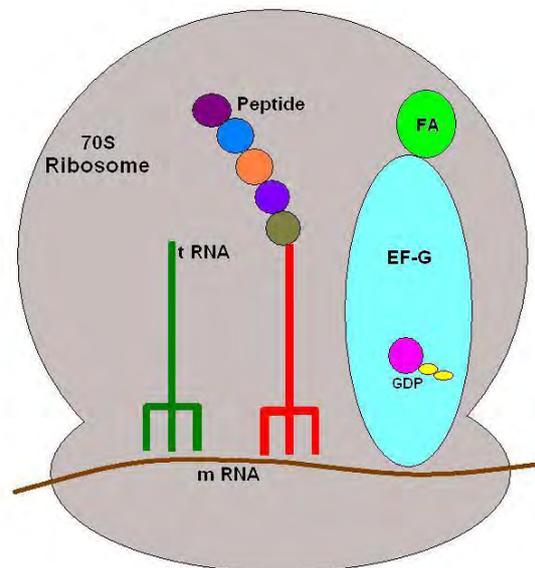# Finding the exact binding site of fusidic acid (FA) in the 70S ribosome -EF-G-GDP-FA complex

Ravi Kiran Koripella

SUMMARY

Fusidic acid (FA) is a narrow spectrum, steroid-like antibiotic that is effective against gram positive bacteria. This antibiotic has drawn much attention these days because of its activity against *Staphylococcus aureus*. This species causes several diseases in humans, which range from minor diseases like skin infections to major diseases like pneumonia and meningitis. *S. aureus* has become resistant to most of the known antibiotics and FA is one among the very few antibiotics that are still working against this bacterium. FA acts by blocking the release of elongation factor G (EF-G)-GDP from the 70S ribosome after the translocation step of protein synthesis and therefore stalls bacterial protein synthesis. The aim of this project was to detect the exact binding site of FA on EF-G.

In the presence of FA, EF-G and GDP can form a tight complex on the 70S ribosome, in which FA is bound to EF-G. However, this complex is stable only in the presence of FA in excess and therefore a pure 70S ribosome-EF-G-GDP-FA complex is difficult to isolate. Specially designed fusidic acid analogues (FAA) that have photoreactive groups but still act like FA were used in stable complex formation. These analogues, when irradiated with ultraviolet light, can crosslink to its target protein EF-G. In order to detect the complex, radioactively labelled components such as [^{3}H]GTP or [^{3}H] FAA were used.

In this project several techniques such as nitrocellulose filter binding assay, gel filtration chromatography, UV-crosslinking, autoradiography and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were used in optimization and purification of 70S ribosome-EF-G-GDP-FA (or FAA) complex. Results from nitrocellulose filter binding assays showed that all the reactants, namely, EF-G, GTP and FA are necessary for the formation of a EF-G-GDP-FA(or FAA) complex on the 70S ribosome. When FA was replaced by FAA, and irradiated with UV light, a stable 70S ribosome-EF-G-GDP-FAA complex was formed, which could be purified by gel filtration chromatography. When a similar complex with [^{3}H]FAA was isolated and analyzed by SDS-PAGE followed by autoradiography, none of the ribosomal proteins but only EF-G were found crosslinked with the [^{3}H]FAA. This result clearly suggested EF-G as the molecular target of FA in the bacterial cell. Whether FA can bind to EF-G in cytosol or not remains an open question in the field.

In order to isolate EF-G crosslinked with FAA, the UV treated 70S ribosome-EF-G-GDP-FAA complex was pelleted through a sucrose cushion by ultracentrifugation and was further separated in a SDS-PAGE. Only FAA-crosslinked EF-G was retained in the complex and was isolated as a gel band. When the FAA-crosslinked EF-G was analyzed by mass-spectroscopy, about ten peptide-peaks with masses different from those of the non-crosslinked EF-G were identified. Among these, at least one peptide should have FAA crosslinked with it. However the identification of the peptide, that is the most probable candidate for FA binding needs further confirmation using complementary approaches.

INTRODUCTION

Controlling bacterial infections with antibiotics is central to modern health care. But increasing bacterial resistance to many of the antibiotics threatens effective therapy. *Staphylococcus aureus* is a common, naturally occurring bacterium in humans, carried by an estimated 30-40% of the population (Peacock *et al*., 2001). It causes many diseases in humans ranging from infections of superficial skin lesions to serious systemic infections such as pneumonia and sepsis (Sibbald *et al*., 2006). In fact, resistance of *S. aureus* to antibiotics was observed very soon after the introduction of penicillin about 60 years ago. In the following years, the ability of bacteria to develop resistance to antibiotics has resulted in the emergence of methicillin-resistant *S. aureus* (MRSA). Right now, only a few antibiotics are still effective against this bacterium. Fusidic acid (FA) is one among them, but emerging resistance is a problem that could limit the options for treatment of staphylococcal infections. To make sure that FA and its derivatives remain effective for the future generations it is necessary to understand the mode of action of this drug and mechanisms that are responsible for bacterial resistance

Fusidic acid :

Fusidic acid (FA) was first derived from the fungus *Fusidium coccineum* and released into the market in the 1960s (Godtfredsen *et al*., 1962). It is a steroid- like molecule having a distinct tetracyclic ring system and chair-boat-chair conformation which is a characteristic of the members of the Fusidane antibiotic group (Fig. 1). FA has potent activity against *Staphylococcus aureus* (Besier *et al*., 2003) including strains resistant to other classes of antibiotics. The current use of FA is primarily in the treatment of skin and eye infections. It is also occasionally used to treat bone and joint infections (Akins and Gottlieb 1999). The molecular target of FA is bacterial elongation factor G.
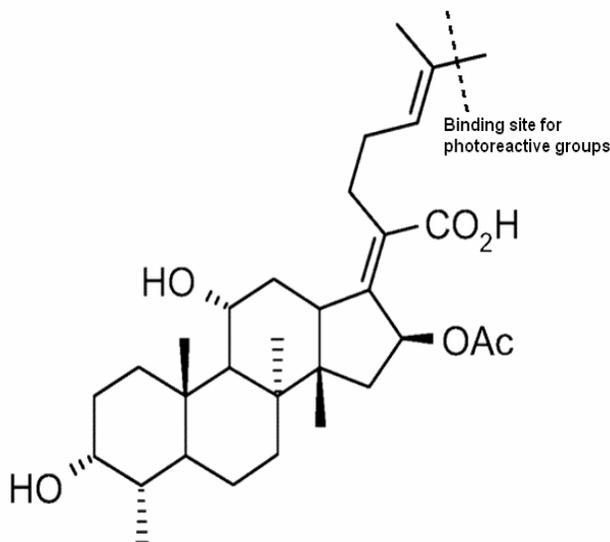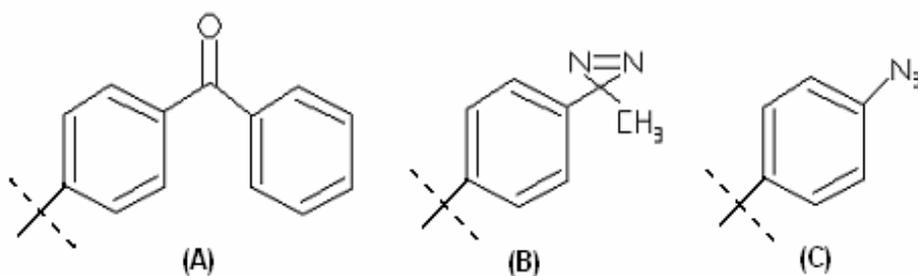

Figure 1: Structure of fusidic acid. The dotted line represents the site on the side chain where the photoreactive groups are coupled to fusidic acid.

Fusidic acid analogues (FAA):

Fusidic acid analogues were developed without affecting the antimicrobial activity of FA. By analyzing the structure-activity relationship, it was shown that the side chain of FA was not involved in the antimicrobial activity (Duvold *et al.*, 2001). Therefore linking aromatic photo-affinity moieties such as benzophenone (Fig. 2a), trifluoromethyldiazirine (Fig. 2b) and azide (Fig. 2c) to the lipophilic part of the side chain of FA did not cause any loss of its antimicrobial activity (Riber *et al.*, 2006). These photoreactive groups can successfully crosslink the fusidic acid analogue to the target protein when irradiated under ultraviolet light. To facilitate the identification of these crosslinked fragments, the analogues were radiolabelled with tritium. The fusidic acid analogue with azide group was used throughout in this study and is referred to as FAA in the rest of the document.

 Figure 2: The structure of the photoreactive groups benzophenone (A), trifluoromethyldiazirine (B) and azide (C). The dotted lines are the sites where these groups are linked to fusidic acid.

