Mechanism and Regulation of Initiation of Protein Synthesis in Eubacteria

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Abstract


Initiation of protein synthesis in E.coli involves several steps, which lead to the formation of the first peptide bond. This process requires three initiation factors: IF1, IF2 and IF3. Using a novel technique of combined light scattering and stopped-flow, we elucidated the importance of IF2-GTP conformation for the recruitment of 50S to 30S pre-initiation complex. Moreover, GTP hydrolysis is essential for IF2 release and later binding of ternary complex. Interestingly, a switch in IF2 affinity to G-nucleotides is induced during 30S pre-initiation complexes formation.

We found that IF1, previously with unknown functions in vitro, increases the rate of naked 70S dissociation by a factor 80 and acts as a fidelity factor in preventing 70S formation containing elongator tRNA instead of fMet-tRNA\textsuperscript{Met}. We showed that RRF/EFG/IF3 split both naked and post-termination complexes while IF1/IF3 split only naked ribosomes. The mechanisms of action of RRF/EFG, the order of their binding to 70S, as well as, the three different conformation of EF-G on the ribosomes are emphasized. Interestingly, 70S formation rate is dependent on the concentration of IF3 and not linear with 50S subunits concentration. We demonstrated that the rate-limiting step in 70S formation is IF3 dissociation from 30S complexes.

The interplay between initiation factors in the rate and accuracy of protein synthesis was thoroughly studied. Using fMet-tRNA\textsuperscript{Met} (initiator tRNA), Met-tRNA\textsuperscript{Met} (non-formylated initiator tRNA) and Phe-tRNA\textsuperscript{Phe} (elongator tRNA), we showed that the major player in the accuracy is IF2 through recognizing the formyl group on fMet-tRNA\textsuperscript{Met}, while IF3 acts by increasing both the on- and off-rate of tRNA from 30S pre-initiation complexes.

Collectively, these novel results describe a comprehensive model of initiation of protein synthesis. In this model, initiation factors increase the rate of fMet-tRNA\textsuperscript{Met} binding to 30S subunits, subsequently, the stabilization of fMet-tRNA\textsuperscript{Met} by IF2 increases the rate of IF3 dissociation. Later, IF2 in GTP conformation allows 50S docking to 30S pre-initiation complex free of IF3 followed by GTP hydrolysis allowing IF2 release for ternary complex to bind and start elongation of protein synthesis.

Keywords: Initiation, Protein Synthesis, Initiation Factors, Light Scattering, Ribosomes

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“The God of heaven Himself will prosper us; therefore we His servants will arise and build” Nehemiah 2:20

To the loving God, the Lord Jesus Christ
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>EF-G</td>
<td>Elongation factor G</td>
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<td>EF-Tu</td>
<td>Elongation factor Tu</td>
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<td>EF-Ts</td>
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<td>GTP</td>
<td>Guanosine triphosphate</td>
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<td>GDP</td>
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<td>GMPPNP</td>
<td>β, γ-Imidoguanosine tri-Phosphate</td>
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<td>GAP</td>
<td>GTPase activating proteins</td>
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<td>GEF</td>
<td>Guanosine nucleotide exchange factor</td>
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<tr>
<td>mRNA</td>
<td>messenger Ribonucleic Acid</td>
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<tr>
<td>ORF</td>
<td>Open Reading Frame</td>
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<tr>
<td>RGS</td>
<td>regulators of G protein signaling</td>
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<td>RF1</td>
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<td>RF3</td>
<td>Release factor 3</td>
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<tr>
<td>RRF</td>
<td>Ribosome Recycling Factor</td>
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<tr>
<td>SD</td>
<td>Shine and Dalgarno</td>
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<tr>
<td>tRNA</td>
<td>transfer Ribonucleic Acid</td>
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Introduction

“Nothing in life is to be feared, it is only to be understood.”

Marie Curie

The Genome

The genome of an organism serves as central storage for the genetic information that is transmitted between generations. Even though species may be completely different on a phenotypic level, the fundamental genetic principles are conserved (Cromie et al., 2001; Lander et al., 2001). According to the classical “central dogma of molecular biology” the expression of genetic information is mediated through the transcription of DNA into RNA that is processed then translated into protein.

Translation:

It is the process by which the genetic information delivered as transcribed mRNA is transformed into an active protein. This process requires several important participants including RNAs like mRNA, tRNA, rRNA and specific protein factors like initiation and elongation factors that are involved in each step of the protein synthesis. Some of these protein factors acts as GTPase like IF2, EF-Tu, EF-G and RF3.

The translation of the genetic information from mRNA to amino acid sequences takes place on a macromolecular complex called “the ribosome”. Ribosomes read nucleotide triplets (codons) on the mRNA and direct the addition of a specific amino acid carried by the cognate tRNA to the growing polypeptide.

The translation is divided into three main stages: initiation, elongation and termination followed by the recycling of the ribosome to enter new cycle of translation of protein synthesis.
Components of the protein synthesis

Ribosomes

Ribosomes are large ribonucleoprotein complexes that are responsible for protein synthesis in all cells (Yusupov et al., 2001). The main catalytic activity is through the RNA, i.e., they are ribozymes (Nissen et al., 2000).

Ribosomes are major components of the cell where, in *Escherichia coli*, during rapid growth, ribosomes constitute about 50% of total dry cell mass. There are several control mechanisms that allow the increase in ribosome concentration during cell growth. In the last two decades, there was a vast work to elucidate the structure of ribosomes using X-ray crystallography and Cryo-electron microscopy. The X-ray structure of 50S at 2.5 and 3.1 resolution (Ban et al., 2000; Harms et al., 2001) and of 30S were recently determined (Schluenzen et al., 2000; Wimberly et al., 2000). The subunit structures have made it possible to reveal details of antibiotics bound to the ribosome from crystallographic data on subunit-antibiotic complexes (Brodersen et al., 2000; Carter et al., 2000) and to study at high resolution the interactions of functional ligands and factors with the 30S and 50S subunits (Carter et al., 2001; Ogle et al., 2001).

The *E. coli* ribosomes are a big macromolecular complex formed mainly of rRNA and different proteins. rRNA makes the backbone of each subunit to which proteins are attached. It acts also as the catalytic part where the main biochemical reactions occur like GTPase activation and peptide bond formation. Those complexes are composed of two subunits, small (30S) subunits, containing 16S rRNA and about 21 ribosomal proteins (S1, S2, ...) and large (50S) subunits, which contain 23S rRNA, 5S rRNA, and 31 ribosomal proteins (L1, L2...).

The ribosome has the unique feature of being a complex molecular machine, with moving parts, many of which are structural elements of rRNA. This molecular movement is inherent for the mechanism of translation (Noller and Baucom, 2002). Each tRNA must move by distances of 20–70 Å during each step of the translocation elongation.
cycle and recently increasing biochemical evidence supports the coupled movement of tRNA to movements within the ribosome structure itself (Noller et al., 2002).

The complete 70S ribosome is formed by association of the 30S and 50S subunits through a network of intermolecular bridges. Its intersubunit space is occupied by the transfer RNAs (tRNAs), whose anticodon base pair with messenger RNA (mRNA) codons in the 30S subunit, whereas their 3'-CCA ends, which carry the growing polypeptide chain and the incoming amino acid, reach into the 50S subunit, the location of the peptidyl transferase center, where peptide bond formation is catalyzed.

There is a clear division of labor between the two subunits of the ribosome (Ramakrishnan, 2002). The small subunit mainly decodes genetic information, while the large subunit is responsible for peptide elongation and protein release.

Different RNA: rRNA, mRNA, tRNA, sRNA and tmRNA

RNA plays an important role in biological systems and carries out a broad range of biological functions. There are three major RNA classes in the cell: rRNA, mRNA and tRNA.

rRNA is a ribozyme, which constitutes the main bulk of the RNA in the cell and forms the backbone of the ribosomes.

mRNA carries the information from DNA in a three-letter genetic code, which are called codons. There are two types of mRNA: leadered and leaderless mRNAs. Leaderless mRNAs start directly with the AUG initiating codon and are present in all kingdoms of life (Moll and Blasi, 2002). Recent studies in E. coli have provided evidence that leaderless mRNAs are devoid of downstream ribosomal recruitment signals (Resch et al., 1996). These mRNA are recognized by a ribosome-fMet-tRNA^fMet (tRNAi) complex and that their translational efficiency can be modulated by components of the translation machinery, i.e., by initiation factors IF2 and IF3 as well as by ribosomal protein S1 (Moll and Blasi, 2002). The leadered mRNA has a shinedalgarano sequence that interacts with the anti-SD sequence on 30S.
tRNA has the main important function of being chemically linked to a particular amino acid and to base pair with a codon in mRNA. Each tRNA is recognized by one and only one amino acyl tRNA synthetase that links only one of the 20 amino acids to a particular tRNA to form aminoacyl-tRNA. This tRNA then recognizes a codon in mRNA and delivers its amino acid to the growing polypeptide chain.

All tRNA molecules have common features:
1- They are formed of 73 to 93 bases long and contain about 7 to 15 modified bases per molecule, which are usually not available for base-pairing.
2- The 5’-end is usually pG and 3’-end is CCA\textsubscript{OH}.
3- The A at the 3’-end is the amino acid attachment site.
4- 50% of nucleotides are base-paired except for 3 loops and the extra arm and the 3-D structure is L-shaped with two major segments of double helix and bases in non-helical regions are involved in tertiary H-bonding interactions.
5- The 3’-CCA is at the end of one arm of the L and the anticodon loop is at the other end.
6- Amino-acyl tRNA activates amino acids by linking them to tRNA in a reaction requiring ATP.

t\textsubscript{f}Met-t\textsubscript{RNA}\textsuperscript{fMet} is the initiator tRNA (Kozak, 1983) who has several specific features that differ from other tRNA in the cell.
1) Initiator tRNA binds directly to the P site of the ribosome in a reaction facilitated by the initiation factors, IF2 in eubacteria and eIF2 in eukaryotes. In contrast, elongator tRNAs bind to the A site of the ribosome.
2) Initiator tRNAs are used exclusively for the initiation of protein synthesis. This is achieved by preventing the binding of initiator methionyl-tRNAs to the elongation factor EF-Tu, which carry aminoacyl-tRNAs to the A site on the ribosome (Stortchevoi et al., 2003).
3) Initiator tRNAs also possess unique sequence and/or structural features that are absent in elongator tRNAs. Some of the unique features of eubacterial initiator are the presence of a base pair mismatch between nucleotides 1 and 72 in the acceptor stem and the presence of 3 G-C pairs.

sRNA, small non-coding RNA, are involved in the gene regulation. Small, untranslated RNA molecules were identified initially in bacte-
ria, but examples can be found in all kingdoms of life (Argaman et al., 2001). They act as co-ordinators of adaptation processes in *Escherichia coli* (Gottesman et al., 2001; Repoila et al., 2003). As a general phenomenon, the abundance of individual sRNAs varies with varying environmental conditions, consistent with a role in cellular adaptation (Gottesman et al., 2001). Many of them act also as regulators of gene expression at a posttranscriptional level, either by acting as antisense RNAs or by interacting with proteins. Recently, it was shown that there are small RNA-encoding genes in the intergenic regions of *Escherichia coli* (Argaman et al., 2001).

