THE FUNCTIONAL ROLE OF ASP50 IN PROTEIN SYNTHESIS ELONGATION FACTOR TU

by

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ABSTRACT

THE FUNCTIONAL ROLE OF ASP50 IN PROTEIN SYNTHESIS ELONGATION FACTOR TU

Elongation factor Tu is an essential enzyme responsible for delivering aminoacyl-tRNA's to ribosomal A-site during protein synthesis. In its active GTP-bound state, it forms an EF-Tu · GTP · aminoacyl-tRNA ternary complex whereas it is inactive in the GDP-bound state. One very unstable and dynamic structural element in the nucleotide binding domain of EF-Tu is the Switch I region, whose structure changes from a β-sheet to an α-helix during the transition from the GTP- to GDP-bound state of EF-Tu. In this thesis, the role of the universally conserved Asp50 residue in the Switch I region in *Escherichia coli* EF-Tu was studied. Mutants of Asp50 to alanine and asparagine were made in EF-Tu and mutant EF-Tu's were studied in their interactions with GDP, GTP and aminoacyl-tRNA, as well as in their activity in poly(Phe) synthesis, GTP hydrolysis and dipeptide formation using *in vitro* kinetic assays. D50A and D50N mutants of EF-Tu were comparable to wild-type EF-Tu with respect to GDP binding and GDP/GTP nucleotide exchange rates. However, the Asp50 mutants have significantly reduced affinity for aminoacyl-tRNA. When analyzed in the presence of high excess of aminoacyl-tRNA, Asp50 mutants were comparable to wild-type EF-Tu in their activity in poly(Phe) synthesis. On the other hand, pre-steady state measurements of GTP hydrolysis and peptide bond formation indicated that the amount of GTP hydrolyzed by the mutants are significantly reduced compared to wild type EF-Tu, even though the rate of GTP hydrolysis was not altered. The results obtained from this work suggest that Asp50 is crucial for a tight GTP conformation of EF-Tu. When Asp50 is mutated, the enzyme easily switches to GDP conformation, explaining the reduced affinity of the Asp50 mutants in aminoacyl-tRNA binding. On the other hand, when ternary complexes with Asp50 mutants reach to the A-site on the ribosomes, some mutant EF-Tu's may switch to their GDP-like conformation, therefore, releasing the aminoacyl-tRNA, which proceed to peptide bond formation without GTP hydrolysis.

ÖZET

ASP50 AMİNOASİDİNİN PROTEİN SENTEZİ ELONGASYON FAKTÖRÜ TU'NUN İŞLEVİNDEKİ ROLÜ

Elongasyon faktörü EF-Tu, protein sentezi sırasında aminoasil-tRNA'yı ribozoma taşımakla sorumlu temel enzimdir. EF-Tu, GTP ile bağlandığı etkin formunda, EF-Tu · GTP · aminoasil-tRNA şeklinde üçlü bir bileşik oluştururken, GDP bağlı formunda etkisizleşir. EF-Tu'nun GTP ve GDP bağlı halleri arasındaki yapısal geçiş sırasında, çok hareketli ve kararsız bir yapıda bulunan Switch I alt bölgesi, şeklini β-sheet'den α-helix'e çevirir. Bu tez çalışmasında, *Escherichia coli* EF-Tu'sunda Switch I bölgesinde bulunan Asp50 aminoasidinin rolü çalışılmıştır. Bu amaçla EF-Tu üzerinde 50. pozisyonda bulunan aspartik asit yerine alanin ve asparajin mutantları yaratılıp bu EF-Tu mutantlarının, GDP, GTP ve aminoasil-tRNA ile ilişkilerinin yanısıra poli(Phe) sentezi, GTP hidrolizi ve dipeptid oluşumundaki aktiviteleri de *in vitro* kinetik deneylerle incelenmiştir. EF-Tu'nun D50A ve D50N mutantları, GDP bağlaması ve GDP/GTP nükleotid değişim hızları açısından doğal EF-Tu'ya benzemektedir. Ancak, Asp50 mutantlarının aminoasil-tRNA ile bağlanmalarında ciddi bir azalma gözlemlenmiştir. Çok yüksek miktarlarda aminoasil-tRNA varlığında incelendiğinde, Asp50 mutantları poli(Phe) sentezini doğal EF-Tu ile karşılaştırılabilinir ölçülerde yerine getirebilmiştir. Öte yandan, GTP hidrolizi ve dipeptid oluşumu açısından incelendiklerinde, GTP hidrolizleme hızları fark göstermemekle birlikte, EF-Tu mutantlarının hidrolizleyebildiği toplam GTP miktarlarının doğal EF-Tu'ya nazaran önemli oranda düşmüş olduğu gözlemlenmiştir. Bu tez çalışmasının sonuçları doğrultusunda Asp50 aminoasidinin kararlı bir EF-Tu.GTP yapısının sağlanması için mutlak gerekliliği olduğu öngörülmüştür. Asp50 aminoasidi mutasyona uğratıldığında, enzimin GDP konformasyonununa geçişinin kolaylaştığı, böylece mutantların aminoasil-tRNA ile bağlanabilmesinin neden zorlaştığı da anlaşılabilmektedir. Öte yandan Asp50 mutantları ile oluşan EF-Tu · GTP · aminoasil-tRNA bileşikleri ribozomun A bölgesine ulaştığında, mutant EF-Tu'ların bazıları GDP yapılarına geri dönebilir, dolayısıyla GTP hidrolizine gerek kalmadan aminoasil-tRNA'yı bırakabilir ve serbest kalan aminoasil-tRNA dipeptid oluşumuna olanak sağlıyor olabilir.

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LIST OF SYMBOLS

bp	Base Pairs
°C	Centigrade degree
fs	Femtosecond
g	Gravity
gr	Gram
kDa	Kilodalton
L	Liter
M	Molar
mA	Milliamper
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
ng	Nanogram
pmol	pico mole
s	Second
V	Volt
µg	Microgram
µl	Microliter
α	Alpha
β	Beta

LIST OF ACRONYMS / ABBREVIATIONS

aa	Amino acid
aa-tRNA	aminoacyl tRNA
A	Alanine
ADP	Adenosine 5'-diphosphate
APS	Ammonium persulphate
ATP	Adenosine 5'-triphosphate
BSA	Bovine serum albumin
D	Aspartic acid
DNA	Deoxyribonucleic acid
DTE	1,4-Dithioerythritol
EDTA	Ethylenediaminetetraacetate
EF-G	Elongation factor G
EF-Ts	Elongation factor Ts
EF-Tu	Elongation factor Tu
GDP	Guanosine 5'-diphosphate
GTP	Guanosine 5'-triphosphate
IPTG	Isopropyl-ß-D-1-thiogalactopyranoside
LB	Luria-Bertani broth
Met	Methionine
Mg^{2+}	Magnesium
mRNA	Messenger RNA
N	Asparagine
N-terminal	Amino terminal
P_i	Inorganic phosphate
PMSF	Phenylmethylsulfonylfluoride
PEP	Phosphoenolpyruvate
RNA	Ribonucleic acid
SDS	Sodiumdodecylsulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TEMED	N,N',N',N'-Tetramethylethylenediamine

Tris	Tris(hydroxylmethyl)aminomethane
tufA	Elongation factor Tu gene
tRNA	Transfer RNA
UV	Ultra Violet

1. INTRODUCTION

1.1. Protein Synthesis

Ribosome is the protein synthesizing machinery in both prokaryotic and eukaryotic cells. Translation of the genetic information from nucleotide sequences carried on messenger RNA (mRNA) to amino acid sequences of proteins being synthesized takes place on this large ribonucleoprotein complex with the help of transfer RNA (tRNA) molecules and several translational protein factors. tRNA molecules act as molecular adaptors in reading the nucleotide triplets (codons) on the mRNA through base pairing with their anticodon stem-loops and carry the amino acid coded by this mRNA codon on their 3'CCA terminal. The ribosome has three binding pockets for tRNA, called the A, P and E sites. The A-site accepts the incoming aminoacyl-tRNA (aa-tRNA) based on the codon-anticodon base pairing between the mRNA and aa-tRNA. When this selection is completed, the amino acid at the 3'-CCA terminal of the aa-tRNA enters the peptidyl transfer center on the ribosome. At the peptidyl transfer center, transpeptidation occurs between the adjacent peptide on the peptidyl-tRNA residing at the P-site and the amino acid on the incoming aa-tRNA residing at the A-site. As a result, amino acid terminus of the incoming tRNA shifts to the P-site, whereas the anticodon loop is still at the A site. This state of the aa-tRNA on the ribosome is called the A/P state. In the next step, the anticodon stem-loop of the aa-tRNA translocates from the A site to the P-site of the ribosome. In parallel with this movement, the deacylated tRNA, which was previously located in the P-site and become empty after transpeptidation, is now pushed to the E-site from which it leaves the ribosome. During the translocation process, the interaction between the tRNA and the mRNA is kept uninterrupted, therefore, the mRNA is also pushed by one codon with this movement (Spirin, 2002). As a result, a new codon to be translated enters the A site of the ribosome and the search for the correct aa-tRNA starts again. This elongation cycle repeats itself until the end of the message on the mRNA defined by stop codons.

During the elongation phase of protein synthesis, a protein factor carries the aa-tRNA to the ribosome's A-site. This essential protein is called the elongation factor Tu

(EF-Tu) in prokaryotic systems (synonym of EF1α, in eukaryotes eEF1α). This enzyme is the main focus of this thesis.

1.2. Role of EF-Tu in Protein Synthesis

EF-Tu is a guanine nucleotide binding protein (G-protein). Like other G-proteins, the activity of EF-Tu is dependent on whether a GDP or a GTP molecule is bound to the protein. In its GTP bound state, EF-Tu is active. EF-Tu · GTP forms a stable ternary complex with aa-tRNA and carries the aa-tRNA molecules to the ribosome's A-site (Krab and Parmeggiani, 1998).

EF-Tu · GTP · aa-tRNA ternary complex binds to the A/T hybrid site of the ribosome and acts as an intermediate in the binding of the aa-tRNA to the A-site of the ribosome. When the cognate codon-anticodon interaction occurs between the aa-tRNA and the mRNA on the ribosome's A-site, ribosome triggers the GTPase activity on EF-Tu. The ribosomal induced GTPase activity of EF-Tu is stimulated by the 50S subunit of the ribosome whereas the 30S subunit applies a modulatory effect on EF-Tu (Krab and Parmeggiani, 1998). GTP hydrolysis drastically changes the EF-Tu structure and facilitates the release of EF-Tu from the ribosome (Yokosawa *et al.*, 1973). When the aa-tRNA is released from EF-Tu, the amino acid terminus of the tRNA moves towards to peptidyl transfer center while the rest of it remains in the A-site of the ribosome. This state is called the A/P state. In the peptidyl transfer center on the ribosome, an ester bond forms between the amino group of the amino acid on the aa-tRNA and the carboxyl group of the polypeptide being synthesized on the peptidyl-tRNA. The EF-Tu · GDP, released from the ribosome, goes into an EF-Tu cycle where it releases the GDP molecule and charges with a new GTP molecule with the help of its nucleotide exchange factor elongation factor EF-Ts (Krab and Parmeggiani, 2002).

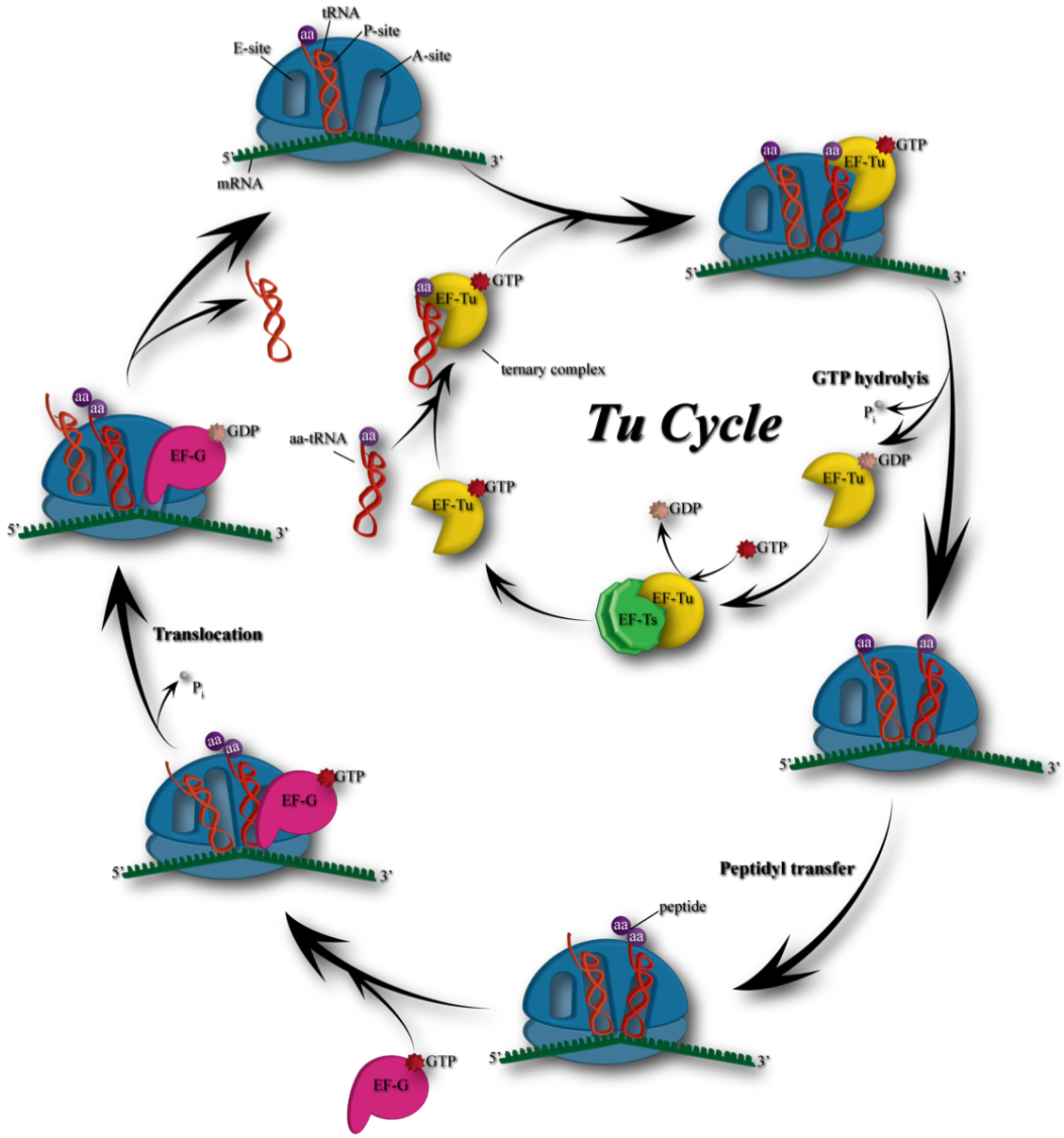

Figure 1.1. Elongation cycle of protein synthesis in bacteria.

1.3. Functional Studies on EF-Tu

Numerous *in vitro* studies have been made on purified EF-Tu protein in order to understand the mechanism of action of this enzyme in protein synthesis. From these studies, it is now known that EF-Tu binds to GDP about a hundred times stronger than GTP (Fasano *et al.*, 1978, Parmeggiani *et al.*, 1987). Comparison of the dissociation rates (k_d) of the guanine nucleotide binding G-domain with GTP and GDP with those of the corre-

sponding intact EF-Tu complexes showed that affinity to GDP and GTP was nearly the same on the G-domain (Parmeggiani *et al.*, 1987). It is also known that chelation of Mg^{2+} ion reduces the affinity for GDP by thousand folds, which shows the importance of the Mg^{2+} ion on nucleotide binding. The presence of guanine nucleotide also seems to be important for the stability of EF-Tu. When the guanine nucleotide is removed from the EF-Tu, the enzyme loses its activity by about 10% every hour (Krab and Parmeggiani, 1998).

Since EF-Tu has a high affinity to GDP, the release of GDP without any nucleotide exchange factors, is as slow as 0.011 s^{-1} (Ruusala *et al.*, 1982). Therefore, EF-Tu requires nucleotide exchange factor elongation factor Ts (EF-Ts). In the presence of EF-Ts, the GDP dissociation rate from EF-Tu increases for about 2000 times (Ruusala *et al.*, 1982). The dissociation of GDP via EF-Ts results in a stable complex of EF-Tu · EF-Ts, and this complex can dissociate by the binding of GTP or GDP molecule to EF-Tu. Since the GTP concentration inside a cell is about 1 mM, about ten times higher than GDP, the dissociation of EF-Tu · EF-Ts complex is driven towards the formation of EF-Tu · GTP binary complex (Krab and Parmeggiani, 1998). The EF-Ts catalysis of GDP/GTP exchange on EF-Tu has a rate of 30 s^{-1}, therefore, is not the rate-limiting step in EF-Tu function in protein synthesis (Ruusala *et al.*, 1982).

Previous studies show that EF-Tu · GTP preferentially interacts with aa-tRNA rather than free tRNA. The affinity increases about 10^5-fold with aminoacylation compared to deacylated tRNA (Miller and Weissbach, 1974, Pingoud *et al.*, 1982). Dissociation constant for aa-tRNA from EF-Tu · GTP is about 5 nM but can vary about 10-fold depending of the nature of the amino acid (Abrahamson *et al.*, 1985, Fasano *et al.*, 1978).

