAs bind to the ribosome and crystallographic studies suggest that this conformational change does not occur when a near-cognate tRNA binds (Ogle, Murphy et al. 2002). It remains unclear why codon-anticodon interactions that would be expected to vary greatly in stability exhibit uniform rejection rates.

Translocation

After the tRNA has been bound to the ribosome in the A/A state and undergone peptide bond formation, it must be translocated to the P-site so a new tRNA may bind to the A-site (Green and Noller 1997). A protein elongation factor, EF-G is a GTPase that catalyzes the translocation of tRNAs through the ribosome. EF-G was first discovered in supernatant fractions that contained GTP hydrolysis activity (thus, the name, EF-G) in the presence of ribosomes and was found to be essential for protein synthesis (Lucas-Lenard and Lipmann 1966; Nishizuka and Lipmann 1966). Early experiments showed that EF-G and EF-T fractions contained complimentary activity (Lucas-Lenard and Lipmann 1966). After a tRNA bound to the ribosome, protein synthesis could not continue until EF-G was added (Haenni and Lucas-Lenard 1968).

When tRNAs first bind to the ribosome, they are not sensitive to reaction with puromycin. Upon addition of EF-G to ribosomes bound with an A and P-site tRNA, the A-site tRNA was converted to a puromycin sensitive state (Skoultchi, Ono et al. 1970). The conversion of the A-site tRNA from a puromycin insensitive state to a puromycin sensitive state by EF-G indicated that EF-G translocates a tRNA from the A-site to the P-site (Skoultchi, Ono et al. 1970). An early formulation of the hybrid states model of translocation described how tRNAs may move through the ribosome (Bretscher 1968). It was hypothesized that tRNAs would first form a hybrid state, being bound to the A-site in one subunit and the P-site in the other subunit (Bretscher 1968). Catalysis by EF-G would translocate the A-site tRNA completely into the P-site (Bretscher 1968).

Chemical protection experiments directly showed how the hybrid states model of translocation works (Figure 1.10A) (Moazed and Noller 1989; Joseph 2003). When a tRNA binds to the A-site and undergoes peptidyl transfer, the acceptor ends of both tRNAs spontaneously translocate relative to the 50S subunit. The deacyl-tRNA that was in the P-site in both subunits is now in the P/E hybrid state (the anticodon is in the 30S P-site and the acceptor end is in the 50S E-site). The peptidyl-tRNA that was present in the A-site is now in the A/P hybrid state. When a tRNA is bound to the same site in both subunits, it is said to be in a "classic state" (as opposed to a hybrid state). EF-G translocates the tRNAs (and mRNA) with respect to the 30S subunit (Joseph and Noller 1998). During this translocation step, EF-G hydrolyzes GTP to GDP and dissociates from the ribosome (Rodnina, Savelsbergh et al. 1997). The

tRNA that was in the P/E hybrid state is now in the E/E classic state and the tRNA that was in the A/P hybrid state is now in the P/P classic state (Moazed and Noller 1989).

Cryo-EM maps of the ribosome with EF-G bound in different states revealed that the 30S and 50S subunits move relative to each other upon binding of EF-G in a conformational change that has been termed ratcheting (Frank and Agrawal 2000). After the hydrolysis of GTP by EF-G, the orientation of the 30S and 50S subunits resets (Frank and Agrawal 2000). Biochemical experiments using FRET have directly observed this inter-subunit rotation during translocation and have suggested that the ratcheted state of the ribosome is identical to the hybrid state (Ermolenko, Majumdar et al. 2007; Ermolenko, Spiegel et al. 2007). Additionally, by restricting inter-subunit rotation by introducing disulfide bridges between the subunits, ratcheting was shown to be required for translocation (Horan and Noller 2007).

The precise mechanism of how EF-G catalyzes the translocation of tRNAs through the ribosome is largely not understood. By monitoring translocation with proflavin 16/17 labeled tRNAs, it was shown that EF-G binds to the ribosome with GTP and that GTP hydrolysis precedes and accelerates translocation (Rodnina, Savelsbergh et al. 1997; Wilden, Savelsbergh et al. 2006) . Interestingly however, EF-G bound with the nonhydrolyzable GTP analog GDPNP was able to stimulate translocation to a lesser degree; and slow, spontaneous translocation occurs even in the absence of EF-G (Spirin 1985; Wilden, Savelsbergh et al. 2006).

EF-G exhibits a relatively low affinity for GDP and GTP (12-40 μM) therefore, exchange of GDP for GTP is expected to occur off of the ribosome without the need of

an exchange factor (Figure 1.10A) (Wilden, Savelsbergh et al. 2006). Crystal structures of EF-G without a nucleotide and with GDP bound appear very similar, leading to the suggestion that EF-G may not undergo large conformational changes upon GTP hydrolysis as observed with EF-Tu (A, Brazhnikov et al. 1994; Czworkowski, Wang et al. 1994). The crystal structure of EF-G in the GTP bound state has not been determined however.

Cryo-EM maps with EF-G bound to the ribosome in different states (GDP, GTP, and inhibited by fusidic acid) have indicated that the interaction between the ribosome and EF-G is greatly altered upon GTP hydrolysis by EF-G (Agrawal, Heagle et al. 1999; Stark, Rodnina et al. 2000). EF-G first interacts with the ribosome in the GTP bound state. In this state, EF-G was found to interact with the L7/L12 stalk of the 50S subunit and the 30S subunit in the cleft between the head and shoulder (Stark, Rodnina et al. 2000). Inihbition of EF-G with the antbiotic fusidic acid is thought to freeze the complex in a state intermediate between the GTP and GDP bound states (Gao, Selmer et al. 2009). Fusidic acid inhibited EF-G was found to shift into the inter-subunit space and orient itself towards the decoding center of the 30S subnit (Stark, Rodnina et al. 2000). After translocation, EF-G was found to move even further into the intersubunit space, contacting both the head and body of the 30S subunit and nearly completely losing contact with the L7/L12 stalk of the 50S subunit (Stark, Rodnina et al. 2000).

The crystal structure of EF-G bound to the ribosome inhibited by fusidic acid has been solved recently (Figure 1.10B) (Gao, Selmer et al. 2009). This structure

shows that EF-G interacts with the ribosome in a similar fashion to ternary complex

(Gao, Selmer et al. 2009). In fact the similarity between EF-G and ternary complex

has lead to the suggestion that they are molecular mimics (Nissen, Kjeldgaard et al.

1995). In the crystal structure of EF-G bound to the ribosome, EF-G interacts closely

with the SRL and extends into the decoding center of the 30S subunit (Gao, Selmer et

al. 2009). A tight loop in EF-G interacts in the minor groove of the codon-anticodon
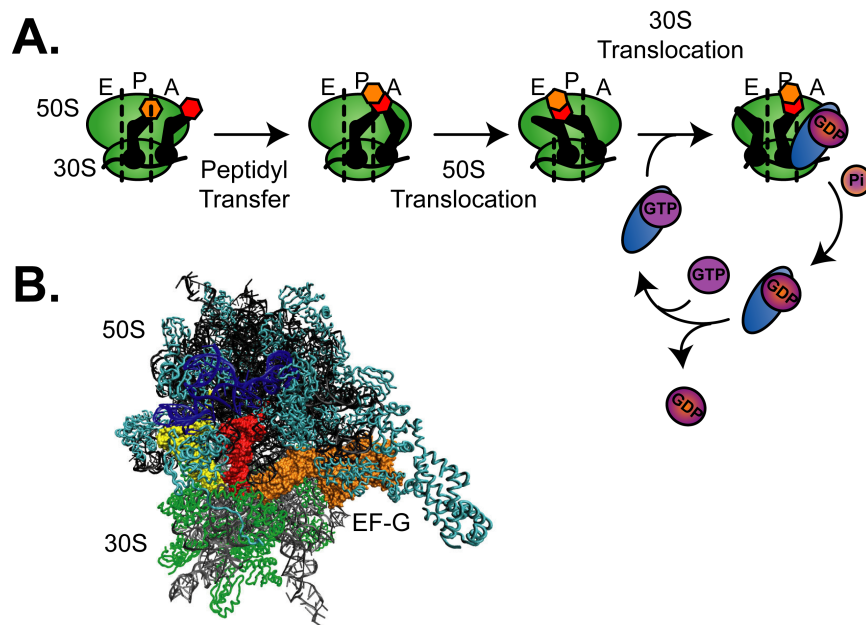
Figure 1.10: Translocation and 70S interactions with EF-G. (A) The series of events of translocation. After peptidyl transfer, the acceptor ends of the tRNAs spontaneously translocate relative to the 50S subunit. The tRNAs are bound in the P/E and A/P hybrid states. EF-G catalyzes translocation of the tRNAs and mRNA, placing the tRNAs in the E/E and P/P classic states. EF-G binds to the ribosome in the GTP form, hydrolyzes GTP, and dissociates from the ribosome in the GDP form. Exchange of GDP for GTP by EF-G occurs off the ribosome without the aid of an exchange factor. (B) The structure of the ribosome with EF-G bound. 23S rRNA (black), 5S rRNA (blue), large subunit proteins (cyan), 16S rRNA (gray), small subunit proteins (green), E-site tRNA (yellow), P-site tRNA (red), EF-G (orange). Protein Data Bank ID 2WRI, 2WRJ (Gao, Selmer et al. 2009).

base pairs of the P-site tRNA (Gao, Selmer et al. 2009). Interaction in the minor groove of the codon-anticodon base pairs has lead to the suggestion that the interactions between A1492, A1493, and G530 of the 16S rRNA with the codon-anticodon base pairs of the A-site tRNA may affect translocation, as EF-G would need to displace these residues to form the observed interactions (Taylor, Nilsson et al. 2007). Biochemical experiments utilizing a fluorescently labeled mRNA to monitor the rate of translocation have shown that these interactions do play an important role in translocation, perhaps affecting the global structure of the ribosome (Studer, Feinberg et al. 2003; Garcia-Ortega, Stephen et al. 2008).

Termination of protein synthesis

During the elongation phase of protein synthesis, the ribosome moves along the mRNA template, catalyzing the polymerization of amino acids until a stop codon enters the A-site. A stop codon in the A-site signals the end of the coding portion of the mRNA and the beginning of the termination phase of protein synthesis (Figure 1.2) (Petry, Weixlbaumer et al. 2008). During the termination phase of protein synthesis, the nascent protein must be released from the ribosome and the ribosome must be recycled so the synthesis of a new protein may be initiated (Kaempfer, Meselson et al. 1968; Petry, Weixlbaumer et al. 2008) .

Peptide release by by release factors

While assigning the identity of codons in the genetic code, three codons were found that did not code for tRNAs (Brenner, Stretton et al. 1965; Zipser 1967). These codons, UAA, UGA, and UAG are known as nonsense codons (Brenner, Stretton et al. 1965; Zipser 1967). Nonsense codons are more commonly called stop codons because they signal the end of protein synthesis (Bretscher, Goodman et al. 1965). Stop codons were found to be read by two protein release factors, release factor 1 (RF1) and release factor 2 (RF2) (Scolnick, Tompkins et al. 1968). RF1 recognizes UAG, RF2 recognizes UGA and both RFs can recognize UAA (Scolnick, Tompkins et al. 1968). Upon recognition of a stop codon RF1 or 2 catalyzes the hydrolysis of the nascent peptide from the P-site tRNA, releasing the protein from the ribosome (Bretscher, Goodman et al. 1965). RF1 and 2 are known as class I release factors because they directly recognize stop codons (Youngman, McDonald et al. 2008). Another class of RFs, class II RFs, are GTPases which remove class I RFs from the ribosome after the release of the nascent peptide (Figure 1.11A) (Petry, Weixlbaumer et al. 2008). Prokaryotes have one class II RF, release factor 3 (RF3) (Petry, Weixlbaumer et al. 2008).

Early studies on the binding of class I RFs to the ribosome showed that the RF interacts with both the decoding center in the 30S subunit and the peptidyl transferase center in the 50S subunit (Tate, Greuer et al. 1990; Brown and Tate 1994; Wilson, Ito et al. 2000; Scarlett, McCaughan et al. 2003). Interaction with these two centers lead

to the suggestion that class I RFs act as molecular mimics of tRNAs (Moffat and Tate 1994). Cryo-EM maps of class I RFs bound to the ribosome verified that RFs do interact simultaneously with both the decoding and the peptidyl transferase centers of the ribosome (Klaholz, Pape et al. 2003; Rawat, Zavialov et al. 2003; Rawat, Gao et al. 2006). Subsequent low resolution crystal structures and recent high resolution crystal structures of class I RFs bound to their cognate stop codons have revealed the molecular details of the interaction of RFs with the ribosome (Figure 1.11B) (Petry, Brodersen et al. 2005; Laurberg, Asahara et al. 2008; Weixlbaumer, Jin et al. 2008).

Genetic experiments identified a tripeptide motif (PVT in RF1 and SPF in RF2) in each class I RF that is essential for the recognition of stop codons. If PVT from RF1 replaced SPF in RF2 then the specificity of the RFs would also be exchanged (Ito, Uno et al. 2000). The ability of these tripeptide motifs to change the specificity of the RF, led to the conclusion that they act as a "tripeptide anticodon", interacting specifically with the stop codon in the A-site of the ribosome (Ito, Uno et al. 2000).

The crystal structure of RF1 and RF2 bound to the ribosome at a cognate stop codon showed that the tripeptide anticodons do interact directly with the stop codon in the decoding center however, there are other interactions that appear to be important in the recognition of stop codons by class I RFs (Laurberg, Asahara et al. 2008; Weixlbaumer, Jin et al. 2008). The anticodon tripeptide motifs show suprisingly few direct interactions with the cognate stop codons of their respective class I RFs in the crystal structures of the ribosomes with an RF bound. The crystal structure of RF1
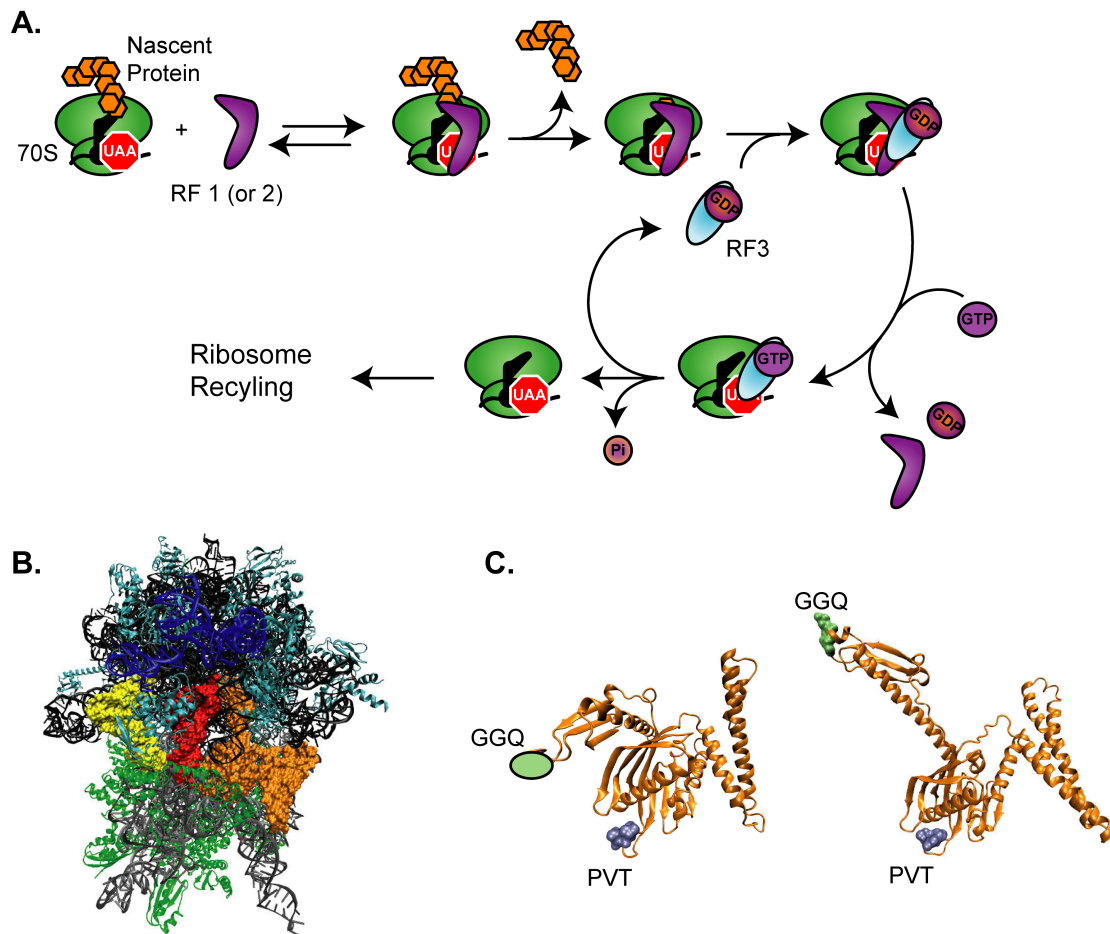
Figure 1.11: Peptide release by release factors. (A) The series of events in peptide release. When a stop codon enters the A-site, RF1 or 2 (purple) binds to the A-site of the ribosome (green). RF1 or 2 then hydrolyzes the nascent protein (orange) from the P-site tRNA (black). RF3 (cyan) then binds in the GDP state. Exchange of GDP for GTP by RF3 occurs on the ribosome and causes the dissociation of RF1 or 2. RF3 then hydrolyzes GTP and dissociates from the ribosome. (B) Structure of RF1 bound to the ribosome. RF1 (orange), 23S rRNA (black), 16S rRNA (gray), 5S rRNA (blue) large subunit proteins (cyan), small subunit proteins (green), E-site tRNA (yellow), P-site tRNA (red), mRNA (purple). Protein Data Bank ID 3D5A and 3D5B (Laurberg, Asahara et al. 2008). (C) RF1 in different conformations on an off the ribosome. RF1 off the ribosome (left) adopts a closed conformation where the GGQ motif (green, disordered) and PVT motif (blue) are close in space. The ribosome bound conformation of RF1 (right) shows the GGQ and PVT motifs more distant, able to interact with the decoding and peptidyl transferase centers simultaneously. Protein Data Bank ID 1RQ0 (Shin, Brandsen et al. 2004).

bound to the ribosome shows that only T186 of the PVT tripeptide anticodon motif of RF1 directly hydrogen bonds with bases of the UAA stop codon (Laurberg, Asahara et al. 2008). Similarly, only S193 of the SPF tripeptide anticodon of RF2 directly hydrogen bonds with the UGA stop codon (Weixlbaumer, Jin et al. 2008). The orientation of the backbone of the class I RFs seems to play an important role in interactions with the stop codons (Laurberg, Asahara et al. 2008; Weixlbaumer, Jin et al. 2008). The tripeptide anticodons are likely extremely important in the organization of the backbone geometry for efficient packing with stop codons. Consistent with biochemical experiments, nucleotides A1492, A1493 and G530 of the 16S rRNA play a significantly different role in RF recognition compared to tRNA selection (Youngman, He et al. 2007). Only A1493 is displaced from its interactions in a vacant A-site (Laurberg, Asahara et al. 2008; Weixlbaumer, Jin et al. 2008). A1913 of the 23S rRNA takes the place of A1493 in stacking with A1492 (Laurberg, Asahara et al. 2008; Weixlbaumer, Jin et al. 2008). This conformational change is likely important for RF binding as A1913 would otherwise clash with the RF (Laurberg, Asahara et al. 2008). The third nucleotide of the stop codon is unstacked from the neighboring nucleotide in the stop codon with a histidine (H193 in RF1 and H202 in RF2) inserted between them (Laurberg, Asahara et al. 2008; Weixlbaumer, Jin et al. 2008). The histidine residue stacks with the middle base of the stop codon (Laurberg, Asahara et al. 2008; Weixlbaumer, Jin et al. 2008). The third base of the stop codon is displaced and stacks with G530 (Laurberg, Asahara et al. 2008; Weixlbaumer, Jin et al. 2008).

The GGQ motif, present in RF1 and RF2, is essential for the catalysis of

peptide release (Frolova, Tsivkovskii et al. 1999). Suprisingly, mutagenesis of the GGQ motif showed that the two glycine residues are absolutely essential for peptide release while mutation of the glutamine was much less deleterious (Mora, Heurgue-Hamard et al. 2003). This was suprising because early, low resolution crystal structures indicated that the glutamine was the only residue from RF that could span the distance to reach A76 to participate in catalysis of peptide release (Petry, Brodersen et al. 2005). Nucleophile partitioning analysis has shown that the glutamine residue is important to select water as the nucleophile in the hydroysis reaction but, has little effect on the activity of the RFs (Shaw and Green 2007).

Catalysis of peptide release is expected to follow an induced fit mechanism (Schmeing, Huang et al. 2005). Portions of the 23S rRNA block access to the peptidyl transferase center, preventing premature peptide release by solvent (Schmeing, Huang et al. 2005). Binding of a deacylated tRNA to the A-site induces conformational changes in the peptidyl transferase center, allowing entry of water molecules and accelerating peptide release (Caskey, Beaudet et al. 1971). When class I RFs bind, they specifically orient a water molecule for attack on the ester bond attaching the nascent protein to the P-site tRNA (Laurberg, Asahara et al. 2008; Weixlbaumer, Jin et al. 2008). High resolution crystal structures of the class I RFs bound to the A-site have also revealed the molecular interactions in the decoding center during peptide release (Laurberg, Asahara et al. 2008; Weixlbaumer, Jin et al. 2008). The two glycine residues appear to be important in allowing a tight turn of the tip of the RFs in the peptidyl tranferase center (Laurberg, Asahara et al. 2008; Weixlbaumer, Jin et al.

2008). Replacement of these residues with any other amino acid would not allow the observed conformation (Laurberg, Asahara et al. 2008; Weixlbaumer, Jin et al. 2008). The glutamine residue is oriented away from the active site and is proposed to form the pocket, organizing a water molecule for attack on the ester bond (Laurberg, Asahara et al. 2008; Weixlbaumer, Jin et al. 2008). The glutamine of the GGQ motif is methylated *in vivo* (Dincbas-Renqvist, Engstrom et al. 2000; Heurgue-Hamard, Champ et al. 2005). The glutamine methylation is proposed to be important for the correct positioning of the glutamine in the peptidyl transferase center but, the difference in activity of the methylated and unmethylated RF is modest (Laurberg, Asahara et al. 2008; Youngman, McDonald et al. 2008).

The crystal structures of free RF1 and RF2 are significantly different from the ribosome bound structures (Petry, Weixlbaumer et al. 2008). In crystal structures of free RF1 and 2, the GGQ and tripeptide anticodon motifs are located too close in space to interact simultaneously with the decoding and peptidyl transferase centers (Figure 1.11C) (Vestergaard, Van et al. 2001; Shin, Brandsen et al. 2004). This observation lead to the proposal that RFs first bind to the ribosome in a closed conformation and open upon binding to the ribosome (Rawat, Zavialov et al. 2003). A MD study supports this conformational change and suggests that protonation of conserved histidines upon binding to the negatively charged environment of the ribosome could cause the expansion of the structure of the RFs (Ma and Nussinov 2004). Solution X-ray scattering (SAXS) experiments have shown that the class I RFs may exist in either the open or closed conformations in solution (Vestergaard, Sanyal

et al. 2005; Zoldak, Redecke et al. 2007). It is unknown what conformation class I RFs adopt when binding to the ribosome.

Compared to tRNA selection, relatively little is known about the mechanism of RF binding to the ribosome and how stop codons are discriminated from sense codons. RFs do not rely on a proofreading system to discriminate stop and sense codons as tRNAs do (Freistroffer, Pavlov et al. 1997; Freistroffer, Kwiatkowski et al. 2000). Studies on stop codon discrimination have focused on the catalytic step of peptide release, quantitating the k_{cat} and K_m of peptide release on a variety of codons (Freistroffer, Kwiatkowski et al. 2000). These studies revealed few obvious rules for the discrimination of stop and sense codons. The first position of the stop codon appears to be the most sensitive to change, resulting in >100 fold defects in the k_{cat} and >2,000 fold defect in the K_m (Freistroffer, Kwiatkowski et al. 2000). The UAU codon turned out to be a hotspot for misreading for RF1 with the most modest defects in k_{cat} and K_m parameters (2.5 and 400 fold defects, respectively) (Freistroffer, Kwiatkowski et al. 2000). For RF2, the UGG codon appeared to be significantly more likely to be misread (8 and 310 fold defect in k_{cat} and K_m respectively) (Freistroffer, Kwiatkowski et al. 2000). All other sense codons tested resulted in ~20 fold defect in k_{cat} and ~1,000 fold defect in K_m (Freistroffer, Kwiatkowski et al. 2000). The greater defect in the K_m parameter suggests that binding of the RFs to sense codons is most important in the discrimination of sense and stop codons (Freistroffer, Kwiatkowski et al. 2000). Since the measurement of K_m is linked to the catalytic activity of the enzyme, a direct binding assay is required to confirm that the discrimination of stop and sense codons

occurs primarily at the binding step.

