CHAPTER 1: Introduction

1.1 Ribosome: protein biosynthesis machinery

The central dogma of molecular biology describes the gene expression pathway from a storage form (DNA) to a functional form (protein) through an RNA intermediate. The last step in this pathway, protein synthesis, is executed by a formidable cellular apparatus. In addition to mRNAs for every protein currently in production, each cell contains tRNAs for (almost) every amino acid it uses to make protein, aminoacyl-tRNA synthetases to charge those tRNAs, an array of facilitating protein factors, and finally ribosomes, the massive enzymes that catalyze mRNA-directed protein synthesis.

Functionally, ribosomes are polymerases. Like DNA polymerase and RNA polymerase, they catalyze the synthesis of biopolymers of a single chemical class, and the sequence of the specific member of that class a ribosome makes is determined by its interaction with a nucleic acid template. The substrates ribosomes consume are aminoacyl-tRNAs, their products are proteins, and their templates are mRNAs.

The ribosomes of all species are a 1:1 complex of two subunits of unequal size, the larger being about twice the molecular weight of the smaller. The molecular weight of the ribosome ranges from about 2.5 x10^6 in prokaryotes to about 4.5 x10^6 in higher eukaryotes, and the typical ribosome is roughly two-thirds RNA and one-third protein. The large subunit (50S) in *Escherichia coli* comprises the 23S (2,904 nt) and 5S rRNA (120 nt), and 33 ribosomal proteins (L1, L2,···, L for large subunit). The small subunit (30S) ribosomes is composed of the 16S rRNA (1,542 nt), and 21 ribosomal proteins (S1, S2,…,S for small subunit; Figure 1.1_1 (Wilson and Nierhaus, 2003)).

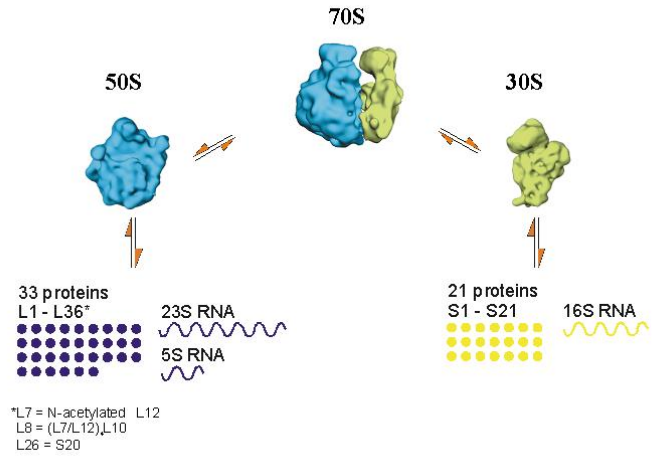

Figure 1.1_1 **Ribosome composition (*Escherichia coli*)**

Eukaryotic ribosomes contain more components and are significantly larger than prokaryotic ones, however eukaryotic ribosomes resemble in both architecture and function the prokaryotic homologues (Dube et al., 1998; Ramakrishnan and Moore, 2001).

Ribosomes are major components of the cell. In *E. coli*, during rapid growth, ribosomes constitute approximately 50% of the total dry cell mass (Jinks et al., 1984; Jinks and Nomura, 1987).

The overall shape of the ribosome was determined by electron microscopy 30 years ago (Lake, 1976). At ~40 Å resolution, the large subunit is crudely a hemisphere about 250 Å in diameter with three projections protruding radially from the edge of its flat face, a large one in the middle (the central protuberance), and two smaller ones at roughly 2 o'clock and 10 o'clock relative to the central protuberance. The central protuberance includes 5S rRNA and its associated proteins (Stöffler and Stöffler-Meilicke, 1984). The small subunit is about 250 Å in length and 150 Å in width in a bird like manner with head, beak, shoulder etc. (Figure 1.1_2).