Like all GTP-binding proteins, EF-Tu has a low intrinsic GTPase activity at a rate about 0.29 hr^{-1} (Krab and Parmeggiani, 1999b). Ribosome acts as the GTPase activating protein for EF-Tu. When the anticodon of aa-tRNA matches the mRNA codon; ribosome induces GTP hydrolysis on EF-Tu. EF-Tu structure changes drastically resulting in the aa-tRNA release in EF-Tu · GDP conformation so that the aa-tRNA can participate in peptide bond formation (Krab and Parmeggiani, 1998, Krab and Parmeggiani, 2002). The rate of the ribosome induced GTPase activity of EF-Tu is about 100 s^{-1} (Bilgin *et al.*, 1992).

1.4. Structure of EF-Tu

EF-Tu in prokaryotic cells has a molecular mass between 40 and 45 kDa. In *Escherichia coli*, its molecular mass is 43.1 kDa (Kjeldgaard and Nyborg, 1992, Krab and Parmeggiani, 2002). EF-Tu consists of three domains, where the first domain contains both α-helices and β-strands, whereas second and third domains consist of β-barrel structures (Song *et al.*, 1999). The N-terminal end of Domain I has six β-strands surrounded by six α-helices, which is a shared structure among all guanine nucleotide binding proteins. The GDP bound state is called the "off-state" and the GTP bound state is the "on-state" of the protein since GTP molecule activates the EF-Tu, and the conformation of Domain I changes between these two states. Domain II and Domain III acts as rigid objects during this conformational change of the protein (Krab and Parmeggiani, 2002).

There are two switch regions in EF-Tu structure that set the limits of the guanine nucleotide-binding site. In order to form a bond between the guanine nucleotides and the EF-Tu molecule, a Mg^{2+} ion that is bound to the β- and γ- phosphates of GTP or β- phosphate of GDP molecule is required (Krab and Parmeggiani, 1998, Krab and Parmeggiani, 2002). The Switch I region consists of residues 50-61 in *Escherichia coli* EF-Tu. This region switches its structure. When EF-Tu is in its on-state it has one α-helix turn, whereas in its off-state this conformation switches to a short antiparallel β-ribbon structure as indicated in Figure 1.2 (Song *et al.*, 1999).

It is known that residues Thr25 and Thr61 directly bind to the Mg^{2+} ion and involved in the nucleotide-Mg^{2+} coordination (Krab and Parmeggiani, 1999b). Also residues Asp50 and Asp80 interact with two water molecules that is bound to Mg^{2+} ion and play an indirect role in the nucleotide-Mg^{2+} coordination (Krab and Parmeggiani, 2002). Mutational studies on EF-Tu showed that mutations at the Thr61 residue decrease the intrinsic GTPase activity of EF-Tu; in contrast, the Asp80 mutants have high intrinsic GTPase activities (Krab and Parmeggiani, 1999b). Thr25 residue was also studied and it was shown that the intrinsic GTPase activity of the Thr25 mutant was reduced by 90% compared to the wild type protein (Krab and Parmeggiani, 1999a).

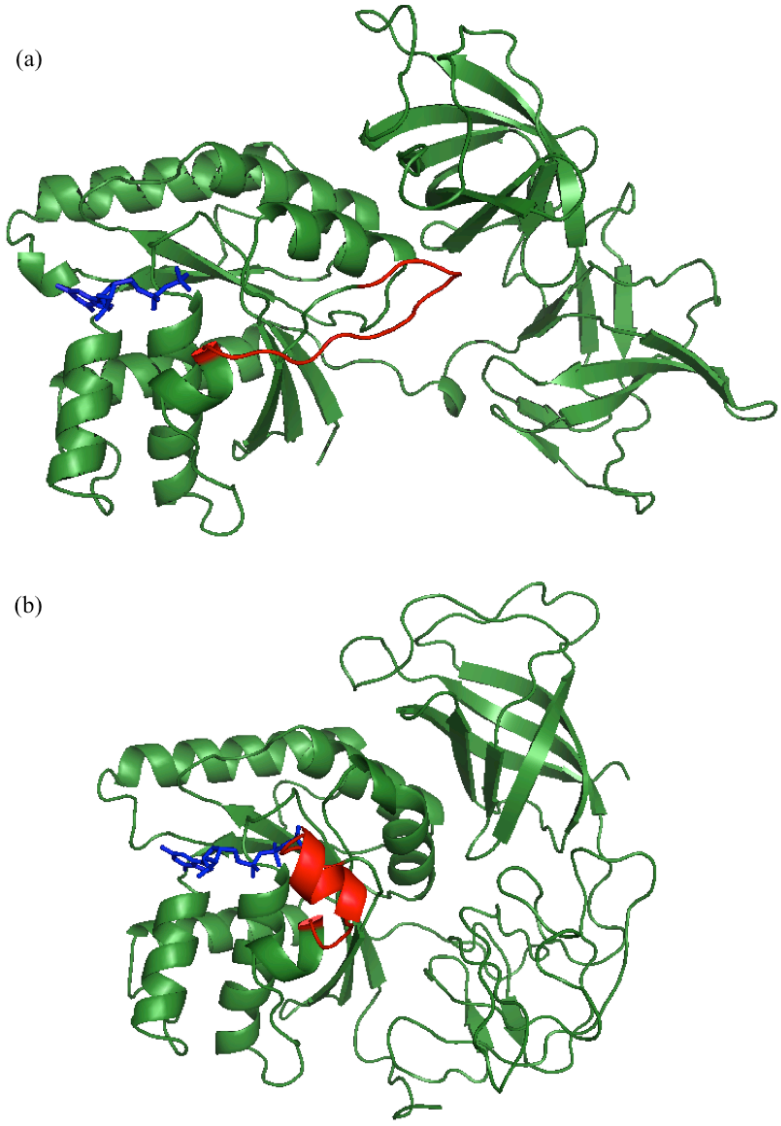

Figure 1.2. Structure of EF-Tu in its GDP (a) and GTP (b) bound states.Switch I region indicated in red and guanine nucleotide in blue color (PDB ID: 1TUI and 2FX3).

Another universally conserved residue, histidine, at 66th position is also located right next to Asp50 in both EF-Tu · GTP and EF-Tu · GTP · aa-tRNA structure. H66A mutation in EF-Tu destabilizes ternary complex (Chapman *et al.*, 2012). These findings emphasize the importance of this region for ternary complex formation.

The importance of the Switch I region located at the GTPase domain of EF-Tu was studied in this thesis work. In particular, the role of the aspartic acid residue located at the 50th position in *Escherichia coli* EF-Tu, which is located in the beginning of the Switch I

region was investigated. This aspartic acid residue is universally conserved in EF-Tu sequences throughout all kingdoms of life (Figure 1.3). Furthermore, this residue is also conserved in *Escherichia coli* EF-G (residue 50), which has a structurally similar GTPase domain with a functionally similar switch region (Kolesnikov and Gudkov, 2002). The structure of the Switch I region changes between the active (GTP-bound) and inactive (GDP-bound) states of EF-Tu (Clark *et al.*, 1999). Asp50 is found to be highly mobile during GTP to GDP transformation on EF-Tu as observed by molecular dynamics simulations (Gül, 2008). Molecular dynamics simulations on the crystal structure of EF-Tu · GTP revealed that the negative charge on the Asp50 and also its size is crucial for the activity of EF-Tu (Gül, 2008).

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                  ....|....| ....|....| ....|....| ....|....| ....|....|
                      50         60         70         73         83
E.Coli            FDQIDNAPEE KARGITINTS HVEYDTP--- ----TRHYAH VDCPGHADYV
T.Aquaticus       YGDIDKAPEE RARGITINTA HVEYETA--- ----KRHYSH VDCPGHADYI
P.Troglodytes     AWVLDKLKAE RERGITIDIS LWKFETS--- ----KYYVTI IDAPGHRDFI
H.Sapiens         AWVLDKLKAE RERGITIDIS LWKFETS--- ----KYYVTI IDAPGHRDFI
F.Catus           AWVLDKLKAE RERGITIDIS LWKFETS--- ----KYYVTI IDAPGHRDFI
M.Musculus        AWVLDKLKAE RERGITIDIS LWKFETS--- ----KYYVTI IDAPGHRDFI
G.Gallus          AWVLDKLKAE RERGITIDIS LWKFETS--- ----KYYVTI IDAPGHRDFI
D.Rerio           AWVLDKLKAE RERGITIDIA LWKFETS--- ----KYYVTI IDAPGHRDFI
D.Melanogaster    AWVLDKLKAE RERGITIDIA LWKFETA--- ----KYYVTI IDAPGHRDFI
C.Elegans         AWVLDKLKAE RERGITIDIA LWKFETA--- ----KYYITI IDAPGHRDFI
S.Cerevisiae      AWVLDKLKAE RERGITIDIA LWKFETP--- ----KYQVTV IDAPGHRDFI
A.Thaliana        AWVLDKLKAE RERGITIDIA LWKFETT--- ----KYYCTV IDAPGHRDFI
O.Japonica        AWVLDKLKAE RERGITIDIA LWKFETT--- ----KYYCTV IDAPGHRDFI
E.Coli_EF-G       AATMDWMEQE QERGITITSA ATTAFWSGMA KQYEPHRINI IDTPGHVDFT
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Figure 1.3. Alignment of EF-Tu (EF-1α in eukaryotes) amino acid sequences around the Switch I region. Switch I residues were shaded in yellow and universally conserved Asp50 showed in red. Amino acids numbered according to *Escherichia coli* sequences.

In fact, Asp50 resides in a region, which can be cleaved by trypsin (Arai *et al.*, 1976). Trypsin cleaves EF-Tu from three amino acids, Arg44, Arg58 and Lys56. Since the cleavage rate of arginines are faster than lysine, the tryptic cleavage of EF-Tu results in a loss of 14 amino acid long polypeptide between residues Arg44 and Arg58 including the conserved Asp50 residue. This cleavage totally disrupts Switch I. Earlier functional studies on trypsin-cleaved EF-Tu were failed to observe any ternary complex formation with aa-tRNA in nitrocellulose filter assays (Arai *et al.*, 1976, Jurnak *et al.*, 1977). Later,

Wittinghofer *et al.*, (1980) reported that trypsin cleavage only reduces the affinity of EF-Tu·GTP to aa-tRNA by using a different assay based on deacylation of aa-tRNA which is not protected by EF-Tu. Masuda *et al.*, (1985) and Ott *et al.*, (1990), on the other hand, used a ribonuclease A protection assay and reported a reduced ternary complex formation ability for EF-Tu after trypsin cleavage.

Crystal structure of the ternary complex indicates that aa-tRNA interacts with all three domains of EF-Tu including the intersection of the three domain interfaces and the GTPase switch regions (Nissen *et al.*, 1995). Both the tryptic cleavage data and the results of this thesis work approve the interaction between Switch I region and the aa-tRNA.

2. PURPOSE

Protein synthesis requires several protein factors including elongation factor Tu, which delivers aa-tRNA to the ribosome from a ternary complex of EF-Tu · GTP · aa-tRNA. EF-Tu is a GTPase protein that contains a conserved GTP binding domain that is similar through all translational GTPase factors from prokaryotes to higher eukaryotes. Even though atomic structures of EF-Tu in complex with all its ligands (GDP, GTP, aa-tRNA and EF-Ts) are known, how GTP hydrolysis is triggered on this enzyme remains to be elucidated. The Switch I region in GTP binding domain of EF-Tu named after its role in switching the protein between its active and inactive states by changing its structure from β-sheets to α-helix. But the triggering mechanism of this conformational switch and GTP hydrolysis is unknown.

The purpose of this thesis is to gather biochemical data in order to understand the role of Switch I region in EF-Tu function. In particular, the function of the universally conserved Asp50 residue in *Escherichia coli* EF-Tu was studied by altering this conserved residue using site directed mutagenesis. Asp50 mutants to alanine and asparagine were made in EF-Tu and the mutant EF-Tu's were studied in their interactions with GDP, GTP and aa-tRNA, as well as their activity in poly(Phe) synthesis, GTP hydrolysis and dipeptide formation were measured in *in vitro* kinetic assays.

3. MATERIALS

3.1. Bacterial Strains

Escherichia coli JM109 (DE3) (Promega, USA) strain is used for the expression of *tufA* gene for both wild type and mutant EF-Tu's. The genotype of the *Escherichia coli* JM109 (DE3) strain is *end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 (r_k^-, m_k^+), *rel*A1, *sup*E44, λ–, Δ*(lac-pro*AB*),* [F′, *tra*D36, *pro*AB, *lac*I^qZΔM15], lDE3.

3.2. Chemicals

Table 3.1. Chemicals used in this study.

Acetic Acid, Glacial	Merck, USA
Acrylamide–N,N'-methylene-bisacrylamide (37.5:1) 40 %	Applichem, Germany
Acrylamide–N,N'-methylene-bisacrylamide (19:1) 40 %	Sigma Aldrich, USA
Adenosine 5'-triphosphate (ATP)	Sigma Aldrich, USA
Agar	Oxoid, United Kingdom
Ammonium persulphate (APS)	Saveen, Sweden
Ampicillin	Sigma Aldrich, USA
β-Mercaptoethanol	Merck, USA
Bromophenol Blue	Merck, USA
Coomassie Brilliant Blue (R-250)	Sigma Aldrich, USA
Dithioerythritol (DTE)	Fluka BioChemika, USA
Guanosine 5'-diphosphate (GDP)	Sigma Aldrich, USA
Guanosine 5'-triphosphate (GTP)	Sigma Aldrich, USA
Isopropyl-ß-D-thiogalactopyranoside (IPTG)	Promega, USA
L-Glutathione reduced	Sigma Aldrich, USA
Methanol	Merck, USA

Table 3.1. Chemicals used in this study (cont.).

N,N,N',N'-Tetramethyl-ethylenediamine (TEMED)	Sigma Aldrich, USA
Phenol Red	Sigma Aldrich, USA
Phenylmethylsulfonylfluoride (PMSF)	Sigma Aldrich, USA
Phospho(enol)pyruvate (PEP)	Sigma Aldrich, USA
Protein Molecular Weight Marker	PageRuler™ Prestained Protein Ladder, Fermentas, USA
Putrescine	Sigma Aldrich, USA
Sodium Dodecyl Sulfate (SDS)	Merck, USA
Spermidine	Sigma Aldrich, USA
Tryptone	Oxoid, United Kingdom
Yeast Extract	Oxoid, United Kingdom

All other laboratory salts are analytical grade and obtained from Merck (USA), Sigma Aldrich (USA) or Riedel-de Haën (Germany).

3.3. Buffers and Solutions

3.3.1. Bacterial Growth

Table 3.2. Media used for bacterial growth.

LB Medium	0.5% (w/v) Yeast Extract 1% (w/v) Tryptone 0.5% (w/v) NaCl 0.2% (w/v) D-Glucose

Table 3.2. Media used for bacterial growth (cont.).

LB Agar	0.5% (w/v) Yeast Extract 1% (w/v) Tryptone 0.5% (w/v) NaCl 1.5% (w/v) Agar 0.2% (w/v) D-Glucose
SOC Medium	0.5% (w/v) Yeast Extract 2% (w/v) Tryptone 10 mM NaCl 2.5 mM KCl 10 mM $MgCl_2$ 10 mM $MgSO_4 \cdot 7H_2O$ 20 mM D-Glucose
TY2 medium	3% (w/v) Tryptone 1% (w/v) Yeast Extract 0.02% (w/v) $MgSO_4 \cdot 7H_2O$ 0.125% (w/v) NH_4Cl 0.299% (w/v) Na_2HPO_4 0.15% (w/v) KH_2PO_4 0.125% (w/v) $Na_2SO_4 \cdot 10H_2O$
Spare Elements (1000 X)	0.2% (w/v) $MgSO_4 \cdot 7H_2O$ 0.1% (w/v) $MnSO_4 \cdot 4H_2O$ 0.06% (w/v) $FeSO_4 \cdot 7H_2O$ 0.06% (w/v) Citric acid

3.3.2. Solutions for SDS-Polyacrylamide Gel Electrophoresis

Table 3.3. Solutions used for SDS-PAGE.

Stacking Gel	5% Acrylamide–N,N'-methylene-bisacrylamide (37.5:1) 60 mM Tris·PO_4 (pH 6.8) 0.1% (w/v) SDS 0.1% (v/v) TEMED 0.1% (w/v) APS
Separating Gel	0.5 M Tris-HCl (pH 8.9) 0.1% (w/v) SDS 0.1% (v/v) TEMED 0.1% (w/v) APS
Electrophoresis Running Buffer	28 mM Tris 220 mM Glycine 0.1% (w/v) SDS
5X Sample Buffer	5% (w/v) SDS 50 mM Tris·PO_4 (pH 6.8) 7 mM β-Mercaptoethanol 50% Glycerol
Protein Lysis Solution	167 mM Tris-HCl (pH 6.8) 0.33 M SDS 10% (w/v) Sucrose 350 mM β-Mercaptoethanol Trace amounts of Bromophenol Blue
Stain Solution	40% Methanol 10% Acetic Acid 0.2% (w/v) Coomassie Brilliant Blue (R-250)
Destain Solution	30% Isopropanol 10% Acetic Acid

3.3.3. Buffers for Column Chromatography

Table 3.4. Buffers used for chromatography.

