

1 Introduction

1.1 Protein synthesis in the prokaryotic cell

Protein synthesis is one of the most essential events in each living cell. Here, the genetic information stored in DNA and transcribed into RNA is translated into a sequence of amino acids forming a polypeptide. In that process, tRNAs are involved bridging the world of proteins with that of nucleic acids. The tRNA has two functional parts: an anticodon stem loop carrying the anticodon complementary to a codon on a mRNA, and the acceptor stem called with the universal CCA-3' end that is covalently attached to the amino acid specific for the codon.

The ribosome contains three tRNA binding sites, two of which are occupied by tRNAs. Peptide bond formation occurs between amino acids attached to the tRNA in the so called A- (aminoacyl) and P- (peptidyl) sites. The third site – the E-site (exit site) - contains always deacylated-tRNA. Few years ago the atomic structure of prokaryotic ribosomes have been resolved (Ban et al., 2000) and since that time we know that the ribosome is a ribozyme, e. g. the catalytic reaction of peptide bond formation is performed by rRNA rather than ribosomal protein as it was previously believed.

The ribosome is a translator of the genetic information with an amazing accuracy. Synthesizing proteins with a rate of 10 to 20 amino acids per second (Dennis and Bremer, 1973; Wilson and Nierhaus, 2003) the ribosome makes one mistake (misincorporation) per 3,000 incorporations (Bouadloun et al., 1983).

Due to its complicated structure the ribosome is a target for many of antibiotics. About 50-60% of all known antibiotics interfere with ribosome or with protein synthesis process. Here, we demonstrate mechanisms of translation inhibition caused by some antibiotics. We also present novel techniques for functional studies of protein synthesis inhibitor.

We describe in detail the structure of the ribosome and two important stages of protein synthesis: (1) initiation of protein synthesis, and (2) the decoding center on the ribosome, as a target for antibiotics impairing the decoding accuracy.

1.1.1 Structure of prokaryotic ribosomes

All ribosomes are composed of two subunits of unequal size. In case of bacterial ribosome the sedimentation constant is 70S, which can be dissociate into a large 50S subunit and a small 30S subunit. Each of ribosomal subunit is a ribonucleoprotein complex. The 50S subunit contains both a 5S and a 23S rRNA (120 nts and 2,904 nts in *E. coli*, respectively), while the 30S subunit contains a single 16S rRNA (1542 nts). The protein fraction consists of 21 different proteins in the small subunit and 33 proteins in the large one.

For the first time the ribosome (“microsome”) was detected in the images of cell cytoplasm from electron microscopy in the late 1940’ (Claude, 1943, for historical details see also Rheinberger, 2004). It could be seen as a black dots swimming in the cell. The significant improvement of the ribosome structure is counted from the beginning of 1980’. The small subunit was described anthropomorphically with a head, connected by a neck to the body with shoulder and platform (Stöffler-Meilicke and Stöffler, 1990). The large subunit presents a more compact structure consisting of a rounded base with three protuberances, termed the L1, central protuberance, and the L7/L12 stalk (Frank, 1989; Stark et al., 1995).

The standard electron microscopy has been improved by embedding the observed object in amorphous water *via* freezing in liquid nitrogen or helium. This method - cryo-electron microscopy (cryo-EM) - was extensively exploited by “ribosomologist” (Frank, 2001). The reconstruction of ribosome from cryo-EM revealed that the rRNAs are located in the core surrounding by exterior proteins. The second feature, which was seen with cryo-EM was a long tunnel (100 Å, the size equal to 30-40 amino acids long chain) through the entire large subunit started around the peptidyl transferase center (Voss et al., 2006). Two potential explanations for the tunnel importance were suggested: (1) In the tunnel early stages of protein folding take place, and (2) probably the nascent polypeptide is prevented here against proteases activity.

The richest information about the ribosome structure was revealed recently from the crystallographic structure (Ban et al., 2000; Brodersen et al., 2002; Cate et al., 1999; Clemons et al., 2001; Harms et al., 2001; Schlutzen et al., 2000; Wimberly et al., 2000; Yusupov et al., 2001). Here, the position of almost each

atom in the ribosome was determined. In principle all structural features of ribosome were only confirmed by high-resolution studies and recognized in much closer view. However, three significant improvements were obtained by X-ray studies: (1) New structural motifs, the minor A motifs, were observed (Nissen et al., 2001). (2) The mechanisms of decoding and peptide-bond formation could be deduced and were found to be performed exclusively by rRNA (Ogle et al., 2003; Schmeing et al., 2005). (3) The structural basis for inhibition mechanisms of many antibiotics could be identified (Brodersen et al., 2000; Carter et al., 2000; Hansen et al., 2002; Pioletti et al., 2001). The regions of rRNA which were previously predicted as separated double strands were now recognized quite often as double-stranded regions stacking end-to-end and forming long quasi-continuous helical structures. Quite often adenine residues are bulged out forming the “A-minor” motif (Nissen et al., 2001). The A-minor motif constitutes an interaction between an adenine residue and the minor groove of an rRNA helix contributed significantly to stability of the tertiary rRNA structure. Four variants of this motif were identified. Type I and II play an important role by being involved in both decoding and formation of peptide bond formation.

1.1.2 The initiation of protein synthesis in bacteria

The first stage of the translation process requires the assembly of ribosomal subunits together with a correct arrangement of mRNA and tRNA on the ribosome. The mRNA has to display its first codon of protein to be expressed exactly at the P-site. The initiation somewhere upstream or downstream from the start codon could result either in truncated or extended protein. The positioning of the mRNA utilizes the untranslated region (UTR) upstream of the start codon, which directs placement of the mRNA on the small subunit. The mechanism is relatively simple and involves a stretch of nucleotides (the Shine-Dalgarno sequence, SD sequence) that interacts with the 3' end of the 16S rRNA (the anti-SD sequence) (Shine and Dalgarno, 1974).