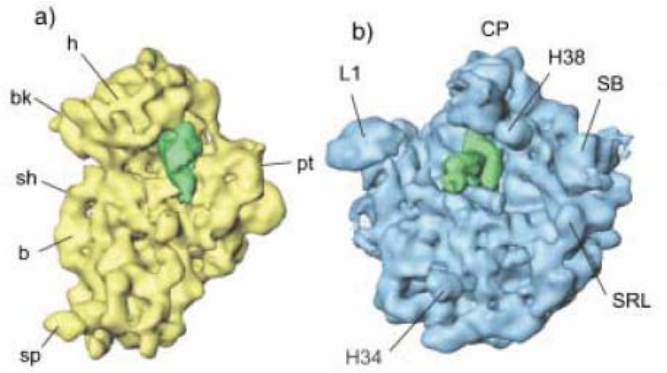

Figure 1.1_2 **The small (a) and large subunit (b) of the bacteria *Escherichia coli* Cryo-electron microscopic reconstructions (Spahn et al., 2001)**
(b, body; bk, beak; h, head; lf, left foot; rf, right foot; pt, platform; sh, shoulder; and sp, spur. Landmarks for the large subunit: CP, central protuberance; L1, L1 protuberance; SB, stalk base; St, L7/L12 stalk; H34, helix 34; H38, helix 38; and SRL, sarcin – ricin loop.)

Looking in more details to the ribosome-dependent protein biosynthesis: the ribosome travels along the mRNA reading the message and synthesizing a protein in a codon-specific manner. A tRNA molecule serves as adapter molecule for the decoding of the genetic information encoded in the mRNA. Initially, two binding sites for tRNAs were proposed for the ribosome (Lipmann, 1963; Watson, 1963; Watson, 1964). The

two sites of this model are the ‘’A’’ site (for aa-tRNA or acceptor site) and a ‘’P’’ site (for peptidyl-tRNA). However, functional studies at the beginning of 80’s (Grajevskaja et al., 1982; Lill et al., 1984; Rheinberger and Nierhaus, 1980; Rheinberger et al., 1981) have demonstrated a third tRNA binding site, the ‘’E’’ site (E for exit) from which deacylated tRNA leaves the ribosome. The E site could be confirmed by neutron scattering, cryo-electron microscopy and X-rays diffraction studies (Agrawal et al., 2000; Nierhaus et al., 1998; Wadzack et al., 1997; Yusupov et al., 2001). This third ribosomal binding site has been found on ribosomes of all kingdoms and seems to be a universal feature of ribosomes (for review see (Blaha and Nierhaus, 2001)). However disagreement exists on several points concerning the importance of the E site (for more details see (Burkhardt et al., 1998; Marquez et al., 2002). Important aspects seem to be the roles of the E site for the accuracy of decoding (Geigenmüller and Nierhaus, 1990) and for the maintenance of the reading frame during translation (Marquez et al., 2004).

Protein synthesis can be divided in three functional phases: (a) initiation, (b) elongation, and (c) termination (Figure 1.1_3).