**tmRNA**, transfer messenger RNA, are used in the rescue of ribosomes during cell starvation or truncation of mRNA. Upon termination of protein synthesis, a stop codon is located into the ribosomal A site followed by binding of a class-1 release factor to the A site of the ribosome and induces a rapid release of the full-length protein (Ivanova et al., 2005; Kisselev and Buckingham, 2000). When the open reading frame (ORF) of the mRNA is truncated, the ribosome will eventually be stalled with a peptidyl-tRNA in the P site and an incomplete codon in the A site, which cannot be recognized by a class-1 release factor. Those stalled ribosomes are rescued by tmRNA, which is RNA molecule that plays the roles of both tRNA (Komine et al., 1994) and mRNA.

The tmRNA, charged with alanine by the alaninyl-tRNA synthetase (Ala-RS) (Ushida et al., 1994) is delivered to a stalled ribosome by elongation factor Tu (EF-Tu) in complex with GTP, analogously to the enzymatic binding of an ordinary aminoacyl-tRNA to the elongating ribosome (Ivanova et al., 2005; Ivanova et al., 2004).

After tmRNA binding to the A site of a stalled ribosome, the unfinished peptide chain is transferred from the P-site tRNA to the alanine of tmRNA in a *trans*-peptidation reaction. The resulting peptide chain tagged with 11 amino acid residues during *trans*-translation is released from the ribosome by a class-1 release factor (RF) bound to the ribosomal A site in response to the stop codon UAA in the ORF of the tmRNA and subsequently rapidly degraded by intracellular proteases (Gottesman et al., 1998).
Ribosomes stalled at a sense codon, due to intracellular deficiency of its cognate aminoacyl-tRNA can also be rescued by tmRNA (Karzai et al., 2000; Roche and Sauer, 1999).

Protein factors

There are several protein factors involved in Eubacterial protein synthesis. They include: (i) initiation factors IF1, IF2 and IF3 involved in initiation of protein synthesis (Kyridies and Woese, 1998). (ii) elongation factors EF-Tu, EF-TS and EF-G required in the elongation step (Wintemeyer et al., 2004). (iii) release factors RF1 or RF2 to release the protein and RF3 for RF1/RF2 recycling.

Factors and Ribosome levels are coordinately controlled in *Escherichia coli* growing at different rates (Howe and Hershey, 1983). Hershey et al. found that the ribosomal proteins and elongation factors were present in equimolar amounts in the cell except L7/L12 and EF-Tu and that the levels of each protein increased in proportion to the growth rate.

G-proteins are an important class of proteins in the cell, which have different conformations depending on whether they are bound to GTP or GDP (Bourne et al., 1991). GTP binding induces the active conformation and the hydrolysis of GTP to GDP+P\(_i\) and converts the protein to the inactive conformation.

Two important factors regulate the GTP-GDP cycle on G-proteins:
1. Guanosine nucleotide exchange factor (GEF) helps both the release of GDP from the nucleotide binding site and the binding of GTP.
2. GTPase activating protein (GAP) that activates the GTPase function of G-proteins.

The conformation changes between the GDP and GTP forms act as a switch (Rodnina et al., 2000), serve a mechanical role or affect the membrane binding properties of the protein (Bourne, 1997).

Rapid protein synthesis in bacteria requires four G proteins IF2, EF-Tu, EF-G, and RF3 (Zavialov and Ehrenberg, 2003) that catalyze all major steps of mRNA translation in a GTP-dependent manner. They
belong to a large class of G proteins that switch between active GTP and inactive GDP forms and participate in diverse cellular processes (Bourne et al., 1991). The GTP is hydrolyzed when the factors contact the ribosomal GTPase center (Ramakrishnan, 2002) and thus the ribosome functions as a GAP for the translation factors (Mohr et al., 2002).

Nucleotides

Two important nucleotides are mainly needed in protein synthesis, ATP and GTP.

**ATP** is required in the activation of amino acid for the aminoacyl-tRNA charging (Carter, 1993). ATP is regenerated from ADP and PEP by the co-operative action of two enzymes: pyruvate kinase (PK) and myokinase (MK).

**GTP** is an important nucleotide used by EF-Tu, EF-G and IF2, which are multidomain GTPases with essential functions in the elongation and initiation phases of translation. They bind to the same site on the ribosome where their low intrinsic GTPase activities are strongly stimulated (Rodnina et al., 2000).

The elucidation of the GTPase mechanisms of several G proteins has been under much investigation. Several groups have demonstrated the structural determinants required for GTP hydrolysis and GTPase activation by GAP or RGS for both the Ras-like proteins and the alpha-subunits of the heterotrimeric G proteins (Coleman et al., 1994a; Coleman et al., 1994b; Scheffzek et al., 1997).

GTP hydrolysis functions to ‘switch off’ the interaction of the G protein with its effectors. Both the translation GTPases IF2 and EF-Tu have similar mechanism of action and use GTP hydrolysis to switch off their activities (Antoun et al., 2003).

The GTPase activity, which is very low for the free factors, is increased by orders of magnitude by binding to the ribosome (Rodnina et al., 2000). The ribosomal contacts that bring about GTPase activation have not been characterized, however there is biochemical evidence suggesting that the ribosomal protein L7/12 is involved in
GTPase activation. Both EF-G and EF-Tu interact with the L7/12 stalk of the 50S subunit, as suggested (Agrawal et al., 1998; Traut et al., 1995).
Moreover, some recent experiments showed that L7/12 as isolated protein strongly stimulates GTP hydrolysis by EF-G (Savelsbergh et al., 2000).

Enzymes

A group of enzymes called **aminoacyl-tRNA synthetases** (aaRS) are involved in the protein synthesis pathways. These enzymes bind their substrates - ATP, amino acids and tRNA - and stabilize the putative transition states in the aminoacylation reaction.

There are 20 known aaRS, which can be divided into two main classes 0 and H (Arnez and Moras, 1997; Carter, 1993). The faithful translation of genetic information depends on the correct matching of amino acids to their cognate tRNAs. Each of aminoacyl-tRNA synthetases is specific for one amino acid and one or more isoacceptor tRNA.

The reaction, called aminoacylation or charging of tRNAs, is carried out in two steps. In the first step, the amino acid is activated by forming aminoacyl-adenylate, and inorganic pyrophosphate while in the second step, the amino acid moiety is transferred to the 3'-terminal ribose of a cognate tRNA, yielding an aminoacyl-tRNA (Arnez and Moras, 1997).

**Special features of these enzymes include:**

1- They bind to tRNA via the inside of the L-shape
2- They have a separate amino acid binding site.
3- They recognize key features of tRNAs called identity elements and each enzyme can distinguish the correct tRNA from all the others via these elements.
4- Some synthetases can ‘proofread’ and reverse step 1 if they adenylate the wrong amino acid.
Process of Protein Synthesis

Initiation

Initiation of protein synthesis in *E.coli* involves several steps, which lead to the formation of the first peptide bond. After termination of protein synthesis, 70S is split by the combined activities of RRF, EF-G and IF3 into its subunits (Freistroffer et al., 1997; Janosi et al., 1998; Janosi et al., 1996). The 30S free from the previous step can bind mRNA and fMet-tRNA^Met^ (the first codon is usually AUG) to form 30S pre-initiation complex in the presence of initiation factors.

Initiation is the key regulatory step in translation. Three initiation factors are needed for this important process, which are IF1, IF2 and IF3. Roles of initiation factors in the formation of the 30S pre-initiation complex have been studied for decades (Boelens and Gualerzi, 2002).

**IF1** is an essential factor for the cell growth (Cumming and Hershey, 1994) but its function *in vitro* was not fully elucidated (Croitoru et al., 2004). IF1 is the smallest of the three bacterial translation initiation factors with a molecular mass of 8.2 kDa in *E. coli*. and is encoded by the *infA* gene (Laursen et al., 2005), whose transcription is not physically linked to any other genes as are *infB* and *infC* (the genes encoding IF2 and IF3) (Dahlquist and Puglisi, 2000).

The structure of IF1 in solution has been determined by NMR spectroscopy (Sette et al., 1997). IF1 is characterized by a five-stranded \( \beta \)-barrel. There is a loop connecting strands three and four that contains a short \( \beta \)-helix but this region shows considerably higher flexibility than the \( \beta \)-barrel. The fold of IF1 is very similar to that found in the bacterial cold shock proteins CspA and CspB and can be identified as the oligomer-binding motif. Several proteins of this family are nucleic acid-binding proteins.

Recently, the functions of IF1 in the dissociation of 70S and in the fidelity process were fully elucidated (Antoun et al., manuscript). IF1 increases the rate of naked 70S dissociation, which is the rate-limiting step for starting initiation of protein synthesis by a factor of 80. Another essential function of IF1 is to prevent formation of 70S contain-
ing elongator tRNA instead of the initiator tRNA (Antoun et al., manuscript).

**IF2** is a G-protein that stabilizes fMet-tRNA$^{\text{Met}}$ (initiator tRNA) binding to 30S subunit (Canonaco et al., 1986; Wintermeyer and Gualerzi, 1983) though some experimental evidence points to the role of IF2 as a fMet-tRNA$^{\text{Met}}$ carrier into this complex (Wu and RajBhandary, 1997).

The pivotal role of IF2 in 50S docking has only recently been seriously appreciated (Antoun et al., 2003; Antoun et al., 2004). Our results are in co-ordination with the results on eIF5B, which is the eukaryotic homologue of IF2 (Lee et al., 2002; Pestova et al., 2000). In these studies using GMPPNP, the importance of both GTP conformation and hydrolysis were confirmed. Our experiments were conducted using the combined biochemical techniques and light scattering (Grunberg-Manago et al., 1975) to determine the rate of 70S formation from 30S ribosomal complexes and 50S subunits. GTP conformation of IF2 allows the fast 70S formation and GTP hydrolysis renders the 70S complex competent in peptidyl transfer. This confirmed the structure and function homology between prokaryotic IF2 and eukaryotic eIF5B.

Since the data obtained in the eukaryotic system was at equilibrium level, the effect of replacing GTP by GDP on the rate of ribosome formation from its subunits was not determined. That is why the importance of the GTP conformation was not illustrated in Eukaryotes.

