1. INTRODUCTION

1.1 The ribosome

The ribosome is a ribonucleoprotein complex found in all living cells responsible for the translation of the genetic code. A typical bacterial ribosome contains more than a quarter of a million atoms and is of a molecular weight of 2.3 million Daltons with a sedimentation coefficient of 70S. It is build of two independent subunits of unequal size (50S of 1.45 and 30S of 0.85 million Daltons, respectively), which associate upon the initiation of protein biosynthesis. In prokaryotes about a third of the ribosomal mass comprises some 58-73 different proteins, depending on the source. The rest are three chains of ribosomal RNA (rRNA), of a total of about 4500 nucleotides, named 23S, 16S and 5S rRNA. The two main tasks during protein synthesis are decoding and peptide bond formation. The decoding is performed by the 30S subunit while the peptide bond formation takes place on the 50S subunit (reviewed in Wilson and Noller, 1998).

The main function of ribosomes is to translate the genetic information encoded in the messenger RNA (mRNA) into proteins. During protein synthesis, ribosomes move along an mRNA molecule, reading one codon at a time. Reading of each codon by the ribosome results in the incorporation of one amino acid into a gradually lengthening protein chain. The amino acids are brought to the ribosome by the transfer RNA (tRNA) molecules, which are the adapter molecules in the translation mechanism. Each of the 20 amino acids used in protein synthesis is linked to a specific kind of tRNA. Ribosomes are thus capable of recognising and binding the right nucleic acid code word specified by the anticodon of the tRNA with its attached amino acid. Once the ribosome reaches a stop codon on the mRNA, translation stops, the ribosomal subunits separate and detach from the mRNA and the completed protein is released.

Structurally, the large subunit, named 50S in prokaryotes, is spherical with a prominent “stalk” and a “central protuberance”. It contains the peptidyltransferase centre that catalyses the peptide bond formation between the incoming amino acid and the growing peptide chain. Whereas the 50S particle is thick and monolithic, the small subunit, named 30S in prokaryotes, is thin and flexible. The 30S accommodates the mRNA decoding centre and is divided into three domains (head, body and platform). Each of these domains contains one of the principal secondary structure domains of 16S rRNA, namely the 3’ major, 5’ and central domains. The 3’ minor domain of the 16S rRNA forms an extended helix (H44 in *E. coli* helix numbering) that runs down the long axis of the 30S subunit surface that interacts with the 50S subunit. All four domain of the 30S particle join at a narrow neck region. The two “active sites” (the decoding centre and the peptidyltransferase centre) face each other across the subunit interface and are functionally linked by the two ends of the tRNA molecule.
The protein synthesis process can be functionally separated in three stages: initiation, elongation and termination.

Initiation. Initiation of translation in prokaryotes (Figure 1) is a complex process that requires the 30S subunit to bind the translation initiation region of an mRNA molecule (Shine-Dalgarno sequence), three translation initiation factors (IF1, IF2-GTP and IF3) and initiator tRNA (reviewed in Gualerzi and Pon, 1990). In eukaryotes the initiation of translation involves in addition to the prokaryotic IFs others initiation factors. At least ten initiation factors are reported in literature for eukaryotic initiation, some of them composed of more than one peptide chain (Kozak, 1999).