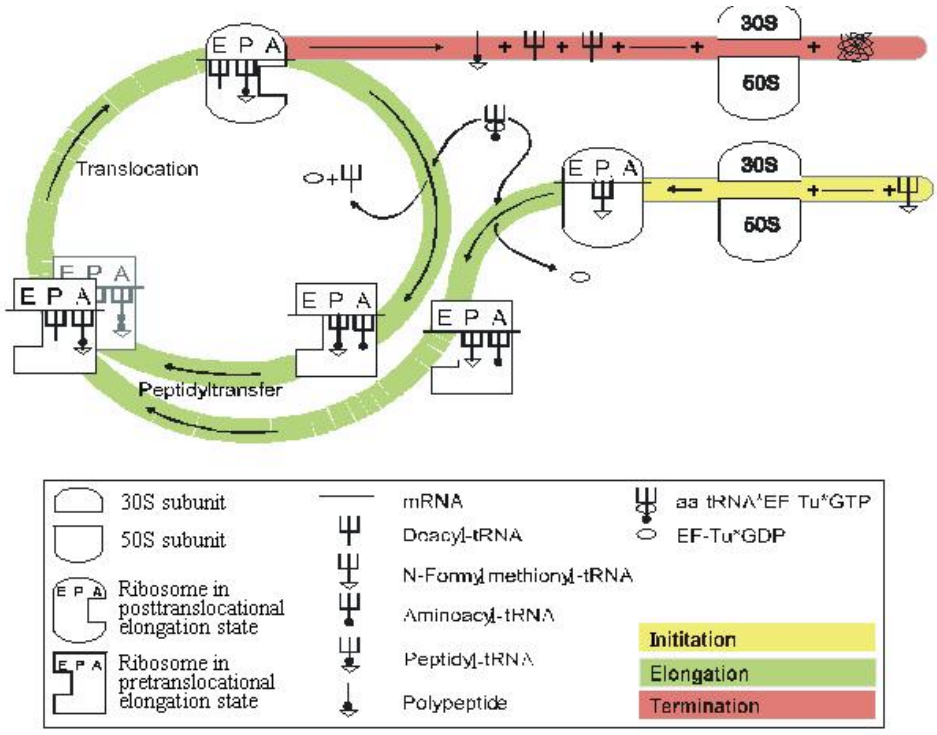

Figure 1.1_3 **The functional phases of the ribosome during protein synthesis**

1.1.1 Initiation

For initiation, 30S small subunit scans on the mRNA untranslated region (UTR) upstream of the start codon and recognizes the start codon with help of a stretch of nucleotides (the Shine – Dalgarno (SD) sequence) on the mRNA that interacts with a complementary sequence within the 3' end of the 16S rRNA (the anti-SD sequence). Then a number of specialist initiation factors have evolved to ensure the initiator fMet-tRNA$_f^{Met}$ binding on the P-Site of the 30S. In bacteria, this process is mediated by three initiation factors, IF1, IF2, and IF3 (reviewed by (Gualerzi et al., 2000)).

IF1 closely mimics the tRNA anticodon stem loop, and correspondingly it has been found to bind to the ribosomal A site (Carter et al., 2001; Dahlquist and Puglisi, 2000). On the other hand, IF3 has been found to interact with 30S ribosomes and interferes with the E site tRNA binding (Dallas and Noller, 2001). It has been proposed that IF1 and IF3 thus might improve the selection of the correct codon AUG exclusively to the P site by blocking the access to the adjacent sites A and E, respectively. IF2 is a G-protein analog to the elongation factor EF-Tu. It assists in fMet-tRNA$_f^{Met}$ binding to the ribosomal P-site (Lockwood et al., 1971) and is stimulated by IF1 (perhaps through direct interaction). Hydrolysis of the GTP is necessary for the translation initiation and triggers the release of IF2 from the ribosome after the association of the large ribosomal subunit (Luchin et al., 1999).

IF3 is a discriminator factor ensuring selection of correct tRNA for the initiation fMet-tRNA$_f^{Met}$, since it destabilizes non-cognate initiation complexes that form at non-canonical codons (Gualerzi and Pon, 1990; La Teana, 1996 ; Meinnel, 1999) . Furthermore IF3 acts in dissociation of 70S complex prior the correct initiation (Blumberg et al., 1979). It follows, that IF3 in addition to its "anti-association" functions fulfills another role during recycling of the ribosomes, namely it triggers the release of the deacylated tRNA from the P site after subunit dissociation (Karimi et al., 1999).

The initiation phase is completed, when the 50S ribosomal subunit associates with the 30S and the fMet-tRNA$_f^{Met}$ located in the ribosomal P site, forming the 70S initiation complex or Pi complex (Pi for initiation).

1.1.2 Elongation

1.1.2.1 General description

The ribosomal elongation cycle describes a series of reactions prolonging the nascent polypeptide chain by one amino acid and driven by two universal elongation factors termed EF-Tu and EF-G in bacteria. EF-Tu brings aa-tRNA to the ribosome in an aa-tRNA • EF-Tu • GTP ternary complex form, delivers the cognate aa-tRNA at the decoding center of the ribosomal A-site and – after EF-Tu dependent GTP cleavage - leaves the ribosome in the EF-Tu•GDP form. After aa-tRNA has been accommodated completely at the A site (the aminoacyl residue is now present at the peptidyltransferase center, PTF), the dipeptide bond will be catalyzed to form, namely the peptide will be transferred to the aminoacyl-tRNA so that the peptide is prolonged by one amino acid residue. Formally, the α-amino group of the aa-tRNA at the A site starts a nucleophilic attack to the carbonyl-residue forming an ester bridge with the P-tRNA. In the last years the molecular mechanism of this central ribosomal activity has been elucidated, and I quote three papers that mark the important progress concerning the molecular details of ribosomal peptide-formation (Erlacher et al., 2005; Schmeing et al., 2005; Trobro and Aqvist, 2005).