The correct location of mRNA on the ribosome is mediated by complementary interaction of RNAs. In contrast to that, the correct tRNA and its location on the P-site are mediated by initiation factors (IFs). The process is performed by three initiation factors: IF1, IF2 and IF3. So far we know the atomic

structure in the complex with ribosome only in respect to IF1 and a domain of IF3 (Carter et al., 2001; Pioletti et al., 2001).

Probably the first initiation factor, which binds to the ribosome, is IF3. It has two identified functions. (1) It acts as an anti-association factor preventing formation of the 70S ribosome from 50S and 30S subunits. (2) It plays an important role in the codon-anticodon discrimination at the P-site (Allen et al., 2005; Sussman et al., 1996). This factor is composed of two domains separated by a long lysine-rich linker. The C-terminal domain (CTD) is sufficient for ribosome binding and is employed in the first function of the factor, the N-terminal domain (NTD) was suggested to be involved in its second function. The binding site of CTD of IF3 located on the ribosome suggests that the anti-association function of IF3 is achieved directly by sterical hindrance (Dallas and Noller, 2001) or indirectly by conformational changes.

The function of IF1 is in spite of a wealth of experimental data. This factor is universal and essential. IF1 is composed of 72 amino acids being the smallest translational factors. Despite the binding of IF1 on the ribosome was detected at the decoding center of the A site (Brock et al., 1998; Carter et al., 2001; Dahlquist and Puglisi, 2000), the exact function of this factor still is unclear. A number of roles have been described for IF1 including (1) subunit association during 70S initiation complex formation, (2) modulating the binding and release of IF2 and (3) blocking the binding of tRNAs to the A-site (review in Gualerzi et al., 2000). Irrespective the role of IF1, its gene - *infA*, is essential for cell viability (Cummings and Hershey, 1994) indicating the importance of IF1.

The structure of IF1 has been determined both alone by NMR spectroscopy (Murzin, 1993) and in the complex with 30S subunit by X-ray crystallography (Pioletti et al., 2001). IF1 contains an oligomer binding (OB) fold motif presents in some protein families (Figure 1a). The architecture of classical OB-fold motif includes a five-stranded β -sheet coiled to form a closed β -barrel. The OB-motif was found in many of ribosomal proteins (S1, S17 and L2), in synthetases as well as in eukaryotic initiation factors. Interestingly, a number of the cold shock proteins (Csp) have high structural homology to IF1 as demonstrated on Figure 1a. The bacterial strain lacking Csp proteins, which is lethal, can be rescued by over-

expression of *E. coli* IF1 (Weber et al., 2001), suggesting that IF1 could assume some of the chaperon activity normally performed by the Csp proteins.

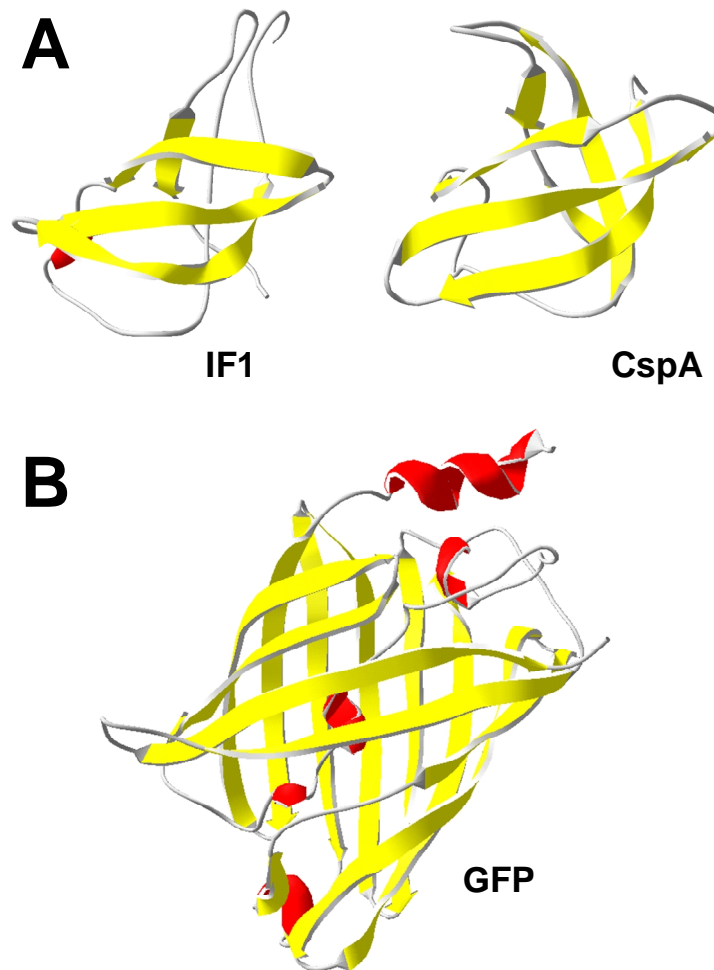


Figure 1: The solution structures of IF1, CspA and GFP. (A) Structural homology between IF1 (PDB: 1ah9) and CspA (PDB: 1mjc), (B) structure of GFP (PDB: 1huy). Yellow color indicates β -sheets and red α -helices.