Elongation factor G (EF-G):

EF-G participates in the elongation phase of bacterial protein synthesis. EF-G in complex with GTP catalyses the translocation step of protein synthesis, where it acts like a motor protein and drives tRNA translocation after GTP hydrolysis (Zavialov *et al.*, 2005). EF-G binds to the translating ribosome and changes its conformation. This conformational change in EF-G brings about the rotation of the smaller subunit with respect to the larger subunit (Agarwal *et al.*, 1999). This in turn leads to the movement of peptidyl tRNA from the A site and deacylated tRNA from the P site to the P and E sites respectively. After GTP hydrolysis, EF-G in complex with GDP dissociates from the ribosome and makes it ready for the next round of elongation.

The detailed structural information of EF-G was first obtained from the bacterium *Thermus thermophilus*, whose EF-G contains 691 amino acid residues (Yakhnin *et al.*, 1989). The EF-G molecule has five domains. Domain I has two subunits that include the GTP binding domain G and G`. Domains I and II are similar to elongation factor-Tu whereas domains III and V are structurally similar to the ribosomal proteins. Domain IV is topologically different from all other domains with a protruding arm (Fig. 3) (Aevarsson *et al.*, 1994).
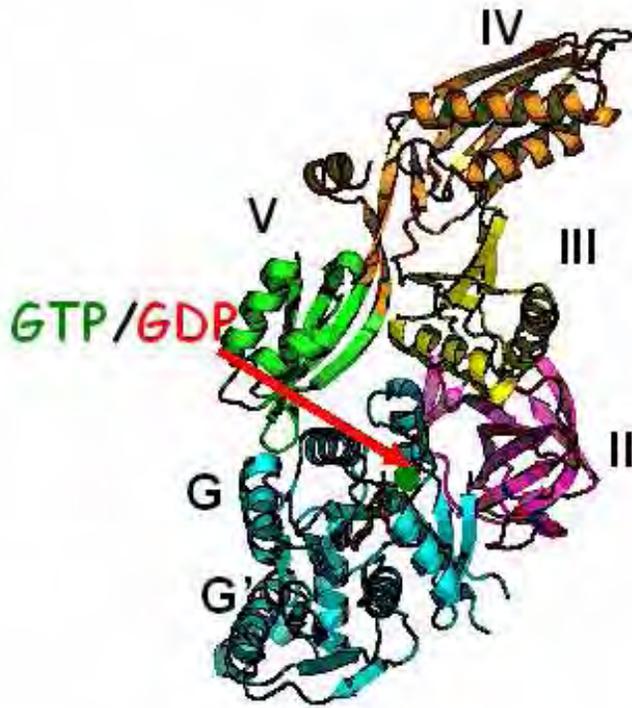
Figure 3: X-ray crystal structure of *Thermus thermophilus* EF-G in complex with GDP. Different domains of EF-G are marked accordingly. (Redrawn by Rastop from PDB, code 1DAR)

How fusidic acid works:

Fusidic acid binds with high affinity to EF-G on the ribosome after GTP hydrolysis (Bodley *et al*., 1969; Hanson et al., 2005) during the tRNA translocation step of protein synthesis. Binding of fusidic acid prevents the release of EF-G-GDP from the ribosome (Willie *et al*., 1975, Johanson *et al*., 1996). As the A-site is still occupied by EFG-GDP, a new ternary complex containing EF-Tu with an amino-acyl tRNA cannot enter the site and hence the ribosome cannot proceed to the next round of elongation, which in turn switches off bacterial protein synthesis.

Fusidic acid resistance:

Mutations in the *fusA* gene, which codes for EF-G, are mainly responsible for conferring resistance to fusidic acid. Structural mapping of mutations causing fusidic acid resistance suggested that the interface between domain G, domain III and domain V is the most likely place where fusidic acid binds (Laurberg *et al*., 2000; Nagaev *et al*., 2001). However, these three domains are quite big and mutations causing fusidic acid resistance are scattered in other domains too, which makes it very difficult to locate the exact binding site with the help of mutational analysis.

Mass-spectrometry:

Mass spectrometry is an anlytical technique that is used to measure the mass to charge ratio of charged particles. This technique is generally used to find the composition and structure of a sample by generating a mass spectrum representing the masses of sample components. The most widely used technique in mass-spectrometry for analyzing biomolecules like proteins, peptides and sugars is the Matrix-assisted Laser Desorption/Ionization (MALDI). In this technique, the ionization is triggered by a laser beam and a matrix is used to protect the biomolecules from being destroyed by direct laser beam and to facilitate vaporization and ionization. For sample preparation, proteins in solution or as SDS-PAGE gel bands are cut into several fragments using proteolytic enzymes such as trypsin or chymotrypsin. The resulting peptides are extracted with acetonitrile, dried under vacuum and dissolved in a small amount of distilled water which are then ready for mass-spectrometry analysis.

A small fraction of the peptides (usually 1 µl) is mixed with a matrix (3,5-dimethoxy-4-hydroxycinnamic acid dissolved in a mixture of highly purified water and acetonitrile) The matrix protects the peptides from the high energy laser beam. The solution containing the peptides and the matrix is spotted on a MALDI plate (a metal plate desighed for this purpose). This plate is then inserted into the vacuum chamber of the mass spectrometer where a pulsed laser beam transfers high amounts of energy into the matrix molecules. The energy transfer is sufficient to promote the transition of matrix molecules and peptides from the solid state into the gas state (ions). The ions are accelerated in the electric field of the mass spectrometer and fly towards an ion detector where their time of flight (TOF) is proportional to their masses. The arrival of ions is detected as an electric signal which is represented as a peak.

AIM:

The aim of this project was to locate the exact binding site of fusidic acid (FA) in the 70S ribosome-EF-G-GDP-FA complex formed *in vitro*. For this, EF-G crosslinked with FA analogue was isolated from the complex and was further analyzed by mass-spectrometry.

RESULTS

Detection of the 70S ribosome-EF-G-[^{3}H]GDP-FA complex by nitrocellulose filter-binding assay:

In the presence of the 70S ribosome, EF-G actively hydrolyses GTP and the EF-G-GDP can be trapped on the 70S ribosome by FA, resulting in a 70S ribosome-EF-G-GDP-FA complex. In order to detect this complex [^{3}H]GTP was used for complex formation and the amount of complex formed was determined by measuring in a scintillation counter the radioactive counts of [^{3}H]GTP retained on a nitrocellulose filter membrane after the complex has filtered through it.

Figure 4 summarizes the results of the nitrocellulose filter binding assay. It shows that the 70S ribosome-EF-G-[^{3}H]GDP-FA complex could be stably formed only when FA was continuously supplied in the complex formation buffer (CFB). When one of the components from the reaction mixture was omitted, no complex formation was seen, indicating that all the components 70S ribosomes, EF-G, GTP and FA were necessary for successful complex formation. When 50S ribosomes were used instead of 70S, limited complex formation was observed suggesting the necessity of the 30S subunit together with the 50S subunit for a good complex formation.
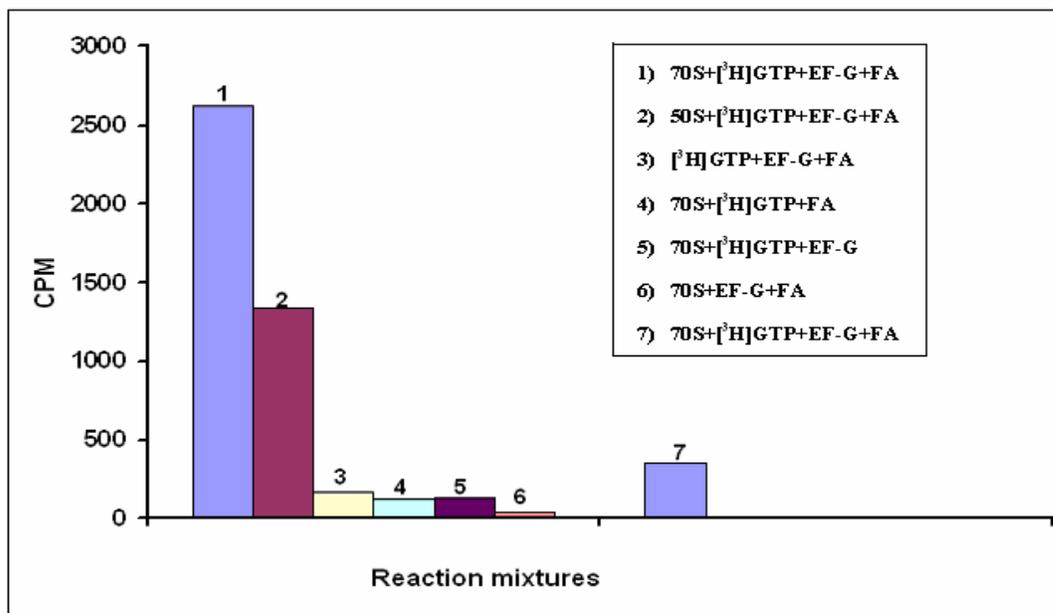

Figure 4: Nitrocellulose filter binding assay to detect 70S ribosome-EF-G-[^{3}H]GDP-FA complex formation:
Reaction mixtures containing varying combinations of 1 μM 70S ribosomes or 50S ribosomes, 2 μM EF-G, 1 μM [^{3}H]GTP and 1 mM FA were incubated on ice for 1 hr for complex formation. After that, the reaction mixtures were filtered through nitrocellulose membrane and washed with 10 ml CFB with FA (bars 1-6) or without FA (bar 7). The radioactive counts from the [^{3}H]GDP trapped on the nitrocellulose filter were measured in the scintillation counter and plotted as bars to indicate the quantity of the ribosomal complex formation. Different reaction conditions in the bars are indicated in the figure by individual color-codes and corresponding numbers.