Upon completion of peptide release, the class I RF remains bound to the A-site and dissociates very slowly (Goldstein and Caskey 1970; Freistroffer, Pavlov et al. 1997) . A class II RF (RF3) is required to accelerate the dissociation of the class I RF from the ribosome after peptide release (Freistroffer, Pavlov et al. 1997). Experiments addressing the mechanism of class I RF removal from the ribosome by RF3 have relied primarily on experiments measuring the turnover of class I RFs in peptide release reactions (Freistroffer, Pavlov et al. 1997). When concentrations of class I RFs are limiting, many turnovers of the class I RF are required in order to observe complete peptide hydrolysis from ribosomes (Freistroffer, Pavlov et al. 1997). Addition of RF3 increases the dissociation of class I RFs from ribosomes, this increases the rate of class I RF turnover and stimulates the observed peptide hydrolysis (Freistroffer, Pavlov et al. 1997). Experiments showing the increased turnover of class I RFs stimulated by RF3 showed that RF3 accelerates the dissociation of class I RFs in a GTP dependent manner (Zavialov, Buckingham et al. 2001). The presence of RF3 had no effect on the peptide hydrolysis reaction in single turnover experiments, indicating that RF3 does not play a role in peptide release by class I RFs (Freistroffer, Pavlov et al. 1997).

The role of GTP in the RF3 mediated dissociation of class I RFs was further studied again using the increased turnover of class I RFs as the experimental assay (Zavialov, Buckingham et al. 2001). It was found that ribosomes complexed with the class I RF act as the guanine nucleotide exchange factor for RF3 (Zavialov,

Buckingham et al. 2001). Based on the fact that the affinity of RF3 for GDP is nearly three orders of magnitude greater that its affinity for GTP, it is presumed that RF3 first binds to posttermination ribosome complexes in the GDP bound state (Zavialov, Buckingham et al. 2001). Excess of RF3 with either GTP or the nonhydrolyzable GTP analog, GDPNP, could support class I RF turnover, indicating that exchange of GDP for GTP occurs on the ribosome and stimulates the dissociation of the class I RF (Zavialov, Buckingham et al. 2001). Limiting concentrations of RF3 with GDPNP could not support turnover of class I RFs indicating that GTP hydrolysis is required for RF3 turnover (Zavialov, Buckingham et al. 2001). RF3 dissociates from the ribosome after the hydrolysis of GTP (Zavialov, Buckingham et al. 2001).   N-terminal truncations of class I RFs could support peptide release but, did not support GTP exchange by RF3 (Laurberg, Asahara et al. 2008; Weixlbaumer, Jin et al. 2008). In the crystal structures of class I RFs bound to the ribosome, the N-terminal region of the RFs are exposed near the surface of the ribosome, available for interaction with RF3 (Figure 1.11A) (Mora, Zavialov et al. 2003). Thus, the ribosome complexed with a class I RF likely provides the binding site for RF3 and stimulates guanine nucleotide exchange (Klaholz, Myasnikov et al. 2004).

A Cryo-EM study of RF3 interactions with ribosome posttermination complexes showed that RF3 can bind in two states (Klaholz, Myasnikov et al. 2004). In the first state, RF3 does not interact extensively with the ribosome (Klaholz, Myasnikov et al. 2004). In this state, RF3 binding and class I RF binding are not mutually exclusive (Klaholz, Myasnikov et al. 2004). In fact the shape of the class I

RF-ribosome complex was suggested to be very complimentary for RF3 binding (Klaholz, Myasnikov et al. 2004). In the second state, RF3 appears to interact much more intimately with the ribosome and overlaps with the binding site of class I RFs (Klaholz, Myasnikov et al. 2004). Transition from the first state to the second state was proposed to occur in a translocation like event including the dissociation of the class I RF and translocation of the P-site tRNA to the E-site (Klaholz, Myasnikov et al. 2004). The interpretation of the location of RF3 binding was based upon fitting of the Cryo-EM density with EF-G (Gao, Zhou et al. 2007).

The crystal structure of RF3•GDP was found to closely resemble EF-Tu•GTP (Gao, Zhou et al. 2007). A more recent, higher resolution Cryo-EM study of RF3 binding to the ribosome shows that RF3•GTP binding does not overlap with class I RF binding and does not translocate the P-site tRNA into the E-site (Gao, Zhou et al. 2007). Instead, conformational changes in the ribosome were observed that are proposed to be the cause of the ejection of the class I RF from the A-site (Gao, Zhou et al. 2007). The P-site tRNA is found to be in the P/E hybrid state (Gao, Zhou et al. 2007). The dissociation of GDP from RF3 alone and in the presence of posttermination ribosome complexes also verified that the posttermination ribosome complex acts as the guanine nucleotide exchange factor for RF3 (Petry, Weixlbaumer et al. 2008).

Ribosome recycling

After class I RFs release the peptide from the P-site tRNA and class II RFs remove the class I RF from the A-site, the ribosome remains with a deacylated tRNA in the P-site, a vacant A-site and mRNA still bound (Kaempfer, Meselson et al. 1968). Early experiments showed that ribosomal subunits are exchanged *in vivo*, indicating that ribosomes are disassembled between different rounds of translation (Hirashima and Kaji 1970; Hirashima and Kaji 1972). A protein factor other than the T and G fractions was shown to be essential for the splitting of subunits upon complete synthesis of a protein (Ishitsuka, Kuriki et al. 1970). EF-G was also shown to be essential for the process of recycling (Ogawa and Kaji 1975; Hirokawa, Kiel et al. 2002). The observation that GTP hydrolysis by EF-G and another protein factor, ribosome recycling factor (RRF) are essential for the splitting of subunits lead to a translocation-like model of ribosome recycling (Selmer, Al-Karadaghi et al. 1999). In this model, RRF bound to the ribosome similar to a tRNA and EF-G catalyzed the movement of RRF through the ribosome, pushing the deacylated P-site tRNA out through the E-site and splitting the subunits. The structure of RRF in solution showed that RRF has nearly identical dimensions to a tRNA (Hirokawa, Kiel et al. 2002; Weixlbaumer, Petry et al. 2007). It was suggested that RRF acts as a functional mimic of a tRNA in line with the translocation-like model of ribosome recycling.

Chemical protection, Cryo-EM, and crystallography have shown that RRF does not bind to the ribosome in the same orientation as a tRNA (Lancaster, Kiel et al.

2002; Agrawal, Sharma et al. 2004; Gao, Zavialov et al. 2005; Weixlbaumer, Petry et al. 2007). RRF interacts primarily with the 50S subunit and overlaps with the tRNA in the A-site and the 50S subunit P-site (but not the 30S P-site) (Gao, Zavialov et al. 2005). The location of RRF bound to the ribosome indicates that the deaclyated tRNA
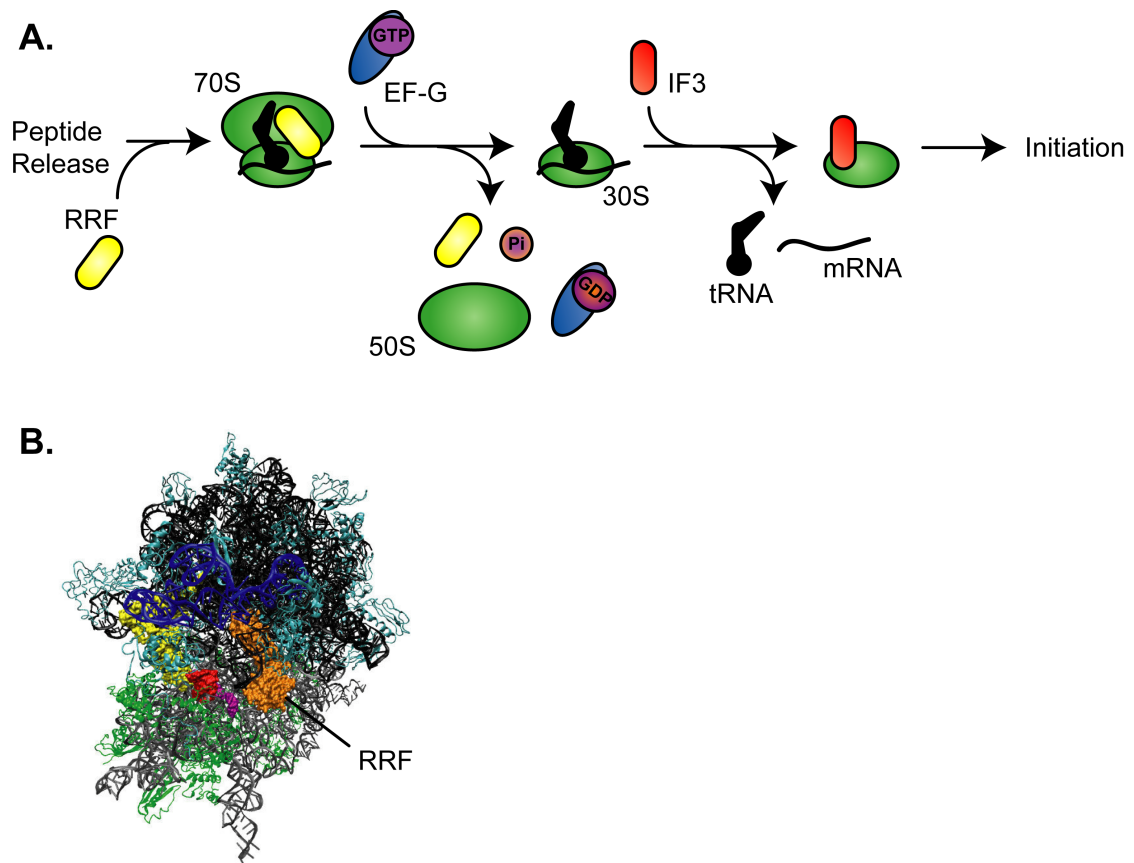
Figure 1.12: Ribosome Recycling. (A) Series of events in ribosome recycling. After peptide release by class I and class II RFs. RRF (yellow) binds with the P-site tRNA (black) in the P/E state. EF-G (blue) binds and hydrolyses GTP (purple). A conformational change in RRF and the ribosome (green) splits the subunits. IF3 (red) binds to the free 30S subunit, dissociating the tRNA. The mRNA also dissociates. The 30S subunit then re-enters the initiation phase of protein synthesis. (B) Crystal structure of RRF (orange) bound to the 70S ribosome. 23S rRNA (black), 5S rRNA (blue), 16S rRNA (gray), large subunit proteins (cyan), small subunit proteins (green), E-site tRNA (yellow), P-site ASL (red), mRNA (purple). Protein Data Bank ID 2V46, 2V47 (Weixlbaumer, Petry et al. 2007).

in the P-site is in the P/E state. Binding of EF-G to the ribosome causes a conformational change in RRF and the ribosome (Toyoda, Tin et al. 2000; Gao, Zavialov et al. 2005). The two domains of RRF rotate relative to each other and are though to disrupt bridges between the two subunits, resulting in the separation of the subunits (Fujiwara, Ito et al. 2004) . Additionally, EF-G variants inactive in translocation are fully active in promoting recycling, indicating that EF-G does not catalyze a translocation-like event during ribosome recycling (Peske, Rodnina et al. 2005).

Kinetic experiments revealed the series of steps in ribosome recycling (Peske, Rodnina et al. 2005). First, RRF binds to the ribosome when a deacylated tRNA is present in the P-site (Moazed and Noller 1989; Hirokawa, Kiel et al. 2002). A deacylated P-site tRNA is bound in the P/E hybrid state, specifically, allowing RRF binding (Peske, Rodnina et al. 2005). EF-G then binds to the ribosome, hydrolyzes GTP, and rotation of a domain of RRF likely disrupts intersubunit bridges, separating the 50S and 30S subunits (Gao, Zavialov et al. 2005). Rotation of a domain of RRF likely disrupts bridges between the two subunits (Peske, Rodnina et al. 2005). GTP hydrolysis by EF-G is required for subunit separation (Martin and Webster 1975). After separation of the subunits, the tRNA remains bound to the 30S subunit (Peske, Rodnina et al. 2005). Binding of IF3 to the 30S subunit accelerates the dissociation of the P-site tRNA from the ribosome (Peske, Rodnina et al. 2005). These kinetic experiments also verified that IF3 acts as an anti-association factor, preventing the premature re-association of the two ribosomal subunits, rather than actively inducing

dissociation of the subunits (Peske, Rodnina et al. 2005). After the subunits have been separated and the 30S subunit is bound by IF3, exchange of mRNAs can occur more easily (Daviter, Gromadski et al. 2006; Petry, Weixlbaumer et al. 2008). The 30S subunit is now ready to re-enter the initiation phase of protein synthesis to begin the synthesis of a new protein.

Thesis goals

During protein synthesis, tRNAs and class I RFs are required to bind to the ribosome based upon a codon presented in the A-site (Rodnina and Wintermeyer 2001; Petry, Weixlbaumer et al. 2008). It is not completely understood how the ribosome is capable of selecting the correct substrate in the A-site with the observed fidelity when the differences among various codons is not always significant.

Pre-steady state kinetics have been invaluable in understanding the mechanism of tRNA selection by the ribosome (Pape, Wintermeyer et al. 1998). Most of our understanding of the intermediates in the process of tRNA selection by the ribosome were identified by one technique (Lovmar and Ehrenberg 2006). In some instances, the identity of the intermediates is controversial and not well established when the primary data is investigated. For example, a nonspecific initial binding intermediate prior to codon recognition by ternary complex on the ribosome would be expected to create competition between cognate and near or non-cognate ternary complexes that would inhibit protein synthesis (Youngman, McDonald et al. 2008). Additionally, no

pre-steady state kinetic assays have been developed to investigate the mechanism of class I RF binding to the ribosome (Gromadski, Daviter et al. 2006). Knowledge of the mechanism of stop codon selection by RFs has been limited to investigation of the catalytic step.

We will investigate the kinetics of codon recognition by ternary complex and release factors using a pre-steady state fluorescence based assay. In the case of ternary complex binding to the ribosome, a new pre-steady state method to measure ternary complex binding to the ribosome will clarify some of the controversial steps in the process of tRNA selection. In the case of stop codon selection by RFs, this will provide the first attempts to understand intermediates in the binding of RFs to the ribosome and the kinetics of RF interaction with stop or sense codons. Additionally, analysis of tRNA and RF codon selection using a similar technique will allow a direct comparison of how a very similar problem is solved by a protein catalyst versus an RNA catalyst.

The original goal of this thesis was to understand how the position and type of mismatch between the codon-anticodon base pairs affects the selection of tRNAs by the ribosome. During the course of studying the association of tRNA^{phe} anticodon mutants binding to the ribosome, it was discovered that the polyamines spermine and spermidine play an important role in the process of ternary complex binding to the ribosome. The work on anticodon mutant tRNA^{phe} binding to the ribosome was put on hold in light of a publication by another lab with similar goals. Instead, we have studied the roles of the polyamines spermine and spermidine in the binding of tRNAs

to the ribosome in an effort to understand why *in vitro* protein synthesis requires high

concentrations of magnesium ions, not present *in vivo*.

Chapter 2: Kinetics of Codon Recognition by Ternary Complex and the Role of

Polyamines

Introduction

A central problem in biology is the decoding of genetic information into functional molecules (Ibba and Soll 1999). Translation of mRNAs into proteins is one of the most important steps in this process (Zaher and Green 2009). mRNAs bound by the ribosome are translated into proteins of a specific amino acid sequence by base pairing between the anticodon of a tRNA with the codon of an mRNA in the A-site of the ribosome (Noller 2006). The amino acid attached to the tRNA specified by the mRNA is then incorporated into the growing polypeptide chain. The process of tRNA selection by the ribosome has been the subject of extensive research but, there are still many poorly understood aspects of this process.

It has been observed that the difference in thermodynamic stability between correct (cognate) and incorrect (near or non-cognate) base pairing interactions is not sufficient to explain the low error rate of protein synthesis (Loftfield 1963). A kinetic proofreading mechanism has been proposed to describe one way the ribosome may be able to obtain greater accuracy than thermodynamically available (Hopfield 1974; Ninio 1975). By separating the selection of tRNAs into two steps, separated by a high energy intermediate, the ribosome is able to increase the accuracy of tRNA selection (Hopfield 1974; Ninio 1975; Thompson and Stone 1977). The first step of kinetic

proofreading is called initial selection (Rodnina and Wintermeyer 2001). Initial selection consists of the binding of ternary complex to the ribosome followed by GTP hydrolysis by EF-Tu (Thompson, Dix et al. 1980). During initial selection, near and non-cognate tRNAs are selected against through faster dissociation of the respective ternary complexes (Thompson, Dix et al. 1981). The second step in kinetic proofreading (called proofreading) consists of the release of tRNA after GTP hydrolysis by EF-Tu and peptidyl transfer, adding the amino acid attached to the A-site tRNA to the growing polypeptide chain (Eccleston, Dix et al. 1985). During proofreading, near and non-cognate tRNAs are discriminated against through dissociation from the ribosome after GTP hydrolysis by EF-Tu in a step called rejection (Thompson 1988).

Many intermediates in the process of ternary complex binding to the ribosome have been identified and are thought to play an important role in the fidelity of protein synthesis (Pape, Wintermeyer et al. 1998). First, ternary complexes are thought to bind to the ribosome in a nonspecific initial binding complex (Rodnina, Pape et al. 1996). In the initial binding interaction, no codon-anticodon interaction takes place and near or non-cognate ternary complex interactions with the ribosome are equivalent to cognate ternary complex interactions with the ribosome (Rodnina, Pape et al. 1996). After the nonspecific initial binding interaction takes place, codon-anticodon base pairs are formed in the codon recognition step (Pape, Wintermeyer et al. 1998). During codon recognition, cognate ternary complex binding is stabilized by interactions in the decoding center, including codon-anticodon base pairing and

interactions with the ribosomal RNA (Ogle, Brodersen et al. 2001). Incorrect ternary complexes dissociate from this step more rapidly (Gromadski, Daviter et al. 2006). Correct codon-anticodon base pairs accelerate GTP hydrolysis by EF-Tu (Rodnina, Fricke et al. 1995). The conformational change of the ribosome and ternary complex that causes faster GTP hydrolysis by EF-Tu is called GTPase activation (Rodnina, Fricke et al. 1995). During GTPase activation and GTP hydrolysis, cognate ternary complexes react more rapidly, helping to maintain a fast overall rate of protein synthesis (Pape, Wintermeyer et al. 1999). Near and non-cognate ternary complexes react more slowly, allowing a greater opportunity to dissociate (Pape, Wintermeyer et al. 1999). After GTP hydrolysis by EF-Tu, the hydrolyzed phosphate must be released in order for EF-Tu to undergo a conformational change that releases the tRNA (Kothe and Rodnina 2006). Upon release of the tRNA by EF-Tu, a correct tRNA will accommodate fully into the A-site of the ribosome and participate in the peptidyl transfer reaction, adding the attached amino acid to the growing polypeptide chain (Pape, Wintermeyer et al. 1998). An incorrect tRNA will likely dissociate from the ribosome after being released by EF-Tu in the rejection step (Pape, Wintermeyer et al. 1999). Accommodation and peptidyl transfer are also accelerated when cognate codon-anticodon base pairing interaction take place relative to near-cognate base pairing interactions (Pape, Wintermeyer et al. 1999). Cognate tRNAs are rapidly accommodated into the ribosome while near-cognate accommodation takes longer, allowing a greater opportunity for incorrect tRNAs to be rejected (Pape, Wintermeyer et al. 1999).

The observed acceleration of GTPase activation/GTP hydrolysis steps and the accommodation/peptidyl transfer steps indicates that the ribosome follows an induced fit mechanism (Pape, Wintermeyer et al. 1999). Only when a cognate substrate binds, certain conformational changes are induced that accelerate the forward reaction steps (Pape, Wintermeyer et al. 1999). Incorrect substrates do not induce the necessary conformational changes, causing them to react more slowly (Pape, Wintermeyer et al. 1999). The slower forward reactions allows a greater opportunity for incorrect substrates to dissociate (Pape, Wintermeyer et al. 1999). In order for an induced fit mechanism to improve the specificity of a reaction, the correct substrate must interact differently from the incorrect substrate in the catalytic step (Post and Ray 1995). Interactions in the decoding center between the rRNA nucleotides A1492, A1493, and G530 appear to play an important role in the induced fit mechanism of tRNA selection by the ribosome (Cochella, Brunelle et al. 2007). A1492, A1493, and G530 undergo a conformational change to interact with the minor groove of the codon-anticodon minihelix in a type I and type II A-minor motif (Ogle, Brodersen et al. 2001). This interaction occurs primarily through the 2'-OH groups of the codon-anticodon base pairs (Ogle, Brodersen et al. 2001). By interacting with the minor groove of the codon-anticodon base pairs in a manner that is sequence independent but, specific for Watson-Crick base pairing geometry, the conformational change of A1492, A1493, and G530 is though to be important for the induced fit mechanism of tRNA selection by the ribosome (Ogle and Ramakrishnan 2005). Additionally, the antibiotic paromomycin, induces the same conformational change of A1492, and A1493 when a

near-cognate tRNA binds to the ribosome (Ogle, Murphy et al. 2002). Paromomycin also causes an acceleration of the rates of GTPase activation/GTP hydrolysis by EF-Tu, and accommodation/peptidyl transfer when near-cognate tRNAs bind to the ribosome (Pape, Wintermeyer et al. 2000). This evidence indicates that nucleotide A1492, A1493, and G530 play a major role in the induced fit mechanism of tRNA selection by the ribosome.

If the ribosome utilizes an induced fit mechanism by monitoring the geometry of the codon-anticodon base pairing interactions in the decoding center of the ribosome, then the conformational change accelerating the rates of GTPase activation/GTP hydrolysis and accommodation/peptidyl transfer must be transmitted from the decoding center to the catalytic centers for each reaction (Zaher and Green 2009). The crystal structure of the 30S subunit with a cognate ASL bound with paromomycin revealed a large conformational change in the ribosome called domain closure (Ogle, Murphy et al. 2002). During domain closure, the head and shoulder of the 30S subunit rotate inward, towards the decoding center (Ogle, Murphy et al. 2002). Domain closure could stabilize tRNA binding or, act as a conformational change required to accelerate forward rates in the induced fit mechanism (Ninio 2006; Schmeing, Voorhees et al. 2009) .

The structural integrity of tRNAs has also been shown to be extremely important to the induced fit mechanism of tRNA selection by the ribosome. Fragmented tRNAs delivered to the ribosome do not accelerate GTPase activation/GTP hydrolysis and accommodation/peptidyl transfer, suggesting that the

signal from the decoding center, inducing faster forward reaction steps may pass through the tRNA (Piepenburg, Pape et al. 2000). The Hirsh suppressor tRNA contains a mutation, disrupting the structure of the anticodon stem of tRNA[trp], allowing this tRNA to read UGA stop codons through the aceleration of GTPase activation/GTP hydrolysis, and accommodation/peptidyl transfer (Hirsh and Gold 1971; Cochella and Green 2005) . The Hirsh suppressor tRNA reinforces the idea that the structure of the tRNA plays an important role in the induced fit mechanism of tRNA selection by the ribosome. Cryo-EM and crystallography have revealed why the structure of the anticodon stem of the tRNA may be extremely important in tRNA selection (Valle, Zavialov et al. 2003; Schmeing, Voorhees et al. 2009). While bound to the ribosome prior to GTP hydrolysis by EF-Tu, the tRNA must adopt a kinked conformation where the tRNA anticodon stem becomes underwound (Valle, Zavialov et al. 2003; Schmeing, Voorhees et al. 2009). This underwound, kinked portion of the tRNA is known as the hinge region (Valle, Zavialov et al. 2003). The hinge region overlaps with the mutations in the Hirsch suppressor tRNA, structurally explaining why these mutations affect the binding of ternary complexes to the ribosome.

