FUNCTIONAL ANALYSIS OF TWO CONSERVED REGIONS OF *ESCHERICHIA COLI* ELONGATION FACTOR G AS STUDIED BY SITE-DIRECTED MUTAGENESIS

DISSERTATION

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By

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ABSTRACT

Elongation factor G (EF-G) catalyzes the translocation step of protein biosynthesis. The function of two conserved regions of *E. coli* EF-G, that are located at the interfaces between domains, was explored by mutating four highly conserved residues, R127, Q128, G461 and E462.

Analysis of R127 mutants revealed that R127 is involved in ribosome binding. No other aspect of the EF-G cycle was significantly affected by mutation of this arginine. Q128 is involved in ribosome-dependent GTP hydrolysis. As a consequence of its defect in ribosome-dependent GTPase activity, the Q128 mutants displayed a reduced specific activity in a turnover protein synthesis assay and increased dissociation from the ribosome. Destabilization of the switch II helix by mutation of the Q128 residue might explain the defects observed in the Q128 mutants.

G461 was shown to be essential for the function of EF-G. Mutation of the G461 residue caused severe defects in the ribosome-dependent GTPase activity, reduced GTP binding, reduced binding to the ribosome and almost no protein synthesis. E462 was involved in the hydrolysis of GTP. Mutants of E462 were functional in all other aspects of the EF-G cycle and had similar protein synthesis activity as wild-type EF-G.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>2.</td>
<td>Materials and Methods</td>
<td>19</td>
</tr>
<tr>
<td>2.1</td>
<td>Mutagenesis of wild-type EF-G</td>
<td>19</td>
</tr>
<tr>
<td>2.2</td>
<td><em>In vivo</em> toxicity assay</td>
<td>24</td>
</tr>
<tr>
<td>2.3</td>
<td><em>In vivo</em> fusidic acid binding assay</td>
<td>24</td>
</tr>
<tr>
<td>2.4</td>
<td>Protein expression and purification</td>
<td>25</td>
</tr>
<tr>
<td>2.5</td>
<td>Purification of <em>E. coli</em> tRNA synthetases</td>
<td>27</td>
</tr>
<tr>
<td>2.6</td>
<td>Preparation of $^{14}$C-Phe-tRNA</td>
<td>27</td>
</tr>
<tr>
<td>2.7</td>
<td><em>In vitro</em> protein synthesis assay</td>
<td>29</td>
</tr>
<tr>
<td>2.8</td>
<td>Ribosome-dependent GTPase assays</td>
<td>30</td>
</tr>
<tr>
<td>2.9</td>
<td>Ribosome-independent GTPase assay</td>
<td>31</td>
</tr>
<tr>
<td>2.10</td>
<td>Assay for the protection of the ribosome from the ribotoxin restrictocin</td>
<td>31</td>
</tr>
<tr>
<td>3.</td>
<td>Role of the C-G helix: Results and Discussion</td>
<td>34</td>
</tr>
<tr>
<td>3.1</td>
<td>Site-directed mutagenesis and protein purification</td>
<td>34</td>
</tr>
<tr>
<td>3.2</td>
<td>Function of the R127 and Q128 mutants <em>in vivo</em></td>
<td>38</td>
</tr>
<tr>
<td>3.3</td>
<td><em>In vitro</em> protein synthesis activity of R127 and Q128 mutant translocases</td>
<td>41</td>
</tr>
<tr>
<td>3.4</td>
<td>Ribosome-dependent GTP hydrolysis</td>
<td>45</td>
</tr>
<tr>
<td>3.5</td>
<td>Ribosome-independent GTPase assay</td>
<td>49</td>
</tr>
<tr>
<td>3.6</td>
<td>Interaction with guanine nucleotides GMPPCP and GDP</td>
<td>49</td>
</tr>
<tr>
<td>3.7</td>
<td>Protection of the ribosome by EF-G</td>
<td>55</td>
</tr>
<tr>
<td>3.8</td>
<td><em>In vitro</em> protein synthesis in the presence of fusidic acid</td>
<td>60</td>
</tr>
<tr>
<td>3.9</td>
<td>Discussion</td>
<td>60</td>
</tr>
</tbody>
</table>

Abstract .......................................................................................................................... ii

List of Tables ................................................................................................................... v

List of Figures ................................................................................................................ vi

List of Abbreviations ........................................................................................................ viii

Chapters:

1. Introduction .................................................................................................................. 1

2. Materials and Methods ............................................................................................. 19
   2.1 Mutagenesis of wild-type EF-G ............................................................................ 19
   2.2 *In vivo* toxicity assay ......................................................................................... 24
   2.3 *In vivo* fusidic acid binding assay ...................................................................... 24
   2.4 Protein expression and purification .................................................................... 25
   2.5 Purification of *E. coli* tRNA synthetases ......................................................... 27
   2.6 Preparation of $^{14}$C-Phe-tRNA ....................................................................... 27
   2.7 *In vitro* protein synthesis assay ....................................................................... 29
   2.8 Ribosome-dependent GTPase assays .................................................................. 30
   2.9 Ribosome-independent GTPase assay .................................................................. 31
   2.10 Assay for the protection of the ribosome from the ribotoxin restrictocin ........ 31

3. Role of the C-G helix: Results and Discussion ...................................................... 34
   3.1 Site-directed mutagenesis and protein purification ............................................. 34
   3.2 Function of the R127 and Q128 mutants *in vivo* .............................................. 38
   3.3 *In vitro* protein synthesis activity of R127 and Q128 mutant translocases .... 41
   3.4 Ribosome-dependent GTP hydrolysis ................................................................. 45
   3.5 Ribosome-independent GTPase assay ............................................................... 49
   3.6 Interaction with guanine nucleotides GMPPCP and GDP ................................ 49
   3.7 Protection of the ribosome by EF-G ................................................................. 55
   3.8 *In vitro* protein synthesis in the presence of fusidic acid ............................... 60
   3.9 Discussion .............................................................................................................. 60


4. Role of the interface between the G-domain and domain III: Results and Discussion 73
   4.1 Site-directed mutagenesis and protein purification ........................................ 73
   4.2 In vivo test of protein function ................................................................. 76
   4.3 In vitro protein synthesis activity of G461 and E462 mutant translocases ....... 79
   4.4 Ribosome-dependent GTP hydrolysis.......................................................... 82
   4.5 Ribosome-independent GTPase assay ......................................................... 84
   4.6 Interaction with guanine nucleotides GMPPCP and GDP ......................... 85
   4.7 Protection of the 23S rRNA by EF-G ......................................................... 87
   4.8 Effect of GMPPCP on the in vitro protein synthesis ............................... 91
   4.9 Discussion ................................................................................................. 91

5. Conclusions ...................................................................................................... 106
   5.1 Importance of the C_G helix ................................................................. 106
   5.2 Stability of the G-domain-domain III interface is important for ribosome
       binding ........................................................................................................ 108

List of references .................................................................................................. 111
LIST OF TABLES

Table                                                                                                                      Page

3.1 *In vivo* tests of toxicity and sensitivity to fusidic acid of the R127 and Q128 mutant translocases                      42

3.2 Specific activity of *in vitro* protein synthesis of wild-type EF-G and the R127 Q128 mutants                           43

3.3 GTPase activities of wild-type EF-G and R127 and Q128 mutant translocases                                           47

3.4 Inhibition constants of GMPPCP and GDP for wild-type EF-G and the R127 and Q128 mutant translocases                    54

4.1 *In vivo* tests of toxicity and sensitivity to fusidic acid of the G461 and E462 mutant translocases                  77

4.2 Specific activity of *in vitro* protein synthesis of wild-type EF-G and the G461 E462 mutants                          81

4.3 GTPase activities of wild-type EF-G and G461 and E462 mutant translocases                                         83

4.4 Inhibition constants of GMPPCP and GDP for wild-type EF-G and the G461 and E462 mutant translocases                  86
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 Schematic representation of the elongation phase of protein synthesis</td>
<td>2</td>
</tr>
<tr>
<td>1.2 Sequence alignment of selected members of the GTPase superfamily</td>
<td>5</td>
</tr>
<tr>
<td>1.3 Structure of GDP-bound EF-G</td>
<td>8</td>
</tr>
<tr>
<td>1.4 Schematic representation of the EF-G cycle</td>
<td>11</td>
</tr>
<tr>
<td>1.5 Sequence alignment of EF-Gs from different organisms showing the two regions that are the focus of this study</td>
<td>14</td>
</tr>
<tr>
<td>2.1 Sequences of the primers used in the site-directed mutagenesis of G461 and E462 of the <em>E. coli</em> EF-G gene</td>
<td>20</td>
</tr>
<tr>
<td>2.2 Strategy for site-directed mutagenesis</td>
<td>21</td>
</tr>
<tr>
<td>2.3 Schematic representation of the ribosome protection assay</td>
<td>32</td>
</tr>
<tr>
<td>3.1 Bacterial growth curves of wild-type EF-G and the R127 and Q128 mutants</td>
<td>36</td>
</tr>
<tr>
<td>3.2 Protein gel of wild-type and mutant EF-Gs</td>
<td>37</td>
</tr>
<tr>
<td>3.3 Structures of fusidic acid and GMPPCP</td>
<td>39</td>
</tr>
<tr>
<td>3.4 Effect of fusidic acid on the EF-G cycle</td>
<td>40</td>
</tr>
<tr>
<td>3.5 <em>In vitro</em> poly(U)-directed protein synthesis</td>
<td>44</td>
</tr>
<tr>
<td>3.6 The ribosome-dependent GTPase cycle under turnover conditions</td>
<td>46</td>
</tr>
<tr>
<td>3.7 Schematic representation of ribosome-independent GTP hydrolysis</td>
<td>50</td>
</tr>
<tr>
<td>3.8 Inhibition of the GTPase activity of wild-type EF-G by GMPPCP</td>
<td>52</td>
</tr>
<tr>
<td>3.9 Inhibition of the GTPase activity of wild-type EF-G by fusidic acid</td>
<td>53</td>
</tr>
</tbody>
</table>
3.10 Effect of GMPPCP on the EF-G cycle .................................................. 57

3.11 Protection of the α-sarcin site of the ribosome by wild-type and mutant translocases in the presence of GMPPCP .............................................. 58

3.12 Effects of fusidic acid on the protection of the ribosome ......................... 59

3.13 Inhibition of the in vitro protein synthesis assay by fusidic acid ................. 62

3.14 Proposed role of arginine 127 of E. coli EF-G in the EF-G cycle ................. 64

3.15 Proposed roles of glutamine 128 of E. coli EF-G in the EF-G cycle .......... 68

4.1 Bacterial growth curves for G461 and E462 mutants .............................. 75

4.2 In vivo test of function of wild-type and mutant translocases................... 78

4.3 In vitro poly(U)-directed protein synthesis of G461 mutants................. 80

4.4 Competitive inhibition of wild-type EF-G and the G461 and E462 mutants by GMPPCP .............................................................. 88

4.5 Protection of the ribosomal 23S rRNA by wild-type EF-G and the G461 and E462 mutant translocases ......................................................... 89

4.6 Effect of fusidic acid on the protection of the ribosome by wild-type EF-G and the G461 and E462 mutant translocases ........................................ 90

4.7 In vitro Protein synthesis activity of wild-type EF-G and the E462 mutants .... 92

4.8 Proposed roles of glycine 461 of E.coli EF-G in the EF-G cycle ............. 97

4.9 Proposed role of glutamic acid 462 of E.coli EF-G in the EF-G cycle ........... 100
LIST OF ABBREVIATIONS

6-His - hexahistidine
A - alanine
ATP - adenosine triphosphate
bp - base pair
Ci - Curie
D - aspartic acid
dATP - deoxyadenosine triphosphate
dNTP - deoxynucleoside triphosphate
DTT - dithiothreitol
E - glutamic acid
EDTA - ethylene diamine tetraacetic acid
EF-2 - elongation factor 2
EF-G - elongation factor G
EF-Ts - elongation factor Ts
EF-Tu - elongation factor Tu
G - glycine
GAC - GTPase activation center
GAP - GTPase activating protein
GDP - guanosine diphosphate
GMPPCP - β,γ-methyleneguanosine 5’-triphosphate

GNRP - guanine nucleotide release protein

GTP - guanosine triphosphate

GTPase - guanosine triphosphatase

H - histidine

IF-2 - initiation factor 2

IPTG - isopropyl-β-D-thiogalactopyranoside

LB - Luria-Bertani

M - molar

mCi - milliCurie

mg - milligram

mL - milliliter

mM - millimolar

mmol - millimole

mRNA - messenger ribonucleic acid

MW - molecular weight

N - asparagine

NEB - New England Biolabs

ng - nanogram

nM - nanoMolar

NTP - nucleoside triphosphate

Nts – nucleotides
CHAPTER 1

INTRODUCTION:

The process by which proteins are synthesized in all living organisms is highly conserved. Protein synthesis occurs in three stages: initiation, elongation and termination. While the overall scheme of the protein synthesis process is known, many details of the mechanism of translation are yet to be determined. Recent advances in technology have allowed a re-examination of the mechanism of this important process.

The elongation step (Fig. 1.1) is the most complicated and least understood aspect of protein synthesis. Even though the classical two-site model (Watson, 1964) used to describe the elongation step of protein synthesis has undergone some recent modifications (Moazed and Noller, 1989, reviewed in Ramakrishnan, 2002), the principle remains the same. Aminoacylated tRNA is brought into the A-site of the mRNA-programmed ribosome as a ternary complex with elongation factor Tu (EF-Tu) and GTP. After GTP hydrolysis, the peptide from the peptidyl tRNA in the P-site is transferred to the amino acid on the aminoacylated tRNA in the A-site by a reaction catalyzed by the ribosome. Following peptidyl transfer, elongation factor G (EF-G) is used to move the ribosome in a process called translocation. Translocation causes the deacylated P-site tRNA to move to the E-site, while the A-site tRNA with the attached peptide moves to
Figure 1.1: Schematic representation of the elongation phase of protein synthesis. Three steps must occur for each amino acid that is to be added to the growing peptide chain. First, EF-Tu brings an aminoacylated tRNA into the A-site of the ribosome. Then, the peptide from the P-site tRNA is transferred to the amino acid on the A-site tRNA. This peptide bond formation step is believed to be rRNA-catalyzed. Finally in a process called translocation, the tRNAs move relative to the ribosome. Translocation is facilitated by EF-G. The vertical lines represent presence of tRNA in the different tRNA-binding sites of the ribosome shown as E, P and A. Numbered circles are successive amino acids in the growing peptide. The mRNA is not shown.
the old P-site. This allows the ribosome to accept the next aminoacylated tRNA into the new A-site.

EF-Tu and EF-G are members of the GTPase superfamily (Bourne et al., 1991). Members of this superfamily are involved in a variety of biological functions (Sprang, 1997) such as proliferation and differentiation of animal cells (Ras), regulation of intracellular signaling pathways (Rho) and in signal transduction (G proteins). All these proteins are active in the GTP-bound form and are inactive in the GDP-bound form or as free protein. Most of these proteins require a specific GTPase activating protein (GAP) which stimulates GTP hydrolysis. The ribosome provides this GAP activity for the elongation factors EF-Tu and EF-G (Kaziro et al., 1972). The mechanism of activation of GTP hydrolysis in EF-G by the ribosome is unknown. In addition to the GAP protein, some members of this superfamily use a guanine nucleotide release protein (GNRP) to help exchange GTP for GDP. EF-Ts is the GNRP for EF-Tu (Arai et al., 1974), EF-G does not require a GNRP since it possesses greater affinity for GTP than GDP (Arai et al., 1977).

There are three regions of homology among all members of the GTPase superfamily (Fig. 1.2). These three regions are involved in binding the guanine nucleotide (Dever et al., 1987). They include the phosphate-binding loop having a consensus sequence of GXXXXGKT/s, the magnesium binding region DXXG, which is a part of the switch II region referred to in later chapters. The third region is NKXD, which binds the guanine ring. A fourth region called the “effector domain” which contains a conserved threonine, is conserved within subgroups of the superfamily (Bourne et al.,
Figure 1.2: Sequence alignment of selected members of the GTPase superfamily. Alignment adapted from Dever et al., 1987, and Bourne et al., 1991. Residues in uppercase are conserved while residues in lowercase are variable. The position of each of these four regions in the *E.coli* EF-G protein is shown by the amino acid number of the first residue of each region. References: 1, Zengel et al., 1984; 2, Ohama et al., 1987; 3, Buttarelli et al., 1989; 4, Lechner et al., 1988; 5, Grinblat et al., 1989; 6, Kohno et al., 1986; 7, Laursen et al., 1981; 8, Brands et al., 1986; 9, Sacerdot et al., 1984; 10, Neuman-Siberberg et al., 1984; 11, Taparowsky et al., 1983; 12, Yeramian et al., 1987; 13, Saxe et al., 1988; 14, Zahraoui et al., 1989; 15, Bobak et al., 1989; 16, Pupillo et al., 1989; 17, Fong et al., 1988.
<table>
<thead>
<tr>
<th>Consensus</th>
<th>Function</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/GxxxxGKT/S</td>
<td>A/GxxxxGKT/S</td>
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<tr>
<td>D----(X)\textsubscript{n}----T</td>
<td>D----(X)\textsubscript{n}----T</td>
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</tr>
<tr>
<td>DxxGH/Q</td>
<td>Mg\textsuperscript{++} binding</td>
<td></td>
</tr>
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<td>NKxD</td>
<td>Guanine ring binding</td>
<td></td>
</tr>
</tbody>
</table>

Translation factors

**E. coli EF-G**
- 16 AhidaGKT
- 50 DwmeqeqeRgIT
- 87 DtpGH
- 141NKmD

**M. luteus EF-G**
- AhidaGKT
- DwmeqeekeRgIT
- DnpGH
- NKmD

**S. platensis EF-G**
- AhidaGKT
- DwmaqereRgIT
- DtpGH
- NKmD

**M. vannielii EF-2**
- AhiahGKT
- DfdeeaeaaRgIT
- DtpGH
- NKvD

**D. melanogaster EF-2**
- AhvdhGKS
- DtrkdeqeRcIT
- DspGH
- NKmD

**Hamster EF-2**
- AhvdhGKS
- DtrkdeqeRcIT
- DspGH
- NKmD

**E. coli EF-Tu**
- GhvdhGKT
- DnapeekaRgIT
- DcpGH
- NKcD

**M. luteus EF-Tu**
- GhvdhGKT
- DsapeekqRgIT
- DapGH
- NKsD

**H. sapiens EF-1**
- GhvdsGKS
- DklkaereRgIT
- DapGH
- NKmD

**E. coli IF-2**
- GhvdhGKT
- tkvasgeaggIT
- DtpGH
- NKiD

Small eukaryotic GTPases

**D. melanogaster Ras 1**
- GpggvGKS
- ydpTI
- DtaGQ
- NKcD

**H. sapiens N-Ras**
- GpggvGKS
- ydpTI
- DtaGQ
- NKcD

**H. sapiens Rho A**
- GdgcGKT
- yvpTV
- DtaGQ
- NKkD

**D. discoidium Sas 1**
- GdsgvGKS
- fitTI
- DtaGQ
- NKcD

**H. sapiens Rab 3A**
- GnsnvGKT
- fvsTV
- DtaGQ
- NKcD

**H. sapiens Arf-1**
- GlgaaGKT
- tipTI
- DvgGQ
- NKqD

**D. discoidium DGα1**
- GagesGKS
- DvlsrtrkTt
- DvgGQ
- NKrD

**H. sapiens G\textsubscript{S}**
- GagesGKS
- DllrcrvtTs
- DvgGQ
- NKqD

*Figure 1.2:* Sequence alignment of selected members of the GTPase superfamily.
All of these conserved motifs of the GTPase superfamily are in domain I (the G-domain) of the 5-domain EF-G structure.