Detection of 70S ribosome-EF-G-[³H]GDP-FA (or FAA) complex by gel filtration chromatography:

Gel filtration chromatography separates molecules based on their size. When the reaction mixture containing 70S ribosomes, EF-G, [³H]GDP and FA or FAA was applied to a sephacryl S-300 column equilibrated with CFB, 70S ribosomes could not enter the pores in the gel-matrix owing to their bigger size and were eluted in the void volume corresponding to fraction no. 5 (Fig. 5, inset). Medium sized molecules such as EF-G entered a few pores and hence had medium speed, so they were eluted after the ribosomes (fraction 6-7). Smaller molecules like GTP and FA or FAA would enter many pores in the gel which greatly reduced their speed and hence they were eluted much later (fraction 8 or higher). A small aliquot of each fraction was put in the scintillation counter and the radioactive counts of [³H]GDP were measured. The counts were plotted as a function of the fraction number (Fig. 5).

The overlap of the OD_{260} peak as well as the [³H]GTP cpm peak in fraction 5 indicates a successful formation of 70S ribosomes-EF-G-[³H]GDP complex with FA and FAA only when FA was supplied with the elution buffer. Further, high counts in fractions 8, 9 10 were from free GTP. The results also show that FAA was more efficient in complex formation compared to FA. Even though non UV treated FA and FAA could form complexes, both required the continuous supply of FA is washing buffer for stable 70S ribosome-EF-G-[³H]GDP-FA (or FAA) complex formation.
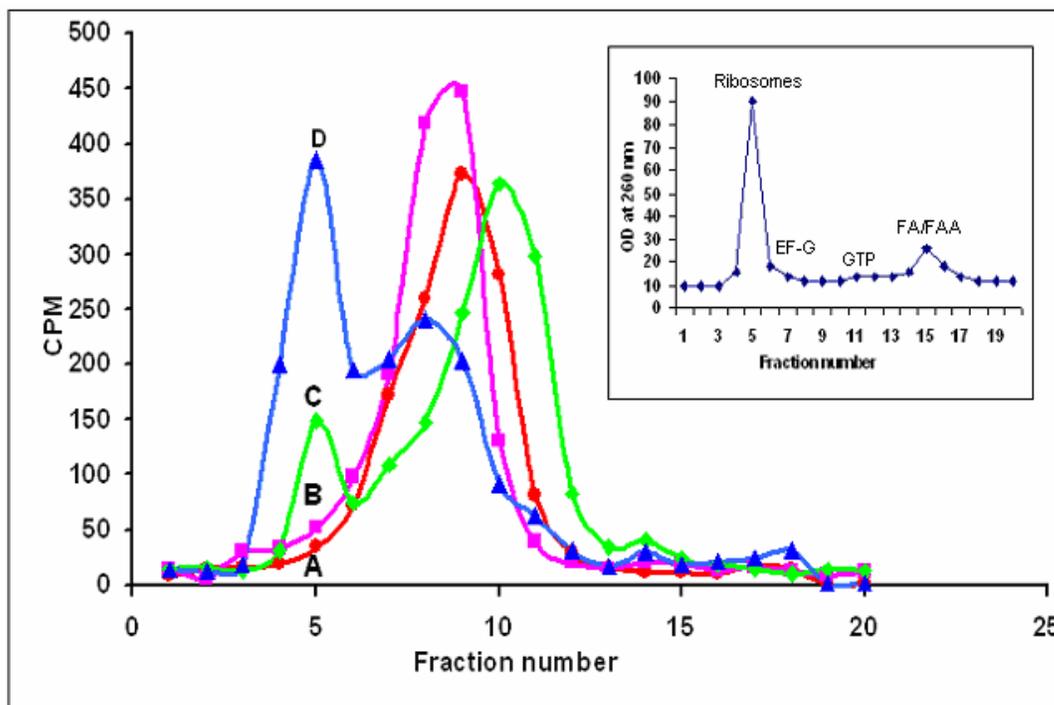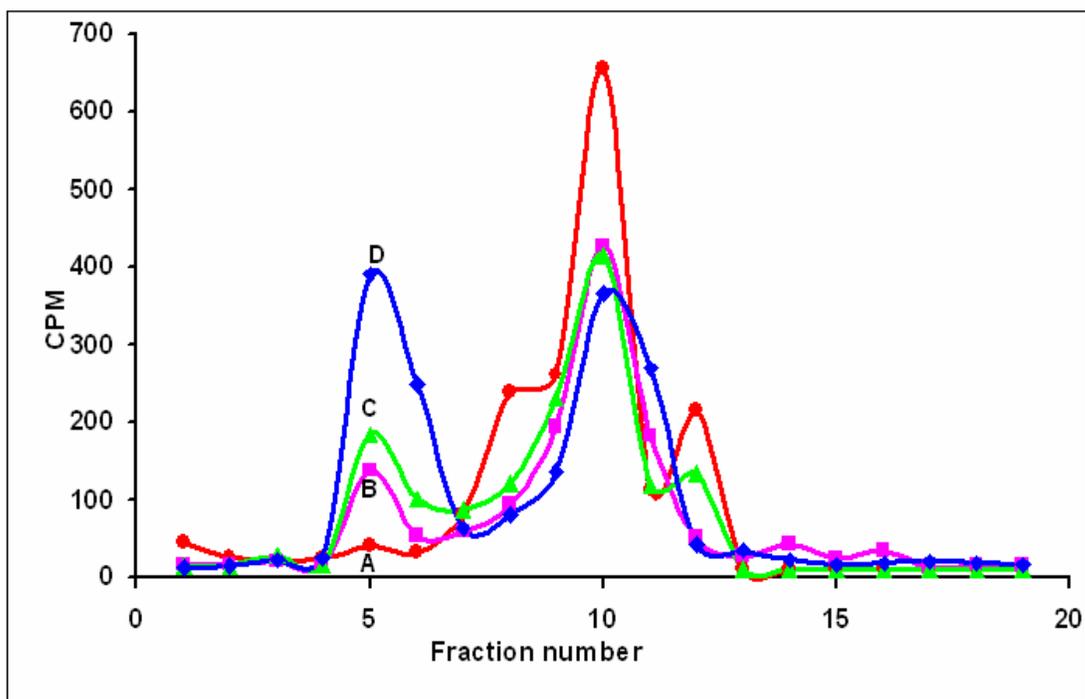
Figure 5: Detection of 70S ribosome-EF-G-[³H]GDP-FA (or FAA) complex by gel filtration chromatography using a Sephacryl S-300 column:
Reaction mixtures containing 1 μM 70S ribosomes, 2 μM EF-G, 1 μM [³H]GTP and 1 mM FA (traces A and C) or FAA (traces B and D) were incubated on ice for 1 hour for complex formation and were applied on a Sephacryl S-300 column equilibrated with CFB without (traces A and B) or with (traces C and D) FA in it. The fractions (10 ml. each) were collected and [³H]GTP counts were measured, which were plotted against the fraction number. In parallel, the OD_{260} of the fractions were monitored to identify the fractions where ribosomes, EF-G, guanidine nucleotides and the free drug were eluted (inset).

Crosslinking of FAA to EF-G in the 70S ribosome-EF-G-[³H]GDP-FAA complex and its detection by gel filtration chromatography:

A fusidic acid analogue with an azide photoreactive group was used for creating a crosslink with its target protein by UV irradiation. Reaction mixtures containing 70S ribosomes, EF-G, [³H]GTP and FA or FAA were incubated on ice for 1 hr and then exposed to UV light for specific time intervals and were then separated on a Sephacryl S 300 column.

Results presented in figure 6 suggested that UV-crosslinking in the reaction mixture containing 70S ribosomes, EF-G, [³H]GTP and FAA lead to the formation of a stable 70S ribosome-EF-G-[³H]GDP-FAA complex, which was intact even though it was washed with a large volume of buffer lacking FA. This was evident from the high radioactive counts in fraction 5 where ribosomes were eluted. The reaction mixture with 70S ribosomes, EF-G, [³H]GTP and FA could not form stable complexes when washed with buffer lacking FA, despite of UV exposure, indicating that normal FA could not be covalently crosslinked to EF-G.