**IF3** has been attributed at least five different roles in the formation of 30S pre-initiation complex (Petrelli et al., 2001). The major roles of IF3 are:

1. Anti-association through preventing 50S binding to the 30S (Grunberg-Manago et al., 1975);
2. Acceleration of fMet-tRNA$^{\text{Met}}$ binding into the 30S pre-initiation complex (Canonaco et al., 1986);
3. Dissociation of pseudo-initiation complexes formed on 30S with elongator tRNAs as well as complexes formed with fMet-tRNA$^{\text{Met}}$ on codons different from AUG, GUG and UUG (Hartz et al., 1989).
This last role of IF3 in the fidelity of initiation at the level of 30S pre-initiation complex formation is by far the most intensively studied (Hartz et al., 1989). We showed that IF3 increases both the on and off rate of tRNA from the 30S pre-initiation complex (Antoun et al, Manuscript).

The timing and mechanism of IF3 removal from 30S subunit upon 70S initiation complex formation is still a matter of controversy. The current view that IF3 is actively ejected from 30S by incoming 50S subunit (Gualerzi, 1986; Gualerzi and Pon, 1990) contradicts the results of earlier experiments showing that fMet-tRNAfMet binding to the 30S-pre-initiation complex results in IF3 dissociation (Vermeer et al., 1973).

We found that IF2 and initiator tRNA destabilize the binding of IF3 to the 30S subunit which leads to its rapid release from 30S pre-initiation complexes. This release is a rate-limiting step in initiation of protein synthesis (Fig.1) and initiator tRNA binding acts like the signal for the association of 50S to the 30S pre-initiation complex to form 70S ready for the elongation step (Antoun et al, Manuscript).

![Fig.1: A Simplified scheme of initiation](image-url)
Elongation step

Translation requires coupled movement of mRNA and tRNA in relation with 70S. This process needs translation elongation factors (EF-Tu, EF-Ts and EF-G), which are the workhorses of protein synthesis on the ribosome (Andersen et al., 2003). They assist in elongating the nascent polypeptide chain by one amino acid at a time.

**EF-Tu** is activated upon GTP binding, and forms a ternary complex with aminoacylated elongator tRNAs (aa-tRNAs). This complex binds to the ribosome after IF2 release (Antoun et al., 2003). On the ribosome, this ternary complex decodes the genetic information by a Watson–Crick-type interaction between the mRNA codon, which is exposed in the ribosomal A-site, and the anticodon of a cognate tRNA (Andersen et al., 2003).

EF-Tu, the most abundant protein in *Escherichia coli*, is a guanine nucleotide-binding protein that in the 'on' state acts as a carrier of amino acyl-tRNA to the ribosome (Weijland et al., 1992). The decoding event triggers the ribosome to induce GTP hydrolysis, and EF-Tu bound to GDP is thus released from the ribosome. This release of EF-Tu leads to repositioning of the aminoacyl-tRNA to promote peptide bond formation.

The loaded aminoacyl-tRNA must have the correct anticodon to base pair with the mRNA codon that is positioned at the A-site. Accuracy during elongation occurs in two steps:

1) Ternary complex carrying the wrong aminoacyl-tRNA is expelled before GTP hydrolysis through a monitoring mechanism by the ribosome. This mechanism involves three important nucleotides A14982, A1493 and G530 (Ogle et al., 2001). These nucleotides help the ribosome to monitor the first two bases of the codon-anticodon helix to discriminate between Watson-Crick base pairing and mismatches (Ramakrishnan, 2002).
2) Proofreading involves release of the aminoacyl-tRNA prior to peptide bond formation (Rodnina and Wintermeyer, 2001).

Once the correct aa-tRNA has been accommodated in the ribosomal A-site, peptide bond formation is rapidly catalyzed by the large subunit. This results in a pre-translocational ribosome complex.

**EF-Ts** recycles back EF-Tu to the GTP form as EF-Ts is the GEF for EF-Tu (Karring et al., 2003). The stimulation of the guanine nucleotide release by EF-Ts is important as the dissociation of GDP otherwise is very slow ($2 \times 10^{-3}$ s$^{-1}$) (Gromadski et al., 2002).

Kirromycin is an antibiotic that inhibits protein synthesis by binding EF-Tu–GTP and preventing the release of EF-Tu–GDP from the ribosome (Parmeggiani and Swart, 1985). It binds at the interface between domain 1 (guanine nucleotide-binding domain, G-domain) and domain 3 of EF-Tu, and locks EF-Tu–GDP in the GTP-bound conformation after hydrolysis of the GTP at the ribosome (Vogeley et al., 2001). Kirromycin and EF-Ts are mutually exclusive in the binding to EF-Tu (Chinali et al., 1977).

**EF-G** is the elongation factor responsible for the translocation step. This process involves the translocation of peptidyl-tRNA from the A site to the P site. During the movement, the mRNA is carried along with the tRNA, and deacylated tRNA dissociates from the P site via the exit (E) site (Wintermeyer et al., 2004).

There are several mechanisms for translocation that have been proposed.

1) Classical model: Binding of EF-G induces the translocation of peptidyl tRNA from the A-site to the P-site. This step is followed by GTP hydrolysis to release EF-G.GDP from the ribosome (Inoue-Yokosawa et al., 1974).

2) Hybrid sites model: Upon peptide bond formation, CCA end of tRNA moves from P-site to E-site resulting in P/E site (Moazed and Noller, 1989). The same occurs for aminoacyl tRNA in the A-site leading to A/P site. This implies the presence of relative motion between subunits. That is to say, the tRNAs move with respect to the large subunit, but do not
move with respect to the small subunit (Moazed and Noller, 1989; Moazed and Noller, 1990).

3) Motor model: GTP hydrolysis occurs before translocation and accelerates the translocation process. EF-G acts as motor protein where the hydrolysis energy is used in translocation (Rodnina et al., 1997).

Cryo-EM data reveal that the A-, P- and E-site tRNAs do not move substantially upon peptide bond formation, arguing against a large-scale conformational change (Valle et al., 2003). Furthermore, it was found that, in catalytically active 50S subunits, there was no movement of an A-site substrate into the P-site following bond formation (Schmeing et al., 2002). These discrepancies can partly be settled by novel single-molecule fluorescence studies of ribosomes. They reveal that tRNAs in the pre-translocational complex and possibly also the post-translocational complex are rapidly exchanging between classical and hybrid states, and that precise experimental conditions might favor one state over the other (Blanchard et al., 2004).

The elongation cycle is repeated until the entire coding sequence of the mRNA is translated and a termination codon appears in the decoding site, whereupon translation is terminated.

Termination step

The final step in protein synthesis is the hydrolysis of the ester bond in peptidyl-tRNA and release of the finished protein (Zavialov et al., 2001).

This occurs when polypeptide release factor RF1 or RF2 binds to the ribosomal A site (Wilson et al., 2000) and activates hydrolysis of the nascent peptidyl-tRNA in prokaryotes (Polacek et al., 2003).

RF1 and RF2, the class I release factors (Scolnick et al., 1968), differ in their recognition specificity of termination codon, where RF1 recognizes the stop codons UAA and UAG and RF2 recognizes UAA and UGA (Kisselev and Buckingham, 2000). Several data demonstrated that release factors are structural mimics of aminoacyl-tRNA (Nakamura et al., 1996). Genetic and biochemical studies indicated
that a conserved Gly-Gly-Gln (GGQ) motif in class-1 release factors interacts with the peptidyl-transferase center on 50S ribosomal subunit leading to the release of protein (Kisselev et al., 2003; Zavialov et al., 2002).

RF3 in bacteria and eRF3 in higher organisms, class II release factors, are G proteins that are not required for peptide release (Freistroffer et al., 1997). Release factor RF3 then catalyses dissociation of RF1 or RF2 from the ribosomal A site (Freistroffer et al., 1997); leaving a post-termination complex (Freistroffer et al., 1997; Zavialov et al., 2001). It was shown recently that this post-termination complex acts as a GEF for the RF3 (Zavialov et al., 2001).

Recycling step

Ribosome recycling is a very fundamental, yet poorly understood, process in protein synthesis. The ribosome which has just released the completed peptide is situated on the mRNA with the termination codon at the A-site and deacylated tRNA on either P-site or E- (exit) site (Kaji et al., 1998).

In the presence of RRF, GTP and EF-G, the post-termination complex is dissociated to the ribosomal subunits with the release of the mRNA and deacylated tRNA (Freistroffer et al., 1997; Janosi et al., 1998; Janosi et al., 1996; Pavlov et al., 1997) in preparation for the next round of polypeptide synthesis.

The disassembly of the post-termination complex is the actual final (fourth) step of protein biosynthesis where RRF is an essential protein for Escherichia coli (Janosi et al., 1994). RRF is composed of two domains, a long three-helix bundle (domain 1) and a three-layer b/a/b sandwich (domain 2) which, combined, strikingly resemble the L-shaped structure of tRNA.

On the basis of this resemblance, it has been proposed that RRF is a structural and functional mimic of tRNA (Selmer et al., 1999). RRF occupies the A/P-site of ribosome (Hirokawa et al., 2002; Lancaster et al., 2002) and orientation of the ribosome-bound RRF (Agrawal et al., 2004) showed that the elbow of RRF is found in an overlapping position at the junction of domains III, IV and V of EF-G, whereas RRF
domain II occupies an overlapping position with domain IV in the GDP (guanosine diphosphate) state of EF-G (Agrawal et al., 2004).

According to this proposal, RRF may bind to the empty A site of the post-translation terminating ribosome in which the P site is already occupied by a deacylated tRNA. Then, EF-G translocates RRF and deacylated tRNA from the A site to the P site and from the P site to the E-site, respectively, in a GTP-dependent manner. These events would be followed by rapid dissociation of EF-G and RRF.

However, the orientation of RRF bound in the ribosome, determined by directed hydroxyl radical probing, is in the ribosomal subunit interface cavity, and this location and orientation differ markedly from any of those observed previously for tRNA (Lancaster et al., 2002).

How RRF interacts with EF-G and disassembles the post-termination ribosome complex is not yet fully understood so we decided to dissect the different steps of recycling.

In our simplified scheme, we showed that RRF binds first to the ribosome followed by EF-G. GTP hydrolysis is stimulated by the ribosome that acts like a GAP and this GTP hydrolysis is extremely fast followed by the dissociation of 70S to its subunits. IF3 binds to the 30S subunit to prevent the rebinding of 50S and now this 30S is ready to start a new cycle of initiation (Fig. 2).