EF-G is a very abundant protein in *E. coli*, making up almost 2% of the total cellular protein (Leder et al., 1969). In *E. coli*, the gene that encodes EF-G is known as the *fus* gene and it is located in the *str* operon along with the genes for EF-Tu and the ribosomal proteins S7 and S12 (Jaskunas et al., 1975a). In the gene and operon nomenclature, “*fus*” refers to the fact that resistance to fusidic acid, an antibiotic, has been mapped to the EF-G gene, and “*str*” refers to the fact that streptomycin resistance maps to the S12 protein. The *fus* gene has been cloned (Jaskunas et al., 1975b) and sequenced (Zengel et al., 1984). It encodes a protein of 704 amino acid residues with a molecular weight of 77,444 Daltons (Zengel et al., 1982).

The crystal structures of the GDP-bound EF-G from *Thermus thermophilus* (Czworkowski et al., 1994, Al-Karadahi et al., 1996) and the nucleotide-free EF-G (Ævarsson et al., 1994) have been solved. In all these structures, various flexible regions like the effector loop and the switch II region of EF-G were resolved. Recently, the structure of a mutant form of EF-G from *T. thermophilus* has been solved (Laurberg et al., 2000) in which domain III of EF-G was resolved. The structure of EF-G in the active GTP-bound form is not yet known. EF-G is a five-domain protein whose overall shape resembles a tadpole (Fig.1.3). The N-terminal domain is the guanine nucleotide binding domain (G-domain) which is similar to those found in ras p21 (Pai et al., 1989), α-transducin (Noel et al., 1993) and EF-Tu (Kjeldgaard et al., 1993). In addition to containing all the conserved elements to bind GTP (Fig1.2), the G-domain also has an
Figure 1.3: Structure of GDP-bound EF-G. A trace of the backbone atoms of *Thermus thermophilus* EF-G with bound GDP and magnesium (Laurberg et al., 2000). Domains 1-5 are red, blue, pink, black and green, respectively. The GDP and the amino acids (R127, Q128, G461, E462) referred to in this study are shown as ball and stick structures. Shown in stick formation are amino acid residues that may interact with those amino acid residues that are the focus of this study. The figure was generated by the program Hyperchem using coordinates (protein database identification is 1FNM) provided by Anders Liljas of Lund University, Sweden.
Figure 1.3: Structure of GDP-bound EF-G.
insert of about 90 residues between helix \( D_G \) and strand \( 6_G \) which is called the G’ sub-domain. This G’ sub-domain is not found in the guanine nucleotide binding domain of other GTPases and it has been proposed that it acts as an internal GNRP for EF-G (ÁEvarsson, 1995).

Domain II of EF-G is structurally similar to the ribosomal translation factors IF-2, EF-Tu and RF-3 (ÁEvarsson, 1995). The structural similarity of the G-domain and domain II of the ribosomal translational factors suggests a common mechanism of interaction of these proteins with the ribosome. Indeed, when the G-domain and domain II of GDP-bound EF-G were compared to GMPPCP-bound EF-Tu extensive homology was observed (Nissen et al., 1995). Since both these proteins interact with post-translational ribosomes, it was proposed that EFG-GDP leaves an “imprint” on the ribosome as it exits thereby allowing EF-Tu-GTP containing a charged tRNA to bind the ribosome (Al-Karadaghi et al., 1996). The homology between these domains is structural, and had not been previously observed at the amino acid level.

Domains III, IV and V of EF-G are unique to EF-G. However when the structure of GDP-bound EF-G is compared to the EF-Tu-GMPPCP-aminoacyl-tRNA structure (Nissen et al., 1995), the structures were almost superimposable, with domains III, IV and V of EF-G, mimicking the structure of the aminoacyl-tRNA. The structure of the ribosome recycling factor (RRF) also mimics a tRNA (Selmer et al., 1999) and it requires EFG to function (reviewed in Janosi et al., 1996). It has been proposed that the
EF-G-RRF complex mimic the presence of two tRNAs in the ribosome. Thus the structural mimicry might involve other phases of protein synthesis (Brock et al., 1998).

Binding of EF-G to the ribosome has been studied by a variety of techniques and the site on the ribosome to which EF-G binds has been elucidated using cross-linking agents (Maassen and Moller, 1974; Maassen and Moller, 1978), chemical footprinting using kethoxal (Moazed and Noller, 1988) and hydroxyl radical probing (Wilson and Noller, 1998a). Recent studies using cryo-electron microscopy have further refined the binding site of EF-G on the ribosome (Agrawal et al., 1998, Agrawal et al., 1999). EF-G binds to the ribosome in a cleft between the 30S and 50S subunits near the base of the L7/L12 stalk with the tip of domain IV of EF-G positioned in the A-site of the 30S subunit of the ribosome (Wilson and Noller, 1998a). Binding of EF-G to the ribosome causes large conformational changes in the ribosome (Agrawal et al., 1999). The binding site of EF-G on the ribosome overlaps the binding site of the EF-Tu ternary complex (Stark et al., 1997) providing further evidence of the molecular mimicry between the elongation factors.

A sequence of steps occurs each time EF-G is involved in translation (Fig 1.4). EF-G binds GTP and adopts a conformation that has higher affinity for the pre-translocation ribosome. The EF-G-GTP complex binds the ribosome and the ribosome stimulates GTP hydrolysis (Rodina et al., 1997). Hydrolysis of GTP is believed to cause changes in conformation of both EF-G and the ribosome (Wilson and Noller, 1998a; Peske et al., 2000) which lead to the movement of the ribosome relative to the mRNA.
Figure 1.4: Schematic representation of the EF-G cycle. The three different proposed conformations of EF-G are represented as a rectangle with one side changing from a V to flat to concave. The presence of tRNAs in the A-site or the P-site of the ribosome is denoted by the letters “A” and “P” respectively. GTP is represented as a dark rectangle, while GDP is represented as an oval.
Once translocation has occurred, the EF-G-GDP complex dissociates from the ribosome and later the GDP dissociates from EF-G allowing EF-G to be recycled.

The effect of GTP binding and hydrolysis on the conformation of the members of this GTPase superfamily can be quite varied (reviewed in Abel and Jurnak, 1996). In some proteins like EF-Tu, the change in conformation is quite dramatic (Kjeldaard et al., 1993). In EF-Tu, the conserved Thr61 (\emph{T. thermophilus} numbering) of the effector loop moves about 17 Å when GTP hydrolysis occurs. On the other hand, in ras p21 the change in conformation on GTP hydrolysis is more subtle (Stouten et al., 1993). Since the structure of EF-G in the GTP bound form is not yet available, the effect of GTP hydrolysis on the conformation of EF-G is unknown. Some studies using small angle X-ray scattering and cryo-electronmicroscopy (Czworkowski et al., 1997, Agrawal et al., 1999) seemed to indicate that there is no major conformational change in EF-G on hydrolysis of GTP. However biochemical studies indicate that EF-G does undergo conformational changes on GTP hydrolysis (Arai et al., 1977, Peske et al., 2000). While the cryo-electronmicroscopy and X-ray scattering studies demonstrate that unlike EF-Tu, EF-G does not undergo major conformational changes on GTP binding and hydrolysis, it is possible that the resolution of the structural studies does not permit the observation of small changes in the conformation of EF-G. When the EFG-GMPP(CH\textsubscript{2})P structure obtained by cryo-electronmicroscopy was compared to the X-ray crystal structure of the EFG-GDP complex (Czworkowski et al., 1994), there was a change in conformation in domains III, IV and V of about 10 Å (Agrawal et al., 1999).
The significance of the conformational changes in EF-G due to GTP lies in the presumed mechanism of translocation. It is currently believed that EF-G helps translocation by causing movement of the ribosomal subunits relative to each other (Spirin, 1985, Wilson and Noller, 1998b, Frank and Agrawal, 2000) though it has also been proposed that EF-G physically displaces tRNA from the A-site (Nissen et al., 1995). GTP hydrolysis is likely to cause small conformational changes to both subunits of the ribosome which leads to translocation (Wilson and Noller, 1998b). This is supported by an earlier study which suggests that GTP hydrolysis occurs before translocation and the energy of GTP hydrolysis is used to drive translocation of the tRNA on the ribosome (Rodina et al., 1997). Further support for the hypothesis of translocation requiring movement of both subunits of the ribosome was obtained from the hydroxyl radical probing study (Wilson and Noller, 1998a) and the cryo-electronmicroscopy study (Agrawal et al., 1998, Agrawal et al., 1999) which indicated that EF-G has numerous contacts with both ribosomal subunits. Thus any change in the conformation of EF-G will be propagated to both subunits of the ribosome (reviewed in Ramakrishnan, 2002).

**Aim and Significance:**

To better understand the significance of the conformational changes in EF-G due to GTP binding and hydrolysis, the role of conserved amino acid residues that lie at the interface between the domains of *E. coli* EF-G was studied. The amino acid residues that form the interfaces between the different domains of EF-G are highly conserved (Fig.1.5).
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<tr>
<td>QxE TxRxRQxx</td>
<td>Helix C\textsubscript{G} of G-domain</td>
<td>121 QsETvwRQan</td>
<td>QsETvwRQad</td>
<td>QsETvwRQad</td>
<td>QsETvwRQae</td>
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<tr>
<td>GxE GELHxx</td>
<td>Interface of G-domain with domains 1 and 3</td>
<td>459 GmGELHld</td>
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Site-directed mutagenesis was used to obtain mutants of some of these conserved amino acid residues and the mutants were tested in various *in vivo* and *in vitro* assays to determine which aspect of the EF-G cycle (Fig. 1.4) was affected by a particular mutant. Knowledge of the roles that these conserved amino acid residues play in the EF-G cycle would allow a better understanding of the mechanism of translocation. Knowledge gained from this study could be extended to the eukaryotic system since the amino acid residues studied are conserved in prokaryotes and archea as well as eukaryotes. Previous *in vivo* studies had indicated that mutations of the amino acid residues at the interfaces between these domains may play a role in allowing EF-G to dissociate from the ribosome (Johanson et al., 1996). It was not clear from the *in vivo* studies whether other aspects of the EF-G cycle were affected by mutation of these amino acid residues.

The amino acid residues R127, Q128, G461 and E462 from the *E. coli* EF-G were chosen for this study. Sequence alignment of various bacterial EF-G homologs and their eukaryotic counterpart EF-2 indicated that these four amino acid residues are highly conserved in both prokaryotes and eukaryotes (Fig. 1.5). Structural studies of the nucleotide-free EF-G (Ævarsson et al., 1994) and of the GDP-bound EF-G (Czworkowski et al., 1994, Al-Karadaghi et al., 1996) from *Thermus thermophilus* had indicated that R127 and Q128 were part of the C helix of the G-domain of EF-G. These two amino acid residues are not present in the corresponding helix of the other GTP binding proteins of this superfamily except for the closely related EF-Tu. This implied that these amino acid residues are unlikely to be involved with the GTP binding and hydrolysis function of EF-G and most probably are involved in a function common to
both EF-Tu and EF-G, such as ribosome binding. Alternatively these amino acid residues could be involved in a function unique to EF-G, that is, translocation. The arginine and glutamine are located at the interface between the G-domain and domain V (Fig. 1.3). In the GDP-bound form of EF-G, the side chain of the glutamine is oriented towards the switch II region of the G-domain while the side chain of the arginine is oriented towards a conserved region of domain V. Any change in conformation brought about by GTP hydrolysis could be transmitted from the switch II region to domain V via the \( C_G \) helix. The crystal structure also indicated a salt bridge between E123 of the \( C_G \) helix and R676 of domain V (Johanson et al., 1996). Mutations to amino acid residues of this \( C_G \) helix cause resistance to the antibiotic fusidic acid (Johanson and Hughes, 1994). Fusidic acid prevents EFG-GDP from dissociating from the ribosome. It has been proposed that mutations to EF-G that cause resistance to fusidic acid are due to the mutant EF-Gs being able to dissociate from the ribosome probably due to their ability to change their conformation despite the presence of fusidic acid (Johanson et al., 1996). The mutations may cause destabilization of the inter-domain interface of these EF-Gs (Laurberg et al., 2000).

The region containing G461 and E462 was not resolved in the earlier structures (Ævarsson et al., 1994; Czworkowski et al., 1994, Al-Karadaghi et al., 1996), probably due to this region being flexible. A crystal structure of the H573A mutant form of GDP-bound EF-G from \( T. \ thermophilus \) that was recently solved (Laurberg et al., 2000) was able to resolve this region of domain III. This structure indicated that G461 and E462 lie at the interface between the G-domain and domain III proximal to the switch II region
of the G-domain. E462 is a part of the helix $B_3$ which is oriented towards a conserved $\beta$ strand of domain V. Thus any change in conformation in the EF-G due to GTP hydrolysis can be propagated to other parts of the protein. Like the amino acid residues of the $C_G$ helix, mutations to the amino acid residues in this region of domain III at the interface between the G-domain and domain III also cause resistance to fusidic acid (Johanson and Hughes, 1994).

The fusidic acid study (Johanson and Hughes, 1994) suggests that these two regions of EF-G may be important for EF-G to be able to transmit the conformational changes due to GTP hydrolysis from the G-domain to other parts of the molecule (Laurberg et al., 2000). The ability to convert the chemical energy of GTP hydrolysis into molecular movement may be crucial to the function of EF-G and to EF-Tu (reviewed in Abel and Jurnak., 1996). Since the conformational change is likely to be involved in translocation, a study of these conserved amino acids might shed light on the mechanism of translocation.

Understanding the roles of amino acid residues at the interfaces of EF-G will also lead to a better understanding of how antibiotics affect EF-G and protein synthesis. These studies will have medical implications, especially at a time when antibiotic-resistant bacterial strains are becoming more common. The study of protein synthesis is also useful in that it will facilitate the development of systems for protein synthesis, needed in large amounts for commercial or medical purposes. A better understanding of protein synthesis would enhance our knowledge of RNA in catalysis (Noller, 1993) as well as
shed light on the importance of RNA-RNA and RNA-protein interactions (reviewed in Brimacombe, 1991).
CHAPTER 2

MATERIALS AND METHODS:

2.1 Mutagenesis of wild-type EF-G:

The *E. coli* fus gene present in the plasmid pPROEX-2A was used as a template for site-directed mutagenesis by the megaprimer method (Sarkar et al., 1990). The plasmid had been constructed by subcloning the 2121 base pair *Cla I* fragment containing the *fus* gene from pTZ19UA into a modified pPROEX-1 vector (Life Technologies) to yield pPROEX-2A (Wolfe, 1999). The construction of pPROEX-2A resulted in an in-frame fusion of a 6-His tag at the N-terminus of EF-G.

Degenerate oligonucleotide primers (Life Technologies) were used to generate multiple mutants of each amino acid residue being studied. The degenerate mutagenic primer G461 was used to generate alanine, proline and serine mutants of glycine 461. It also encodes a silent mutation that generates a *Sst I* restriction site in the plasmid. The degenerate primer E462 was used to generate E462D and E462H. This primer introduces a *Xba I* restriction site via a silent mutation. The primer RAP1 anneals with the *fus* gene in the region that codes for domain II of EF-G, while the RAP 2 primer anneals to pPROEX-2A downstream of the *fus* gene. The sequences of the primers used are shown in figure 2.1, and the overall megaprimer strategy is shown in figure 2.2.
Figure 2.1: Sequences of the primers used in the site-directed mutagenesis of G461 and E462 of the E. coli EF-G gene. The bases in boldface represent the various bases that could be present at that site (V = A, C, G; B = C, G, T; S = C, G). The bases that cause a silent mutation or the required mutation are underlined.
**Figure 2.2**: Strategy for the site-directed mutagenesis of *E. coli* EF-G by the PCR megaprimer method (Sarkar et al; 1990).
All PCR reactions were performed in a Perkin-Elmer Cetus DNA Thermal Cycler. Each cycle of the PCR reaction consisted of one minute at 94°C, one minute at 55°C and one minute per kilobase pair at 72°C. Typically 30 cycles were used for each reaction. After completion of the 30 cycles, an additional extension of seven minutes at 72°C was performed. VENT DNA polymerase (New England Biolabs) was used in all PCR reactions. This enzyme had been used previously for site-directed mutagenesis in our laboratory (Wolfe, 1999) as well as in other studies (Juncosa-Ginesta et al., 1994). Reactions were performed in a total volume of 100 µl containing 1X NEB reaction buffer, 1 unit of VENT DNA polymerase, 0.02 ng/µl of linearized pPROEX-2A (digested with NgoA IV) as template, 0.5 µM of each primer, 400 µM of each dNTP, and 0.1 µg/µl non-acetylated BSA. The VENT DNA polymerase was added after the other reactants had been pre-heated at 94°C for two minutes and immediately chilled on ice.

