Ribosomal translocation in real time

Method development to Applications

CHANG-IL KIM
Translational elongation is the process in which the ribosome adds one amino acid at a time to the nascent peptide chain. As the ribosome elongates the peptide chain by 14 - 20 amino acids per second and performs hundreds of such cycles per protein, ‘elongation’ is one of the most crucial steps in translation. During elongation, the ribosome must move precisely by one codon along the mRNA after peptidyl transfer. This step is known as ‘ribosomal translocation’, which is catalyzed by the elongation factor G (EF-G). Despite extensive research, the exact sequence of events in translocation is still unclear. Thus, development of an in vitro assay, which would allow precise kinetic measurement of the steps involved in ribosomal translocation, is highly important. In 2003, a research group led by Simpson Joseph designed a fluorescence-based assay to monitor translocation in stopped-flow using a short mRNA labeled with fluorescent-dye pyrene at the 3’ end. Although optimized for the highest fluorescence change, this assay showed significantly slower translocation than what has been measured by conventional translocation assays. Moreover, when performed with a popular peptidyl tRNA analog, it produced complex multiphasic kinetics. Therefore, this assay was not only limited for deriving physiologically relevant rates, but also the analysis of the rates from the kinetic data was challenging. In this thesis, we have optimized the fluorescent-mRNA based stopped-flow translocation assay, by carefully calibrating it with the functional tripeptide formation assay performed in quench-flow. First, we identified the most suitable mRNA length that is optimal for both the fluorescence signal and the rate of translocation. Further, by systematically varying temperature, Mg\[^{2+}\], EF-G and tRNA analog concentration, we have derived the ideal condition for obtaining near monophasic kinetics in stopped-flow, which allows determination of the translocation rate in an unambiguous manner. This optimized assay has further been tested in different contexts involving translocation, which include (i) characterization of the GTPase deficient EF-G mutants, (ii) studying the effect of non-hydrolyzable GTP analogs, (iii) evaluating the effect of extension of the C-terminal tail of the ribosomal protein S13 in the decoding center, and (iv) understanding the mechanism of action of a novel aminoglycoside antibiotic ‘arbekacin’ in translation. These studies altogether provide deep understanding for how different factors can modulate ribosomal translocation.

Keywords: ribosome, EF-G, pyrene mRNA, aminoglycoside, ribosomal protein, GTP hydrolysis

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List of Papers

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Introduction

Bacterial Ribosome

All the living cells, by the definition of “life”, metabolize, grow, regulate and reproduce. All these “life” activities are progressed and controlled by the genetic information encoded in the sequence of deoxyribonucleic acid (DNA). The information in DNA should be transcribed into messenger RNA (mRNA), followed by translation into functional proteins, which catalyze the metabolism inside the cells, regulate the cell signaling and ligand binding or provide the cellular structure for the cells to “live”. The whole process of transferring information of DNA into proteins is known as the ‘central dogma’ of molecular biology (Crick, 1970).

Ribosomes are large macromolecular machines that translate the genetic information encoded in mRNAs into the functional proteins. In both prokaryotes and eukaryotes, ribosomes are composed of two unequal subunits, which further comprise ribosomal RNAs (rRNA) and ribosomal proteins (r-proteins). Based on the sedimentation coefficient (S), the bacterial ribosome is called the 70S ribosome, while the two subunits are called 50S (large), 30S (small) subunit respectively (Figure 1). The 50S subunit is composed of 23S rRNA (2900 nucleotides), 5S rRNA (120 nucleotides) and 31 proteins; the 30S subunit is composed of 16S rRNA (1540 nucleotides) and 21 proteins. Both subunits of the ribosome contain three tRNA (transfer RNA) binding sites, called the A, P, and E sites. The A site and the P site are the binding sites of the aminoacyl tRNA and peptidyl tRNA, respectively. The E site stands for ‘exit site’, from which the deacylated tRNAs exit the ribosome (Figure 1).

The ribosome contains two important functional sites; decoding center (DC) in the 30S subunit and peptidyl transfer center (PTC) in the 50S subunit (Figure 1). While DC maintains the fidelity of translation by accurate selection of the cognate tRNAs from the large pool of competing non-cognate and near-cognate tRNAs (Schmeing, Voorhees et al., 2009, Wilson & Nierhaus, 2003), PTC catalyzes efficient peptide bond formation, during which the polypeptide
is transferred from the P-site tRNA to the amino acid on the A-site tRNA. In *Escherichia coli* (*E. coli*), the PTC is comprised of three conserved bases U2585, A2451 and C2063 of 23S rRNA (*E. coli* numbering) while the DC is comprised of A1492, A1493 and G530 of 16S rRNA (Schmeing et al., 2009, Wilson & Nierhaus, 2003).

**Figure 1.** Structure of the bacterial ribosome (70S) and its large (50S) and small (30S) subunit depicting three tRNA binding sites (A, P, and E site) and the functional centers DC (decoding center) and PTC (peptidyl transferase center). The 23S rRNA is in silver, the r-proteins of the large subunit in purple, the 16S rRNA in blue-gray and the small subunit proteins are in green. The three tRNAs bound to the A, P and E sites are in red, the mRNA is magenta. The illustrations are based on cryo-EM structure of *E. coli* ribosome, adopted from PDB:5IQR.

**Ribosomal translation**
Ribosomal translation involves four major steps; (i) initiation – during which the initiator tRNA binds to the P-site of the mRNA programmed 30S subunit and the ribosomal subunits assemble, (ii) elongation – during which an aminoacyl tRNA binds to the mRNA at the A site, then peptide bond forms resulting in transfer of the peptide chain from the P-site tRNA to the A-site tRNA, and finally the peptidyl tRNA translocates to the P site along with the
mRNA thereby allowing a new round of elongation until a stop codon reaches the A site, (iii) termination – during which the nascent peptide chain is hydrolyzed from the peptidyl tRNA and released from the ribosome and (iv) recycling – during which the ribosomal subunits dissociate and prepare for the next round of initiation. In all four steps, several protein factors, including GTP hydrolyzing factors or translational GTPases, cooperate with the ribosome to facilitate accurate and efficient translation (Figure 1). The major translation factors are initiation factors 1, 2 and 3 (IF1, IF2 and IF3), elongation factors -Tu, -Ts, -G and -P (EF-Tu, EF-Ts, EF-G and EF-P), release factors 1, 2 and 3 (RF1, RF2 and RF3) and ribosome recycling factor (RRF). Efficient and accurate translation depends on action of these translational factors in definite sequence, which is detailed below.

Initiation
In bacteria, initiation of translation starts by binding of the mRNA to the 30S subunit. It is often facilitated by a specific sequence in the mRNA upstream the start codon (AUG), called the Shine-Dalgarno sequence (SD) (Shine & Dalgarno, 1974, Yusupov, Yusupova et al., 2001). The SD sequence interacts with a complimentary sequence at the 3’-end of 16S rRNA, which ensures the positioning of the mRNA in such a way that the start codon (usually AUG or GUG) is correctly placed in the P site. Correct positioning of the mRNA is important for efficient and accurate initiation; it allows binding of the initiator tRNA to the P site. The efficiency and accuracy of initiation often depend on concerted action of the three initiation factors. IF3 binds to the E site of the free 30S subunit in order to prevent premature and nonfunctional association of 50S with it. Similarly, IF1 occupies the A site of 30S subunit and prevents binding of the incoming aminoacyl-tRNA (aa-tRNA) as well as the initiator tRNA to the A site. IF2 is a GTPase factor. It binds to the 30S subunit as IF2•GTP and ensures stable binding of the initiator tRNA in the P site.

A special initiator tRNA is involved in prokaryotic translational initiation. This tRNA is charged with N-formylated methionine and is called as fMet-tRNA_{Met}. IF2 recognizes the fMet and enables specific binding of fMet-tRNA_{Met} to the P site (Antoun, Pavlov et al., 2006). Other features like 3G:C base pair motif in the anticodon stem loop of tRNA_{Met} confer discrimination against elongator tRNA_{Met} (Varshney, Lee et al., 1993). Positioning of fMet-tRNA_{Met} with the help of IF2 on the P site (Roy, Liu et al., 2018) and the
correct formation of base pairing between the anticodon and the start codon completes the formation of 30S pre-initiation complex. The last step of initiation is to form 70S initiation complex (70S IC). This is achieved by association of the 50S subunit with the 30S pre-initiation complex. Here, IF2 constructs a docking site for the 50S by interacting with the L7/L12 stalk. GTP hydrolysis by IF2 is not needed for subunit association. However, GTP hydrolysis reduces affinity of IF2 to the ribosome and fMet-tRNA^{Met}, and IF2•GDP dissociates. (Andreeva, Belardinelli et al., 2018, Mandava, Peisker et al., 2012). Although the exact sequence of the events is not fully established, the other initiation factors IF1 and IF3 also leave the complex. The finally formed 70S initiation complex containing fMet-tRNA^{Met} in the P site and empty A site enters into the elongation step of protein synthesis. The steps are summarized in Figure 2.

**Elongation**

The ‘elongation’ step takes the longest time of all in overall synthesis of a protein, as this step repeats for every amino acid in the peptide chain until the ribosome reaches a stop codon (Figure 2). Elongation is divided into two sub steps; (i) decoding and peptidyl transfer, and (ii) coupled mRNA-tRNA translocation. Two major translational GTPase factors, EF-Tu and EF-G, mediate these two steps. The GTPase factors alternate between the GTP bound active state and GDP bound inactive state. While EF-Tu is a guanosine nucleotide exchange factor (GEF) for EF-Tu, no protein factor has been identified as GEF for EF-G. It has been proposed that 70S ribosome might play a role as GEF for EF-G (Wieden, Gromadski et al., 2002, Zavialov, Hauryliuk et al., 2005a).