Figure 6: Isolation of a stable UV-crosslinked 70S ribosome-EF-G-GDP-FAA complex by gel-filtration chromatography using Sephacryl S 300 column:
Reaction mixtures containing 1 μM 70S ribosomes, 2 μM EF-G, 1 μM [³H]GTP and 1 mM FA (traces A and C) or FAA (traces B and D) were incubated on ice for 1 hour. After UV-treatment they were separated on a Sephacryl S 300 column with (traces A and B) or without (traces C and D) FA in the running CF buffer. Fractions were collected and the radioactive counts from the [³H]GTP of respective fractions were plotted against the fraction number.

Standardization of the conditions for the formation of 70S ribosome-EF-G-[³H]GDP-FA and 70S ribosome-EF-G-[³H]GDP-FAA complexes:

In order to standardize the conditions for the formation of 70S ribosome-EF-G-[³H]GDP-FA and 70S ribosome-EF-G-[³H]GDP-FAA complexes, several experiments were done by varying concentrations of each of the primary components of the complex. These are 70S ribosomes, EF-G, fusidic acid and GTP respectively. In case of the complexes with FA, the extent of complex formation was monitored by the nitrocellulose filter binding assay, whereas the crosslinked 70S ribosome-EF-G-[³H]GDP-FAA complex formation was standardized with the help of gel-filtration chromatography. The data from these experiments are presented in the Appendix. In summary, 1 μM 70S ribosomes, 2 μM EF-G, 1 μM GTP and 1 mM FA or FAA together resulted in optimal complex formation. It was also shown that optimal crosslinking could be obtained if the samples were exposed to UV light four times, one minute each.

Separation of EF-G crosslinked with [³H]FAA from a 70S ribosome-EF-G-GDP-[³H]FAA complex and its detection by autoradiography:

A reaction mixture containing 70S ribosomes, EF-G, GTP and [³H]FAA was crosslinked under UV light and separated on a sodium dodecyl sulphate (SDS) polyacrylamide gel and subjected to autoradiography. Due to the detergent activity of SDS, the 70S ribosome complex was disintegrated into individual proteins and EF-G was well-separated from the ribosomal proteins as the biggest single protein-band that migrated most slowly on the gel. By comparing with the bands of the standard marker, it was concluded that the EF-G band was followed by the S1 protein from the 70S ribosome (Fig. 7 a). Smaller molecules like GTP and [³H]FAA migrated much faster and their bands could be seen near the bottom of the gel. The gel was dried and subjected to autoradiography to detect bound and free [³H]FAA molecules. When the film was autoradiographed, [³H] was seen only in the EF-G band and not with any other ribosomal protein bands, indicating that the [³H]FAA was covalently bound to EF-G (Fig. 7 b, lane 3).

In a similar set up only EF-G was crosslinked with [³H]FAA. The sample was analyzed by SDS-PAGE and was autoradiographed. Also here, a significant amount of [³H]FAA was seen covalently bound to EF-G (Fig 7 b, lane 4). This was the first evidence that a fusidic acid analogue (and therefore most probably fusidic acid as well) can bind to EF-G off the ribosome. In the autoradiogram, no radioactivity was seen in the EF-G bands of the non-crosslinked samples (Fig. 7 b, lanes 1 and 2). In all the samples free [³H]FAA was found in the bottom of the gel.

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A)

B)
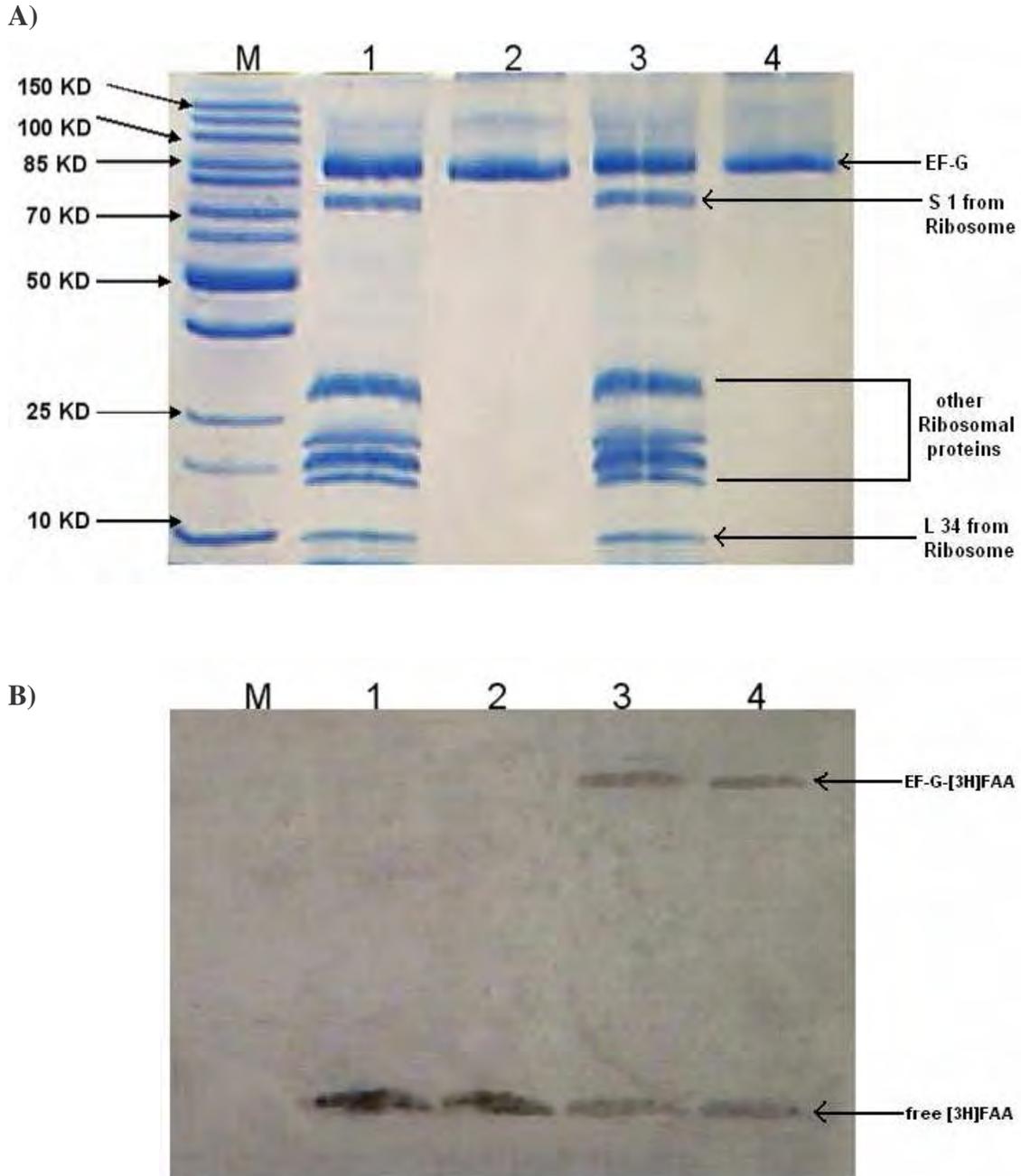
**Figure 7: Separation of the components of 70S ribosome-EF-G-GDP-[³H]FAA complex by SDS-PAGE (A) followed by autoradiography (B):**
(A) The 70S ribosome-EF-G-GDP-[³H]FAA complex and free EF-G mixed with [³H]FAA was treated with UV for crosslinking. The resulting samples were loaded (lanes 3 and 4 respectively) on a SDS-polyacrylamide gel and electrophoresis was conducted to separate the components. As a control the same samples without UV-treatment were loaded in lanes 1 and 2 respectively. The molecular weight markers are loaded in lane marked M. (B) The gel was then dried and autoradiographed.