Binding Buffer	50 mM Tris pH 7.6 (adjusted with HCl) 300 mM KCl 10 mM $MgCl_2$ 0.5 mM DTE 10 µM GDP 0.1 mM PMSF
Elution Buffer	50 mM Tris pH 8.0 (adjusted with HCl) 300 mM KCl 10 mM $MgCl_2$ 10 mM Glutathione 0.5 mM DTE 10 µM GDP 0.1 mM PMSF
P50 Buffer	20 % Glycerol 50 mM K-Phosphate (pH 7.5) 0.5 mM DTE 10 µM GDP 0.1 mM PMSF
Polymix Buffer	95 mM KCl 5 mM NH_4Cl 5 mM $MgAc_2 \cdot 4H_2O$ 0.5 mM $CaCl_2 \cdot 2H_2O$ 8 mM Putrescine 1 mM Spermidine 5 mM K-Phosphate (pH 7.5) 0.5 mM DTE

3.3.4. Stock Solutions for Non-Denaturing PAGE

Table 3.5. Solutions for non-denaturing PAGE.

Running Buffer	0.1 mM GTP 1 mM DTE in PAGE buffer
10X PAGE Buffer	100 mM MOPS 50 mM Mg acetate 650 mM NH_4 acetate 10 mM EDTA pH 6.65 (adjusted with KOH)
Polyacrylamide gel	10% Acrylamide–N,N'-methylene-bisacrylamide (19:1) 0.1% (w/v) APS 10 µM GTP 25 µM DTE 0.1% (v/v) TEMED in PAGE buffer

3.4. Equipment

Table 3.6. List of equipment used in this study.

Autoclave	Model MAC-601, Eyela, Japan Model ASB260T, Astell, UK
Balances	Mettler-Toledo ML54, Switzerland
Bead Beater	Model 1107900, Biospec Products, USA
Bioreactor	Applicon ADI 1010, Netherlands
Centrifuges	Beckman J2-MC Centrifuge, USA Beckman J2-21 Centrifuge, USA
Deep Freezers (-20°C)	Arçelik, TR

Table 3.6. List of equipment used in this study (cont.).

Deep Freezers (-80°C)	Sanyo Ultra Low, UK Thermo Scientific, USA
Electrophoresis Equipment	Mini-PROTEAN 3 Cell, BIO-RAD, USA
Ice Machine	Scotsman Inc. AF20, ITALY
Magnetic Stirrers	IKA and Heidolph, Germany
Micro-centrifuge	Eppendorf 5417R, Germany
Ovens	Gallenkamp 300, UK
pH Meter	Jenway 3010, USA
Pipettes	Pipetman, Gilson, USA
Pipettor	Pipetman, Gilson, USA
Power Supply	BMC workshop, Uppsala, Sweden
Refrigerator (4°C)	Arçelik, TR
Rotors	Beckman JS-7.5, USA Beckman JA-14, USA Beckman JA-20, USA
Spectrophotometer	Agilent 8453, USA Nanodrop
Vortex	IKA and Heidolph, Germany
Water Baths	Grant, UK Bibby, UK
Water Purification Systems	UHQ II Elga, UK

4. METHODS

4.1. Expression of *tufA* gene and Its Mutants in *Escherichia coli* JM109 (DE3) Cells

4.1.1. Preparation of *Escherichia coli* JM109 (DE3) Competent Cells

Escherichia coli JM109 (DE3) cells were streaked out on LB agar plates and cultured at 37°C for overnight. A single colony was selected from the plate and used for inoculation of a 2 ml liquid SOC medium. Cells were cultured at 37°C in an orbital shaker at 150 rpm for overnight. In the next day, the overnight culture was used for the inoculation of a 50 ml SOC medium with 1:100 dilution. The growth was monitored by measuring the absorbance at 5 nm at 30 minutes intervals. When the OD_{600} value was reached to 0.8, the cells were harvested by centrifugation at 1400 x g for 10 minutes. After its supernatant was discarded, the cells were suspended gently in 25 ml of ice-cold 50 mM $CaCl_2$, and were transferred to a 50 ml centrifuge tube. The suspension was left on ice for 30 minutes and then the cells were collected by centrifugation as above. Cells were suspended in 5 ml ice-cold 50 mM $CaCl_2$ containing 10% glycerol. 100 µl aliquots were placed into 1.5 ml sterile microcentrifuge tubes and the cells were dipped immediately into liquid nitrogen for quick freezing. Competent cells were stored at -80°C until use.

4.1.2. Transformation of *Escherichia coli* JM109 (DE3) Cells

Escherichia coli JM109 (DE3) competent cells (100 µl) were thawed on ice and 2 µl (about 100-200 ng) of plasmid DNA was added. The cells were left on ice for 30 minutes and then heat shocked at 42°C for one and a half minutes. The cells were then placed on ice for two minutes and 1 ml of liquid LB medium was added. The cells were incubated at 37°C for one hour. From this culture, 500 µl of cells were spread on LB plates containing ampicillin at a final concentration of 150 µg/ml in order to select the transformants. Plates were incubated overnight at 37°C. The next day, some of the appearing

colonies were once more re-streaked on LB-ampicillin plates in order to purify the transformants.

4.1.3. Harvesting *Escherichia coli* JM109 (DE3) Cells Expressing *tuf*A Gene

Escherichia coli JM109 (DE3) cells that were transformed with pGEX-TEV-*tuf*A plasmid were cultured in 50 ml liquid LB medium containing ampicillin (150 µg/ml) for overnight. Overnight culture (30 ml) was used for the inoculation of 3 L TY2 medium containing 150 µg/ml ampicillin and 1.3% (w/v) D-Glucose. Cells were grown at 37°C in a bioreactor with ~1000 rpm mixing and with continuous aeration. Cell growth was monitored by taking 1 ml samples from the culture and measuring the OD_{600} values. When the OD_{600} value is about 7.5, 1 ml of sample was taken to a 1.5 ml microcentrifuge tube and placed on ice (as a control for uninduced cells (no IPTG). IPTG was added to the rest of the culture to a final concentration of 1 mM and growth was continued for 3 hours at 37°C. After that, 1 ml of sample was taken to a 1.5 ml microcentrifuge tube placed on ice (as a positive control for induced cells with IPTG). The cells were harvested by centrifugation at 10000 x g for 20 minutes. After the supernatant is discarded, the cells were suspended in 300 ml of ice cold 0.9% NaCl solution and transferred into 50 ml centrifuge tubes. The samples were centrifuged under the same conditions, in order to wash the leftover growth medium. After this centrifugation, the cell pellet was kept frozen at -80°C until its use for EF-Tu purification.

4.1.4. Extraction of Proteins from Cells for Expression Analysis on SDS-PAGE

Samples taken from the cell culture before and after the IPTG induction (1 ml each) were centrifuged at 14000 x g for five minutes using a microcentrifuge. The supernatants were discarded and the cell pellets were suspended in 100 µl of 10% Trichloroacetic acid (TCA). The suspensions were incubated on ice for 10 minutes in order to allow the precipitation of proteins and then centrifuged at 14000 x g for two minutes. The supernatants were discarded carefully by using a micropipette and 1 ml of acetone was added to each pellet. Samples were vortexed and centrifuged once more at 14000 x g for two minutes. The supernatants were discarded and the samples were heated to 60°C until the pellets dry.

100 µl of protein lysis solution was added to each sample. Samples were vortexed and stored at -20^{o}C until electrophoresis.

4.2. SDS - Polyacrylamide Gel Electrophoresis (PAGE)

For SDS-PAGE analysis, 10 µl of each sample was mixed with 2.5 µl 5X Sample Buffer and boiled at 95°C for two minutes. The samples were then applied to the wells of an SDS-PAGE gel (10% polyacrylamide (37.5:1) gel with a 4% stacking gel) and electrophoresed at 50 mA until the marker dye reaches to the bottom. After electrophoresis, the gel was stained with the Coomassie Blue staining solution for about one hour with mild agitation at 50 rpm on a rotary shaker. Samples were destained using destain solution for overnight.

4.3. Purification of EF-Tu Protein

4.3.1. Preparation of the Cell Lysates

About 30 g of frozen *Escherichia coli* JM109 (DE3) cells that expressed EF-Tu were thawed on ice and suspended in about 120 ml of binding buffer. Nitric acid washed and sterilized glass beads (0.1 mm in diameter) were also washed with ice-cold binding buffer. The cell suspension was mixed with an equal volume of beads in the 80 ml vessel of the Bead-Beater (Biospec Products) for cell disruption. Bead-Beater vessel was placed into an ice-chamber to keep the samples ice-cold throughout the runs. The Bead-Beater was run for 30 seconds for three times. Between each run, samples were allowed to cool for two minutes. The beads were allowed to precipitate at the bottom of the vessel and the liquid phase was transferred to 50 ml centrifuge tubes. This procedure was repeated until all the cell suspension was used. The cell lysate was transferred to centrifuge tubes and centrifuged at 30000 x g for 30 minutes at 4°C. The supernatant was transferred to new centrifuge tubes and centrifugation repeated once more to clear the supernatants further. Supernatants were saved, combined and the total volume of this S30 fraction was recorded.

4.3.2. GST Affinity Chromatography

5 ml GSTrap HP (GE Healthcare) column was first equilibrated with binding buffer at a speed of 1 ml/min. After equilibration, the S30 fraction of the cell lysate was applied to the column at a speed of 0.5 ml/min. About 80 ml of binding buffer was passed through the column until the OD_{280} value reaches to the base line. After the column was washed, the EF-Tu protein was eluted with an elution buffer that contains 10 mM glutathione during which 2 ml fractions were collected. All processes were conducted at 4°C. Fractions were kept on ice and 10 µl from each fraction was analyzed by SDS-PAGE and the fractions containing EF-Tu were pooled.

4.3.3. TEV Protease Digestion of the GST Tag

After pooling the fractions containing EF-Tu, its approximate protein concentration was determined spectrophotometrically from the OD_{280} value of the sample and the total amount of protein was calculated. TEV protease was added at a 1:100 ratio (w/w) of TEV protease/total protein of the pooled fraction. This sample was dialyzed (Spectra/Por 2, 12-14 KDa MWCO, Spectrum Labs) against 1 L binding buffer at 4°C for overnight. On the next day, both samples from dialysis and the undigested sample were analyzed electrophoretically on SDS-PAGE in order to check whether the GST tag was cleaved from the EF-Tu protein.

4.3.4. Removal of the GST Tag

The TEV digested and dialyzed sample was re-applied to the very same 5 ml GSTrap HP (GE Healthcare) which was re-equilibrated with the binding buffer at a speed of 0.5 ml/min. The flow-through fractions (2 ml each) were collected as TEV-cleaved EF-Tu. The GST column was further washed with 50 ml of binding buffer and GST tag was eluted by passing about 20 ml elution buffer at 1 ml/min speed. From each fraction, 10 µl of samples were analyzed electrophoretically by SDS-PAGE. The EF-Tu containing frac-

tions were pooled and kept on ice until it is further purified by size exclusion chromatography. All chromatography processes were performed at 4°C.

4.3.5. Size Exclusion Chromatography

The fractions from the second GSTrap chromatography were concentrated using Vivaspin 20 (30 kDa MWCO, Sartorius Stedim) concentrators. Centrifugations were done at 4°C at 4600 x g. The concentrated sample (in about 5 ml) was applied to AcA 44 (Ultragel) gel filtration column (120 cm in length, 2.4 cm in diameter), which is equilibrated with P50 buffer. Elution speed was 20 ml/h and 10 ml fractions were collected. Samples were analyzed electrophoretically by SDS-PAGE and EF-Tu containing fractions were pooled and concentrated appropriately using Vivaspin 20 as described above. All steps were performed at 4°C.

4.3.6. Dialysis with Polymix Buffer and Storage of EF-Tu

Concentrated EF-Tu sample was dialyzed against polymix buffer containing 10 μM GDP and 0.1 mM PMSF overnight at 4°C using Spectra/Por 2 Dialysis Membrane (12-14 kDa MWCO, Spectrum Labs). Next day, the dialysis was continued for about three to four hours after changing with freshly made buffer. After dialysis EF-Tu samples were stored at -80°C in 100 μl aliquots.

4.4. Bradford Assay

The total protein concentration of the purified EF-Tu proteins were determined by Bradford assay (Bradford, 1976). In order to obtain a standard curve, a series of dilutions from a 1 mg/ml stock solution of Bovine Serum Albumin (BSA) between 1 μg to 8μg protein in a final volume of 100 μl were prepared in 0.15 M NaCl. To these protein samples 1 ml of Bradford reagent was added with 30 seconds intervals. Samples were vortexed and left at room temperature. After 10 minutes, OD_{595} values of the samples were measured again with 30 seconds intervals, so that all samples had exactly identical incubation times.

As a blank for spectrophotometry, 100 µl of 0.15 M NaCl solution in 1 ml Bradford reagent was used. In parallel with to the standard curve measurements, an appropriate dilution series of EF-Tu was also prepared in 0.15 M NaCl in a final volume of 100 µl and these samples were also processed as above. For both measurements, OD_{595} values were plotted as a function of BSA or EF-Tu in volumes. The slopes of the lines were compared. By using the known concentration of the BSA and the dilution ratios, the total protein concentration of the EF-Tu samples were calculated.

4.5. GDP Binding Assay

EF-Tu concentration which is capable of binding GDP was determined using GDP binding assay (Ehrenberg *et al.*, 1990a). In this assay, different volumes of EF-Tu (from 0 to 10µl) were added to a final volume of 100 µl in polymix buffer containing 30 nmol [^{3}H]-GDP (from a 5 mM stock, specific activity ~6 cpm/pmol). The samples were incubated at 37°C for 15 minutes in order to allow full exchange of GDP on EF-Tu, then the tubes were placed on ice. After adding about 4 ml of ice-cold polymix buffer, the samples were filtered quickly through nitrocellulose filters (Protran BA 85, Schleicher & Schuell). The filters were washed twice with ~4 ml ice-cold polymix buffer and then placed into scintillation vials. In order to measure the specific activity, two samples, with a 5 µl or a 10 µl of [^{3}H]-GDP (from the 5mM stock used above) pipetted directly on filters were used. Vials containing filters were dried at 110°C for 15 min, cooled and 4 ml of scintillation cocktail was added to the vials. The radioactivity on the filters was counted by using a liquid scintillation counter (Packard Instruments, USA). The specific activity (as cpm/pmol) of the [^{3}H]-GDP was calculated using the measured radioactivity (as cpm) and the known amount of the [^{3}H]-GDP (in pmols) of the samples taken directly onto filters from 5 mM [^{3}H]-GDP stock. Using this specific activity, the amount of [^{3}H]-GDP retained on the filters for each sample (in pmols) was calculated. The amount of GDP on the filters was plotted as a function of EF-Tu (in volumes). From which, the concentration of [^{3}H]-GDP on EF-Tu was determined.

4.6. Nucleotide Exchange Assay

Nucleotide exchange assay was performed as described earlier (Ehrenberg *et al.*, 1990a). Two EF-Tu mixes were prepared with about 500 pmol EF-Tu (in 48.5 µl). Here the amount of EF-Tu was calculated from the Bradford assay. The EF-Tu mixes contained also ~25 pmol [^{3}H]-GDP (45ci/mmol) in polymix buffer. To one of the EF-Tu mixes, 1500 pmol GDP was added (+GDP); to the other EF-Tu mix distilled H_2O was added (-GDP) in 1.5 µl. A charging mix in 50 µl containing 2 mM ATP, 20 mM PEP, 2 mM GTP, 5 µg pyruvate kinase, and 0.3 µg myokinase in polymix buffer was also prepared. All mixes were incubated separately at 37°C for 15 minutes. An equal volume of charging mix was added to the EF-Tu mix (-GDP) at 37°C. 20 µl of samples were taken at time points varying from 15 to 900 seconds and placed into microcentrifuge tubes on ice containing 20 µl of 20% HCOOH. The same procedure was repeated with the other EF-Tu mix containing 1500 pmol GDP (+ GDP).

All samples were centrifuged at 4°C at 14000 x g for 15 minutes. Supernatants were saved and kept at -20°C until use. From each sample, 10 µl of the supernatant was applied to thin layer plate (Polygram Cel^{3000} PI, Macherey-Nagel). To each spot, 1 µl of marker containing 10 mM GDP and 10 mM GTP was also applied. Thin layer chromatography buffer was 0.5 M KH_2PO_4 (pH 3.5). After about two and a half hours, when the buffer front reached the top of the plate, chromatography was stopped and the chromatography plate was completely dried using a hairdryer. The GDP and GTP spots were visualized under UV_{254} and marked with a pencil. Spots were cut from the plate and placed into scintillation vials. After adding 4 ml of scintillation cocktail to each vial, radioactivity of the samples was counted.

The ratios of $[\,^3\mathrm{H}]\mathrm{GDP}/([\,^3\mathrm{H}]\mathrm{GDP} + [\,^3\mathrm{H}]\mathrm{GTP})$ for each time point was calculated for (–) GDP samples ($\Delta r_0(t)$) and for (+)GDP samples ($\Delta r_1(t)$). The ratio at the 900 seconds sample was subtracted from all points as a background, since nearly all GDP on EF-Tu should have been replaced with a GTP after this time. Natural logarithms (*ln*) of the calculated ratios were plotted as a function of time. The dissociation constant (k_d) for GDP from EF-Tu was calculated from the slope of the lines (which should be the same for (-)

GDP and for (+) GDP samples). The total amount of GDP in each sample, on the other hand, was determined by using the formula: $G_T = \frac{G_0}{(\Delta r_0(t)/\Delta r_1(t))-1}$ where G_T is the total amount of GDP and G_0 was the amount of added GDP (1500 pmol). From the amount of GDP used in an assay, $G_T \times \Delta r_0(t)$ equation gives the amount of GDP on EF-Tu molecule in the assay. By dividing this number to the total volume of the EF-Tu added to the assay, the concentration of EF-Tu was calculated.