EF-Tu•GTP brings aa-tRNAs to the empty A site of the ribosome, where cognate tRNAs are accepted and near- and non-cognate tRNAs are rejected by the ribosome in the process called “initial selection” (Fislage, Zhang et al., 2018, Ieong, Uzun et al., 2016). During the delivery of aa-tRNA, tRNA anticodon stem loop interacts with the mRNA codon in the A site by complementary base pairing, while the other end of tRNA remains bound to EF-Tu to prevent peptide bond formation before correct decoding of the mRNA codon (Valle, Zavialov et al., 2003a). The DC of the ribosome has three universally conserved bases of 16S rRNA, A1492, A1493 and G530 (E. coli numbering), known as the ‘monitoring bases’. These bases flip-out upon binding of the aa-
tRNA and form hydrogen bonds with the minor groove formed by correct codon-anticodon base pairing (Ogle, Brodersen et al., 2001). The adjacent adenines 1492 and 1493 interact with the first and second positions of the codon-anticodon pair, while G530 interacts with the second and third positions (Fislage et al., 2018, Loveland, Demo et al., 2017). Recent cryogenic electron-microscopy (cryo-EM) based structural studies demonstrate that the hydrogen bond network involving these three monitoring bases is the key to the correct aa-tRNA selection. When the cognate tRNA forms stable complementary base pairing with the A-site codon on mRNA, GTP hydrolysis is triggered in EF-Tu, which is followed by its release from the ribosome (Ogle, Carter et al., 2003, Rodnina, Gromadski et al., 2005). After EF-Tu•GDP release, the aa-tRNA moves and rotates about 70 Å so that its 3’-end can reach the PTC. This process is called “tRNA accommodation”. In some occasions near- or non-cognate tRNA can bypass initial selection. However, there is a high chance that these tRNAs fail in the accommodation step and are rejected. This additional mode of accuracy, which allows rejection of the incorrect tRNAs even after GTP hydrolysis by EF-Tu is called “proof reading”.

After the accommodation of the aa-tRNA to the A site peptide bond is formed between the carboxyl group (-COOH) of the polypeptide at the P site and the amino group (-NH₂) of the amino acid in the A site. The peptidyl transfer is a function of the 23S rRNA in the PTC, which makes the ribosome a ribozyme. After peptidyl transfer, the ribosome holds peptidyl tRNA in the A site and a deacylated tRNA in the P site. At this point, the ribosome is ready for translocation and the ribosomal complex is called a pre-translocation (pre-T) complex.

For the next step of peptide elongation, pre-T complex should translocate by one codon so that the tRNAs at the P site and A site move to the E and P site respectively. The pre-T complex spontaneously fluctuate between so called “hybrid” and “classical” state, primarily suggested by chemical foot printing assay (Jamiolkowski, Chen et al., 2017, Kim, Puglisi et al., 2007, Moazed & Noller, 1989) and then confirmed by single molecule studies (Fei, Bronson et al., 2009, Fei, Kosuri et al., 2008, Flis, Holm et al., 2018, Ning, Fei et al., 2014, Wasserman, Alejo et al., 2016). In the “classical” state, the tRNAs remain in the A/A and P/P state with respect to both the 30S and the 50S subunits. However, in the “hybrid” state, 3’-end of the tRNAs bound to the A site and P site of the 30S, move to the P site and E site in the 50S, respectively.
This is accompanied by the counter clockwise rotation of the ribosomal subunits with respect to each other by about 10°, which is known as “ribosome ratcheting” (Agirrezabala, Liao et al., 2012, Frank & Agrawal, 2000, Frank, Gao et al., 2007).

![Diagram of bacterial translation](image)

Figure 2. Overview of bacterial translation in four steps. Several translation factors cooperate along the four major steps, namely initiation, elongation, termination and recycling.

Current structural and functional studies suggest that “ribosome ratcheting” is the first step of ribosomal translocation, which ensures stable binding of EF-G•GTP on the ribosome. Translocation cannot happen if the ribosome ratcheting was blocked (Horan & Noller, 2007). However, completion of translocation requires back ratcheting of the ribosomal subunits after GTP hydrolysis on EF-G (Chen, Tsai et al., 2012, Ermolenko & Noller, 2011, Frank et al., 2007, Ling & Ermolenko, 2016). Apart from overall subunit ratcheting, the
ribosome goes through extensive conformational changes during translocation. One of the big rearrangements is the swiveling of the 30S head domain by about 20° with respect to the rest of the 30S, which swivels back synchronously or after the mRNA-tRNA translocation (Guo & Noller, 2012, Ratje, Loerke et al., 2010, Wasserman et al., 2016). L1 stalk of the 50S subunits also moves in and out of the E site while interacting with the deacylated tRNA (Fei et al., 2008). Thus, ribosomal translocation requires orchestration of several complex events involving conformational dynamics of the ribosome, tRNAs and EF-G.

Efficient translocation requires EF-G, even though the ribosome can translocate at rather low rate in factor independent way (Cukras & Green, 2005, Gavrilova & Spirin, 1971, Pestka, 1968). EF-G is a GTPase protein and binds to the pre-T ribosome in GTP form. EF-G prefers binding to the ribosome at the hybrid state (Blanchard, Kim et al., 2004). Alternatively, it pushes the equilibrium of the classical/hybrid state to the hybrid state upon binding to the ribosome (Ermolenko, Majumdar et al., 2007, Fei et al., 2008, Kim et al., 2007, Munro, Altman et al., 2010a, Sharma, Adio et al., 2016, Spiegel, Ermolenko et al., 2007, Valle, Zavialov et al., 2003b). Prompt GTP hydrolysis by EF-G stabilizes the ratcheted conformation and enables EF-G•GDP to move close to A site in the 30S (Spiegel et al., 2007). As a result the A-site tRNA move to the P site together with the mRNA and the P-site tRNA also moves to the E site. The mRNA-tRNA movement is the heart of translocation and depends on GTP hydrolysis by EF-G. However, this is independent of release of inorganic pyrophosphate after GTP hydrolysis (Zavialov, Mora et al., 2002). As the mRNA-tRNAs translocate, ribosome return to the classical state by back-ratcheting. This movement is loosely coupled with back swiveling of the 30S head, moving in of the L1-stalk and release of the E-site tRNA (Cornish, Ermolenko et al., 2009, Ermolenko & Noller, 2011, Guo & Noller, 2012, Wasserman et al., 2016). In the back ratcheted state of the ribosome, EF-G•GDP loses its affinity to the ribosome and dissociates leaving empty A site for the next elongation of the peptide chain.

Termination and Recycling

Peptide elongation by ribosome continues until it encounters one of the three stop codons UAA, UAG and UGA in the A site. There are no tRNAs available,
which read the stop codons, instead release factors recognize them and cata-
yze the hydrolysis of the polypeptide from the P-site tRNA. In bacteria, there
are two classes of release factors. The class-I release factors include RF1 and
RF2, which recognize the stop codons in a semispecific manner. While UAA
codon is read by both RF1 and RF2 (Scolnick, Tompkins et al., 1968), UAG
is read by only RF1 and UGA is read by RF2. The specificity of stop codon
recognition is guaranteed by the different recognition motifs in RF1 and RF2;
P(A/V)T motif in RF1 and SPF motif in RF2. Earlier report shows that the
release factors lose specificity when these motives are exchanged (Ito, Uno et
al., 2000). The release factors use a conserved GGQ motif for peptide release
from the peptidyl tRNA (Korostelev, Asahara et al., 2008, Zhou, Korostelev
et al., 2012). The Q of the GGQ motif is methylated, which facilitates fast
peptide release. The class-I release factors bind to the ribosome in the closed
conformation when the SPF/P(A/V)T motif and GGQ motif are separated by
only 25 Å (Fu, Indrisiunaite et al., 2019, Graf, Huter et al., 2018, Korostelev
et al., 2008, Svidritskiy & Korostelev, 2018, Vestergaard, Sanyal et al., 2005).
Upon correct recognition of the stop codon, the factors change conformation
to “open” state, when the helix carrying GGQ motif extends till PTC and the
distance between the two above mentioned motives becomes ~75 Å (Jin,
Kelley et al., 2010, Rawat, Gao et al., 2006). The release factors release the
nascent polypeptide chain from the peptidyl tRNA by ester bond hydrolysis.
Once the peptide is released, class-I release factors need to leave the ribosome.
Then the class-II release factor RF3 come into action. RF3 is another GTPase
protein, which has comparatively high affinity to GDP than GTP by three or-
ders of magnitude (Zavialov, Buckingham et al., 2001). RF3•GDP binds to
the post-termination ribosome with RF1/RF2 bound to it. Ribosome binding
allows exchange of GDP to GTP on RF3. As soon as RF3 binds to GTP, it
facilitates ratcheting of the ribosomal subunits (Gao, Zhou et al., 2007,
Pallesen, Hashem et al., 2013). As a result, RF1 and RF2 lose their contact
with the ribosome and dissociates readily (Gao et al., 2007, Shi & Joseph,
2016, Zavialov et al., 2001). Finally, GTP hydrolysis by RF3 leads to change
in its conformation. RF3•GDP loses its affinity for the ribosome and dissoci-
ates (Zavialov et al., 2001) leaving the ribosome with only a deacylated tRNA
in the P site.

Ribosome recycling is necessary to ensure continuity of protein synthesis in
the cell. For that a special factor called the ribosome recycling factor (RRF)
acts in coordination with EF-G (Borg, Pavlov et al., 2016, Hirashima & Kaji,
1973, Zavialov, Hauryliuk et al., 2005b). RRF resembles an inverted tRNA in its 3D structure. RRF binds to the A site of the ribosome carrying a deacylated tRNA in the P site. Then EF-G•GTP binds to it and the ribosome ratchets causing conformational change on the ribosome similar to that in elongation. Next EF-G hydrolyzes GTP and the ribosome back ratchets. As a result of these conformational dynamics, the ribosomal subunits split and IF3 binds to the 30S subunit to ensure subunit separation. The ribosomal subunits are then ready for next round of translation initiation.

Ribosome as a target of Antibiotics

Antibiotics are the natural or semi-synthetic compounds which kill or inhibit the growth of bacteria. Since the discovery of penicillin, antibiotics have become the integral part of human lives for their widespread use in modern medicine. Most of the antibiotics inhibit the growth of susceptible bacteria by affecting their cell wall, nucleic acids and protein synthesis. Early penicillins, cephalosporins, monobactams and glycopeptide antibiotics essentially target the bacterial cell wall. Inhibitors for nucleic acids synthesis include sulfonamides and trimethoprim (on folate synthesis), quinolones (on DNA gyrase) and rifampin (on RNA polymerase). Likewise, tetracyclines, aminoglycosides, macrolides and chloramphenicol inhibit the protein synthesis.