The first step of the mutagenesis procedure involved a PCR reaction using either of the degenerate primers and RAP1. The approximately 380 bp product was purified by gel electrophoresis on a 1.5% NuSieve GTG agarose gel (FMC Biochemical) and it was used as a megaprimer in a second round of PCR along with the RAP2 primer. This generated a 1200 bp PCR product that was used in a third round of PCR with primers RAP1 and RAP2 to obtain more product. The 1.2 kb PCR product was digested with Pml1 and Kpn21, and sub-cloned into pPROEX-2A from which the corresponding Pml1 / Kpn21 fragment had been removed (Fig. 2.2). E. coli DH5α competent cells were transformed with plasmids containing the mutated fus gene by electroporation and the transformants were selected on repressing media containing 20 mM glucose and 0.1
mg/ml ampicillin (Sigma). The pPROEX-2 vector is under the control of a lac promoter, thus glucose could be used to repress the expression of the mutant EF-G. It had been shown that overexpression of some mutants of EF-G is deleterious to the bacterial host (Wolfe, 1999). Single colonies were inoculated into 10 ml LB media containing 20 mM glucose and 0.1 mg/ml ampicillin and grown overnight at 37°C. The plasmid DNA was extracted by the alkaline lysis mini-prep method (Birnboim et al., 1979).

The G461 mutants were screened for the presence of a SstI restriction site in the fus gene, while the E462 mutants were screened for the presence of a XbaI site within the fus gene. Clones containing the mutated fus gene were prepared for DNA sequencing by the method of Kraft (1988) and were sequenced by the Dideoxy sequencing method (Sanger et al., 1977) using α-35S-dATP (Amersham) and a Sequenase 2.0 DNA sequencing kit (United States Biochemical). The 1.2 kb region of pPROEX-2A between the regions to which the RAP1 and RAP2 primers anneal was sequenced (Fig. 2.2). The sequencing gel contained 8% acrylamide, 8 M urea in 1X TBE solution (Asubel et al., 1987). The gels were dried and exposed to film (Kodak XOMAT) for 72 hours at -70°C. Three clones containing alanine, proline and serine mutations of glycine 461 were obtained, while two clones containing aspartic acid and histidine mutations of glutamic acid 462 were obtained.

The R127E, R127Q, Q128E and Q128N mutant derivatives of E. coli EF-G had been prepared earlier by Xiao Chun Liu in our laboratory. Michael Wolfe in our laboratory had prepared the G21D mutant of E. coli EF-G used as a negative control, since it cannot bind guanine nucleotides.
2.2 *In vivo* toxicity assay:

Overexpression of some mutants of EF-G is toxic to the cells (Wolfe, 1999). An *in vivo* toxicity assay was performed to determine if the mutants of EF-G used in this study could be overexpressed in *E. coli*. Single colonies of *E. coli* DH5α cells containing the pPROEX-2A or one of the mutant plasmids were obtained by spreading 50 µl of each culture onto separate LB-GA plates. The expression of the EF-G alleles was induced by streaking the single colonies onto various types of media followed by overnight incubation at 37°C. Clones were streaked onto LB-GIA plates (LB with 20 mM glucose, 0.3 mM IPTG and 0.1 mg/ml ampicillin) as well as LB-IA plates (LB with 0.3 mM IPTG and 0.1 mg/ml ampicillin). Glucose represses the expression of EF-G, which has the *fus* gene contained on a plasmid that is under the control of a *lac* promoter, thus cells containing the pPROEX-2A plamids should survive. IPTG induces the overexpression of EF-G from the pPROEX-2A plasmid and if the mutant EF-G is toxic, the cells will not survive.

2.3 *In vivo* fusidic acid binding assay:

The ability of the EF-G mutant translocases to bind to the ribosome *in vivo* was screened for their ability to convert the fusidic acid resistant host to sensitivity. Fusidic acid is an antibiotic that freezes the EF-G on the ribosome, thereby killing the cell. Fusidic acid sensitivity (as in wild-type EF-G) is dominant to resistance. The fusidic acid resistant host *E. coli* NO863 (Nomura and Engbaek, 1972), was transformed with the pPROEX-2A plasmid (containing wildtype EF-G) and the plasmids containing the
mutant alleles of EF-G. The conversion of the NO863 host to a sensitive phenotype when streaked onto LB media containing 0.3 mM IPTG, 0.1 mg/ml ampicillin and 0.5 mM fusidic acid (Sigma) would suggest that the mutant EF-G is able to bind ribosomes.

2.4 Protein Expression and Purification:

The specific binding of 6-His EF-G to a nickel column was used to purify overexpressed wild-type and mutant EF-G and separate them from native EF-G. The solubility of the expressed proteins was initially checked on a small scale. Five ml aliquots of LB-A were inoculated with 50 l of an overnight LB-GA culture of DH5α containing pPROEX-2A or each of the mutant plasmids, and the cultures were grown at 37°C until the A590 0.5. IPTG (0.6 mM) was then added into each LB-A culture and the cultures were incubated for another 3 h at 37°C. The cells were harvested and lysed by alternatively freezing at -70°C for 5 min and then thawing the cells at 42°C for 5 min. Proteins present in each supernatant were visualized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a discontinuous buffer system (Laemmli., 1970).

For large-scale protein preparation, one-liter LB-A cultures of DH5α containing the expression vector pPROEX-2A or each of the mutant plasmids R127E, R17Q, Q128E, Q128N, G461A, G461P, G461S, E462D, E462H and G21D were grown at 37°C until A590 0.5. IPTG (0.6 mM) was then added into each LB-A culture to induce protein expression using the lac operator and promoter of the pPROEX-2A and allowed to grow for an additional 3 h. Cells were collected by centrifugation, washed in PBS (50 mM Na2HPO4, 300 mM NaCl) before being fast frozen and stored at -70°C. The cells were
weighed, added to a mortar along with 1.5X the cell weight of washed aluminum oxide powder and ground to a homogenous paste to lyse the cells. Ten ml PBS containing 0.1 mM PMSF (Life Technologies) was added and the sample was centrifuged at 10,000xg for 20 min. 3 µg/ml DNAse I (Sigma) was added and a clear solution was obtained by centrifugation at 20,000xg for 20 min followed by centrifugation at 30,000xg for 30 min.

Histidine-tagged proteins were recovered by loading 10 ml of each supernatant onto a 1 ml HiTrap Chelating column (Pharmacia) containing NiSO4. The flow-through was reloaded on the column to optimize binding of the histidine-tagged protein to the nickel column. The column was washed with 10 ml of PBS containing 0.1 mM PMSF and the protein was eluted using a gradient of 0-200 mM imidazole. 1ml fractions were collected in a Frac-100 fraction collector (Pharmacia). The protein content of the eluent was checked by a Uvicord SII UV detector (Pharmacia). Immediately after collecting the eluent, 1 mM DTT was added to each fraction to stabilize the protein. 5 µl aliquots of every third fraction eluted from the column after the start of the imidazole gradient were run on 8% SDS-PAGE gels to verify the presence of EF-G. The fractions that contained the most EF-G were pooled and dialyzed overnight with 10 mM Tris-HCl pH 7.5, 1 mM DTT and 0.1 mM PMSF. The proteins were quantitated by the Bradford method (Bradford, 1976) with the Coomassie Plus Protein Assay Reagent (Pierce).
2.5 Purification of *E. coli* tRNA synthetases:

*E. coli* tRNA synthetases for use in the preparation of \(^{14}\text{C}\)-phenylalanyl-tRNA were isolated according to previously published methods (Muench et al., 1966, Ravel et al., 1971). *E. coli* MRE600 cells were grown at 37°C in 3X 1.5 liter cultures of LB media until A\(_{650}\) 0.3. The cells were then harvested by centrifugation and washed in 500 ml of Tris buffer (20 mM Tris-HCl pH 7.4, 10 mM MgCl\(_2\) and 1 mM DTT). The cells were ground with three times the weight of alumina. The paste was suspended in about 20 ml 10 mM Tris-HCl pH8.0, 10 mM MgCl\(_2\), 10% glycerol, centrifuged at 14000xg for 20 min at 4°C followed by two high speed centrifugations for 20 min at 31000xg and at 41000xg in order to get rid of debris. The supernatant was dialyzed against two changes of 500 ml dialysis buffer (40 mM Tris-HCl pH 7.7, 6 mM β-mercaptoethanol).

2.6 Preparation of \(^{14}\text{C}\)-Phe-tRNA:

The classical method to determine protein synthesis activity of an elongation factor, involves the polymerization of \(^{14}\text{C}\)-phenylalanine using a poly(U)-directed message and tRNA\(_{\text{Phe}}\) (Nirenberg, 1963). Phenylalanyl-tRNAs need to be charged with \(^{14}\text{C}\)-phenylalanine for this assay. Small scale (100µl) charging reactions were performed to check the activity of the *E. coli* tRNA synthetase as well as to optimize the reaction conditions. Five large scale charging reactions were performed as described (Ravel et al., 1971). Each 10 ml reaction contained 1 ml 1 M Tris-HCl pH 7.7, 1 ml 0.1 M MgCl\(_2\), 2.36 mM ATP, 15 µl β-mercaptoethanol, 20 mg *E. coli* MRE600 tRNA (Boehringer Mannheim), 10 µl \(^{14}\text{C}\)-phenylalanine (NEN Life Science Products, specific activity 505
mCi/mmmole), 0.1 mg/ml phenylalanine to give a total (hot + cold) of 100 nmol phenylalanine, and 1 ml dialyzed *E. coli* tRNA synthetase preparation. A 5 µl aliquot was removed from each reaction mixture, spotted onto a #34 Glass fiber filter (Schleicher & Schuell) and the amount of radioactivity in each reaction was determined in a Liquid Scintillation Counter (LKB Wallac, model 1209 Rackbeta). The reactions were mixed, incubated for 15 min at 37°C and then placed on ice. A 50 µl aliquot was removed and a cold TCA precipitation was performed to determine the amount of phenylalanine incorporated into Phe-tRNA. 1 ml 2 M KOAc, pH6.0 and 11 ml liquefied phenol was added to each reaction and vortexed for 4 min. The two layers were separated by centrifugation at 27000xg for 15 min at 4°C. The aqueous layer was removed and to it 10% of the volume of 2 M KOAc, pH6.0 and 2 volumes of cold 100% ethanol were added. The samples were mixed by inversion, kept for two hours at -20°C and the pellet was collected after centrifugation. Each pellet was resuspended in 5 ml of 0.2 M KOAc, then 10 ml of ice cold 100% ethanol was added and left at -20°C for 1 h. The final pellet containing the $^{14}$C-Phe-tRNA was recovered by centrifugation and stored at -70°C. Before use, the pellet was resuspended in 100 µl 10 mM potassium succinate pH 6.0. A small aliquot was precipitated by TCA, filtered through a glass fiber filter and the amount of $^{14}$C-Phe-tRNA was determined in a liquid scintillation counter.
2.7 *In vitro* Protein Synthesis Assay:

The ability of wild-type EF-G and mutants of EF-G to function *in vitro* is determined by measuring the polymerization of $^{14}$C-phenylalanine in the presence of ribosomes and EF-Tu using a poly(U)-directed message. *E. coli* ribosomes and elongation factor Tu used in this assay had been previously prepared in our laboratory by Michael Wolfe following published procedures (Ravel et al., 1971, Ravel et al., 1968). Each 100 µl reaction contained 50 mm Tris-HCl, pH 7.8, 8 mM MgCl$_2$, 90 mM NH$_4$Cl, 100 µM spermine, 1 mM DTT, 2.5 mM GTP (Sigma), 5 µg poly uridylic acid (Sigma), 35 pmoles $^{14}$C-Phe-tRNA (specific activity 143 mCi / mmole), 80 µg ribosomes, 7.5 µl of EF-Tu preparation and 6-His EF-G as indicated. The 6-histidine tag does not significantly interfere with the assay (Wolfe, 1999). The non-hydrolyzable GTP analog GMPPCP and the antibiotic fusidic acid were as listed. After incubating the reactions at 37°C for 15 min to allow polyphenylalanine to be formed, 2 ml of 5%TCA was added and the reactions were incubated at 90°C for 10 min to hydrolyze the aminoacyl bond. The reactions were cooled to room temperature and filtered through glass fiber filters using a filtration apparatus (Millipore). Each reaction tube was rinsed twice with 1% TCA and filtered through the corresponding filter. The filters were dried and the radioactivity was quantitated by counting in 5 ml of Ecoscint-A liquid scintillation cocktail (National Diagnostics) for ten minutes in a liquid scintillation counter (LKB Wallac). The picomoles of $^{14}$C-Phenylalanine incorporated was plotted versus micrograms of 6-His EF-G. The slope is the specific activity of the 6-His EF-G.
2.8 Ribosome–Dependent GTPase Assays:

The GTPase activity of EF-G is greatly enhanced by ribosomes (Rodnina et al., 1997). To determine the ability of wild-type EF-G and the different mutants of EF-G to hydrolyze GTP in the presence of ribosomes, a ribosome-dependent GTPase assay was performed. Each reaction in a final volume of 25 µl contained 50 mM Tris-HCl pH 7.8, 8 mM MgCl₂, 90 mM NH₄Cl, 100 µM spermine, 1 mM DTT, and unless otherwise indicated, 0.5 µM 6-His EF-G and 0.25 µM ribosomes. GTP concentration was varied from 0 – 2 mM unless indicated otherwise. The competitive inhibitors GMPPCP and GDP were as listed. The reactions were incubated at 37°C. At various time points, 2.5 µl aliquots of each reaction were removed, added to 2.5 µl of formic acid and kept on ice. The GTPase activity was measured by monitoring the hydrolysis of α⁻³²P-GTP (Amersham). The samples were loaded onto PEI-cellulose plates (Sigma) and α⁻³²P-GDP was separated from α⁻³²P-GTP by ascending thin-layer chromatography in 0.6 M potassium phosphate, pH 3.5 (Randerath et al., 1966). The TLC plates were dried and exposed to a phosphorimager screen and the amount of radioactivity was quantitated on a 445 SI Phosphor Imager (Molecular Dynamics). The amount of GTP hydrolyzed was calculated and plotted versus time. The velocity of each reaction was obtained from the slope of the plot. Either a double reciprocal plot (Lineweaver and Burk, 1934) or an Eadie-Hofstee plot (Hofstee, 1959) was used to determine the rate constants.
2.9 Ribosome-Independent GTPase Assay:

Wild-type EF-G has the ability to hydrolyze GTP, but the intrinsic GTPase activity is much lower than the intrinsic GTPase activity of other members of the GTPase superfamily. It had been shown that isopropanol increases the intrinsic GTPase activity of EF-G (De Vendittis et al., 1986; Masullo et al., 1989). In this study, 10% isopropanol was used to increase the GTPase activity of wild-type EF-G and the EF-G mutants to a level at which the activity could be measured. 4 µM 6-His EF-G and 2 mM GTP (saturating) were added to a buffer containing 50 mM Tris-HCl pH 7.8, 8 mM MgCl₂, 90 mM NH₄Cl, 100 µM spermine, 1 mM DTT. 5 µl of isopropanol was added and the final volume made up to 50 µl with distilled water. Trace amounts of α-³²P-GTP was added and the reactions were incubated at 37°C for 16 h. Every 4 h, a 2.5 µl aliquot of each reaction was removed and added to 2.5 µl of formic acid. The GTP hydrolyzed was monitored as described above (2.8). The \( V_{\text{max}} \) of the reaction was obtained from the slope of a plot of pmole GTP hydrolyzed versus time. The turnover number of the reaction was calculated by dividing \( V_{\text{max}} \) by the amount of 6-His EF-G used.

2.10 Assay for the protection of the ribosome from the ribotoxin restrictocin:

EF-G binds to specific regions of the ribosome (Wilson and Noller, 1998a, Agrawal et al., 1999). The sarcin-ricin loop of the 23S rRNA is a highly conserved sequence to which EF-G binds. This sarcin-ricin loop is also the site at which various ribotoxins cleave the 23S rRNA (Wool, 1984). The binding of EF-G to the ribosome protects the 23S rRNA from cleavage by the ribotoxins α-sarcin and restrictocin. The
Figure 2.3: Schematic representation of the assay for the protection of the ribosome from the ribotoxin restrictocin. Lane 1 is completely protected 23S rRNA, lane 2 corresponds to 23S rRNA that has been completely cut by restrictocin, while lane 3 represents a partial cleavage of the RNA by restrictocin. The sarcin-ricin loop is indicated by the letter ‘S’. The probe is $^{32}$P-labeled SarcII primer, which is complimentary to a region near the 3’ end of 23S rRNA.
protection of the 23S rRNA from cleavage by the ribotoxins can be used as a measure of EF-G binding to the ribosome (Fig.2.3). Each reaction contained 2 mM GTP (or GMPPCP), 2.5 pmole ribosomes (6.5 µg), and varying amounts of 6-His EF-G in a final reaction volume of 25 µl of buffer (20 mM Tris pH 7.7, 10 mM NH₄Cl, 10 mM MgCl₂, 3 mM β-mercaptoethanol). The components were mixed and incubated on ice for 10 min. 0.5 µg of restrictocin (Sigma) was added to each reaction followed by incubation at 37°C for 10 min. The reactions were stopped by adding 75 µl of RNA extraction buffer (10 mM Tris pH 7.7, 40 mM NH₄Cl, 10 mM MgCl₂, 200 mM NaCl, 7 mM β-mercaptoethanol, 1% SDS) and the RNA was extracted following the procedure of Spedding (Spedding, 1990). The RNA pellet was dissolved in 50 µl distilled water. T4 kinase was used for 5’ end labeling of the Sarc II probe with γ-³²P ATP (Amersham) according to the instructions from the manufacturer (Invitrogen). The ³²P end labeled probe was then annealed to 4 µl of each purified rRNA in 2% SSC by first incubating at 75°C for 10 min followed by incubation at 37°C for 30 min. 2 µl of 6X TBE gel loading dye (Novex) was added to each tube and the reactions were loaded on a precast 8% TBE gel (Invitrogen). After electrophoresis for 1 h at 200V, the gel was wrapped in Saran wrap, exposed to a phosphorimager screen and the amount of uncut 23SrRNA and the α - fragment present in each reaction were quantified on a phosphorimager (Fig.2.3). In a separate experiment, 1 mM fusidic acid was added to each reaction in addition to the 2 mM GTP.