Separation and detection of EF-G-FAA from 70S ribosome-EF-G-GDP-FAA complex by a combination of sucrose cushion ultracentrifugation and SDS-PAGE:

A sucrose cushion is a solution containing very high amounts of sucrose which makes it very dense. When a mix of 70S ribosomes, EF-G, GTP and FAA was loaded on top of a 30% sucrose cushion and centrifuged at 85000 x g, only the 70S ribosomes could pass into this cushion and settle as a pellet because of their high mass. Medium-sized molecules like EF-G and smaller molecules like GTP and FAA could not pass into the dense cushion and thus remained in the supernatant. The 70S ribosome-EF-G-GDP-FAA complex, both non-crosslinked and UV-crosslinked were loaded on top of 30% sucrose cushion, subjected to ultracentrifugation and the pellets were analyzed by SDS-PAGE. As expected, the EF-G band was seen only in the crosslinked case, indicating that EF-G was retained on the 70S ribosome due to FAA crosslinking (Fig. 9). No EF-G band was observed from the sucrose cushion pellet of non-UV-treated 70S ribosome-EF-G-GDP-FAA sample (figure 8). The EF-G band from the crosslinked sample was subjected to further analysis.
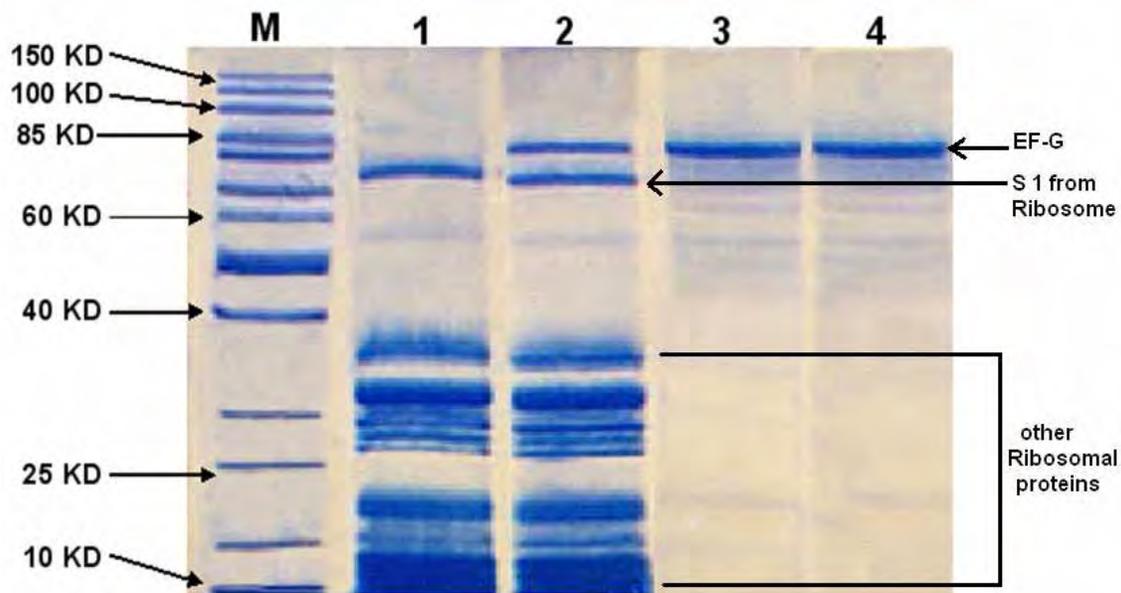

Figure 8: SDS-PAGE analysis of crosslinked and non crosslinked 70S ribosome-EF-G-GDP-FAA complexes centrifuged through a sucrose cushion:
70S ribosomes, EF-G, GTP and FAA were mixed in optimal concentrations to form the 70S ribosome-EF-G-GDP-FAA complex. Half of the complex was treated with UV for crosslinking. Both the non-crosslinked and the crosslinked samples were centrifuged at 85000 x g through a 30% sucrose cushion. The pellets (Lane 1 and 2 respectively) and the supernatants (Lanes 3 and 4 respectively) were subjected to SDS-PAGE. EF-G had been added in excess for stable complex formation.

Mass-spectrometry analysis of the gel bands containing EF-G crosslinked to FAA:

Gel bands containing UV-treated free EF-G and FAA-crosslinked EF-G isolated from the ribosomal complex were excised and sent for mass-spectrometry analysis. Both the bands were extensively digested by trypsin and then analyzed by MALDI-TOF. The mass spectra (Fig. 9) generated from both the samples were carefully compared to identify individual peaks with significant mass difference. About ten peptides were identified to be different in the two samples, which are presented in the mass table (Table 1). At least one of these peptides, which was possibly VPRIAFVNK should contain the FAA bound on it. For final identification, this peak along with some other peaks needs to be further analyzed by MS/MS analysis.
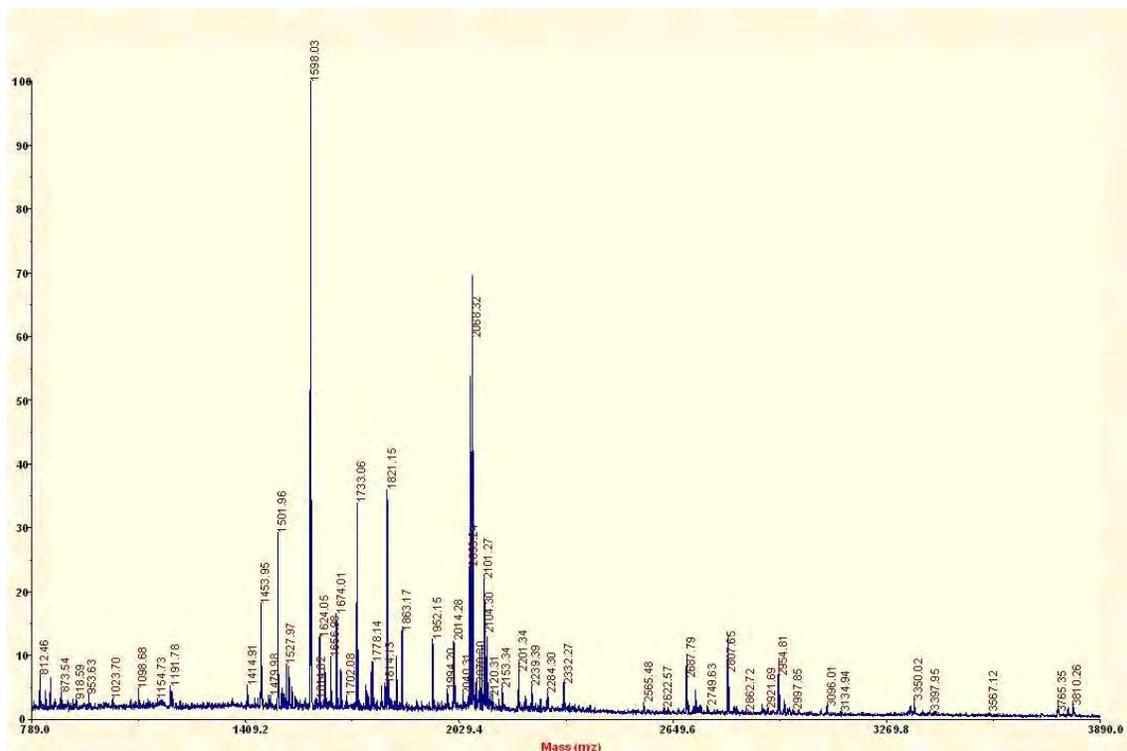
Figure 9: Mass spectrum of the UV treated EF-G-FAA:
The EF-G-FAA crosslinked sample was isolated as a gel-band and subjected to trypsin digestion. Further, the digested peptides were run in MALDI-TOF and individual mass-peaks were separated. The intensity of different peaks were plotted on Y-axis and the masses of various peptides in ascending order were plotted on X-axis. The mass of each peptide is represented by the number on its respective peak.

Table 1: Comparative analysis of the peptides from the crosslinked and non-crosslinked EF-Gs.

Mass of peptides with significant difference (in Da)	Mass of peptides after subtracting mass of FAA (506.3396 Da)	Peptides from free EF-G that were close matches	Sequence of the peptide which was the closest match
896.474	390.1344	393.1915	
1282.687	776.3474	774.4468	
1525.904	1019.5644	—	
1549.807	1043.4674	1043.6360	VPRIAFVNK
1570.757	1064.4174	1062.5578	
1712.922	1206.5824	—	
1758.875	1252.5324	1383.7954	
1886.971	1380.6314	—	
2831.357	2325.0714	—	
3485.646	2979.3064	—	

V - valine**, P -** proline**, R -** arginine**, I** - isolucine**, A -** alanine
F - phenylalanine**, N -** asparagine, **K -** lysine

DISCUSSION

Several antibiotic-drugs used now-a-days target the bacterial ribosome or different translation factors involved in bacterial protein synthesis. But the growing number of resistant bacteria has driven scientists to a constant quest to identify or design new compounds with potent antibacterial activity. In this quest two things become highly important; (i) a clear understanding of the binding site and the mechanism of action of the existing antibiotics, and (ii) the development of new drugs, more effective and specific, based on the mechanistic knowledge of the existing drugs. It may sound surprising yet true thateven in this age of molecular biology the binding site and the mode of action of many antibiotics are not known. One such antibiotic is fusidic acid, a small steroid molecule, widely used since last few decades to treat infections arising from gram-positive bacteria such as *Staphylococcus aureus*.