Ribosome is as an attractive antibiotic target because it catalyzes the process of protein synthesis in bacteria. Many clinically useful antibiotics interfere with bacterial protein synthesis by binding at highly conserved functional centers of the ribosome. This binding either stabilizes the ribosomal conformation or inhibit the binding of translational factors leading to defective translation. Several modes of action of antibiotics on ribosome has been inferred from structural and functional analysis. Since elongation step is most repetitive and ribosome stays long time in this phase, most of the translation inhibitors target this step (Wilson, 2014).

Aminoglycosides (e.g. Streptomycin) and tuberactinomycin (e.g. viomycin) drugs bind with high affinity to the A site of the 30S subunit, particularly to the conserved helix 44 (h44) of 16S rRNA. The binding of the aminoglycosides causes the monitoring bases A1493 and A1492 to flip out to the “locked” position, which in turn leads to erroneous selection of the tRNAs (miscoding).
Some aminoglycosides can also block ribosomal translocation or directly inhibit initiation (Davis, 1987, Kotra, Haddad et al., 2000, Lin, Zhou et al., 2018, Wilson, 2014), mRNA-tRNA translocation (Feldman, Terry et al., 2010, Lin et al., 2018, Tsai, Uemura et al., 2013). Likewise, tetracyclines bind to helix 34 of decoding center and inhibit the binding of aminoacyl-tRNAs. Chloramphenicol, Oxazolidinones (e.g. linezolid), and lincosamides (e.g. Clindamycin) bind to peptidyl transfer center where they prevent binding of amino acid side chain of aminoacyl tRNAs (Lin et al., 2018). Macrolides (e.g. erythromycin) and streptogramins bind to the nascent peptide exit tunnel (NPET) and constrict the diameter of tunnel leading to obstruction in peptide elongation (Kannan, Kanabar et al., 2014, Polikanov, Aleksashin et al., 2018). Apart from this, only few antibiotics (for e.g. kirromycin and fusidic acid) bind directly to the translation factors thereby interfering with their function (Borg, Holm et al., 2015, Koripella, Chen et al., 2012, Parmeggiani, Krab et al., 2006).

Emergence of resistance determinants against these translation inhibitors is of main concern. The most common mean of resistance is through development of drug inactivating enzymes or target site modification by bacteria (D’Costa, King et al., 2011). Since most of the bacteria contain multiple copies of the rRNA operon, aminoglycoside resistance by target site mutation is uncommon. However, enzymatic drug modification or 16S rRNA modification (e.g. methylation) are common mechanisms of aminoglycoside resistance. To curb these problems, there have been several attempts to design semisynthetic derivative of prototype drug with enhanced stability and activity. Arbekacin sulfate (Abk), a rationally designed semisynthetic aminoglycoside was discovered as a derivative of dibekacin (Kondo, 1994) (Figure 3). Previous studies show it has enhanced antibiotic activity and it is less susceptible to the aminoglycoside modifying enzymes (Nakamura, Yamaguchi et al., 2015, Watanabe, Yanagihara et al., 2012) thereby maintaining potency against multidrug-resistant (MDR) pathogens.

Figure 3. Chemical structure of Arbekacin
Elongation factor G

EF-G is a bacterial elongation factor associated with the TRAFAC (named after translation factor) class of the P-loop GTPases (Bourne, Sanders et al., 1991, Leipe, Wolf et al., 2002). In *E. coli*, EF-G contains 704 amino acids forming five distinct domains namely G (I), II, III, IV and V (Figure 4A). Domain I resembles a canonical G domain conserved across diverse GTPases. It is responsible for binding and hydrolyzing GTP to GDP. The G domain is a prototypical Rossmann fold, comprising five highly conserved motifs G1 (or P-loop), G2 (Switch-I), G3 (Switch-II), G4 and G5 (Figure 4B).

The conserved histidine (H91 in *E. coli* EF-G) after DXXG motif (G4) is known to play a crucial role in GTPase activation (Li, Liu et al., 2015) and GTP hydrolysis, its mutation impairs GTP hydrolysis and subsequent Pi release (Cunha, Belardinelli et al., 2013, Holtkamp, Cunha et al., 2014, Koripella, Holm et al., 2015). In the available crystal structures of EF-G in free-state, this crucial histidine is flipped away from the guanine nucleotide bound at the active site (Hansson, Singh et al., 2005, Laurberg, Kristensen et al., 2000). The intrinsic GTPase activity of EF-G is feeble like Elongation Factor Tu (EF-Tu). In EF-Tu, it is proposed that a “hydrophobic gate” consisting of residues from P-loop and Switch I, occlude the catalytic histidine from acquiring a catalytically competent state (Voorhees et. al., 2010). A similar scenario could also explain the poor intrinsic GTPase activity of EF-G, where isoleucines (18th and 60th) act as the hydrophobic gate.

A comparison of the high resolution structures of 70S pre-translocation and post-translocation complexes reveal the different orientations of the catalytic histidine prior and post GTP hydrolysis (Gao, Selmer et al., 2009, Lin, Gagnon et al., 2015, Mace, Giudice et al., 2018, Zhou, Lancaster et al., 2013). While it remains oriented towards the γ-phosphate before GTP hydrolysis, it flips away from the active site after GTP hydrolysis, which in turn facilitates Pi release (Brilot, Korostelev et al., 2013, Chen, Feng et al., 2013, Gao et al., 2009, Koripella et al., 2015, Zhou et al., 2013).

Upon binding of EF-G•GTP to the ribosome, domain IV together with domain III and V, moves 25 to 30° relative to the G domain and domain II. This movement allows positioning of the tip of the domain IV near the A-site codon in the mRNA during translocation (Chen et al., 2013, Gao et al., 2009, Zhou et al., 2013). On the ribosome, Domain II and V of EF-G interact with 30S (ri-
bosomal protein S4, S12 and 16S rRNA) and 50S (helix 43 and 44) respectively during translocation, whereas domain III contacts both subunits possibly sensing the subunit rotation (classical/hybrid).

Figure 4. (A) EF-G structure shown in domains (PDB: 2XEX) (B) G domain of EF-G (PDB: V49O)

GTP hydrolysis by EF-G is crucial for efficient tRNA-mRNA translocation. The same has been demonstrated by characterizing several GTPase deficient mutants of EF-G, especially those with mutations in the catalytic histidine (H91 in *E. coli*) (Cunha et al., 2013, Holtkamp et al., 2014, Koripella et al., 2015). Despite that, the exact role of GTP hydrolysis is still being debated. It is suggested that EF-G acts like a motor protein and uses the energy from GTP hydrolysis to translocate mRNA-tRNAs. This is because translocation slows down significantly (about 50 times) when EF-G bound with non-hydrolyzable GTP analog is used in translocation (Rodnina, Savelsbergh et al., 1997). However, results produced by other groups showed that translocation with EF-G in the presence of GDP•Pi analogs yield similar rates as with GTP. These authors claim that GTP hydrolysis by EF-G is needed for structural rearrangement to leave the ribosome (Ermolenko & Noller, 2011, Salsi, Farah et al., 2016). It will require systematic investigation in future combining multiple approaches to sort out the actual role of GTP hydrolysis in ribosomal translocation.

*In vitro* kinetic tools for studying translational elongation

Till date, ensemble fast kinetic methods, namely quench-flow and stopped-flow have been used extensively to study the mechanisms of elongation of
protein synthesis. These studies mostly use reconstituted bacterial translation system composed of individually purified 70S ribosome, all major translocation factors, all tRNA synthetases, tRNA-bulk and mRNA, in highly optimized, near-physiological buffer condition. The reconstituted translation systems are optimized carefully for in vivo like rate and accuracy (Holm, Borg et al., 2016, Jelenc & Kurland, 1979, Johansson, Zhang et al., 2012).

In a quench-flow, equal volumes of two reactants are mixed rapidly in the reaction loop and incubated at constant temperature until the reaction is quenched by the quencher (normally formic acid is used to precipitate the ribosome) (Figure 5A). The reaction time can be set as short as few milliseconds (dead time of the quench-flow is normally 1-2 ms), and the products from the quenched reaction are analyzed either in high performance liquid chromatography (HPLC) or thin-layer chromatography (TLC) with radioactivity detection. Most commonly, radioactively labeled amino acids are used for peptide bond formation experiments, where the di-/tri-/longer peptides can be separated in the reversed phase chromatography in HPLC and detected with radiography (Holm et al., 2016). For GTP hydrolysis experiments radioactive GTP is used. After the reaction, the GTP and GDP are separated in TLC or with ion exchange chromatography and quantified by using the radioactivity of the respective peaks/bands. The mean time for the translocation including all the steps starting from EF-G binding, GTP-hydrolysis, couple mRNA-tRNA translocation and Pi release and EF-G release can be measured precisely (Figure 6).

![Figure 5](image_url) Schematic description of quench-flow (A) and stopped-flow (B)

In a stopped-flow, the mechanism of the detection is often light scattering and fluorescence (Figure 5B). Due to difference in particle size, the 70S ribosome
show higher light scattering than the individual subunits. This property is typically used to monitor subunit association during initiation and 70S dissociation during ribosome recycling (Antoun, Pavlov et al., 2004, Mandava et al., 2012, Pavlov, Antoun et al., 2008). Studies of the elongation steps using a stopped-flow normally employ fluorescence dye labeling to either tRNA, mRNA, the ribosomal proteins or the elongation factors (Adio, Sharma et al., 2018, Blanchard et al., 2004, Goyal, Belardinelli et al., 2017, Perla-Kajan, Lin et al., 2010, Rodnina et al., 1997, Salsi, Farah et al., 2014, Studer, Feinberg et al., 2003, Volkov & Johansson, 2019). Fluorescence changes upon binding, release or movement are monitored in real time to determine the rates and the sequence of the events. Since elongation involves several structural arrangements between and inside the subunits, fluorescence resonance energy transfer (FRET) has also been used in stopped-flow where two ribosomal proteins located within the Forster distance have been labeled with the donor and the acceptor dye pairs. The change in the FRET signal against time reflects the dynamics of the labeled components and provides informational about ribosomal dynamics (Salsi, Farah et al., 2015, Wang, Caban et al., 2015, Wang, Pulk et al., 2012, Wasserman et al., 2016).

![Scheme of the fast kinetic studies on translational elongation](image)

Figure 6. Scheme of the fast kinetic studies on translational elongation. Mean time for the translocation ($\tau_{\text{translocation}}$) is calculated as the subtraction of mean times of the two peptide bond formation ($\tau_{\text{p1}}$ and $\tau_{\text{p2}}$) from total tripeptide formation time ($\tau_{\text{tripeptide}}$).