Using Fusidic acid, we could analyze the different structure of EF-G that occurs during recycling. Fusidic acid is an antibiotic that allows both the GTP hydrolysis and the dissociation of 70S; however, it prevents GDP release from EF-G and recycling of the factor.
Fig. 2: Simplified Model for Protein Synthesis
Fusidic acid

Fusidic acid is a steroid-like antibiotic isolated from the fungus Fusidium Coccineum (Godtfredsen et al., 1962). It belongs to a family of naturally occurring antibiotics known as fusidanes having in common a tetracyclic ring system with the unique chair±boat±chair conformation, a carboxylic acid-bearing side chain linked to the ring system at C17 via a double bond and an acetate group at C16 (Fig. 3). The fusidanes show a high degree of antibacterial activity and have a similar spectrum. Fusidic acid is commonly used against Gram-positive bacterial infections especially staphylococci.

The mechanism of action of this antibiotic is through inhibition of protein synthesis by stalling EF-G on the ribosome after translocation (Hansson et al., 2005; Stark et al., 2000). It binds with high affinity to EF-G on the ribosome after GTP hydrolysis and therefore prevents the release of EF-G•GDP from the ribosome (Bodley et al., 1969).

EF-G is an important GTPase that is involved in two important steps in protein synthesis, the elongation and recycling. EF-G enhances the translocation of tRNA from the A-site to P-site, leaving the A-site
empty for another ternary complex (EF-Tu.tRNA.GTP) to bind (Nilsson and Nissen, 2005; Wintermeyer et al., 2001). EF-G also acts synergistically with RRF during recycling steps (Pavlov et al., 1997).

So the inhibitory activity of Fusidic acid on cell growth is probably through acting on these two steps of protein synthesis.

A significant number of the mutations conferring strong Fusidic acid resistance have been mapped at the interfaces between domains G, III and V of EF-G. These mutants also have higher and lower affinity for GTP respectively than wild-type EF-G (Hansson et al., 2005).

Accuracy

Two important characters for the translation of protein synthesis are the rate and accuracy of this process. The genetic information is transferred with an error rate $10^8$-$10^{10}$ and the infidelity of *E.coli* RNA Polymerase is $10^{-5}$ (Blank et al., 1986; Rosenberger and Foskett, 1981).

For the ribosome to synthesize functional proteins, it has to decode the mRNA in short time and with low error frequency. One major type of translation errors is **missense errors** through incorporation of an amino acid different from the encoded one. It is estimated that the missense error frequency is $10^{-4}$ (Kurland and Gallant, 1996).

The translation of the genetic code on ribosomes has to select one substrate “the cognate” and avoid processing a set of similar non-cognate substrates. The cognate and non-cognate are chemically very similar.

The accuracy process is divided to two steps: initial selection and proof-reading.

**Initial selection:** Pauling (Freistroffer et al., 2000; Pauling, 1957) was among the first to reflect on the errors that must occur during protein synthesis because of the limited maximum differences in interaction standard free energies between different pairs of amino acids, and he concluded that amino acid substitutions theoretically should occur
at frequencies in the percent range. Though later shown to be overly pessimistic (Fersht, 1981), these predictions led to theoretical studies showing that enzymatic selection can, under certain conditions, be more accurate than the intrinsic selectivity of a single step (Freistroffer et al., 2000).

The accuracy (A) of simple enzyme selection schemes, defined as the probability of correct product formation divided by the probability of an error at equal concentrations of cognate and non-cognate substrates (Freistroffer et al., 2000), is limited by the standard free energy difference between the transition state leading to product formation and ground state for cognate (ΔG‡) and non-cognate (ΔG°) substrates by the inequality

\[ A = \frac{k_w}{k_{cub}} / k_{cub} / k_{M} \leq e^{-(\Delta G^{\text{ext}} - \Delta G^*)/RT} = e^{\Delta G/RT} \]

The precision of codon translation depends on differences in H-bond energies between cognate and non-cognate tRNA-codon interactions, though other interactions like steric repulsion may also be important.

**Kinetic Proof-reading:** Biochemical experiments show that the precision of codon translation is achieved by kinetic proofreading (Ruusala et al., 1982; Thompson et al., 1980), where a moderate ΔΔAG value is used twice or more in successive selection steps (Ehrenberg and Blomberg, 1980; Hopfield, 1974). To satisfy the rules of thermodynamics, such reactions must be accompanied by extra dissipation of free energy, provided during translation by the hydrolysis of GTP on EF-Tu (Ruusala et al., 1982; Thompson et al., 1980).

Another example that uses proofreading is the aminoacyl-tRNA synthetases that recognize both tRNA and amino acid. Cognate tRNAs have a greater intrinsic affinity for the binding site, so they are bound more rapidly and dissociate more slowly. Following binding, the enzyme scrutinizes the tRNA that has been bound.

If the correct tRNA is present, binding is stabilized by a conformational change in the enzyme. This allows aminoacylation to occur rapidly. If the wrong tRNA is present, the conformational change does not occur. As a result, the reaction proceeds much more slowly; this
increases the chance that the tRNA will dissociate from the enzyme before it is charged. This type of control is called kinetic proofreading.

There are two stages at which proofreading of an incorrect aminoacyl-adenylate may occur during formation of aminoacyl-tRNA.

1- The non-cognate aminoacyl-adenylate may be hydrolyzed when the cognate tRNA binds. This mechanism is used predominantly by several synthetases, including those for methionine, isoleucine, and valine.

2- Some synthetases use chemical proofreading at a later stage. The wrong amino acid that is actually transferred to tRNA, is then recognized as incorrect by its structure in the tRNA binding site, and so is hydrolyzed and released. The process requires a continual cycle of linkage and hydrolysis until the correct amino acid is transferred to the tRNA.

**Light Scattering**

Static light scattering is a classical method for the determination of molecular masses and can be used to obtain information on particle shape and interactions, and even on superstructures. The molecular weight of *Escherichia coli* ribosomes has been determined by analysis of scattered light (Igarashi et al., 1973).

By using a novel technique of combining light scattering with stopped-flow, we could monitor both the association of ribosomal subunits and the dissociation of 70S after their rapid mixing in an SX-18MV stopped flow instrument (Bio-sequential SX-18MV, Applied Photophysics, Leatherhead, UK) equipped with Xenon arc light source (Antoun et al., 2003; Antoun et al., 2004; Hennelly et al., 2005).

Special precautions are needed to remove dust particles and avoid air bubbles. The reaction mixtures were spun for 3 min at 14000 rpm before they were loaded into the syringes of the stopped-flow instrument and pre-incubated at the specified times (Antoun et al., 2003; Antoun et al., 2004).

After rapid mixing, light scattering at 436 nm at a right angle to the illuminating light was recorded as a function of time.
Kinetics of macromolecular complex formation analyzed by stopped-flow and light scattering

In a typical light scattering experiment to monitor a binary complex formation \( C \) between particles of type \( A \) and \( B \), a solution containing particles \( A \) is rapidly mixed with a solution containing particles \( B \) and the intensity of light scattered perpendicular to the beam of illuminating light is recorded as a function of time.

Initially, the mixture contains particles \( A \) and \( B \) at concentrations denoted \( a(0) \) and \( b(0) \), respectively, while the concentration, \( c(0) \), of complex \( C \) is zero. The scattering intensity, \( I(t) \), at a time \( t \) after the mixing is the sum of the scattering intensities from free \( A \)-particles, free \( B \)-particles and \( C \)-complexes:

\[
I(t) = a(t) \cdot I_A + b(t) \cdot I_B + c(t) \cdot I_C \quad [1]
\]

\( a(t) \) and \( b(t) \) here are the concentrations of free particles \( A \) and \( B \), \( c(t) \) is the concentration of complexes, \( C \). \( I_A, I_B \) and \( I_C \) are the scattering intensities per unit concentration for the corresponding particles and complexes.

Since, for every \( C \) complex that is formed, one particle \( A \) and one particle \( B \) are consumed, \( a(t) \) and \( b(t) \) are related to the initial concentrations \( a(0) \) and \( b(0) \) as: \( a(t) = a(0) - c(t) \) and \( b(t) = b(0) - c(t) \). Introducing these mass relations in expression [1] gives

\[
I(t) = I(0) + c(t) \cdot \Delta I_C, \quad [2]
\]

Where \( \Delta I_C \) is the increase in light scattering intensity when the two particles \( A \) and \( B \) form a complex \( C \).

For particles with dimensions much smaller than the wavelength of the illuminating light, the scattering intensity of a particle is proportional to the square of its molecular mass and does not depend on particle shape (Grunberg-Manago et al., 1975). Since the complex \( C \) between particles \( A \) and \( B \) is just a bigger particle, \( \Delta I_C \) can be estimated as

\[
\Delta I_C = I_C - I_A - I_B =
Z \cdot (M_A + M_B)^2 - Z \cdot M_A^2 - Z \cdot M_B^2 = 2 \cdot Z \cdot M_A \cdot M_B \quad [3]
\]
\( M_A \) and \( M_B \) are the molecular masses of the \( A \) and \( B \) particles, respectively, and \( Z \) is a proportionality coefficient for the particular experimental set up. Relation [2] shows that the increase, \( I(t)-I(0) \), in scattering intensity with time is directly proportional to the concentration \( c(t) \) of formed complexes. When complex formation has reached equilibrium, the plateau value, \( I_{eq} \), of the scattered intensity is given by

\[
I_{eq} = I(0) + c_{eq} \cdot \Delta I_c
\]  

Combining the experimentally measured parameters \( I(0) \), \( I(t) \) and \( I_{eq} \), and using the relations [2] and [4] one gets:

\[
\frac{I(t)-I_{eq}}{I(0)-I_{eq}} = \frac{I(0)+c(t) \cdot \Delta I_c - I(0) - c_{eq} \cdot \Delta I_c}{I(0) + c(0) \cdot \Delta I_c - I(0) - c_{eq} \cdot \Delta I_c} = \frac{c(t) - c_{eq}}{c(0) - c_{eq}} = f_c(t) \quad [5]
\]

The time evolution of the function \( f_c(t) \), which is one at time zero and zero at infinite time, contains all kinetic information about the complex formation. The ratio \( f_c(t) \) is the difference between the current and the equilibrium concentration of the complex \( C \), normalized to the value of this difference at time zero.

Notice that \( f_c(t) \) can be obtained from the experimentally measured intensities \( I(0) \), \( I(t) \) and \( I_{eq} \) without knowledge of the absolute value of \( \Delta I_c \). Therefore, kinetic experiments can be interpreted without knowledge of the coefficient \( Z \) in relation [3], which depends on the experimental set up. An extended and more detailed description of light scattering theory and its experimental applications can be found in (Antoun et al., 2004).