The sequence of the Sarc II probe used was: ATCTCGGGGCAAGTTTTCG.
CHAPTER 3

ROLE OF THE C\textsubscript{G} HELIX: RESULTS AND DISCUSSION:

3.1 Site-directed mutagenesis and protein purification:

The amino acid residues R127 and Q128 are part of the C\textsubscript{G} helix (Al-Karadaghi et al., 1996). This helix has contact with both the switch II helix as well as conserved regions of domain V (Laurberg et al., 2000). The arginine and glutamine amino acid residues of this C\textsubscript{G} helix are highly conserved in the EF-G (EF-2) of both prokaryotes and eukaryotes (Fig. 1.5), but not in other members of the GTPase superfamily suggesting that these amino acid residues may be involved in functions that are specific to the elongation step of protein synthesis. To test this possibility, mutant derivatives of EF-G containing alterations at either the R127 or Q128 positions were produced and tested for their ability to function in the different steps of the EF-G cycle (Fig.1.4).

The pPROEX-2A plasmids containing the R127E, R127Q, Q128E, Q128N mutations of the \textit{E. coli} EF-G gene were prepared by site-directed mutagenesis by Xiao Chun Liu. The 6-His tag present in the pPROEX-2A expression vector was used to separate the overexpressed wild type and mutant EF-Gs from the endogenous EF-G (Schmitt et al., 1993, Crowe et al., 1994). This technique had been used previously to purify elongation factors (Borowski et al., 1996, Worix et al., 1995, Wolfe, 1999). In addition to the 6-His tag, the pPROEX –2A vector encodes an additional 23 amino acid
residues fused to the amino-terminal of the EF-G protein. The amino-terminal region upstream of the phosphate-binding loop is not conserved among EF-G (EF-2) proteins of different organisms (Kohno et al., 1986, Creti et al., 1994). It had been shown that wild-type EF-G containing the 6-His tag was functional in vivo. The 6His-EF-G also had similar activity as the native EF-G in protein synthesis and GTP hydrolysis assays in vitro (Wolfe, 1999).

Bacterial growth curves (Fig. 3.1) were monitored to check for toxicity after induction of protein expression with IPTG. In addition, plasmids containing the wild type and mutant translocases were streaked onto inductive plates impregnated with inducer (LB containing ampicillin and IPTG). The tests for toxicity of the mutant EF-Gs on induction were necessary since a previous study in our laboratory had shown that a mutation of a single amino acid residue produced a protein that was toxic to the cell (Wolfe, 1999). Other studies with mutant EF-Tu had shown similar results (Gumusel et al., 1990, Zeidler et al., 1995). The observation that growth curves for wild-type and mutant EF-Gs are essentially identical suggests that overexpression of the R127E, R127Q, Q128E and Q128N mutant translocases was not harmful to the cell.

The proteins were purified by binding to a nickel chelating column and the EF-Gs were eluted from the column using an imidazole gradient. The homogeneity of each protein prepared was ascertained by electrophoresing a sample on an 8% SDS-polyacrylamide gel, followed by staining with Coomassie Blue (Fig 3.2). Wild-type EF-G and R127E, R127Q, Q128E, Q128N mutants were overexpressed to a high level, with yields of about 1 mg/g of cells (wet weight) and were soluble in aqueous solutions.
Figure 3.1: Bacterial growth curves of wild-type EF-G and the R127 and Q128 mutants. Wild-type 6-His EF-G and the R127E, R127Q, Q128E, Q128N mutants in broth cultures were induced for protein expression. Absorbance at 590 nm was monitored. To induce expression of the translocases, IPTG was added at the time indicated by an arrow: wild-type EF-G (diamond); R127E (square); R127Q (triangle); Q128E (rectangle); Q128N (circle).
Figure 3.2: Protein gel of wild-type and mutant EF-Gs. The purified proteins were run on an 8% SDS-polyacrylamide gel and stained with Coomassie Blue. Lanes 1 and 14, Benchmark protein ladder; lane 2, crude protein lysate; lane 3, wild-type EF-G; lane 4, R127E; lane 5, R127Q; lane 6, Q128E; lane 7, Q128N; lane 8, G461A; lane 9, G461P; lane 10, G461S; lane 11, E462D; lane 12, E462H; lane 13, G21D.
No precipitation phenomena or other stability problems were observed as long as DTT was present in the solution. Protein folding of wild-type EF-G and these mutants has been checked by circular dichroism spectroscopy (Fiala, unpublished).

3.2 Function of the R127 and Q128 mutants *in vivo*:

The steroid antibiotic fusidic acid (Fig. 3.3) is an inhibitor of protein synthesis. Fusidic acid inhibits translation by preventing the release of EF-G-GDP from the ribosome after translocation (Willie et al., 1975, Frank and Agrawal, 2000). Fusidic acid stabilizes the EF-G-GDP-ribosome complex (Bodley et al., 1970b, reviewed in Gale 1981) preventing recycling of the ribosome (Fig. 3.4). In the presence of fusidic acid, EF-G-GDP cannot adopt the conformation required to release from the ribosome (Baca et al., 1976). Strains of *E. coli* resistant to fusidic acid have been reported (Kinoshita et al., 1968, Tocchini-Valentini et al 1968). Resistance is usually due to a mutation in the *fus* gene which codes for EF-G (Bernardi et al., 1970, Richter-Dalfors et al., 1990, Johanson et al., 1994). Sensitivity to fusidic acid is dominant over resistance (Jaskunas et al., 1975a) because polysomes become blocked even in the presence of fusidic acid-resistant EF-G. When a fusidic acid-resistant host is transformed with wild-type EF-G from a sensitive strain of *E. coli*, transformed cells become sensitive to fusidic acid. The sensitive phenotype of the transformed cells indicates that the fusidic acid-sensitive EF-G is being expressed and is capable of binding to the ribosome *in vivo*. 
Figure 3.3: Structures of Fusidic acid and GMPPCP
Figure 3.4: Effect of fusidic acid on the EF-G cycle. Fusidic acid prevents the dissociation of the EF-G-GDP complex from the ribosome. The three different proposed conformations of EF-G are represented as a rectangle with one side changing from a V to flat to concave. The presence of tRNAs in the A-site or the P-site of the ribosome is denoted by the letters “A” and “P” respectively. GTP is indicated as a dark rectangle, while GDP is represented as an oval.
We have used the dominance of fusidic acid sensitivity in a rapid screen to determine whether EF-G mutant translocases can bind ribosomes. A fusidic acid-resistant strain of *E. coli* NO 863 (Nomura and Engbaek, 1972) was transformed with plasmids containing the wild-type, R127E, R127Q, Q128E or Q128N mutant EF-G. The cells were plated on inducible media containing 0.5 mM fusidic acid. Both the R127 mutants were sensitive to fusidic acid at the concentration of fusidic acid used, indicating that they can bind ribosomes *in vivo*. Transformed NO863 cells containing the Q128 mutant translocases were resistant to fusidic acid (Table 3.1), suggesting that the mutants themselves conferred fusidic acid resistance, or they failed to bind ribosomes.

3.3: *In vitro* protein synthesis activity of R127 and Q128 mutant translocases:

When evaluating mutant proteins, it is essential to examine the overall effect of the mutation on the protein’s ability to sustain its physiological function and later dissect each aspect of the protein’s function to determine specific defects. The poly(U)-dependent protein synthesis assay determines the overall ability of EF-G to function in translation. An artificial messenger RNA, polyuridylic acid poly(U), is often used for the determination of *in vitro* protein synthesis activity (Nirenberg et al., 1961). When phenylalanine and the other components of protein synthesis are supplied, the poly(U) RNA directs the synthesis of polyphenylalanine. The $^{14}$C-polyphenylalanine produced can be detected as described in materials and methods. The assay was performed under turnover conditions using saturating amounts of EF-Tu and limiting amounts of EF-G.
Table 3.1: *In vivo* tests of toxicity and sensitivity to fusidic acid. *E. coli* DH5α cells containing a plasmid-encoded EF-G gene were streaked on inductive media and grown overnight at 37°C. Ability of the cells to grow indicated that the EF-G was not toxic to the cell. Sensitivity to Fusidic acid was checked by streaking *E. coli* NO863 cells bearing the a plasmid-encoded EF-G gene on inducing media containing Fusidic acid, cells were allowed to grow overnight at 37°C. Growth of colonies on LB plates containing Fusidic acid indicated resistance to Fusidic acid.

<table>
<thead>
<tr>
<th>6His EF-G</th>
<th>Toxicity (<em>in vivo</em>)</th>
<th>Sensitivity to Fusidic acid (<em>in vivo</em>)</th>
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<tbody>
<tr>
<td>Wild-type EF-G</td>
<td>Non-toxic</td>
<td>Sensitive</td>
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<tr>
<td>R127E</td>
<td>Non-toxic</td>
<td>Sensitive</td>
</tr>
<tr>
<td>R127Q</td>
<td>Non-toxic</td>
<td>Sensitive</td>
</tr>
<tr>
<td>Q128E</td>
<td>Non-toxic</td>
<td>Resistant</td>
</tr>
<tr>
<td>Q128N</td>
<td>Non-toxic</td>
<td>Resistant</td>
</tr>
</tbody>
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Table 3.2: Specific activity of \textit{in vitro} protein synthesis of wild-type EF-G, and the R127 and Q128 mutants. Specific activities are expressed as pmole phenylalanine polymerized per pmole EF-G in 15 min. The Specific activities were obtained from the slope of a plot of pmole $^{14}$C-phenylalanine polymerized versus $\mu$g of EF-G used. Statistical analysis of the data was performed using the hetroscedastic t-test (Harris, 1995). The difference in the data obtained for the wild-type EF-G and the mutants was statistically significant.

<table>
<thead>
<tr>
<th>6His-EF-G</th>
<th>\textit{In vitro} protein synthesis specific activity</th>
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<tbody>
<tr>
<td>Wild-type EF-G</td>
<td>19.59 ± 0.59</td>
</tr>
<tr>
<td>R127E</td>
<td>22.24 ± 2.13</td>
</tr>
<tr>
<td>R127Q</td>
<td>16.65 ± 3.72</td>
</tr>
<tr>
<td>Q128E</td>
<td>5.32 ± 0.72</td>
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<tr>
<td>Q128N</td>
<td>5.68 ± 0.28</td>
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Figure 3.5: *In vitro* poly(U)-dependent protein synthesis. Protein synthesis assays were conducted in 100 µl reactions containing 35 pmole $^{14}$C-Phenylalanine-tRNA as described in the Materials and Methods. Amounts of $^{14}$C-Phenylalanine polymerized are shown. Data were corrected by subtracting a blank value from each point. The data represent the average of three trials. Specific activities were calculated as the slopes of the linear range of the plots. Wild-type EF-G (diamonds); R127E (squares); R127Q (triangles); Q128E (rectangles); Q128N (circles).
The R127E and R127Q mutants sustained the same rate of protein synthesis as wild-type EF-G (Table 3.2). This agreed with the results of the in vivo fusidic acid assay. The Q128E and Q128N mutants showed about 30% of the activity compared to wild-type EF-G (Fig. 3.5). The reduction in the protein synthesis activity of the Q128 mutants were apparently not affected by the identity of the amino acid substitution at this position since both Q128E and Q128N had similar activity.

3.4: Ribosome-dependant GTP hydrolysis:

For EF-G to function as a translocase, it needs to be able to perform various tasks (Fig. 1.4). To better understand how a particular mutation affects EF-G, each step of the EF-G cycle must be examined. Towards this end, various assays were performed using the mutant translocases. These assays include ribosome-dependent GTP hydrolysis, ribosome-independent GTPase, guanine nucleotide binding, and ribosome protection. To the extent possible, the reaction conditions were similar in all assays to determine the effect of each mutation under a constant set of conditions.

Unlike other GTP binding proteins of the GTPase superfamily, but similar to other GTP-binding protein synthesis factors, EF-G does not have a GTPase activating protein (GAP). It is believed that the ribosome provides a GTPase activation center that serves as the GAP for EF-G and a variety of translation factors (Modolell et al., 1971, Cundliffe 1971). The mutant translocases were therefore tested for their ability to catalyze the hydrolysis of GTP in a ribosome-dependent GTPase assay. EF-Tu, aminoacylated-tRNAs and poly(U) were withheld from the reaction, thus the tranlocation
Figure 3.6: The ribosome-dependent GTPase cycle under turnover conditions. The three different proposed conformations of EF-G are represented as a rectangle with one side changing from a V to flat to concave. GTP is indicated as a dark rectangle, while GDP is represented as an oval.
Table 3.3: GTPase activities of wild-type and mutant translocases. The velocities were calculated from a plot of pmole GTP hydrolyzed versus time using a substrate range of 0 – 1 mM GTP. The Km values were determined by the method of Eadie-Hofstee (Hofstee, 1995). The plots had correlation coefficients ≥ 0.95. The turnover numbers were determined at a saturating substrate concentration of 2 mM GTP by dividing the maximal rate (pmole GTP/sec) by the amount of EF-G(pmole) present. 10% isopropanol was used to obtain the turnover number in the ribosome-independent GTPase assay. Standard deviations are given. The data represent the results from triplicate sets of experiments.
step of the EF-G cycle is bypassed (Fig. 3.6). The steady-state kinetic parameters Km and kcat were determined for the R127 mutants and the Q128 mutants (Table 3.3). Since the assays were under steady-state conditions, any step of the EF-G cycle except translocation might be affected by a particular mutant.

The Km of the R127E, R127Q and Q128E mutants was about 2-fold higher than wild-type EF-G. The K_\text{m} of the Q128N mutant was similar to that of wild-type EF-G. The increased K_\text{m} of these mutants is unlikely to affect their activity in the \textit{E. coli} cells since intracellular GTP levels in the related \textit{Salmonella typhimurium} bacteria have been estimated at 923 \( \mu \text{M} \) (Neuhard and Nygaard, 1987). This is about 14-fold higher than the K_\text{ms} of the R127 and Q128 mutant translocases. The R127 and Q128 residues in helix C_G are about 20 \( \text{Å} \) away from the GTP binding region, thus mutations of these residues in helix C_G would not be predicted to have a direct effect on the binding of GTP.

The turnover numbers for the two R127 mutants were about 80\% that of the wild-type EF-G. The Q128N mutant was about 63\% as active as the wild-type EF-G, while the Q128E mutant had only 32\% of the ribosome-dependent GTPase activity compared to wild-type EF-G. The reduced kcat of these mutants could be due to a variety of reasons such as improper binding of GTP, or an improper binding of the EF-G–GTP complex to the ribosome to receive GTPase stimulation. The reduced activity could also be due to a lower rate of dissociation of GDP from the EF-G, or of EF-G from the ribosome. The Q128 mutants are interesting since the level of ribosome-dependent GTPase activity seems to be dependent on the identity of the substituted amino acid. It is possible that Q128 of \textit{E. coli} EF-G has a role in GTP hydrolysis.
3.5: **Ribosome-independent GTPase assay:**

EF-G has a very low GTPase activity in the absence of ribosomes. Such low activity cannot be measured accurately (Parmeggiani and Sander, 1981, Raimo et al., 1995). Certain alcohols like isopropanol (DeVendittis et al., 1986), or high salt concentrations (Masullo et al., 1994), can increase the GTPase activity to a level at which it can be measured. Isopropanol was used in the reaction to eliminate all ribosome binding steps as well as the translocation step from the EF-G cycle (Fig. 3.7). Such an assay is designed to investigate GTP binding to EF-G, GTP hydrolysis and GDP dissociation from EF-G. All four mutants being studied displayed a reduced intrinsic GTPase activity. The 2-fold decrease in ribosome-independent GTPase activity by these mutants is not as dramatic as the 20-fold decrease in ribosome-independent GTPase activity reported for a mutant EF-G in which the mutation directly affected the mechanism of GTP hydrolysis (Wolfe, 1999). This may indicate that the R127 and Q128 residues are not directly involved in the hydrolysis of GTP, though a defect in the positioning of the GTP for its hydrolysis cannot be ruled out for these mutant EF-G proteins.

3.6: **Interaction with guanine nucleotides GMPPCP and GDP:**

The ability of EF-G to bind GTP and release GDP are important aspects of its function in the EF-G cycle. Equilibrium dialysis would be the method of choice to directly determine the binding constants of the nucleotides to EF-G. However, it is not practical due to the relatively high $K_d$ values for GTP ($1.0 \times 10^{-4} \text{M}$) and GDP ($5.0 \times 10^{-5}$
Figure 3.7: Schematic representation of ribosome-independent GTP hydrolysis in the presence of isopropanol. Isopropanol enhances the intrinsic ability of EF-G to hydrolyze GTP. The three different proposed conformations of EF-G are represented as a rectangle with one side changing from a V to flat to concave. GTP is indicated as a dark rectangle, while GDP is represented as an oval.
M (Arai et al., 1977). Alternative methods used to determine the binding of nucleotides to the translocase have been reported (Masullo et al., 1994, Rodnina et al., 1997, Martemyanov et al., 2001). These methods use the inhibition by a non-hydrolyzable analog of GTP to study the binding of GTP by EF-G. It has been reported that EF-G prefers certain GTP analogs over others (Parmeggiani and Sander, 1981). GMPPCP (Fig. 3.3), was used in this study as it had been shown to bind EF-G the best among the GTP analogs studied (Parmeggiani and Sander, 1981). GMPPCP is a competitive inhibitor of GTP (Fig. 3.8) and thus the inhibitory constant, $K_i$, is also the binding constant ($K_d$) of GMPPCP for EF-G. Similarly, GDP is a competitive inhibitor of GTP (Fig. 3.9) and the $K_i$ of GDP for EF-G can be determined by measuring the inhibition of the ribosome-dependent GTP hydrolysis by GDP.