In recent years, single molecule FRET (smFRET) has become popular in kinetic measurement of protein synthesis, which also requires site-specific incorporation of the fluorescent dyes. The distance change in real time of two or three dyes is recorded in fluorescent microscopes (Cornish et al., 2009, Jamiolkowski et al., 2017, Juette, Terry et al., 2016, Wasserman et al., 2016). The advantage of this technique over the stopped-flow technique is that it allows more precise analysis of reactants in heterogeneous system and the short-lived intermediate state. However, sensitivity of the dye to the environment and possible damage in the molecules by the usage of high power laser can be limiting factor for this technique for certain applications.
Aim of the thesis

This thesis aims to optimize a fluorescent-mRNA based real time translocation assay. Further aims are to characterize effects of different factors, which affect ribosomal translocation, using this assay. These factors include EF-G mutations, r-protein modification, different tRNAs and antibiotics.

- The length and the position of the pyrene on the mRNA have been optimized by careful calibration with the functional translocation assay by tripeptide synthesis in a quench-flow. (Paper I)
- The external factors such as temperature, Mg$^{2+}$ concentration in the buffer, concentrations of EF-G, NAc-Phe-tRNA$^{\text{Phe}}$ have been optimized for better signal and reliable data analysis. (Paper I)
- The effects of EF-G mutations, GTP hydrolysis and nucleotide binding on the ribosomal translocation are analyzed using optimized translocation assay. (Paper I & II)
- The role of the extended tail of the ribosomal protein S13 from *Thermus thermophilus* has been investigated in relation with ribosomal translocation and recycling. (Paper III)
- The mode of action of the novel aminoglycoside antibiotic arbekacin on translocation. (Paper IV)
The present work

**Optimization of pyrene-labeled mRNA based translocation assay**

In 2003, Studer et al. developed a clever assay to monitor tRNA/mRNA translocation in real time by using pyrene labeled mRNA (Studer et al., 2003), which has been used in numerous studies thereafter (Dorner, Brunelle et al., 2006, Ermolenko & Noller, 2011, Feinberg & Joseph, 2006, Guo & Noller, 2012, Khade & Joseph, 2011, Salsi et al., 2016). For that, they labeled 3’ end of the mRNAs of various lengths with fluorescent dye pyrene and tested those by translocating N-acetylated Phe-tRNA\(^{\text{Phe}}\) (NAc-Phe-tRNA\(^{\text{Phe}}\)) – a peptidyl tRNA analog, on a Pre-T complex by adding EF-G.

The idea was that when translocation takes place, the mRNA moves by one codon and as a result the pyrene residue enters inside the ribosome or comes closer to it (Figure 7). This leads to the change in its local environment and further change in its fluorescence intensity. Thus, the mRNA-tRNA translocation can be followed directly in real time by monitoring the change in the pyrene fluorescence in a stopped-flow instrument equipped with fluorescence detector. The mRNA+9 (Figure 8) showed the largest difference in fluorescence intensity between the Pre-T and post-translocation (Post-T) complex, further experiments were performed with this construct. However, this truncated mRNA should be validated further in order to use in our fast kinetic tripeptide formation studies.

![Figure 7. Schematic description on EF-G dependent translocation using pyrene labeled mRNA.](image-url)
Optimization of pyrene-mRNA for functional kinetic studies in quench-flow

In this work, we have tested the pyrene labeled mRNAs of different lengths +9 to +12, and unlabeled +9 and +10 (Figure 8) in quench-flow based tripeptide formation assay in parallel to the stopped flow based translocation assay (Figure 9A). The mRNAs, encode for tripeptide Met-Phe-Leu, were designed and named based on the Studer et al., 2003. The process includes two peptide bond formation steps and one translocation step driven by EF-G. The di- and tripeptide formation experiments were conducted in quench-flow, where an elongation factor mix containing the respective ternary complexes (TC) was rapidly mixed with the 70S IC containing mRNA and fMet-tRNA^{Met} in the P site. By fitting the kinetic data as described in the in Paper I, the mean times of the first peptide bond formation ($\tau_{p1}$), second peptide bond formation ($\tau_{p2}$) and tripeptide formation ($\tau_{tripeptide}$) were determined. The mean time of a full translocation reaction ($\tau_{translocation}$) was calculated as [$\tau_{tripeptide} - (\tau_{p1} + \tau_{p2})$]. In parallel, the kinetics of translocation starting from the 70S IC was followed in stopped-flow, where the fluorescence change of the 3’ pyrene labeled mRNAs was monitored in real time. The mean time $\tau_{fluor}$, indicated total time of all events starting from the 70S IC up to and including mRNA movement. The mean time for mRNA movement, $\tau_{mRNA}$, was determined by subtracting $\tau_{p1}$ from $\tau_{fluor}$.

![Figure 8. Sequence of different length of mRNA constructs. ⊙ represents the pyrene dye at the 3'-end of mRNAs. Shine-Dalgarno (SD) sequence is in grey font color and mRNAs code for tripeptide fMet-Phe-Leu.](image)

Using quench-flow, we have followed the kinetics of formation of fMet-Phe-Leu formation with six mRNAs (labeled & unlabeled). Two mixes, mix A containing 70S initiation complex (1 µM) with fMet-tRNA^{Met} in the P site and mix B containing ternary complexes of Phe and Leu (4 µM each), and EF-G (10 µM), were mixed rapidly for tripeptide synthesis. The reaction mixture was quenched at given time points, and treated for further analysis in Reversed Phase-High Performance Liquid Chromatography (RP-HPLC) (C18 column)
with on-line radiation detection (Holm et al., 2016) (Figure 9B). The proportion of fMet-Phe-Leu was fitted into three consecutive step model to determine $\tau_{\text{tripeptide}}$ (Borg & Ehrenberg, 2015, Holm et al., 2016). Similarly, the mean time for the first peptide bond formation ($\tau_{p1}$) and the second peptide bond formation ($\tau_{p2}$) were measured as described (Borg et al., 2015).

The mean time value of the first peptide bond formation ($\tau_{p1}$) was identical for all six mRNAs, accounted for about 31 ms, indicating that neither the mRNA length or 3’-modification of the mRNAs did not affect this step (Table 1A). However, the mean time of fMet-Phe-Leu tripeptide formation ($\tau_{\text{tripeptide}}$) and the mean time of formation of the second peptide bond ($\tau_{p2}$) varied for the mRNAs. The tripeptide formation was noticeably slower for mRNA+9 and mRNA+9 nodye ($315 \pm 15$ ms and $368 \pm 20$ ms respectively) than other mRNAs ($261 \pm 15$ ms, $244 \pm 12$ ms, $255 \pm 18$ ms and $217 \pm 27$ ms respectively for mRNA+10 nodye, mRNA+10, mRNA+11 and mRNA+12) (Figure 9B and Table 1A). Similarly, mRNA+9 and mRNA+9 nodye were slower in the second peptide bond formation (Table 1A). The mean time for translocation ($\tau_{\text{translocation}}$), which contains multi- sub-steps between the two peptidyl transfer was determined by subtracting $\tau_{p1}$ and $\tau_{p2}$ from $\tau_{\text{tripeptide}}$. $\tau_{\text{translocation}}$ for all other mRNAs was faster than for mRNA+9 by about 50 ms, indicating the defects of the short mRNA for translocation. However, these defects are improved by the addition of just one additional base at the 3’- end of the mRNA in mRNA+10, which is as fast as the longer mRNAs.

The same 70S IC with pyrene labeled mRNA were deployed in stopped-flow to determine mRNA-tRNA translocation by following fluorescence upon EF-G addition. Same mixes A and B were rapidly mixed after incubating at 37 °C for 15 min. The fluorescence traces showed an initial small increase followed by a predominant monophasic decrease (Figure 9C). The traces were analyzed as described in Materials and Methods in paper I, and the mean time for mRNA movement ($\tau_{\text{mRNA}}$) was determined. It should be noted that $\tau_{\text{mRNA}}$ is supposedly shorter than $\tau_{\text{translocation}}$ as the mRNA-fluorescence based assay is insensitive about EF-G release and ribosomal rearrangement prior to next EF-Tu TC binding (Figure 9A). $\tau_{\text{mRNA}}$, measured by following pyrene fluorescence, did not vary between mRNAs, indicating the steps for the defects of mRNA+9 are post mRNA movement (Figure 9D and Table 1A). In relation to the fluorescence intensity data in (Studer et al., 2003), the amplitude of fluorescence change varied largely. Compared the amplitude for mRNA+9, mRNA+11 changed the fluorescence by only about half, whereas mRNA+12
produced mere changes in the fluorescence intensity thus reliable data could not be achieved from fitting (Figure 9C).

Figure 9. (A) Schematic representation of the peptide elongation cycle on the ribosome starting from the 70S IC. (B) Kinetics of the tripeptide formation in quench-flow starting from the 70S IC. (C) Fluorescence trace of pyrene upon EF-G addition to the 70S initiation complex. (D) Mean time values of each step for tripeptide formation in quench-flow and fluorescence measurement in stopped-flow. The values of three-colour stacked bar accounts for the mean time of tripeptide formation ($\tau_{\text{Tripeptide}}$), and purple bar accounts for $\tau_{\text{trans}}$, acquired from stopped-flow measurement.
From the results above, we have concluded that mRNA+10 is the optimal construct for both tripeptide formation and fluorescence based translocation assay, as it still generates similar change in fluorescence intensity as the +9 mRNA and yet produces a high rate of translocation and tripeptide formation - similar to the longer mRNAs. In contrast, the pyrene labeled mRNA+9 is particularly defective as it is the slowest of all in tripeptide formation and especially in the translocation sub-steps after the mRNA-tRNA movement. These sub steps potentially include Pi release, ribosome rearrangement (backratcheting) and EF-G release. We also realized that these steps altogether require longer time (85 ± 18 ms additional time for mRNA+10) than the actual mRNA-tRNA movement (~50 ms).

Table 1. The mean time of different steps of elongation starting from either 70S or pre-T complex measured by quench-flow and stopped-flow.