Kinetics of dissociation of 70S ribosomes in the presence of initiation factors

\[
70S \xrightarrow{k} 30S + 50S,
\]

With effective rate constants \( k \) and \( q \) that depend on the presence and concentrations of initiation factors. It can be shown that the slow rate of 70S formation for the kinetic scheme in conditions of excess of IF3 and 50S subunits over 30S subunits is given by the following approximate relation:

\[
k_{obs} \approx -q_2 \frac{k_1 \cdot [50S]}{k_1 \cdot [50S] + k_2 \cdot [IF3]}
\]
Where $k_1$ and $k_3$ are genuine second order association rate constants for 50S and IF3, respectively. Therefore, a plot of $1/k_{\text{obs}}$ versus IF3 concentration should give a straight line because:

$$\frac{1}{k_{\text{obs}}} \approx \frac{1}{q_3} \left( \frac{k_1 \cdot [50S] + k_3 \cdot [IF3]}{k_1 \cdot [50S]} \right) = \frac{1}{q_3} + \frac{1}{k_1 \cdot [50S] \cdot K_3} [IF3]$$

**Fig. 4:** Example of 70S dissociation determined by light scattering technique coupled with stopped-flow.
AIMS

Our aim of this study was to determine the effects of Initiation Factors on the rate and accuracy of initiation of protein synthesis. We studied each factor by itself and also different combinations to elucidate the interplay of factors that leads to accurate and efficient initiation.

We tried to focus on the following questions:

1. What is the role of GTP in initiation of protein synthesis?
2. What are the functions of IF1 and why it is essential for the cell?
3. How 70S formation occurs and what are the factors affecting this rate?
4. What is the rate-limiting step in initiation of protein synthesis?
5. How the initiation factors assist in obtaining fast and accurate initiation?
6. How RRF and EF-G work synergistically to dissociate post-termination complexes?
7. How the initiation process recognize the initiator tRNA (fMet-tRNA\(^{1\text{Met}}\))? 

Using the results and answers of these important questions, we could obtain a comprehensive model of initiation of protein synthesis.
RESULTS AND DISCUSSION

Importance of GTP conformation of IF2 and GTP hydrolysis (Paper I, II)

To study the roles of IF2 and GTP in initiation of protein synthesis, we used an in vitro system for mRNA translation with purified components from *E. coli* (Pavlov et al., 1997; Zavialov et al., 2001). We decided to study the effect of IF2 in the formation of 30S pre-initiation complex and the subsequent step of 50S docking to 30S to allow formation of competent 70S.

IF2 is a GTPase protein like EF-Tu (Rodnina et al., 2000) and Ras (Friedman and Devary, 2005). The importance of GTP was under question by work that claimed that GTP has no function in protein synthesis and it can be replaced by GDP without affecting this important process (Tomsic et al., 2000).

We started by determining the effect of G- nucleotide on the extent of fMet-tRNA$^{f\text{Met}}$ binding to 30S. As shown in the figure below, the extent of fMet-tRNA$^{f\text{Met}}$ bound to 30S pre-equipped with a strong SD mRNA, IF1, IF2 and IF3 depends on the amount of IF2 present and the type of G-nucleotide (Fig. 5). GTP allows the optimum binding of fMet-tRNA$^{f\text{Met}}$ to 30S pre-initiation complexes. GMPPNP (the non hydrolysable analogue of GTP) gives similar extent of fMet-tRNA$^{f\text{Met}}$ binding while in presence of GDP, the extent of fMet-tRNA$^{f\text{Met}}$ bound to 30S is 70% the optimum amount.
Fig. 5: Extent of fMet-tRNA$^{\text{Met}}$ binding to 30S pre-initiation complexes in presence of different combinations of IF2 and G-nucleotide

Next, we decided to study the mutual effect of IF2 and GTP on their binding properties and how fMet-tRNA$^{\text{Met}}$ and/or 30S subunits, supplemented with mRNA, IF1 and IF3, affected the binding of either GDP or GTP to IF2. As shown in Fig. 1, paper I, fMet-tRNA$^{\text{Met}}$ stabilizes the binding of GTP to IF2 in the 30S pre-initiation complexes. The binding of GDP to IF2 was independent of the presence of initiator tRNA and/or 30S subunits. The binding of GTP to IF2 (Fig. 1b) was weak in the absence of fMet-tRNA$^{\text{Met}}$ or 30S subunits and strong in the presence of both fMet-tRNA$^{\text{Met}}$ and 30S subunits ($K_d=2\mu M$) (Antoun et al., 2003).

After formation of the pre-initiation 30S complex, the next step in initiation of eubacterial protein synthesis is association of the ribosomal subunits and formation of the 70S ribosome (Blumberg et al., 1979; Grunberg-Manago et al., 1975). We decided to measure the effect of IF2 and the G-nucleotide on the rate of formation of 70S ribosomes from ribosomal subunits. To determine the rate of 70S formation, we used a novel method that was improved in our group, which is light scattering. This is a direct way to assess 70S formation from subunits (Grunberg-Manago et al., 1975), since the scattered intensity from each type of macromolecular complex is proportional to its concentration and to the square of its molecular weight.
Upon replacing GDP with GTP, subunit association was increased 20 fold. However, the rate in presence of GMPPNP was comparable to GTP case. To further support the results of light scattering, a dipeptide assay was conducted.

Once 70S ribosomes have been formed, IF2 must dissociate to allow for binding of a ternary complex. Peptide bond formation, which is very fast on post-initiation ribosomes (Tomsic et al., 2000), was used to monitor the rate of initiation of protein synthesis in the presence of GTP, GDP or GMPPNP. Fig. 3 in paper I shows that peptide bonds were made much more rapidly in the presence of GTP than GDP as expected from light scattering data. We have shown already that 70S formation proceeds the same with GMPPNP as with GTP but the rate of peptide bond formation was almost zero in the presence of GMPPNP and very fast in the presence of GTP (Fig. 3b). This indicates that IF2 was able to promote rapid subunit association in the presence of GMPPNP, but remained bound to the 70S ribosome, thereby blocking subsequent association of ternary complex and peptide bond formation (Antoun et al., 2003).

We showed further that IF2•GMPPNP dissociation rate from 70S is rate limiting for the subsequent dipeptide formation (Fig. 4, paper I). Since different biochemical groups use different conditions and buffers in their in vitro system. We decided therefore to check the effect of different temperatures and buffers by using 2 different conditions Polymix at 37°C or TMK at 20°C. We discovered that the buffer composition and temperature affects GTP to GDP exchange on IF2 in complex with 30S.

We used these results to examine whether these different experimental conditions could explain the discrepancy between our results showing the importance of GTP in initiation of protein synthesis and the claims in (Tomsic et al., 2000). From the data in Fig. 5, it can be demonstrated that the disappearance of GTP importance in their results is due to the presence of small amounts of GTP. We also found that lowering the temperature from 37°C to 20°C and increasing the Mg²⁺ concentration from 5 to 7 mM increased the relative affinity of GTP to IF2 even further and dramatically slowed down the exchange of GTP for GDP. This elucidates the importance of purifying all components including G-nucleotides before using in experimental design. We have
developed in our laboratory a method to purify and analyze different G-nucleotides.

The main important conclusions concerning the GTP importance in initiation of protein synthesis are as following:

1- $f$Met-tRNA$_{f}$Met enhances the affinity of GTP to IF2 on the 30S subunit.

2- IF2 in complex with either GTP or the non-cleavable analogue GMPPNP promotes fast association of the ribosomal subunits, while their association in the presence of GDP is slow.

3- GTP hydrolysis is essential for rapid removal of IF2 from initiated 70S ribosomes.

We further studied the effect of Thiostrepton on IF2. Thiostrepton is an antibiotic that acts on EF-G. Some results claim that Thiostrepton binding to the ribosome does not interfere with factor binding or with single-round GTP hydrolysis but inhibits the function of EF-G in subsequent steps, including release of inorganic phosphate from EF-G after GTP hydrolysis, tRNA translocation, and the dissociation of the factor from the ribosome (Rodnina et al., 1999).

We found that adding Thiostrepton inhibited the rate of 70S formation, this inhibition is not similar to that in presence of GDP as shown in Fig. 6. In addition, Thiostrepton seems to be without significant effects on IF2 recycling.

Fig. 6: The association of 50S to 30S pre-initiation in presence of IF2·GTP (■), IF2·GDP (▲) or IF2·GTP with Thiostrepton (●).
Characterization of the GTP Binding Properties of Initiation Factor 2 (Paper VI)

The importance of GTP for initiation has recently become a matter of great controversy because of two conflicting reports about the role of GTP in initiation.

A major difference between the two sets of rather similar experiments conducted in the two above mentioned studies were the temperature and the buffer composition of the reaction mixture. In the first study a simple TMK buffer system was used and experiments were conducted at 20°C while in the second study the experiments were conducted in Polymix buffer (PM) at 37°C.

Another more subtle difference between experimental set ups used in those two studies was that a commercial GDP was used in the first, while an additionally purified GMP and GTP free GDP (Zavialov et al., 2001) was used in experiments conducted in the second study. In this communication, we demonstrated that GTP contaminations present in commercial preparations of GDP are the major source of discrepancy between the reported results.

When bound to the 30S ribosomal subunit in the presence of fMet-tRNA^fMet, the affinity of IF2 to GTP increases dramatically (Antoun et al., 2003). We measured the ratio between equilibrium dissociation constants of GTP and GDP from IF2 at different experimental conditions. We obtained 5 times difference in affinities between GTP and GDP in PM buffer at 37°C and 30 fold differences in TMK buffer at 20°C. One can easily calculate that in TMK at 20°C with only 3% GTP contamination in GDP, the prepared 30S pre-initiation complex would contain around 50% IF2 in GTP form and 50% of IF2 in GDP form. On the other hand, in PM buffer at 37°C with the same contaminated GDP, less than 10% of 30S pre-initiation complexes would contain IF2 in GTP form.

We confirmed these results with light scattering association experiments conducted at the two different temperatures in PM and TMK buffers.
With pure GDP, the rate of association of 50S subunits with 30S initiation complexes is very slow in all experimental conditions. Adding GTP increases the rate dramatically in all buffer conditions. As expected, the addition of 2% GTP to purified GDP results in a noticeable increase in the rate of subunit association, this effect is dependant on the temperature and buffer composition. At 20°C in TMK buffer, the 70S complex formation had two distinct phases, a fast one with a rate corresponding to the rate of formation of 70S complexes with GTP and a much slower phase.

A close similarity between results obtained by light scattering (Fig.2) and dipeptide formation experiments (Fig.3) was obtained and indicated that docking of 50S to the 30S pre-initiation complex is the rate limiting step in the sequence of events that leads to dipeptide formation.