After peptide-bond formation EF-G•GTP enters, triggers the translocation of the two tRNAs from A- and P-site (Pre-translocational state or PRE state) to the P- and E-sites (Post-translocational state or POST state), respectively, the GTP is hydrolyzed and EF-G• GDP leaves ribosome. Now the A-site is free and a new cycle starts.

In cytoplasm, EF-Tu·GDP is regenerated to EF-Tu·GTP by the nucleotide exchange factor EF-Ts. Then EF-Tu·GTP will bind aa-tRNA obeying the "Thermodynamic Compensation" rule (LaRiviere et al., 2001) so that every kind of aa-tRNA has about the same affinity to EF-Tu·GTP.

The second factor EF-G does not have any nucleotide exchange factor like EF-Ts because it has similar affinity to GDP and GTP, but in cell level, the molar ratio of GDP to GTP is 1:10 so that EF-G·GDP will be automatically exchanged to GTP form. EF-G lowers the activation energy barrier of about 120 kJ/mol between the PRE and POST states of the ribosome (Schilling-Bartetzko et al., 1992_a), although in the absence of

EF-G ribosomes can perform translocation at ~1000-fold lower rates (Bergemann and Nierhaus, 1983; Gavrilova et al., 1976).

1.1.2.2 Models for the elongation cycle: tRNA translocation motif

Two models have been proposed to explain how the elongation cycle proceeds.

According to the "Hybrid-Site Model" (Moazed and Noller, 1989b) translocation moves those tRNA-parts that are bound to 50S subunits after peptide-bond formation, whereas the tRNA parts bound to 30S subunits follow only during the translocation reaction. This mechanism generates hybrid states for the two tRNAs at the PRE state after peptide bound formation and before translocation, for example P/E and A/P sites. A peptidyl-tRNA is thought to be at the A/P sites, if the tRNA is still at the A site of the 30S subunit, but has moved on the 50S subunits to the P site (Moazed and Noller, 1989b; Wilson and Noller, 1998). EF-G dependent translocation moves the states mentioned above to the E/E and P/P, respectively (Noller et al., 2002).

The second model, the α-ε model, is based on the tRNA contact patterns on the ribosome in combination of the observation that A and E-sites are allosterically coupled through negative cooperativity: when aa-tRNA binds to the A-site, the affinity of deacylated tRNA to the E-site drops and tRNA leaves the ribosome. In contrast, when the deacylated tRNA occupies the E site, the affinity of the A-site is low which enables the ribosome to select the correct ternary complex (Geigenmüller and Nierhaus, 1990). This model also incorporates the finding that deacylated tRNA at the E-site undergoes codon-anticodon interaction. However, following translocation thus forming the respective POST-complex, the contact patterns of the A-site tRNA before translocation and the P-site tRNA after translocation did not change. The interpretation was that micro-topography on the ribosome did not change during translocation. These results argue for a movable ribosomal domain that binds tightly the tRNAs at A and P sites and moves them to the P and E sites during the translocation reaction (Dabrowski et al., 1995; Dabrowski et al., 1998). This domain contains two tRNA binding regions that have been termed "α" for the tRNA contact pattern displayed at the A and P site before and after translocation, respectively; and "ε" for the tRNA contact pattern present at the P

site before translocation and at the E site after. It follows that at the A site only α can appear and at the E site only ε (Wilson et al., 2002).

1.1.3 Termination

The synthesis of the polypeptide chain continues until a stop codon (UAA, UAG or UGA) is invading the A site. Protein factors, called release factors (RF), will recognize the stop codon and will release the nascent polypeptide chain from the ribosomes and then recycle the ribosomes for the next initiation. Two classes of release factors are distinguished: Class I do not consume energy and are specific decoding factors that are responsible for the hydrolysis of the peptidyl-tRNA. RF1 and RF2 belong to this group, the factors recognize UAG and UGA respectively; both RF's overlap in the recognition of the termination codon UAA. Class I RF's promote hydrolysis of the ester bond between the polypeptide and the P site tRNA on the ribosome. RF1 and RF2 genes (*prfA* and *prfB*) have been shown to have a high similarity at the amino acid level (Caskey et al., 1984; Craigen et al., 1985; Weiss et al., 1984). In eukaryotes and in archaea only a single class I factor, eRF1 (and aRF1) have been identified that recognizes all three-stop codons (reviewed by (Wilson et al., 2002).