Interestingly, the binding to the decoding center of the A-site induced a flip out of A1492 and A1493 from the helix 44 (Ogle et al., 2001). This is reminiscent of the situation where these residues are flipped out due to binding of cognate tRNA to the A-site as well as due to binding of the antibiotic paromomycin. Despite that IF1 blocks the A-site, in the course of the 30S subunit initiation this is probably not its real function, especially if we take into a count that the 30S subunit has exclusively one binding site, namely the P-site (Gnirke and Nierhaus, 1986; Hartz et al., 1988).

The third bacterial IF is IF2. This factor – complexed with GTP - binds specifically the initiator tRNA fMet-tRNA and directs it to the 30S subunit. Delivery of the initiation tRNA by IF2 is enhanced also by IF1 (see reviewed by Wilson and Nierhaus; Wilson and Nierhaus, 2003).

The final product of protein initiation is a 70S ribosome containing mRNA with the AUG codon at the P-site and an initiator tRNA. Such a ribosome is ready to accept the ternary complex aa-tRNA•EF-Tu•GTP according to the codon displaced at the A-site and thus to extend the nascent polypeptide.

1.1.3 Elongation of translation – decoding of translation

After the initiation stage, the ribosome enters the elongation cycle, where in one cycle of the elongation single amino acid is incorporated into the nascent polypeptide. According to the codon present at the A-site the ribosome has to select the correct or cognate aa-tRNA among 41 contestants present in *E. coli*. It happens that a wrong aa-tRNA carrying an anticodon similar to the cognate one is selected. With respect to a distinct codon three to four aa-tRNAs can be mistakenly selected (near-cognate aa-tRNAs); the remaining 90% of tRNAs have a dissimilar anticodons and are termed non-cognate tRNAs.

An aminoacylated-tRNA (aa-tRNA) is not a substrate for the selection process but rather the ternary complex aa-tRNA•EF-Tu•GTP. In spite of the large complex with ~72 kDa and therefore many unspecific interaction points with the ribosome, the selection rests solely on the anticodon with only ~1 kDa. A-site occupation proceeds in two steps: (1) the interaction of codon-anticodon and (2) flipping the of aa-tRNA to the A-site (accommodation). During the initial decoding step (1), the A-site is in a low affinity state (as a consequence of reciprocal interaction between A- and P-site, for details see Blaha and Nierhaus, 2001; Spahn and Nierhaus, 1998), which restricts the interaction of the ternary complex to codon-anticodon interactions, thus excluding general and unspecific contacts of the aa-tRNA and EF-Tu. By restricting the binding surface of the ternary complex to the discriminating feature (anticodon), the binding energy is both small and approximately equal to the discrimination energy. The second step, accommodation into the A-site, requires release of the aa-tRNA from the ternary complex, which is triggered by the release of Pi after GTP hydrolysis.

Accommodation utilizes nondiscriminatory binding energy to dock the aa-tRNA precisely into the A-site attaching the aminoacyl residue into the PTF center on the 50S subunit.

We consider two aspects of the selection process: (1) discrimination against the 90% non-cognate aa-tRNAs, and (2) discrimination of the cognate against the near-cognate aa-tRNAs. The fact that the non-cognate aa-tRNA are never selected could be shown to be due to the reciprocal linkage between A and E site. This is caused - as mentioned above - by reducing the ternary complex interactions with the ribosome to codon-anticodon interaction selection. Therefore, the A site does not exist for the non-cognate aa-tRNAs, since the anticodon is dissimilar to the cognate one and outside of the anticodon the ternary complex shows only negligible interactions with the ribosome (for review see Wilson and Nierhaus, 2006).

How then the ribosome discriminates the cognate ternary complex against the near-cognate ones? A model of such a process was for the first time suggested by Potapov about 20 years ago (Potapov, 1982). The model assumes that the ribosome recognizes the codon-anticodon duplex by sensing the stereochemical correctness in respect to Watson-Crick base pairing and the location of the phosphate-sugar backbone in the structure. To verify it, Potapov has prepared the mRNA carrying a DNA codon at one of the three ribosomal sites (Potapov et al., 1995). It was found that a deoxycodon at the A-site was disastrous for tRNA binding, whereas a deoxycodon at the P-site had no effect on the tRNA binding site.

Recently, crystallographic studies have shed more light on the decoding mechanism with respect of cognate versus near-cognate ternary complexes (Ogle et al., 2001). The binding of mRNA and cognate tRNA induces two major conformational changes in the decoding center (A-site). The two universally conserved adenine residues A1492 and A1493 of the internal loop of h44 flip out (Figure 2), while the universally conserved base G530 switches from the conformation *syn* to *anti*. The base A1493 recognizes the first base pair of codon-anticodon helix in the A-site by the interaction of hydrogen bonds with the first position of the codon-anticodon duplex. The second base pair is also monitored by OH interactions, but in this case by two bases, namely A1492 and G530. Thus, it