4.7. Gel-Shift Assay on Non-Denaturing PAGE for Ternary Complex Formation Between EF-Tu · GTP · Phe-tRNA^{Phe}

Gel-shift assay was performed as described earlier (Bilgin and Ehrenberg, 1995). A non-denaturing polyacrylamide gel containing 5% polyacrylamide (19:1) in Gel-Shift Buffer with freshly added 10 µM GTP and 1mM DTE was prepared and casted into a 0.5 mm thick and 20 cm long gel plate. After polymerization, gel was kept at 4°C until electrophoresis. The gel was pre-run for one hour at 30 mA (~100V) at 4°C. During this time, 200 pmol of EF-Tu in polymix buffer containing 1 mM ATP, 10 mM PEP, 1 mM GTP, 5 µg pyruvate kinase, 0.3 µg myokinase, 0.3 mM [^{14}C]-Phe (specific activity ~6 cpm/pmol), and 30 U of Phe-tRNA synthetase (PheRS) was mixed with tRNA^{Phe} in varying amounts between 0 to 400 pmol and incubated at 37°C for 15 minutes for ternary complex formation. After 15 minutes the samples were placed on ice. From these samples 20 µl was mixed with 2 µl of 50% glycerol containing traces of Bromophenol Blue as a marker. The samples were applied directly to non-denaturing PAGE gel and run at 50 mA (~150V) for two and a half hour. After electrophoresis, the gel was stained and destained as described for SDS-PAGE.

In order to calculate the amount of Phe-tRNA^{Phe} present in each sample, from the remaining of the samples left on ice after 15 minutes of incubation at 37°C, 20 µl was taken into tubes containing about 5 ml of 10% ice-cold TCA. The samples were filtered through GF/C (Whatman) filters. Filters were washed with 5 ml ice-cold 10% TCA twice and finally with 5 ml of ice-cold isopropanol. The filters were dried at 110°C, cooled and 4 ml of scintillation cocktail was added. The radioactivity retained on the filters was counted.

4.8. RNAse A Protection Assay

The dissociation constant of Phe-tRNA^{Phe} from EF-Tu was measured using RNAse A protection assay (Bilgin *et al.*, 1992). The EF-Tu · GTP · Phe-tRNA^{Phe} ternary complex was prepared in 90 µl in polymix buffer containing 0.3 mM [^{14}C]-Phe, 1 mM ATP, 10 mM PEP, 1 mM GTP, 5 µg pyruvate kinase, 0.3 µg myokinase, 10 U PheRS, 2 µM tRNA^{Phe}, and varying concentrations of EF-Tu (between 0.4 µM to 2 µM). Samples were incubated at 37°C for 15 minutes to allow aminoacylation and ternary complex formation and then placed to 20°C. 20 units of RNAse A in 10 µl polymix buffer was added to the ternary complex and immediately 50 µl aliquots were taken out from this mixture into tubes containing 5 ml ice cold 10% TCA at time points between 10 seconds to 50 seconds. Samples were left on ice until filtering through GF/C (Whatman) filters. Filters were washed with 5 ml ice-cold 10% TCA twice and then with 5 ml of ice-cold isopropanol. Filters were placed into scintillation vials, dried at 110°C and 4 ml of scintillation mix was added to each vial. The radioactivity retained on the filters was counted, from which the total amount of intact Phe-tRNA was calculated. Since all free aa-tRNA was cleaved by RNAse A, the radioactivity retained on the filter corresponds to the amount of the ternary complex. For each concentration of EF-Tu, the amount of ternary complex was calculated from the radioactivity retained on the filters and plotted against time. From the extrapolation of the lines, fit for every concentration of EF-Tu, the total ternary complex present at time=0 was determined. K_D was determined using a Scatchard plot, in which 1/slope gives the K_D for ternary complex.

4.9. Determination of Binding Constant for Phe-tRNA^{Phe} to EF-Tu · GTP via Exchange of Differentially Labeled Phe Amino Acids

This assay measures the exchange of radioactivity on the aminoacyl tRNA in the ternary complex at 37°C. Ternary complex was initially formed by Phe-tRNA^{Phe} by using [^{14}C]-Phe. When Phe-tRNA^{Phe} was dissociated from the ternary complex, free Phe-tRNA^{Phe} can be deacylated by the action of Phe-tRNA synthetase and can be reacylated using [^{3}H]-Phe (Tapio *et al.*, 1990). An initial charging mix prepared in 50 µl polymix containing 0.6 mM [^{14}C]-Phe, 2 mM ATP, 20 mM PEP, 2 mM GTP, 5 µg pyruvate kinase, 0.3

μg myokinase, 4 U PheRS and 20 μM $tRNA^{Phe}$. The charging mix divided into two parts and for the first part 90 pmol [^{3}H]-Phe in 5 μl added and total volume adjusted to 100 μl with polymix buffer. After incubation of this mixture, which contains both [^{14}C]-Phe and [^{3}H]-Phe from the beginning, for 15 minutes at 37°C, the specific activity for [^{3}H]-Phe was determined (~~35 cpm/pmol). To the second part of the charging mix, varying concentrations of EF-Tu (between 2 μM to 10 μM for wild type and 7 μM to 40 μM for the mutants) added in 45 μl polymix buffer. The mix containing EF-Tu incubated at 37°C for 15 minutes until ternary complexes with [^{14}C] labeled Phe formed. Later 90 pmol [^{3}H]-Phe (preheated to 37°C) in 5 μl added in the mixture in order to initiate the reaction. The reaction times kept at 20 minutes for tubes containing 2 μM to 3μM wild type EF-Tu and 7 μM to 9 μM mutant EF-Tu. Other tubes kept for 40 minutes at 37°C in order to exchange [^{3}H]-Phe with [^{14}C]-Phe on ternary complexes. Reactions were stopped after adding 10% ice-cold TCA on tubes. The samples were filtered through GF/C (Whatman) filters and were processed and counted as described above.

During the reaction the [^{3}H]-Phe on ternary complexes increased exponentially: [[^{3}H]-Phe] = [[^{14}C]-Phe] x $(1 - e^{kt})$ where k is the average of deacylation rates k_f and k_b for free (T_f) and tRNA bound EF-Tu · GTP (T_b).

$$k = \frac{(k_f[T_f] + k_b[T_b]}{Total\ \text{tRNA}^{\text{Phe}}}$$

k_f determined in the absence of EF-Tu and k_b determined where EF-Tu is present at maximum concentration. The bound and free EF-Tu fractions for all samples were calculated and the dissociation constant K_D for Phe-$tRNA^{Phe}$ and EF-Tu was determined from the Scatchard plot of the experiment.

4.10. Poly(Phe) Synthesis Assays

Poly(Phe) synthesis was assayed for wild type and for mutant EF-Tu's in ribosome cycle as well as in EF-Tu cycle experiments (Ehrenberg *et al.*, 1990b, Tapio *et al.*, 1990).

In these assays the ribosome was initially programmed for protein synthesis on poly(U) and the reactions started by adding a factor mix containing EF-Tu · GTP · Phe-tRNA^{Phe} ternary complex. The reaction mixes also contained excess EF-G and EF-Ts in the presence of an energy pump to ensure full recharging of GTP and ATP from PEP, and the presence of excess Phe and Phe-tRNA synthetase to ensure re-aminoacylation of tRNA^{Phe}. In the ribosome cycle, the ribosome concentration and in the EF-Tu cycle, EF-Tu concentration was kept low at a rate-limiting level.

Burst assay (ribosome cycle) was performed as EF-Tu titrations at a constant ribosome concentration. An initial ribosome mix was prepared, in 50 µl polymix buffer containing 50 pmol ribosomes (20% active), 60 pmol N-Ac[^{3}H]-Phe-tRNA^{Phe} (specific activity ~240 cpm/pmol) and 20 µg poly(U). A Factor mix was prepared in 50 µl polymix buffer containing 0.6 mM [^{14}C]-Phe, 2 mM ATP, 20 mM PEP, 2 mM GTP, 5 µg pyruvate kinase, 0.3 µg myokinase, 200 U PheRS, 50 pmol EF-Ts, 90 pmol EF-G, 20 µM tRNA^{Phe} and varying amounts of EF-Tu between 10 to 300 pmol. Both mixes were separately incubated at 37°C for 15 minutes. The reactions were started by adding 50 µl of ribosome mix onto a 50 µl factor mix. Reaction times were varied over 9 to 35 seconds, depending on the EF-Tu concentration, in order to obtain the same length of poly(Phe) chains as the product. Reactions were stopped by adding 5 ml 10% ice-cold TCA on the reaction mixture.

EF-Tu cycle measurements were performed as ribosome titrations at a constant EF-Tu concentration. A ribosome mix was prepared, in 50 µl polymix buffer containing ribosomes between 50 to 500 pmol, Ac[^{3}H]-Phe-tRNA^{Phe} between 60-600 pmol (specific activity ~240 cpm/pmol) and 20-200 µg poly(U) mRNA. A factor mix was prepared, in 50 µl polymix buffer, same as in EF-Tu titrations above, but the amount of EF-Ts was increased to 1000 pmol and EF-Tu was kept at 10 pmol. Both mixes were pre-incubated at 37°C. Reactions were started by adding 50 µl factor mix onto 50 µl ribosome mix. The reaction times were varied between 14 to 33 seconds depending on the ribosome concentration and reactions were stopped by adding 5 ml 10% ice-cold TCA on samples.

For both experiments, the samples were boiled for 15 minutes, cooled and filtered through Whatman glass fiber (GF/C) filters. Filters were washed twice by 5% TCA and

ones with isopropanol and placed into scintillation vials. The filters were dried for 30 minutes at 110°C, cooled and 4 ml of scintillation mix with solubilizer was added to each vial. The radioactivity retained on the filters was counted. [^{3}H] counts used for determining the number of poly(Phe) chains formed and [^{14}C] counts used for the total Phe added on the chains. The ratio of these values gives the chain lengths, which are about 25 amino acids in these conditions, and their division by time equals to the rates of poly(Phe) synthesis.

4.11. Rate of GTP Hydrolysis on EF-Tu and Dipeptide Formation

Because ribosome induced GTP hydrolysis on EF-Tu and dipeptide formation are fast, the rates of GTP hydrolysis and dipeptide formation were measured using a quench-flow equipment. Quench flow experiments were performed as described in (Bilgin *et al.*, 1992) and (Johansson *et al.*, 2008) with some modifications. 70S ribosomes were prepared with f[^{3}H]-Met-tRNA^{fMet} in the P site and a Phe codon (UUU) programmed on the A site, by incubation of 200 pmol MRE600 70S ribosomes, 300 pmol initiation factor 1 (IF1), 150 pmol initiation factor 2 (IF2), 300 pmol initiation factor 3 (IF3), 400 pmol mRNA (MFTI) and 400 pmol f[^{3}H]Met-tRNA^{fMet} in 50 µl polymix buffer containing 1 mM ATP, 10 mM PEP, 1 mM GTP, 2.5 µg pyruvate kinase and 0.15 µg myokinase for 15 minutes at 37°C. A factor mix was also prepared in 40 µl polymix buffer containing 0.4 mM Phe, 2000 or 8000 pmol tRNA^{Phe}, 2 mM ATP, 10 mM PEP, 40 U Phe-tRNA synthetase (PheRS), 2.5 µg pyruvate kinase and 0.15 µg myokinase. A third EF-Tu mix prepared in 10 µl which contains 600 pmol EF-Tu, 600 pmol [^{3}H]-GDP. Initially EF-Tu and [^{3}H]-GDP was incubated at 37°C for 10 minutes in order to exchange cold GDP molecules on EF-Tu with [^{3}H]-labeled ones. At that time other components of the factor mix was also incubated at 37°C for 10 minutes for the aminoacylation of tRNA^{Phe}. The EF-Tu mix was added on the factor mix and the incubation continued for 15 minutes at 37°C to allow the formation of the ternary complexes. Using a temperature controlled quench-flow instrument (RQF-3, KinTek Corporation) at 37°C, equal volumes of ribosome and factor mixtures were mixed for the indicated reaction times and the reactions were quenched using formic acid (17% final concentration). Quenched samples, collected in microcentrifuge tubes were left on ice until the completion of all samples. Samples were centrifuged at 4°C at 14000 x g for 20 minutes. Supernatants and pellets were saved separately. The supernatants were applied to

a MonoQ ion exchange column (10 cm, 10 µm particle size, GE Healthcare, UK) connected to an HPLC in order to separate [^{3}H]-GDP from [^{3}H]-GTP. Radioactivity on the GDP and GTP peaks were counted by on-line scintillation counting (βRAM3, INUS) and analyzed by using Laura Lite 3 (Lablogic) software (Pavlov *et al.*, 2009). The pellets of the samples, on the other hand, were dissolved in 100 µl of 0.5 M KOH and incubated at 37°C for 10 minutes in order to solubilize amino acids and dipeptides from tRNAs. The samples were neutralized by the addition of 20 µl 100% formic acid. Samples were centrifuged again at 4°C at 14000 x g for 20 minutes to precipitate the deacylated tRNAs and proteins. The supernatants were applied to a C18 column (25 cm, 5µm particle size, Merck, USA) connected to an HPLC in order to separate f[^{3}H]-Met from f[^{3}H]Met-Phe. Radioactivity was monitored by on-line scintillation counting and analyzed as above.

In order to determine the amount of free GDP in the factor mix at the beginning of the reaction, a known amount of cold-GDP (about 360 pmol) was added into a factor mix for both wild type and Asp50 mutant EF-Tu and these samples were processed as for the other samples. Using the GDP / (GDP+GTP) ratio of the samples with or without GDP addition together with the known amount of cold-GDP added to (+GDP) samples, the amount of cold-GDP present in the (-GDP) samples can be calculated (Bilgin *et al.*, 1992). Multiplying the GDP / (GDP+GTP) ratio with the cold-GDP present in the assays gives the exact amount of GTP hydrolysis for each time point.

The probability of a EF-Tu molecule in GTP hydrolysis can be written as $g(t)=1-\frac{GDP(t)}{GDP(\infty)}$, where GDP(t) is the amount of GDP hydrolysis at any time point, whereas $GDP(\infty)$ is the amount of GDP hydrolyzed at long times, which can be obtained from a plot of GDP hydrolyzed as a function of time. The average time for GTP hydrolysis (τ_{GTP}) was calculated by using the integral $\tau_{GTP} = \int_0^\infty g(t)\, dt$. Since the GTP hydrolysis is a single exponential, the logarithmic plot of g(t) gives a straight line where the slope equals to $k_{GTP} = {}^1/_{\tau_{GTP}}$.

Similarly, the probability of ribosome in a state of peptidyl transfer can be written as h(t)=1-$\frac{\text{dip(t)}}{\text{dip}(\infty)}$. where dip(t) is the amount of dipeptide formed at any time point whereas dip(∞) is when it reaches to a plateau value at long times. which can be obtained from a plot of dipeptides as a function of time. The average time of peptidyl transfer was also calculated by using the integral $\tau_{dip} = \int_0^\infty h(t)\, dt$. The logarithmic plot of h(t) does not give a straight line since there are several steps passed during peptidyl transfer. In order to calculate the average dipeptide formation time, the integral of time delay in the short times ($0 \leq t \leq t_s$) calculated separately by using the integrate tool in OriginPro 8 SR4 software. For longer times ($t > t_s$), h(t) is approximated by equation $h(t) = H_0 \times e^{-k_H t}$ where the natural logarithmic plot gives the straight line (H_0 is the intercept of the y-axis and k_H is the slope of the straight line and t_s accepted as 10 ms from the experimental data) (Bilgin *et al.*, 1992). So the average time for peptidyl transfer is calculated from;

$$\tau_{dip} = \int_0^\infty h(t)\, dt = \int_0^{t_s} h(t)dt + \int_{t_s}^\infty (H_0 \times e^{-k_H t})dt = \int_0^{t_s} h(t)dt + \frac{H_0}{k_H} \times e^{-k_H t_s}$$

5. RESULTS

5.1. Expression of *tufA* Gene and Its Mutants in *Escherichia coli*

The *tuf*A gene in the pGEX vector is under the control of IPTG-inducible *tac* promoter. The *Escherichia coli* JM109 (DE3) cells hosting the pGEX-TEV-tufA plasmid efficiently express EF-Tu after IPTG induction (Figure 5.1). The molecular size of the protein appeared after IPTG induction is in accordance with the expected size of the recombinant protein (43.3 kDa EF-Tu + 27.5 kDa GST tag).