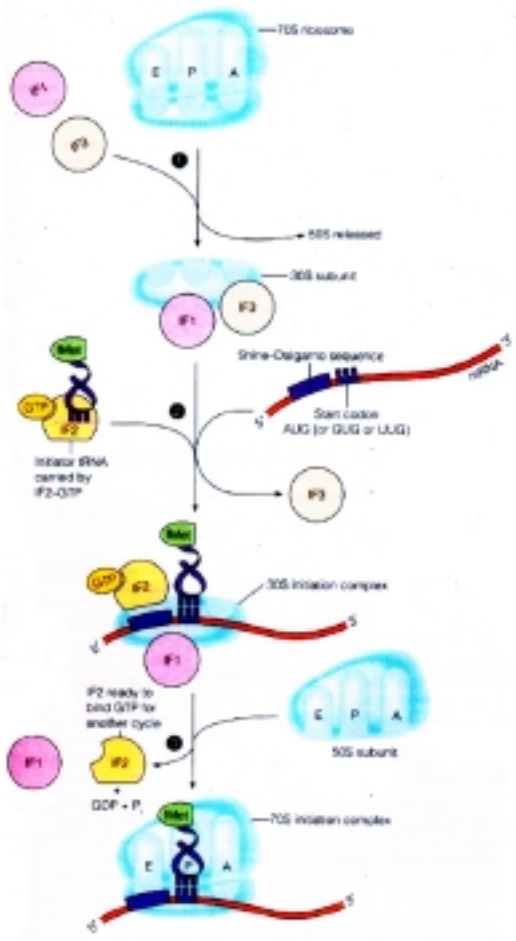

Figure 1. *Schematic representation of the initiation steps in prokaryotes.*

The key intermediate of initiation is the 30S initiation complex consisting of the 30S ribosomal subunit, mRNA, fMet-tRNA^{fMet} and the initiation factors IF1, IF2·GTP and IF3. During initiation complex formation fMet-tRNA^{fMet} and mRNA, containing the appropriate initiation signals, bind to the 30S subunit in random order (Wu et al., 1996). The initiation factors influence the kinetic of the formation of the 30S initiation complex and enhance its stability. Concomitantly release of IF1 and IF3 and association of the 50S subunit to the 30S initiation complex gives rise to the 70S initiation complex. Upon

subunit association the GTPase of IF2 is activated, GTP is hydrolysed to GDP and phosphate and IF2 is released. At this stage the fMet-tRNA^{fMet} is located in the ribosomal P-site ready to form the first peptide bond with the second coded aminoacyl tRNA, brought to the ribosomal A-site by elongation factor Tu (EF-Tu·GTP) and the elongation process can start.

Elongation. The elongation of protein synthesis can be divided in three steps (Figure 2). Starting from the 70S initiation complex where the initiation tRNA is bound to the ribosome in the P-site, in the first step of elongation aminoacyl-tRNA (aa-tRNA) binds to the A-site in a codon dependent manner. This reaction requires an elongation factor (EF-Tu in bacteria), GTP and aa-tRNA to form a high-affinity ternary complex (EF-Tu·tRNA·GTP). This ternary complex brings the aa-tRNA to the ribosomal A-site. After codon recognition, GTP hydrolysis and dissociation of EF-Tu·GDP from the ribosome, the 3' end of aa-tRNA accommodates in the peptidyl transferase centre and immediately enters the peptidyl transfer reaction, the second step of the elongation cycle. Formation of the peptide bond results in deacylated tRNA in the P-site and peptidyl-tRNA in the A-site. The third step of the cycle, translocation, is catalysed by another elongation factor (EF-G in bacteria) which hydrolyses GTP during the reaction. During translocation, peptidyl-tRNA is translocated from the A-site to the P-site, while deacylated tRNA is transferred from the P-site to the E-site from where it dissociates and exits the ribosome. Thus, translocation restores the initial state of the ribosome which then enters the next round of elongation It was also shown that the A and E-sites are coupled by a negative allostery. The occupation of either the A or E-site lowers the affinity of the other site (reviewed in Burkhardt et al., 1998).