The Class II release factors are non-decoding and energy consuming factor. The RF3 belongs to this class. It stimulates the termination process in a GTP dependent manner.

RF3 in *E. coli* is not essential; knockout strains of its gene *prfC* gene are viable. The main function of RF3 is to support removal of the class I RF's from the ribosome using GTP hydrolysis, once the peptide hydrolysis has taken place (Freistroffer et al., 1997; Zavialov et al., 2001). In other words, the post-termination complex is the substrate for RF3 that stimulates the recycling of RF1 and RF2 (Freistroffer et al., 1997).

1.1.4 Recycling

After the oligopeptide cleavage of the P-site peptidyl-tRNA by the class I RFs has occurred, the ribosome is found in a post termination complex, i.e., with RF1 or RF2

located in the ribosomal A site, and one deacylated tRNA bound at the P site and probably another one at the E site. RF3 in a GDP form (Zavialov et al., 2001) binds to the post termination complex and nucleotide exchange on the ribosome promotes the dissociation of the decoding factors from the ribosome. Subsequent hydrolysis of the GTP on RF3 triggers its dissociation after it has accelerated the removal of the decoding RF's from ribosomes (Freistroffer et al., 1997).

Once the decoding factors and the RF3 have left the ribosome, the RRF (Ribosome Recycling Factor) mediates the ribosome recycling.

1.2 Translational errors and two tRNAs on the ribosome

During protein synthesis the ribosome produces errors. These errors have been classified as: (1) processivity errors; (2) missense errors and (3) loss of the correct reading frame (frameshifting). A processivity error is defined as the release (drop-off) of a prematurely short peptidyl-tRNA from the ribosome. Premature termination is also a cause of processivity error. Release of the peptidyl-tRNA from the ribosome could occur, if a sense codon is mis-recognized as a termination signal, although false stop constitutes a very low proportion of processivity errors (Jorgensen and Kurland, 1990). A missense error results from the incorporation of an incorrect aminoacyl residue into the nascent peptide. Generally this kind of mistake is not harmful, i.e., most amino acid substitutions do not eliminate protein function, since most often the cognate amino acid is misread by a chemically similar one due to the organization of the genetic code. In contrast, a shift in the reading frame results in an immediate loss of the genetic information usually leading to non-functional proteins.

As mentioned before, during the elongation cycle the ribosome oscillates between two major states: the PRE and the POST states. During the elongation cycle there are, at all times, two tRNAs bound on the ribosome, i.e. in P and A sites in the PRE-state and in E and P sites in the POST state.

According to the allosterical coupling, the negative allostery between the E and A sites reduce the chance for non-cognate tRNAs to bind to the ribosomal A site (Geigenmüller and Nierhaus, 1990; Nierhaus, 1990). An important signal for the ribosome to adopt a POST state is obviously codon-anticodon interaction at the

ribosomal E site, since a near-cognate tRNA at the E site does not reduce the error of aa-tRNA selection at the A site (Geigenmüller and Nierhaus, 1990). Additionally, crystal structure analyses have shown that the E-site tRNA makes extensive contact with the small subunit (Yusupov et al., 2001) and POST complexes with tRNAs bound to E and P sites can be isolated by centrifugation through sucrose cushion without loss of deacylated tRNA from the E site (Wadzack et al., 1997) proving that the tRNA at the E site is bound in a stable fashion.

High ionic strength caused by e.g. increased Mg^{2+} concentrations are also known to induce errors (W. Szaflarski and K. H. Nierhaus, unpublished). High concentrations of cations shield the negative phosphate charges of the rRNA and thus condense the 3D structure of the ribosome. A consequence is that the ribosome will lose the flexibility required for conformational changes, and it is conceivable that movements such as the EF-G dependent translocation might not be successful in all the cases and thus the ribosome might not reach the canonical POST state, but rather becomes stuck during the course of a translocation reaction. If stuck ribosomes accumulate the cell will die. However, wild type cells (e.g. E. coli K-12 MG1655 strain used in this study) can cope with an increase of the ionic strength by addition of Mg^{2+} reaching concentrations of 100 mM or higher, or K+ with concentrations of 200 mM or higher. We will demonstrate in this thesis that the protein LepA is required to fulfill this astonishing achievement.