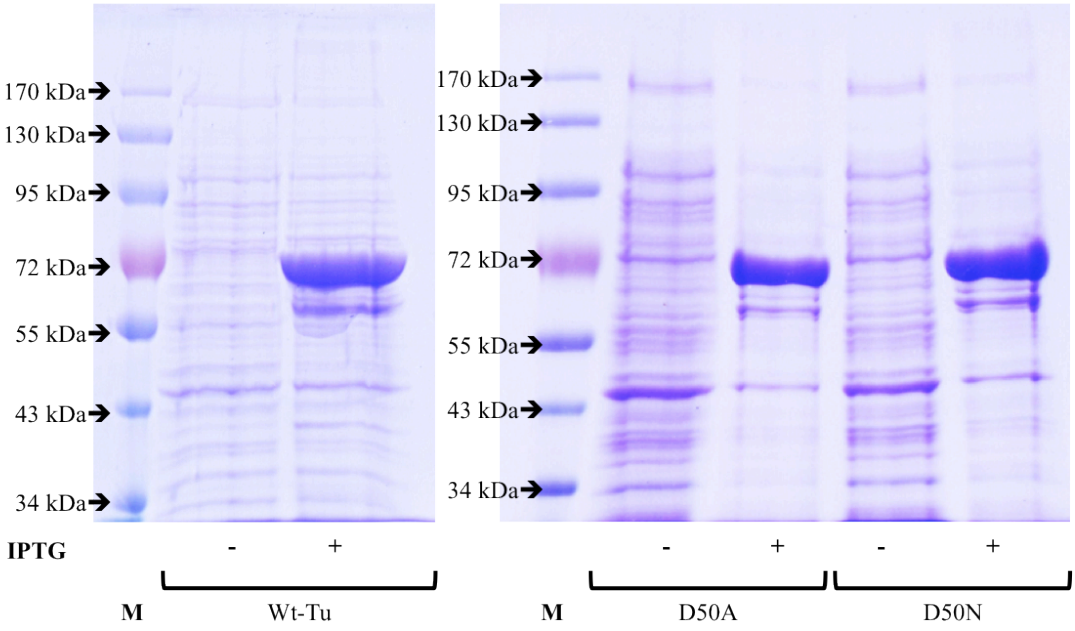

Figure 5.1. Overexpression of *tufA* gene in *Escherichia coli* (JM109 (DE3)) cells. (M, PageRuler SM#0671 protein marker)

5.2. Purification of EF-Tu Protein

Escherichia coli cells that have over expressed wild type EF-Tu and Asp50 mutants were purified via two GST-affinity chromatography steps followed by a size exclusion chromatography step. The purification profile of wild type EF-Tu was shown on SDS-PAGE in as a representative image for all purifications (Figure 5.2).

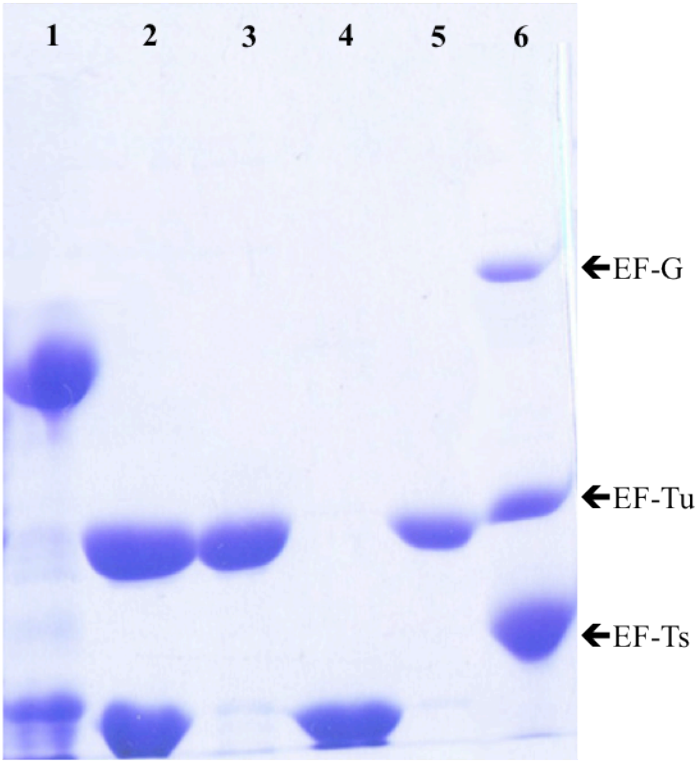

Figure 5.2. Purification profile of EF-Tu.(1, undigested protein, eluted from the first GST-affinity column with glutathione; 2, after TEV digestion; 3, flow-through from the second GST-affinity column after digestion; 4, glutathione wash of the second GST-affinity column after digestion, 5, after AcA 44 size exclusion column; 6, EF-G, EF-Tu and EF-Ts mix used as molecular size marker)

5.3. Dissociation Rate (k_d) of EF-Tu for GDP

Nucleotide exchange assay measures the concentration of the purified EF-Tu but also the dissociation rate (k_d) of EF-Tu for GDP (Ehrenberg *et al.*, 1990a). Since the presence of EF-Ts would dramatically change this k_d value, this experiment also verifies the absence of EF-Ts contamination in the purified EF-Tu. The concentrations of EF-Tu and its mutants were determined by three different assays. Bradford assay, GDP binding assay and nucleotide exchange assay were all comparable meaning that nearly 100% of the isolated protein is in its active form. Table 5.1 summarizes the k_d values measured for both the wild type EF-Tu and for the mutants. Asp50 mutations do not significantly alter the k_d values.

Table 5.1. Dissociation rates for EF-Tu molecules determined from nucleotide exchange assays.

	Wt-Tu	**D50A**	**D50N**
k_d (s^{-1})	0.013	0.016	0.011

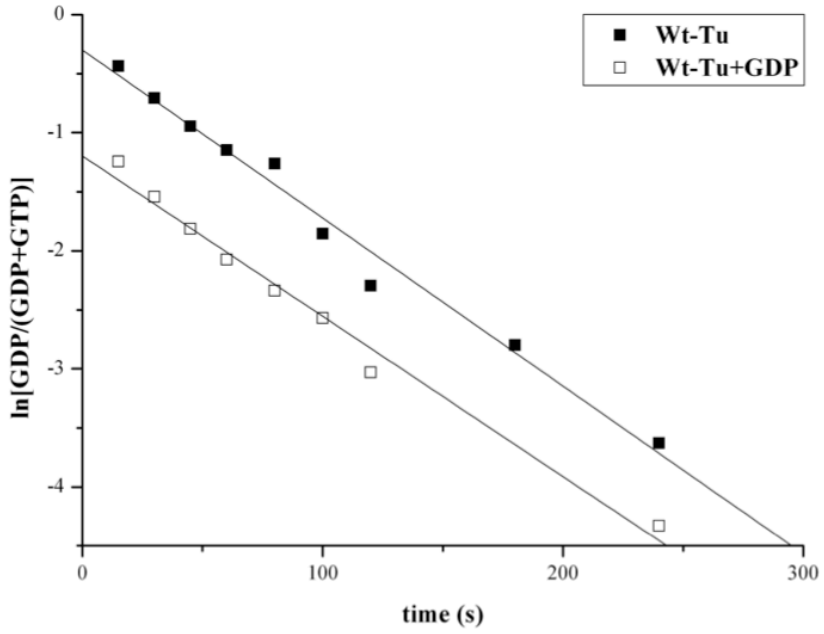

Figure 5.3. Nucleotide exchange on EF-Tu. Ln-plot of the fraction of GDP on EF-Tu as a function of time for (a) wild type, (b) D50A and (c) D50N mutant EF-Tu.

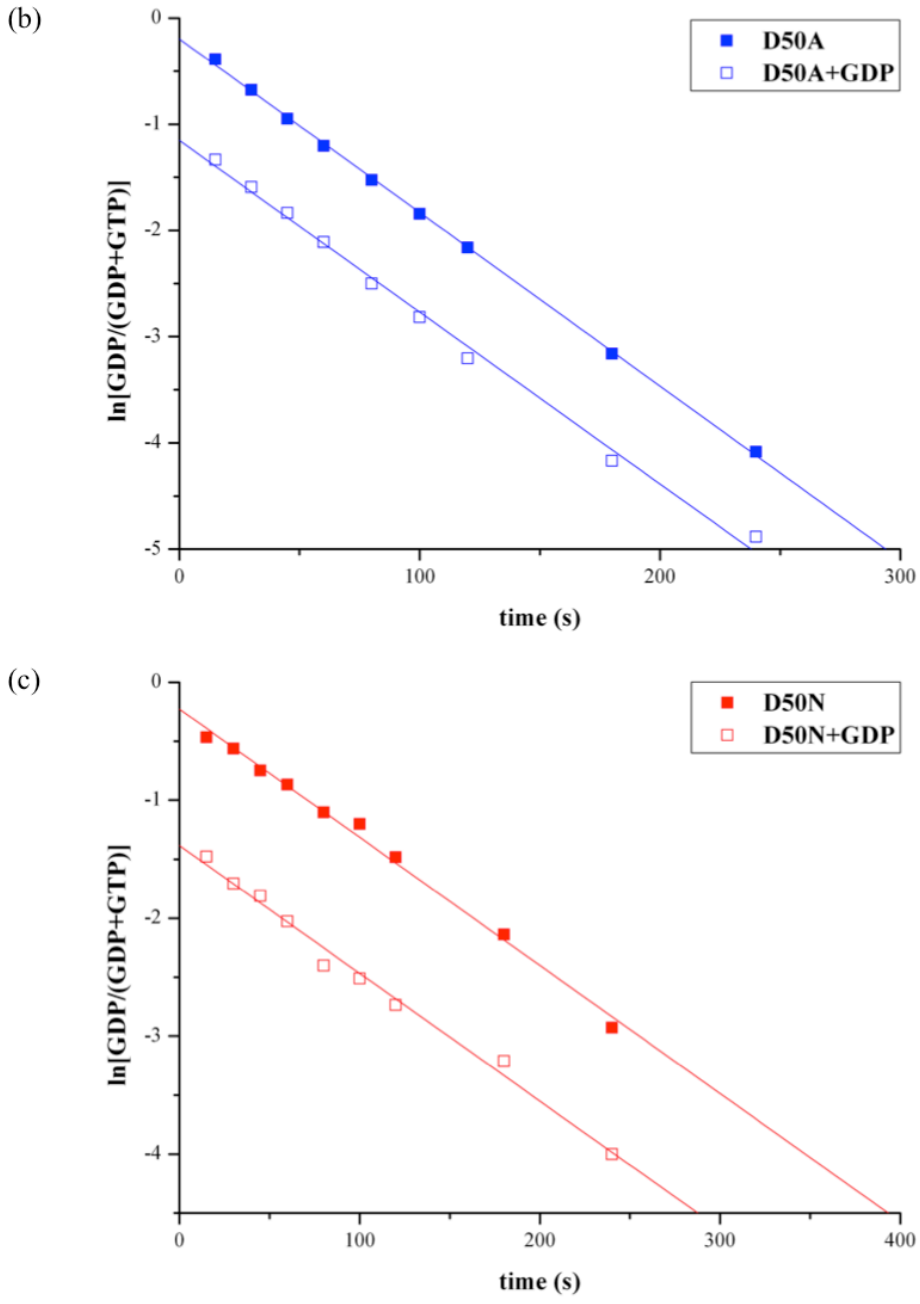

Figure 5.3. Nucleotide exchange on EF-Tu. Ln-plot of the fraction of GDP on EF-Tu as a function of time for (a) wild type, (b) D50A and (c) D50N mutant EF-Tu (cont.).

5.4. Ternary Complex Formation Between EF-Tu · GTP and Phe-tRNA^{Phe}

Non-Denaturing PAGE was used to analyze the ternary complex formation for both wild type and mutant EF-Tu's. In this experiment, since tRNA^{Phe} is negatively charged, the ternary complex has a higher mobility than free EF-Tu, therefore migrates faster in the gel. Figure 5.4 shows that while the wild type EF-Tu completely forms a stable ternary complex, the Asp50 mutants failed to do so. For both D50A and for D50N variants, a smear instead of a sharp band indicates that their ternary complexes are much less stable than the wild type under identical assay conditions.

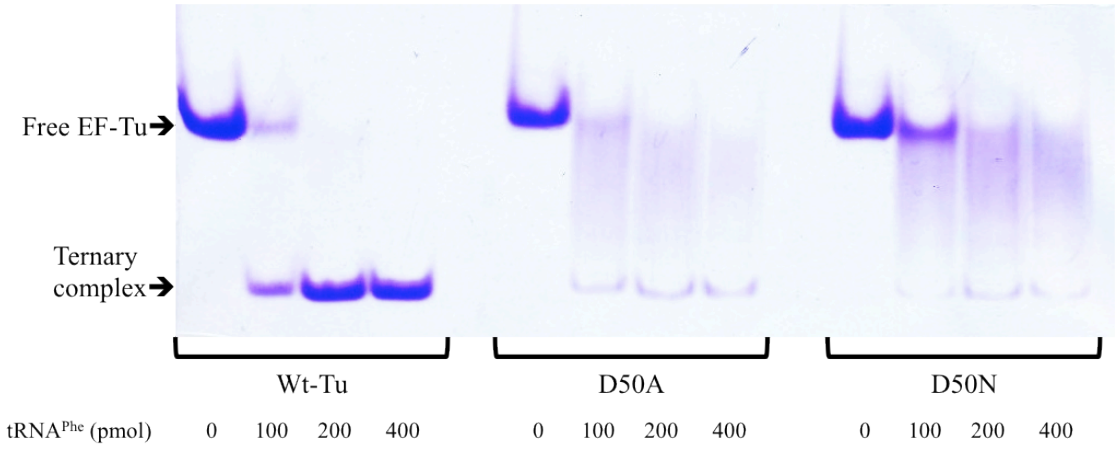

Figure 5.4. Ternary complex formation between EF-Tu · GTP · Phe-tRNA^{Phe}. Non-denaturing PAGE was performed as Phe-tRNA^{Phe} titrations in the presence of 200 pmol of EF-Tu.

5.5. Determination of Binding Constant for Phe-tRNA^{Phe} to EF-Tu · GTP

The dissociation rate of the EF-Tu · GTP · Phe-tRNA^{Phe} ternary complex was determined in two different ways at two different temperatures. The first method, RNAse A protection assay, was based on the observation that the aminoacyl end of a Phe-tRNA^{Phe} was protected in a ternary complex by EF-Tu against RNAse A digestion, whereas the dis-

sociated Phe-tRNA^{Phe} was digested by the enzyme. The dissociation rate of Phe-tRNA^{Phe} from EF-Tu is too fast to be measured at 37°C, therefore this experiment was performed at 20°C. The rates of dissociation of Phe-tRNA^{Phe} from EF-Tu (K_D) for Asp 50 mutants were determined from the Scatchard plots (Figure 5.5) and summarized in Table 5.2.

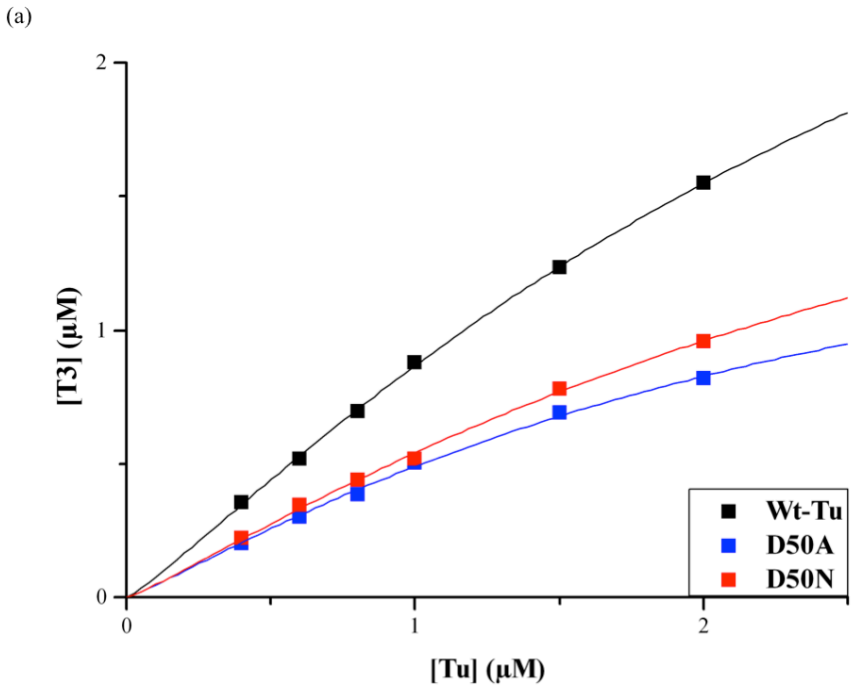

Figure 5.5. Determination of dissociation constants for Phe-tRNA^{Phe} and EF-Tu · GTP from RNAse A protection experiment at 20°C. (a) The concentration of ternary complex [T3] was plotted as a function of EF-Tu concentration [Tu]. (b) The Scatchard plot from (a). The inverse of the slope of the lines give the dissociation constant K_d, and the intercepts at the x-axis give the stoichiometry of the ternary complex.