The Alignment/Misalignment hypothesis provides an explanation for how the structure of the hinge region plays an important role in decoding (Sanbonmatsu 2006). tRNAs must have increased flexibility in one direction, allowing them to adopt the A/T state where anticodon end of the tRNA is present in the 30S subunit A-site and the acceptor end of the tRNA bound to EF-Tu (Sanbonmatsu 2006). tRNAs must also have a rigid structure, allowing proper placement of the tRNA within the ribosome

(Sanbonmatsu 2006). Disruptions in the decoding center between the codon-anticodon base pairs and their interaction with the rRNA may result in a misalignment of the tRNA which would be translated to a much greater disruption in the catalytic centers for GTP hydrolysis and peptide bond formation due to the stiffness of the tRNA and the large distance between the recognition site (decoding center) and the catalytic sites (GTPase activation center and peptide transfer center) (Sanbonmatsu 2006). By disrupting the structure of the hinge region of the tRNA however, the tRNA may be able to adopt a more reactive conformation despite disruptions in the decoding center alignment.

The current model for tRNA binding by the ribosome provides a series of events that occur when ternary complexes react with the ribosome (Pape, Wintermeyer et al. 1998). The induced fit model of tRNA selection has provided an attractive mechanism explaining how the ribosome may maintain a high rate of protein synthesis while maintaining the necessary fidelity (Pape, Wintermeyer et al. 1999). Serious questions have been raised however for both the series of events that occurs when tRNAs bind to the ribosome and the induced fit model for tRNA selection (Ninio 2006; Johansson, Lovmar et al. 2008). The nonspecific initial binding step of ternary complex binding to the ribosome is not well understood. Cognate, near-cognate, and non-cognate ternary complex interact equivalently with the ribosome during initial binding (Rodnina, Pape et al. 1996). Protein synthesis must always operate with an excess of incorrect substrate *in vivo* (Johansson, Lovmar et al. 2008). The excess of incorrect ternary complexes would be expected to act as competitive inhibitors of

ternary complex binding to the ribosome, causing the rate of protein synthesis to be slowed below the observed rate (Johansson, Lovmar et al. 2008). The initial binding step also has not been observed using any other technique, including smFRET (Blanchard, Gonzalez et al. 2004). Additionally, the current mechanism of ternary complex binding to the ribosome does not allow for dissociation of the ternary complex at many intermediate steps (Ninio 2006). There is no reason why ternary complex could not dissociate from the ribosome during phosphate release for example which was found to occur more slowly than originally expected (Kothe and Rodnina 2006). The induced fit mechanism of tRNA selection by the ribosome has also been called into question (Ninio 2006). In nearly all cases used to prove the induced fit mechanism, an increase in the dissociation rate of the incorrect tRNA accompanied the slower forward reaction steps (Gromadski and Rodnina 2004; Daviter, Gromadski et al. 2006; Gromadski, Daviter et al. 2006). It is not entirely clear if the forward reaction steps appear to be slower due to multiple binding and dissociation events of the incorrect substrate or if the rate constants of the actual chemical steps are in fact slower.

Another persistent problem in *in vitro* studies of protein synthesis has been that reaction conditions that have allowed optimum activity require magnesium ion concentrations greater than observed *in vivo* (Jelenc and Kurland 1979; Bartetzko and Nierhaus 1988) . tRNA selection has been found to be particularly sensitive to the amount of magnesium ions in the reaction (Thompson, Dix et al. 1981; Gromadski and Rodnina 2004). Too much magnesium causes a decrease in the fidelity of protein

synthesis while too little magnesium impairs the binding of tRNAs to the ribosome (Rodnina, Pape et al. 1996; Gromadski and Rodnina 2004). More recently, conditions have been used to study tRNA selection by the ribosome that approach the concentration of magnesium ions found *in vivo* (Gromadski and Rodnina 2004). In these studies however, it is unclear that ribosomes are fully active in protein synthesis. The observed rate of peptide bond formation is nearly 10-fold slower than the expected rate of protein synthesis (Gromadski and Rodnina 2004; Johansson, Bouakaz et al. 2008). Additionally, the active ribosome complexes must be prepared under conditions of elevated magnesium ion concentration (Gromadski and Rodnina 2004). Polyamines have been used to improve the activity of ribosome complexes under low magnesium ion concentrations but, the concentration of polyamines used has been significantly lower than their concentrations *in vivo* (Martin and Ames 1962; Igarashi and Kashiwagi 2000; Gromadski and Rodnina 2004).

Polyamines have been shown to improve the activity of protein synthesis *in vitro* and have a sparing effect for the amount of magnesium ions required (Igarashi, Sugawara et al. 1974). Two polyamines present *in vivo* that appear to have the greatest effects on protein synthesis are spermine and spermidine (Takeda 1969). Polyamines have been shown to play a role in many interactions with nucleic acids, for example, they have been shown to affect RNA folding pathways (Koculi, Thirumalai et al. 2006). Polyamines are also present in both ribosomal subunits at specific sites (Xaplanteri, Petropoulos et al. 2005). Polyamines have been shown to have a specific effect on tRNAs however. tRNA aminoacylation has been found to be

stimulated by polyamines and proceed with improved fidelity (Loftfield, Eigner et al. 1981; Peng, Kusama-Eguchi et al. 1990).   Fluorescence, gel mobility, and limited RNAse digestion assays have shown that polyamines affect the conformation of tRNAs in solution (Nilsson, Rigler et al. 1983; Peng, Kusama-Eguchi et al. 1990; Amarantos and Kalpaxis 2000).   Spermine is an essential additive for the crystallization of tRNA (Kim, Quigley et al. 1971; Ichikawa and Sundaralingam 1972; Ladner, Finch et al. 1972).   Difference maps have shown that two spermines bind to the core of tRNAs (Quigley, Teeter et al. 1978).   One spermine is specifically bound in the helix of the anticodon stem of tRNA[phe] between the phosphate backbone of the nucleotides that must remain flexible for the tRNA to adopt the kinked intermediate during tRNA selection by the ribosome (Schmeing, Voorhees et al. 2009).

In order to better understand the mechanism of tRNA selection by the ribosome, we have developed a new fluorescence based method to monitor the interaction of tRNAs in the decoding center of the 30S subunit. Using this method we hope to clarify the mechanism of tRNA selection by the ribosome. We have also found that polyamines, specifically spermine and spermidine can stimulate the binding of tRNAs to the ribosome at least as well as unphysiologically high magnesium concentrations.

Results

Fluorescence based method to monitor tRNA binding to the A-site

In order to study the binding of ternary complex to the ribosome, we developed a fluorescence based method to monitor codon recognition. Ribosome initiation complexes (IC) were programmed with mRNAs labeled at the 3' end with the fluorescent dye pyrene and tRNA^{fMet} was bound to the P-site. This model mRNA has been previously used in translocation studies with the dye attached to the 3' end of the mRNA, three nucleotides from the codon in the A-site (Studer, Feinberg et al. 2003). This places the dye near the A-site between the head and shoulder of the 30S subunit according to crystal structures of the ribosome with mRNA bound (Figure 2.1A) (Yusupova, Jenner et al. 2006).

Addition of different A-site substrates showed increases in the fluorescence emission intensity of the pyrene probe, indicating that the probe is sensitive to codon recognition in the 30S subunit A-site. Addition of ternary complex to fluorescently labeled IC led to an increase in fluorescence emission intensity of pyrene (Figure 2.1B). Binding of tRNAs alone resulted in the same fluorescence change (data not shown). Binding of ternary complexes containing H84A EF-Tu also resulted in the same increase in fluorescence intensity (Figure 2.1C). H84A EF-Tu is deficient in GTP hydrolysis therefore, the increase in fluorescence upon binding of H84A EF-Tu containing ternary complexes to the ribosome indicates that the observed change in
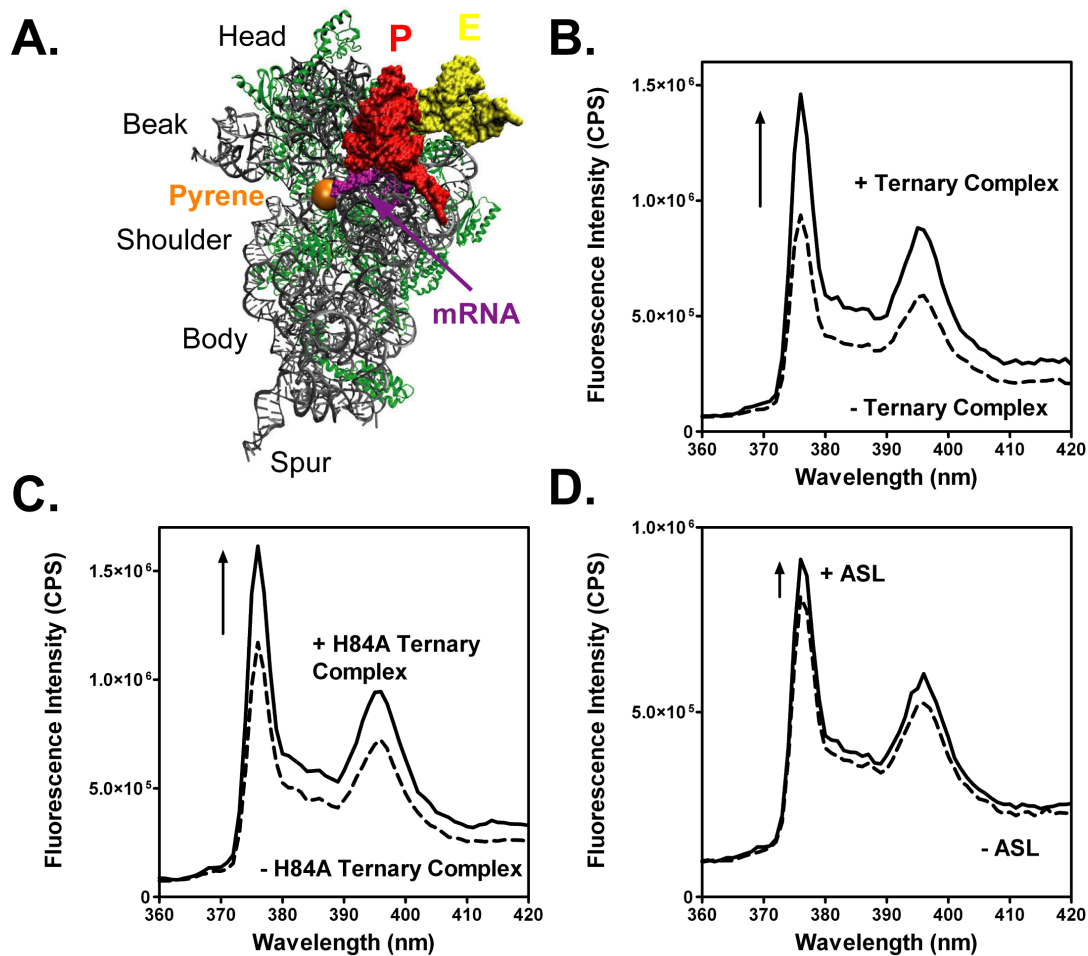
Figure 2.1: Fluorescence signal to monitor tRNA binding to the ribosome. (A) Crystal structure of the 30S with mRNA bound showing the approximate location of the pyrene probe (orange bead). 16S rRNA (gray), small subunit proteins (green), E-site tRNA (yellow), P-site tRNA (red), mRNA (purple). (B) Fluorescence emission scans showing the fluorescence intensity of the pyrene probe before and after addition of ternary complex. (C) Fluorescence emission scans showing the fluorescence intensity of the pyrene probe before and after the addition of ternary complex containing H84A EF-Tu which is deficient in GTP hydrolysis. (D) Fluorescence emission scans showing the fluorescence intensity of the pyrene probe before and after the addition of an ASL.

fluorescence is due to a step prior to GTP hydrolysis by EF-Tu or an event that occurs independently of GTP hydrolysis by EF-Tu (Scarano, Krab et al. 1995; Daviter, Wieden et al. 2003). According to the current mechanism of ternary complex binding to the ribosome, this indicates that codon recognition is likely the cause of the fluorescence change (Figure 1.7). In order to verify that codon recognition causes an increase in the fluorescence of pyrene, an anticodon stem loop (ASL) analog of tRNA was added to fluorescently labeled initiation complexes and an increase in fluorescence was observed (Figure 2.1D). The increase in fluorescence observed upon addition of saturating amounts of ASL was less than with full-length tRNA substrates. Nevertheless, this indicates that the presence of the anticodon of the tRNA in the A-site results in an increase in the fluorescence intensity of the pyrene probe. Addition of non-cognate ternary complex results in little to no increase in the fluorescence of the pyrene probe, consistent with their poor binding (addressed further in chapter 4).

Effect of polyamines on ternary complex binding

Polyamines have previously been shown to have an effect on the efficiency of protein synthesis (Igarashi, Sugawara et al. 1974). Magnesium is commonly used to improve the efficency of *in vitro* protein synthesis (Lamborg and Zamecnik 1960; Tissieres, Schlessinger et al. 1960; Nathans and Lipmann 1961). We have examined the effect of the polyamines spermine and spermidine and Mg^{2+} ions on the association of cognate and near-cognate ternary complexes to the ribosomal A-site. Cognate

ternary complexes were mixed with fluorescently labeled IC in buffers containing varying concentrations of spermine. As the concentration of spermine was increased, the rate of the fluorescence change increased until the complete fluorescence change had occurred before the first time point could be measured (Figure 2.2A). The effect of spermine appeared to be saturated at 0.5 mM but, because the complete fluorescence change had occurred within 10 seconds, higher concentrations could have a greater effect that was not observable.   Increasing concentrations of spermine appeared to have similar effect when ternary complexes were added to ribosomes programmed with the near-cognate codon CUC in the A-site (Figure 2.2B).   Under near-cognate conditions, saturation of the polyamine effect (faster fluorescence change) also appeared to occur at 0.5 mM spermine however, the fluorescence change occurred more slowly and intermediate time points could be measured.   Taken together, it appears that the effect of spermine on the binding of ternary complexes to the ribosome appears to be saturated at 0.5 mM. This agrees well with the content of spermine in cells which as been measured to be ~1.0 mM (Igarashi and Kashiwagi 2000).

Similar experiments were performed varying the concentrations of another common polyamine, spermidine. Increasing the concentration of spermidine in the ternary complex binding reactions had a similar effect to spermine however, the improvement in binding appeared to require a higher polyamine concentration of 2.5 mM to reach saturation (Figure 2.2C). Similarly, binding of ternary complex to near-cognate CUC programmed ribosomes showed a spermidine concentration dependence
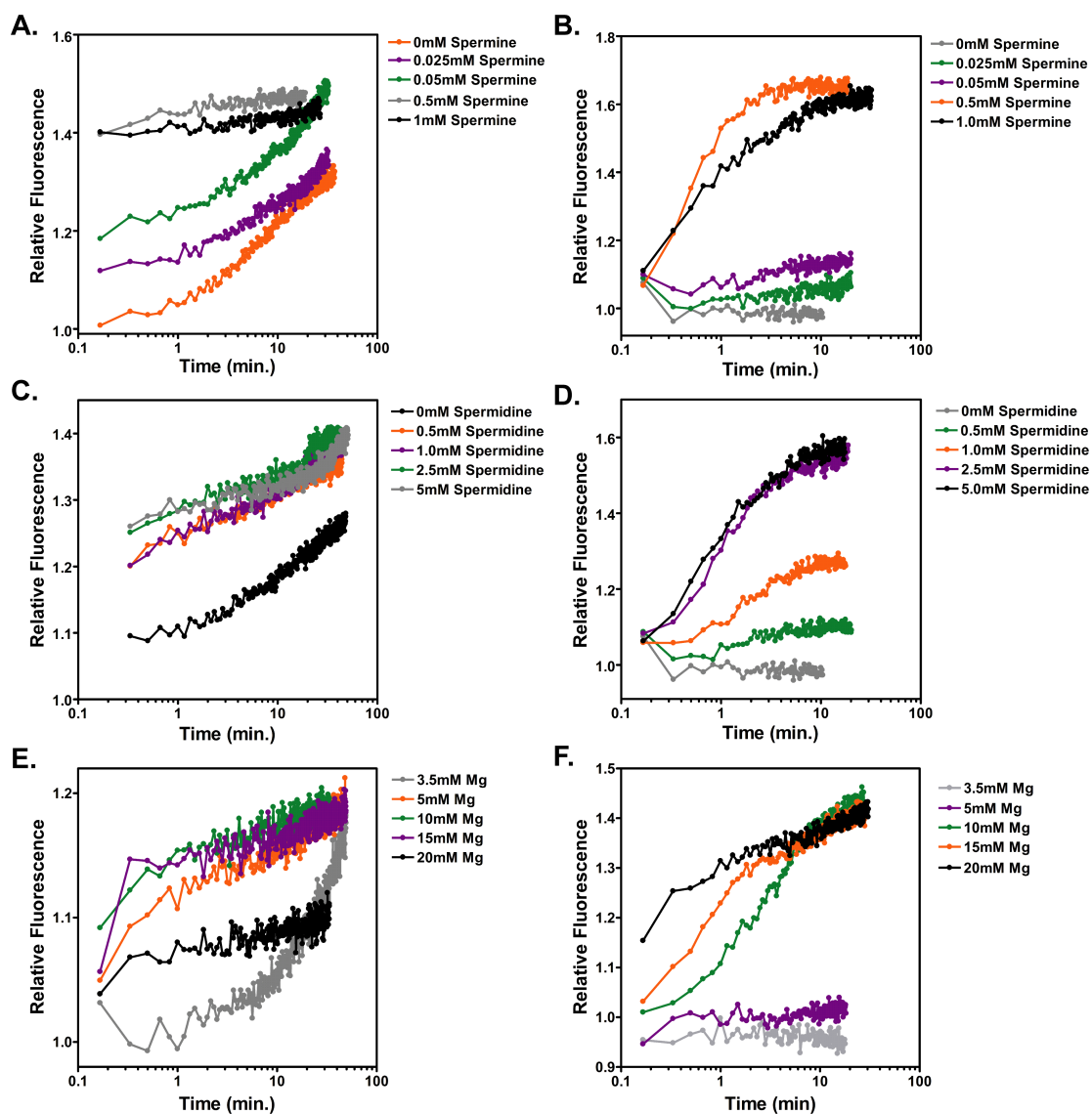
Figure 2.2: Effect of spermine, spermidine, and magnesium on the binding of cognate and near-cognate ternary complex. Fluorescence timecourses were measured upon addition of ternary complex to fluorescently labeled initiation complex with either cognate (UUU) codon (A, C, E) or near-cognate (CUC) codon (B, D, F) codons in the A-site with varying concentrations of spermine (A, B), spermidine (C, D) or magnesium (E, F).

that saturated at 2.5 mM (Figure 2.2D). The saturation of the effect of spermidine on ternary complex binding to the ribosome also agrees well with the cellular concentration of spermidine which has been measured at ~7 mM in *E. coli* (Igarashi and Kashiwagi 2000). It has long been known that Mg^{2+} can improve the binding of tRNAs to the ribosome and can decrease the specificity of decoding (Szer and Ochoa 1964; Thompson, Dix et al. 1981). Therefore, we also tested the effect of increasing Mg^{2+} concentrations on the binding of ternary complexes to the ribosome on cognate and near-cognate codons. Increasing the concentration of Mg^{2+} increased the rate of the fluorescence change observed when ternary complexes were mixed with ribosomes programmed with a cognate UUU codon (Figure 2.2E). At 5-10 mM Mg^{2+} concentrations, a further increase in the rate of fluorescence change could not be observed because the fluorescence change occurred too rapidly to be measured using manual mixing techniques. The fluorescence change upon binding of ternary complex to ribosomes programmed with the near-cognate CUC codon was also measured under varying Mg^{2+} concentrations. As the concentration of Mg^{2+} was increased, the rate of fluorescence change also increased (Figure 2.2F). The rate of fluorescence change, indicating the binding of ternary complex to a near-cognate codon, appears to increase without saturating up to 20 mM Mg^{2+}.

Kinetics of ternary complex binding to the A-site

Polyamines appear to have a significant effect on the binding of ternary

complex to the ribosome. Magnesium ions have been previously shown to affect the binding of ternary complex to the ribosome (Szer and Ochoa 1964; Gromadski and Rodnina 2004). In order to better understand how polyamines effect the binding of tRNAs to the ribosome, we measured the kinetics of ternary complex binding to the ribosome in a stopped-flow fluorimeter under a subset of the buffer conditions used in the above experiments.

Ternary complexes were mixed with fluorescently labeled initiation complexes in a stopped-flow fluorimeter to measure the time course of fluorescence change in buffers containing 0.5 mM spermidine (Low buffer), 0.5 mM spermine (spermine buffer), 5.0 mM spermidine (spermidine buffer), and 20 mM Mg^{2+} (Mg^{2+} buffer). The Low, spermine, and spermidine buffers each also contained 3.5 mM Mg^{2+}. These reaction conditions were chosen based on physiological polyamine concentrations (Igarashi and Kashiwagi 2000), buffers used in previous work on tRNA selection (Gromadski and Rodnina 2004) and the above experiments measuring the dependence of ternary complex binding on polyamines (Fig. 2.2). In all four buffer conditions, a biphasic increase in fluorescence was observed in the stopped-flow time courses (Fig. 2.3A). Stopped-flow time courses were fit to a sum of two exponentials equation to determine two observed reaction rates. Fitting with a single exponential equation was insufficient to describe the data as indicated by a plot of the residuals (Fig. 2.3B). The residuals of a single exponential fit showed major runs of point above and below the best fit curve while the two exponential fit showed a much more random distribution of points across the best fit line (Fig. 2.3B). Observed rates of fluorescence change

were determined at varying concentrations of ternary complex. Plotting the observed rate of the first phase against ternary complex concentration showed a linear concentration dependence in the high spermine, spermidine and Mg^{2+} buffers (Figure 2.3C). In Low buffer however, a hyperbolic relationship was observed between observed rate and ternary complex concentration. A linear concentration dependence indicates a second order reaction where the slope of the line is equal to the second order association rate constant (Johnson 1986). A hyperbolic concentration dependence indicates a first order conformational change is rate limiting (Johnson 1986). This data indicates that increased counter ion concentrations (polyamines or Mg^{2+}) cause a second order fluorescence change. According to control reactions, a fluorescence change in pyrene can be observed due to the codon recognition step. This indicates that polyamines spermine and spermidine, and Mg^{2+} improve ternary complex binding so that codon recognition occurs as a second order association reaction rather than a first order conformational change as previously reported. The association rate constant of ternary complex binding in spermine buffer was 84 μM^{-1} s^{-1}, 86 μM^{-1} s^{-1} in spermidine buffer, 66 μM^{-1} s^{-1} in Mg^{2+} buffer (Table 2.1). In low buffer, the codon recognition step displayed first order kinetics (saturating at 5 s^{-1}), indicating that an initial binding intermediate prior to codon recognition is possible under the appropriate conditions (Fig. 2.3C) (Table 2.1). Interestingly, physiological concentrations of the polyamines spermine and spermidine actually increased the association rate of ternary complexes to the ribosome greater than even unphysiologically high concentrations of Mg^{2+}.
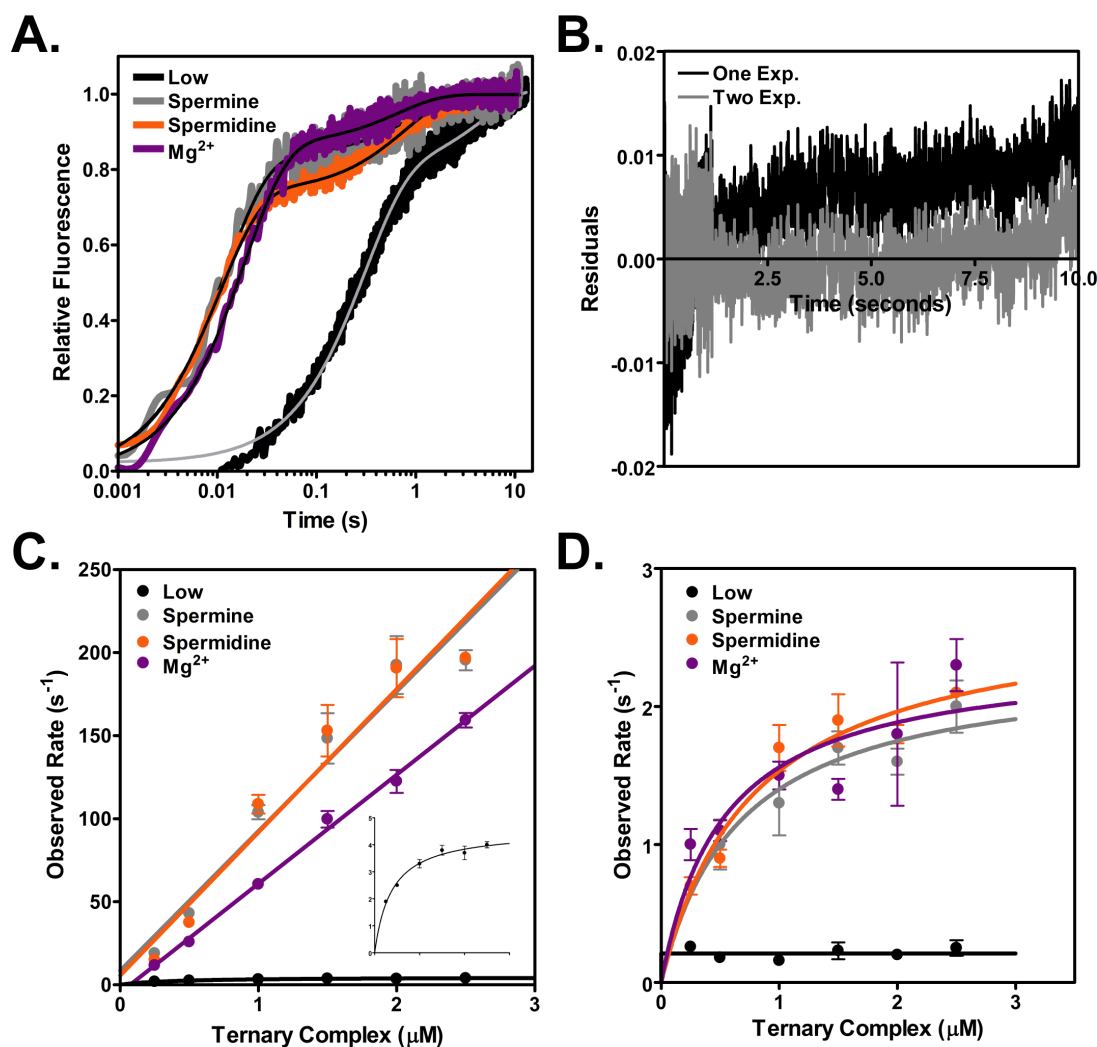
Figure 2.3: Kinetics of ternary complex binding to the ribosome. (A) Stopped-flow time courses of the fluorescence change observed upon mixing ternary complex with fluorescently labeled initiation complex in each buffer. (B) Example of residuals of fitting data from stopped-flow time courses to a single (black) or double (gray) exponential equation. (C) Concentration dependence of the observed rate of the first phase of the fluorescence change in stopped-flow time courses showed linear concentration dependence in spermine (gray), spermidine (orange), and magnesium (purple) buffers. The concentration dependence of the first phase of the fluorescence change in low buffer (black) was significantly slower and displayed a hyperbolic concentration dependence (inset). (D) Concentration dependence of the observed rates of the second phase of the fluorescence change in stopped-flow timecourses. Observed rates showed a hyperbolic concentration dependence.