**IF1 an essential factor both *in vivo* and *in-vitro* (Paper VII)**

IF1 is an essential factor in *E. coli in vivo* (Cummings and Hershey, 1994) while its functions *in vitro* remained obscure (Croitoru et al., 2004). It has been reported that IF1 slightly accelerates dissociation of empty 70S ribosomes into subunits (Grunberg-Manago et al., 1975) as well as formation of the 30S pre-initiation complex at low temperature (Pon and Gualerzi, 1984). Neither of these effects appear to be significant enough to account for the essential nature of IF1. That is why; we tried to understand the essential functions of IF1 *in vitro*.

Using Stopped-flow experiments with light scattering, we could show for the first time the importance of IF1 *in vitro*. IF1 has two important functions:

**First**, it increases the dissociation rate of 70S ribosomes by a factor of 80 (Fig.1, IF1 paper).

**Second**, it acts as a fidelity factor by strongly inhibiting formation of 70S initiation complexes containing deacylated elongator tRNA, aminoaacylated elongator tRNA or Met-tRNA_{Met}. Rapid subunit association in the presence of IF1 strictly requires fMet-tRNA_{Met}. 

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Another less important function of IF1 is the enhancement of fMet-tRNA^{fMet} binding rate to 30S subunits, thereby speeding up formation of translation competent 70S initiation complexes and reducing ribosome sequestration.

Addition of IF1 increases the dissociation rate constant for the splitting of 70S ribosomes to subunits from about 0.002 s\(^{-1}\) to 0.15 s\(^{-1}\) at saturating IF1 concentration and increases the extent of 70S dissociation. When the concentration of IF3 was varied between 1 and 5 µM at a fixed concentration of IF1, there was no change in the IF1 dependent dissociation kinetics, which is in clear contrast to the 70S dissociation by RRF and EF-G, which is inhibited by increasing IF3 concentration (Antoun et al., manuscript).

We showed also that free IF2 and fMet-tRNA^{fMet} bind separately to the 30S subunit, which is in agreement with the results of (Boelens and Gualerzi, 2002) since adding fMet-tRNA^{fMet} prior to IF2 reduces the total rate of initiation of protein synthesis.

**Interplay between initiation factors and fMet-tRNA^{fMet} in initiation of eubacterial protein synthesis (Paper IV)**

The roles of the initiation factors and fMet-tRNA^{fMet} in the formation of 70S initiation complex from 50S subunits and 30S pre-initiation complexes was studied using our purified components in combination with the stopped flow light scattering technique.

We show that there is interplay between IF2, IF3 and fMet-tRNA^{fMet} that determines the rate and fidelity of formation of 70S initiation complexes, which are competent in translation.

IF3 prevents non-competent 70S formation. The binding of fMet-tRNA^{fMet} into the 30S pre-initiation complex acts like a signal to allow IF3 dissociation and 50S docking to 30S pre-initiation complex. We showed that 50S binds to IF3 free 30S pre-initiation complex, which is in fast equilibrium with IF3.
IF2 increases the rate of 70S initiation complex formation by a factor of 1000 and makes it compatible with the rate of initiation in vivo.

We discovered several important conclusions concerning the interplay of initiation factors:

First, initiation factors accelerate the rate of fMet-tRNA^{Met} binding to the 30S subunit. The association rate constants for fMet-tRNA^{Met} binding to the 30S•mRNA complex and to IF1•30S•RNA complex increases from 0.03 μM⁻¹s⁻¹ to 12.5 μM⁻¹s⁻¹ when all factors are added. This corresponds to a 400 fold increase in the rate of fMet-tRNA^{Met} binding to the 30S•mRNA complex.

In absence of all initiation factors or with IF1 presence on 30S, the calculated equilibrium dissociation constants (K_d) for fMet-tRNA^{Met} are surprisingly low around 4 nM. Adding IF3 alone or in combination with IF1 greatly destabilizes fMet-tRNA^{Met} binding to the 30S while the addition of IF2 stabilizes fMet-tRNA^{Met} on the 30S subunit. Remarkably, IF2 stabilization compensates almost exactly the IF3 destabilization effects in the presence or absence of IF1. This compensation results in a similar K_d for fMet-tRNA^{Met} in the absence of all factors, in the presence of both IF2 and IF3 or in the presence of all three initiation factors.

Initiation factors have, however, a profound kinetic effect of fMet-tRNA^{Met} binding (Table I).

Second, binding of fMet-tRNA^{Met} to the 30S pre-initiation complex regulates the docking of 50S subunit.

The 50S docking to 30S•mRNA complex without fMet-tRNA^{Met} results in the formation of “empty” 70S ribosomes. We found that addition of IF3 or IF3 with IF2 to the 30S•mRNA results in a sharp decrease in the rate of 70S formation from subunits. Remarkably, the addition of IF3 together with IF1 or addition of all three initiation factors together to the 30S•mRNA complex in the absence of fMet-tRNA^{Met} completely blocks 70S ribosomes formation from subunits.

Addition of fMet-tRNA^{Met} together with IF2 results in drastic acceleration of 50S docking. With IF2 and fMet-tRNA^{Met} the subunit association is reasonably fast with IF3 and becomes very fast upon IF3 removal. This allows us to conclude that the binding of fMet-tRNA^{Met}
to the 30S•mRNA complex containing all initiation factors serves as a signal for 50S docking.

**Third**, IF3 has to dissociate to allow 50S docking. The association of naked 30S and 50S subunits follows the second rate association law (Antoun et al., 2004; Wishnia et al., 1975). However in the presence of all initiation factors, the rate does not follow the second order reaction law. Removing IF3 allows the docking reaction to follow the above-mentioned law and these data suggested that IF3 dissociation from 30S pre-initiation complex is the rate-limiting step in the 70S formation process (Antoun et al., manuscript).

This deviation from the second order binding kinetics can be explained by the following kinetic scheme.

\[
30S * f\text{Met} * IF2 + 50S \xrightarrow{k_1} 70S + IF3 \xleftarrow{q_3} 30S * f\text{Met} * IF2 * IF3
\]

This scheme implies that IF3 dissociation from the 30S subunit is required to allow the docking of the 50S subunit and that 50S does not actively displace IF3 from 30S. We suggest that fMet-tRNA^fMet binding to 30S produces two important effects:

(a) fMet-tRNA^fMet forces IF2 in the conformation that is compatible with fast 50S docking

(b) fMet-tRNA^fMet presence destabilizes the binding of IF3 to the 30S subunit by increasing the dissociation rate of IF3.

From previous results, we showed that fMet-tRNA^fMet binding to 30S pre-initiation complex is 400 fold less stable than its binding to 30S•mRNA. From detailed balance considerations (Fersht, 1997), such a destabilizing effect of IF3 on fMet-tRNA^fMet binding should be matched by an equal destabilizing effect of fMet-tRNA^fMet on IF3.

The apparent rate of subunit association is

\[
k_{\text{obs}} \approx -q_3 \cdot \frac{k_1 \cdot [50S]}{k_1 \cdot [50S] + k_3 \cdot [IF3]} \]
In this work, we presented a coherent picture of the interplay between initiation factors and fMet-tRNAfMet during later stages of initiation of protein synthesis in eubacteria.

1) fMet-tRNA^{fMet} and IF3 are two main switches operating in the last stage of the initiation process that regulate the formation of translation competent 70S complexes. IF3 presence on 30S is required to block the formation of non-productive 70S complexes that do not contain bound fMet-tRNA^{fMet}. In addition, IF3 presence accelerates fMet-tRNA^{fMet} binding to the 30S subunit. This binding, in turn, relieves IF3 block and allows a relatively fast docking of 50S subunits to the 30S pre-initiation complexes.

2) The presence of IF2 in its GTP form together with fMet-tRNA^{fMet} in 30S pre-initiation complex is pivotal for 50S docking since IF2•GTP accelerates the rate of docking 30-50 times in the absence of IF3 and even more in IF3 presence.

3) We show that 50S binding to 30S pre-initiation complexes containing IF3 deviates considerably from second order binding reaction and that this binding is inhibited by increased IF3 concentrations. These observations strongly support the model in which IF3 should leave the 30S subunit before 50S subunit can bind and that the tight binding of fMet-tRNA^{fMet} to the initiation codon increases the rate of IF3 departure from 30S.

The Interplay of Initiation Factors in the Rate and Accuracy of Eubacterial Protein Synthesis (Paper V)

In this part of the project, we emphasize the roles of initiation factors in the rate and accuracy of initiation of protein synthesis.

Initiation factors affect the accuracy of protein synthesis at two levels:

(I) at the level of 30S pre-initiation complexes formation, they affect the binding properties of different tRNA to 30S by favoring initiator tRNA over elongator tRNA. (II) at the level of 70S formation, they allow the fast association of 50S subunit to the 30S ribo-
somal complexes in case of the proper initiator tRNA (fMet-tRNA$^{fMet}$) compared to a very slow rate in the presence of non-initiator tRNA.

(I) Fidelity at the level of 30S pre-initiation complex

It requires both the fast binding of fMet-tRNA$^{fMet}$ to 30S binding and the fast dissociation of the elongator tRNA from 30S pre-initiation complexes. Therefore, we decided to compare the kinetics and thermodynamics of 30S pre-initiation complex formation in the presence of the initiator tRNA (fMet-tRNA$^{fMet}$), an elongator tRNA (Phe-tRNA$^{Phe}$) or non-formylated initiator tRNA (Met-tRNA$^{fMet}$). The kinetic and thermodynamic parameters, which we monitored, include:

1. $k_a$ (association rate constant of tRNA to 30S):
   - Without initiation factors; there is no significant difference between the $k_a$ of different tRNA, which is slow $\approx 0.031 \text{ M}^{-1} \text{s}^{-1}$ (Table I). Upon adding IF2, there was pronounced enhancement in $k_a$ of fMet-tRNA$^{fMet}$ to 30S to $0.335 \text{ M}^{-1} \text{s}^{-1}$ without any significant effect on the $k_a$ of elongator tRNA (Phe-tRNA$^{Phe}$) or $k_a$ of non-formylated initiator tRNA to 30S. The presence of all initiation factors in the 30S complex results in a dramatic increase in the $k_a$ of fMet-tRNA$^{fMet}$ by a factor of 400 from 0.031 $\text{ M}^{-1} \text{s}^{-1}$ without factors to 12.5 $\text{ M}^{-1} \text{s}^{-1}$ with all factors. For the elongator tRNA (Phe-tRNA$^{Phe}$) or non-formylated initiator tRNA, the presence of all factors has much less pronounced effects.