The inhibitory constant of GMPPCP for EF-G was about 3-fold higher than wild-type in the R127E, R127Q and Q128N mutants. The Q128E mutant has a slightly higher $K_i$ for GMPPCP (Table 3.4). These results indicate that all four mutants probably have a slight defect in the binding of GTP. While this defect in GTP binding might affect the $k_{cat}$ of these mutant translocases, the actual effect of this defective binding of GTP by these proteins on the hydrolysis of GTP is likely to be minimal since the $k_{cat}$ of these proteins were calculated using 2 mM GTP, this causes the equilibrium to be shifted towards GTP binding. The inhibitory constant of GDP for EF-G was lower in the Q128 mutants, about the same as wild-type EF-G in the R127E mutant and 2-fold higher in the R127Q mutant. These observations imply that the defect observed in the ribosome-
Figure 3.8: Inhibition of the GTPase activity of wild-type EF-G by GMPPCP. The ribosome-dependent GTPase activity of wild-type EF-G was measured in the presence or absence of GMPPCP (no GMPPCP (diamonds); 10 µM GMPPCP (squares); 20 µM GMPPCP (triangles);) as described in the Materials and Methods. Three sets of experiments were performed with no GMPPCP and in the presence of 10 µM GMPPCP. One experiment was performed with 20 µM GMPPCP. (a). Michaelis-Menten plot of velocity versus substrate concentration. (b). Lineweaver-Burk plots of 1/V versus 1/S was used to determine the $K_i$ of GMPPCP using the formula $K_p = K_m(1+([I]/K_i))$, where $K_p$ is the apparent $K_m$ obtained at the different inhibitor concentrations [I]. The $K_i$ of GMPPCP was obtained for each of the mutant translocases in parallel experiments. (See Table 3.4).
Figure 3.9: Inhibition of the GTPase activity of wild-type EF-G by GDP. The ribosome-dependent GTPase activity of wild-type EF-G was measured in the presence or absence of GDP (no GDP (diamonds); 100 µM GDP (squares); 200 µM GDP(triangles)) as described in the Materials and Methods. Three sets of experiments were performed with no GDP and with 200 µM GDP. One experiment was performed with 100 µM GDP. (a). Michaelis-Menten plot of velocity versus substrate concentration. (b). Lineweaver-Burk plots of $1/V$ versus $1/S$ was used to determine the $K_i$ of GDP using the formula $K_p = K_m(1+([I] / K_i))$, where $K_p$ is the apparent $K_m$ obtained at the different inhibitor concentrations $[I]$. The $K_i$ of GDP was obtained for each of the mutant translocases in parallel experiments. (See Table 3.4).
<table>
<thead>
<tr>
<th>6His EF-G</th>
<th>$K_i$ GMPPCP ($\mu$M)</th>
<th>$K_i$ GDP ($\mu$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type EF-G</td>
<td>$1.61 \pm 0.19$</td>
<td>$32.10 \pm 2.47$</td>
</tr>
<tr>
<td>R127E</td>
<td>$4.02 \pm 0.11$</td>
<td>$33.92 \pm 3.29$</td>
</tr>
<tr>
<td>R127Q</td>
<td>$5.35 \pm 0.02$</td>
<td>$64.22 \pm 0.95$</td>
</tr>
<tr>
<td>Q128E</td>
<td>$8.99 \pm 0.18$</td>
<td>$24.22 \pm 0.60$</td>
</tr>
<tr>
<td>Q128N</td>
<td>$4.68 \pm 0.25$</td>
<td>$20.21 \pm 1.39$</td>
</tr>
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Table 3.4: Inhibition constants ($K_i$) of GMPPCP and GDP for wild-type EF-G and the R127 and Q128 mutant translocases. The velocities were calculated from a plot of pmole GTP hydrolyzed versus time using a substrate range of 0–100 $\mu$M GTP. Triplicate sets of experiments were performed using either 10 $\mu$M or 20 $\mu$M GMPPCP and 100 $\mu$M or 200 $\mu$M GDP. The $K_m$ and the apparent $K_m$ ($K_p$) were determined from Lineweaver-Burk plots with correlation coefficients $\geq 0.95$. Standard deviations are given.
dependent GTPase assay with the R127 mutant translocases is not due to an increased affinity of these mutants for GDP. The slightly better binding of GDP by the Q128 mutants compared with wild-type might explain their reduced ribosome-dependent GTPase activity, though other possibilities exist.

3.7: Protection of the ribosome by EF-G:

The ribotoxins α-sarcin and restrictocin cleave the bond between nucleotides 2661 and 2662 of *E. coli* 23S rRNA (Hausner et al., 1987, Gluck and Wool, 2002). Nucleotides 2661 and 2662 are within a highly conserved region of the sarcin-ricin loop (SRL) of domain VI of the 23S rRNA (Leffers et al., 1988). It has been suggested that this region might be part of the ribosomal GTPase (Moazed et al., 1988). This site has been localized to the L6-L14 region of the 50S subunit of the ribosome, close to the EF-G binding site (Ban et al., 1999). It has been proposed to interact with EF-G through contacts near the interface of the G-domain and domain V (Wilson and Noller, 1998b).

The presence of EF-G protects ribosomes from cleavage by these ribotoxins (Fig. 2.3) in both prokaryotes and eukaryotes (Leffers et al., 1988, Brigotti et al., 1989, Miller and Bodley, 1991). The level of protection provided by EF-G could be used to determine the binding of EF-G to the ribosome (Wolfe, 1999). Binding to the ribosome is an essential step in the EF-G cycle, and the ribosome greatly enhances the rate of GTP hydrolysis by EF-G. Characterization of mutant forms of EF-G should include the possibility that the mutant EF-G might be defective in binding to the ribosome. In this study, the protection
afforded by the mutant translocases to the ribosome was studied using the ribotoxin restrictocin (from *Aspergillus restricticus*).

In the presence of GTP, wild-type EF-G and the mutant EF-Gs did not protect the ribosome from cleavage by restrictocin. This may indicate a defect in binding to the ribosome, or it may result from a high turnover of EF-G, where after GTP hydrolysis the EF-G-GDP complex dissociates from the ribosome leaving the ribosome vulnerable to cleavage by restrictocin. Two methods were used to slow the dissociation of EF-G from the ribosome. In initial experiments, GMPPCP was used in ribosome protection assays, to slow the dissociation of EF-G from the ribosome. GMPPCP is a non-hydrolyzable analog of GTP and will stall the EF-G cycle at the GTPase step (Fig 3.10). Better protection of the $\alpha$-sarcin site in the presence of GMPPCP was observed because the conformational change needed for the release of EF-G-GDP from the ribosome cannot take place (Kaziro, 1978). To account for the possibility that reduced binding of GMPPCP to EF-G might affect the binding of EF-G to the ribosome, a second set of experiments was performed using GTP and the antibiotic fusidic acid. Fusidic acid binds to EF-G and stabilizes the EF-G-GDP complex on the ribosome (Fig. 3.4), thus providing greater protection of the ribosome from cleavage by restrictocin.

In the presence of GMPPCP, the Q128N mutant protects the 23SrRNA similarly to the wild-type EF-G, while the R127E, R127Q, Q128E mutants do not protect the ribosome as well as wild-type EF-G (Fig. 3.11). As in the case with GMPPCP, the Q128N mutant protected the ribosome just as well as the wild-type EF-G while the R127E, R127Q, and Q128E mutants provided less protection (Fig. 3.12).
Figure 3.10: Effect of GMPPCP on the EF-G cycle. GMPPCP is a non-hydrolyzable analog of GTP and inhibits the GTP hydrolysis step of the EF-G cycle. The three different proposed conformations of EF-G are represented as a rectangle with one side changing from a V to flat to concave. The presence of tRNAs in the A-site or the P-site of the ribosome is denoted by the letters “A” and “P” respectively. GTP is indicated as a dark rectangle, while GDP is represented as an oval.
Figure 3.11: Protection of the α-sarcin site of the ribosome by wild-type and mutant translocases. Proportion of 23S rRNA protected from cleavage by restrictocin. Reactions contained 2 mM GMPPCP (except the control that contained 2 mM GTP), 2.5 picomole ribosomes, 0.5 µg restrictocin and varying amounts of EF-G as indicated. The assay was conducted as described in the Materials and Methods. The results are the average of triplicate sets of data, the error bars indicate the standard deviation between the sets of data. Wild-type EF-G (diamonds); R127E (large squares); R127Q (triangles); Q128E (rectangles); Q128N (circles), wild-type EF-G with GTP (small squares).
Figure 3.12: Effect of Fusidic acid on the protection of the \(\alpha\)-sarcin site of the ribosome by wild-type and mutant translocases. Proportion of 23S rRNA protected from cleavage by restrictocin. Reactions contained 1 mM Fusidic acid and 2 mM GTP (except the control that contained only 2 mM GTP), 2.5 picomole ribosomes, 0.5 \(\mu\)g restrictocin and varying amounts of EF-G as indicated. The assay was conducted as described in the Materials and Methods. The results are the average of triplicate sets of data, the error bars indicate the standard deviation. Wild-type EF-G (diamonds); R127E (large squares); R127Q (triangles); Q128E (rectangles); Q128N (circles), wild-type EF-G with GTP (small squares).
The data observed for the R127 mutants were surprising since both these mutants seem to have similar activity to the wild-type EF-G in the protein synthesis assay as well as the ribosome-dependent GTPase assay. It is possible that the slight decrease observed in the ribosome-dependent GTPase assay for these two mutants might be due to the defect in ribosome binding seen in this experiment. The Q128 mutants have been shown to be insensitive to fusidic acid in an in vivo assay using 0.5 mM fusidic acid. However in this in vitro assay which used 1 mM fusidic acid, both the Q128 mutants showed some protection, that is they both bound to the ribosome. This implies that the resistance to fusidic acid is dependent on the amount of fusidic acid present, that is, the lower amount of fusidic acid used in the in vivo assay was probably not sufficient to freeze enough of the Q128 mutants on the ribosome to affect cell viability.

3.8: In vitro protein synthesis in the presence of fusidic acid:

The Q128 mutants were insensitive to fusidic acid in vivo but sensitive to the drug in vitro. To clarify this apparent contradiction, another in vitro experiment was performed. In this assay, a wide range of fusidic acid concentrations was used. It seemed that at lower concentrations of fusidic acid the Q128 mutants are relatively resistant to the drug, but at higher concentrations these mutants are sensitive to fusidic acid (Fig. 3.13).

3.9: Discussion:

This work analyzed the effects of mutations of two conserved residues of E. coli EF-G. Both of these residues are located in the third helix (C₆) of the guanine nucleotide
binding domain of EF-G (Fig. 1.3)(Ævarsson et al., 1994, Czworkowski et al., 1994, Al-Karadaghi et al., 1996). This helix is highly conserved in all classes of organisms (Fig. 1.5) (Creti et al., 1994). Despite the high level of conservation of the amino acids of this helix, very little work has been done to characterize this region of EF-G. Most of the studies of EF-G have focused either on those regions of the G-domain involved with GTP binding and hydrolysis (Sharer et al., 1999, Wolfe, 1999, Mohr et al., 2000), or on the regions of the EF-G believed to be involved in the binding of the ribosome (Hou et al., 1994b, Martemyanov et al., 1999, Savelsbergh et al., 2000a). Recent cryo-electron microscopy studies have suggested that GTP hydrolysis by EF-G causes large conformational changes in the ribosome (Agrawal et al., 1998, Agrawal et al., 1999). These studies suggest that inter-domain interactions between the G-domain and domains III and V of EF-G allow the EF-G to undergo conformational changes that drive translocation. This is in agreement with an earlier hydroxyl radical probing study which suggested that GTP hydrolysis causes a rearrangement of the G-domain near its interfaces with domains III and domain V. This slight conformational change in domains III and V could cause a shift in domain IV, the tip of which has been mapped in the A-site of the 30S ribosomal subunit (Wilson and Noller, 1998a). The repositioning of domain IV of EF-G was proposed to allow it to interact with conserved regions of the ribosome which caused the ribosome to be unlocked, thus leading to translocation (Spahn and Nierhaus, 1998, Wilson and Noller, 1998a, Frank and Agrawal, 2000). This current model of translocation highlights the importance of the inter-domain contacts in the mechanism of translocation. Another indication that this C_g helix is important for EF-G function, is the
Figure 3.13: Inhibition of the *in vitro* protein synthesis assay by Fusidic acid. Protein synthesis assays using wild-type EF-G, Q128E and Q128N were conducted as described in the Materials and Methods. 10 pmole $^{14}$C-phenylalanine was used along with saturating EF-Tu. Reactions were performed in the presence of 2.5mmGTP and varying amounts of Fusidic acid as indicated. The results are the average of triplicate sets of data, the error bars indicate the standard deviation between the sets of data. Wild-type EF-G (diamonds); Q128E (squares); Q128N (circles).
observation that most of the EF-G mutants that are fusidic acid resistant have mutations affecting amino acid residues of the C_G helix or to the amino acid residues of the interface between domains III and the G-domain (Johanson and Hughes, 1994, Laurberg et al., 2000).

We propose that the role of arginine 127 is in ribosome binding (Fig.3.14). This concept is supported by the weaker protection of the ribosome from cleavage by restrictocin in the presence of these mutants (Figs. 3.11 and 3.12). Arginine 123 of T. thermophilus EF-G corresponding to R127 of E. coli EF-G, is oriented towards a conserved region of domain V and it is in proximity to the highly conserved glycine 651 (E. coli numbering)(Al-Karadaghi et al., 1996, Laurberg et al., 2000. Glycine 651 with which arginine 127 is believed to interact has been shown to be important for ribosome binding (Wilson and Noller, 1998a). Mutations of this arginine probably affect the link between the C_G helix of the G-domain and the region of domain V which is believed to be responsible for binding the ribosome (Agrawal et al., 1998). The defect in the protection of the ribosome could be due to a defect in either EF-G-GTP binding to the ribosome, or EF-G-GDP dissociation from the ribosome or even a combination of the two.

It is unlikely that the R127E and R127Q mutants affect the ability of the mutant EF-G-GTP complexes to bind to the ribosome. If mutation of R127 did cause a defect in the binding of the EF-G-GTP complex to the ribosome, it would also cause a reduction in the ribosome-dependent GTPase activity as well as reduce the protein synthesis activity of these mutants. Since the R127E and R127Q mutants have similar protein synthesis
Figure 3.14: Proposed role of arginine 127 of *E. coli* EF-G in the EF-G cycle. The arginine residue is believed to help EF-G bind to the ribosome. Mutations of this residue allow the EF-G-GDP complex to dissociate from the ribosome faster than the wild-type EG-G-GDP complex. The three different proposed conformations of EF-G are represented as a rectangle with one side changing from a V to flat to concave. The presence of tRNAs in the A-site or the P-site of the ribosome is denoted by the letters “A” and “P” respectively. GTP is indicated as a dark rectangle, while GDP is represented as an oval.
activity as wild-type EF-G (Table 3.2), it implies that, at the very least, the R127 mutant EF-G proteins bind the ribosome and are capable of translocation. Furthermore, the R127E and R127Q mutant EF-Gs do not seem to have a dramatic defect in the binding of GMPPCP and GDP (Table 3.4), suggesting that the conformation of the mutant proteins is not distorted to a level that affects binding of these nucleotides.

The analogous arginine in the EF-Tu from *T. aquaticus* is oriented towards domain III in EF-Tu in both the GDP-bound and the GTP-bound forms (Kjeldgaard et al., 1993, Nyborg et al., 1996). The similar orientation of the side chain might imply a role of this conserved arginine that is shared by both EF-Tu and EF-G. One possibility is that the correct positioning of this arginine is needed for stimulation of the GTPase of both EF-Tu and EF-G by the ribosome. This is unlikely as both the R127E and R127Q mutants of EF-G did not have a dramatic decrease in their ribosome-dependent GTPase activity (Table 3.3). If the arginine residue was directly involved in the hydrolysis of GTP, one would expect to see a higher GTPase activity in the more conservative R127Q mutant compared to the R127E mutant. The slightly reduced ribosome-dependent GTPase activity could be explained by inefficient binding of GTP as noted in the slight decrease in affinity for GMPPCP. Both the R127 mutants had reduced intrinsic GTPase activity. Due to the low level of GTP hydrolysis even in the presence of isopropanol, it is not unusual to see mutants with 2 to 4-fold decreases in intrinsic GTPase activity (Jacquet and Parmeggiani, 1988). This assay is useful in identifying more dramatic decreases in the intrinsic GTPase activity (Wolfe, 1999).
The R127E and R127Q mutants are sensitive to fusidic acid in vivo. Fusidic acid is believed to stabilize the EF-G-GDP complex on the ribosome by preventing a conformational change required for the EF-G-GDP to dissociate from the ribosome (Laurberg et al., 2000). Both R127 mutants are defective in ribosome binding and would be expected to be fusidic acid resistant (Johanson et al., 1996, Wolfe, 1999). It is possible the ribosome protection assay, which measures the protection of the sarcin-ricin loop of 23S rRNA of the 50S ribosome, is not sensitive enough to predict whether a mutant translocase can bind to the ribosome. This sarcin-ricin loop is involved in the binding of EF-G and it has been shown to interact with the region of domain V containing the conserved glycine 651 (Wilson and Noller, 1998a, Agrawal et al., 1999). Arginine 127 is in position to interact with glycine 651 (Laurberg et al., 2000). Any mutations to R127 could affect the binding of this region of domain V with the sarcin-ricin loop, which would expose this loop to cleavage by restrictocin even though the rest of the EF-G were still bound to the ribosome by numerous other contacts between EF-G and both subunits of the ribosome (Wilson and Noller, 1998a, Agrawal et al., 1999). Since the EF-G is still bound to the ribosome, it could be sensitive to fusidic acid, but the ribosome binding assay in the presence of fusidic assay would not be able to demonstrate this sensitivity to fusidic acid by the R127 mutant translocases.

Glutamine 124 of the *T. thermophilus* EF-G is oriented towards the switch II helix in the GDP-bound structure of EF-G (Al-Karadaghi et al., 1996). In the GDP-bound form of EF-G, glutamine 128 binds to the carbonyl group of valine 92 of the switch II helix (Johanson et al., 1996). This is in contrast to the orientation of this residue in EF-Tu. In
both the GDP-bound and the GTP-bound form of EF-Tu (Kjeldgaard et al., 1993, Nyborg et al., 1996), the side chain of this glutamine is oriented towards domain III of EF-Tu, similar to the orientation of the adjacent arginine residue. This might indicate that this glutamine residue is involved in different functions in EF-Tu and EF-G. EF-Tu is known to undergo a dramatic change in conformation on hydrolysis of GTP (Kjeldgaard et al., 1993, Berchtold et al., 1993), yet the orientation of this glutamine is not altered. It is plausible to expect that the orientation of glutamine 128 in the active GTP-bound form of EF-G remains oriented towards the switch II helix of the G-domain given that EF-G does not undergo as dramatic a change in conformation when GTP is hydrolyzed (Czworkowski and Moore, 1997, Agrawal et al., 1999).