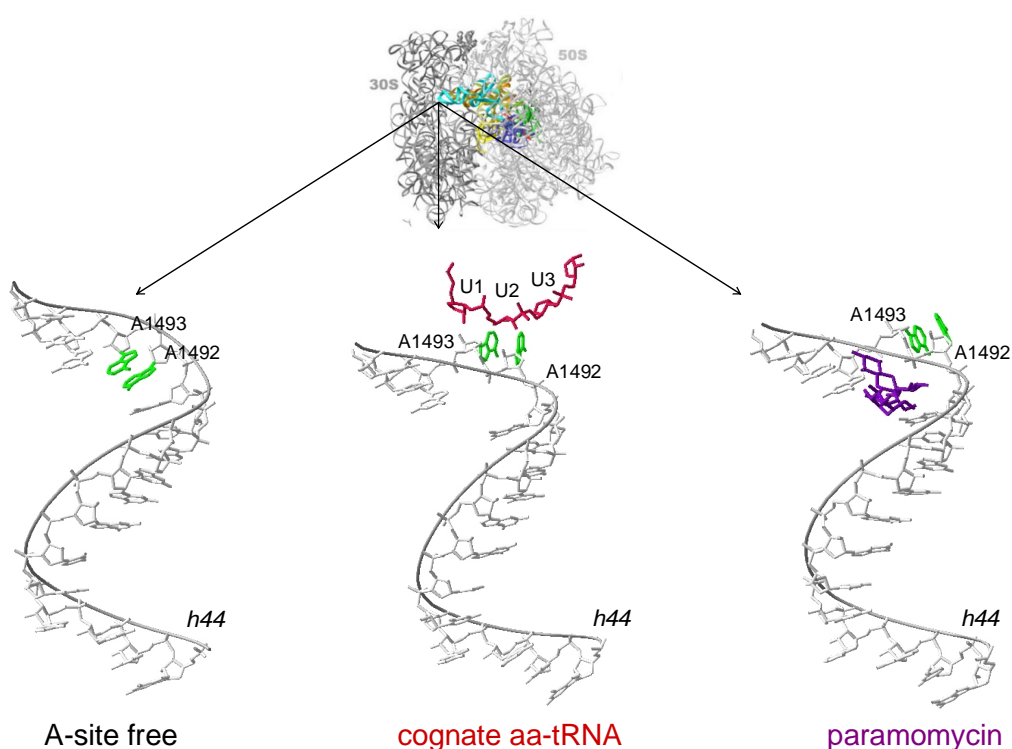


Figure 2: The flip out of A1492 and A1493 in the decoding center upon binding of a cognate tRNA and paramomycin. In dark-gray showed the 16S rRNA. The base residues of A1492 and A1493 indicated in green. The anticodon (U1, U2 and U3) indicated as red and the antibiotic puromycin as violet.

seems that the monitoring of the middle base pair of a codon-anticodon duplex is more rigid than the first base pair. In contrast, the third position is less rigorously monitored. This is in agreement with observation that the third position of anticodon is the so called *wobble* position allowing accommodation in the presence of a suboptimal base pair. Some antibiotics like paramomycin can interfere with the decoding process and artificially flip out the A1492 and A1493 mimicking a cognate tRNA at the A-site and in consequence causes incorporation of the incorrect amino acids into the nascent peptide chain (Figure 2).

Accommodation of aa-tRNA at the A-site triggers peptide-bond formation at the PTF center. During PTF reaction the nucleophilic α -amino group of the A-tRNA attacks the carbonyl group of the peptidyl residue of the P-tRNA, which is linked through an ester bond to the tRNA moiety. This results in the formation of a tetrahedral intermediate, which leads to formation of a peptide bond. As a result,

the aa-tRNA becomes a peptidyl-tRNA extended by one aminoacyl residue at the A site, leaving the deacylated tRNA at the P site.

After peptide bond formation the complex mRNA•tRNA(P-site)•peptidyl-tRNA(A-site) is translocated from the PRE to POST state, resulting in a deacylated tRNA at the E-site and peptidyl-tRNA at the P-site. Translocation is mediated by the elongation factor G (EF-G) belonging to the G-protein family as IF2, RF2 (release factor 2) and EF-Tu (Czworkowski et al., 1994). A spontaneous translocation on the ribosome could be observed (Gavrilova and Spirin, 1971; Pestka, 1969), but the rate is about three orders of magnitude slower than that in the presence of EF-G (Bergemann and Nierhaus, 1983; Cukras et al., 2003).

1.1.4 The Allosteric Three-site Model for the ribosomal elongation cycle

The allosteric three-site model is characterized by two features of the elongation cycle (Rheinberger and Nierhaus, 1986): (1) The ribosome contains three tRNA-binding sites A (aminoacyl-tRNA site), P (peptidyl-tRNA site) and E (exit site specific for deacylated tRNA). (2) Two of these sites (A and E) are allosterically linked *via* a negative cooperativity, i.e. occupation of one site decreases the tRNA affinity of the other and *vice versa*. Thus, the model defines two states of the elongating ribosomes, the pre-translocational state (PRE-state), with A and P as the high affinity sites and the post translocational state (POST-state), with P and E as high affinity sites. In both states, two tRNAs are present at the respective sites during translocation.

The model implies that two types of A-site occupation have to be distinguished (Figure 3). One is the A-site occupation of the 70S initiation complex with only P-site filled with fMet-tRNA, the E site is free. This type of occupation is termed i-type (i for initiation) (Hausner et al., 1988). The second type is the e-type, e for elongation: an A-site occupation during an elongation cycle deals with a ribosomal complex carrying two tRNAs, viz. a peptidyl-tRNA and a deacylated tRNA at the P- and E-sites, respectively. i- and e-types of A-site binding are functionally not equivalent: The e-type requires much higher activation energy (120 kJ/mol) as compared to about 60 kJ/mol (Schilling-Bartetzko et al., 1992). Hausner and coworkers have further demonstrated that aminoglycosides block A-

site tRNA binding of the e-type in contrast to that of the i-type (Hausner et al., 1988).