The concentration dependence of the second phase of the fluorescence change was also determined (Fig. 2.3D). Plotting the observed rate of the second phase of the fluorescence change against ternary complex concentration showed a hyperbolic concentration dependence, consistent with a first order conformational change (Figure 2.3D). The rate of this conformational change is much faster in the presence of higher concentrations of spermine, spermidine or Mg^{2+}. The second step saturated at rates of 2.3 s^{-1} in spermine buffer, 2.7 s^{-1} in spermidine buffer, and 2.4 s^{-1} in Mg^{2+} buffer. In low buffer, the second phase saturated at the significantly lower rate of 0.2 s^{-1} (Table 2.1).

Table 2.1: Rates of ternary complex binding

Buffer	k_{on} ($\mu M^{-1}\ s^{-1}$)	$k_{obs1}{}^{y\text{-}int}$ (s^{-1})	k_{max1} (s^{-1})	$K_{1/2}{}^{obs1}$ (μM)	k_{max2} (s^{-1})	$K_{1/2}{}^{obs2}$ (μM)
Low			5	0.4	0.2	-
Spermine	84	9			2.3	0.7
Spermidine	86	6			2.7	0.8
Mg^{2+}	66	-			2.4	0.5

Domain closure in ternary complex binding

The above analysis of the kinetics of ternary complex binding to the ribosome assumed that the biphasic fluorescence change observed in stopped-flow time courses was due to a two step binding mechanism in which the enzyme and substrate first undergo a second order association followed by a conformational change of the

enzyme-substrate complex (mechanism I. Fig. 2.4A). In the case of ternary complex binding to the ribosome, the ternary complex is considered to be the substrate and ribosome IC is considered to be the enzyme. Biphasic binding time courses are commonly interpreted using this two step binding mechanism (Johnson 1992; Hsieh and Fierke 2009). Other mechanisms of enzyme substrate interaction can produce biphasic kinetics (Cayley, Dunn et al. 1981; Hsieh and Fierke 2009) (Fig. 2.4A). If the free enzyme (or substrate) is present in an equilibrium between two conformations, with only one conformation capable of binding substrate, the first phase of a fluorescence change would be caused by the binding of substrate to the enzyme in the conformation that is competent for substrate binding. The second phase would be caused by the isomerization of the enzyme that is not competent to bind substrate, followed by substrate binding which will pull the equilibrium into the bound enzyme-substrate complex (mechanism II. Fig. 2.4A). Two different binding pathways due to two populations of enzyme or substrate could also produce biphasic time courses if the reaction kinetics of each pathway are significantly different (mechanism III. Fig. 2.4A).

In order to differentiate among the above three mechanisms, we have analyzed the concentration dependence of the amplitudes of each phase of the stopped-flow time courses and the amplitudes of the fluorescence change when different substrates are added to fluorescently labeled IC (Fig. 2.4). In stopped-flow time courses of ternary complex binding to the ribosome, the total fluorescence change was partitioned into two phases with the first phase contributing ~75 % of the total

fluorescence change and the second phase contributing ~25 % of the total fluorescence change under each of the buffer conditions tested (Fig. 2.4B). The contribution of each phase to the total fluorescence change remained constant regardless of the concentration of ternary complex tested (Fig. 2.4B). The fact that each phase contributes the same fraction of the total fluorescence change regardless of the ternary complex concentration indicates that the biphasic fluorescence change is not due to an isomerzation of ternary complex between conformations that are or are not capable of binding to the ribosome as in mechanism II, or an isomerization of ternary complex with different binding properties as in mechanism III. If ternary complex undergoes a conformational change, the amplitude of the slow phase would be expected to decrease as the concentration of ternary complex increases, due to the fact that the population of ternary complex that is competent to bind or binds more rapidly would be increased so that it would bind completely, causing the amplitude of the slow phase to decrease. Isomerization of the ribosome is also not likely to be the cause of the biphasic kinetics due to the fact that release factor binding using the same system does not display biphasic kinetics. The kinetics of release factor binding to the ribosome are investigated in chapter 3.

Analysis of the amplitudes of fluorescence change supports a two step binding mechanism with ternary complex binding to the ribosome in a second order association step followed by a conformational change of the ribosome and ternary complex. Based upon the location of the pyrene probe, we hypothesized that domain closure of the 30S subunit could be the cause of the fluorescence change associated

with the conformational change after ternary complex association (Fig. 2.1, 1.8). Domain closure of the 30S subunit occurred only when a cognate ASL was bound to the ribosome in the presence of paromomycin (Ogle, Murphy et al. 2002). When an ASL was added to fluorescently labeled IC, the observed fluorescence change was not as great as when ternary complex bound (Fig. 2.4C). The full fluorescence change was observed when ASL was bound to ribosomes in the presence of paromomycin (Fig. 2.4C). Paromomycin alone caused only a negligible fluorescence change and did not have any effect on the observed fluorescence change when ternary complex bound to the ribosome (Fig. 2.4C). The change in fluorescence when ASL binds to the ribosome indicates sensitivity to the codon recognition step of ternary complex binding to the ribosome. The additional increase in fluorescence observed when ASL bound in the presence of paromomycin indicates that the probe is also sensitive to domain closure of the 30S subunit.

Comparing the contribution of each phase of the stopped-flow time courses to the fluorescence change when ASL or ASL and paromomycin are bound to the ribosome indicates that the first phase of the fluorescence change is due to codon recognition and the second phase of the fluorescence change is due to domain closure of the 30S subunit (Fig. 2.4D). The first phase of the stopped-flow time courses contributed approximately 75 % of the total fluorescence change in all buffers (Fig. 2.4D). The second phase of the stopped-flow time courses contributed approximately 25 % of the total fluorescence change (Fig. 2.4D). When ASL and paromomycin bound to the ribosome, approximately 70 % of the total fluorescence change could be

Figure 2.4: Analysis of biphasic fluorescence change. (A) Three possible reaction schemes that yield biphasic kinetics. I. A two step binding mechanism with a second order association between the enzyme and substrate, followed by a first order conformational change of the enzyme-substrate complex. II. The enzyme may fluctuate between an active and inactive conformation with the substrate only able to bind to one conformation of the enzyme. III. The enzyme may be present in two conformations with each conformation displaying significantly different reaction kinetics with the substrate. (B) Concentration dependence of the amplitude of the first phase (closed points) and second phase (open points) from fitting the stopped-flow time courses in each buffer. Error bars are one standard deviation. Low ($\bullet$), Spermine ($\blacktriangledown$), Spermidine ($\blacksquare$), Magnesium ($\blacklozenge$). (C) Increase in fluorescence intensity upon addition of the indicated A-site substrate to fluorescently labeled initiation complex. (D) Fraction of the fluorescence change in stopped flow time courses attributed to the first phase (white bars) and second phase (gray bars) compared with the fraction of total fluorescence change when ASL binds to fluorescently labeled initiation complexes that is attributed to ASL binding or the additional increase observed when ASL binds with paromomycin.

attributed to ASL binding alone and 30 % could be attributed to the additional fluorescence change observed when ASL bound in the presence of paromomycin (Fig. 2.4D). The correlation between the two phases of the stopped-flow time courses and the different contribution of ASL with and without paromomycin to the observed fluorescence change indicates that in the stopped-flow time courses, the first phase corresponds to codon recognition and the second phase corresponds to domain closure.

Miscoding under different couterion conditions

One major concern in the process of decoding is how the ribosome miscodes, or accepts tRNAs on the wrong codon. Increased concentrations of Mg^{2+} have been shown to increase the error rate of protein synthesis (Szer and Ochoa 1964). Given that polyamines also appear to improve the binding of ternary complexes to the ribosome, it is important to understand if they cause an increase in the error rate of protein synthesis. In order to determine if we see an increase in miscoding under these different buffer conditions, we measured ternary complex binding to near cognate (CUC) codons under single and multiple turnover conditions and compared this with free tRNA binding (Figure 2.5).

Time courses of fluorescence change were first measured under multiple turnover conditions. Under these conditions, there is a large excess of GTP and

catalytic amounts of EF-Ts, allowing turnover of EF-Tu so that ternary complex may be reformed after GTP hydrolysis by EF-Tu. Under these conditions, a slower fluorescence change is observed when compared to ternary complex binding to cognate mRNAs (compare Figure 2.3A and Figure 2.5). The slower fluorescence change could be due to an inherently slower binding process or due to ternary complexes turning over multiple times in order to allow tRNAs to bind on an incorrect codon. In order to determine which of these possibilities is occurring, time courses under multiple turnover conditions were compared with time courses taken under conditions allowing only a single turnover of ternary complexes. Under these conditions, no EF-Ts is present so, turnover of ternary complexes is limited by dissociation of GDP from EF-Tu which is very slow in the absence of the exchange factor (Wagner, Simon et al. 1995). The rate of fluorescence change under single turnover conditions on near-cognate mRNAs is significantly slower than under multiple turnover conditions, indicating that the turnover of EF-Tu helps to force near-cognate tRNAs into the A-site when no other options are present. Even under single turnover conditions, it is still possible that the slower rate of fluorescence change is due to an inherently slower binding process mediated by EF-Tu or that free tRNAs could bind to the ribosome after GTP hydrolysis by EF-Tu. Comparison of tRNA binding under single turnover conditions to binding of free tRNAs to near cognate initiation complexes shows a similar rate of binding. This shows that under single turnover conditions, tRNAs actually bind as free tRNAs. tRNAs that are rejected in the proofreading step may still bind to the ribosome as free tRNAs since ternary

complex cannot be regenerated under these conditions. There appears to be no major loss in fidelity in either buffer condition tested because, there is not a significant amount of tRNA accepted into the A-site on one turnover of ternary complex. It is possible however that the differences in miscoding in each reaction system are too small to be resolved using this experiment.
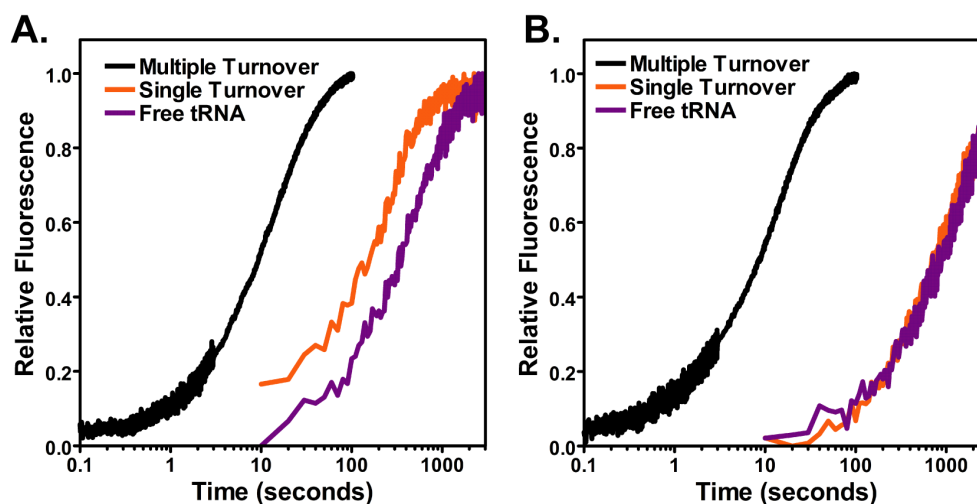
Figure 2.5: **Binding of near-cognate ternary complex to the ribosome.** Fluorescence time courses shown when mixing tRNAs with near-cognate initiation complexes, programmed with a CUC codon in the A-site under various conditions. Multiple turnover (black) corresponds to conditions where ternary complex may be regenerated after GTP hydrolysis by EF-Tu. Single turnover (orange) corresponds to conditions where ternary complex may not be regenerated after GTP hydrolysis. Free tRNA (purple) shows the fluorescence change observed when phe-tRNA^{phe} is mixed with fluorescently labeled near-cognate initiation complex in the absence of EF-Tu. (A) Fluorescence time courses using spermine buffer. (B) Fluorescence time courses using Mg^{2+} buffer.

Discussion

We have developed a new fluorescence based method to monitor the binding of ternary complex to the ribosome. Using this method, we have shown that physiological concentrations of polyamines are capable of stimulating the binding of ternary complex at least as well as unphysiologically high concentrations of magnesium, commonly used for *in vitro* translation studies.

Polyamines have previously been shown to improve the efficiency of and to reduce the Mg^{2+} content required for *in vitro* protein synthesis (Martin and Ames 1962; Igarashi, Sugawara et al. 1974). Polyamines have also been shown to specifically affect the conformation of tRNA and improve the fidelity of aminoacyl-tRNA synthesis (Loftfield, Eigner et al. 1981; Nilsson, Rigler et al. 1983; Amarantos and Kalpaxis 2000). Difference maps of the crystal structure of tRNA[phe] showed that there are two spermines specifically bound to the core of the tRNA (Quigley, Teeter et al. 1978). One spermine is bound between the phosphate backbones of the anticodon stem of tRNA[phe] in the hinge region (Quigley, Teeter et al. 1978). The flexibility of the hinge region of tRNAs is extremely important in allowing the tRNA to bind to the ribosome in the A/T state (Valle, Zavialov et al. 2003; Sanbonmatsu, Joseph et al. 2005; Schmeing, Voorhees et al. 2009). Additionally, the flexibility of this region has been suggested to play a role in the rate enhancement of GTPase activation/GTP hydrolysis and accomodation/peptidyl transfer (Cochella and Green 2005). Spermine or spermidine bound to the anticodon stem of the tRNA may be important for allowing

the tRNA to adopt the kinked conformation required by the A/T state. A specific interaction between the polyamines and tRNA rather than simply a charge shielding effect is supported by the fact that significantly more total charge must be added in the case of Mg^{2+} in order to see the observed acceleration of the association of ternary complex to the ribosome. A more general charge shielding effect likely does play a role in the stimulation of RNA-RNA interactions by polyamines however.

Our data shows that mechanism of ternary complex association to the ribosome follows a two step binding mechanism. First, the codon recognition step occurs as a second order process. During the codon recognition step, the anticodon end of the tRNA enters the 30S subunit A-site. After codon recognition, a conformational change occurs that is consistent with the observations of domain closure from 30S subunit crystal structures (Ogle, Murphy et al. 2002). The association rate constant of ternary complex binding to the ribosome is in good agreement with the rates measured using other techniques (Pape, Wintermeyer et al. 1998). The conformational change after binding occurs with significantly slower kinetics, indicating that domain closure is likely a late step in the binding of ternary complex to the ribosome. Domain closure has been proposed to play a role in the acceleration of the rates of GTPase activation and GTP hydrolysis (Schmeing, Voorhees et al. 2009). The kinetics of domain closure measured here suggests it does not play a role in GTPase activation or GTP hydrolysis but, likely is important in the accommodation step, perhaps helping to position a cognate tRNA correctly for the peptidyl transfer reaction or "locking" the tRNA into the A-site (Ninio 2006; Sanbonmatsu 2006) .

Reactions of near-cognate ternary complex with ribosomes indicated that a vast majority of near-cognate ternary complexes are rejected by the ribosome under conditions of physiological polyamine concentrations and high Mg^{2+} concentrations, most likely after GTP hydrolysis by EF-Tu. These tRNAs were then able to bind to the ribosome without EF-Tu, in agreement with the proposal that so-called shortcut events would decrease the fidelity of protein synthesis (Ninio 2006). High Mg^{2+} concentrations have previously been shown to decrease the fidelity of protein synthesis therefore it is possible that the method used is not sensitive enough to observe modest differences in fidelity. A direct competition assay, allowing more realistic competition among tRNAs would be a more accurate measure of fidelity in each buffer system. Direct competition experiments similar to those that have been used to study DNA polymerase would likely shed a great deal of light on the fidelity of protein synthesis (Bertram, Oertell et al. 2010).

The present work shows some discrepancies with the current models of ternary complex binding to the ribosome. In most cases, these discrepancies can clarify the nature of some of the intermediates in ternary complex binding to the ribosome. The currently favored model of ternary complex binding to the ribosome indicates that there is a nonspecific initial binding intermediate prior to the formation of the codon recognition complex (Rodnina, Pape et al. 1996; Daviter, Gromadski et al. 2006). Codon recognition has been proposed to occur as a first order conformational change of the initial binding complex (Pape, Wintermeyer et al. 1998). The initial binding intermediate has been criticized because it allows near or non-cognate ternary complex

to act as competitive inhibitors of protein synthesis and would be expected to cause extremely slow protein synthesis under *in vivo* conditions (Johansson, Lovmar et al. 2008). Direct competition experiments have shown that near or non-cognate ternary complexes do not significantly slow protein synthesis (Bilgin, Ehrenberg et al. 1988). Our data indicates that the codon recognition step occurs as a second order process, indicating that the first interaction between ternary complex and ribosomes may actually be a specific interaction. If the codon-anticodon interaction is part of the first interaction between ternary complex and ribosomes, near or non-cognate ternary complexes would be expected to be significantly less effective in competing with cognate ternary complex for ribosome binding. Furthermore, the kinetics of the codon recognition step was previously measured through a fluorescence change observed with proflavin labeled tRNAs bound to the ribosome (Pape, Wintermeyer et al. 1998). The fluorescence change was proposed to occur due to a conformational change in the D-loop, where the fluorescent label was located (Rodnina, Fricke et al. 1994). A conformational change induced in the tRNA upon ribosome binding is by definition a first order reaction therefore, the kinetic mechanism of codon recognition cannot be expected to be accurately determined using this technique unless it can be demonstrated that the conformational change of the tRNA causing the fluorescence change occurs significantly more rapidly than the codon recognition event itself. Additionally, changes in reaction conditions that affect the conformational change of the tRNA may not necessarily affect the codon-anticodon interaction in the same way. A more direct method to monitor codon-anticodon interactions is desirable.

Despite questions about the measurements of the codon recognition step, a codon independent interaction between ribosomes and ternary complex has been clearly demonstrated (Rodnina, Pape et al. 1996). In less favorable binding conditions (low buffer) we observe that codon recognition occurs as a first order conformational change, in agreement with the observation that a codon independent binding step may occur. Current models of ternary complex binding indicate that the initial binding step can bind only one ternary complex and that near or non-cognate ternary complex would be expected to competitively inhibit cognate ternary complex binding (Pape, Wintermeyer et al. 1998; Johansson, Lovmar et al. 2008). The initial binding step has been shown to occur even when the A-site of the ribosome is occupied with a tRNA, indicating that a ternary complex bound in the initial binding step may in fact not block the A-site from ternary complex binding (Rodnina, Pape et al. 1996). Models of the L7/L12 stalk proteins indicate that the L7/L12 stalk may help to recruit ternary complex to the ribosome (Kothe, Wieden et al. 2004; Diaconu, Kothe et al. 2005). Consistent with these findings, we hypothesize that the L7/L12 stalk may loosely interact with multiple ternary complexes in the initial binding interaction, increasing the local concentration of ternary complex. Initial binding in this way would also be compatible with both our kinetic measurements of codon recognition and previously measured kinetics of codon recognition and would not result in as severe competition between near or non-cognate ternary complex and cognate ternary complex. In this mode of initial binding, near or non-cognate ternary complex could directly dissociate from the A-site in the codon recognition step. Once a ternary complex is passed to the

A-site from the initial binding interaction, another ternary complex could rapidly take the vacant position, forcing an incorrect ternary complex to dissociate directly from the codon recognition complex rather than being required to pass back into the initial binding interaction, an event that has also been proposed to inhibit protein synthesis (Ninio 2006).

Much remains to be learned about the mechanism of ternary complex binding to the ribosome. Future work will focus on measurement of the chemical steps of ternary complex binding to the ribosome in an effort to verify the mechanism proposed here. Additionally, a more accurate measurement of the fidelity of protein synthesis and understanding the mechanism of near-cognate tRNA rejection by the ribosome are necessary.

Chapter 3: Kinetics of Stop Codon Recognition by Release Factor 1

Introduction

Termination of protein synthesis is triggered when the nearly universal stop codons UAA, UAG, or UGA enter the decoding center of the small ribosomal subunit (Brenner, Stretton et al. 1965). Recognition of a stop codon by class I release factors (RF) leads to peptidyl-tRNA hydrolysis and the release of the newly synthesized protein from the ribosome (Capecchi 1967). In bacteria, the stop codons in the mRNA sequence are recognized by two release factors: RF1 and RF2 (Scolnick, Tompkins et al. 1968). RF1 recognizes UAA and UAG, while RF2 recognizes UAA and UGA (Scolnick, Tompkins et al. 1968). In eukaryotes, a single release factor (eRF1) recognizes all three stop codons (Konecki, Aune et al. 1977). Stop codons are recognized by RFs with remarkably high accuracy (error frequency of 1 x 10^{-3} to 1 x 10^{-6}), even without a proofreading mechanism, indicating that the RFs have a sophisticated mechanism for distinguishing the three stop codons from the sixty-one sense codons (Jorgensen, Adamski et al. 1993; Freistroffer, Kwiatkowski et al. 2000).

RF1 and RF2 consist of 4 domains (Weixlbaumer, Jin et al. 2008). Genetic and

biochemical studies identified a 'tripeptide anticodon' motif in domain 2 of RF1 and RF2 [P(A/V)T in RF1 and SPF in RF2] that is important for stop codon recognition (Ito, Uno et al. 2000). Additionally, a universally conserved GGQ motif located in domain 3 of RF1 and RF2 is important for peptide-tRNA hydrolysis suggesting that RF1 and RF2 spans the ~75 Å distance between the decoding and the peptidyl transferase centers (Frolova, Tsivkovskii et al. 1999). This was confirmed by hydroxyl-radical probing experiments (Wilson, Ito et al. 2000; Scarlett, McCaughan et al. 2003), cryoelectron microscopy (cryo-EM) (Klaholz, Pape et al. 2003; Rawat, Zavialov et al. 2003; Rawat, Gao et al. 2006) and crystal structures of RF1 or RF2 bound to the ribosome (Petry, Brodersen et al. 2005). In contrast, crystal structures of unbound RF1 and 2 show the factors in a closed conformation with the tripeptide anticodon and GGQ motif only 25Å apart (Vestergaard, Van et al. 2001; Shin, Brandsen et al. 2004). This has led to the suggestion that RFs bind to the ribosome in a closed conformation and extend into the peptidyl transferase center after binding (Rawat, Zavialov et al. 2003). Solution X-ray scattering (SAXS) experiments show that RF 1 and 2 exist in an ensemble of open and closed forms in solution (Vestergaard, Sanyal et al. 2005; Zoldak, Redecke et al. 2007). What conformation RFs are in when binding to the ribosome and what conformational changes they undergo remain open questions.