We propose that Q128 helps to stabilize the switch II region of the G-domain. Destabilization of the switch II helix could affect multiple steps of the EF-G cycle (Fig. 3.15), such as GTP hydrolysis, translocation, and dissociation of the EF-G-GDP complex from the ribosome. This hypothesis is supported by the observation that mutations to this glutamine residue affect the GTP hydrolysis activity of the mutant translocases (Table 3.3). The mutant translocase containing the more conservative Q128N mutation has a higher catalytic efficiency compared to the Q128E mutant. Since the identity of the amino acid at this position seems to be relevant to the ribosome-dependent GTPase activity, it implies that the Q128 residue makes a specific interaction that is needed for the hydrolysis of GTP. Due to its distance of about 20 Å from the GTP binding site, the Q128 mutant probably has an indirect role in the mechanism of GTP hydrolysis such as stabilization of the switch II helix. The insertion of a negative charge within the
Figure 3.15: Proposed roles of glutamine 128 of *E. coli* EF-G in the EF-G cycle. The glutamine residue is believed to affect both GTP hydrolysis as well as translocation. Mutations to this residue allow the EF-G-GDP complex to dissociate from the ribosome faster than the wild-type EG-G-GDP complex. The three different proposed conformations of EF-G are represented as a rectangle with one side changing from a V to flat to concave.
hydrophobic region of switch II could cause destabilization of the switch II helix. In EF-Tu, the switch II region is involved in orienting the magnesium ion required for GTP hydrolysis (Nyborg et al., 1996). It is likely that the switch II region of EF-G plays a similar role in the stabilization of the magnesium ion needed for GTP hydrolysis given their similar orientation in both proteins (Nyborg et al., 1996, Al-Karadaghi et al., 1996). The slight decrease in the affinity for GTP and the slight increase in the affinity for GDP observed in these mutants are unlikely to be responsible for the reduced activity seen in the ribosome-dependent GTPase assay (Table 3.4).

Since it has been shown that GTP hydrolysis drives translocation (Rodnina et al., 1997), the reduced activity of these mutants in the in vitro protein synthesis assay is to be expected (Table 3.2). However, unlike in the ribosome-dependent GTPase assay, where the identity of the substituted residue affected the catalytic efficiency, protein synthesis activity in both mutants was similar (Fig. 3.5). This suggests that the Q128 mutants are unable to undergo the conformational change needed for translocation (Agrawal et al., 1999, Ramakrishnan, 2002). Thus not only did the Q128E and Q128N mutants have a defect in ribosome–dependent GTP hydrolysis, they probably had a second defect, a reduced ability to change their conformation after GTP hydrolysis and before the translocation step. This conformational change is probably due to GTP hydrolysis and has been observed in many members of the GTPase superfamily such as ras and EF-Tu (Berchtold et al., 1993). Since in the Q128 mutants, one of the links between the GTP binding region of EF-G and the rest of EF-G is missing, transmission of a change in conformation in the GTP binding region to the rest of EF-G will be affected. Thus the
shift in the position of domain IV which is believed to cause translocation (Wilson and Noller, 1998a, Agrawal et al., 1999) might not occur as efficiently as in wild-type EF-G, causing an additional decrease in the protein synthesis rate due to defective translocation.

Further evidence of the inability of these Q128 mutants to change their conformation after GTP hydrolysis was obtained from the ribosome protection assay. The protection afforded to the ribosome by the Q128N mutant in the presence of GMPPCP (Fig. 3.11), indicated that this mutant binds efficiently to the ribosome and that the defect in protein synthesis is probably due to a decrease in the ability of this mutant EF-G to change its conformation, thereby preventing translocation. Such changes in the conformation of EF-G after binding to the ribosome have been suggested (Abel and Jurnak, 1996) and some intermediate ribosome bound states have been observed in EF-Tu (Rodnina et al., 1995).

Binding to the ribosome is an important part of the EF-G cycle. Though EF-G is not needed for translocation (Gavrilova et al., 1976), the binding of EF-G to the ribosome greatly increases the rate of protein synthesis by stimulating the rate of GTP hydrolysis by EF-G (Modolell et al., 1971). This suggests that the ability for protein synthesis is inherent to the ribosome, and that EF-G might aid protein synthesis by lowering a kinetic barrier upon binding to the ribosome (reviewed in Ramakrishnan, 2002).

There are several reasons why a mutation might confer fusidic acid resistance. The interaction between EF-G and the ribosome might be altered, or the EF-G might have reduced affinity for the antibiotic due to a change in the binding site for fusidic acid. The most likely cause of fusidic acid resistance is probably due to the ability of the
translocase to dissociate from the ribosome despite the presence of fusidic acid (Johanson et al., 1996). The Q128E and Q128N mutants are resistant to fusidic acid in vivo. In vitro, the Q128E mutant is resistant to fusidic acid, while the Q128N mutant is sensitive to fusidic acid (Fig. 3.12). This is consistent with earlier studies that found that a Q128H mutant was resistant to fusidic acid (Johanson and Hughes, 1994). Other work done in our laboratory had indicated that the Q128E mutant was fusidic acid resistant in vivo as well as in vitro (Wolfe, 1999).

Mutations of the corresponding glutamine in E.coli EF-Tu (Q124) cause resistance to kirromycin by destabilization of the inter-domain contacts, thus allowing the transition to the GDP-bound conformation of the protein which can dissociate from the ribosome (Abdulkarim et al., 1994, Anborgh et al., 1991). The ribosome binding studies reported here indicated that the Q128E mutant of EF-G has a defect in ribosome binding in the presence of fusidic acid and GMPPCP (Figs. 3.11 and 3.12). In the Q128E mutant, the negative charge near the switch II region due to the mutation of glutamine 128 to glutamic acid may change the conformation of this mutant EF-G such that it affects its binding to the ribosome. This probably caused the observed resistance to fusidic acid and the ability of this mutant to rescue a toxic phenotype of EF-G (Wolfe, 1999). The Q128N mutant protects the ribosome equally as well as the wild-type EF-G, and furthermore in the in vitro assay, the Q128N mutant was sensitive to fusidic acid. Sensitivity to the drug is probably due to the higher concentration of fusidic acid used in the ribosome protection assay. The direct relationship between fusidic acid sensitivity and the concentration of drug used was demonstrated in an in vitro protein synthesis assay (Fig. 3.13). The in vivo
and *in vitro* studies of the effect of fusidic acid on the Q128 mutant showed that at low levels of fusidic acid, the Q128 mutants are resistant to fusidic acid.

It has been reported (Martemyanov et al., 2001) that there is a direct correlation between fusidic acid sensitivity and GMPPCP binding. This has been substantiated by the Q128E results. The Q128E mutant has been shown to be fusidic acid resistant *in vivo* (Wolfe, 1999, this study), and this mutant also has a lower affinity for GMPPCP. This might indicate that GTP binding to EF-G might help fusidic acid to function as an inhibitor. This is plausible because on binding GTP, the EF-G-GTP complex would bind to the ribosome allowing fusidic acid to stabilize the EF-G-GDP complex on the ribosome (Laurberg et al., 2000). It is still not clear whether fusidic acid binds to EF-G or the ribosome, however since the chromosomal mutations that cause fusidic acid resistance map to the EF-G gene, it has been suggested that EF-G is the target for fusidic acid (Johanson and Hughes, 1994).

Thus Q128 of *E. coli* EF-G is probably involved in stabilization of the switch II helix. Loss of this contact with switch II, may cause a reduction in the ribosome-dependent GTPase activity due to destabilization of the magnesium ion needed for GTP hydrolysis. The reduced protein synthesis activity in both Q128 mutants is probably due to both a reduced GTPase activity as well as the inability to change their conformation, which is required for translocation. Mutation of glutamine to glutamic acid also caused the mutant EF-G to dissociate from the ribosome, conferring fusidic acid resistance to this mutant.
CHAPTER 4

ROLE OF THE INTERFACE BETWEEN THE G-DOMAIN AND DOMAIN III:

RESULTS AND DISCUSSION:

4.1: Site-directed mutagenesis and protein purification:

The two amino acid residues G461 and E462 are part of a highly conserved region of domain III of EF-G that form the interface between the G-domain and domain III (Fig. 1.5). Apart from the observation that most mutations that confer resistance to fusidic acid are located in this region (Johanson and Hughes, 1994), very little is known about the function of the amino acid residues at this interface. Since this region has not been resolved in wild-type EF-G (Czworkowski et al., 1994, Al-Karadaghi et al., 1996), it is difficult to predict what interactions might be involved between the amino acid residues in this region and other regions of EF-G. However, the high conservation of the amino acid residues at this interface with the G-domain, in close proximity to the switch II helix (Laurberg et al., 2000), suggests that these amino acid residues may be involved in stabilizing the switch II region, and possibly transmit conformational changes due to GTP hydrolysis from the G-domain to other domains of EF-G. To study these possibilities, site-directed mutagenesis was used to produce mutant derivatives of glycine 461 and glutamic acid 462, which are at the center of this conserved region of domain III. These mutant derivatives were then tested for their ability to function in protein synthesis and in the various steps of the EF-G cycle (Fig. 1.4).
Plasmids containing the G461A, G461P, G461S, E462D, E462H mutations to the *E. coli* EF-G gene were prepared by site-directed mutagenesis as described in chapter 2. VENT DNA polymerase, which contains proofreading activity, was used to reduce the possibility of unwanted PCR-generated mutations. The lack of secondary mutations was confirmed by DNA sequencing of the plasmids containing the mutations to the EF-G gene. DH5α cells were transformed with the plasmids and grown at 37°C in LB media containing ampicillin. The growth of these cells was monitored (Fig. 4.1) to detect the presence of toxic mutations by streaking *E. coli* cells containing these mutant plasmids onto inductive media containing IPTG. None of the G461 or E462 mutants were toxic (Table 4.1).

The mutant proteins containing a 6-His tag were purified using a nickel-chelating column. The purity of each protein was determined by running aliquots of each on a SDS-polyacrylamide gel and visualizing the proteins by staining with Coomassie Blue Stain (Fig. 3.2). All the mutant EF-G proteins obtained were soluble in aqueous solutions. The protein yield obtained with the G461P mutant plasmid was lower than that obtained from the wild-type EF-G plasmid, while the protein yield from the other mutants was comparable to wild-type EF-G. Circular dichroism spectroscopy has been used to demonstrate that these mutant translocases were folded correctly (Fiala, unpublished).
Figure 4.1: Bacterial growth curves. Induction of wild-type 6His EF-G and the G461A, G461P, G461S, E462D, E462H mutants in broth cultures during protein expression. Absorbance at 590 nm were monitored. To induce expression of the translocases, IPTG was added at the time indicated by an arrow. Wild-type EF-G (diamonds); G461A (squares); G461P (triangles); G461S (light rectangles); E462D (dark rectangles); E462H (circles).
4.2: *In vivo* test of protein synthesis:

The *fus*\(^{-}\) host *E. coli* NO863 (Nomura and Engbaek, 1972) was transformed with the plasmids containing wild-type EF-G, G461A, G461P, G461S, E462D and E462H translocases and their expression on media containing fusidic acid was examined. The E462D and E462H mutants were able to induce fusidic acid sensitivity in the *fus*\(^{-}\) host cells (Fig. 4.2). The ability of these E462 mutants to induce fusidic acid sensitivity indicated that the E462D and the E462H mutant translocases were stable, able to bind the ribosome and participate in ribosome associated activities *in vivo*. The G461A, G461P, G461S EF-G mutants were resistant to fusidic acid *in vivo*. This could be due to a number of reasons. It is unlikely that the G461 mutant translocases are unstable *in vivo*, since the levels of soluble G461A, G461P, G461S and wild-type EF-G obtained from crude protein preparations were found to be similar (data not shown). The G461 mutant translocases may not bind to the ribosomes *in vivo* due to an inability to adopt a conformation required for ribosome binding, because of a defect in either GTP binding or the hydrolysis of GTP. Another possibility is that these mutant translocases bind to the ribosomes but are able to dissociate from the ribosomes despite the stabilizing effect of fusidic acid for the EF-G-GDP complex on the ribosome. This would imply that these G461 mutant EF-Gs are functional and fusidic acid resistant *in vivo*. This would not be surprising, since it had been reported that mutations that confer fusidic acid resistance are usually located at domain interfaces (Johanson and Hughes, 1994).
<table>
<thead>
<tr>
<th>6His EF-G</th>
<th>Toxicity (\textit{in vivo})</th>
<th>Sensitivity to Fusidic acid (\textit{in vivo})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type EF-G</td>
<td>Non-toxic</td>
<td>Sensitive</td>
</tr>
<tr>
<td>G461A</td>
<td>Non-toxic</td>
<td>Resistant</td>
</tr>
<tr>
<td>G461P</td>
<td>Non-toxic</td>
<td>Resistant</td>
</tr>
<tr>
<td>G461S</td>
<td>Non-toxic</td>
<td>Resistant</td>
</tr>
<tr>
<td>E462D</td>
<td>Non-toxic</td>
<td>Sensitive</td>
</tr>
<tr>
<td>E462H</td>
<td>Non-toxic</td>
<td>Sensitive</td>
</tr>
</tbody>
</table>

Table 4.1: \textit{In vivo} tests of toxicity and sensitivity to fusidic acid of the G461 and E462 mutant translocases. \textit{E. coli} DH5\(\alpha\) cells containing a plasmid-encoded wild-type EF-G gene or one of the mutant translocase genes were streaked on inductive media and grown overnight at 37°C. Ability of the cells to grow indicated that the EF-G was not toxic to the cell. Sensitivity to fusidic acid was checked by streaking \textit{E. coli} NO863 cells bearing the plasmid-encoded wild-type EF-G gene or one of the mutant EF-G genes on inductive media containing fusidic acid, cells were allowed to grow overnight at 37°C. Lack of growth of colonies on LB plates containing fusidic acid indicated sensitivity to fusidic acid.
**Figure 4.2:** *In vivo* test of function of wild-type and mutant translocases. Fusidic acid resistant *E. coli* NO863 cells were transformed with the wild-type EF-G gene in pPROEX-2 plasmid, E462D plasmid, E462H plasmid or the parent vector pPROEX-2. Cells containing the different plasmids were streaked onto inductive media containing fusidic acid and allowed to grow overnight at 37°C. Since sensitivity is dominant to resistance, cells expressing a functional EF-G protein from a fus\(^s\) strain, will not survive.
4.3: *In vitro* protein synthesis activity of the G461 and E462 mutant translocases:

The activity of the G461 and E462 mutant translocases was tested in an *in vitro* protein synthesis assay. This assay tests the ability of the mutant translocases to perform poly(U)-directed protein synthesis. The mutagenesis of glycine 461 to alanine, proline or serine severely diminished the ability of these translocases to incorporate phenylalanine. The protein synthesis activity of the most conservative mutation (glycine to alanine) had a specific activity of about 1% of that of wild-type EF-G (Fig. 4.3, Table 4.2). The other two mutants had an even greater reduction in their respective specific activities, with the G461P mutant EF-G having protein synthesis activity that was barely above background. Since this assay was performed at a much higher GTP concentration (2.5 mM) than the concentration of GTP that is normally used for such experiments (Nirenberg, 1963, Nishizuka and Lipmann, 1966), insufficient GTP is not likely to be the reason for the reduced protein synthesis activity. However, an inability of these G461 mutants to bind GTP would cause a dramatic decrease in protein synthesis since the translocases need to bind GTP in order to bind to the ribosome (Rodnina et al., 1997). The protein synthesis activity of the E462D and E462H mutant translocases was similar to that of wild-type EF-G. The results of the *in vitro* protein synthesis assay correlate well with those of the *in vivo* ribosome-binding assay using fusidic acid. In both assays the G461 mutants have demonstrated an inability to function on the ribosome, while the E462D and E462H mutant translocases had activities similar to that of wild-type EF-G.
Figure 4.3: *In Vitro* poly(U)-directed protein synthesis of G461 mutants. Protein synthesis assays were conducted in 100 µl reactions containing 35 pmoles $^{14}$C-Phenylalanine-tRNA as described in the Materials and Methods. Amounts of $^{14}$C-Phenylalanine polymerized were as indicated. Data was corrected by subtracting a blank value from each point. The data represent the average of three trials. Specific activities were calculated as the slopes of the linear range of the plots. Wild-type EF-G (diamonds); G461A (squares); G461P (triangles); G461S (light rectangles); E462D (dark rectangles); E462H (circles).
Table 4.2: Specific activity of *in vitro* protein synthesis of wild-type EF-G, and the G461 and E462 mutants. The specific activities of wild-type and mutant EF-Gs are shown. Specific activities are expressed as pmole phenylalanine polymerized per pmole EF-G in 15 minutes. The specific activities were obtained from the slope of a plot of pmole $^{14}$C-phenylalanine polymerized versus µg of EF-G used. The difference in the data obtained for the wild-type EF-G and the G461 mutants was statistically significant.