1.1.5 Termination of protein synthesis

The last step of protein synthesis is termination. The ribosome stops the translation when a stop codon enters the decoding center at the A site. Three stop codons exist (UAA, UAG and UGA), and the characteristic feature of them is that

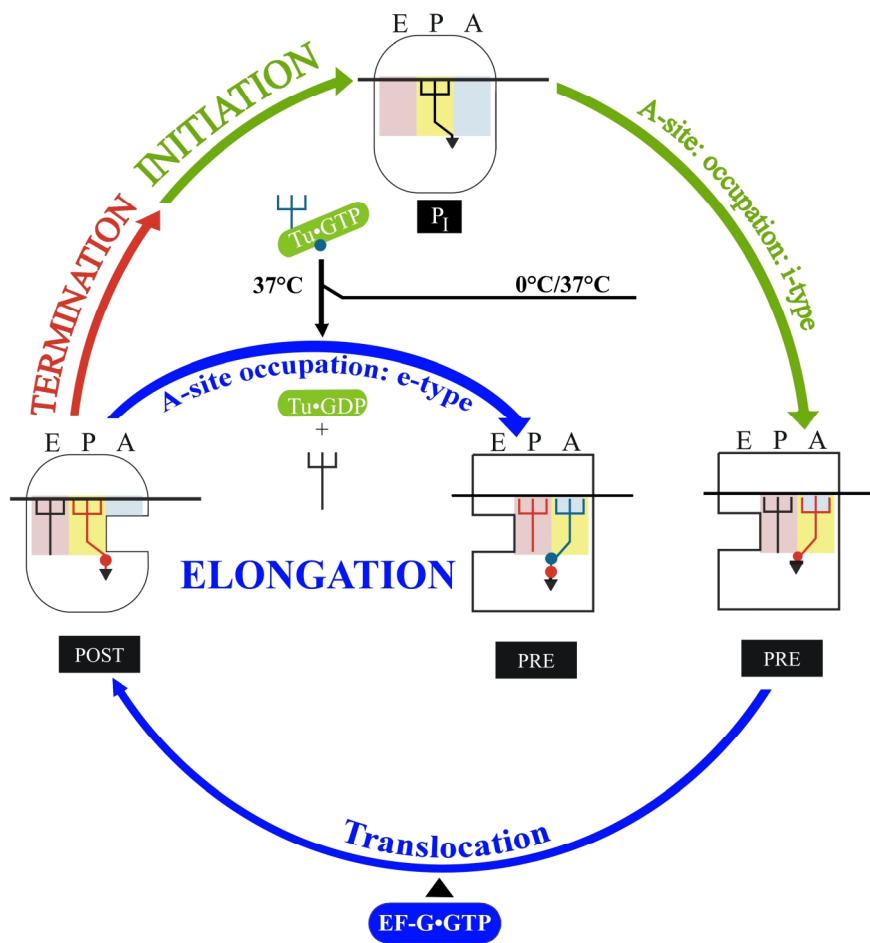


Figure 3: The two types of A-site occupation according to the allosteric three-site model (Hausner et al., 1988), modified). The i-type occupation follows the initiation reactions, the E-site is free. The e-type occupation occurs after an elongation cycle, and is characterized by an occupied E-site.

they do not have corresponding aa-tRNA. When a stop codon is at the A site, the release factors (RF) releases the nascent polypeptide chain from the ribosome and the ribosome recycles for a next initiation.

1.2 Cell-free protein synthesis systems - RTS 100 and RTS 500

Coupled *in vitro* transcription-translation systems offer a great potential both as an analytical tool and a method for efficient production of recombinant proteins in amounts of several mg per ml. Cell-free protein synthesis provides advantages over conventional *in vivo* protein expression method: First of all, *in vitro* systems can direct most of the metabolic resources of the cell extract towards the production of one protein. While *in vivo* expression of proteins occurs in concert with numerous physiological activities, cell-free translation takes place without the need to support processes required for cell viability. Secondly, the lack of cell wall barrier is another advantage, for in an open system there is the opportunity to create an optimal environment for expression of proteins by directly manipulating the reaction conditions. For example, system allows for incorporation of isotope-labeled amino acids (^{15}N , ^{13}C , ^{35}S) for NMR studies as well as incorporation of unnatural amino acids for protein design. Thirdly, cytotoxic proteins can be produced in cell-free systems.

Depending on the reactors in which the reaction is performed, several types of cell-free systems can be distinguished: batch system, continuous flow (Spirin et al., 1988), semi-continuous system (Kim and Choi, 1996), and hollow fiber membrane reactors (Jewett, 2002). In the batch system the reaction mix contains all the necessary components for transcription and translation as well as the synthesized products. In the course of our study with the bacterial *Escherichia coli* system we used both a batch system and semi-continuous reactors (Roche ProteoMaster; RTS 100/500 *E. coli* HY Kit). The latter contains two chambers for the reaction and for the feeding mix, respectively, separated by a semi-permeable membrane. The reaction chamber houses the machinery for mRNA and protein production, together with the DNA template. The chamber with the feeding mix is ~10 volumes larger than that of the reaction and supplies nucleotide-triphosphates (NTPs) and amino acids, and removes by-products. The final product, the protein, accumulates in the reaction chamber.

The essential component of the system is the cell-free extract containing most of the cellular cytoplasmic compounds necessary for protein synthesis, such as ribosomes, translational factors, tRNA synthetases and tRNAs. Furthermore, usually the RNA polymerase (RNAP) from the T7 bacteriophage is used. The gene of interest, flanked by a T7 RNAP promoter and terminator, is introduced into the system on a plasmid or as linearised double-stranded DNA.

Besides the above mentioned advantages of the *in vitro* system, there are certain difficulties to express genes in the prokaryotic-based systems. A major drawback is, as shown here, the unsatisfying low activity of the synthesized protein seen for the well soluble GFP protein, which ranges between 30 to 70% and impairs therefore subsequent structural and functional studies.

In this work we solve this major *in vitro* expression problem and report conditions under which high yields of synthesized proteins with up to 100% activity are achieved.