A. Reaction starting from 70S IC, the elongation mix contains natural peptidyl tRNA

<table>
<thead>
<tr>
<th>mRNA</th>
<th>τₚ₁ (ms)</th>
<th>τtranslocation (ms)</th>
<th>τₚ₂ (ms)</th>
<th>τtripeptide (ms)</th>
<th>τfluor (ms)</th>
<th>τmRNA (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MFL+9 nodye</td>
<td>32 ± 2</td>
<td>236 ± 15</td>
<td>100 ± 6</td>
<td>368 ± 20</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MFL+9</td>
<td>30 ± 2</td>
<td>180 ± 18</td>
<td>119 ± 15</td>
<td>315 ± 13</td>
<td>79 ± 9</td>
<td>49 ± 9</td>
</tr>
<tr>
<td>MFL+10 nodye</td>
<td>32 ± 3</td>
<td>154 ± 16</td>
<td>75 ± 6</td>
<td>261 ± 15</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MFL+10</td>
<td>31 ± 3</td>
<td>137 ± 16</td>
<td>76 ± 10</td>
<td>244 ± 12</td>
<td>83 ± 6</td>
<td>52 ± 7</td>
</tr>
<tr>
<td>MFL+11</td>
<td>31 ± 3</td>
<td>158 ± 20</td>
<td>66 ± 8</td>
<td>255 ± 18</td>
<td>93 ± 2</td>
<td>62 ± 4</td>
</tr>
<tr>
<td>MFL+12</td>
<td>33 ± 3</td>
<td>128 ± 28</td>
<td>56 ± 6</td>
<td>217 ± 27</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

B. Reaction starting from pre-TC with NAc-Phe-tRNA_Phe in the A site

<table>
<thead>
<tr>
<th>mRNA</th>
<th>τtranslocation + τₚ₂ (ms)</th>
<th>τmRNA (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MFL+9</td>
<td>2381 ± 227</td>
<td>112 ± 6</td>
</tr>
<tr>
<td>MFL+10</td>
<td>1667 ± 139</td>
<td>119 ± 7</td>
</tr>
</tbody>
</table>

Translocation with pre-T complex containing NAc-Phe-tRNA_Phe
The pre-T complex containing natural peptidyl tRNAs in the ribosomal A site is inherently unstable on the timescale of typical laboratory work. Thus, tRNAs charged with N-acetylated amino acids are commonly used as A-site
peptidyl tRNA analogs to mimic the pre-T complex. The binding of such analogs is a factor-independent equilibrium process and the substrates can therefore be supplied in large excesses (Dorner et al., 2006, Ermolenko & Noller, 2011, Feinberg & Joseph, 2006, Feldman et al., 2010, Guo & Noller, 2012, Juette et al., 2016, Khade & Joseph, 2011, Salsi et al., 2016, Studer et al., 2003). We designed a set of experiments to compare translocation of a pre-T complex formed by pre-equilibration with a commonly used peptidyl tRNA analog NAc-Phe-tRNA\(^\text{Phe}\), to a pre-T complex containing natural fMet-Phe-tRNA\(^\text{Phe}\), in continuous progression (without pre-equilibration), starting from the 70S IC.

Four pyrene-labeled mRNAs were deployed in stopped-flow measurement for mRNA-tRNA translocation with pre-T complex instead of 70S IC. The translocation displayed a biphasic fluorescence decay similar to previous reports (Ermolenko & Noller, 2011, Feinberg & Joseph, 2006, Guo & Noller, 2012, Khade & Joseph, 2011, Salsi et al., 2016), and absent of the initial increase presented in the reaction when 70S IC was a starting complex of translocation reaction. The amplitude also varied similarly to the reaction with 70S IC, mRNA+11 and +12 achieved little fluorescence change during translocation, furthermore unreliable for analysis (Figure 10A). The fast phase of the fluorescence trace with mRNA+9 and +10 accounted for 80 to 90% of the total amplitude, which rate was used to determine the mean time of mRNA translocation (\(\tau_{\text{mRNA}}\)). mRNA +10 translocated at a similar rate as +9, but faster in the elongation cycle (peptide bond formation and translocation) measured in quench-flow, validating that mRNA +10 is the optimal mRNA for functional assays (Table 1B). Compared to the rate of the translocation starting from IC, pre-T complex delivered slower translocation by about half rate (~50 ms and ~120 ms for 70S IC and pre-T respectively) despite the two reaction had identical buffer condition and components (Table 1B and Figure 10B). This may be due to the inability of NAc-Phe-tRNA\(^\text{Phe}\) for mimicking the peptidyl-tRNA, as the ribosome sense the peptidyl chain of the A-site tRNA before translocation (Frank & Agrawal, 2000).
Optimization of the reaction conditions for better data analysis

One bottleneck of the earlier protocol of the pyrene-mRNA based translocation assay was that it always produced bi-phasic kinetics with a major slow phase of unknown origin. This made mean time estimation very challenging. We have here optimized the conditions for the translocation starting from the pre-T complex so that a near monophasic fluorescence change can be obtained. Concentrations of NAc-Phe-tRNA$^{\text{Phe}}$, EF-G and Mg$^{2+}$, and the temperature are the factors, which influence fluorescence change in the translocation reaction. By titrating NAc-Phe-tRNA$^{\text{Phe}}$ in the reaction, we have found that the affinity of the NAc-Phe-tRNA$^{\text{Phe}}$ to the 70S pre-T complex is low at physiological Mg$^{2+}$ concentration and 37 °C. When it was titrated in the mix of the 70S ribosome with mRNA, deacylated tRNA$^{\text{fMet}}$ in the P site and empty A site, the rates of fast and slow phase as well as the proportion of the amplitudes did not differ much. The total amplitude did rather vary, the higher concentration of NAc-Phe-tRNA$^{\text{Phe}}$ yielded higher amplitude. We have determined the $K_D$ value (2.7 ± 0.2 µM) of NAc-Phe-tRNA$^{\text{Phe}}$ binding to the A site of the ribosome, by titrating NAc-Phe-tRNA$^{\text{Phe}}$ in the EF-G mix. The proportion of the properly formed 70S pre-T complex calculated from the $K_D$ value actually matched with the amplitude data above, validating the fact that NAc-Phe-tRNA$^{\text{Phe}}$ has poor affinity to the A site of the 70S ribosome.

By titrating EF-G, we have found that high concentration of EF-G in the translocation reaction is important for pushing the proportion of the fast phase as well as the rate of the translocation. Same trend was observed with higher temperature and lower Mg$^{2+}$ concentration (Table 2). The specific cause of
higher proportion of the fast phase in these conditions could not be determined. However, we notice that the biphasic curves are normally reported in the literature, whenever the pre-T complex has been allowed to pre-equilibrate. This is irrespective of whether the reaction was done with peptidyl tRNA analogs (Ermolenko & Noller, 2011, Feinberg & Joseph, 2006, Guo & Noller, 2012, Salsi et al., 2016, Walker, Shoji et al., 2008) or natural tRNAs (Cunha et al., 2013, Feldman et al., 2010, Mohr, Wintermeyer et al., 2000, Rodnina et al., 1997). In contrast, when the pre-T complex was formed by continuous progression from the 70S IC and translocated immediately, the fluorescence decay was monophasic (Borg et al., 2015). Previous report (Xie, 2014) have suggested that such biphasic curves likely represent different translocation rates from the two conformational states of the pre-T complex which are known to populate at equilibrium (Blanchard et al., 2004, Cornish, Ermolenko et al., 2008, Kim et al., 2007). One of these is “translocation competent”, which yields the fast phase. The other group is incompetent in translocation and therefore yields slow phase. It is likely that EF-G binding, low Mg$^{2+}$ and higher temperature facilitate the transition to the majority of the pre-T complex to a “translocation competent” state. In summary, we have identified the best reaction conditions for the pyrene-mRNA based translocation assay. We suggest that high concentration of NAc-Phe-tRNA$^{\text{Phe}}$, EF-G, low concentration of free Mg$^{2+}$ and high temperature are optimal conditions for translocation assay with NAc-Phe-tRNA$^{\text{Phe}}$.

Significance of the optimized assay is (i) optimization of the length of the mRNA by calibration with the functional assay, (ii) to enable the comparing quench-flow and stopped-flow assay for understanding sub-steps in ribosomal translocation, (iii) investigation of translocation starting from 70S IC and pre-T complex and (iv) to suggest the optimal conditions for translocation with NAc-Phe-tRNA$^{\text{Phe}}$. 

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Table 2. Effects of varying EF-G and Mg\textsuperscript{2+} concentration and temperature on mRNA-tRNA movement during ribosomal translocation with NAc-Phe-tRNA\textsuperscript{Phe}

<table>
<thead>
<tr>
<th></th>
<th>$k_1$ (s\textsuperscript{-1})</th>
<th>$k_2$ (s\textsuperscript{-1})</th>
<th>$\frac{A_1}{(A_1+A_2)}$</th>
<th>$\tau_{mRNA}$ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. EF-G (μM)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>2.8 ± 0.5</td>
<td>0.6 ± 0.05</td>
<td>0.49 ± 0.03</td>
<td>357 ± 67</td>
</tr>
<tr>
<td>1.25</td>
<td>4.4 ± 0.1</td>
<td>0.92 ± 0.01</td>
<td>0.76 ± 0.06</td>
<td>227 ± 5</td>
</tr>
<tr>
<td>2.5</td>
<td>6 ± 0.2</td>
<td>0.72 ± 0.1</td>
<td>0.84 ± 0.04</td>
<td>167 ± 6</td>
</tr>
<tr>
<td>5</td>
<td>8.4 ± 0.5</td>
<td>0.66 ± 0.2</td>
<td>0.88 ± 0.06</td>
<td>119 ± 7</td>
</tr>
<tr>
<td>10</td>
<td>10.3 ± 0.3</td>
<td>0.4 ± 0.07</td>
<td>0.91 ± 0.02</td>
<td>97 ± 3</td>
</tr>
<tr>
<td><strong>B. Temperature (°C)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>8.4 ± 0.5</td>
<td>0.66 ± 0.2</td>
<td>0.88 ± 0.06</td>
<td>119 ± 7</td>
</tr>
<tr>
<td>30</td>
<td>3.2 ± 0.1</td>
<td>0.56 ± 0.02</td>
<td>0.74 ± 0.01</td>
<td>313 ± 10</td>
</tr>
<tr>
<td>25</td>
<td>1.25 ± 0.06</td>
<td>0.30 ± 0.01</td>
<td>0.62 ± 0.01</td>
<td>800 ± 38</td>
</tr>
<tr>
<td>20</td>
<td>0.35 ± 0.07</td>
<td>0.09 ± 0.02</td>
<td>0.47 ± 0.02</td>
<td>2857 ± 571</td>
</tr>
<tr>
<td><strong>C. Extra Mg\textsuperscript{2+} (mM)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5.8 ± 1.3</td>
<td>0.71 ± 0.01</td>
<td>0.66 ± 0.01</td>
<td>172 ± 39</td>
</tr>
<tr>
<td>2</td>
<td>3.2 ± 0.2</td>
<td>0.43 ± 0.03</td>
<td>0.62 ± 0.03</td>
<td>313 ± 20</td>
</tr>
<tr>
<td>3</td>
<td>1.9 ± 0.2</td>
<td>0.28 ± 0.11</td>
<td>0.52 ± 0.02</td>
<td>526 ± 55</td>
</tr>
<tr>
<td>5</td>
<td>0.8 ± 0.3</td>
<td>0.17 ± 0.04</td>
<td>0.48 ± 0.05</td>
<td>1250 ± 469</td>
</tr>
<tr>
<td>10</td>
<td>0.19 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>0.49 ± 0.01</td>
<td>5263 ± 277</td>
</tr>
</tbody>
</table>
Application of optimized translocation assay