<table>
<thead>
<tr>
<th>6His-EF-G</th>
<th><em>In vitro</em> protein synthesis specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type EF-G</td>
<td>19.59 ± 0.59</td>
</tr>
<tr>
<td>G461A</td>
<td>0.29 ± 0.01</td>
</tr>
<tr>
<td>G461P</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>G461S</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>E462D</td>
<td>20.76 ± 1.22</td>
</tr>
<tr>
<td>E462H</td>
<td>18.33 ± 2.77</td>
</tr>
</tbody>
</table>
4.4: **Ribosome-dependent GTP hydrolysis:**

The ability of the G461 and E462 mutant translocases to hydrolyze GTP in a ribosome-dependent assay was measured under steady-state conditions and their Km and kcat values were determined. Consistent with the protein synthesis results, the G461 mutant translocases had severely reduced GTPase activity that was, in the case of the G461P mutant, barely above background (Table 4.3). As in the protein synthesis assay, the G461A mutant was the most active while the G461P mutant was the least active. The G461S mutant had similar GTPase activity as that of the G461A mutant. The ribosome-dependent GTPase assay was repeated to confirm that the activities observed for the G461 mutants were consistent and were above background (data not shown). The control used in these experiments was the inactive G21D mutant of EF-G. This mutant of EF-G cannot bind GTP due to the mutation in the phosphate-binding loop of EF-G. The extreme reduction in the ribosome-dependent GTPase activity of the G461 mutants might indicate a defect in GTP hydrolysis similar to that of the G21D mutant, that is the inability to bind GTP. Defects in the catalytic step might also reduce the GTPase activity of these mutants. The Km for these G461 mutants was 15-fold higher than the Km for wild-type EF-G. The Km data again indicated a problem with either the binding of GTP, or with the GTP hydrolysis step of the EF-G cycle. Other problems such as dissociation of the EF-G-GDP complex from the ribosome, or the release of GDP from these mutant translocases could also cause the high Km values that were observed for these mutants.
Table 4.3: GTPase activities of wild-type EF-G and the G461 and E462 mutant translocases. The velocities were calculated from a plot of pmole GTP hydrolyzed versus time using a substrate range of 0 – 2 mM GTP. The Kms were determined by the method of Eadie-Hofstee. The plots had correlation coefficients ≥ 0.95. The turnover numbers were determined at a saturating substrate concentration of 2 mM GTP by dividing the maximal rate (pmole GTP/sec) by the amount of EF-G (pmole) present. Standard deviations are given. The data represent the results from triplicate sets of experiments.

<table>
<thead>
<tr>
<th>6His EF-G</th>
<th>Km (µM)</th>
<th>Ribosome-dependent kcat (min⁻¹)</th>
<th>Ribosome-independent kcat (min⁻¹)*10⁻³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type EF-G</td>
<td>31.92 ± 1.19</td>
<td>50.53 ± 1.61</td>
<td>0.284 ± 0.02</td>
</tr>
<tr>
<td>G461A</td>
<td>401.03 ± 8.34</td>
<td>8.14 ± 0.64</td>
<td>0.138 ± 0.00</td>
</tr>
<tr>
<td>G461P</td>
<td>496.37 ± 8.47</td>
<td>0.572 ± 0.05</td>
<td>0.097 ± 0.01</td>
</tr>
<tr>
<td>G461S</td>
<td>430.45 ± 19.00</td>
<td>8.66 ± 0.63</td>
<td>0.117 ± 0.01</td>
</tr>
<tr>
<td>E462D</td>
<td>330.77 ± 9.58</td>
<td>17.58 ± 1.01</td>
<td>0.149 ± 0.00</td>
</tr>
<tr>
<td>E462H</td>
<td>370.20 ± 10.30</td>
<td>17.69 ± 0.70</td>
<td>0.177 ± 0.01</td>
</tr>
</tbody>
</table>
The E462D and E462H mutants also had reduced ribosome-dependent GTPase activity. Both these mutant translocases had a 10-fold increase in their respective Kms (Table 4.3). As with the G461 mutant translocases, the increased Km might indicate a defect in either GTP binding or the catalytic step of GTP hydrolysis.

4.5: Ribosome-independent GTPase activity:

The ribosome-independent GTP hydrolysis of the G461 and the E462 mutant translocases was determined in the presence of 10% isopropanol as described (chapter 2). All the mutants had ribosome-independent GTPase activity about 2-fold lower than that of wild-type EF-G (Table 4.3). The decrease in ribosome-independent GTPase activity of the G461A, G461P, and G461S mutant translocases is not as much as the decrease in their ribosome-dependent GTPase activities. This suggests that a ribosome-associated defect is the probable cause of the reduced ribosome-dependent GTPase activity and protein synthesis activity of the G461 mutant translocases. The reduction in the ribosome-dependent GTPase activity and the ribosome-independent GTPase activity of the E462 mutant translocases is about the same. This suggests that the substitution of this residue has caused a change in conformation of the translocase such that the intrinsic ability for GTP hydrolysis by these mutants is responsible for the reduced GTPase activity.
4.6: Interactions with guanine nucleotides:

The decreased ribosome-dependent GTPase activity of the G461 and possibly the E462 mutant translocases could be due to a defect in the binding of GTP, a defect in the binding of these translocases to the ribosome, a defect in the mechanism of GTP hydrolysis, or an increased affinity for GDP. To determine which of these possibilities is responsible for the defects observed in the G461 and E462 mutants, the interaction of these mutants with the GTP analog GMPPCP, as well as their interaction with GDP was studied. Both GMPPCP and GDP are competitive inhibitors of GTP (Figs. 3.8 and 3.9), therefore their respective dissociation constants could be determined by measuring the inhibitory constant of each. GDP may inhibit the GTP hydrolysis at two steps of the EF-G cycle: GDP competes with GTP for the nucleotide binding site of free EF-G, and it also helps shift the equilibrium of the dissociation of GDP from the EF-G-GDP complex towards the EF-G-GDP complex thereby delaying recycling of the EF-G. We were interested in determining whether a defect in GDP binding could explain an observed defect in the rate of GTP hydrolysis, rather then the precise step at which the defect in GDP binding affected the GTPase rate. All the G461 and E462 mutants had reduced affinity for GDP (Table 4.4), thus tight binding to GDP was not responsible for the decreased ribosome-dependent GTPase activity of these mutant translocases.

The E462D and E462H mutant translocases had a slightly lowered affinity for GMPPCP (Table 4.4). The decrease in affinity for GMPPCP by these E462 mutants was similar to that observed for the R127 and the Q128 mutant translocases discussed earlier. Since the E462 mutants had similar affinity for GMPPCP as the R127 and Q128 mutants
<table>
<thead>
<tr>
<th>6His EF-G</th>
<th>$K_i$ GMPPCP (µM)</th>
<th>$K_i$ GDP (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type EF-G</td>
<td>1.61 ± 0.19</td>
<td>32.10 ± 2.47</td>
</tr>
<tr>
<td>G461A</td>
<td>ND</td>
<td>95.56 ± 5.84</td>
</tr>
<tr>
<td>G461P</td>
<td>ND</td>
<td>135.01 ± 7.69</td>
</tr>
<tr>
<td>G461S</td>
<td>ND</td>
<td>129.64 ± 15.08</td>
</tr>
<tr>
<td>E462D</td>
<td>4.23 ± 0.15</td>
<td>83.13 ± 4.61</td>
</tr>
<tr>
<td>E462H</td>
<td>5.02 ± 0.25</td>
<td>53.23 ± 4.77</td>
</tr>
</tbody>
</table>

Table 4.4: Inhibition constants ($K_i$) of GMPPCP and GDP for wild-type EF-G and the G461 and E462 mutant translocases. The velocities were calculated from a plot of pmole GTP hydrolyzed versus time using a substrate range of 0 – 1 mM GTP. Triplicate sets of experiments were performed using 10 µM GMPPCP for the E462 mutants, and either 100 µM or 200 µM GDP for the different G461 and E462 mutants. The Km and the apparent Km (Kp) were determined from Lineweaver-Burk plots with correlation coefficients ≥ 0.95. The $K_i$ of GMPPCP for the G461 mutants could not be determined and is denoted as ND. Standard deviations are given.
but the Km of the E462 mutants was much higher than the Km values of the R127 and Q128 mutants, the E462 mutant translocases probably have a defect at a step after GTP binding has taken place. The inhibitory constant of GMPPCP for the G461 mutants could not be accurately determined by the competition assay as the data obtained was not reproducible. Therefore, to study the effect of GMPPCP on the ribosome-dependent GTPase activity of these G461 mutants, an inhibition study using different concentrations of GMPPCP was done. GMPPCP did cause some inhibition of the GTPase activities of the G461 mutant translocases (Fig. 4.4) but the level of inhibition was lower than that of wild-type EF-G. This might indicate that the G461 mutants do bind some GTP. The reduced level of inhibition by these mutants provided further evidence of a defect in a ribosome-associated activity for these mutants. The defect in the ribosome-associated activity could be either decreased ribosome binding, or a stimulation of the GTPase activity by the ribosome. Since cells expressing these mutant translocases survive, as evidenced from the toxicity assay, it is unlikely that these translocases are frozen on the ribosomes. The E462D and E462H mutants behaved similar to the wild-type EF-G in this assay.

4.7: Protection of the 23S rRNA by EF-G:

The ability of the G461 and E462 mutant translocases to protect the 23S rRNA from cleavage by restrictocin was used as an *in vitro* test for ribosome binding. GMPPCP and fusidic acid were used to slow the dissociation of the EF-G-GDP complex from the ribosome. In both cases the E462 mutant translocases protected the ribosomes to the same
Figure 4.4: Competitive inhibition of the GTPase of wild-type EF-G, G461A, G461P, G461S, E462D and E462H by GMPPCP. GTP hydrolysis assays were performed as described in the Materials and Methods. Reactions contained 5 µg EF-G, 80 µg ribosomes, 2 mM GTP and GMPPCP as indicated. Wild-type EF-G (diamonds); G461A (squares); G461P (triangles); G461S (X); E462D (dark rectangles); E462H (circles).
Figure 4.5: Protection of the ribosomal 23S rRNA by wild-type EF-G and the G461 and E462 mutant translocases. Proportion of 23S rRNA protected from cleavage by restrictocin. Reactions contained 2 mM GMPPCP (except the control that contained 2 mM GTP), 2.5 picomole ribosomes, 0.5 µg restrictocin and varying amounts of EF-G as indicated. The assay was conducted as described in the Materials and Methods. The results are the average of triplicate sets of data, the error bars indicate the standard deviation between the sets of data. Wild-type EF-G (diamonds); G461A (squares); G461P (triangles); G461S (light rectangles); E462D (X); E462H (circles); wild-type EF-G with GTP (dark rectangles).
Figure 4.6: Effect of fusidic acid on the protection of the ribosomal 23S rRNA by wild-type EF-G and the G461 and E462 mutant translocases. Proportion of 23S rRNA protected from cleavage by restrictocin. Reactions contained 1 mM fusidic acid and 2 mM GTP (except the control that contained only 2 mM GTP), 2.5 picomole ribosomes, 0.5 µg restrictocin and varying amounts of EF-G as indicated. The assay was conducted as described in the Materials and Methods. The results are the average of triplicate sets of data, the error bars indicate the standard deviation between the sets of data Wild-type EF-G (diamonds); G461A (squares); G461P (triangles); G461S (light rectangles); E462D (X); E462H (circles); wild-type EF-G with GTP (dark rectangles).
extent as wild-type EF-G (Figs. 4.5 and 4.6). The G461 mutants did not protect the ribosome from cleavage by restrictocin. This is in agreement with the earlier observation that the defects in the ribosome-dependent GTPase activity and the protein synthesis activity of these mutants are due to a defect in ribosome binding.

4.8: Effect of GMPPCP on the *in vitro* protein synthesis activity:

In an *in vitro* protein synthesis assay, the E462D and E462H mutant translocases had similar activity as wild-type EF-G. However, the ribosome-dependent GTPase activity of these mutants was only 35% that of wild-type EF-G. There could be several reasons to explain these observations. It is possible that a reduced GTPase activity may be sufficient for these proteins to perform in the *in vitro* protein synthesis assay. Alternatively, GTP binding might be sufficient to enable these mutants to function in protein synthesis. To check these possibilities, an *in vitro* protein synthesis assay was performed in the presence of GTP or GMPPCP using the E462D, E462H and wild-type EF-G (Fig. 4.7). No protein synthesis activity was observed in the presence of GMPPCP. This indicated that some GTP hydrolysis is needed for protein synthesis. This confirms an earlier report that GTP hydrolysis drives translocation (Rodnina et al., 1997).

4.9: Discussion:

The roles of the G461 and E462 residues within domain III of *E. coli* EF-G were investigated. The E462 residue is part of the helix B3, which forms the inter-domain interface with domain V as well as the switch II region of the G-domain. The G461
Figure 4.7: *In vitro* protein synthesis of wild-type EF-G, E462D and E462H. Protein synthesis assays were conducted as described in the Materials and Methods. 10 pmole \(^{14}\)C-phenylalanine-tRNA was used along with saturating EF-Tu. Amounts of protein assayed were as indicated. Reactions were performed in the presence of 2.5 mmGTP (a), or 2.5 mM GMPPCP (b). G21D was used as the control. The results are the average of triplicate sets of data, the error bars indicate the standard deviation between the sets of data. wild-type EF-G (diamonds); E462D (squares); E462H (circles); G21D (triangles).
residue is part of the loop preceding helix B3 (Laurberg et al., 2000). Since this region was not resolved in the earlier structures of EF-G (Ævarsson et al., 1994, Czworkowski et al., 1994, Al-Karadahi et al., 1996). It was interpreted to indicate that this region of domain III is flexible in EF-G. The amino acid residues that form this interface with other domains are highly conserved (Fig. 1.5), implying that this region of the EF-G is important for the participation of EF-G in protein synthesis. This was supported by the observation that removal of domain III reduced the ribosome-dependent GTPase activity of this deletion mutant as well as affected its ability to participate in translocation (Martemyanov and Gudkov, 2000). To gain a better understanding of how this region of EF-G affects the ribosome-dependent GTPase activity of EF-G, various experiments were performed on mutant derivatives of the G461 and E462 residues.

Our results indicate that any of three mutations of the glycine at position 461 of EF-G caused a severe impairment of the \textit{in vitro} protein synthesis activity. The \textit{in vitro} protein synthesis assay measures the overall function of the EF-G in the EF-G cycle, therefore this result could be interpreted as a defect in any step of the EF-G cycle. These include the inability of the mutant translocases to bind GTP, bind to the ribosome or receive activation from the ribosome for the GTPase activity of EF-G. A defect in the chemistry of GTP hydrolysis or the inability to undergo conformational changes required for translocation could also cause reduced protein synthesis activity. It is also possible that an inability of the EF-G-GDP complex to dissociate from the ribosome or that a tight binding of GDP by the mutant translocases might reduce protein synthesis activity.
The 10-fold reduction in the ribosome-dependent GTPase activity of these G461 mutant translocases (Table 4.3) was much greater than the 2-fold decrease in the ribosome-independent activity of these mutants. This suggested that a ribosome-associated defect was caused by mutation of glycine 461. This hypothesis was supported by the observation that tight binding of GDP is not responsible for the reduced GTPase rate, since all three G461 mutants have lower affinity for GDP compared to wild-type EF-G. Thus the defect caused by mutation of glycine 461 is likely due to reduced GTP binding, defective binding of these mutants to the ribosome, or a decrease in ribosome stimulation of their GTPase activity. The ribosome-dependent GTPase activity of these mutants was not reduced to the same degree as that of wild-type EF-G in the presence of GMPPCP (Fig.4.4). This result indicated that there was some defect in the binding of GTP by these G461 mutants, which could explain the higher Km values obtained for these translocases in the presence of GTP. However, since some inhibition of the GTPase activity was observed, it implied that the initial steps of the EF-G cycle occur in these mutants. That is, the G461 mutant translocases are able to bind GTP to some limited extent, and the EF-G-GTP complex could then bind to the ribosome. The ribosome protection assay indicated that these mutant translocases do not protect the sarcin-ricin loop of 23S rRNA from cleavage by restrictocin (Figs. 4.5 and 4.6). This indicated that in addition to the defect in the binding of GTP by these mutants, these translocases were also defective in effective binding to the ribosome.

The region of domain III containing the G461 and E462 amino acid residues was not resolved in the earlier structures (Ævarsson et al., 1994, Czworkowski et al., 1994, Al-
Karadahi et al., 1996). In addition, this region is not present in EF-Tu (Nyborg et al., 1996). A recent GDP-bound structure of a mutant EF-G was solved in which domain III was resolved (Laurberg et al., 2000). In this structure, G461 was positioned at the interface between domain III and the switch II helix of the G-domain. The authors suggested that a mutation of the conserved glycine 459 (E. coli numbering) in this region might cause destabilization of the G-domain switch II helix due to the insertion of histidine 464 into the interface (Laurberg et al., 2000). A similar destabilization of this interface might occur due to a mutation of G461 residue. It is likely that mutations to G461 destabilize switch II of the G-domain thereby reducing the ability of these mutants to bind GTP, since the switch II is probably involved in the stabilization of the magnesium ion needed for GTP hydrolysis (Knudsen and Clark, 1995). This indirect effect on the G-domain conformation would explain the higher Km observed for these mutants. In addition, the destabilization of the interface might cause these mutant translocases to adopt a conformation that could disrupt the binding of the EF-G-GTP or of the EF-G-GDP complex to the ribosome. A mutant EF-G that is unable to properly orient the GTP might prevent the ribosome from activating GTP hydrolysis of these translocases, causing a greater reduction of the ribosome-dependent GTPase activity than the reduction of the ribosome-independent GTPase activity. The results obtained for these mutants (Table 4.3) seem to support such a hypothesis.
These mutants fail to protect the ribosome in the presence of GMPPCP or fusidic acid. The lack of protection of the ribosome implies that these mutant translocases dissociate from the ribosome faster than wild-type EF-G. The dissociation of the mutant translocases from the ribosome might occur before the conformational changes necessary for translocation. That these mutant translocases dissociate from the ribosome before translocation is supported by the observation that the protein synthesis activity of these mutants is inhibited to a greater extent than the inhibition of their ribosome-dependent GTPase activity. Such effects on ribosome-dependent GTPase activity and the protein synthesis activity due to destabilization of the inter-domain interface and switch II have been reported (Abdulkarim et al., 1994, Knudsen et al., 2001).

The likely sequence of events for these G461 mutants in the EF-G cycle is shown in Figure 4.8. These mutants probably have a reduced binding of GTP. When the mutant EF-G-GTP bind to the ribosome, the GTP is probably not positioned correctly to allow activation of GTP hydrolysis by the ribosome due to the destabilization of the switch II helix. The EF-G-GTP (or EF-G-GDP, if GTP hydrolysis has occurred) dissociates from the ribosome before the translocation step due to the destabilization of the EF-G-GDP-ribosome complex brought about by changes to the interface between domain III and the region of the switch II helix.