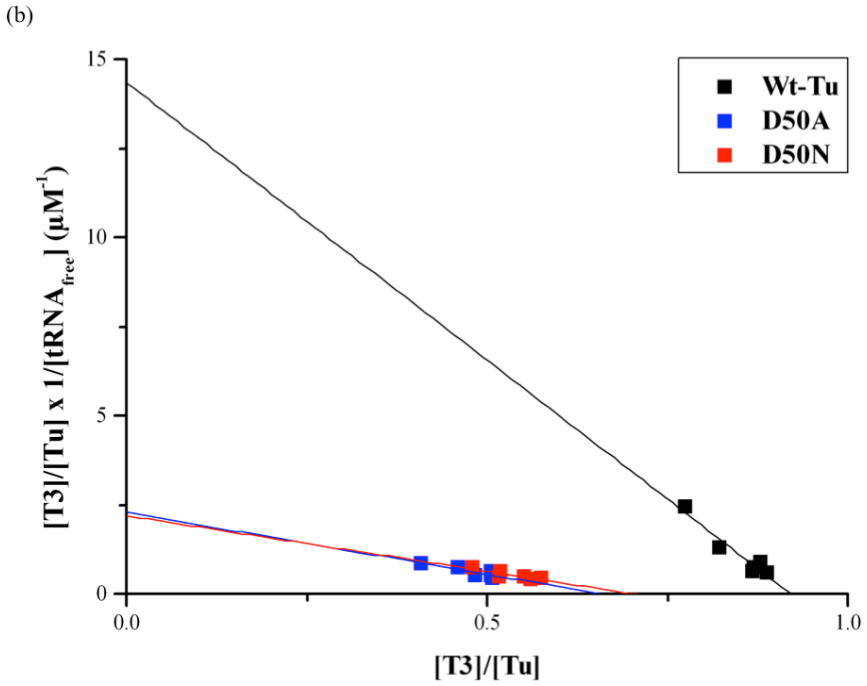

Figure 5.5. Determination of dissociation constants for Phe-tRNA^{Phe} and EF-Tu · GTP from RNAse A protection experiment at 20°C. (a) The concentration of ternary complex [T3] was plotted as a function of EF-Tu concentration [Tu]. (b) The Scatchard plot from (a). The inverse of the slope of the lines give the dissociation constant K_d, and the intercepts at the x-axis give the stoichiometry of the ternary complex (cont.).

Table 5.2. The dissociation constant of Phe-tRNA^{Phe} from ternary complex as determined from RNAse A protection experiments for wild type and mutant EF-Tu.

	Wt-Tu	**D50A**	**D50N**
***K*$_D$ (µM)**	0.060 ± 0.006	0.313 ± 0.035	0.324 ± 0.035

The second method for the determination of K_D is based on the exchange of Phe-tRNA^{Phe} on the ternary complex in the presence of two differentially labeled Phenylalanine amino acids in the mixture that can aminoacylate tRNA^{Phe}. The ternary complex was initially formed with [^{14}C]-Phe and than [^{3}H] labeled Phe was added on the mixture in high amounts. Since the exchange of radioactivity on the ternary complex is dependent on the dissociation of the complex, the rate of dissociation was measured from the exchange of radioactivity. This experiment performed at 37°C and dissociation rates were summarized in Table 5.3.

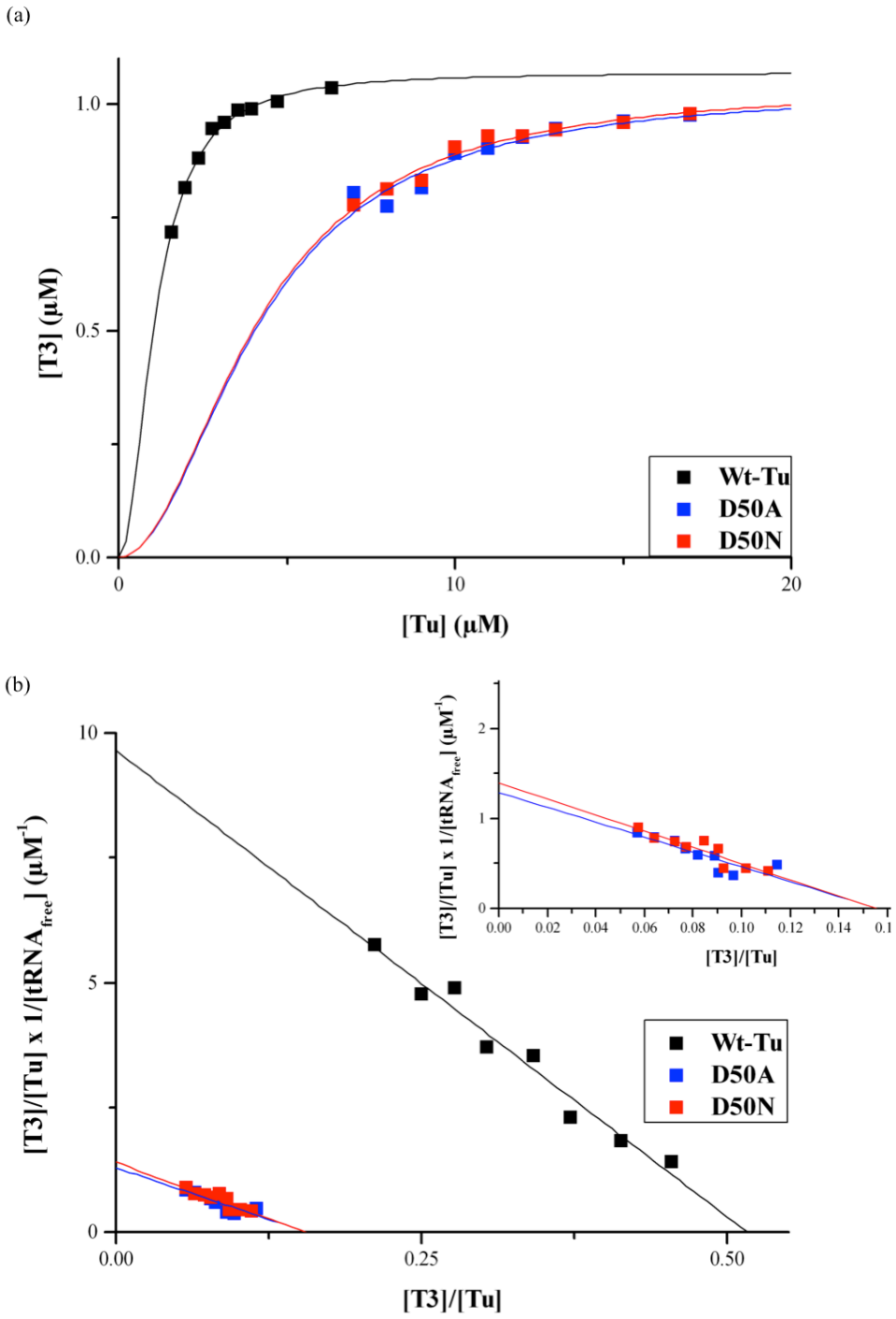

Figure 5.6. Determination of dissociation constants for Phe-tRNA^{Phe} and EF-Tu · GTP from deacylation protection experiments at 37°C. (a) The concentration of ternary complex [T3] was plotted as a function of EF-Tu concentration [Tu]. (b) The Scatchard plots from (a). The inverse of the slope of the lines give the dissociation constant K_d, and the intercepts at the x-axis give the stoichiometry of the ternary complex.

Table 5.3. The dissociation constant of Phe-tRNA^{Phe} from ternary complex as determined from EF-Tu protection of aminoacylated tRNA^{Phe} experiments for wild type and Asp50 mutants.

	Wt-Tu	**D50A**	**D50N**
K_D **(μM)**	0.050 ± 0.006	0.115 ± 0.009	0.120 ± 0.013

5.6. Poly(Phe) Synthesis Assays

EF-Tu titrations were made at fixed and rate limiting concentration of ribosomes. The extend of poly(Phe) synthesis as a function of EF-Tu was shown in Michaelis-Menten and Eadie-Hofstee plots (Figure 5.7). Comparisons of the k_{cat} and k_{cat}/K_m values for Asp50 mutants with wild type EF-Tu were given in Table 5.4. No significant difference was observed between wild type EF-Tu and the Asp50 mutants.

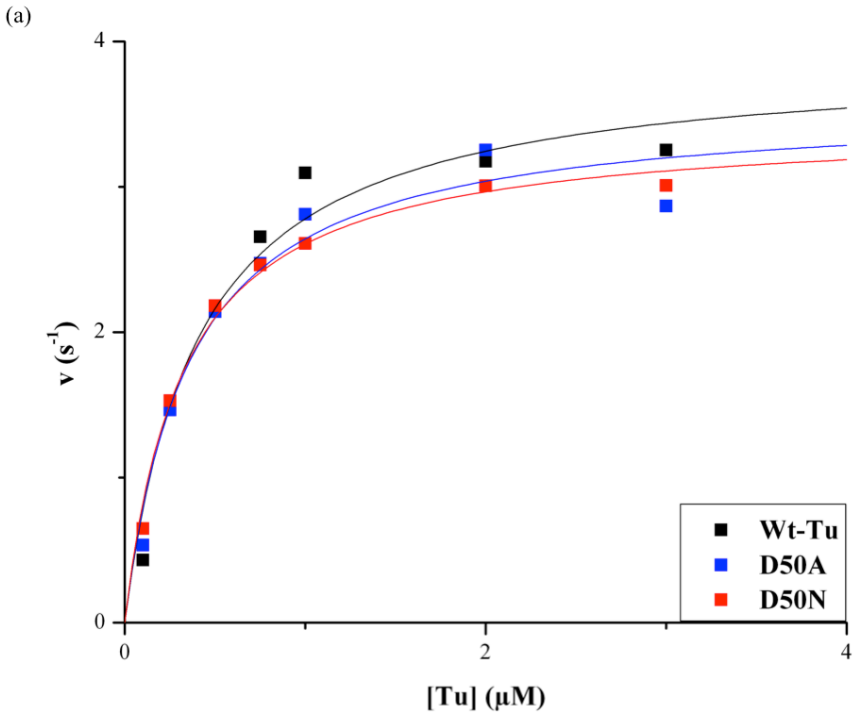

Figure 5.7. EF-Tu titration at rate limiting ribosome concentration in poly(Phe) synthesis. (a) Michaelis-Menten (b) Eadie-Hofstee plots. The rate for the ribosome cycle (k_{cat}) for EF-Tu and Asp50 mutants were obtained from the intercept at the ordinate axis of the Eadie-Hofstee plot. The intercept at abscissa gives the second order rate constant k_{cat}/K_m for the association of EF-Tu to the ribosome.

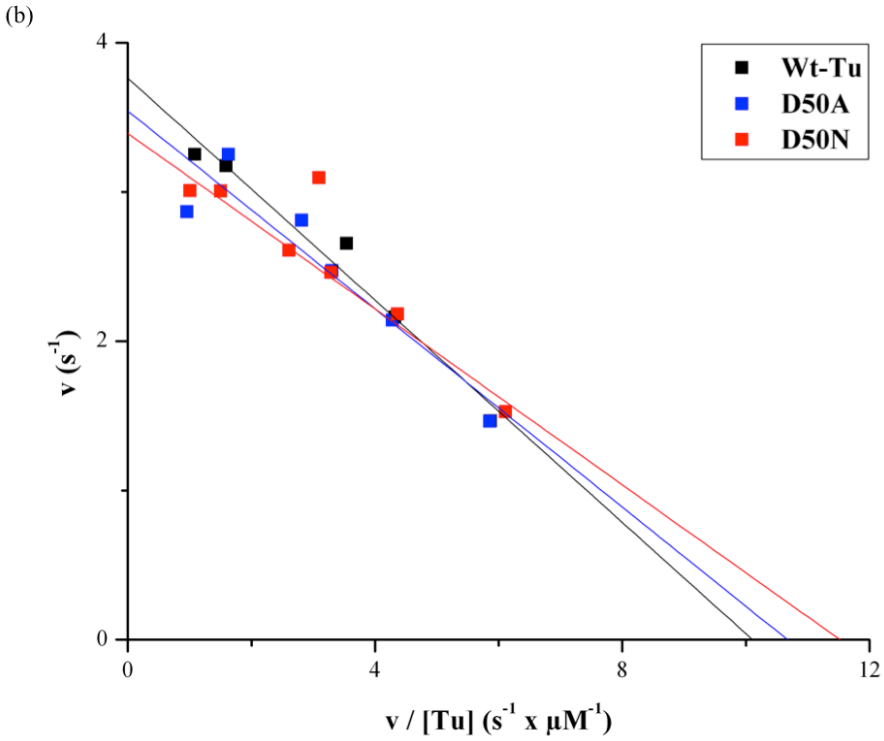

Figure 5.7. EF-Tu titration at rate limiting ribosome concentration in poly(Phe) synthesis. (a) Michaelis-Menten (b) Eadie-Hofstee plots. The rate for the ribosome cycle (k_{cat}) for EF-Tu and Asp50 mutants were obtained from the intercept at the ordinate axis of the Eadie-Hofstee plot. The intercept at abscissa gives the second order rate constant k_{cat}/K_m for the association of EF-Tu to the ribosome (cont.).

Table 5.4. k_{cat} and k_{cat}/K_m values for ribosome cycle in the presence of wild type and Asp50 mutants of EF-Tu.

	Wt-Tu	**D50A**	**D50N**
k_{cat} (s^{-1})	3.61 ± 0.21	3.62 ± 0.11	3.50 ± 0.15
k_{cat}/K_m ($\mu M^{-1} \times s^{-1}$)	9.94 ± 0.24	10.7 ± 0.03	11.8 ± 0.39

Poly(Phe) synthesis was performed at limiting and fixed concentrations of EF-Tu as ribosome titrations in the presence of excess amounts of EF-G and EF-Ts in order to measure the maximal cycling rate of wild type and mutant EF-Tu's. The results of these experiments were given as Michaelis-Menten and Eadie-Hofstee plots (Figure 5.8). No differences in these kinetic parameters were observed between wild type and the mutant EF-Tu's.

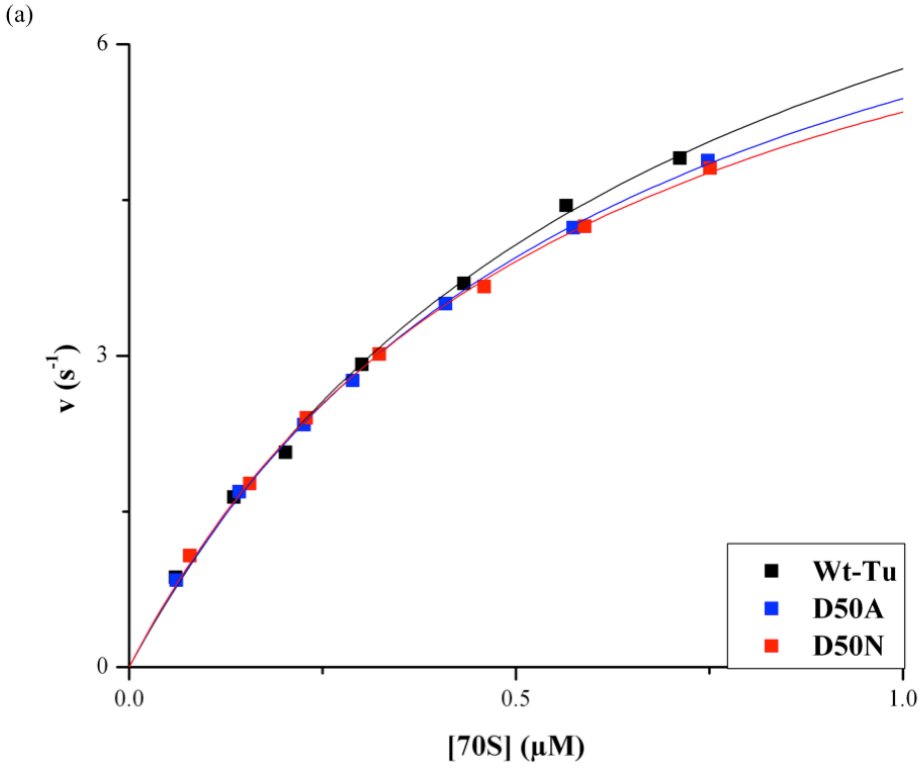

Figure 5.8. Ribosome titrations at rate limiting EF-Tu concentration in poly(Phe) synthesis. (a) Michaelis-Menten (b) Eadie-Hofstee plots. The intercept at the ordinate axis of the Eadie-Hofstee plot gives the maximal cycling rate of EF-Tu (k_{cat}) and the intercept at the abscissa gives the second order association rate constants for EF-Tu to the ribosome (k_{cat}/K_m).

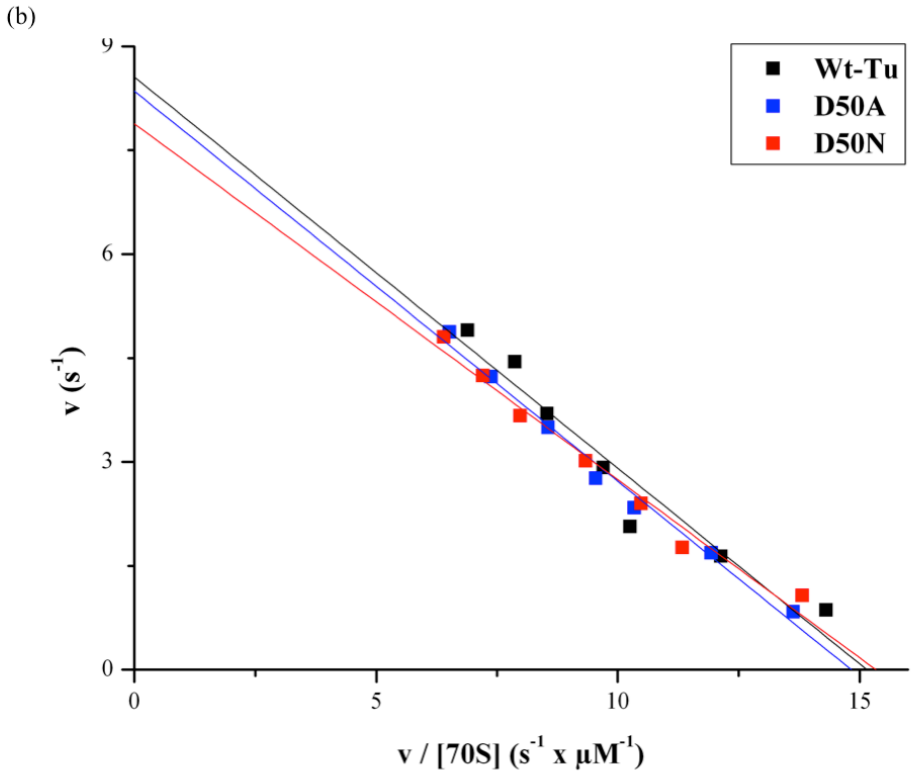

Figure 5.8. Ribosome titrations at rate limiting EF-Tu concentration in poly(Phe) synthesis. (a) Michaelis-Menten (b) Eadie-Hofstee plots. The intercept at the ordinate axis of the Eadie-Hofstee plot gives the maximal cycling rate of EF-Tu (k_{cat}) and the intercept at the abscissa gives the second order association rate constants for EF-Tu to the ribosome (k_{cat}/K_m) (cont.).