The optimized translocation assay based on the pyrene-labeled mRNA has been employed for the investigation of the effect of different factors on the mRNA-tRNA translocation studies. These are non-hydrolyzable GTP analogs (Paper I), tRNA variable loop (this thesis), EF-G mutants (Paper I and II), ribosomal protein S13 (Paper III) and antibiotic Arbekacin (Paper IV).

Non-hydrolyzable GTP analogs in translocation

Translational G-proteins play an important role in the initiation, peptidyl transfer, ribosomal translocation, peptide release, and ribosome recycling. In most cases they facilitate the translation when they are bound to GTP. The GDP or GDPNP (non hydrolyzable analog of GTP) bound form cannot efficiently assist the ribosomal translation, indicating the necessity of GTP binding and GTP hydrolysis for their function. Like all other translational GTPases, EF-G undergoes conformational change in the GTP and GDP bound forms (Chen et al., 2013, Lai, Ghaemi et al., 2017, Lin et al., 2015, Munro, Wasserman et al., 2010b). When GTP is replaced with non-hydrolyzable GTP analogs, the protein cannot conduct GTP hydrolysis and hence stays in the “on” state for much longer time. Thus, these GTP analogs are normally used to investigate the role of GTP hydrolysis by EF-G in translocation. It has also been used in many ribosomal translocation studies (Ermolenko & Noller, 2011, Flis et al., 2018, Rodnina et al., 1997, Salsi et al., 2016). Previous studies using same non-hydrolyzable GTP analog, one result showed significant defects by more than one order of magnitude supporting the role of GTP hydrolysis as promoting ribosomal translocation (Rodnina et al., 1997). The other data showed comparable rates of translocation with the analog to the EF-G•GTP or less than two times defects. This result led to the conclusions stating that of GTP hydrolysis is necessary for EF-G release from the 70S ribosome (Ermolenko & Noller, 2011, Rodnina et al., 1997, Salsi et al., 2016). Such data discrepancy has not been investigated properly, different buffer and reaction condition made it difficult to compare.

Three non-hydrolyzable GTP analogs (GDPNP, GDPCP and GTP-γ-S) were added in the optimized translocation assay with a Pre-T complex formed with NAc-Phe-tRNA^{Phe}. This strategy was essential to avoid another GTPase translation factor, EF-Tu, which would otherwise be required to deliver the aa-tRNAs. With non-hydrolyzable GTP analogs, EF-G translocated almost 50
time slower than with GTP, in accordance with earlier report (Rodnina et al., 1997). EF-G with GTP-γ-S was the least defective among the three analogs tested, producing 2-4 times faster translocation than with GDPNP and GDPCP, due to the higher rate of GTP hydrolysis compared to the other two analogs (Kuhle & Ficner, 2014). Surprisingly, GDP showed same degree of defect as GDPNP (at a EF-G concentration of 5 µM), which was apparently unexplainable.

In order to investigate the scenario further, we titrated EF-G in translocation reaction together with GTP, GDPNP and GDP. The rates were plotted against EF-G concentration in the Michaelis Menten plots. For the reactions with GTP the \( k_{cat} \) and \( K_M \) were 11.9 ± 0.9 s\(^{-1}\) and 2.2 ± 0.4 µM. The reaction was highly inefficient with GDPNP. The \( K_M \) increased by about five fold, but the \( k_{cat} \) decreased about 20 times. Interestingly, EF-G with GDP produced higher \( k_{cat} \) (3.4 ± 2.4 s\(^{-1}\)) than with GDPNP. It is ~four times slower than the \( k_{cat} \) with GTP. But, the reaction could not be saturated even at 20 µM concentration (unlike GTP or GDPNP), and the \( K_M \) was as high as 70 µM, ~35 fold higher than with GTP (Figure 11). We speculate that EF-G•GDP is defective in the formation of “translocation competent” ribosome complex, showing high \( K_M \) value. However, our results with GDP and GDPNP suggest that translocation can occur, albeit inefficiently, even in the absence of GTP hydrolysis. Being said that the possibility that the cause of defect with GDP and GDPNP is different, cannot be nullified. Thus, further investigations will be required for further clarification.

![Figure 11. Michaelis Menten plots of the translocation rate by titration of EF-G with either GTP (A), GDPNP (B), or GDP (C)](image)

Possible importance of tRNA body in elongation
A tRNA is an adapter molecule, pivotal for translation of mRNA to proteins. It acts as a physical link between mRNA codons and the amino acid sequence
in the peptide through its unique structure. The tRNAs have L-shaped three dimensional structure which locates the anticodon loop and the acceptor stem to the DC in 30S subunit and the PTC in 50S subunit, respectively. Each tRNA has its unique anticodon and attaches specific amino acid at the acceptor stem. This specificity is conferred by the corresponding aminoacyl tRNA synthetase which endows accuracy and fidelity to the translation. During translation by ribosome, aminoacylated tRNA in the form of ternary complex with EF-Tu•GTP enters the A site in the ribosome, move (translocate) after peptidyl transfer from A to P site and again P to E site before finally exiting the ribosome. Several studies have investigated the interaction of tRNA with either rRNA or ribosomal proteins (Abdurashidova, Tsvetkova et al., 1991, Bocchetta, Xiong et al., 2001, Demeshkina, Jenner et al., 2010, Shetty, Shah et al., 2017), but little is known about their role in translocation, especially in relation with the length of the variable loop of the tRNAs.

Table 3. Translocation and peptide formation with different tRNAs in the A site.

<table>
<thead>
<tr>
<th>Codon</th>
<th>tRNA</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_M$ (µM)</th>
<th>$k_{cat}/K_M$ (µM$^{-1}$s$^{-1}$)</th>
<th>Codon mismatch</th>
<th>Nucleotides in variable loop</th>
</tr>
</thead>
<tbody>
<tr>
<td>UUC</td>
<td>Phe</td>
<td>25.5 ± 0.63</td>
<td>1.5 ± 0.07</td>
<td>17 ± 0.84</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>UUU</td>
<td>Phe</td>
<td>27.5 ± 0.88</td>
<td>2.5 ± 0.18</td>
<td>11 ± 0.86</td>
<td>yes</td>
<td>0</td>
</tr>
<tr>
<td>GCA</td>
<td>Ala</td>
<td>29.5 ± 0.5</td>
<td>1.6 ± 0.1</td>
<td>18.4 ± 1.2</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>UAC</td>
<td>Tyr</td>
<td>43.7 ± 2</td>
<td>2.1 ± 0.13</td>
<td>21.4 ± 1.62</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td>CUG</td>
<td>Leu1</td>
<td>11.4 ± 0.73</td>
<td>1.9 ± 0.17</td>
<td>6 ± 0.64</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>AGC</td>
<td>Ser</td>
<td>33.1 ± 2</td>
<td>2 ± 0.19</td>
<td>16.6 ± 1.86</td>
<td>-</td>
<td>17</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Codon</th>
<th>tRNA</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_M$ (µM)</th>
<th>$k_{cat}/K_M$ (µM$^{-1}$s$^{-1}$)</th>
<th>tRNA abundancy</th>
<th>Nucleotides in variable loop</th>
</tr>
</thead>
<tbody>
<tr>
<td>UUC</td>
<td>Phe</td>
<td>92.1 ± 9.8</td>
<td>0.91 ± 0.17</td>
<td>101.2 ± 21.8</td>
<td>1.6</td>
<td>0</td>
</tr>
<tr>
<td>UUU</td>
<td>Phe</td>
<td>100 ± 13.6</td>
<td>2.84 ± 0.62</td>
<td>35.2 ± 9.1</td>
<td>1.6</td>
<td>0</td>
</tr>
<tr>
<td>GCA</td>
<td>Ala</td>
<td>81.8 ± 9.4</td>
<td>2.47 ± 0.46</td>
<td>33.1 ± 7.2</td>
<td>5.04</td>
<td>0</td>
</tr>
<tr>
<td>UAC</td>
<td>Tyr</td>
<td>36.7 ± 3.4</td>
<td>1.48 ± 0.28</td>
<td>24.8 ± 5.2</td>
<td>3.14</td>
<td>7</td>
</tr>
<tr>
<td>CUG</td>
<td>Leu1</td>
<td>38.3 ± 4.4</td>
<td>4.24 ± 0.81</td>
<td>9 ± 2</td>
<td>6.94</td>
<td>10</td>
</tr>
<tr>
<td>AGC</td>
<td>Ser</td>
<td>30.6 ± 1.8</td>
<td>1.26 ± 0.16</td>
<td>24.3 ± 3.4</td>
<td>2.01</td>
<td>17</td>
</tr>
</tbody>
</table>
Using the experimental set up with fluorescent mRNA as explained above, we have investigated the movement of mRNA-tRNA with five different tRNAs – tRNA\textsuperscript{Phe} and tRNA\textsuperscript{Leu\_1}, tRNA\textsuperscript{Ala}, tRNA\textsuperscript{Tyr} and tRNA\textsuperscript{Ser}. The number of nucleotides in the variable loop is listed in Table 3. While tRNA\textsuperscript{Ser} has longest variable loop (17 nucleotides), the tRNA\textsuperscript{Phe} and tRNA\textsuperscript{Ala} have none. In case for Phe, two different mRNAs were applied, one of them has wobble mismatch with tRNA\textsuperscript{Phe}. The translocation reaction started from the 70S IC with fMet-tRNA\textsuperscript{P\_Met} in the P site and empty A site. In the translocation reaction, different tRNAs bind and move from A to P site, and the translocation rate ($k_{mRNA}$) was determined as $1/\tau_{mRNA}$ as described above (Figure 9A). Michaelis Menten parameters $k_{cat}$ and $K_M$ were estimated by titrating EF-G in the reactions (Table 3A). Our results summarized in Table 3A showed that tRNA\textsuperscript{Leu\_1} is slowest in translocation compared to all other tRNAs. However, tRNA\textsuperscript{Ser} was faster in translocation than tRNA\textsuperscript{Leu\_1}. Thus, no correlation between the translocation rate and the variable loop size could be found. Rather tRNA\textsuperscript{Tyr} translocated faster than the tRNAs without the variable loop.