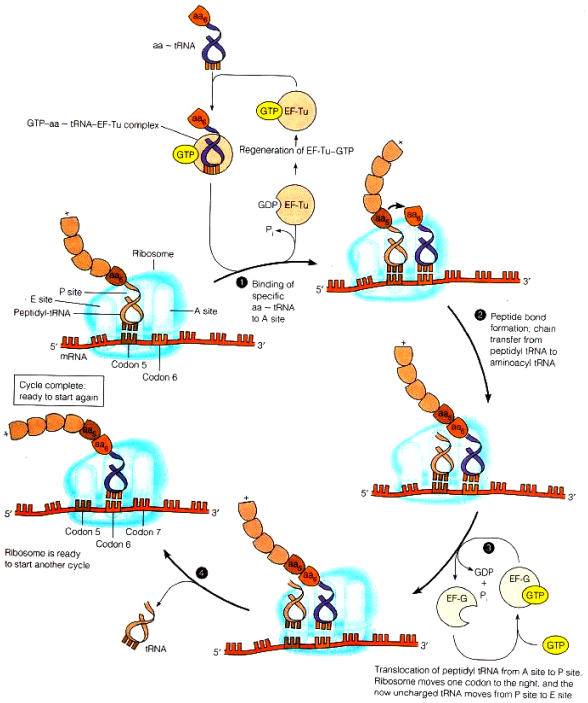

Figure 2. *Schematic representation of the elongation steps in prokaryotes.*

Termination. The termination of translation (Figure 3) occurs once the prokaryotic codon-specific release factors RF1 and RF2 recognise a messenger-encoded stop signal (UAA or UAG for RF1 and UAA or UGA for RF2) and promote the hydrolysis of peptidyl-tRNA in the P-site of the ribosome, releasing the polypeptide chain (reviewed in Nakamura et al., 2000).

The dissociation of RF1 and RF2 from the ribosomal A-site is promoted by release factor 3 (RF3) in a GTP-dependent reaction. To guarantee a pool of free subunits able to participate in the translation cycle, breakdown of the termination complex and recycling of the ribosome are required. This process is catalysed by the ribosome recycling factor (RRF) and the elongation factor G (EF-G) (Pavlov et al., 1997; Kaji et al., 1998). RF3 is able to substitute EF-G in ribosome recycling by dissociating deacylated tRNA with RRF. The ribosome recycling step has been confirmed to be essential in protein synthesis since RRF inactivation was bacteriostatic in the exponential growth phase and bactericidal during the transition between the stationary and growing phases (Kim et al., 2000).