Table 5.5. k_{cat} and k_{cat}/K_m values for EF-Tu cycle for both wild type and Asp50 mutants of EF-Tu.

	Wt-Tu	**D50A**	**D50N**
$\boldsymbol{k_{cat}}$ **(s^{-1})**	8.93 ± 0.52	9.02 ± 0.94	9.08 ± 1.69
$\boldsymbol{k_{cat}/K_m}$ **(μM^{-1} x s^{-1})**	17.1 ± 2.52	15.9 ± 1.56	16.4 ± 1.53

5.7. Ribosome Induced GTP Hydrolysis on EF-Tu and Dipeptide Formation

The ribosome induced hydrolysis rate of GTP on EF-Tu as well as the rate of dipeptide formation on ribosomes were measured by using a quench-flow equipment at 37°C. Since Asp50 mutants are deficient in aa-tRNA binding, these measurements were performed at two different tRNA concentrations, 20 μM and 80 μM $tRNA^{Phe}$ (Figure 5.9 and Figure 5.10 respectively).

The rates of GTP hydrolysis were calculated from the *ln*(g(t)) vs. time graphs in which the slopes of the straight lines give the rate of GTP hydrolysis. The results were summarized in Table 5.6. Both Asp50 mutants have nearly the same rate of GTP hydrolysis. However, interestingly, the extent of GTP hydrolysis is significantly lower (two to three fold) for Asp50 mutants compared to wild type EF-Tu.

Dipeptide formation on the ribosomes include the time for EF-Tu binding to the ribosome, the time for GTP hydrolysis on EF-Tu and the time for peptide bond formation between fMet and Phe amino acids at the peptidyltransfer center. Therefore, it is not possible to measure the rate of dipeptide formation; rather, the total time elapsed for dipeptide formation was measured.

The *ln*(h(t)) vs. time graphs were plotted. By taking the integral of these curves, the total time for dipeptide formation for wild type and mutant EF-Tu's were calculated. The total time elapsed for dipeptide formation was comparable for wild type and mutant EF-Tu’s.

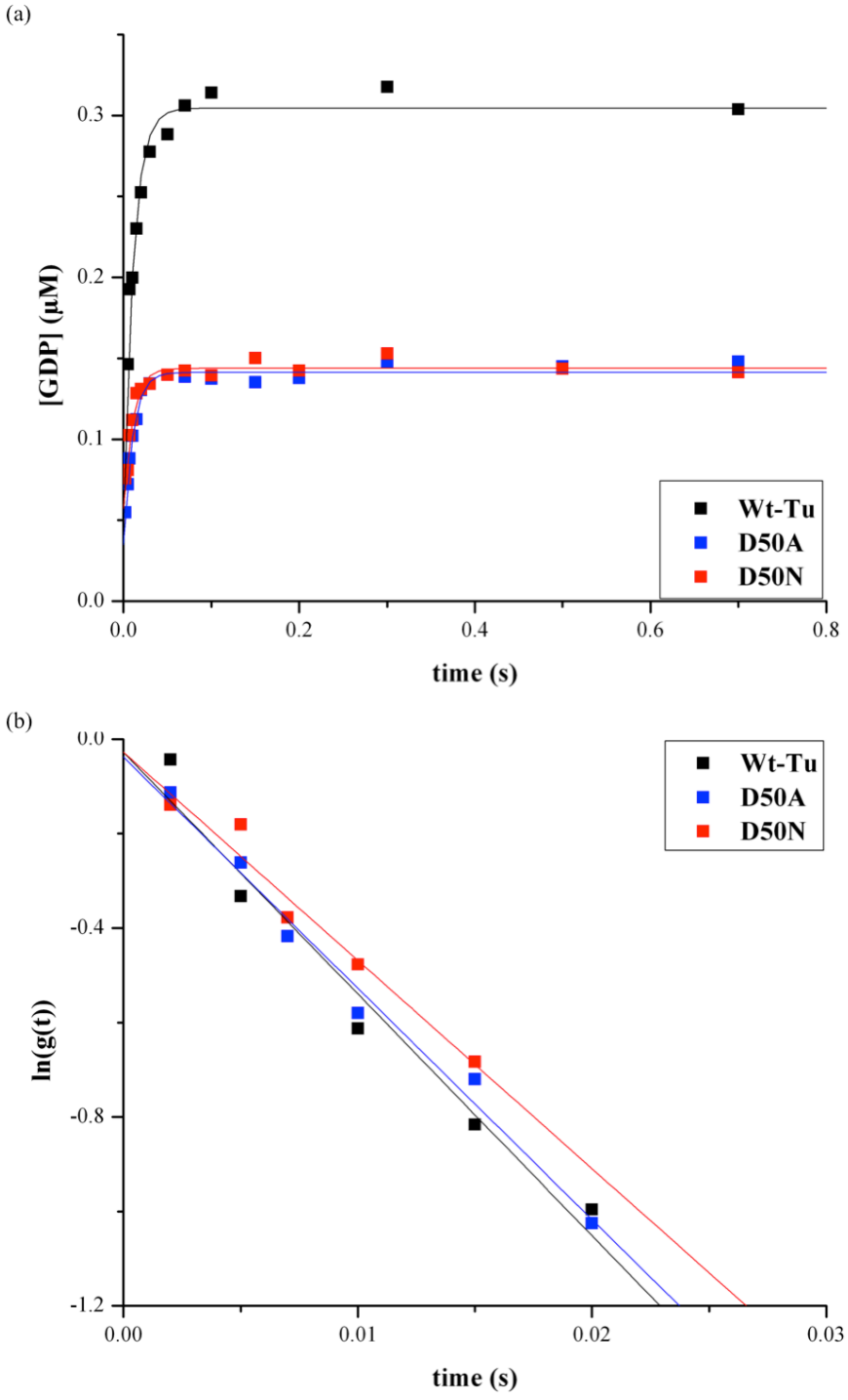

Figure 5.9. Rate of ribosome induced GTP hydrolysis on EF-Tu for wild type and Asp50 mutants in the presence of 20 μM Phe-tRNA^{Phe}. (a) The concentration of hydrolyzed GDP (GDP(t)) was plotted as a function of time. (b) *ln*(g(t)) versus time graph of (a) where the slope gives the $k_{GTP.}$

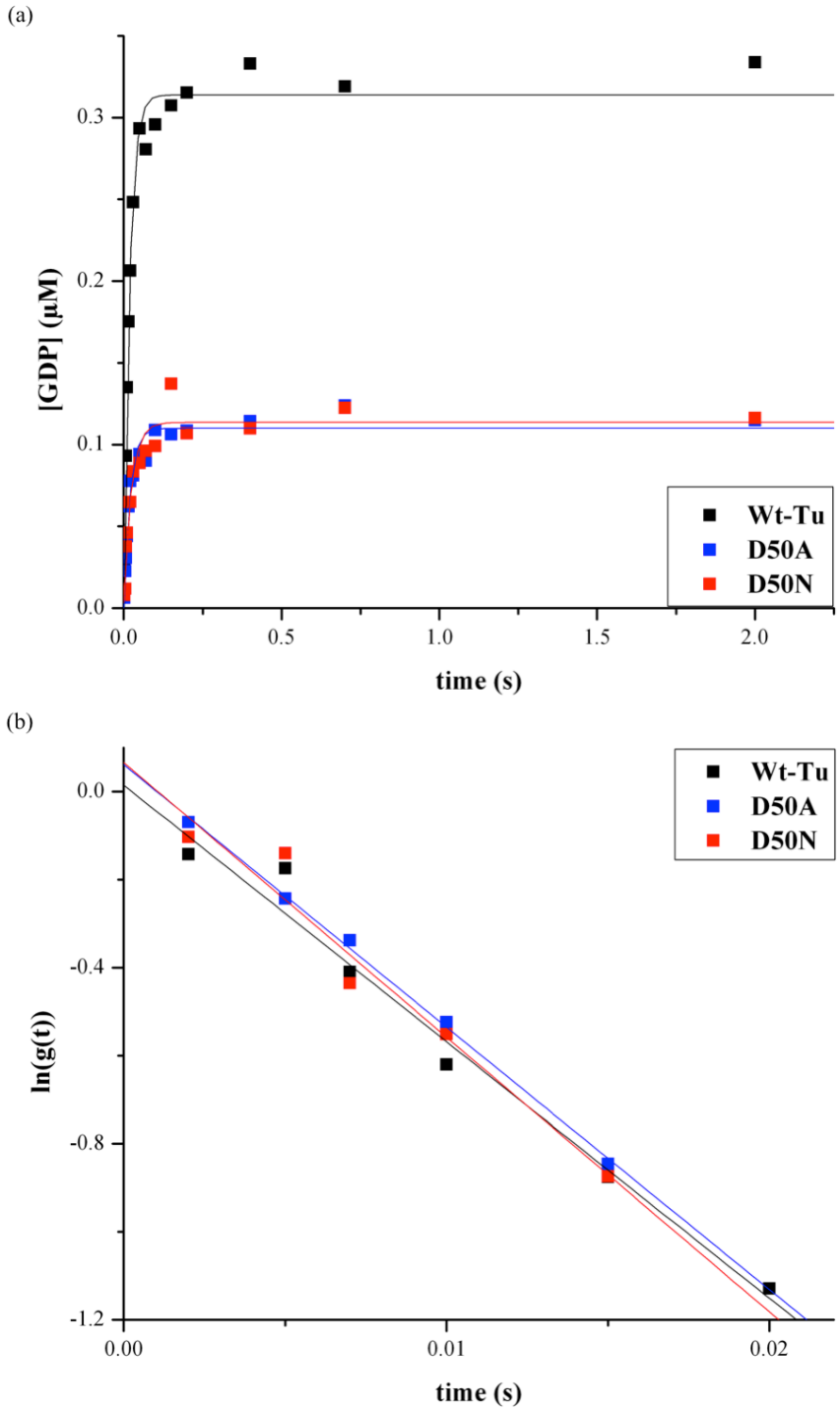

Figure 5.10. Rate of ribosome induced GTP hydrolysis on EF-Tu for wild type and Asp50 mutants in the presence of 80 μM Phe-tRNA^{Phe}. (a) The concentration of hydrolyzed GDP (GDP(t)) was plotted as a function of time. (b) *ln*(g(t)) versus time graph of (a) where the slope gives the $k_{GTP.}$

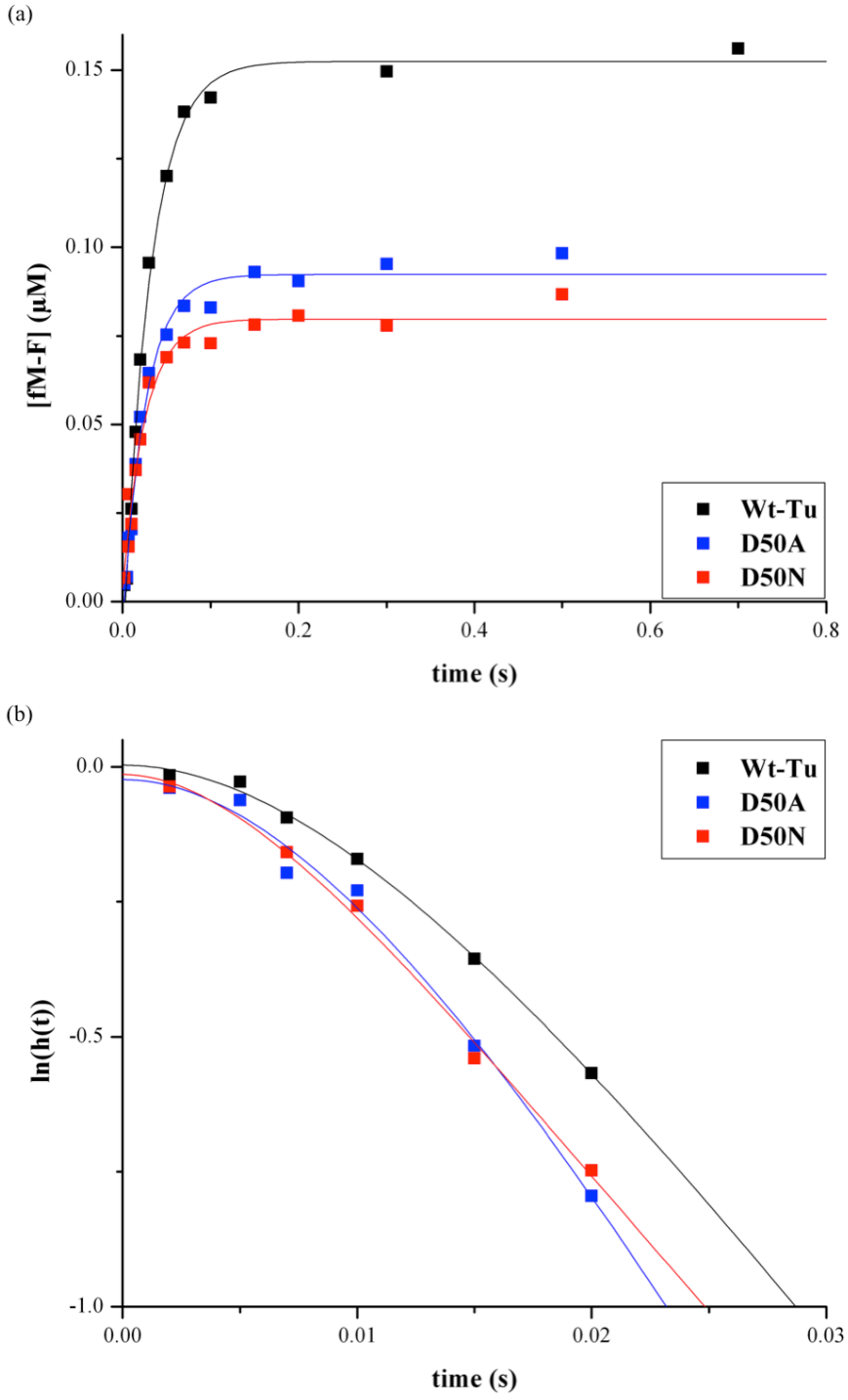

Figure 5.11. The dipeptide formation on ribosomes in the presence of 20 μM $tRNA^{Phe}$. (a) The concentration of dipeptides formed (dip(t)) was plotted as a function of time. (b) *ln*(h(t)) versus time graph of (a) where the integrate of the curve gives the k_{dip} for EF-Tu.

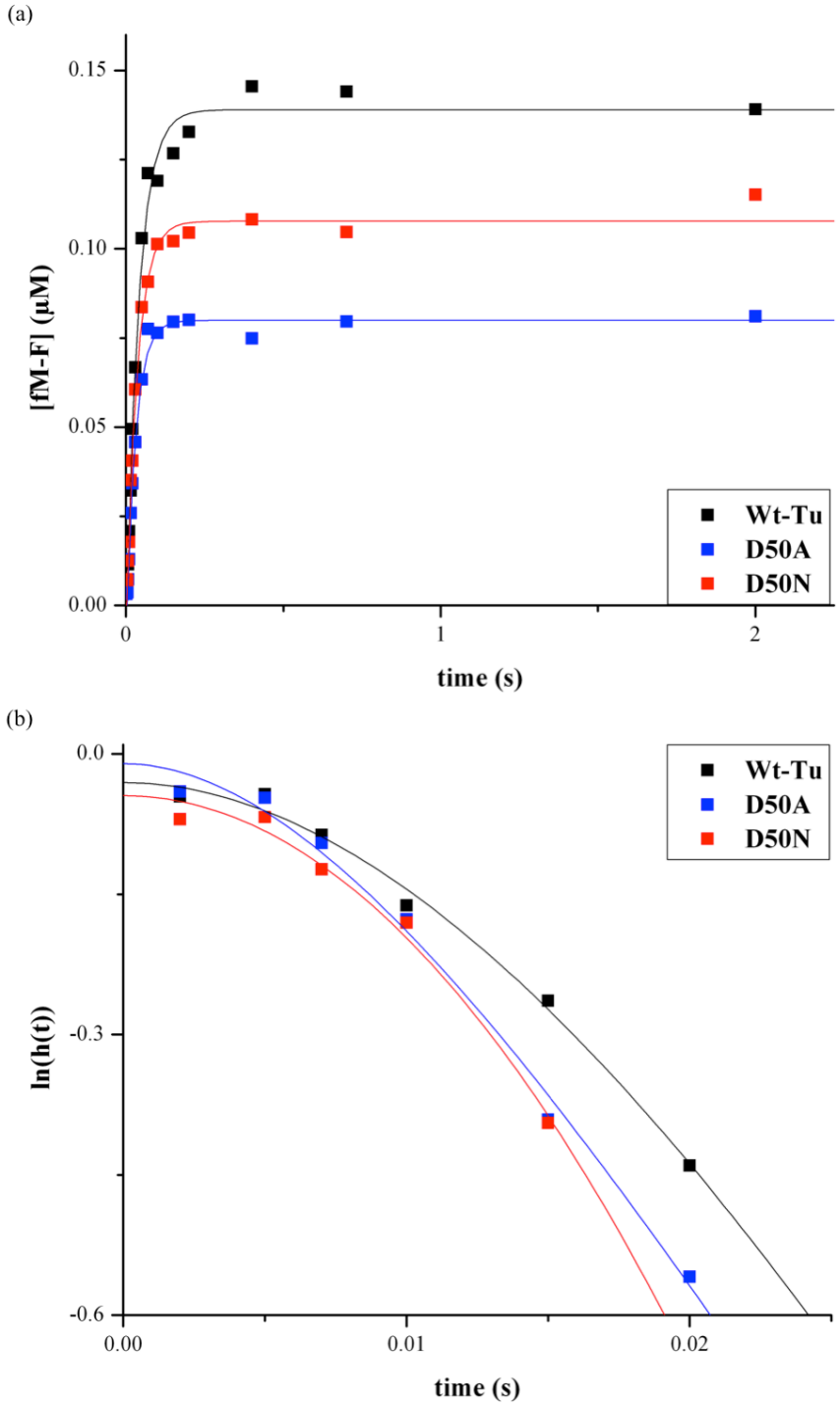

Figure 5.12. The dipeptide formation on ribosomes in the presence of 80 µM $tRNA^{Phe}$. (a) The concentration of dipeptides formed (dip(t)) was plotted as a function of time. (b) *ln*(h(t)) versus time graph of (a) where the integrate of the curve gives the k_{dip} for EF-Tu.