2. $k_d$ (dissociation rate constant of tRNA from 30S complexes):
   - Without initiation factors; there is no significant difference between the $k_d$ of different tRNA. Addition of IF3 resulted in a one to two hundred fold increase in the $k_d$ of different tRNA. Adding IF1 with IF3 has more a striking effect on $k_d$. In contrast to the effect of IF2 on $k_a$, addition of IF2 had only a marginal effect on $k_d$ in the case of Phe-tRNA$^{Phe}$ and small effect in the case of fMet-tRNA$^{fMet}$ (Table I).
   IF3 acts in a similar way on both cognate and non-cognate tRNA by increasing their rate of dissociation from 30S pre-
initiation complexes of by more than 100 fold, which is in contrast with previous claims concerning the effect of IF3 on the dissociation rate of fMet-tRNA^{fMet} and Phe-tRNA^{Phe}. This demonstrates that IF3 affects the accuracy through a kinetic mechanism by increasing the on and off rates of initiator and elongator tRNA from 30S pre-initiation complexes.

3. \( K_d \) (dissociation constant of tRNA):

The formylation of fMet-tRNA^{fMet} has a pronounced role for its high binding affinity to the 30S pre-initiation complexes in the presence of all initiation factors. The addition of factors destabilizes complexes containing non-formylated tRNAs. Remarkably, the addition of IF2 to fMet-tRNA^{fMet} containing complexes reverses the effect of IF1 and IF3 making the affinity of fMet-tRNA^{fMet} with all three initiation factors present much the same as in the absence of all factors.

### Table I

<table>
<thead>
<tr>
<th>IF1</th>
<th>IF2</th>
<th>IF3</th>
<th>( k_{a, f} )</th>
<th>( k_{d, f} )</th>
<th>( k_{a, t} )</th>
<th>( k_{d, t} )</th>
<th>( K_{d, f} )</th>
<th>( K_{d, t} )</th>
</tr>
</thead>
<tbody>
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<td>+</td>
<td>12.5</td>
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<td>2.7</td>
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<td>0.15</td>
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<tr>
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<td>-</td>
<td>+</td>
<td>0.24</td>
<td>0.11</td>
<td>460</td>
<td>0.22</td>
<td>0.16</td>
<td>730</td>
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<td>-</td>
<td>+</td>
<td>+</td>
<td>1.65</td>
<td>0.006</td>
<td>3.9</td>
<td>0.19</td>
<td>0.07</td>
<td>142</td>
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<tr>
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<td>-</td>
<td>+</td>
<td>0.18</td>
<td>0.015</td>
<td>86</td>
<td>0.24</td>
<td>0.05</td>
<td>104</td>
</tr>
<tr>
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<td>+</td>
<td>-</td>
<td>1.35</td>
<td>1.5 \times 10^{-4}</td>
<td>0.11</td>
<td>0.022</td>
<td>2.1 \times 10^{-4}</td>
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<td>-</td>
<td>-</td>
<td>0.035</td>
<td>3.5 \times 10^{-4}</td>
<td>10</td>
<td>0.024</td>
<td>3.5 \times 10^{-4}</td>
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<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>0.335</td>
<td>1.8 \times 10^{-4}</td>
<td>0.5</td>
<td>0.024</td>
<td>1.2 \times 10^{-4}</td>
<td>5</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.031</td>
<td>1.1 \times 10^{-4}</td>
<td>3.6</td>
<td>0.026</td>
<td>1.2 \times 10^{-4}</td>
<td>4.6</td>
</tr>
</tbody>
</table>

Association rate constant \( (k_a) \) are given in units \( \mu \text{M}^{-1} \text{s}^{-1} \); dissociation rate constants \( k_{d,f} \) are in \( \text{s}^{-1} \) and equilibrium dissociation constants \( K_{d,f} \) are in \( \text{nM} \). Rate constants and their standard deviation were calculated using non-linear regression program Origin 7 (OriginLab Corp.). Standard deviations were in most cases around 5% of their values and never exceeded 15%.

We discovered that a three nucleotide increase in the distance between the P-site codon and the SD sequence is responsible for the observed 10 times lower affinity of Phe-tRNA^{Phe} to the 30S•mRNA in the absence of initiation factors. Thus, 30S do not possess an intrinsically higher affinity to the initiator tRNA compared with that for the elongator Phe-tRNA^{Phe}. Moreover, the position effect observed for fMet-tRNA^{fMet} and Phe-tRNA^{Phe} in the absence of initiation factors is mainly due to increased \( k_d \) from non-optimally placed P-site codons.
These results indicate also that tRNA affinity to the 30S•mRNA complex is very sensitive to the mRNA nature.

(II) Fidelity at the level of 70S formation

We studied the effects of initiation factors on the rate of 70S formation from 30S pre-initiation complex and 50S subunits. In the presence of all factors, the rate of 70S formation differs by a factor of 700 between fMet-tRNA\textsuperscript{tMet} and elongator tRNA containing complexes (Table II, paper V).

The presence of IF3 on 30S especially together with IF1 considerably slows the rate of 50S docking in presence or absence of IF2. Without fMet-tRNA\textsuperscript{tMet} the formation of 70S is completely blocked in presence of IF1 and IF3. Addition of fMet-tRNA\textsuperscript{tMet} together with IF2 results in drastic acceleration of 50S docking to 9 µM\textsuperscript{-1} s\textsuperscript{-1} (Table II, paper V).

IF2 has several roles on the kinetic and thermodynamic parameters of fMet-tRNA\textsuperscript{tMet}: it increases the rate of its binding to 30S pre-initiation complexes and decreases fMet-tRNA\textsuperscript{tMet} dissociation rate from 30S complexes. Moreover, we showed that IF2 increases the rate of 70S formation from 30S complexes containing fMet-tRNA\textsuperscript{tMet} and 50S subunits. These collective effects make IF2 the major player in the accuracy and selection of proper initiator tRNA through recognition of the formyl group.
Effects of Initiation factors on the accuracy parameters

\[
\begin{align*}
30S \cdot \text{mRNA} \cdot \text{IFs} + \text{tRNA} & \overset{k_d}{\underset{k_c}{\longrightarrow}} 30S \cdot \text{mRNA} \cdot \text{IFs} \cdot \text{tRNA} + 50S \\
& \overset{k_c}{\underset{k_d}{\longrightarrow}} 70S \cdot \text{mRNA} \cdot \text{tRNA}
\end{align*}
\]

(1A)

The effective rate of different tRNAs incorporation in the 70S initiation complex in this model is described by a \(k_{\text{cat}}/K_m\)

\[
\frac{k_{\text{cat}}}{K_m} = \frac{k_c}{k_c + k_d}
\]

(2A)

The accuracy parameter (A) is defined as a ratio of \(k_{\text{cat}}/K_m\) for the cognate and non-cognate substrates. Surprisingly, we showed that omission of any one of three initiation factors results in a striking drop in accuracy of initiation. Omission of IF1 in initiation process leads to a more than a 100 fold drop in the accuracy of initiation even in presence of the 2 “main” initiation factors IF2 and IF3. Another surprising conclusion is that the efficiency of initiation parallels its accuracy. A high accuracy can be achieved either by decreasing the non-cognate \(k_{\text{cat}}/K_m\) or by increasing the cognate \(k_{\text{cat}}/K_m\).

Several important conclusions can be depicted

**First important conclusion** is that initiation factors have different mechanisms of action in favoring the binding of fMet-tRNA\(^{\text{fMet}}\). IF2 acts at the level of 30S pre-initiation complex through recognizing the formyl group of fMet-tRNA\(^{\text{fMet}}\). IF3 enhances both the rate of binding of tRNA to 30S pre-initiation complex and the rate of dissociation of tRNA from 30S pre-initiation complexes irrespective of the type of tRNA. In the step of 70S formation, IF3 decreases the rate of 70S formation differently according to the tRNA present.

**Second conclusion** that we obtained from our results is that there is important interplay between factors in increasing the accuracy of initiation of protein synthesis.
Third conclusion is that the position of the codon from the SD sequence influences the binding properties of tRNA to 30S pre-initiation complex.

RRF and EF-G: Essential Functions in 70S Ribosomes Dissociation in Eubacteria (Paper VIII)

Stopped flow experiments with light scattering show that RRF and EF-G can open efficiently naked 70S in the presence of low concentrations of IF3 but less efficiently upon increasing IF3 concentration. Addition of deacylated tRNA per se increases 70S dissociation rate. Post-termination complexes as well as naked 70S can be dissociated efficiently in the presence of GTP, substitution with GDP or the non-hydrolysable analogue GMPPNP, causes a severe decrease in the dissociation rate.

Both RRF and EF-G slightly increase the rate of 70S dissociation but they act synergistically on the rate and extent of dissociation of naked 70S ribosomes.

We showed an important interplay between IF3 and RRF with EF-G on dissociation of naked 70S ribosomes since increasing the concentration of IF3 from 1 µM to 5 µM (Fig. 4A, 4B) greatly decreases the rate of naked 70S dissociation from 0.13 s\(^{-1}\) to 0.013 s\(^{-1}\). The rate of IF1 induced ribosomal dissociation however does not depend on IF3 concentration and remains around 1 min\(^{-1}\) at high and low IF3 concentrations.

Splitting of Post-termination 70S ribosomes containing deacylated tRNA in P-site by RRF EF-G

The Post-termination ribosomal complexes that contain deacylated tRNA in the P-site can be opened only by RRF and EF-G. These results show that only naked ribosomes can be efficiently opened by IF1 and IF3 (Antoun et al., manuscript), while RRF together with EF-G
are required to open post-terminated ribosomes. Furthermore, we showed that pre-termination complexes are resistant to the IF1 or RRF and EF-G dissociation actions.

The order of binding of RRF and EF-G

By fast kinetics, we could prove that RRF binds before EF-G to 70S ribosomes to obtain the fastest rate of dissociation. There is a decrease in the rate of 70S dissociation upon the binding of EF-G prior to RRF to 70S.

The G nucleotide effects on the dissociation

Since EF-G is a GTPase protein, we studied the effect of type of G-nucleotide and the GTP hydrolysis on the rate and extent of dissociation of both 70S naked, deacylated tRNA containing complexes and post-termination complexes. The substitution of GTP by GDP or GMPPNP causes a huge decrease in rate of 70S dissociation in all the above-mentioned cases.

Fusidic acid and EF-G

During translocation, EF-G can hydrolyze GTP in presence of fusidic acid but fusidic acid prevents the release of EF-G from the ribosomal complex. Therefore, we studied the effect of the fusidic acid on the dissociation of both naked and post-termination complexes by RRF and EF-G. In recycling, Fusidic acid allows both the GTP hydrolysis and the dissociation of 70S but does not allow the recycling of EF-G from the ribosome. We found that EF-G•GDP•Fusidic acid•50S makes strong complex after GTP hydrolysis that releases GDP slowly.

**A Time-resolved Investigation of Ribosomal Subunit Association (Paper III)**

Using a novel, fast chemical-modification technique to provide time-resolved details of 16S rRNA structural changes that occur as
bridges are formed between the ribosomal subunits as they associate, we could determine the rRNA dynamics.