Recent x-ray crystal structures of RF1 or RF2 bound to the ribosome have revealed in exquisite detail the structural basis for stop codon recognition (Laurberg, Asahara et al. 2008; Weixlbaumer, Jin et al. 2008). The 'anticodon tripeptide' motif in

RF1 and RF2 interact precisely with the stop codons in the decoding center (Figure 3.1). Interestingly, the structures showed that other residues in RF1 and RF2, in addition to the tripeptide motif, are also important for stop codon recognition. The first position of the stop codon (U1) interacts with a conserved glycine in domain 2 of RF1 or RF2. Additionally, specific hydrogen bonds are formed by U1 with conserved residues in the tripeptide motifs of RF1 and RF2. These interactions strongly discriminate against a purine and also explain the preference for a uridine at the first position. The second position of the stop codon (A2 or G2) stacks against conserved residues in the RFs and forms hydrogen bonds with the threonine or serine in the tripeptide motifs of RF1 or RF2, respectively. However, it is not clear how the RFs discriminate against pyrimidines at the second position, other than the loss of packing interactions. Finally, the third position of the stop codon (A3 or G3) is unstacked from the second position of the codon by a histidine from RF inserted between the two bases and stacks instead on G530 of the 16S rRNA. The third position forms several hydrogen bonds with specific residues in RF1 or RF2, which explains the preference for an adenine or a guanine by RF1 and the preference for adenine by RF2.

While the x-ray crystal structures provide a rationale for specific recognition of stop codons by RF1 and RF2, it is not known how the dynamics of RF binding is influenced by stop and sense codons in the ribosome. This is an important problem because the kinetics of RF association and dissociation must be finely tuned so that stop codons are efficiently recognized without inhibiting the elongation phase of protein synthesis by competing with aminoacyl-tRNAs for binding to the A site. A

landmark study with a variety of sense codons that differed by a single nucleotide from the stop codon showed that the catalytic rate constant (k_{cat}) of peptide release was reduced by 2- to 180-fold, while the K_M of RF1 increased by 400- to 3,000-fold (Freistroffer, Kwiatkowski et al. 2000). However, this study did not monitor RF binding directly but relied on K_M measurements to distinguish between defects in binding from catalysis. Recent studies suggest that the conformational changes induced by RF1 with stop codons versus sense codons are different leading to differences in the k_{cat} of peptide release (Youngman, He et al. 2007). This makes it difficult to interpret the molecular basis for the observed changes in K_M and k_{cat} with ribosomes having sense codons in the A site.

Recently, the effect of the antibiotic paromomycin on peptide release has been clarified. Paromomycin binds to the decoding center of the 30S subunit and causes conformational changes that would create steric clashes with release factor 1 upon binding to the A-site (Ogle, Brodersen et al. 2001; Laurberg, Asahara et al. 2008). Crystal structures and studies looking at the effect of paromomycin on the catalysis of peptide release suggest that paromomycin acts as a competitive inhibitor of peptide release (Youngman, He et al. 2007).

In order to directly monitor the interaction of RF1 with the ribosome, we have developed a fluorescence based, pre-steady state kinetic assay for RF allowing determination of binding association and dissociation (Figure 3.1B). Our kinetic studies show that the rate of association of RF1 is not significantly affected with a stop or sense codon in the decoding center. In contrast, the rate of dissociation of RF1

differs by as much as a 4,000-fold depending on whether a stop or sense codon is present in the decoding center. Interestingly, the binding kinetics of RF1 does not always correlate with the rate of peptide hydrolysis suggesting that conformational changes, following stop codon recognition, are important for preventing premature termination on sense codons. We have also measured the binding of RF1 to the A-site in the presence of paromomycin.
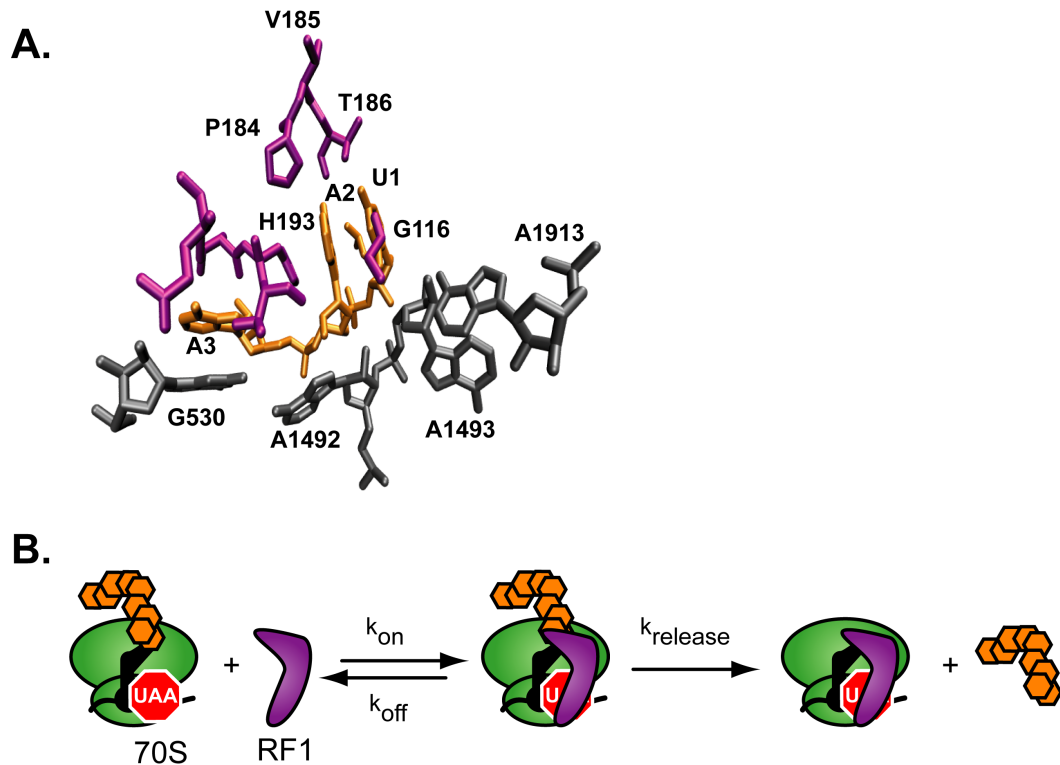
Figure 3.1: Interaction of RF1 with the ribosome. (A) Recognition of the stop codon in the decoding center by RF1. Stop codon $U_1A_2A_3$ (orange), RF1 residues (purple), and bases in 16S rRNA (gray). (B) A kinetic model for RF1 binding to the ribosome followed by hydrolysis of the newly synthesized protein attached to the P site tRNA. Ribosome (green), mRNA (black), P site tRNA (black) with attached protein (orange hexagons), and RF1 (purple).

Results

Fluorescence based method to measure RF1 binding to the ribosome

Rapid kinetic methods have been very valuable for understanding the mechanism of translation initiation, tRNA selection, translocation, and ribosome recycling (Rodnina, Fricke et al. 1994; Studer, Feinberg et al. 2003; Peske, Rodnina et al. 2005; Studer and Joseph 2006). However, no rapid kinetic methods have been developed to examine the intermediates in the mechanism of stop codon recognition by RF1 or RF2 (Youngman, McDonald et al. 2008). We have developed a fluorescence based method to determine the pre-steady state kinetics of RF1 binding to the ribosome. We attached the fluorescent probe, pyrene, to the 3' end of a short mRNA (Figure 3.2A). The pyrene dye is located 3 bases away from the A-site codon where the release factor binds. Based upon crystal structures, the probe would be located in the mRNA channel between the head and shoulder of the 30S subunit, approximately 25 Å away from the middle position of the codon in the A-site (Yusupova, Jenner et al. 2006).

To monitor the binding of RF1 to the ribosome, we formed release complexes (RC) by sequentially adding pyrene-labeled mRNA and tRNA$^{\text{fMet}}$ to 70S ribosomes. The mRNA has a start codon (AUG) at the first position and a stop codon (UAA) at the second position. Binding of tRNA$^{\text{fMet}}$ to the P site will position the stop codon in the A site. Upon addition of RF1 to release complexes, an increase in the fluorescence

intensity of the pyrene probe was observed (Fig. 3.2B). A likely explanation for the increase in fluorescence intensity is the exclusion of solvent from the ribosomal A-site upon binding of RF1. Direct interactions between the probe and RF1 appear unlikely based on the X-ray crystal structures.

Affinities of RF1 for stop and sense codons

Four model mRNAs were synthesized in order to investigate the mechanism of RF1 discrimination of stop and sense codons (Figure 3.2A). The sense codons were selected based upon interactions observed in the crystal structure of RF1 bound to a UAA stop codon (Laurberg, Asahara et al. 2008), previous biochemical data on release factor discrimination (Freistroffer, Kwiatkowski et al. 2000), and work done on tRNA selection (Gromadski and Rodnina 2004) in order to facilitate comparison between these processes. The UAA stop codon was chosen in order to characterize the correct stop codon recognition pathway. The second mRNA has a CAA codon in the A site. The single incorrect C in the first position should not create any obvious steric clashes with the release factor but will result in loss of hydrogen bonding with the release factor in this position. A third mRNA with a UUU A-site codon was chosen because UUU is by far the most commonly used codon for in vitro ribosome experiments and other than loss of packing interactions with the release factor, it is unclear from crystallography how RF1 discriminates against pyrimidines in the second and third positions of the A-site codon (Laurberg, Asahara et al. 2008). The last mRNA tested

had a CUC codon in the A-site, which is different from a stop codon at all three positions while still avoiding obvious steric clashes with the ribosome or release factor.

Previous work addressing discrimination of sense codons from stop codons by RF1 at the binding step was primarily done by measuring the K_M of peptide release. As K_M measurements are dependent on catalytic activity, discrimination of stop and sense codons by RF1 at the binding step have never been addressed independent of catalysis (Youngman, McDonald et al. 2008). In order to determine the affinity of RF1 for stop and sense codons, we performed fluorescence titrations of RF1 with ribosomes programmed with each of the above mRNAs (Figure 3.2C). Increasing amounts of RF1 were added to a fixed concentration of RC programmed with each of the mRNAs. The fluorescence emission was measured after each addition of release factor. In parallel, a blank titration was performed to account for increase in fluorescence due to light scattering under conditions of high protein concentration. The affinity of RF1 for a UAA stop codon programmed A-site could not be accurately determined due to the extremely tight binding affinity of the release factor for this codon. Titrations at the lowest measurable concentration of labeled termination complexes showed that the K_D of RF1 for UAA programmed ribosomes was less than 3.5 nM. Fluorescence titrations performed on each of the sense codons showed at least 1000-fold increase in the K_D of RF1 for the A-site. Among sense codons, RF1 was found to bind most tightly to the UUU codon with a K_D of 1.6 μM. Release factor
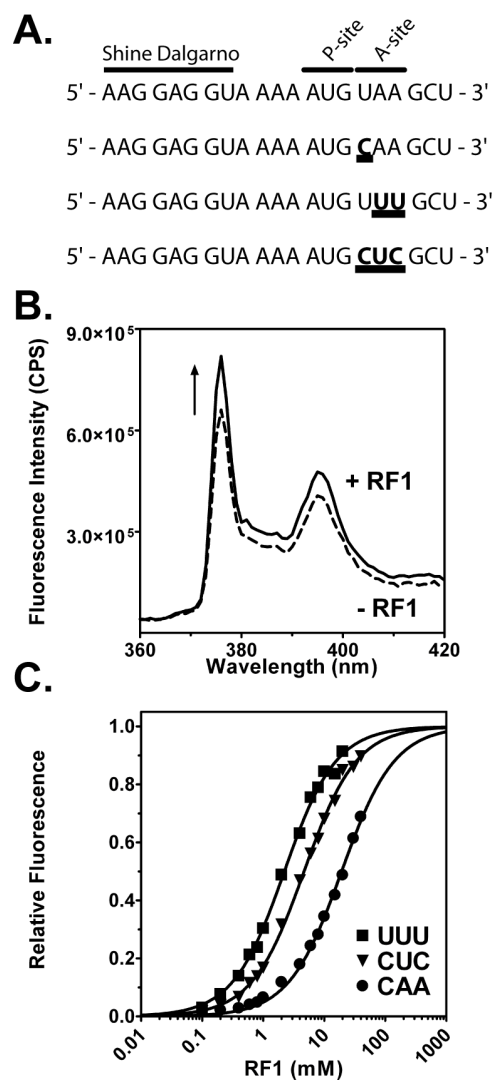
Figure 3.2: Fluorescence assay to monitor RF1 binding. (A) Sequence of four model mRNAs used to measure RF1 binding to stop or sense codons. The Shine-Dalgarno sequence, P-site, and A-site codons are labeled. Changes from the UAA stop codon are shown in bold and underlined. (B) Increase in fluorescence intensity due to RF1 binding. Fluorescence emission scans before and after addition of RF1 to release complex with a UAA stop codon in the A site. (C) Examples of fluorescence titrations to determine the K_D of RF1 for sense codons: UUU, CUC, and CAA. Data were analyzed by fitting to a quadratic equation and normalized from zero to one based upon the best fit line.

bound less stably to the CUC codon with a K_D of 7 μM. Surprisingly, RF1 bound least stably to the CAA codon, with a K_D of 15 μM, even though it varies by only one nucleotide from a cognate stop codon.

Kinetics of RF1 binding to stop and sense codons

In order to investigate the kinetics of RF1 binding to stop and sense codons, we measured time courses of fluorescence change with a stopped-flow fluorimeter. Release complexes were mixed with varying amounts of RF1 and the increase in fluorescence intensity was monitored over time. Time courses of the fluorescence change were described well by single exponential fits, which were used to determine the observed rate of RF1 binding to the ribosome (Figure 3.3A). Plotting the observed rate of fluorescence change versus release factor concentration showed a linear relationship (Figure 3.3B). The linear concentration dependence indicates a second order reaction with the slope of the line equal to the association rate constant and the y-intercept equal to the dissociation rate constant (Johnson 1986). Release factor 1 bound to UAA programmed ribosomes with a rate constant of 34 $\mu M^{-1}s^{-1}$. This association rate constant was reduced by two-fold or less in the cases of sense codons CAA, UUU or CUC, indicating that the association of the factor to the A-site is not a significant source of stop versus sense codon discrimination. Due to the small value of the y-intercept of concentration dependence curves of RF1 binding to the UAA stop codon and errors magnified by extrapolation of the data back to the y-axis, the values

of the dissociation rate constants were calculated from directly measured values of K_D and k_{on} (Table 3.1). Dissociation rates were found to vary over a nearly 15-fold range among sense codons and overall to be at least 100-fold faster than dissociation from the UAA stop codon. Trends in dissociation rates were the same when comparing y-intercepts and calculations from K_D measurements.

Kinetics of peptide release on stop and sense codons

Peptide release time courses were performed by forming release complexes containing each mRNA to be tested and $[S^{35}]$fMet-tRNA^{fMet} in the P-site, then mixing release complexes with saturating amounts of RF1. RF1-catalyzed release of $[S^{35}]$fMet was analyzed by electrophoretic TLC (eTLC) and phosphorimaging. In order to verify that saturation had been reached, time courses were also performed with half the concentration of RF1 with identical kinetics obtained. As expected, peptide release was significantly slower on sense codons than on the stop codon, however, the kinetics of peptide release surprisingly did not follow the same trends as binding (compare Figure 3.3C and Table 3.1). Peptide release was catalyzed with a rate of 9.0×10^{-2} s^{-1} on the cognate UAA stop codon, which is similar to previously determined rates of release (Youngman, He et al. 2007). Among sense codons, RF1 was able to most efficiently catalyze peptide release on the CAA sense codon to which it bound least stably ($k_{release} = 9.9 \times 10^{-4}$ s^{-1}). Peptide release on the CAA sense codon was 10-15 times faster than on UUU or CUC sense codons ($k_{release} = 9.4 \times 10^{-5}$ s^{-1} and 7

x 10^{-5} s^{-1} for UUU and CUC, respectively).

Paromomycin inhibits binding of RF1

The antibiotic paromomycin has been reported to be a competitive inhibitor of peptide release (Youngman, He et al. 2007). The binding of RF1 to the ribosome in the presence of paromomycin was not directly measurable however (Youngman, He et al. 2007). We have performed fluorescence K_D titrations to determine the affinity of RF1 for the A-site on a UAA stop codon in the presence of 100 µM paromomycin. Paromomycin was found to strongly decrease the affinity of RF1 for the A-site. The K_D was increased to 11 ± 2 µM in the presence of 0.1 mM paromomycin (Figure 3.4). This is consistent with the function of paromomycin as a competetive inhibitior of peptide release but, does not exclude other inhibition mechanisms. Stopped-flow timecourses of RF1 binding to the A-site were performed to clarify the binding mechanism of RF1 with paromomycin bound to the A-site. Due to the poor affinity of RF1 for the A-site when paromomycin was bound, the kinetics of binding could not be accurately determined and should be a subject of future research.
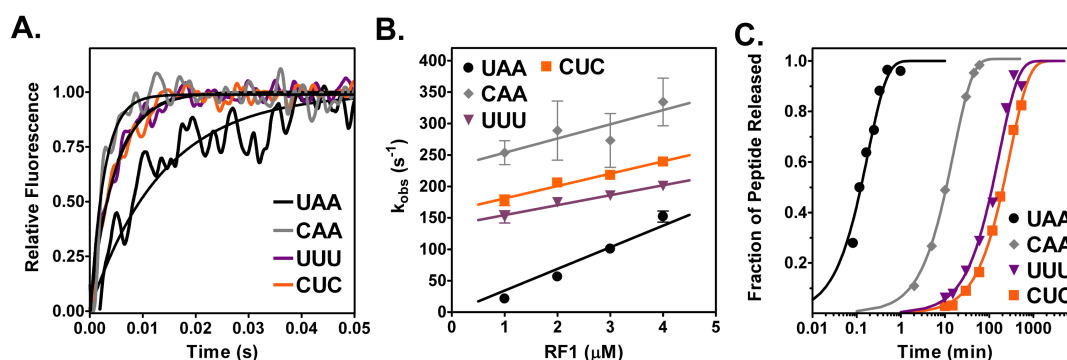
Figure 3.3: Kinetics of RF1 binding to stop and sense codons. (A) Stopped-flow time course of RF1 (2.0 µM) binding to release complexes (0.25 µM) with UAA, CAA, UUU, or CUC codon in the A site. The time courses were fit to a single exponential equation to determine the observed rate of RF 1 binding (k_{obs}). (B) Concentration-dependence of RF1 binding. Observed rates are plotted versus RF1 concentration for UAA, CAA, UUU, and CUC. Plots were fit to a linear equation to determine the association (k_{on}) and dissociation (k_{off}) rate constants. In the case of UAA, the y-intercept was constrained to be a positive value (C) Examples of peptide release time course at saturating RF1 concentrations. Peptide release from release complexes with UAA, CAA, UUU, or CUC codons in the A site are indicated. The concentration of RF1 was 9 µM for the stop codon and 200 µM for the sense codons. Data were normalized and fit to a single exponential equation to determine the rate of peptide release ($k_{release}$).

Table 3.1: Kinetic and thermodynamic parameters for RF1 binding

Codon	K_D (µM)	k_{on} (µM^{-1} s^{-1})	k_{off} (s^{-1})[a]	$k_{release}$ (s^{-1})
UAA	< 0.0035	34	< 0.1	$9.0 \times 10^{-2} \pm 0.1 \times 10^{-2}$
CAA	15 ± 5	23	348	$9.9 \times 10^{-4} \pm 0.9 \times 10^{-4}$
UUU	1.6 ± 0.3	16	25	$9.4 \times 10^{-5} \pm 0.4 \times 10^{-5}$
CUC	7 ± 3	20	127	$7 \times 10^{-5} \pm 1 \times 10^{-5}$

a, k_{off} was calculated from K_D and k_{on}.
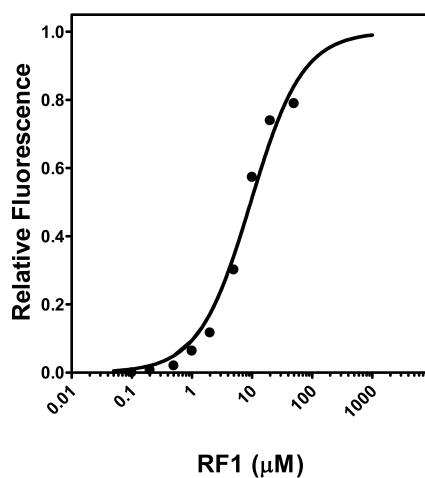
Figure 3.4: Inhibition of RF1 binding by paromomycin. Fluorescence titration of RF1 on mRNA with a UAA stop codon in the A-site in the presence of 0.1 mM paromomycin. Titrations were produced in triplicate and data were fit to a quadratic equation and the K_D was determined to be 11 ± 2 μM.

Discussion

The simplest kinetic model for peptide release involves two steps: (1) Binding of RF1 or RF2 to ribosomes with a stop codon in the A site and (2) Release of the newly synthesized protein by the catalytic activity of RF1 or RF2 (Figure 3.1B). Much work has been done investigating the mechanism of peptide release but this work has focused exclusively on the catalytic step (Youngman, McDonald et al. 2008). In order to differentiate the contribution of binding and catalysis to correct stop codon selection by a release factor, we have developed a fluorescence-based method to monitor RF1 binding to the ribosome. Fluorescence titrations on four model mRNAs designed to disrupt interactions with RF1 in a variety of ways were performed to look at the affinity of release factor for sense codons. As expected, RF1 bound best to the

correct UAA stop codon but, surprisingly, had the lowest affinity for the CAA codon, which only has a single base change from a cognate stop codon, compared to UUU and CUC which have 2 and 3 base changes, respectively. The binding kinetics of RF1 to the A-site was also determined. Association of release factor to the A-site was only slightly affected by the codon. In contrast, dissociation rates were found to increase by at least 100-fold when a stop codon was replaced with a sense codon. Furthermore, dissociation from sense codons varied over a 15-fold range. In agreement with the equilibrium K_D measurements, the rate of dissociation of RF1 was greater from ribosomes with the CAA than CUC codon in the A site.

It is unclear why RF1 would bind less stably to CAA than to CUC. Both have the first, and most sensitive, position of the codon changed from U to C but the two remaining nucleotides are correct for a stop codon in CAA and incorrect for a stop codon in CUC. The crystal structure of RF1 bound to a UAA stop codon shows that the second and third nucleotides in the codon are unstacked with a histidine residue from the release factor inserted between them (Laurberg, Asahara et al. 2008). It appears from the structural data that this unfavorable event could at least be partially compensated for by stacking of A in the second position of the codon with H193 of RF1 and stacking of the third position A with G530 of the 16S rRNA. The difference in stability of RF1 binding to a CAA or CUC codon however suggests that unstacking of the AA residues is not entirely compensated for by these alternative interactions. Although, thermodynamically unfavorable, unstacking of the AA residues could be evolutionarily conserved if it plays an important role in conformational changes

associated with the catalytic step of peptide release. This does appear to be the case as peptide release is catalyzed more efficiently on a CAA codon than CUC despite the less favorable binding interactions.

Consistent with our results, a previous study on release factor fidelity showed that RF1 is most sensitive to changes in the first position of the stop codon (CAA or CAG). A CAA codon in the A-site increased the K_M of peptide release by 2,000-fold and decreased the k_{cat} by 180-fold (Freistroffer, Kwiatkowski et al. 2000). We observed a greater than 4,000-fold defect in K_D and a 90-fold defect in peptide release. The difference in the effect of CAA on the K_D and K_M is likely due do the fact that the active state of peptide release is at least somewhat induced on the CAA codon, resulting in less RF1 required to complete hydrolysis. This agrees with our observation that a lower concentration RF1, relative to the K_D, was required to reach saturation on a CAA codon than the other sense codons tested (data not shown).