It is known from previous biochemical experiments that fusidic acid blocks EF-G on the ribosome after tRNA translocation and GTP hydrolysis (Hanson *et al*., 2005). The fact that it binds to EF-G and not the ribosome was recently demonstrated by Leo Pharma AB, in collaboration with Sanyal group (Riber *et a*l. 2006), yet the identification of the binding site of fusidic acid was beyond the scope of that work. In this project, the aim was to detect the exact binding site of fusidic acid in bacterial translation machinery, which will in turn aid significantly to my understanding of how this drug works.

A usual way of identifying the binding site of a drug or an inhibitor is to look for the resistance mutations on the target molecule. In case of fusidic acid the resistance mutations are found mostly in the *fusA* gene which codes for EF-G in bacteria (Johanson and Hughes, 1994). However, EF-G is a fairly big molecule (~80 kDa) with five domains and the scattered occurrence of the fusidic acid resistance mutations in different domains of EF-G makes the prediction of fusidic acid binding site more complex and unreachable by mutational analysis.

In the present study, I have used one of the three photo-affinity labeled fusidic acid analogues, reported earlier by Riber *et al*. to create UV-induced covalent crosslinking of fusidic acid in its target site on EF-G. This fusidic acid analogue (FAA) has azide as the reactive side chain (Fig. 2), which has been used successfully in other crosslinking studies (Robinette *et al*., 2006). The mass of the FAA is 607.36 Da. When exposed to UV, it looses two nitrogen atoms from the azide group resulting in a covalent bond at its target site. In general, azide groups are highly reactive and have the potential of crosslinking with the peptide backbone as well as the amino acid side-chains (Vodovozova, 2007). Due to this high reactivity, the specificity of azide-crosslinking often has been questioned and an optimal ratio of the target molecule to the azide-drug is essential (Scott and Richard, 1994).

The specificity of the FAA binding to EF-G has been achieved by crosslinking it in a functional complex on the ribosome. The ribosome is known to stimulate the intrinsic GTPase activity of EF-G (Nagel *et at*., 1993). When EF-G and GTP are incubated with 70S ribosome and fusidic acid, EF-G hydrolyses GTP to GDP, and then fusidic acid traps EF-G-GDP on the 70S ribosome. This complex (70S ribosome-EF-G-GDP-FA) is stable only in the presence of fusidic acid in excess. But, when FAA was used instead of FA and crosslinked with UV, a stable complex (70S ribosome-EF-G-GDP-FAA) could be isolated even in the absence of fusidic acid. When this complex was made with radiolabelled FAA and was analyzed by SDS-PAGE followed by autoradiography, only EF-G and none of the ribosomal proteins or RNA was found to retain FAA (Fig. 7). This result indicated that FAA binding to EF-G was specific. It should be mentioned that FAA crosslinking was also obtained with free EF-G, off the ribosome, although it was not possible to exclude the possibility of nonspecific binding in that case.

Mass-spectrometry analysis of the crosslinked EF-G-FAA revealed VPRIAFVNK as the possible peptide sequence in EF-G for FAA binding. When all the domains of EF-G were searched for the location of this peptide, it was found to be present in the domain G. However, the exact location of the binding site on this peptide is still a question and a detailed MS/MS analysis is required to pinpoint the residues on EF-G where fusidic acid binds. The outcome of this project will bring significant insight to our understanding about how fusidic acid works as an antibacterial drug.

MATERIALS AND METHODS

Strains and Plasmids:

1) *E. coli* JE-28: A His_6 tag had been inserted in frame at the C-terminus of ribosomal protein L12 directly on the chromosome using genetic recombineering.

2) *E. coli* BL-21(DE3)•pET30fus A: pET30 has a cloned His_6 tagged *fus A* gene under the control of T7 promoter for over-expressing EF-G.

Purification of His_6-tagged 70S ribosomes:

The His_6-tagged 70S ribosomes were purified from *E. coli* JE-28 cells (glycerol stock provided by the laboratory) through affinity chromatography. These cells were grown in 1 L Luria-Bertani (LB) broth (10 g NaCl, 5 g yeast extract and 10 g tryptone) + kanamycin (50 µg/ ml) at 37ºC until the turbidity of the culture reached 1.5 at 600 nm. The cells were then centrifuged at 5000 x g for 30 minutes at 4ºC. After resuspending the cells in a lysis buffer (20 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 150 mM KCl, 30 mM NH_4Cl, 1 µM protease inhibitor (PMSF) and 1 µg/ ml DNAse I), they were loaded into the pressure cell of the French press and a high pressure (7-8 tons) was applied in order to break the cells. The lysate was centrifuged at 18000 x g for 40 minutes at 4ºC.

A His_6-trap column (GE Health Care, UK) was connected to an AKTA prime chromatography system (provided by laboratory). After equilibrating the column with lysis buffer, the supernatant from the centrifuged cell lysate was loaded. When the supernatant passes through this affinity column, all the His_6-tagged 70S ribosomes were trapped by the Ni^{2+} resin due to specific binding whereas all the non-specific binders were eluted. The column was then washed with lysis buffer that had 5 mM imidazole to get rid of any weakly bound molecules. The 70S ribosomes were finally eluted by washing the column with lysis buffer that had 150 mM imidazole. The final elutant containing the 70S ribosomes was loaded to dialysis bags and dialyzed against lysis buffer until the imidazole was completely separated from the ribosomes. The ribosomes were then concentrated by loading them on top of a 30% sucrose cushion (20 mM Tris-HCl (pH 7.5), 500 mM NH_4Cl, 10.5 mM Mg acetate, 0.5 mM EDTA, 1.1 M sucrose and 3 mM 2-mercaptoethanol) and centrifuged at 30000 x g for 12 hours at 4ºC in an ultra centrifuge. They were finally resuspended in 1 x polymix (5 mM NH_4Cl, 95 mM KCl, 0.5 mM $CaCl_2$, 8 mM putrescine, 1 mM spermidine, 1 mM dithioerythritol (DTE) and 5 mM potassium phosphate,) and stored at -80ºC.

Purification of His$_6$-tagged EF-G:

The His$_6$-tagged EF-G was purified from *E.coli* BL-21(DE3)•pET30fus A cells (provided by the laboratory) through affinity chromatography. The His$_6$-tagged EF-G was purified with the help of a His$_6$-trap column in the similar way as with the His$_6$-tagged 70S ribosomes. The final eluate containing His$_6$-tagged EF-G was dialyzed against lysis buffer to separate imidazole from EF-G. The EF-G was finally concentrated by loading to AMICON ultra centrifugal filter tubes (GE Health Care, UK) and centrifuged at 3000 x g for 30 minutes. It was finally resuspended in 1 x polymix and stored at -80ºC.

Nitrocellulose filter binding assay:

Complex formation mixtures were prepared with the following components: 1 µM 70S ribosomes, 2 µM EF-G, 1 µM [^{3}H]GTP (GE Healthcare, Sweden) and 1 mM fusidic acid (FA) (LEO Pharma, Denmark) in a complex formation buffer (CFB) (10 mM Tris-HCl (pH 7.5), 10 mM NH$_4$Cl and 10 mM Mg acetate). These mixtures were incubated on ice for 1 hour and then filtered through a nitrocellulose filter (0.45 µM, Protran BA 85) presoaked in CFB, followed by washing with 10 ml CFB. The filters were then immersed in 10 ml liquid scintillation cocktail for membrane filters (Packard Bioscience BV, Netherlands) and left under shaking for 1 hour. The radioactive count from the trapped [^{3}H]GDP in the 70S ribosome-EFG-[^{3}H]GDP-FA complex was measured in a Beckman LS6500 multipurpose scintillation counter. A positive control was set by filtering the complex through nitrocellulose filter (presoaked in CFB containing 0.3 mM fusidic acid) followed by washing with CFB containing 0.3 mM fusidic acid. Several negative controls were set by omitting one of the components from the complex formation mixture.

UV crosslinking:

The UV crosslinking was done in a Bio-Rad GS Genelinker-UV chamber which was equipped with UV lamps. Reaction mixtures were made with 1 µM 70S ribosomes, 2 µM EF-G, 1 µM [^{3}H]GTP and 1 mM FAA (LEO Pharma, Denmark) in complex formation buffer and incubated in eppendorf tubes on ice for 1 hour. The GS Genelinker-UV chamber was set to crosslinking mode and the eppendorf tubes containing the reaction mixtures were kept inside the UV chamber. The UV lamps were switched on and the tubes were left inside the chamber for 1 minute to allow crosslinking. This was followed by a rapid cooling step where the samples were incubated on ice for 3 minutes. This process was repeated 4 times in order to achieve crosslinking of FAA to EF-G in the 70S ribosome-EFG-[^{3}H]GDP-EF-G complex. A negative control was set by replacing FAA with FA in the complex formation mixture.