1.3 Inhibitors of protein synthesis in the prokaryotic cell

Due to its complexity the ribosome can be a target for interfering molecules causing disruption of protein synthesis. Indeed, every step of translation can be a target for antibiotics, although with different degree of specificity. Antibiotics are defined as low-molecular-weight metabolic products, usually below 2 kDa, which are produced by microorganisms in order to limit the population of their neighborly and in consequence to win the struggle for life. Here, in this thesis the term antibiotic is used to encompass natural and non-natural chemical compounds that exhibit inhibitory effects against particular microorganisms.

Antibiotics target an astonishing variety of cell processes. However, 50-60% of them interfere with the translational apparatus. Here, we focus our interest on a few selected antibiotics, where we could contribute to the current understanding of their mode of action.

1.3.1 Kasugamycin

Kasugamycin (Ksg) was discovered during the early 1960's in Japan. Japanese looked for a substance which could inhibit the rice blast disease caused by the fungus *Piricularia oryzae*. Among others the substance was discovered

and demonstrated to block the growth of the fungus. The substance was called kasugamycin from the name of Kasuga shrine at Nara City (Umezawa et al., 1965). Ksg is an aminoglycoside antibiotic (Suhara et al., 1966) shown to be active against a wide variety of microorganisms, but exhibited a low toxicity against plant, human, fish and animals, and was therefore accepted for agricultural usage

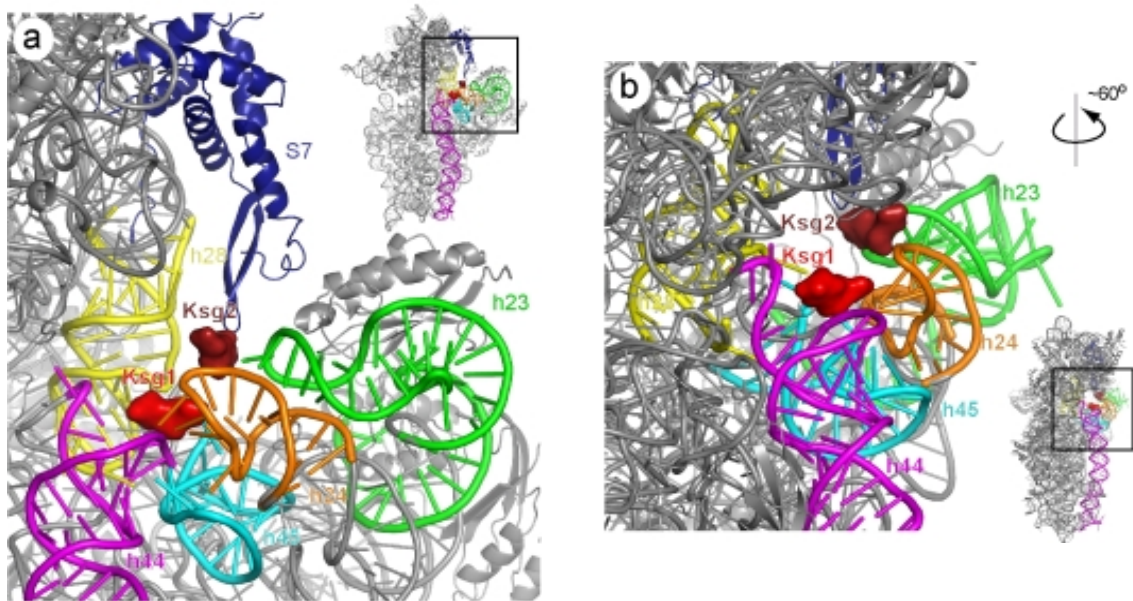


Figure 4: The primary and secondary Ksg binding sites on the 30S subunit. (a) Overview of the two Kasugamycin binding positions on the *T. thermophilus* 30S subunit. Ksg1 (light red spacefill) is located at the top of h44 (magenta) spanning between h24 (orange) and h28 (yellow), whereas Ksg2 (dark red) interacts with the tip of the β -hairpin of ribosomal protein S7 (dark blue) on the head and approaches h23 (green) on the platform of the 30S subunit. (b) Overview as in (a) but rotated anticlockwise by 60°

(Hamada et al., 1965; Takeuchi et al., 1965).

Ksg inhibits poly(U)-dependent poly(Phe) synthesis on bacterial *E. coli* ribosomes *in vitro*, but unlike other aminoglycosides Ksg does not induce translation misreading (Masukawa et al., 1968; Tanaka et al., 1966b) read-through or frameshift (Cassan et al., 1990) - in fact increased translation fidelity has been reported in some cases (Van Buul et al., 1984). This is consistent with the absence of streptamine moiety common in aminoglycosides, such as streptomycin or neomycin (See Appendix), which induce translation misreading (Davies et al., 1964). Many studies have shown that Ksg inhibits the binding of the initiator fMet-tRNA to the P-site of mRNA-programmed 30S subunits, but not the binding of

alanyl-tRNA to the A site of 70S ribosomes, leading to the classification of Ksg as an initiation inhibitor (Okuyama et al., 1971; Poldermans et al., 1979). Unlike canonical mRNAs, the translation *in vivo* of leaderless mRNAs, i.e. those starting directly with a 5' AUG, is not affected by the presence of Ksg (Chin et al., 1993; Moll and Bläsi, 2002) casting doubt on the general validity of the assumption that Ksg is a blocker of fMet-tRNA binding to the P site..

Our co-workers have determined the structure of Ksg bound to the *Thermus thermophilus* 30S ribosomal subunit at resolution of 3.3 Å. They observed two binding sites for Ksg, both of which are on the intersubunit side of the 30S subunit. In the primary binding site Ksg sits at the top of h44, spanning between h24 and h28 of the 16S rRNA, whereas in the secondary position Ksg interacts with h23, h24 and the β -hairpin extension of the ribosomal protein S7 (Figure 4).