Measurement of peptide release rates showed that the efficiency of catalysis of peptide release on sense codons is not directly related to the affinity of release factor to the A-site. Among sense codons tested, peptide release was most efficiently catalyzed on a CAA sense codon, which had the lowest affinity for RF1. Especially interesting is the 10-fold higher rate of peptide release on CAA compared to CUC codon. A potential explanation for why this occurs comes from X-ray crystallography. Stacking of the third residue in the codon with G530 of 16S rRNA is more favorable with a purine than a pyrimidine. By unstacking the third residue from the rest of the codon, the backbone of the mRNA is distorted. A1492 comes out of its helix and

packs against this distorted mRNA backbone. Movement of A1492 is important for release factor function as it opens up space for A1913 of the 23S rRNA to stack with A1493 of the 16S rRNA, allowing RF1 to undergo its putative extension into the peptidyl transferase center after binding or to bind in the fully extended form (Laurberg, Asahara et al. 2008). Without this conformational change, A1913 would block binding of RF1. A purine in the third codon position could be important to precipitate the conformational changes seen in the decoding center, explaining why peptide release is better on a CAA codon than CUC despite the greater binding defects.

It has often been suggested that release factors act as molecular mimics of tRNAs (Youngman, McDonald et al. 2008). However, there are clear differences in the pathway of codon selection by release factors and tRNAs. First, release factors are able to discriminate stop from sense codons without a high energy intermediate, which is required for tRNA selection by the kinetic proofreading mechanism (Freistroffer, Kwiatkowski et al. 2000; Rodnina and Wintermeyer 2001). Second, there appears to be no nonspecific binding intermediate limiting the association or dissociation of RF1 from the ribosome as has been suggested in the mechanism of tRNA selection (Rodnina, Fricke et al. 1994). This is important as the uniformity in RF1 association rates indicates that RF1 is able to scan each codon position and only remains stably bound when a stop codon is encountered. If dissociation rates of RF1 on sense codons were not sufficiently fast, an overall inhibition of protein synthesis would be observed. Third, the affinity of RF1 for all codons is not as predictable as Watson-Crick base

pairing between tRNAs and mRNA. We have seen that single base changes from a cognate stop codon can result in a 10-fold lower affinity than when two bases are changed even in the limited set of sense codons tested.

Decoding by release factors and tRNA selection do appear to share an induced fit mechanism, however. In tRNA selection, Watson-Crick base pairing between the anticodon of the tRNA and codon of the mRNA in the A-site of the ribosome induces an acceleration of the forward rates of GTP hydrolysis by EF-Tu and peptidyl transfer (Daviter, Gromadski et al. 2006). Modulation of these rates could be achieved through acceleration of the conformational changes leading to catalysis or improvement in the transition state stabilization. Similarly, for codon selection by RF1, an increase in the rate of peptide hydrolysis is seen independently from defects in the binding step. It seems reasonable that an active site such as the peptidyl transferase center would be dependent upon conformational changes caused by substrate binding because it must be able to accept a variety of very distinct substrates and catalyze two distinct reactions depending upon interactions 75 Å away in the decoding center, while protecting the currently bound substrate from spontaneous hydrolysis by solvent (Schmeing, Huang et al. 2005).

Paromomycin has previously been reported to act as a competitive inhibitor of peptide release (Youngman, He et al. 2007). This is consistent with the crystal structure of RF1 bound to the ribosome (Laurberg, Asahara et al. 2008). The release factor occupies space required by conformational changes induced in the A-site when paromomycin binds. These are indirect observations however, direct observation of

RF1 binding in the presence of paromomycin is necessary to clarify the mechanism of inhibition of peptide release. Fluorescence K_D titrations showed a nearly 3,000 fold increase in the K_D of RF1 for the A-site with paromomycin present. This is consistent with the current model of inhibition but, does not distinguish from other inhibition mechanisms. Paromomycin and RF1 could both be bound at the same time but, each could destabilize the binding of the other. Stopped-flow time courses to determine the kinetics of RF1 binding in the presence of paromomycin would clarify this mechanism. Determining the kinetics of RF1 binding with paromomycin bound to the A-site should be a subject of future work.

We have shown that steps after the simple recognition of the codon by release factor are important in selection of the correct stop codon. Changes in the measured value of the $k_{release}$ step could encompass conformational changes proposed to occur after release factor binding such as full opening of RF after binding, decoding center rearrangement, changes in the peptidyl transferase center to accommodate the RF and attacking water molecule, or misalignment of catalytic residues in the peptityl transferase center leading to poor transition state stabilization (Rawat, Zavialov et al. 2003; Schmeing, Huang et al. 2005). Determination of relevant reaction intermediates and their role in correct stop codon selection is essential to understand the early steps of termination of protein synthesis.

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Chapter 4: Preparation of Complexes for Ribosome Kinetics and Initial

Discrimination Experiments

Introduction

The decoding step of protein synthesis consists of a multistep binding mechanism that allows the ribosome to quickly and accurately discriminate correct from incorrect tRNAs based upon base pairing interactions in the A-site of the 30S subunit between the anticodon of the tRNA and the codon of the mRNA (Pape, Wintermeyer et al. 1998). During this process, tRNAs bind to the ribosome as a ternary complex consisting of an aminoacylated tRNA, a protein elongation factor, EF-Tu, and GTP. The aminoacylation state of tRNAs can be quite labile and the tRNA is protected from deacylation by binding to EF-Tu (Ling, So et al. 2009). Due to the labile nature of the aminoacyl linkage to the tRNA and the ability of tRNAs to bind to the ribosome in any state (ternary complex, aminoacylated, or deacylated), we must be certain to have efficient ternary complex formation conditions so the binding kinetic are not complicated with multiple types of substrates binding to the ribosome.

The fidelity of protein synthesis is approximately 10^{-3}-10^{-4} (Zaher and Green 2009). The ribosome incorporates an incorrect amino acid once in every 1,000-10,000 codons. How the ribosome is able to maintain this fidelity has been a subject of intense study especially since it was first observed that the thermodynamic difference

in base pairing between correct (Watson-Crick) and incorrect base pairs is not sufficient to explain the high accuracy of protein synthesis (Davies, Gilbert et al. 1964). Since this observation, several mechanisms have been proposed to explain how the ribosome attains such high fidelity.

One way to improve the fidelity of protein synthesis would be for the ribosome to provide a special environment in which the difference in stability between correct and incorrect base pairs was amplified (Gorini and Kataja 1964). Crystal structures of an ASL bound to the 30S subunit shed light on the mechanism of how the ribosome is able to provide this environment. Bases of the 16S rRNA interact across the minor groove of the codon-anticodon helix, hydrogen bonding to the 2'-OH groups of the codon-anticodon base pairs. This allows the ribosome to monitor the geometry of the minor groove so that Watson-Crick base pairs are selectively stabilized over other base pairing interactions (Ogle, Brodersen et al. 2001).

The ribosome has also been shown to employ a kinetic proofreading mechanism in order to improve fidelity (Thompson and Stone 1977). In a kinetic proofreading mechanism, there are two substrate selection steps, separated by a high energy intermediate (Hopfield 1974; Ninio 1974). In tRNA selection, the first step is called initial selection, where the ternary complex first binds to the ribosome (Thompson, Dix et al. 1980). EF-Tu then hydrolyzes GTP (the high energy intermediate) (Thompson, Dix et al. 1980). The second selection step is known as proofreading. After GTP hydrolysis, EF-Tu releases the tRNA and the tRNA may either dissociate from the ribosome or accommodate fully into the A-site and undergo

peptidyl transfer with the P-site tRNA (Thompson and Stone 1977).

More recently, the above two mechanisms have been linked, suggesting that the ribosome uses an induced fit mechanism in tRNA selection to improve fidelity (Pape, Wintermeyer et al. 1999). The chemical steps of GTP hydrolysis and peptidyl transfer occur more rapidly when a cognate tRNA binds to the A-site (Pape, Wintermeyer et al. 1999). Conformational changes that occur only when a cognate tRNA binds have been proposed to cause the acceleration of the rates of these reactions (Daviter, Gromadski et al. 2006). The rRNA-tRNA-mRNA interactions observed in the 30S crystal structures have been proposed to be important in these conformational changes (Ogle, Brodersen et al. 2001; Ogle, Murphy et al. 2002). Some experiments have confirmed that interactions between the decoding center rRNA and codon-anticodon base pairs are important in the rate acceleration of GTP hydrolysis and peptidyl transfer (Cochella, Brunelle et al. 2007).

The effect of the position and type of mismatch between the tRNA and mRNA on the selection of tRNAs is fundamental to the question of how the ribosome selects correct tRNAs. Understanding how these mismatches are accepted by the ribosome will allow further testing of the A-minor interactions observed in 30S crystal structures. We have used a new fluorescence based method to monitor ternary complex binding to the ribosome. Using this technique, we have assessed the relative binding of incorrect ternary complexes based upon the position and type of mismatch between the tRNA and mRNA.

Results

Ternary Complex Formation

tRNAs bind to ribosomes as ternary complexes (Ravel, Shorey et al. 1968). In order to study the mechanism of tRNA selection, we must be able to form stable ternary complexes. Other labs have reported that over expressed EF-Tu may be only marginally active (Sanderson and Uhlenbeck 2007). In order to asses the activity of our purified EF-Tu in forming ternary complex, we performed native gel analysis of ternary complexes (Bilgin, Ehrenberg et al. 1998). EF-Tu was incubated with increasing amounts of HPLC purified phe-tRNA[phe] in an attempt to saturate EF-Tu with tRNA and determine how much of the over expressed EF-Tu is active (Figure 4.1A). Most of the EF-Tu was able to be shifted, indicating that a major portion of the over expressed EF-Tu was active. A significant excess of aminoacylated tRNA was required to shift the EF-Tu however. Performing the titration in reverse, fixing the concentration of phe-tRNA[phe] and increasing the concentration of EF-Tu shows that not all of the aminoacylated tRNA is able to be shifted even with ten fold excess of EF-Tu (Figure 4.1B). During ternary complex formation, aminoacylated tRNAs are incubated for one hour at pH 7 or above. At this pH, aminoacylated tRNAs can be spontaneously deacylated. Deacylation of the purified phe-tRNA[phe] could explain why EF-Tu is not able to shift all of the tRNAs. To asses the aminoacylation state of the tRNAs, a deacylation time course was run where HPLC purified, aminoacylated

tRNAs were incubated with or without EF-Tu and run on an acid gel to determine how quickly the tRNAs deacylate spontaneously (Figure 4.1C). Within 30 min, nearly all of the tRNAs had been deacylated under both conditions, showing that ternary complex was not effectively formed due to tRNA deacylation. To maintain the aminoacylation state of phe-tRNA[phe] phenylalanyl tRNA synthetase (PheRS) was included in the ternary complex formation reaction and the aminoacylation state of tRNA[phe] was assessed up to one hour (Figure 4.1D). PheRS was able to keep the tRNA aminoacylated for over one hour. Gel shift of ternary complexes, including PheRS to maintain the phe-tRNA[phe] shows that increasing amounts of EF-Tu was effectively able to shift nearly all of the tRNAs (Figure 4.1E). This analysis was performed using tRNA[phe] purchased from Sigma. The over expressed, purified tRNA[phe] as outlined in the materials and methods and used in the experiments in previous chapters provided tRNAs that could be more completely shifted into the ternary complex band (data not shown).

Aminoacylation of tRNA[phe] Anticodon Mutants

In order to determine how the ribosome discriminates based upon the position and type of mismatch between the tRNA and mRNA codon-anticodon base pairs, a set of tRNA[phe] anticodon mutants were prepared. PheRS recognizes the anticodon of the tRNA in order to aminoacylate it (Bruce and Uhlenbeck 1982; Sampson, Behlen et al. 1992). Special conditions needed to be developed in order to allow the
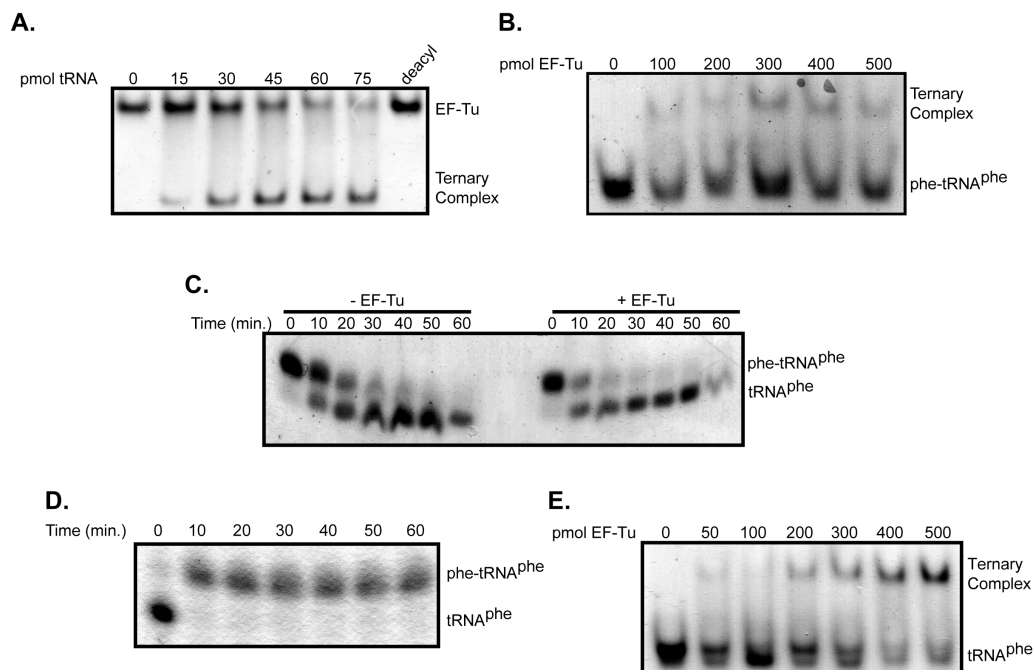
Figure 4.1: Analysis of ternary complex formation. (A) Native gel of ternary complex. 37.5 pmol of EF-Tu was incubated with the indicated amount of HPLC purified phe-tRNA^{phe}. (B) 50 pmol of phe-tRNA^{phe} was incubated with indicated amounts of EF-Tu. (C) Acid gel analysis of phe-tRNA^{phe} deacylation. HPLC purified phe-tRNA^{phe} was incubated with or without EF-Tu to see if tRNAs remain aminoacylated during the ternary complex incubation. At indicated time points 3 M NaC$_2$H$_2$O$_2$ pH 5.2 was added to prevent further deacylation before running on the gel. In both cases, tRNAs were almost completely deacylated within 30 min. (D) Acid gel analysis of tRNA^{phe} aminoacylation. When phenylanalyl tRNA synthetase (PheRS) was used to aminoacylate the tRNAs, the aminoacylation state of the tRNAs was maintained beyond 60 min. (E) Gel shift of ternary complex formation with PheRS to aminoacylate the tRNAs. Ternary complex formation was much more efficient when PheRS was present to ensure the aminoacylation state of the tRNAs.

aminoacylation of the tRNA^{phe} anticodon mutants. Acid gel analysis of the aminoacylation of tRNA^{phe} mutants under standard aminoacylation conditions confirmed that changing the anticodon resulted in less efficient aminoacylation by PheRS (Figure 4.2A). Increasing the concentration of Mg^{2+} to very high concentrations has been reported to reduce the specificity of PheRS (Loftfield, Eigner et al. 1981). Performing the aminoacylation reactions with 50 mM Mg^{2+} showed that the mutant tRNAs could be aminoacylated to a large extent (Figure 4.2B).
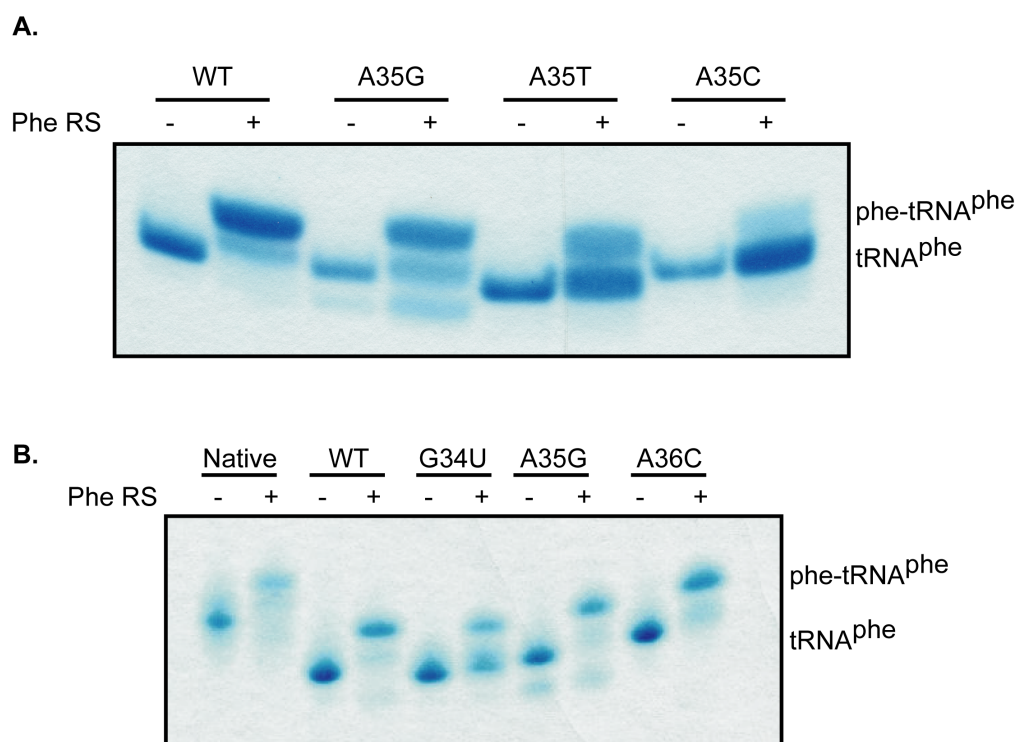
**Figure 4.2: Aminoacylation of tRNA^{phe} Anticodon Mutants.** Acid gel analysis of the aminoacylation of anticodon mutant tRNA^{phe} transcripts. (A) Under standard aminoacylation conditions, some of the mutations in the anticodon of tRNA^{phe} prevented aminoacylation. (B) Addition of 50 mM MgCl$_2$ to the aminoacylation reaction allowed the aminoacylation of all tRNA^{phe} anticodon mutants.

Discrimination of tRNAs by Mismatch Position and Type

In order to begin to understand how the position and type of mismatch between the codon-anticodon base pairs affects the likelihood of miscoding, a variety of tRNA^{phe} anticodon mutants were produced. The relative binding level of the mutants was assessed by mixing ternary complexes containing the mutant or wild type tRNA with initiation complexes programmed with a short model mRNA labeled at the 3' end with the fluorescent dye pyrene. An increase in fluorescence intensity is observed when ternary complexes bind to the A-site. Comparing the extent of fluorescence change when mutant or wild type tRNAs are added to fluorescently labeled initiation complexes allows us to see, relatively, which tRNAs bind better to the A-site and thus, are more likely to be misincorporated. By using tRNA mutants, we will be able to asses the likelihood of miscoding based upon the position and type of mismatch alone, excluding effects from changing the tRNA body which have been shown to affect proper coding (Ledoux, Olejniczak et al. 2009). In addition, by changing the anticodon of the tRNA^{phe} we are able to asses the most conservative GU wobble mismatches which are theoretically, the most stable mismatches (Ogle and Ramakrishnan 2005). Previous studies have only assessed the binding of more severe mismatches with tRNA^{phe} because the codon in the A-site was changed (Gromadski, Daviter et al. 2006). The correct codon for tRNA^{phe} is UUU so if the nucleotides of the codon are changed, binding of tRNAs with GU wobble base pairs cannot be assessed.

First, we used mutants at position 36 of the tRNA to see the effects of the type of mismatch on miscoding. Binding of these mutant tRNAs to the A-site results in a GU wobble, UU mismatch, or CU mismatch between the tRNA and mRNA (Figure 4.3A). Fluorescence emission scans were measured before and after the addition of ternary complexes containing the indicated tRNA mutant to initiation complexes programmed with fluorescently labeled mRNA (Figure 4.3B-D). The percent increase in fluorescence intensity upon addition of ternary complex was determined in order to compare the binding efficiency (Figure 4.3E). As predicted, a GU wobble base pair at the first position of the codon-anticodon base pairing interaction resulted in the least significant defect in binding of the ternary complex. A UU mismatch resulted in a greater binding defect but, a CU mismatch appears to be the most deleterious to binding. The slight decrease in fluorescence observed in the case of A36C is due to the slight dilution of initiation complexes upon addition ternary complex which was not corrected for.

In order to asses the effect of the position of the mismatch on the likelihood of miscoding, we measured the fluorescence change when ternary complexes containing tRNAs that would form a UU mismatch with each position of the mRNA were added to fluorescently labeled initiation complexes (Figure 4.4A). Fluorescence emission scans were taken before and after the addition of ternary complexes containing the indicated tRNA (Figure 4.4B-D). The greatest increase in fluorescence intensity among the mutant tRNAs was observed with a mismatch in the third position (G34U). A mismatch in the middle position (A35U) created the largest defect, with a slight
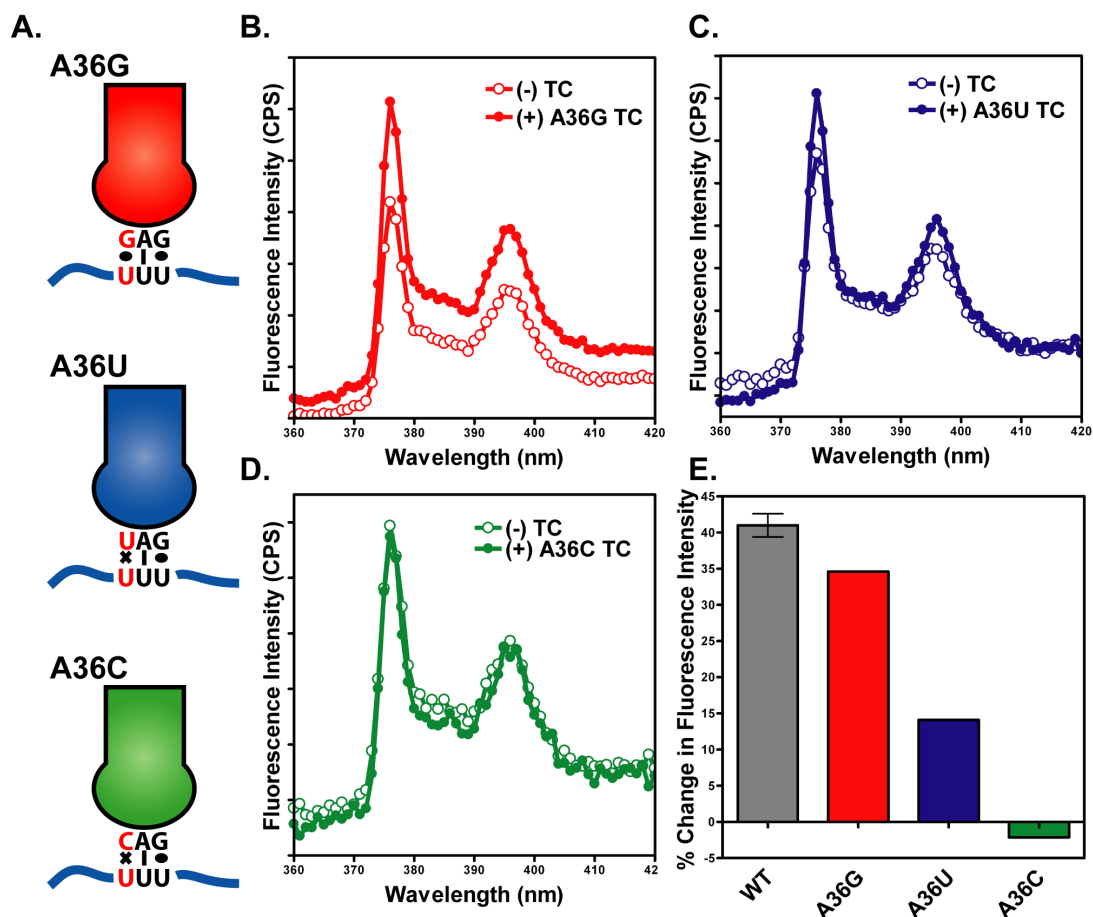
Figure 4.3: Discrimination based upon type of mismatch. (A) Three possible mismatches in the first position of the codon-anticodon base pairs. A G-U wobble, U-U mismatch, and C-U mismatch were tested. (B-D) Fluorescence emission scans of pyrene before and after the addition of ternary complexes containing the indicated tRNA[phe] mutant. (E) Quantitation of the percent increase in fluorescence upon addition of each mismatched ternary complex. Mismatched ternary complexes can be compared to the increase in fluorescence observed upon addition of a cognate ternary complex (WT).

decrease is observed due to uncorrected dilution of the fluorescent initiation complex. Under these conditions, it appears that A35U tRNA did not bind at all to the A-site. A36U, creating a mismatch in the first position of the codon-anticodon duplex resulted in an intermediate defect. (Figure 4.4E).