Most mutations that cause fusidic acid resistance occur in this region of domain III that forms an interface with the G-domain (Johanson and Hughes, 1994, Laurberg et al., 2000). Fusidic acid resistance may be caused by reduced affinity for the antibiotic by EF-G, interference with conformational changes or by an alteration of the interaction
**Figure 4.8:** Proposed roles of glycine 461 of *E. coli* EF-G in the EF-G cycle. The glycine residue is believed to affect GTP binding, GTP hydrolysis as well as the translocation step. Mutations to this residue allow the EF-G-GDP complex to dissociate from the ribosome faster than the wild-type EG-G-GDP complex. The three different proposed conformations of EF-G are represented as a rectangle with one side changing from a V to flat to concave.
between EF-G and the ribosome (Johanson et al., 1996). In the G461 mutant translocases it appears that the mutant translocases dissociate from the ribosome due to decreased EF-G-ribosome stability, and this confers fusidic acid resistance. Mutation of the adjacent amino acid residues G459, M460, L463 and H464 (E. coli numbering) in the related EF-Gs from *S. aureus* and *S. typhimurium* confer fusidic acid resistance in these organisms (Laurberg et al., 2000). It is possible that mutations to any of these residues cause a similar destabilization of the interface between domain III and the G-domain, which reduces the stability of binding of the EF-G-GDP on the ribosome, allowing these mutants to dissociate from the ribosome even in the presence of fusidic acid.

There is another possible explanation for most of the results observed for the G461 mutant translocases. This region of domain III might interact directly with the ribosome. Domain III and domain V of EF-G have a similar double split β-α-β fold observed in a number of ribonucleotide binding proteins such as the snRNP protein U1A (Nagai et al., 1990) and the ribosomal protein L22 (Unge et al., 1998). It has also been proposed that domain III might mimic the acceptor stem of Cys-tRNA positions 1 to 6 and positions 66 to 73 of aminoacylated tRNA bound to EF-Tu-GTP (Nissen et al., 1996). The acceptor stem of aminoacylated-tRNA is bound to specific loops in the peptidyl transferase center of 23S rRNA (Nissen et al., 2000). Hydroxyl radical mediated footprinting studies provided distance constraints which helped to place domain III in a position to interact with the 50S subunit (Wilson and Noller, 1998a). In a cryo-electron microscopy study (Agrawal et al., 1998), the EF-G-GDP structure (Czworkowski et al., 1994) was superimposed on the cryo-electron map of the ribosome. Since domain III of
EF-G was not resolved in this structure, thus possible interactions of this region of domain III with the ribosome could not be determined.

If this region of domain III is responsible for the binding to the ribosome, it could explain most of the observed deficiencies for the G461 mutants except for their reduced ability to bind GMPPCP. However, since EF-G has an elongated structure (Ævarsson et al., 1994), it is unlikely that a defect in ribosome binding to one region of the EF-G would affect its binding to the numerous other sites on EF-G (Wilson and Noller 1998a). Thus it seems more likely that the defects of the G461 mutant translocases in binding to the ribosome are due to the destabilization of the interface between domain III and the G-domain.

Mutation of glutamic acid 462 to aspartic acid and to histidine affected its ribosome-dependent GTPase activity (Fig 4.9, Table 4.3). The GTPase activity was reduced by a similar level in both E462D and E462H. The relative reduction in the ribosome-dependent GTPase activity between the E462D and E462H mutant translocases suggested that the identity of the residue at position 462 is important for its function in EF-G. Both of these E462 mutants also had a 10-fold increase in the respective Km values (Table 4.3). This might be due to either a defect in the binding of GTP or to a defect in the GTP hydrolysis. However, since the Ki of GMPPCP binding for each of these mutant translocases was about 3-fold lower than that for wild-type EF-G, it is unlikely that at the saturating concentration of GTP used in this assay, a slight implied defect in GTP binding would affect the GTPase rate. Rather it suggests that the GTP
Figure 4.9: Proposed role of glutamic acid 462 of *E. coli* EF-G in the EF-G cycle. The glutamic acid residue is believed to affect GTP hydrolysis by EF-G. The three different proposed conformations of EF-G are represented as a rectangle with one side changing from a V to flat to concave.
cleavage step is affected. Similar results have been reported in other EF-G mutants (Mohr et al., 2000)

A defect in GDP release is not likely to be the reason for the reduced GTP hydrolysis rates of these mutants as both E462D and E462H have lower affinity for GDP compared to wild-type EF-G (Table 4.2). The E462 mutants possess the same level of activity in the protein synthesis assay as the wild-type EF-G, and they were able to inhibit the ribosome-dependent GTPase assay to the same extent as the wild-type EF-G (Fig. 4.4). These E462 mutant translocases were also able to protect the 23S rRNA from cleavage by restrictocin to a similar level as wild-type EF-G. These results suggest that both EF-G E462D and E462H are defective in the GTP hydrolysis step of the EF-G cycle. The defect in GTP hydrolysis in these mutants could be due to a slight change in conformation of these translocases that would reduce the intrinsic GTPase activity of these mutants. The results obtained in the ribosome-independent GTPase assay support such a postulate since the ribosome-independent GTPase activity of both these mutants was lower than that of wild-type EF-G.

Another possibility is that GTP is not positioned correctly for activation of the GTP hydrolysis activity by the ribosome. A recently solved structure of the GDP-bound form of a mutant derivative of EF-G in which domain III had been resolved (Laurberg et al., 2000), indicated that the side chain of E462 is oriented towards a conserved region of domain V. E462 was said to form an interaction with phenylalanine 663 and glycine 664 of domain V (Fig. 1.3)(Laurberg et al., 2000). The glutamic acid at position 462 and the
glycine at position 664 of the *E. coli* EF-G are highly conserved among all EF-Gs and EF-2s (Cammarano et al., 1992). However, in the EF-2 of *Sulfolobus acidocaldarius* (Schroder and Klink 1991) and in the EF-2 of *Desulfurococcus mobilis* (Creti et al., 1994), the glutamic acid at this position in domain III is replaced by phenylalanine and threonine respectively. In both organisms, the corresponding glycine of domain V is not conserved. Since both the E462 and the G664 residues are highly conserved in most EF-Gs it suggests that the E462 of domain III makes an important contact with G664 of domain V. In the E462D and E462H mutant translocases, loss of this contact with glycine 664 may increase the flexibility of the glutamic acid residue, which, due to its close proximity to the switch II helix, might destabilize the bound-GTP. This would prevent the ribosome from activating the GTPase activity of these translocases. Since EF-G interacts with the ribosome at numerous sites of both the 50S and 30S ribosomal subunits (Hou et al., 1994a, Ævarsson et al., 1994, Wilson and Noller, 1998, Agrawal et al., 1998), it is possible that a defect causing an improper activation of GTP hydrolysis by the ribosome, will not affect the overall binding of these mutant translocases to the ribosome. The possibility of having defective stimulation of the GTPase by the ribosome while bound to the ribosome is supported by the similar protection afforded to the ribosome by wild-type EF-G and the mutant translocases (Figs. 4.5 and 4.6).

Further evidence that wild-type EF-G, E462D and E462H translocases have similar binding to the ribosome was obtained by *in vitro* and *in vivo* experiments involving fusidic acid. All three translocases were sensitive to fusidic acid *in vivo* (Table 4.1). These translocases were also sensitive to fusidic acid in an *in vitro* ribosome
protection assay (Fig. 4.6). These results indicated that the E462 mutant translocases bind and dissociate from the ribosome just as efficiently as wild-type EF-G. Most mutations that confer fusidic acid resistance are located in this region of domain III (Johanson et al., 1996). Mutations to the residues on either side of this glutamic acid in the closely related EF-Gs from *S. aureus* and *S. typhimurium* confer fusidic acid resistance (Laurberg et al., 2000). It was surprising that mutations of the E462 residue did not confer fusidic acid resistance. Destabilization of the inter-domain interface which favors dissociation of the EF-G-GDP complex from the ribosome was reported to cause resistance to fusidic acid or kirromycin among elongation factors (Abdulkarim et al., 1994, Wolfe, 1999). Perhaps, the orientation of the side chains of these residues is an important determinant in the ability of mutations to confer resistance to these antibiotics. The side chain of E462 is oriented towards glycine 664 of domain V, and consequently any instability caused by mutations to this glutamic acid may be spread out throughout domain III, thereby reducing the destabilization of the switch II helix at the interface between domain III and the G-domain.

There have been suggestions that conformational changes occurring in the G-domain on hydrolysis of GTP may be passed through the inter-domain interfaces to the other domains of EF-G, causing the translocation of the tRNAs (Wilson and Noller, 1998, Agrawal et al., 1999). From the current structural data available for EF-G (Ævarsson et al., 1994, Al-Karadaghi et al., 1996, Laurberg et al., 2000), E462 seems to be the only residue among the conserved amino acids of the domain III – G-domain interface whose side chain is in a position to affect a change in the conformations of domains V and
domain IV in response to changes in the G-domain. Since the mutation of this residue did not affect the rate of protein synthesis, it implies that these E462 mutant translocases are capable of participating in the translocation step of the EF-G cycle. Thus, any conformational changes due to GTP hydrolysis would have to be transmitted via the C_G helix directly to domains V and IV and not through the interface of the G-domain with domain III. However, since the active GTP-bound structure of EF-G is not yet available, there may be other interactions between this region of domain III and domain V that may participate in the transmission of the signal from the G-domain.

To understand how these E462 mutant translocases could have similar levels of protein synthesis as wild-type EF-G and yet have reduced ribosome-dependent GTPase activity, an in vitro protein synthesis assay was performed in the presence of GMPPCP to test whether GTP binding is sufficient for these mutant translocases to function in protein synthesis. The protein synthesis activities of the E462 mutants were inhibited by GMPPCP to the same extent as the wild-type EF-G (Fig 4.7). The result obtained indicated that the low level of ribosome-dependent GTPase activity of these E462 mutants was sufficient for efficient protein synthesis. This suggests that the GTPase activity and the translocation step may be uncoupled as suggested by the earlier models of translocation. However, a recent report indicated that GTP hydrolysis drives translocation (Rodnina et al., 1997). Another possible explanation is that the defect in the GTPase activity of these mutants is masked by a slower step in protein synthesis. Since the GTPase activity was measured under turnover conditions, a masking of the defect in the ribosome-dependent GTPase of these E462 mutants would imply that the slow step
was either the first step involving EF-Tu, or in the second step involving the peptidyl transferase center of the ribosome (Fig. 1.1). Mutants of EF-Tu that display the same characteristics as that of the E462 mutant translocases have been reported (Zeidler et al., 1995, Zeidler et al., 1996). Thus the postulated slow step of protein synthesis is not in the EF-Tu cycle, suggesting that the peptide transfer step is the slow step of protein synthesis. Since the actual chemistry of peptide transfer is believed to be spontaneous (Nishizuka and Lipmann 1966, Pape et al., 1998), it is possible that the slow step of protein synthesis involves accommodation of the aminoacyl end of A-site tRNA into the peptidyl transferase center of the 50S ribosomal subunit (reviewed in Ramakrishnan 2002).
CHAPTER 5

CONCLUSIONS:

5.1: Importance of the C\textsubscript{G} helix:

Arginine 127 of \textit{E. coli} EF-G was found to be involved in the binding of EF-G to the ribosome. The proposed interaction with glycine 651 of domain V supports such a hypothesis, as it had been reported that the region around this glycine is important for binding to the ribosome (Wilson and Noller, 1998a). Loss of this interaction allowed the EF-G-GDP complex to dissociate faster from the ribosome as indicated by less protection afforded to the ribosome by these mutant translocases. Mutation of this arginine residue did not destabilize the interface between the G-domain and domain V since the mutants were able to perform all the functions of EF-G in the EF-G cycle at a level comparable to the wild-type EF-G. The region containing G651 has been implicated in the binding to the sarcin-ricin loop of 23S rRNA (Wilson and Noller, 1998a). It is possible that loss of this interaction with the sarcin-ricin loop due to a mutation of arginine 127 exposes the sarcin-ricin loop to cleavage by restrictocin even though the EF-G remains bound to the ribosome by its numerous other contacts. This would explain why the arginine mutant translocases were functional in all other aspects of the EF-G cycle.
Glutamine 128 was found to be involved with the ribosome-dependent GTP hydrolysis by EF-G. The reduction of the ribosome-dependent GTPase activity was not due to reduced GTP binding or increased affinity for GDP by the Q128E and Q128N mutant translocases. Instead, it appears that GTP is not positioned correctly in these mutants, preventing proper stimulation of the GTPase activity of EF-G by the ribosome. The improper positioning of GTP by the Q128 mutants is probably due to the destabilization of the switch II helix, as it had been reported that glutamine 128 interacts with the switch II helix (Laurberg et al., 2000). In addition to affecting the ribosome-dependent GTPase activity, the destabilization of the switch II helix by these Q128 mutants appeared to cause a slight conformational change in the structure of these mutant translocases that affected their ability to bind to the ribosome. The increased ability of these mutants to dissociate from the ribosome probably conferred resistance to fusidic acid to these translocases and reduced their activity in protein synthesis.

The current model for translocation by EF-G involves a signal, brought about by conformational changes in the G-domain due to hydrolysis of GTP, being transmitted through domains III and V, which cause a shift in domain IV. The shift in the position of domain IV is believed to effect translocation (Wilson and Noller, 1998a, Agrawal et al., 1999, reviewed in Ramakrishnan, 2002). To transmit such a signal between the different domains of EF-G, the inter-domain contacts are likely to play a pivotal role. The $C_G$ helix of the G-domain forms inter-domain contacts with domain V (Ævarsson et al., 1994, Czworkowski et al., 1994, Al-Karadaghi et al., 1996). It has been proposed that the interactions between the $C_G$ helix and domain V are important for the proper functioning
of EF-G (Liljas et al., 1995). There are numerous contacts between the switch II helix and the C\textsubscript{G} helix (Al-Karadaghi et al., 1996), Q128 being one of the interactions between these two helices. There are two main contacts between the C\textsubscript{G} helix and domain V. R127 is in proximity to G651 of domain V, while E123 is known to interact with R676 (Wolfe, 1999). Any changes in conformation in the GTP binding region of the G-domain brought about by GTP hydrolysis will be sensed by the switch II helix and transmitted via the C\textsubscript{G} helix to domain V and then to domain IV.

This study supports the role of Q128 in the transmission of such a signal from the G-domain to domain V, since reduced protein synthesis activity was observed in both the Q128E and the Q128N mutant translocases. The R127 mutant translocases, however, were as efficient as wild-type EF-G in protein synthesis. This implied that arginine 127 is probably not involved in the signal transduction. If the hypothesis of signal transduction is correct, the E123-R676 interaction may be crucial for the function of EF-G in translocation. It is likely that the signal from the G-domain caused by the hydrolysis of GTP is transmitted from the switch II helix to Q128, the signal is then transmitted from the C\textsubscript{G} helix to domain V via the E123-R676 interaction.

5.2: Stability of the G-domain – domain III interface is important for ribosome binding:

A glycine at position 461 of the \textit{E. coli} EF-G is essential for its ability to function in GTP hydrolysis and protein synthesis. It probably helps EF-G undergo the necessary conformational changes needed for translocation. Mutation of this glycine probably caused destabilization of the switch II helix, which affects almost all the steps of the EF-
G cycle, including GTP binding and positioning of the GTP to receive stimulation from the ribosome. This caused a severe defect in the ability of these G461 mutant translocases to hydrolyze GTP and function in protein synthesis. The G461A, G461P and G461S mutants were insensitive to fusidic acid, probably due to the reduced binding of these translocases to the ribosome. The exact reason for the importance of G461 in EF-G function is unclear due to the absence of structural information for the active GTP-bound EF-G and because this domain is missing in the closely related EF-Tu (Berchtold et al., 1993, Kjeldgaard et al., 1993).

In contrast to G461, the E462D and E462H mutants were sensitive to fusidic acid and bound the ribosome as well as wild-type EF-G. Mutations to this glutamic acid residue did not cause a dramatic defect in the overall function of the mutant translocases in protein synthesis, probably because the effect of the mutation is dissipated throughout domain III and has less of an effect on the stability of switch II compared to the mutations of G461. The E462 mutant translocases probably caused the GTP to be positioned such that the ribosome could not efficiently stimulate GTP hydrolysis. Thus it appears that the stability of the G-domain–domain III interface is important for EF-G. A similar result had been reported for EF-Tu (Abdulkarim et al., 1994), where mutation of Q124 destabilized the interface between domain III and the G-domain of EF-Tu.

Current structural data of EF-G (Ævarsson et al., 1994, Czworkowski et al., 1994, Al-Karadahi et al., 1996, Laurberg et al., 2000), suggest that a signal that causes translocation could be passed from the switch II region of the G-domain to domain V through E462, which appears to interact with G664 of domain V (Laurberg et al., 2000).
However, since the E462 mutant translocases have similar protein synthesis activity as the wild-type EF-G \textit{in vitro}, it is unlikely that glutamic acid 462 is involved in the transmission of a signal from the G-domain to domain IV via the intermediate domains. This does not exclude the possibility that other amino acids from this interface (including G461) are involved in the transmission of such a signal from the G-domain to domain V. Since the E462D and the E462H mutant translocases have similar protein synthesis activity as wild-type EF-G and yet had a reduced ribosome-dependent GTPase activity, it suggests that the slow step of protein synthesis is not in the EF-G cycle.

There are several possible reasons why mutations to the highly conserved R127 and E462 amino acids have similar activity as wild-type EF-G in \textit{in vitro} protein synthesis. Arginine 127 and glutamic acid 462 may be located at “hot spots” at protein interfaces (Bogan and Thorn, 1998), that is, they are located adjacent to critical residues of the protein (Q128 and G461). Another possibility is that the R127 and E462 residues are genetically selected for by providing a more stable form of EF-G. R127 interacts with G651 while E462 interacts with G664. Neither of these contacts is essential for protein synthesis, but they probably help stabilize EF-G. A third possibility is that the R127 and E462 residues may be involved in a function of EF-G other than translocation. It has recently been reported that the interaction between EF-G and RRF is required for the recycling of the ribosomes (Rao and Varshney, 2001). The arginine and glutamic acid might be involved in the recycling of ribosomes.
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