Together with the functional data presented in the results section we demonstrated a new model explaining the inhibitory effect of Ksg on the binding of P-site tRNA during canonical translation initiation, but lack of initiation during 70S-type translation of leaderless mRNAs.

1.3.2 Edeine and Pactamycin

Both antibiotics, edeine (Ede) and pactamycin (Pct) were proposed to target the initiation step of protein synthesis (for details see review: Gale et al., 1981). Ede is a product of *Bacillus brevis* being composed of β -tyrosine residue attached to a C-terminal spermidine-like moiety (See Appendix). Both drugs are universally with respect to their activities against ribosomes from all domains of organisms. We demonstrate (see result section 3.3.2, page 89) that Ede inhibits specifically mRNA-directed binding of aa-tRNAs to both 30S subunit and 70S ribosomes (Dinos et al., 2004).

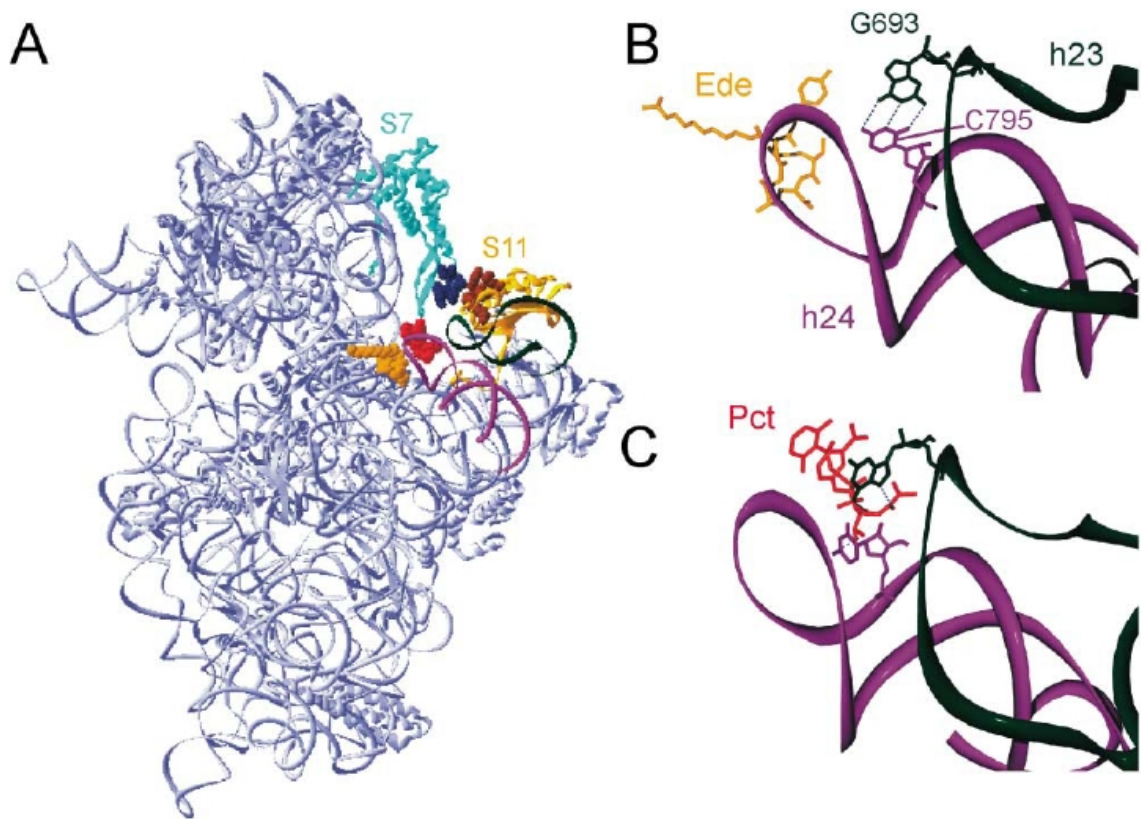


Figure 5: The binding site of edeine and pactamycin on the 30S subunit

(a) Relative binding positions of edeine (dark yellow) and pactamycin (red) on the *T. thermophilus* 30S subunit, with the tip of h23 (dark green) and h24 (purple) of the 16S rRNA and the ribosomal protein S7 (cyan) and S11 (yellow). Residues that constitute the interaction between S7 (dark blue) and S11 (brown) are shown in spacefill representation.

(b) Close-up view showing the hydrogen bond formed between residue G693 of h23 and C795 of h24 upon binding of edeine (yellow) to the 30S subunit (PDB: 1HNX; Brodersen et al., 2000).

(c) Close-up view of the pactamycin binding site, illustrating the hydrogen bond interactions between G693 and C795 and pactamycin (red) (PDB: 1I95; Pioletti et al., 2001). Coloring of helices and bases as in (b).

The crystal structure of Ede with the 30S ribosomal subunit from *T. thermophilus* has been resolved (Pioletti et al., 2001). It revealed the binding site for this antibiotic to be located between the P and E sites (Figure 5). In details, the drug binds between h24, h28, h44 and h45 inducing base-pair formation between C795 and G693 at the tips of h24a and h23b, respectively (Figure 5b). The same region of 16S rRNA was found to be protected by chemical modification upon tRNA binding to the ribosomal P-site (Moazed and Noller, 1987). The location of the P-site was then revised by (Yusupov et al., 2001), since these bases are part of the E-site. From the atomic structure of ribosome it is known that the tRNA

binding at the P-site requires the base pair induction between C795 and G693 (so called open conformation). Because Ede also induced conformational changes causing C795-G693 base pair formation, the mode of inhibition of Ede may be indirect, i.e., Ede binding may inducing the closed conformation (C795-G693) mimicking a ribosome already containing a P-site tRNA and therefore prevent the associated conformational changes necessary for stable binding of tRNA to the P-site.