The most stable base pair other than a Watson-Crick base pair is a GU wobble (Leontis, Stombaugh et al. 2002). GU wobble base pairs are expected to have a thermodynamic stability similar to an AU base pair but, the backbone geometry is different, affecting the backbone positioning and stacking with the neighboring bases (Battle and Doudna 2002). Ternary complexes containing mutant tRNAs that would result in a GU wobble base pair in each position were assessed for binding based on their increase in fluorescence (Figure 4.5A). The tRNA mutated in position 34 actually results in an AU base pair between the tRNA and mRNA in the third position. The WT tRNA case however accomodates a GU wobble in the third position of the codon-anticodon duplex. Fluorescence emission scans were taken before and after the addition of ternary complexes containing the indicated tRNA mutant to fluorescently labeled initiation complexes (Figure 4.5B-D). The trends observed for the effect of the position of the mismatch on the fluorescence change for UU mismatches was also true for GU wobble base pairs. With an AU base pair in the third position, a slightly greater increase in fluorescence was actually observed than even the wild type case. It is unlikely that binding of this tRNA is better than wild type so, it is likely that the greater fluorescence increase is due to more stable position of the mRNA due to more complete base pairing rather than better binding. A GU wobble in the middle
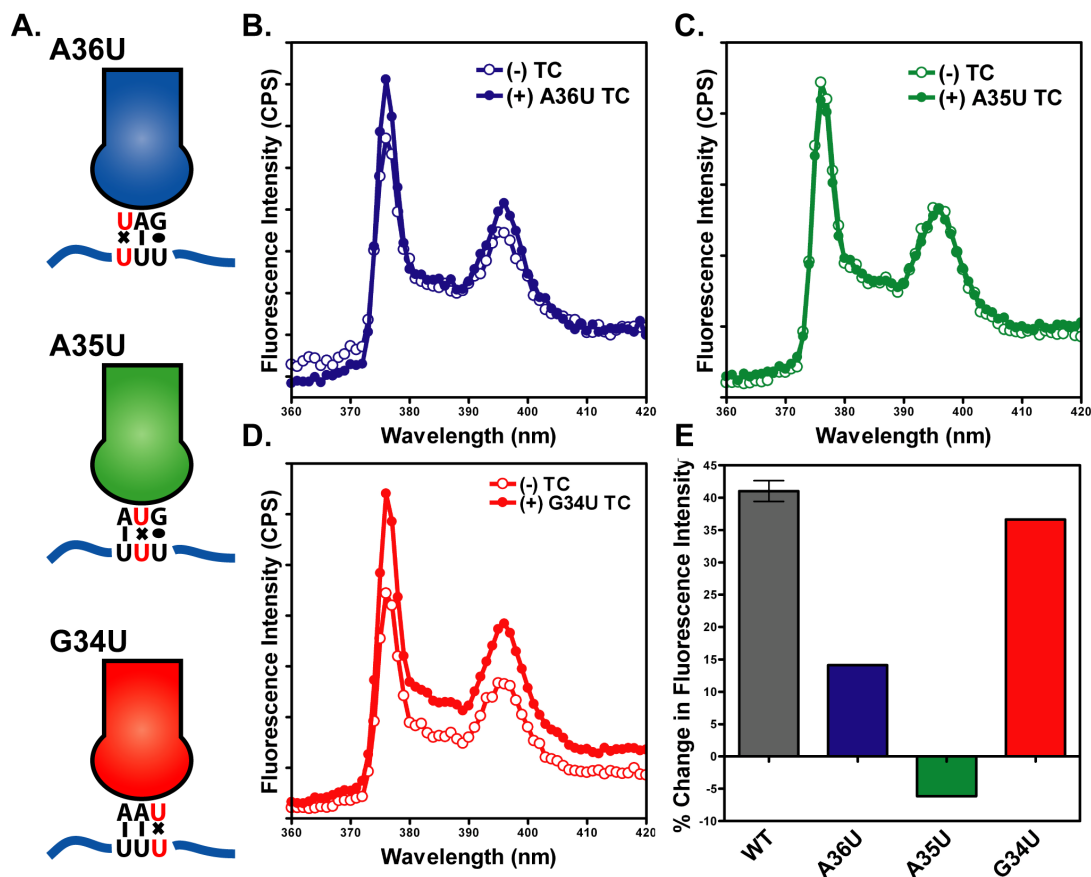
Figure 4.4. Discrimination based on mismatch position (UU mismatches). (A) U-U mismatches were tested at each position of the codon-anticodon base pairs. (B-D) Fluorescence emission scans of pyrene before and after the addition of ternary complexes containing the indicated tRNA[phe] mutant. (E) Quantitation of the percent increase in fluorescence upon addition of each mismatched ternary complex. Mismatched ternary complexes can be compared to the increase in fluorescence observed upon addition of a cognate ternary complex (WT).
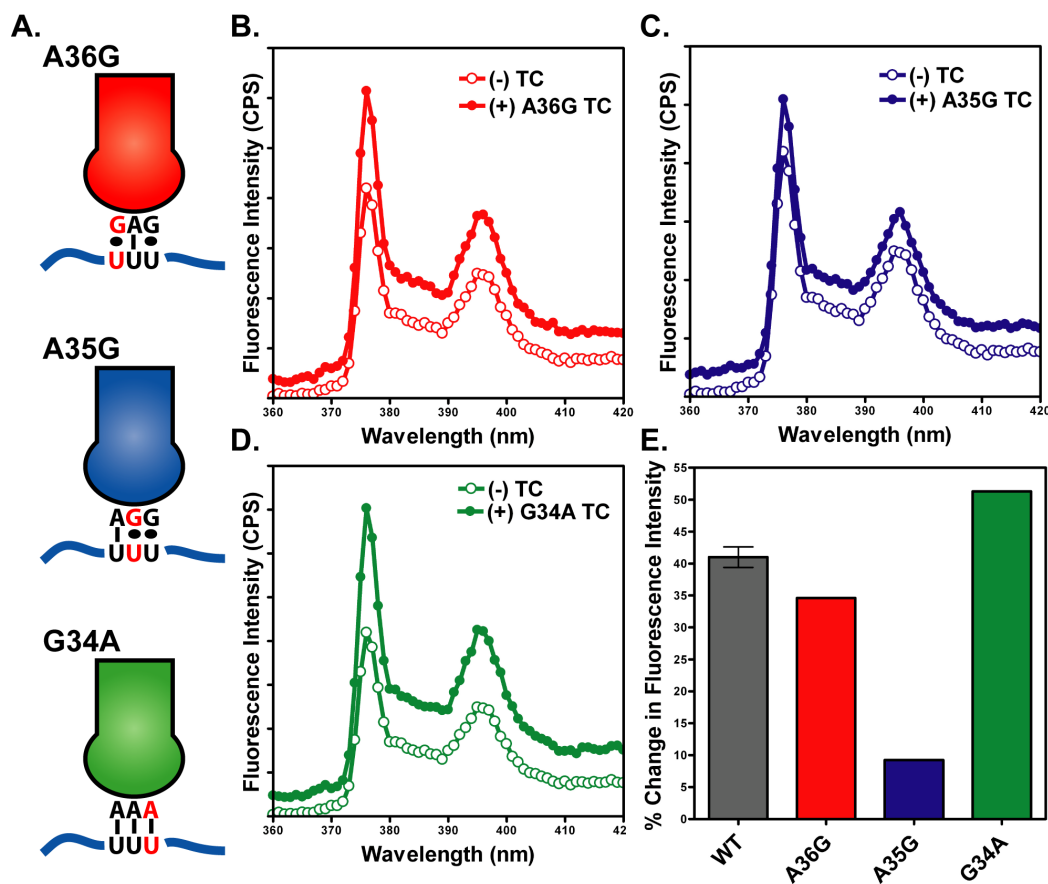
Figure 4.5: Discrimination based on mismatch position (GU wobbles). (A) G-U wobble base pairs (A-U base pair in the third position, WT contains a G-U wobble in the third position) were tested at each position of the codon-anticodon base pairs. (B-D) Fluorescence emission scans of pyrene before and after the addition of ternary complexes containing the indicated tRNA[phe] mutant. (E) Quantitation of the percent increase in fluorescence upon addition of each mismatched ternary complex. Mismatched ternary complexes can be compared to the increase in fluorescence observed upon addition of a cognate ternary complex (WT).

position resulted in the greatest defect, and a GU wobble in the first position was again intermediate (Figure 4.5E).

Discussion

In order to investigate the mechanism of ternary complex selection by the ribosome, several problems had to be overcome. First, conditions under which ternary complexes could be maintained stably needed to be developed. Originally, tRNAs were aminoacylated in separate reactions then HPLC purified and mixed with EF-Tu in order to form ternary complexes. Very little tRNA appeared to be bound in ternary complexes under these conditions as, much of the tRNA becomes deacylated and will no longer be bound by EF-Tu. Spontaneous deacylation of tRNAs can be a significant issue in forming uniform complexes for studying the ribosome. In order to overcome this issue, we included phenylalanyl tRNA synthetase in the ternary complex formation reactions in order to maintain the aminoacylated state of the tRNA. Under these conditions, tRNAs remained aminoacylated and were able to more efficiently form ternary complexes with EF-Tu.

In order to understand how the binding of tRNAs to the ribosome is affected by the position and type of mismatch between the codon anticodon base pairs, we created a library of tRNA^{phe} anticodon mutants, allowing us to asses the defects in binding without changing the body of the tRNA. Phenylalanyl tRNA synthetase recognizes the anticodon of tRNA^{phe} in the aminoacylation reaction, thus, some mutants were not

efficiently aminoacylated under the standard reaction conditions (Peterson and Uhlenbeck 1992). In order to trick the synthetase into aminoacylating the tRNA[phe] anticodon mutants, we included 50 mM Mg^{2+} in the reaction buffer. Increased Mg^{2+} reduced the specificity of the synthetase sufficiently to allow the mutant tRNAs to be aminoacylated.

Producing and aminoacylating the mutant tRNAs allowed us to asses the relative binding to the ribosome based upon the position and type of mismatch between the tRNA and mRNA duplex using our new fluorescence based assay to monitor ternary complex binding to the ribosome. Upon binding of ternary complexes to the ribosome an increase in the fluorescence intensity of pyrene attached the the 3' end of a short mRNA is observed. By comparing the increase in fluorescence intensity when ternary complexes containing each of the mutant tRNAs was added to fluorescently labeled initiation complexes, we could determine the relative defect in binding based on the position and type of mismatch. In general, it appears that GU wobble base pairs are most likely to be misread followed by UU mismatches, with CU mismatches being the least likely to be misread. GU wobble base pairs are thermodynamically most similar to Watson-crick base pairs so it is not surprising that these mismatches appeared to result in the least binding defect (Battle and Doudna 2002).

In order to asses the effect of mismatch position on incorrect binding, tRNAs that would result in a UU mismatch or a GU wobble base pair were mixed with fluorescently labeled initiation complexes and the increase in the fluorescence of the

pyrene probe was compared. Both sets of incorrect tRNAs (UU mismatches or GU wobble base pairs) gave similar trends. The middle position of the codon-anticodon mini helix was most sensitive to disruption. Disrupting the base pairing interactions in the first position appeared to have a less deleterious effect. The third position, also known as the wobble position due to its ability to accept wobble base pairs was the least sensitive to disruption of the codon-anticodon interactions (Crick 1966).

Measurement of the relative increase in fluorescence when each tRNA is bound at one concentration is not the best way to monitor defects in binding. The differences in the extent of the fluorescence change could be due to differences in the fully bound state in individual complexes, not in the degree of binding. In order to more accurately determine the binding defects, equilibrium fluorescence titrations would be preferred to quantitatively assess the defects in binding thermodynamics. Even with the above caveat, we believe these results do accurately reflect the relative affinities of these mutant tRNAs for the A-site. Similar trends are observed when the effects of different mismatches are used to asses effect of the position of mismatches and when different positions are used to asses the effects of the types of mismatches. It is unlikely that intrinsic differences in the final fluorescence intensity of completely bound complexes would coincidentally show the same trends in all cases. It is likely therefore that these trends do reflect the relative effect of the position and type of each mismatch on ternary complex binding to the ribosome.

The fact that the position and type of mismatch between the codon anticodon base pairs may have a significant effect on tRNA binding to the ribosome contradicts

one thorough study suggesting that tRNAs are selected uniformly, with the position and type of mismatch between the codon-anticodon interaction not significantly changing the likelihood that a tRNA would be accepted or rejected by the ribosome (Gromadski, Daviter et al. 2006). There are several problems with this study however. Mismatches were made by changing the codon of the mRNA in the A-site and assessing the binding of tRNA[phe] containing ternary complexes (Gromadski, Daviter et al. 2006). This combination allows only highly unfavorable mismatches to be made. These mismatches would be expected to not only be highly unfavorable themselves but to also disrupt base pairing interactions of their nearest neighbor (Sanbonmatsu and Joseph 2003). Future work will focus on determination of the kinetics and thermodynamics of these tRNA[phe] anticodon mutants binding to the ribosome in order to completely understand how the ribosome discriminates cognate tRNAs from near or non-cognate.

Despite the lack of quantitative data on the binding of the tRNA[phe] anticodon mutants, our results do support interactions seen in the crystal structure between the 16S rRNA and the codon-anticodon mini helix (Ogle, Brodersen et al. 2001). Crystal structures of the 30S subunit with an anticodon stem loop analog bound to the A-site showed that three bases in the 16S rRNA change conformation to interact with the minor groove of the codon-anticodon mini helix (Ogle, Brodersen et al. 2001). A1492 and A1493 appear to measure the minor groove of the first and second base pairs in the codon-anticodon interaction, selectively stabilizing Watson-Crick base pairs (Ogle, Brodersen et al. 2001). G530 interacts with the third position in the codon-anticodon

base pairs but in a manner that would not be specific for Watson-Crick geometry (Ogle, Brodersen et al. 2001). The observed interactions explain how GU wobble base pairs are discriminated against except in the third position of the codon-anticodon interaction. The difference in sensitivity of each position to mismatches is also explained by these interactions in the crystal structure. The middle position has the most extensive interactions with the rRNA which is why mismatches at this position are most deleterious to binding (Ogle, Brodersen et al. 2001). The minor groove is monitored by the rRNA in the first position as well but not as extensively, consistent with the observation that mismatches in the first position have a less severe impact on binding (Ogle, Brodersen et al. 2001). The geometry of the third position is not monitored. This is consistent with the observation that GU wobble base pairs are easily accepted by the ribosome in this position. Our data supports the theory that the ribosome provides a special environment that selectively stabilizes Watson-Crick base pairs, allowing a greater selectivity than would be expected from the thermodynamic stability difference between cognate and near-cognate codon-anticodon interactions alone (Davies, Gilbert et al. 1964; Ogle, Brodersen et al. 2001).

Chapter 5: Materials and Methods

Buffers

Low buffer consists of 50 mM Tris-HCl (pH 7.5), 70 mM NH_4Cl, 30 mM KCl, 3.5 mM $MgCl_2$, 0.5 mM spermidine, 8 mM putrescine, and 2 mM DTT (Gromadski and Rodnina 2004). Spermine buffer consists of 50 mM Tris-HCl (pH 7.5), 70 mM NH_4Cl, 30 mM KCl, 3.5 mM $MgCl_2$, 0.5 mM spermine, 8 mM putrescine, and 2 mM DTT. Spermidine buffer consists of 50 mM Tris-HCl (pH 7.5), 70 mM NH_4Cl, 30 mM KCl, 3.5 mM $MgCl_2$, 5 mM spermidine, 8 mM putrescine, and 2 mM DTT. Magnesium buffer consists of 50 mM Tris-HCl (pH 7.5), 70 mM NH_4Cl, 30 mM KCl, and 20 mM $MgCl_2$ (Gromadski, Daviter et al. 2006). Buffer for peptide release experiments consists of 20 mM Hepes-KOH (pH 7.6), 6 mM $MgCl_2$, 150 mM NH_4Cl, 4 mM 2-mercaptoethanol, 0.05 mM spermine, and 2 mM spermidine (Bartetzko and Nierhaus 1988).

mRNA Labeling and Purification

mRNAs were purchased from Dharmacon and labeled with pyrene succinimide as previously described (Studer, Feinberg et al. 2003).

Transcription Reactions and Purification

tRNA^{phe} transcripts were prepared by runoff transcription. 250 µg of plasmid (purified using Qiagen Megaprep) was digested with BstN1 in 500 µL for 5 hours at 60 °C. Digestion was verified by running a sample 9 µL on 1.5 % agarose gel. Digestion reactions were extracted 1:1 with phenol:chloroform:isoamyl alcolhol, then extracted 1:1 with choloroform, then ethanol precipitated with 1 mL ethanol at -80 °C for 1 hour. DNA was pelleted by centrifuging at 4 °C for 30 min, then washed with 70 % ethanol. The supernatant was decanted and the pellet dried under vacuum. Dried DNA was resuspended in 200 µL water and mixed with 2.5 mM DTT, 1.25 mM NTP mix, 5 mM GMP, and 20 µL purified T7 RNA Polymerase and transcription buffer (40 mM Tris-HCl pH 7.5, 20 mM MgCl$_2$ 2 mM Spermidine, 0.1% Triton X100) in 2 mL final volume. Transcription reactions were incubated at 37 °C overnight for at least 12 hours. Transcription reactions were then extracted twice with 1:1 chloroform, then ethanol precipitated with 2.5 volumes of ethanol at -80 °C for 1 hour. Samples were pelleted as above then resuspended in 100 µL loading buffer per microcentrifuge tube and purified on a 10% acrylamide gel. tRNA bands were identified by UV shadowing, cut out, and eluted in 600 µL elution buffer overnight at 4 °C. Eluted tRNAs were extracted with 1:1 chloroform three times, ethanol precipitated as above and resuspended in 100 µL water.

tRNA^{phe} Purification

     tRNA^{phe} was either purchased from Sigma or purified from overexpression. Overexperession tRNA^{phe} was purified as follows. DH5α cells were transformed with pBsPhe plasmid and plated on 2xYT plates. Competent cells were thawed on ice. 1 μL of plasimid prepared using a Qiagen miniprep kit was added to 100 μL of competent cells. The cells and DNA were incubated on ice for 30 min then heat-shocked at 42 °C for 90 seconds. 1 mL of 2xYT broth was added to the cells than incubated at 37 °C for one hour without shaking. 50 or 100 μL of transformed cells were then spread onto 2xYT-Ampicillin (Amp) (50 mg/L) plates and incubated at 37 °C overnight.

     500 mL 2xYT-Amp (50 mg/L) were then inocculated with a single colony from overnight plates and incubated on a 37 °C shaker for 20 hours. Cells were harvested by centrifuging at 5,000 g for 20 min at 4°C. The cell pellet was resuspended in 8.5 mL lysys buffer (1mM Tris-Cl (pH 7.5), 10 mM Magnesium Acetate) for every 1 L of cell culture. The resuspended cell pellet was transferred to oak ridge tubes and water saturated phenol was added at a one to one ratio. Cells were than vortexed for 30 seconds two times with a 30 second rest between. Samples were then centrifuged 12,000 RPM in a Beckman JA17 rotor for 1 hour at 20 °C. The aqueous phase was transferred to new tubes and ethanol precipitated with 2.5 volumes of ice cold ethanol for 30 min. on ice. Samples were then centrifuged at 15,000 RPM in a Beckman JA17 rotor for 30 min at 4 °C to collect precipitate. The supernatant was decanted and

pellets were allowed to air dry. Pellets were resuspended in 4 mL 1 M NaCl and centrifuged at 10,000 RPM for 30min at 4 °C in a Beckman JA17 rotor. The supernatant was collected and ethanol precipitated as above. Pellets from the ethanol precipatation step were then resuspended in 4 mL 1 M Tris-Cl pH 8.0 and incubated at 37 °C for 2 hours to deacylate the tRNAs. Samples were then ethanol precipitated again as above. Pellets were resuspended in loading buffer (20 mM $NaC_2H_3O_2$ (pH 5.2), 10 mM $MgC_2H_3O_2$, 2 M $(NH_4)_2SO_4$) and purified on a phenyl column on a linear gradient buffer A (20 mM $NaC_2H_3O_2$ (pH 5.2), 10 mM $MgCl_2$, 1.5 M $(NH_4)_2SO_4$ to buffer B (20 mM $NaC_2H_3O_2$ (pH 5.2), 10 mM $MgCl_2$) (Figure 5.1). Fractions corresponding to the last peak were pooled and 2x the volume of Q buffer A (50 mM $NaC_2H_3O_2$ (pH 5.2)) was added. Samples were then purified on a Q column using a linear gradient from Q buffer A to Q buffer B (50 mM $NaC_2H_3O_2$ (pH 5.2), 1 M NaCl). A single peak elutes and those fractions are pooled and ethanol precipitated as above except the centrifugation step was performed in 1.5 mL microcentrifuge tubes. The final pellets were resuspended in approximately 400 μL water. tRNAs were then purified on a C18 column using a nonlinear gradient from C18 buffer A (20 mM Tris-Acetate (pH 5.2), 400 mM NaCl, 10 mM $MgC_2H_3O_2$) to C18 buffer B (20 mM Tris-Acetate (pH 5.2), 400 mM NaCl, 10 mM $MgC_2H_3O_2$, 60% MeOH) (Figure 5.2). The following gradient was employed.

% B	Time (min)
0	5
0 → 34	5
34	10
34 → 46	5
46	10

Fractions containing tRNA phe were pooled and ethanol precipitated as above. The final pellet was resuspended in approximately 200 μL water and quantitated by A260.
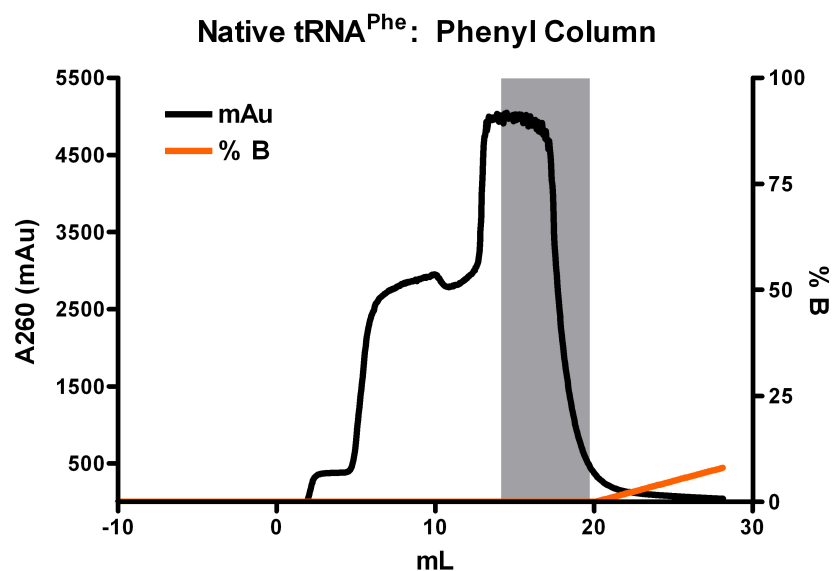
Figure 5.1: Phenyl column purification of native tRNA[phe]. Extracted cell lysate was loaded onto a 5 mL phenyl column and eluted with a linear gradient. Shaded region shows fractions combined containing tRNA[phe].
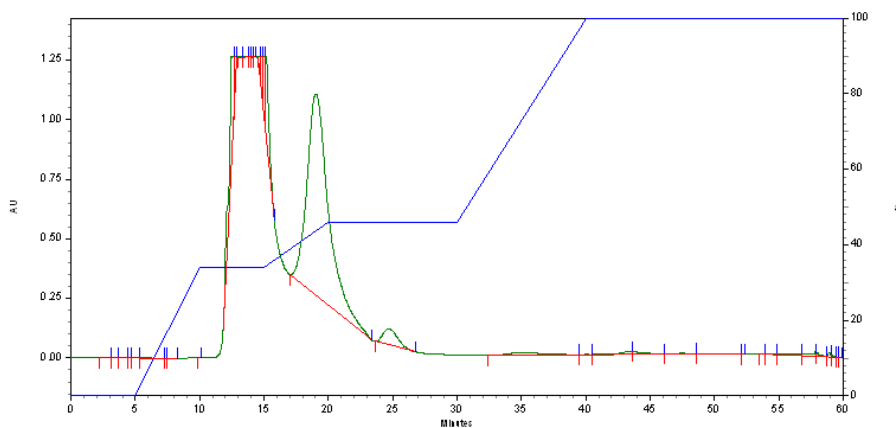
Figure 5.2: C18 HPLC purification of native tRNA^{phe}. Absorbance profile of C18 purification of native tRNA^{phe} the second peak contains tRNA^{phe} and was collected and purified as described.