Column Chromatography:

1 µM 70S ribosome, 2 µM EF-G, 1 µM [^{3}H]GTP and 1 mM fusidic acid analogue (FAA) were mixed in CFB and incubated on ice for 1 hour followed by UV crosslinking. A gel filtration column (Sephacryl S300) was connected to the AKTA prime chromatography system and different parameters were set as follows: UV output range 0.2, flow rate 1 ml/min, fraction size 0, buffer valve position 1. The column was washed with water followed by equilibration with CFB. The crosslinked sample was taken in a Hamilton syringe and injected into the column through valve 3. The fraction size was set to 5 ml and the column was then washed with CFB. Several fractions were collected into 10 ml plastic tubes and 200 µl from each fraction was mixed in 5 ml liquid scintillation cocktail for proteins and peptides (Zinsser analytic, Great Britain). After shaking for 10 minutes, the radioactive counts from [^{3}H]GTP from different samples were measured in a Beckman LS6500 scintillation counter. A crosslinked sample that was washed with CFB containing 0.3 mM FA served as positive control whereas non-crosslinked samples and samples that had FAA replaced by FA served as negative controls.

Sodium dodecyl sulphate - polyacrylamide gel electrophoresis:

A 12% polyacrylamide gel was a combination of two layers of gels that were cast on top of each other. The bottom layer was cast by pouring 10 ml resolving gel (2.5 ml of 1.5 M Tris-HCl (pH 8.8), 3.3 ml of H_2O, 4.0 ml of 30% acrylamide: N-N'-methylene-bis-acrylamide (37.5:1), 0.1 ml of 10% SDS, 0.1 of ml 10% ammonium persulphate and 0.004 ml of TEMED) into a glass plate sandwich and allowed to solidify for 30 min. 2 ml of stacking gel (0.25 ml of 1 M Tris-HCl (pH 6.8), 1.4 ml of H_2O, 0.33 ml of 30% acrylamide: N-N'-methylene-bis-acrylamide (37.5:1), 0.02 ml of 10% SDS, 0.02 ml of 10% ammonium persulphate and 0.002 ml of TEMED) was poured on top of solidified resolving gel, a comb was inserted and the gel was allowed to solidify for 20 min. Then the glass plate sandwich was placed in a buffer tank filled with running buffer (15 g Tris-HCl (pH 2.3), 72 g glycine and 5.0 g SDS per 1L distilled water).

Reaction mixtures were made with 1 µM 70S ribosome, 2 µM EF-G, 1µM GTP and 1 mM [^{3}H]FAA in CFB and incubated on ice for 1 hour. After UV treatment, 30 µl of each sample was mixed with 10 µl of loading dye (10 ml dye contained 2.4 ml of 1M Tris-HCl (pH 6.8), 3 ml of 20% SDS, 3 ml of 100% glycerol, 1.6 ml of β mercaptoethanol and 0.006 g of bromophenolblue) and loaded into the wells of stacking gel. A protein size standard loaded in one of the wells served as control and a non crosslinked sample served as a negative control. Electrophoresis was done for 45 minutes at 200 V. The gel was then immersed in a staining dye (100 ml of dye contained 90 ml of methanol: water (1:1), 10 ml of glacial acetic acid and 0.25 g of Coomassie Brilliant Blue), boiled in a microwave oven for 30 seconds and incubated at 37 ºC for 1 hour. For destaining, the was immersed in a destaining solution (I L solution contained 120 ml of isopropanol and 50 ml of glacial acetic acid), boiled in a microwave oven for 1 minute and left on a shaker until the gel got cleared and different bands on the gel could be seen clearly.

Autoradiography:

After SDS-PAGE, the gel was immersed in 1 M sodium citrate for 30 minutes and vacuum dried for 1 hour. The dried gel was taken to dark room and placed on top of a high performance chemiluminescence film which was then kept inside an autoradiographic cassette and left in dark for 10 days. The chemiluminescence film was then fed to an Optimax X-ray film processor which developed the chemiluminescence film into an autoradiogram.

Centrifugation on a sucrose cushion:

100 µl of reaction mixture was made with 1 µM 70S ribosome, 2 µM EF-G, 1 µMGTP and 1 mM FAA in CFB and incubated on ice for 1 hour, crosslinked under UV light and loaded on top of 100 µl of 30% sucrose cushion in micro-centrifuge tubes. A non crosslinked sample served as the negative control. The samples then were centrifuged in a Sorvall RC150 GX ultra micro-centrifuge at 85000 x g for 45 minutes. After the centrifugation, the supernatants were separated from the pellets with a micro-pipette and 30 µl of CFB was added to each of the pellets to resuspend them.

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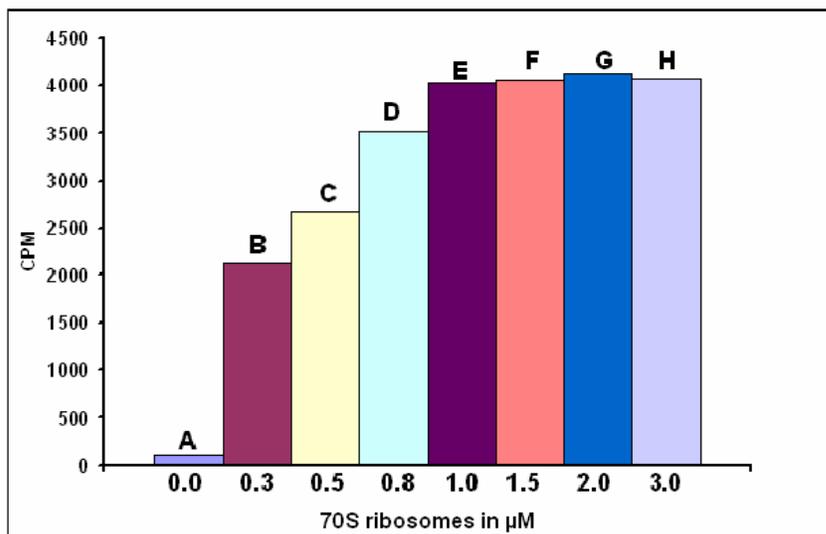
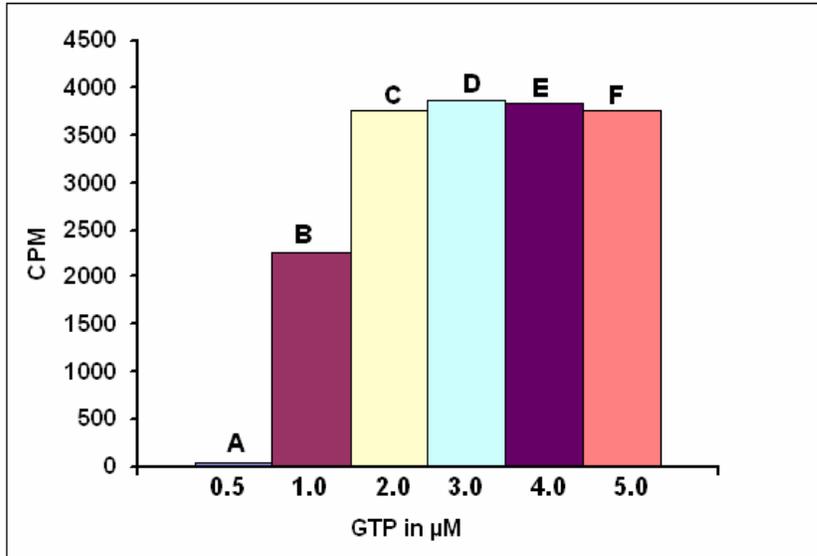**Figure 11: Nitrocellulose filter binding assay to detect 70S ribosome-EF-G-[³H]GDP-FA complex formation when different concentrations of 70S ribosomes were used:**
Reaction mixtures containing 1 μM [³H]GTP, 2 μM EF-G, 1 mM FA and varying concentrations of 70S ribosomes were incubated on ice for 1 hour and then they were filtered through nitrocellulose membrane and washed with 10 ml CFB with FA. The radioactive counts from the [³H]GDP trapped on the nitrocellulose filter were measured in the scintillation counter and plotted as bars to indicate the quantity of the ribosomal complex formation. Different reaction conditions in the bars were indicated by individual color-codes and corresponding alphabets.
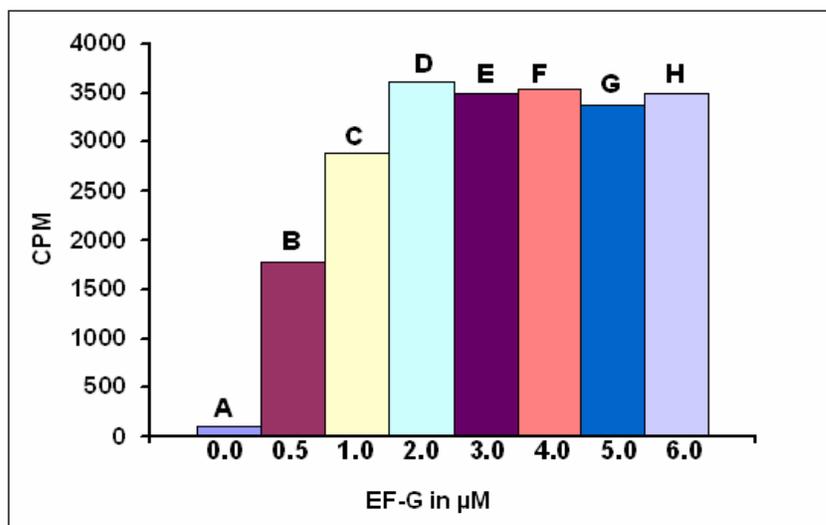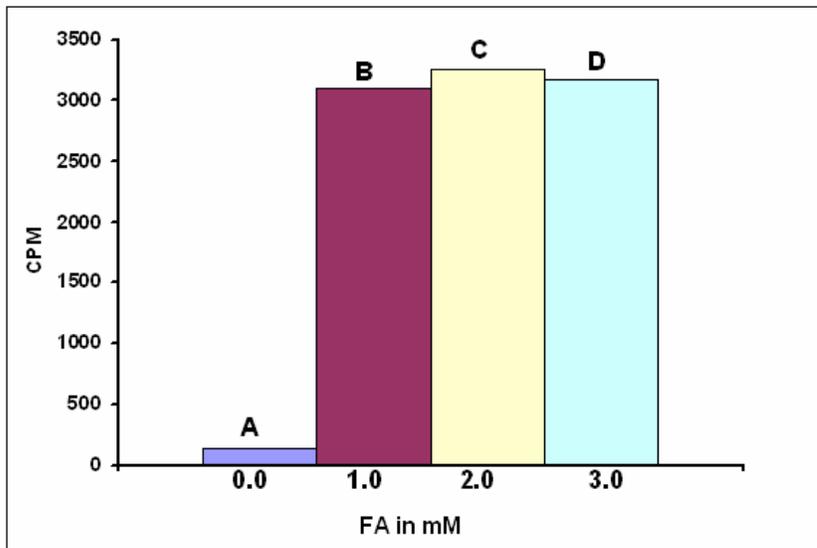