Pct is a well-known antibiotic extensively studied from 1970s on. Similar to Ede it universally inhibits prokaryotic as well as eukaryotic ribosomes. This fact eliminated it from clinical usage. The crystal structure of the 30S subunit from *T. thermophilus* in the complex with Pct has been resolved (Brodersen et al., 2000). The single binding site of Pct was in excellent agreement with the biochemical data. Two distal rings of Pct stack upon each other, one interacts with G693 at the tip of h23 of 16S rRNA, while the central ring interacts with C795 and C796 in h24 (Figure 5c). Interestingly, the Pct binding site corresponds remarkably well with the base pair induced when Ede binds to the 30S subunit (compare with Figure 5 b and c). Moreover, it was demonstrated by (Dinos et al., 2004) that Ede blocked fMet-tRNA binding to the P-site and astonishingly the effect of tRNA binding can be relieved by increasing concentration of Pct.

Pct originally was termed as an initiation inhibitor because of an accumulation of putative pre-initiation complexes in the presence of the drug. A systematic study showed that Pct did not affect initiation but rather the translocation step (Dinos et al., 2004). Interestingly, Pct is a tRNA-specific inhibitor of translocation. It blocked significantly translocation of Met-tRNA, Val-tRNA or Lys-tRNA in contrast to Phe-tRNA, where little or no inhibition was observed. This finding was in the agreement with the lack of inhibition of Pct on poly(U)-dependent poly(Phe) synthesis in contrast to a very strong inhibition of poly(A)-dependent poly(Lys) synthesis.

Here, we have used a very efficient, optimized *in vitro* coupled-translation system for functional studies of Ede and Pct.

1.3.3 Antibiotics interfering with the ribosomal A-site

The decoding center is located on the 30S subunit. The binding sites of many antibiotics could be identified on that subunit in crystallographic studies. We will focus our interest on the mechanisms of action of two antibiotics, namely tetracycline (Tet) and paromomycin (Par).

Independent studies revealed at least two binding sites of Tet on the 30S subunit (Brodersen et al., 2000; Pioletti et al., 2001). The primary binding site seems to be located around A-site and is responsible for blocking protein synthesis. Tet is composed of four fused rings (See Appendix) which interact predominantly with the 16S rRNA through the oxygen atoms located along one side of the molecule. The oxygen atoms form hydrogen bonds with the exposed sugar phosphate backbone of helix h34. Thus, the hydrophilic side of the tetracycline interacts with the 16S RNA, while the hydrophobic side is located towards the lumen of the A site. This is surprising, since interaction between two molecules is usually through their hydrophobic regions. The primary binding site of tetracycline overlaps with the position of the A site tRNA after accommodation, and thus the mechanism of action of tetracycline most probably results from a direct inhibition of aa-tRNA during the accommodation step of A-site binding.

Paromomycin belongs to the aminoglycosides family of antibiotics and increases misreading. The binding site for paromomycin, determined by X-ray crystallography, involves contacts with h44 of the 16S rRNA (Brodersen et al., 2000; Ogle et al., 2001). The binding of paromomycin induces the universally conserved residues A1492 and A1493 to flip out of the helix h44 in the fashion reminiscent of that observed during binding of cognate aa-tRNA to the A-site (compare with section 1.1.3, page 22). This conformational change is caused by insertion of one of four rings (ring I) of paromomycin (See Appendix) into h44. In this position, ring I mimics a nucleotide base - it stacks with G1491 and hydrogen bonds with A1408. The stability of this conformation is further reinforced by hydrogen-bonding interaction between ring I and the backbone of the flipped-out A1493. Significantly, rings I and II of paromomycin are found in some members of other aminoglycosides, such as the neomycin, gentamycin and kanamycin families, which suggests that misreading caused by these aminoglycosides operates through similar mechanism.

Streptomycin (Str) is structurally related to the aminoglycoside family of antibiotics (See Appendix) and also increases misreading. Despite this common feature, Str binds to a distinct site on the ribosome and therefore mediates its inhibitory and misreading effect by an unrelated mechanism. Str has a single binding site on the 30S subunit that connects helices from all four different domains of 16S rRNA, namely h1, h18, h27 and h44, and interacts with ribosomal protein S12 (Carter et al., 2001). Comparison of crystal structures of 30S subunits with respect to interaction of the codon-anticodon duplex with the decoding center in the cases of cognate and near-cognate tRNA, respectively, led to the proposal that selection of the correct i.e. cognate-tRNA by the ribosome requires a transition from an open to a closed form (Ogle et al., 2002). The binding of Str stabilizes the closed form and, by doing so, explains the lower translation fidelity, since it facilitates the selection of a near-cognate tRNA and subsequently the incorporation of the corresponding amino acid.

1.3.4 Novel Ribosome Inhibitor (NRI)

The search for new unknown antibiotics has led to the recent discovery by Abbott Laboratories of a series of novel ribosome inhibitors (NRI), (Dandliker et al., 2003), structurally related to the antibacterial quinolones. The quinolones are well known as DNA gyrase and topoisomerase IV inhibitors, therefore it was surprising that small modification in the structure of the drugs were sufficient to turn them to protein synthesis inhibitors. Moreover, NRI blocked the growth rate of a range of various bacteria, both Gram-positive and Gram-negative bacteria, including a number of common respiratory pathogens, while human cell-lines were not affected (Dandliker et al., 2003). Probably the most essential observation is that NRI inhibits translation using a completely new mechanism because NRI-resistant strains exhibit no cross-resistance with other translation inhibitors. Here, we analyse the misreading potential of NRI.