Ribosome Purification

Tight coupled 70S ribosomes were isolated from E. coli MRE600 cells, essentially as described (Powers and Noller 1991).

RF1 Purification

His-tagged RF1 was purified essentially as described in the QIAexpressionist manual (Qiagen). Fractions containing RF1 were pooled and concentrated in an Amicon 10 kDa cutoff filter. Buffer exchange was performed in the Amicon filter to greater than 3,000 fold dilution of the unretained buffer. RF1 was then quantitated by the Bradford assay, flash frozen in liquid nitrogen, and stored at -80 °C.

EF-Tu Purification

EF-Tu was purified using the IMPACT-CN system (Bio-rad). 1 L of Luria Broth (LB) with ampicillin was inocculated with 1 mL of cells from saturated overnight cultures. Cultures were incubated at 37 °C shaking until OD_{600} was 0.5-0.8. Protein expression was induced with the addition of 0.5 mM IPTG. Cultures were then incubated overnight shaking at 15 °C. Cells were harvested by centrifuging at 5,000 g for 10 min at 4 °C. Cells were resuspended in 50 mL cold cell lysis buffer (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, 0.1% Triton-X 100) and lysed by sonication. The crude cell extract was then centrifuged at 20,000 g for 30 min at 4°C and the cleared lysate was loaded onto a 20 mL chitin column by gravity flow. The column was then washed with 200 mL (10 column volumes) of column buffer (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, 30 μM GDP). 60 mL (3 column volumes) of clevage buffer (20 mM Tris-HCl (pH 8.0), 500 mM NaCl, 1 mM EDTA, 30 μM GDP, 50 mM DTT) was then passed over the column to initiate the clevage of the protein from the column. The column was then incubated at room temperature for 16 hours. The cleaved protein was washed from the column using 45 mL of column buffer. The eluate was concentrated in a 10 kDa amicon filter and washed with column buffer to a dilution factor of greater than 200-fold to remove excess DTT. EF-Tu was then concentrated down to a volume less than 300 μL, and flash frozen to be stored at -80 °C.

The purity of EF-Tu was assessed by running SDS-PAGE gel and coomassie

staining. One band could be seen corresponding to EF-Tu and a second band corresponding to EF-Ts. The activity of EF-Tu was assessed using a native gel assay for tRNA binding (Bilgin, Ehrenberg et al. 1998).

Nucleotide Free EF-Tu Purification

Nucleotid-free EF-Tu was purified using the same method as above with a few modifications. After passing the cleared lysate over the chitin column, the beads were first washed with column buffer containing no EDTA and incubated in that buffer for 1 hour to remove EF-Ts. The column was then washed with column buffer containing no $MgCl_2$ and incubated in that buffer for 1 hour to remove GDP. Clevage buffer was then passed over the column and the purification proceeded exactly as above.

Acid Gel to verify tRNA aminoacylation

The aminoacylation of tRNAs was assessed by acid gel analysis. tRNAs were loaded onto a 6.5 % 8 M urea gel with 0.1 M $NaC_2H_2O_2$ (pH 5.2) and run at 4 °C 11 watts, for 13.5 hours. The gel was then stained with methylene blue.

Aminoacylation and HPLC Purification of tRNA^{phe}

For some experiments, aminoacylated tRNAs were HPLC purified prior to

being used. Prior to aminoacylation reactions, tRNAs were prefolded by heating to 60 °C for 3 min. 10 mM $MgCl_2$ was added then slow cooled to room temperature. $tRNA^{phe}$ was then aminoacylated by adding 30 µL purified phenylananyl tRNA synthase, to pre-folded tRNA in charging buffer (250 mM Tris-HCl pH 7.8, 8 mM $MgCl_2$, 1 mM phenylalanine, 3 mM ATP, 30 mM Kcl, 1 mM DTT) with a final volume of 300 µL. Aminoacylation of anticodon mutant tRNAs was carried out under 50 mM $MgCl_2$. Aminoacylated tRNAs were HPLC purified as described for the C18 purification of native $tRNA^{phe}$ but the peak for aminoacylated $tRNA^{phe}$ appears slightly later.

Ternary Complex Formation

Ternary complexes were formed by incubating 1 mM phenylalanine, 3 mM ATP, 1 mM GTP, 3 mM phosphoenol pyruvate, 0.25 mg/mL pyruvate kinase, 3 % phenylalanyl-tRNA synthetase, 15 µM EF-Tu, and 5 µM $tRNA^{phe}$ in the buffer to be used for each experiment and incubated at 37°C for 1 hour. Ternary complexes were then diluted down to the working concentration for each particular experiment. The concentration of ternary complex was taken to be the concentration of tRNA in the reaction.

Gel Shift of Ternary Complex Formation

The efficiency of ternary complex formation was meausured using a native gel. Ternary complexes were formed as described then mixed with 0.1 volume of 50 % glycerol with trace amounts of bromphenol blue. Samples were then loaded onto a 5 % acrylamide native gel and run at 80 mA, 200 V for 1 hour at 4 °C. The native gel buffer was 20 mM MES, 20 mM $MgC_2H_3O_2$, 130 mM $NH_4C_2H_3O_2$, with the final pH adjusted to 6.7 using NaOH. Gels were stained with either methylene blue or coomassie blue. This gel shift was adapted from a previously published procedure (Bilgin, Ehrenberg et al. 1998)

Initiation/Release complex Formation

Initiation/Release complexes were formed by heat activating 0.25 μM tight-coupled 70S ribosomes at 42 °C for 10 min. Ribosomes were then cooled to 37 °C for 10 min. 0.33 μM pyrene labeled mRNA was added and incubated for 10 min. at 37 °C. 0.5 μM tRNA^{fMet} was then added and incubated at 37 °C for 30 min.

tRNA Mutagenesis

tRNA mutagenesis was performed using the pAlter site directed mutagenesis kit from BioRad. Mutagenesis was carried out according to the manufacturer's

provided protocols. The following primers were used to produce the mutant tRNAs.

Mutation	Primer (5'-3')
A36T	ACCAAGGACACAAGGATTATCAATCCTTTGCTC
A36C	ACCAAGGACACAAGGATTGTCAATCCTTTGCTC
A36G	ACCAAGGACACAAGGATTCTCAATCCTTTGCTC
A35T	ACCAAGGACACAAGGATTTACAATCCTTTGCTC
A35C	ACCAAGGACACAAGGATTTGCAATCCTTTGCTC
A35G	ACCAAGGACACAAGGATTTCCAATCCTTTGCTC
G34T	ACCAAGGACACAAGGATTTTAAATCCTTTGCTC
G34A	ACCAAGGACACAAGGATTTTTAATCCTTTGCTC
G34C	ACCAAGGACACAAGGATTTTGAATCCTTTGCTC

Fluorimeter Experiments

Fluorescence emission scans were performed with a Fluoromax-P (J. Y. Horiba, Inc. USA) using an excitation and emission bandpass of 1 nm. Samples were excited at 343 nm and emission scans from 360 to 420 nm were taken before and after the addition of the indicated amounts and type of substrates.

K_D Titrations: RF1 Binding

Equilibrium K_D titrations were performed by mixing the indicated amounts of RF1 with 0.25 µM release complexes in a 1 mL fluorescence cuvette at 25 °C. The samples were excited with 343 nm wavelength light and the emission at 376 nm was

read 5 min after mixing. In parallel, the fluorescence emission of RF1 added to buffer was measured and subtracted from the data to account for light scattering at high protein concentrations. Data were fit to the equilibrium K_D equation below using Graphpad Prism as described.

$$Y=m*((K+R+X)-sqrt((K+R+X)^2-4*R*X))/(2*R)$$

m = maximum fluorescence signal

$K = K_D$

R = Release complex concentration

Stopped-flow Measurements

Stopped-flow measurements were performed at 25 °C on a μSFM-20, BioLogic stopped-flow instrument. The samples were excited at 343 nm (band pass 10 nm) and the fluorescence emission was measured at 376 nm after passing a longpass filter 361 AELP (Omega Optical, VT, USA) installed in front of the detector. 0.25 μM (final concentration) initiation or release complexes were mixed with varying amounts of RF1 or ternary complex. Time courses of release factor binding were fit to the first-order rate equation ($Y = b + C*exp(-k*x)$). Time courses of ternary complex binding were fit to a sum of two exponentials equation ($Y = b + C1*exp(-k1*x) + C2*exp(-k2*x)$).

Peptide Release Assay

Release complexes for peptide release assays were formed by heat activating 1.0 μM 70S at 42 °C for 10 min. Samples were then cooled to 37 °C for 10 min. 1.3 μM mRNA was added and incubated at 37 °C for 10 min. In parallel, tRNA^{fMet} was aminoacylated by mixing 2 μM tRNA^{fMet}, 3 μM [^{35}S] methionine, 0.4 mM N10-formyltetrahydrofolic acid, 3 mM ATP, 10 % MetRS, and 10 % MTF. MetRS and MTF were purified as previously described (Shimizu et al., 2001). The aminoacylation reaction was then mixed with the 70S/mRNA complex and the incubation was continued for 30 min at 37 °C. Excess [^{35}S] Met was washed away by filtration in a Microcon YM-100 centrifugal filter to a dilution of greater than 1,000 fold and the volume was adjusted to obtain pre-termination complexes with a final concentration of 0.5 μM.

Peptide release time courses were performed by mixing 0.25 μM (final concentration) release complex with varying amounts of RF1. Reactions were quenched with 25% formic acid, spotted on a TLC, separated and analyzed as described previously (Feinberg and Joseph 2006).

Chapter 6: Conclusions and Future Directions

Codon dependent binding of substrates to the ribosome is an essential step in the translation of genetic information into proteins (Zaher and Green 2009). tRNAs must specifically interact with the codon displayed in the A-site of the ribosome and contribute the attached amino acid to the growing polypeptide chain in order to synthesize a protein of the correct amino acid sequence (Daviter, Gromadski et al. 2006). Release factors must specifically recognize stop codons in the A-site of the ribosome in order to end the synthesis of a protein and release the fully synthesized protein from the ribosome (Youngman, McDonald et al. 2008). After over 50 years of study, many question regarding the recognition of codons in the A-site of the ribosome remain unanswered.

In order to study how the ribosome accurately selects substrates based on the identity of the codon in the A-site, we have developed a fluorescence based method that is sensitive to the binding of tRNAs or release factors to the A-site of the ribosome. Ribosomes programmed a with short model mRNA labeled at the 3' end with the fluorescent dye pyrene exhibited a greater fluorescence emission intensity of the probe when a tRNA or release factor is bound to the A-site (Figure 2.1, 3.2). The approximate location of the pyrene probe is between the head and shoulder of the 30S subunit, three nucleotides away from the codon in the A-site (Figure 2.1). Based on crystal structures, direct interaction between the tRNA or RF and the pyrene probe is unlikely. The cause of the fluorescence increase may be due to reduction in collisional

quenching due to solvent exclusion from the A-site when tRNAs or RFs bind. Whether or not reduced collisional quenching is the cause of the increase in fluorescence intensity has not been investigated directly however.

Stopped-flow timecourses monitoring the fluorescence of the pyrene probe showed a biphasic fluorescence change when ternary complex binds to the ribosome (Figure 2.3A, B). Analysis of the concentration dependence of the observed rates of each phase of the fluorescence change showed that the first phase of the fluorescence change exhibits a linear concentration dependence, consistent with the bimolecular association of ternary complex to the ribosome (Figure 2.3C) (Johnson 1992). The second phase of the fluorescence change shows a hyperbolic concentration dependence, consistent with a conformational change after binding (Figure 2.3D) (Johnson 1992). A two step binding mechanism such as this is often invoked to describe biphasic kinetics however, this is not the only mechanism of binding that may result in biphasic time courses (Fierke, Johnson et al. 1987; Hsieh and Fierke 2009). Isomerization of the ribosome or ternary complex between conformations either capable or incapable of interacting or resulting in different binding pathways could also result in biphasic kinetics if the isomerization step is slow relative to the binding step (Fierke, Johnson et al. 1987; Heidary, Gross et al. 1997; Heidary, O'Neill et al. 2000). Analysis of the concentration dependence of each the amplitude of each phase of the fluorescence change rules out isomerization of ternary complex as the cause of the biphasic fluorescence change (Figure 2.4) (Fierke, Johnson et al. 1987; Hsieh and Fierke 2009). The partition of the total fluorescence change between the first and

second phase of the fluorescence change did not change with increasing concentrations of ternary complex. If an isomerization of the ternary complex was responsible for the biphasic kinetics, the slow phase of the fluorescence change would be expected to be reduced as the total concentration of ternary complex is increased (Fierke, Johnson et al. 1987). Isomerization of the ribosome cannot be entirely ruled out as a cause of the biphasic fluorescence change however, it is unlikely due to the fact that the fluorescence change observed when RF1 binds to the ribosome can be described well by a single exponential equation (Figure 3.3).

A two step binding mechanism is supported by ruling out alternative mechanisms expected to result in a biphasic fluorescence change. Further experiments revealed the substeps of ternary complex binding to the ribosome responsible for each phase of the fluorescence change. Crystal structures of the 30S subunit with an ASL bound in the presence or absence of the miscoding antibiotic paromomycin revealed a large conformational change of the 30S subunit known as domain closure (Ogle, Murphy et al. 2002). Binding of an ASL to fluorescently labeled initiation complexes showed an increase in fluorescence that was not as great as when a full length tRNA bound (Figure 2.1D). Binding of an ASL in the presence of paromomycin however resulted in the same fluorescence change observed when a full length tRNA bound to the ribosome, indicating that the fluorophore is sensitive to the codon recognition and domain closure steps of ternary complex binding to the ribosome (Figure 2.4C). The fraction of the total fluorescence change observed when an ASL alone bound to the ribosome agrees well with the first phase of the fluorescence change observed in

stopped-flow time courses, indicating that the first phase of the fluorescence change is due to codon recognition (Figure 2.4D). The increase in fluorescence observed only when ASL bound in the presence of paromomycin correlated well with the second phase of the fluorescence change observed in stopped-flow time courses, indicating that the cause of the second phase of the fluorescence change is domain closure of the 30S subunit.

High magnesium concentrations are commonly used in in vitro translation experiments in order to increase the activity of ribosomes (Salas, Miller et al. 1967). High magnesium concentrations have been shown to reduce the fidelity of protein synthesis (Thompson, Dix et al. 1981). The polyamines spermine and spermidine have been shown to improve the efficiency of and reduce the magnesium requirement of translation in vitro (Igarashi, Hashimoto et al. 1982). Using our fluorescence based method to monitor ternary complex binding to the ribosome, we have shown that the polyamines spermine and spermidine stimulate the binding of ternary complex to the ribosome at physiological levels at least as well as even unphysiologically high concentrations of magnesium (Figure 2.3). Spermine has been shown to bind to the anticodon stem of tRNA^{phe} in the hinge region that must adopt a kinked conformation upon binding of ternary complex to the ribosome, prior to GTP hydrolysis by EF-Tu (Quigley, Teeter et al. 1978; Amarantos and Kalpaxis 2000; Valle, Zavialov et al. 2003; Schmeing, Voorhees et al. 2009). Spermine and spermidine may help the tRNA to adopt the conformation of the kinked intermediate. The increase in the efficiency of cognate ternary complex binding to the ribosome does not appear to severely

compromise the fidelity of ribosomes as, incorporation of near-cognate ternary complex was not detectable in single turnover experiments (Figure 2.5).

The finding that the codon recognition step of ternary complex binding to the ribosome obeys second order kinetics indicates that the first interaction between the ribosome and ternary complex is a codon specific step. Previously, it was proposed that prior to codon recognition, a nonspecific initial binding step precedes codon recognition (Rodnina, Pape et al. 1996). The initial binding complex was proposed to be equivalent for cognate, near-cognate, or non-cognate ternary complexes. The proposal of a nonspecific initial binding complex has been criticized because incorrect ternary complexes could act as competitive inhibitors of translation, slowing the rate of translation to much lower than is observed in vivo (Johansson, Lovmar et al. 2008). Our data indicates that there is not a nonspecific initial binding complex and that codon recognition occurs as a second order process. The two lines of research may be reconciled with the following model of ternary complex association to the ribosome. The L7/L12 stalk consists of multiple copies of the L7/L12 proteins that have been shown to interact with elongation factors and are important for protein synthesis (Kothe, Wieden et al. 2004). It has been proposed that multiple copies of these proteins may act to increase the local concentration of ternary complex (Diaconu, Kothe et al. 2005). Interaction of multiple ternary complexes with the L7/L12 stalk may be the interaction that occurs during the initial binding step. Transfer of the ternary complex to the ribosome A-site is the codon recognition step (Figure 6.1). If transfer from L7/L12 is rapid and occurs among multiple ternary complexes, it could

exhibit apparent second order kinetics. Once a ternary complex is transferred to the A-site of the ribosome, a new ternary complex would be expected to occupy the now vacant site in the L7/L12 stalk. If all ternary complex binding sites in the L7/L12 stalk are occupied, dissociation of incorrect ternary complex from the ribosome would be expected to occur directly from the A-site rather than the ternary complex passing back into the L7/L12 initial binding complex. This model resolves a second criticism with the previous model of ternary complex binding to the ribosome. Specifically, based on the current model of tRNA selection, incorrect ternary complexes would be passed back and forth between the codon recognition and initial binding conformations multiple times before dissociating, resulting in a significant inhibition of protein synthesis (Ninio 2006).
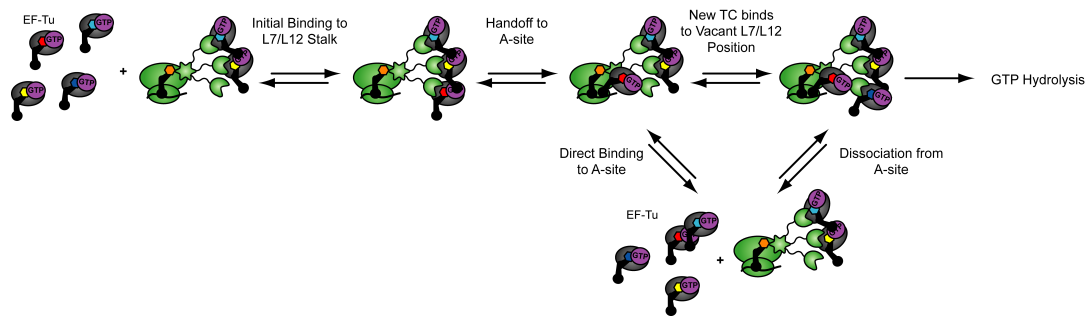
Figure 6.1: A model for initial binding of ternary complex to the ribosome. Initial binding may occur through interaction of ternary complex with L7/L12 stalk proteins. Hand off from the L7/L12 stalk to the ribosome A-site constitutes the codon recognition step. The free position on the L7/L12 stalk may now bind a new ternary complex, preventing the passage of a incorrect ternary complex back to the initial binding state prior to dissociation. Alternatively, direct binding to the ribosome A-site could occur. After codon recognition, ternary complex proceeds to catalyze GTP hydrolysis.

Using our fluorescence based method, we have also assessed effect of the position and type of mismatch between the tRNA and mRNA on the relative binding of ternary complex to the ribosome. We have made each possible single nucleotide substitution in the anticodon of tRNA[phe] and measured the change in fluorescence of the pyrene probe observed when mixing ternary complex with tRNA[phe] anticodon mutants upon binding to a UUU codon normally coding for phenylalanine (Figure 4.3, 4.4, 4.5). The relative increase in the fluorescence intensity of the probe when each anticodon mutant tRNA bound to the ribosome was assumed to be indicative of the extent of the ternary complex binding. Consistent with previous studies, it was found that mismatches between the codon-anticodon base pairing interaction in the middle position had the most deleterious effect (Ito and Igarashi 1986). Mismatches in the first position did not exhibit as severe of a binding defect but, were more deleterious than mismatches in the third position (Ito and Igarashi 1986). GU wobble base pairing interactions were found to be the most well tolerated followed by UU mismatches with CU mismatches being the most deleterious to binding (Ito and Igarashi 1986).

Future work on tRNA selection should focus on fully characterizing the kinetics of near-cognate ternary complex binding to the ribosome using the library of tRNA[phe] anticodon mutants. Initial experiments indicate that some mismatches may be well tolerated by the ribosome and it is not understood how the ribosome may discriminate against very conservative codon-anticodon mismatches. We have identified for the first time in solution experiments the domain closure step however, the role of domain closure in the selection of cognate tRNAs is not known. Interations

in the decoding center between the rRNA and codon-anticodon base pairs have been proposed to be important in precipitating the conformational change of domain closure (Ogle, Murphy et al. 2002). The effect of these interactions on domain closure of the 30S subunit should be studied to understand the role of domain closure on tRNA selection by the ribosome. Finally, we have shown that the polyamines spermine and spermidine greatly improve the binding of ternary complex to the ribosome. Similarly, magnesium has been shown to improve the binding of tRNAs to the ribosome. While magnesium decreased the fidelity of protein synthesis, it is not well known what the effect of polyamines is on the fidelity of protein synthesis. Future work investigating the effect of polyamines on the binding of near-cognate ternary complex to ribosomes will reveal if polyamines reduce the specificity of protein synthesis.

The fluorescent dye pyrene attached to the 3' end of a model mRNA was also found to be sensitive to the binding of class I RFs to the ribosome. A significant problem in the understanding of stop codon recognition by class I RFs was the fact that no intermediates in the process of peptide release had been identified (Youngman, McDonald et al. 2008). How release factors recognize stop codons was limited to measuring effects on the catalysis of peptide release (Freistroffer, Kwiatkowski et al. 2000). Using our fluorescence based method, we have determined the binding kinetics and thermodynamics of RF1 binding to its cognate stop codon, UAA and several sense codons (Hetrick, Lee et al. 2009). The association of RF1 to the A-site was found to be the same regardless of the identity of the codon in the A-site,

indicating that the association step is not a significant source of discrimination (Figure 3.3B). The dissociation rate of RF1 from the ribosome was found to be greatly accelerated when binding to sense codons (Table 3.1). Measuring the equilibrium dissociation constant of RF1 for stop and sense codons showed that RF1 binding did not discriminate stop and sense codons in a predictable way (Figure 3.2C). For example, RF1 bound more stably to a CUC codon than to a CAA codon even though CAA varies by only one nucleotide from a stop codon and CUC varies by all three nucleotides.

Measurement of the catalysis of peptide release on stop and sense codons showed that the catalysis of peptide release did not follow the same trends as binding (Figure 3.3C). RF1 catalyzed peptide release on CAA, which varies by only one nucleotide from its cognate stop codon, most efficiently among the sense codons tested. The discrepancy between binding and catalysis lead to the suggestion that RFs use an induced fit mechanism and that conformational changes in the decoding center, induced by RF binding, allow peptide release to occur more efficiently. Crystal structures of class I RFs show that they may adopt a closed conformation when free in solution (Vestergaard, Van et al. 2001; Ma and Nussinov 2004). Solution X-ray scattering experiments show that RF1 and 2 are capable of sampling both the closed conformation observed in structures of free RF1 or 2 and the open conformation observed when RF1 or 2 binds to the ribosome (Vestergaard, Sanyal et al. 2005; Zoldak, Redecke et al. 2007) . Future work should focus on what conformation class I RFs adopt when binding to the ribosome and the role of conformational changes in the

catalysis of peptide release.

A fundamental problem in understanding codon selection by tRNAs or release factors is the comparison of dissociation rates and forward reaction rates (Johnson 2008; Tummino and Copeland 2008). Typically, it is assumed that the observed saturation rate for forward reactions is the first order rate constant for the reaction being measured. The assumption that the saturation of forward rates is the first order rate constant for the reaction being measured is fundamental to the interpretation that selection of tRNAs by the ribosome follows an induced fit mechanism where correct ternary complexes and RFs catalyze forward reaction steps faster than incorrect ternary complexes or RFs. When the dissociation rate of ternary complex or RFs from the ribosome are faster than the forward reaction rates, the substrate would be expected to bind and dissociate multiple times before successfully catalyzing the forward reactions of GTP hydrolysis or peptide release (Ninio 2006). If only a small fraction of substrates remain bound long enough to successfully catalyze a reaction, then the rate of downstream reactions would be expected to be slowed because substrate must bind and dissociate many times before successful catalysis may occur (Johnson 2008). The comparison of forward chemistry steps and dissociation rates is essential in understanding how correct substrates are selected by the ribosome and in many biochemical reactions (Tummino and Copeland 2008). Pre-steady state kinetics and fluorescence based methods monitoring reaction kinetics and conformational changes is essential in understanding the chemistry of enzyme catalyzed reactions.

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