Figure 12: Nitrocellulose filter binding assay to detect 70S ribosome-EF-G-[³H]GDP-FA complex formation when different concentrations of EF-G was used:
Reaction mixtures containing 1 μM 70S ribosomes, 1 μM [³H]GTP, 1 mM FA and varying concentrations of EF-G were incubated on ice for 1 hour and then they were filtered through nitrocellulose membrane and washed with 10 ml CFB with FA. The radioactive counts from the [³H]GDP trapped on the nitrocellulose filter were measured in the scintillation counter and plotted as bars to indicate the quantity of the ribosomal complex formation. Different reaction conditions in the bars were indicated by individual color-codes and corresponding alphabets.
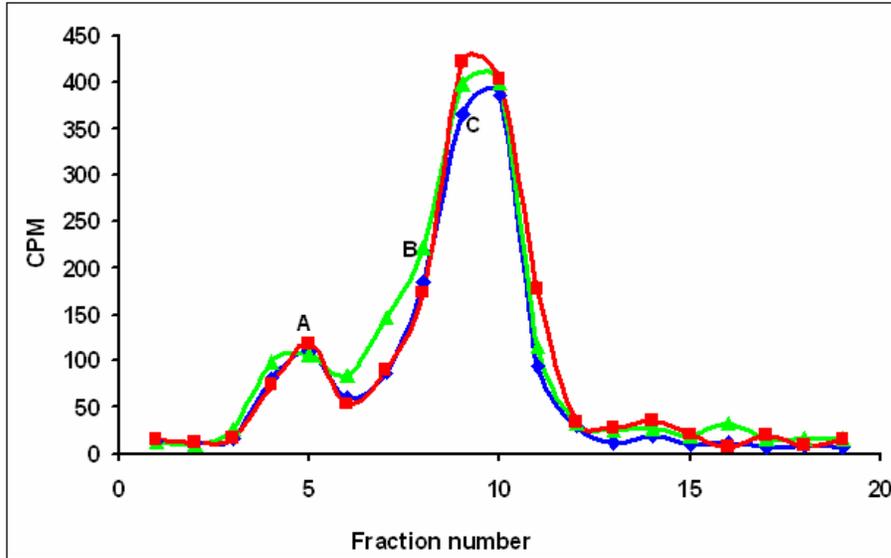
Figure 13: Nitrocellulose filter binding assay to detect 70S ribosome-EF-G-[³H]GDP-FA complex formation when different concentrations of GTP was used:
Reaction mixtures containing 1 µM 70S ribosomes, 2 µM EF-G, 1 mM FA and varying concentrations of [³H]GTP were incubated on ice for 1 hour and then they were filtered through nitrocellulose membrane and washed with 10 ml CFB with FA. The radioactive counts from the [³H]GDP trapped on the nitrocellulose filter were measured in the scintillation counter and plotted as bars to indicate the quantity of the ribosomal complex formation. Different reaction conditions in the bars were indicated by individual color-codes and corresponding alphabets.


Figure 14: Nitrocellulose filter binding assay to detect 70S ribosome-EF-G-[³H]GDP-FA complex formation when different concentrations of FA was used:
Reaction mixtures containing 1µM 70S ribosomes, 2 µM EF-G, 1 µM[³H]GTP and varying concentrations of FA were incubated on ice for 1 hour and then they were filtered through nitrocellulose membrane and washed with 10 ml CFB with FA. The radioactive counts from the [³H]GDP trapped on the nitrocellulose filter were measured in the scintillation counter and plotted as bars to indicate the quantity of the ribosomal complex formation. Different reaction conditions in the bars were indicated by individual color-codes and corresponding alphabets.

Figure 15: Detection of 70S ribosome-EF-G-[³H]GDP-FAA complex by gel filtration chromatography when different concentrations of FAA was used:
Reaction mixtures containing 1 μM 70S ribosomes, 2 μM EF-G, 1 μM [³H]GTP and different concentrations of FAA (1 mM in trace A, 2mM in trace B and 3 mM in trace C) were incubated on ice for 1 hour for complex formation. After UV treatment they were seperated on a Sephacryl S-300 column equilibrated with CFB without FA in it. The fractions (10 ml. each) were collected and [³H]GTP counts were measured, which were plotted against the fraction number.
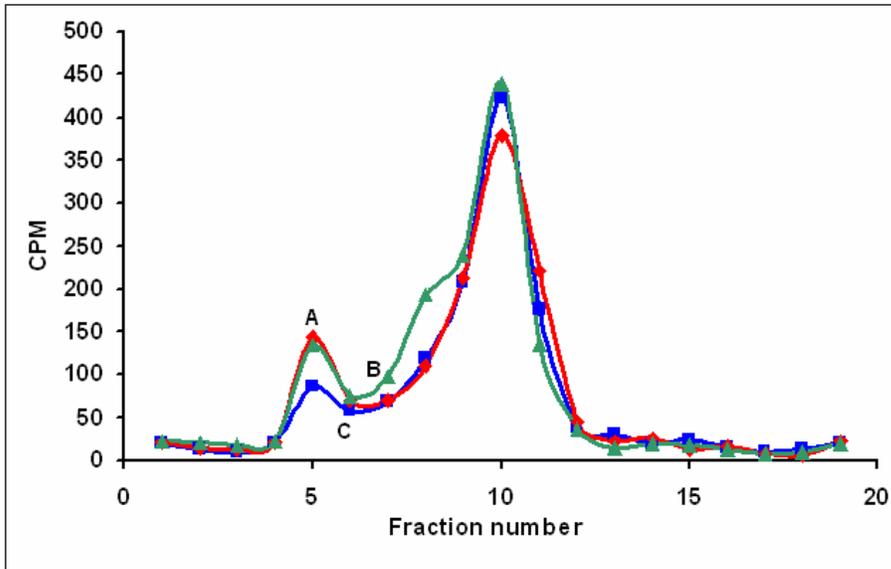
Figure 16: Detection of 70S ribosome-EF-G-[³H]GDP-FAA complex by gel filtration chromatography when different UV exposure times were used:
Reaction mixtures containing 1 μM 70S ribosomes, 2 μM EF-G, 1 μM [³H]GTP and 1 mM FAA were incubated on ice for 1 hour and then exposed to UV light for 1 minute several times (4 times for trace A, 8 times for trace B and 2 times for trace C) for stable complex formation. After UV treatment they were seperated on a Sephacryl S-300 column equilibrated with CFB without FA in it. The fractions (10 ml. each) were collected and [³H]GTP counts were measured, which were plotted against the fraction number.