Interestingly, we have found the correlation of the variable loop with the rate of peptide bond formation (Table 3B). While Phe-tRNA\textsuperscript{Phe} and Ala-tRNA\textsuperscript{Ala} can incorporate to the A site and form dipeptide rapidly, other three aa-tRNAs with variable loops were substantially slower by 60-70 %. It is most likely that these tRNAs require longer time to accommodate, since actual chemical reaction should be same as $pK_a$ values of these amino acids, which are not very different. However, extensive investigation is required for consolidating this conclusion. Additionally, it is worthwhile to pay attention about the correlation between $K_M$ of peptide bond formation reaction and the tRNA abundancy inside the cell (Dong, Nilsson et al., 1996).

Investigation of EF-G mutants

For understanding the mechanism of translocation by EF-G, GTP hydrolysis, Pi release and conformational change during translocation, mutations have been introduced in EF-G in many studies (Cunha et al., 2013, Holtkamp et al., 2014, Koripella et al., 2015, Li et al., 2015, Mohr et al., 2000, Peske, Matassova et al., 2000, Rodnina et al., 1997, Salsi et al., 2014). The mutation in the catalytic histidine to alanine (H91A) has confirmed the defects in GTP hydrolysis and subsequent Pi release (Koripella et al., 2015, Rodnina et al., 1997). This mutant is also grossly defective in translocation, specifically on
30S (Cunha et al., 2013), in accordance with the translocation data with non-hydrolyzable GTP analogs. We have further deployed H91 mutants – H91E and H91Q in the translocation assay starting from the pre-T complex with NAc-Phe-tRNA_{Phe}. The defects were more extensive than GTP analogs by about 2 to 5 times. Furthermore, H91E revealed rather long initial increase phase compared to H91Q mutant, which is also not visible in the translocation reaction starting from 70S IC.

Various mutation was further introduced in the G domain of EF-G, in the position of R28 (R28A), R58 (R58A) and two threonine in the 23rd and 24th position (T23-24A) which associates the function of guanosine nucleotide binding and GTP hydrolysis in the vicinity of P loop and switch I. Mutation of the two threonine (T23-24A) rendered EF-G completely deficient in guanosine nucleotide binding, presenting apo state of EF-G. T23-24A mutant further annihilates Pi release and translocation. When R28A and R58A mutants were titrated in translocation assay starting from the 70S IC, $k_{cat}$ could not be estimated as the rate did not saturate at highest possible concentrations (20 µM). At the concentration of 10 µM, R28A and R58A mutants translocated by 15 to 30 fold slower than the wild type for R28A and R58A respectively. However, surprisingly, R28A was completely impaired in Pi release similarly to the T23-24A mutant. In addition, R28A mutant had similar affinity towards GDP compared to the wild type EF-G, while $K_D$ for R58A was one order of magnitude smaller indicating either tight binding or faster exchange of nucleotides.

The inconsistency in the Pi release kinetics for two arginine mutants suggests that Pi release is not coupled with translocation. It can occur before or after the translocation, not implying with previous findings (Peske et al., 2000). However, our results indicate that the binding of guanosine nucleotide is absolutely vital for EF-G function similar to other G-proteins.

Role of the extended C-terminal tail of the ribosomal protein S13 in protein synthesis

Ribosomal protein S13 is a 118 amino acids long protein in *E. coli*, located in the head part of the 30S subunit. Crystal structures of the bacterial ribosome show several interactions of the S13 protein at the subunit-interface; it plays an important role in forming intersubunit bridges between the subunits. The bridge 1a is formed between helix 38 of 23S rRNA and the 93rd amino acid
of S13, while bridge 1b is formed by the N-terminus of S13 and the large subunit protein bL5. Furthermore, Its C-terminal tail makes contact with the anticodon stem loop of the P-site tRNA (Yusupov et al., 2001), thereby potentially affecting the speed and accuracy of translocation. Previous studies have demonstrated important role of S13 protein in bacterial fitness and translation. It was shown that the chromosomal deletion of S13 protein either induces cell death or creates critical fitness defects in *E. coli* (Cukras & Green, 2005, Shoji, Dambacher et al., 2011). Moreover, small deletion at the C-terminal tail causes noticeable fitness defects in *E. coli*, possibly resulting from reduced affinity towards the P-site tRNA to the ribosome (Cukras & Green, 2005, Hoang, Fredrick et al., 2004).

The length of the C-terminal tail of S13 varies in different bacteria. While most bacteria have a short C-terminal tail like in *E. coli*, some bacteria have a longer tail. One such example is *Thermus thermophilus*, which has seven extra amino acids in the tail compared to *E. coli*. The sequence of S13 protein from *E. coli* and *T. thermophilus* have high similarities, the C-terminal domain contains 20-30 identical amino acids. The C-terminus tail of S13 contains RKGPRK sequence followed by different extensions; four amino acids in *E. coli* and eleven in *T. thermophilus*, ending with K in both. When compared for their structure on the ribosome, the longer tail of *T. thermophilus* S13 clearly extends between the P-site and the A-site tRNA (Figure 12) suggesting its potential role in hindering the movement of the tRNAs during translocation. The occurrence of multiple positively charged amino acids in the extended tail of S13 further strengthens the likelihood that it will interact more with the tRNAs, thereby influencing their movement, particularly during translocation.

To understand the implication of the C-terminal extension of the S13 protein in translocation, we have created several variations of the S13 tail in the chromosome of the *E. coli* JE28 strain (Ederth, Mandava et al., 2009) (Figure 13A). The parental strain JE28 carries tetra his-tagged ribosomes, where the nucleotides coding hexa-histidine tags are fused at the chromosomal locus of the *rplK* gene coding for the ribosomal protein L7/12. The his-tags allow easy pull-down of the intact ribosomes by affinity chromatography. For modification of the S13 tail, red-recombineering was done at the *rpsM* locus, where ampicillin resistance (*amp*<sup>+</sup>) cassette was used as a reporter gene. The S13 modified strains are named with the suffix CIK28. To ensure that the reporter
gene does not affect bacterial growth, only $amp^R$ cassette was also introduced in the same locus. This strain is called CIK28.

![Figure 12](image)

Figure 12. Superposition of T. thermophilus S13 (blue) on the structure of E. coli S13 (pink). Gray cartoons indicate three tRNAs bound to the A-, P- and the E-site. The structures are adopted from PDB 4K0L and PDB 3E1C. The ribosome components other than S13 have been removed to demonstrate the location of the extended tail of the S13 protein.

To investigate the impact of the length of S13 tail on bacterial growth and tRNA translocation, either four terminal amino acids of *E. coli* S13 were deleted (CIK28d), or the C-terminal tail was extended partially or fully with the sequence similar to that of *T. thermophilus*, resulting into CIK28b and CIK28c strains, respectively. Alternatively, the *E. coli* S13 tail was extended by three and seven alanines resulting into CIK28a3 and CIK28a7 strains, respectively. In order to investigate the importance of the positive charge in the S13 tail, last two lysines of the *E. coli* S13 tail were altered into either uncharged alanine (CIK28aa) or negatively charged glutamic acid (CIK28ee). Also, all positively charged amino acids of the *T. thermophilus*-like tail in CIK28c were exchanged with alanine resulting into the CIK28ca strain. The strains and the modifications are illustrated in Figure 13A.

The 70S ribosomes from *E. coli* S13 mutants were purified by affinity chromatography, and further dissociated into the subunits following the existing protocol (Ederth et al., 2009). As S13 protein is essential for interactions between the subunits, we have measured subunits association and 70S dissociation kinetics with the 30S subunits from the CIK28 strains. The alterations in the S13 tail did not change the rate or extent of subunit association or 70S
dissociation. Likewise, the rate of peptide bond formation (meantime ~18 msec) was also not changed. However, some of these modifications significantly affected bacterial fitness and tRNA translocation.

Figure 13. S13 tail modification in bacteria E. coli and their implications in the bacterial growth and translocation. (A) The list of the engineered E. coli strains with S13 C-terminal tail modifications. (B) Parallel bar diagram illustrating generation time of the engineered CIK28 strains measured in LB at 37 °C. (C) Pyrene-mRNA based translocation with the ribosomes from the CIK28 strains as indicated. (D) The rate of translocation estimated by fitting the fluorescence curves (C) with double exponential function.

The generation time of CIK28 was around 26 min similar to the host strain JE28 (23.6 ± 0.7 min). Thus, incorporation of the amp cassette did not influence the growth rate. However, deletion of the last four amino acids of E. coli S13 (CIK28d) or their replacement with eleven amino acids as in T. thermophilus (CIK28c) or with alanines (CIK28ca and CIK28a7) showed significant growth defect with generation time prolonged by 9 – 10 minutes (~35 min). The CIK28ee strain with double negative charge introduced in the tail replacing two positive charges, showed the largest growth defect (46.5 ± 1.9 min). The strain CIK28a3 also showed some defect. However, all other strains with intermediate extension (CIK28b) or with alanines replacing the terminal lysines (CIK28aa) did not show alterations of the growth rate. The growth rates are shown as bar diagram in Figure 13B.
Modification of S13 C-terminal tail showed varying degrees of defect on EF-G catalyzed translocation (Figure 13C). Deletion of the C-terminal tail (CIK28d), addition of three or seven alanines (CIK28a3 and CIK28a7), and alteration of the electric charge (CIK28aa and CIK28ee) did not affect the rate of translocation compared to the CIK28 ribosomes (25.2 ± 1.5 s⁻¹). However, interestingly only *T. thermophilus*-like extension of S13 slowed down the translocation, exact tail modification from *T. thermophilus* (CIK28c) translocated at the slowest rate (11.4 ± 0.8 s⁻¹). The translocation rate of CIK28ca and CIK28b ribosomes were 14.8 ± 1.7 s⁻¹ and 18.4 ± 1.8 s⁻¹ respectively, which were intermediate between CIK28c and JE28. Translocation rates for other strains are shown in bar diagram (Figure 13D).