Our results suggest that key regions of 16 S rRNA, necessary for decoding and tRNA A-site binding, are structurally altered in a time-dependent manner by association with the 50 S ribosomal subunits.

The connections between the subunits serve a vital role, since, in addition to holding the ribosome together, they are likely conduits for signal transmission between the 30 S decoding subunit and the 50 S catalytic subunit (Hennelly et al., 2005).

We studied three different regions of 16 S rRNA in the 30 S ribosomal subunit that are involved in inter-subunit bridges: helix 23 (the 700 region), helix 27 (the 900 region), and helix 44 (the 1400 region). These regions represent 16 S nucleotides participating in four separate bridges (B2a, B2c, B5 and B7a) that form between the subunits. The 16 S rRNA was then extracted, sequenced using primer extension, and the resulting time-dependent reactivity of various bases monitored.

The results of this time-dependent study of ribosomal subunit association show changes in reactivity proceeding more rapidly at positions known to be involved in decoding as well as those involved in ribosome stability. Mainly, changes in helix 27 precede those in helix 44, with the exception of A1408, which changes at the same rate as some residues in H27. These results support a model of association in which the initial contacts include the bridges B2c and B2a of the 30 S subunit. Following this, protective interactions proceed rapidly in bridge B5 and an interaction in H44 with H27 (A1413). Finally, sites more removed from the decoding center, bridge B7a and a residue near the loop of H44, base A1441 become fully protected. Furthermore, the time-resolved reactivity of bases in H27 point to a conformational change within or including this helix.

The regions of 16 S rRNA containing bridges B2a and B2c undergo conformational rearrangement, in response to the 50 S subunit, characteristic of an induced fit between the two subunits.
The model of initiation from a system biology approach

We collected the data of the fMet-tRNA<sup>fMet</sup> binding properties to 30S pre-initiation complexes in presence of different combinations of initiation factors. We combined them with the results of 70S formation rate and the effect of factors on this important step.

Using this vast array of data obtained through different experiments, we made a mathematical model to describe the initiation process. To check the relevance and accuracy of this model, we conducted experiments in vitro in parallel and we obtained similar rates of protein synthesis in our experiment as the rates calculated by the mathematical model as shown in Fig. 7.

![Formation of dipeptides at different IF3 concentrations](image)

Fig.7: comparison of the rate of initiation from experimental design and the calculated using mathematical model
The model equations and parameters values

The mathematical model used the following parameters for the calculation of the protein synthesis rate:

1- The association rate constant of subunits ($k_s$)
2- 50S association rate to 30S+fMet ($k_i$)
3- 70S dissociation to 50S and 30S subunits ($q_s$)
4- fMet-tRNA$^{\text{fMet}}$ association ($k_4$) and dissociation rate constant ($q_4$) to/from 30S or 30S in presence of IF3
5- fMet-tRNA$^{\text{fMet}}$ association ($k_1$) and dissociation rate constant ($q_1$) to/from 30S or 30S in absence of IF3
6- IF3 association ($k_2$) and dissociation rate constants ($q_2$) to/from free 30S.
7- IF3 association ($k_3$) and dissociation rate constants ($q_3$) to/from free 30S or 30S with fMet-tRNA$^{\text{fMet}}$

The different 30S complexes as depicted in Fig. 8 are:
- 30S bound to 50S as 70S
- 30S free
- 30S with IF3
- 30S with both fMet-tRNA$^{\text{fMet}}$ and IF3
- 30S with fMet-tRNA$^{\text{fMet}}$
- 30S containing fMet-tRNA$^{\text{fMet}}$ bound to 50S as 70S

Fig. 8: A simplified scheme of initiation used in mathematical modeling
CONCLUDING REMARKS

The aim of this project was to clarify the roles of initiation factors IF1, IF2 and IF3 in initiation of translation in prokaryotes. We started with elucidating the role of IF2 and GTP in initiation of translation in *E.coli*. We have found that GTP conformation of IF2 is important for fast docking of 50S subunits to 30S pre-initiation complexes. We have also demonstrated that GTP hydrolysis on IF2 is necessary to render the assembled 70S initiation complex competent in the formation of the first peptide bond. These results establish a very close functional similarity between IF2 and its eukaryotic counterpart - the initiation factor eIF5B both at the subunit-joining step of the initiation complex formation and at the next step when GTP hydrolysis is used to remove IF2/eIF5B from the ribosome.

Next, we showed the function of IF1 *in vitro* and its interaction with IF2. IF1 is essential for cell viability but a clear function was not attributed to this factor previously. We studied how this factor affects both association of subunits and dissociation of 70S and how it helps the control mechanism for the proper formation of competent 70S complexes.

The regulation mechanism of IF3, and how the level of IF3 will affect both the rate and accuracy of translation was fully illustrated through our experiments. Putting these data together, we can depict the mechanism of initiation of protein synthesis and the order of steps. 70S is opened by RRF, EF-G and IF3 or with IF1 and IF3 then new mRNA binds to the 30S subunit. IF2 binds to 30S and recruits fMet-tRNA$_{Met}$. The binding of IF2 and fMet-tRNA$_{Met}$ increase the rate of IF3 dissociation, which is followed by 50S docking to 30S pre-initiation complex.

After 70S formation, GTP is hydrolyzed on IF2 and IF2 leaves the 70S as IF2•GDP. The 70S complex is now ready to accept the ternary complex (EF-Tu•charged elongator-tRNA•GTP).
Regleringen av proteinsyntesens initiering i Eubacteria och dess mekanistiska förklaring.

Proteinsyntesen är den process genom vilken polypeptidkedjor byggs utifrån informationen i mRNA-molekylerna. Denna process kräver flera viktiga komponenter såsom mRNA, tRNA och ribosomer, och delas in i tre faser: initiering, elongering och terminering. Bakterieribosomer är sammansatta av två delar; lilla (30S) delen och stora (50S) delen. Den lilla delen har som huvuduppgift att avkoda den genetiska informationen, medan den stora delen ansvarar för peptidelongering och frisläppande av proteinet. fMet-tRNA\textsuperscript{fMet} är det tRNA som är specifikt för initiering av proteinsynthesen och har flera specifika egenskaper som skiljer det från andra tRNA. fMet-tRNA\textsuperscript{fMet} binder direkt till P-sätet på ribosomen.

Initiering av proteinsyntesen i \textit{E. coli} sker i flera steg som leder fram till bildningen av den första peptidbindningen. 70S delas upp i sina delar genom de kombinerade aktiviteterna hos RRF, EF-G och IF3. I närvaro av initieringsfaktorer kan sedan den fria 30S-delen binda mRNA och fMet-tRNA\textsuperscript{fMet} och bilda ett 30S-preinitieringskomplex. För att ta reda på hur de olika initieringsfaktorerna samverkar till att ge en effektiv och korrekt initiering, har vi studerat de olika initieringsfaktorers betydelse och dessutom alla kombinationer av dem. Vi har fokuserat på att ge svar på följande frågor med hjälp av en ny teknik som kombinerad ljusspridning med stopped-flow (Stopped-flow light scattering).

1- Vad har GTP för roll i initiering av proteinsyntes?

IF2 i komplex med antingen GTP eller den oklyvbara analogen GMPPNP främjar en snabb association mellan de ribosomala delar, medan association i närvaro av GDP är långsamt. Hydrolys av GTP är nödvändig för att snabbt avlägsna IF2 från initierade 70S-ribosomer.
2- Vilka är IF1:s funktioner och varför är den nödvändig för cellen?

IF1 har två viktiga funktioner. Dels ökar IF1 dissociationshastigheten för 70S-ribosomer med en faktor 80 och dels fungerar den som en kontrollfaktor genom att kraftigt motverka bildandet av 70S-initieringskomplex utan initierings tRNA.

3- Vilka roller spelar IF3?

Den agerar anti-associations faktor, den accelererar bindningen av fMet-tRNAfMet till 30S pre-initieringskomplex, och den spelar en viktig roll för proteinsyntesens effektivitet då den ökar både association och dissociationshastigheten för tRNA till 30S pre-initieringskomplex.

4- Hur sker 70S formering, vilka faktorer påverkar dess hastighet och på vilka sätt bidrar initieringsfaktorerna till snabb och noggrann initiering?

Vi visar att det är ett samspel mellan IF2, IF3 och fMet-tRNAfMet som bestämmer hastigheten och noggrannheten i formeringen av translationskompetenta 70S initieringskomplex. Initieringsfaktorerna accelererar bindningshastigheten för fMet-tRNAfMet till 30S delen. IF3 förhindrar bildning av icke-kompetenta 70S ribosomer och bindningen av fMet-tRNAfMet till 30S pre-initieringskomplexet fungerar som en signal för att IF3 skall lämna komplext, vilket i sin tur tillåter dockning av 50S delen. Initieringsfaktor IF2 ökar hastigheten för bildning av 70S initieringskomplex med en faktor 1000, vilket gör hastigheten kompatibel med motsvarande in vivo värde.

5- Vilket är det hastighetsbegränsande steget för initiering av protein syntes?

Att IF3 skall lämna 30S kplexett är det step. Vi visar att 50S bara binder IF3 fria 30S.

6- Hur samverkar RRF och EF-G för att dissociera post-termineringskomplex?

Vid slutsteget för proteinsyntesen, släpps den nybildade polypeptiden fri från tRNA av ett speciellt protein ”release-factors”. Såväl post-termineringskomplexet som nakna 70S
kan effektivt delas upp i sina två delar av RRF/EF-G i närvaro av GTP men ej i närvaro av GDP eller GMPPNP. Vi visar att RRF binder först till 70S för att sedan följas av EF-G. De olika EF-G strukturen illustreras. Vi klargör mekanismen för hur antibiotikan fucidinsyra fungerar i återvinningssteget och visar på likheten med dess mekanism i translokation.

7- Hur känner initieringsprocessen igen initierings tRNA ($tRNA^{fMet}$)?

IF2 har den viktigaste funktionen för noggrannhet och val av korrekta initierings tRNA genom att den känner igen formylgruppen. IF3 påverkar noggrannheten genom en kinetisk mekanism genom att öka både associations- och dissociationshastigheterna för både initierings- och elongerings-tRNA till 30S pre-initieringskomplexet. Formeringen av 70S ribosomer från 30S pre-initieringskomplex är 700 gånger snabbare för initiatortRNA än för övriga tRNA I närvaro av alla tre initieringsfaktorerna.

Sammanfattningsvis har vi klargjort rollerna för initieringsfaktorerna IF1, IF2 och IF3 i prokaryot translationsinitiering. Genom att sätta samman dessa data har vi fått en detaljerad bild av initiering av proteinsyntesen, vilken är ämnet för denna avhandling.
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