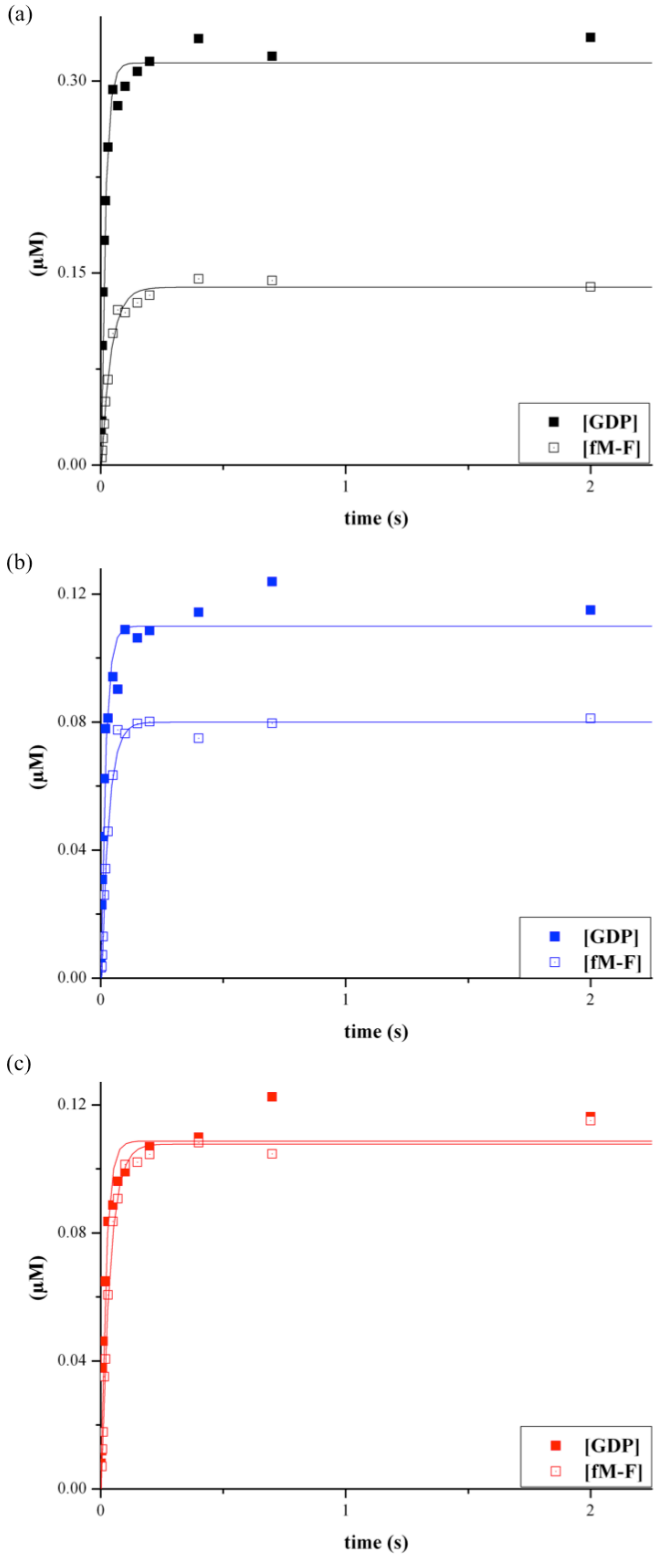

Figure 5.13. Stoichiometry of GTP hydrolysis on EF-Tu to dipeptide bond formation. The amount of GTP's hydrolyzed and the dipeptides formed as a function of time for wild type EF-Tu (a), D50A (b) and D50N (c) was plotted as a function of time.

Table 5.6. Comparison of the kinetics of GTP hydrolysis and dipeptide formation for wild type and mutant EF-Tu's.

In the presence of 20 µM $tRNA^{Phe}$			
	Wt-Tu	**D50A**	**D50N**
τ_{GTP} (ms)	19.5	20.4	22.7
τ_{dip} (ms)	46.5	40.9	38.3
Stoichiometry*	2.01	1.53	1.80
In the presence of 80 µM $tRNA^{Phe}$			
	Wt-Tu	**D50A**	**D50N**
τ_{GTP} (ms)	18.5 ± 1.94	16.8 ± 0.07	17.3 ±1.84
τ_{dip} (ms)	46.8 ± 2.73	40.0 ± 1.00	42.6 ± 7.24
Stoichiometry*	2.07 ± 0.25	1.18 ± 0.28	0.94 ± 0.18

* Stoichiometry for GTP hydrolysis per peptide bond formation

6. DISCUSSION

In this thesis, the role of the conserved Asp50 residue in EF-Tu function was studied. Two mutants of Asp50 were made; one of the substitutions was from aspartic acid to alanine, an unpolar residue smaller in size. The other substitution was to asparagine, a polar amino acid like aspartic acid but uncharged. Recombinant proteins, including the wild type EF-Tu were constructed with a TEV protease cleavable GST tag allowing an efficient two-step affinity purification of the EF-Tu's. Furthermore, a gel filtration step was included in order to make sure that EF-Tu's are fully depleted of any EF-Ts contamination. GDP binding and nucleotide exchange assays were used in order to measure the active concentrations of the EF-Tu's. When compared to protein content measured from Bradford assay, both mutants and the wild type EF-Tu's were found fully active. These assays also showed that the EF-Tu mutants of Asp50 were indistinguishable from the wild type EF-Tu in terms of GDP binding and GDP/GTP nucleotide exchange rates. Nucleotide exchange rates further confirmed the absence of EF-Ts in the EF-Tu preparations.

Three different assays were utilized to study the ability of the Asp50 mutants in ternary complex formation: a qualitative mobility shift assay using non-denaturing PAGE for ternary complex formation (Bilgin and Ehrenberg, 1995), and two quantitative assays for protection of the aminoacyl bond by EF-Tu. The first of the latter two assays is a modified version of the deacylation protection assay (Tapio *et al.*, 1990) and the other assay is an RNAse A protection assay (Bilgin and Ehrenberg, 1995). All three assays clearly showed that Asp50 mutants of EF-Tu have significantly reduced affinity for aa-tRNA and indicated the importance of Asp50 in aa-tRNA binding.

RNAse A protection assays were performed at 20°C because measurements at 37°C by hand mixing the components were not possible due to fast dissociation rates. In this assay, the dissociation constant for the binding Phe-tRNA^{Phe} to wild type EF-Tu · GTP was 0.060 μM at 20°C. Both Asp50 mutants have about five times higher K_D's than the wild type EF-Tu.

In order to validate the results of the RNAse A protection assay at 37°C, a different assay based on the aa-tRNA synthetase derived deacylation of aa-tRNA was used (Tapio *et al.*, 1990). In this assay, K_D for Phe-tRNA^{Phe} to wild type EF-Tu · GTP was 0.05 μM, which is comparable to earlier measurements (Tapio *et al.*, 1990). For the mutant EF-Tu's, a two-fold reduction in the affinity of aa-tRNA to EF-Tu · GTP was observed.

Both RNAse protection assay and deacylation protection assays clearly showed that both Asp50 mutants of EF-Tu have reduced affinity for aa-tRNA. On the other hand, K_D observed from RNAse A protection assay was nearly the same as in the deacylation protection assay for wild type EF-Tu, even though these two assays were performed at 20°C and 37°C respectively. One should have expected a higher affinity at lower temperatures. Both of these assays were indirect measurements for the dissociation of aa-tRNA from ternary complex based on the protection of the aminoacyl-bond on aa-tRNA by EF-Tu. One possible explanation between the overestimates of the RNAse protection assays (or underestimates of the deacylation protection assay) may be related to the EF-Tu concentrations used in these experiments. The working range of the EF-Tu concentrations in the deacylation protection assays were significantly higher than those used in the RNAse A protection assays. Furthermore, the deacylation protection experiments showed significant sigmoidal behavior at low concentrations of EF-Tu (Figure 5.6). With no explanation given, this sigmoidal behavior was also observed earlier (see Tapio *et al.*, (1990) Figure 4 therein) for wild type EF-Tu. The distortions in the Scatchard plots due to the sigmoidal behavior at low concentrations of EF-Tu may contribute to the differences between the two measurements.

RNAse A protection and deacylation protection assays also give information about the stoichiometry of the ternary complex: stoichiometry of EF-Tu to aa-tRNA in ternary complex was about 2:1 for wild type EF-Tu at 37°C whereas it was about 1:1 at 20°C, as previously reported by Bilgin and Ehrenberg, (1995). However, the stoichiometry was significantly higher for Asp50 mutants than that of wild type EF-Tu in both assays (6.7:1 in deacylation protection assay at 37°C, 1.4:1 in RNAse protection assay at 20°C). This may indicate the difficulty of mutant EF-Tu's in binding aa-tRNA for ternary complex formation, even at high concentrations (20-25 μM) of Phe-tRNA^{Phe}, in line with the sigmoidal

binding curves for aa-tRNA in EF-Tu titrations (see above). For Asp50 mutants, Scatchard plots may be distorted further due to more pronounced sigmoidal behavior at low concentrations of EF-Tu.

One observation, contrasting the idea that EF-Tu mutants are unable to form ternary complex, comes from poly(Phe) synthesis measurements. In EF-Tu cycle measurements in the presence of high concentrations of aa-tRNA, same as the aa-tRNA concentration used in the protection experiments (20 μM), the second order association rate constant (k_{cat}/K_{m}) for ternary complex with ribosomes was identical for wild-type EF-Tu and the Asp50 mutants. This observation indicates that Asp50 mutants and wild type EF-Tu could form similar amount of ternary complex that can be processed further by the ribosomes. In fact, the rate of ribosome induced GTP hydrolysis for the Asp50 mutants is nearly identical to wild-type EF-Tu. On the other hand, surprisingly, Asp50 mutants hydrolyze two- to three-fold less GTP compared to wild type EF-Tu. Reduced amount of GTP hydrolysis could have been explained if less ternary complex reaches to ribosomes for the Asp50 mutants. However, burst experiments contradict this view because k_{cat}/K_{M} values for the Asp50 mutants were comparable to wild type (see above).

Alternatively, even though same amount of ternary complex reaches the ribosomal A-site, after codon-anticodon interaction takes place, mutant EF-Tu's may release aa-tRNA's prior to GTP hydrolysis (because they have low affinity for aa-tRNA). Because EF-Tu · GTP has little affinity to ribosomes without aa-tRNA bound (Kawakita *et al.*, 1974), after EF-Tu · GTP release aa-tRNA could participate in peptide bond formation. Preparing ternary complexes in the presence of non-hydrolysable GTP analogs can test this scenario. If the above scenario is correct, one should observe peptide bond formation independent of GTP or non-hydrolysable GTP analog is present in the ternary complex for Asp50 mutants.

Another probable scenario could be as follows: the Switch I region, which is distorted by Asp50 mutations, is not strict to the bound nucleotide as for the wild-type EF-Tu. Therefore even in the GTP bound mutant EF-Tu's, Switch I can change conformation and bring the EF-Tu into GDP conformation. This would explain the non-denaturing gel re-

sults, where Asp50 mutants are unable to form stable ternary complexes with aa-tRNA. The reduced aa-tRNA affinities of the Asp50 mutants observed in protection experiments also support this view. Ternary complexes brought to the ribosomes in the presence of high excess of EF-Tu mutants may undergo conformational switch and without hydrolyzing GTP, could switch to GDP conformation and release aa-tRNA.

The crystal structure of ternary complex showed that the Switch I region of EF-Tu is in interaction with aa-tRNA in the ternary complex and helps to trap the tRNA on EF-Tu (Nissen *et al.*, 1995). In the inactive state of EF-Tu where GDP is bound, the Switch I region opens and leaves the tRNA (Polekhina *et al.*, 1996). The crystal structural data on EF-Tu was recently investigated further using molecular dynamics simulations. In these simulations, starting from the crystal structure of EF-Tu · GTP, the positioning of Asp50 in the Switch I region next to the GTP binding site was simulated using Particle Mesh Ewald Molecular Dynamics (PMEMD) up to 43 ns with a time step of 2 fs (Gül, 2008).

Figure 6.1 shows the coordinates at 0, 20 and 43 ns from these simulations (Gül, unpublished observations). This data indicates the possible role of Asp50 in the GTP binding conformation of EF-Tu, where Asp50 and Thr61 coordinates Mg^{2+}, keeping the Switch I region in the α-helical form. During the simulation, Asp50 moves towards the position at its GDP conformation. Even though these simulations are too short to observe the final GDP conformation, and the necessity to be further investigates remains, it is likely that Asp50 is crucial for a tight GTP conformation. When Asp50 is mutated, its coordination with Thr61 through Mg^{2+}, therefore, will be lost, leading to an easier switch to GDP conformation.

Whether the conformational change of the Switch I region could take place without GTP hydrolysis on EF-Tu requires further work.

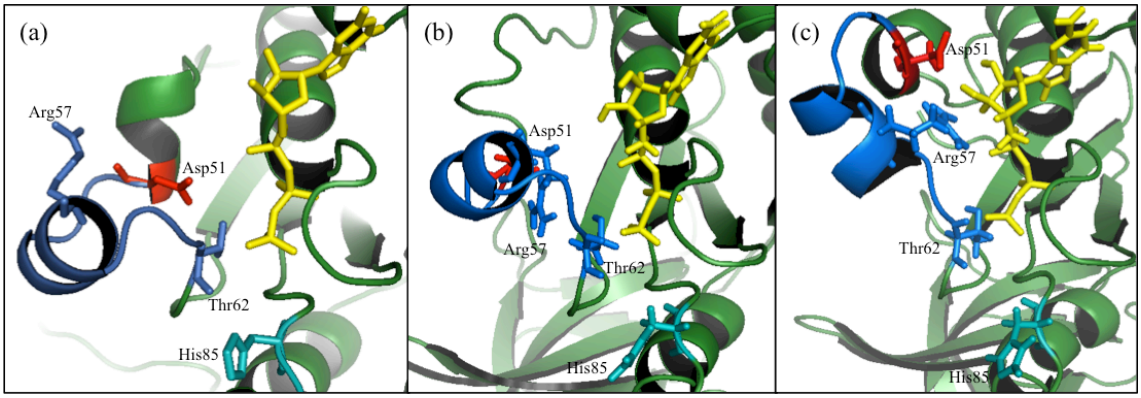

Figure 6.1. Images from Particle Mesh Ewald Molecular Dynamics (PMEMD) simulation of *Thermus aquaticus* EF-Tu · GTP at 0 ns (a), 20 ns (b) and 43 ns (c). The Switch I region showed in blue where the Asp51 residue in red and GTP in yellow.

At last, the fast-kinetic measurements on the ribosome induced GTP hydrolysis and peptide bond formation in this thesis work also confirmed the earlier measurements of the stoichiometry of GTP hydrolysis per peptide bond formation: Using poly(Phe) system a stoichiometry of 2:1 for GTP hydrolysis to peptide bond formation had been reported (Ehrenberg *et al.*, 1990b). Later, these measurements were questioned, with the suggestion that the 2:1 stoichiometry is an artifact of the poly(U) system where the hydrolysis of the additional GTP might be due to the translation of the stretch of uridines (Rodnina and Wintermeyer, 1995). However, in this thesis work, one of the same mRNA construct (mMFTI) was used as in the work of Rodnina and Wintermeyer, (1995) experiments, yet the stoichiometry for GTP hydrolysis per peptide bond formation for wild type EF-Tu was found to be strictly 2:1. The stoichiometry of the ternary complex for wild type EF-Tu at 37°C as measured from deacylation protection experiments (Figure 5.6) supports the existence of a pentameric complex instead of a ternary complex carrying the aa-tRNA onto ribosomes. However, how two GTP's are hydrolyzed on the ribosome remains to be studied.

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