Our results clearly show that longer CTD tail in CIK28c and CIK28ca reduce the speed of single round of translocation by three times. Since translocation is the key event in every elongation cycle, these results explain why these strains show slower growth rate. These mutants can be crucial for establishing the role of translocation in the context of bacterial growth and fitness. Perhaps the ribosomes in thermophilic bacteria require a special break to regulate the speed of translocation, for which the S13 tail evolved as a longer peptide. However, the implication of so many positive charges in the S13 tail in translocation is not clear. We see reduction in the growth rate when the terminal lysines are replaced with glutamic acid, but not when exchanged with alanines. Thus, although it seems that the positive charges are required for interaction and stabilization of the tRNAs their actual role can be primarily architectural, meaning that the positive charges ensure stable association of the relatively unstructured tail of the S13 protein with the negatively charged ribosomal RNA. Lastly, the strain with terminal truncation did not show slower translocation although it showed similar growth defect as the strains with the extended S13 tail. Thus, the origin of growth defect in this strain must not be via translocation. Instead, the shortening might affect the stability of the P-site tRNA (Hoang et al., 2004). All our experimental data did not show direct correlation between translocation and bacterial fitness. We speculate that since as the cell growth is the complicated process executed in combination of many factors including but not limited to ribosomal translation.
Antibiotic Arbekacin on translocation

Antibiotic arbekacin is a semisynthetic aminoglycoside, belongs to the kanamycin family, and originally synthesized from dibekacin in 1970s. It was clinically introduced in Japan in 1990s, and rather successful in the effort to overcome the antibiotic resistance. The modes of action of arbekacin in inhibiting protein synthesis in bacteria has not fully understood, however it was anticipated in comparison with other similar aminoglycosides. It is believed that arbekacin binds to the common aminoglycoside binding site in the 30S, and affects the accuracy of elongation as well as translocation (Feldman et al., 2010, Romanowska, Reuter et al., 2013, Tsai et al., 2013).

In this study we have investigated the effects of arbekacin on translocation by adding it in the optimized fluorescent-mRNA based translocation assay. As expected from the results with other aminoglycoside and tuberactinomycins, arbekacin showed strong inhibition of translocation. By fitting the translocation data, the fraction of the ribosome inhibited with arbekacin was estimated and plotted against arbekacin concentration. The inhibition constant was determined from the midpoint of the transition. When ribosomes were pre-incubated with arbekacin, the $K_I$ was as low as about 0.4 µM. However, it increased to $2 \pm 0.3$ µM ($K_{IC}$) when added in the EF-G mix. It means that pre-incubation of ribosome with arbekacin is certainly beneficial for the drug action. Further, from the rate of the slow phases the dwelling time of the drug could be estimated. Unlike viomycin (Holm et al., 2016), The dwelling time of arbekacin decreased with increasing EF-G concentration (Figure 14). Moreover, at a given EF-G concentration the dwelling time of the drug did not change with increasing arbekacin concentration (from 0.25 µM to 20 µM). It means that (i) arbekacin competes with EF-G for ribosome binding, but (ii) it cannot bind back to the ribosome once EF-G binds to it. This results clearly indicates that for effective inhibition arbekacin must bind to the ribosome prior to EF-G binding and it cannot bind back once it gets detached by competitive binding of EF-G. We have also followed the role of arbekacin in inducing error in translation and inhibiting ribosome recycling. These results are included in paper IV.
Figure 14. Effects of Arbekacin on translocation. (A) The translocation mean time at varying concentrations of EF-G and arbekacin. (B) Translocation rates in inhibition by arbekacin are plotted over EF-G concentration.

From the translocation data, the arbekacin mode of action on translocation can be explained as follows (Figure 15). $k_p$ is the rate constant for uninhibited 70S to bind to EF-G after formation of peptide bond, which was estimated as 50 s$^{-1}$ (from the rate of dipeptide ML formation in the experiments and fast binding of EF-G to the 70S pre-T complex). From the inhibition constant when arbekacin was incubated with the 70S ($K_{IC} = 2 \pm 0.3$ µM) and $k_p$, $k_1$ was determined as $k_p / K_{IC} = 25 \pm 4$ µM$^{-1}$ s$^{-1}$. As $K_i$ (0.4 µM) is the ratio of $k_1$ and $k_1$, $k_1$ (10 s$^{-1}$) can be estimated. $k_{mRNA}$ was estimated as $k_{cat}$ of translocation (11.4 ± 0.7 s$^{-1}$, Table 3A) while $k_g$ (0.007 µM$^{-1}$ s$^{-1}$) is EF-G concentration dependent step and determined from the slop value of the translocation plot over arbekacin concentration (Figure 14B). It is suggested that arbekacin is a tight binder and EF-G strives to push out arbekacin for successful translocation. Arbekacin binds to the 70S and stabilizes the “flipped off” conformation of the three monitoring bases at the DC, which is a translocation incompetent conformation (Feldman et al., 2010, Munro et al., 2010b). Moreover, it has less affinity to the ratcheted conformation of 70S stabilized by EF-G.

Figure 15. Kinetic model for arbekacin inhibition on translocation. 70S* represents the post-T complex. Abk = arbekacin
Conclusions and future perspective

This thesis mainly aimed at the optimization of the pyrene-labeled mRNA based translocation assay for high fluorescence signal and high in vivo-like rate both in quench-flow and stopped-flow measurement. The optimized conditions we suggest are mRNA+10, translocation starting from IC and higher concentrations of EF-G, NAc-Phe-tRNA^Phe, low concentration of free Mg^{2+} in the solution and higher temperature (preferably 37 °C) in the translocation starting from pre-T complex with NAc-Phe-tRNA^Phe. We have also suggested the proper analysis of biphasic curve of translocation by increasing the amplitude proportion of the fast phase. In the subsequent studies applying the optimized the translocation assay, we have confirmed the importance of GTP hydrolysis, nucleotide binding to the EF-G using non-hydrolysable GTP analogs and EF-G mutants. Furthermore, the C-terminal tail of the ribosomal protein S13 from Thermus thermophilus was revealed to affect the translocation in a manner of length and charge interaction. The mode of action of novel antibiotic arbekacin was explained kinetically.

The future steps from this thesis is to truly determine the role of GTP hydrolysis not only in connection with the mRNA-tRNA movement but also structural rearrangements in EF-G and the ribosome such as subunit ratcheting and back-ratcheting, and swiveling action of 30S head in reference to the body. The sequence of the action is also important for better understanding of the EF-G directed ribosomal translocation. The optimized real time translocation assay opened an opportunity for the investigation of the mechanism of antibiotics targeting translocation and other factors affecting translocation such as ribosomal proteins and modification of RNAs.
Ribosomen är ett makromolekylärt maskineri vars struktur består utav tre ribosomala RNA:n och tiotal ribosomala proteiner. Dess proteinskapande funktion är livsavgörande för celler. Detta, då proteiner är viktiga molekyler i kroppen; nästan alla enzymer som katalyserar biokemiska reaktioner i kroppen är proteiner samt att de även bygger upp vävnader, muskler och signalreceptorer. Proteiner består vanligtvis utav hundratals aminosyror, och ribosomen använder dessa som byggstenar utifrån informationen den hämtar från den genetiska blåkopian mRNA.


Genom kalibering av fluorescerande mRNA med tripeptidsyntesanalys, har vi optimiserat den korrekta positionen av färgen så att den kan utnyttjas tillförlitligt i fortsatta studier. Vi har också utvecklat de optimala förhållandena för pålitliga mätningar inom translokeringen, som temperatur, koncentrationen av tRNA och EF-G samt magnesiumjonkoncentrationen i
bufferten. Genom att använda detta optimiserade protokoll har vi undersökt mutationer i EF-G som påverkar hydrolysering av GTP och translokering.

I wish to express my deepest gratitude to my supervisor Suparna Sanyal for accepting me in her group and for the great supervision. The past years are great experiences for me, we have gone through thick and thin together. I have gained more knowledge as well as become a better person, and I can never imagine this without your intelligence and kind-hearted support.

Thank you, Måns Ehrenberg and my co-supervisor Magnus Johansson, for your kind support, it will never be forgotten.

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Feinberg JS, Joseph S (2006) Ribose 2'-hydroxyl groups in the 5' strand of the acceptor arm of P-site tRNA are not essential for EF-G catalyzed translocation. RNA 12: 580-8


Jelenc PC, Kurland CG (1979) Nucleoside triphosphate regeneration decreases the frequency of translation errors. Proc Natl Acad Sci U S A 76: 3174-8


Mohr D, Wintermeyer W, Rodnina MV (2000) Arginines 29 and 59 of elongation factor G are important for GTP hydrolysis or translocation on the ribosome. EMBO J 19: 3458-64
Munro JB, Altman RB, Tung CS, Cate JH, Sanbonmatsu KY, Blanchard SC (2010a) Spontaneous formation of the unlocked state of the ribosome is a multistep process. Proc Natl Acad Sci U S A 107: 709-14


Schmeing TM, Voorhees RM, Kelley AC, Gao YG, Murphy FVt, Weir JR, Ramakrishnan V (2009) The crystal structure of the ribosome bound to EF-Tu and aminoacyl-tRNA. Science 326: 688-694
Shine J, Dalgarno L (1974) Identical 3' terminal octanucleotide sequence in 18S ribosomal ribonucleic acid from different eukaryotes. A proposed role for this sequence in the recognition of terminator codons. Biochem J 141: 609-15
the ribosome as seen by cryo-electron microscopy. Nat Struct Biol 10: 899-906
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