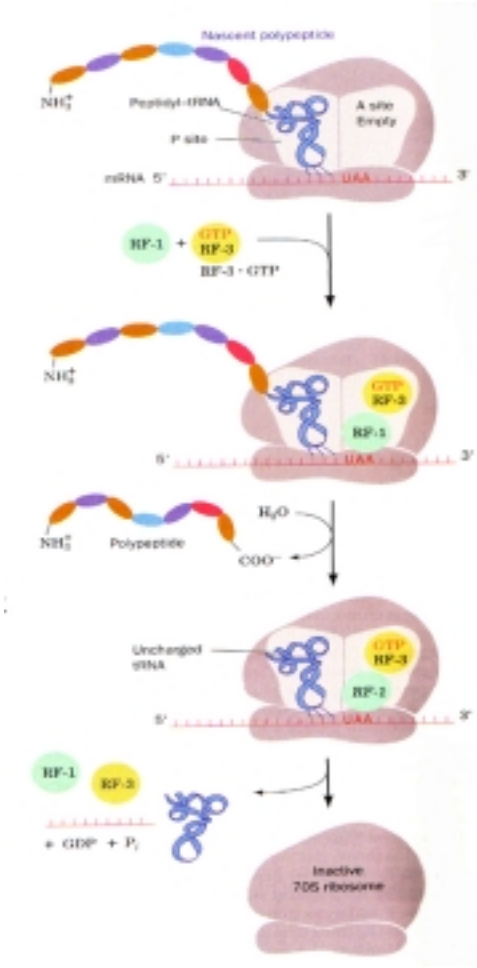

Figure 3. *Schematic representation of the termination steps in prokaryotes.*

1.1.1. Initiation factors

Here we describe in more details the initiation factors, and in particular IF3, since IF3 is one of the subjects of this work.
Of the three initiation factors the least is known about IF1. It appears to increase the affinities and activities of the other two factors and is required for cell viability in *E. coli* (Cummings and Hershey, 1994). It has also been suggested from foot printing data, and lately proved by structural data, that IF1 may mimic the A-site bound tRNA and thereby can help to prevent aa-tRNA from binding during initiation (Moazed et al., 1995; Carter et al., 2001).
The function of IF2 is to stimulate the binding of fMet-tRNA^{fMet} to ribosomes during formation of 30S and 70S initiation complexes followed by hydrolysis of GTP to GDP and phosphate. It appears that IF2 binds to the ribosome essentially by protein-protein interactions covering a large part of the head and platform of the 30S ribosomal subunit and, in addition, interacts with the L7/L12 stalk in the 50S subunit (Moreno et al., 1999).
The role of IF3 in translation initiation has been studied extensively. It was originally identified as a dissociation factor because it binds the 30S subunit with high affinity and, thereby, prevents the formation of 70S couples. This binding results in a shift in the equilibrium toward the dissociation of 70S ribosomes into 50S and 30S subunit (Godefroy-Colburn et al., 1975). IF3 has also been shown to select initiator tRNA over other aa-tRNAs on both natural and poly(U) mRNA substrates (Berkhout et al., 1986; Hartz et al., 1989; Risuleo et al., 1976). Further experiments showed that this selection arises from a recognition of the anticodon loop and the three unique G:C base pairs of the anticodon stem of the initiator tRNA (Hartz et al., 1990). These studies also suggest that IF3 may discriminate at the third position in the initiation codon on ribosome-bound mRNA.
A feature of IF3 is that all prokaryotic *infC* genes, known so far, begin with the unusual AUU or AUA initiator codon (Sacerdot et al., 1982). These rare triplets may be used to slow down the expression of some selected genes. In the model proposed by Gold (Gold et al., 1984) *infC* mRNA is translated in an IF3-independent manner due to the abnormal initiator triplet. The model predicts that when the IF3 level is low relative to the number of 30S ribosome subunits, IF3-independent translation of *infC* mRNA will be preferred to IF3-dependent translation of other cellular mRNAs, causing a relative increase in IF3 level. This model is supported from *in vivo* experiments (Butler et al., 1986) and mutation studies either in the initiation codon or in the Shine-Dalgarno sequence (Butler et al., 1987; La Teana et al., 1993).

1.2. Antibiotics acting on the ribosome

Antibiotics are extremely versatile in their modes of action. They have three main bacterial targets: bacterial cell-wall biosynthesis (i.e. lactamases, vancomycin), bacterial protein synthesis (i.e. macrolides, tetracyclines, aminoglycosides, oxazolidinones) and bacterial DNA replication and repair (i.e. fluoroquinolones) (reviewed in Walsh, 2000). Therefore, the ribosome is an important target for a wide variety of antibiotics. Most ribosomal antibiotics work by binding to specific sites on the ribosome and interfering

with its function during protein synthesis. In most cases, the target is ribosomal RNA rather than proteins (Gale et al., 1981).
Many antibiotics, such as streptomycin and tetracycline, were of great clinical importance when they were first discovered, but unfortunately strains of bacteria with resistance to these drugs have become common place, limiting their effectiveness (Neu, 1992). Some other ones, such as edeine or pactamycin, inhibit the initiation of protein synthesis in all phylogenetic kingdoms (Altamura et al., 1988), hence have little clinical relevance. Anyway, the universal effect of these antibiotics on initiation implies that structural elements important for the initiation process are universally conserved in prokaryotes and eukaryotes.
Structural information about ribosomal antibiotic-binding sites was first obtained by NMR studies of complexes of antibiotic agents with fragments of 16S rRNA (Fourmy et al., 1996). Recently, the high resolution structures of the ribosomal subunits (Ban et al., 2000; Schlünzen et al., 2000; Wimberly et al., 2000) have facilitated detailed elucidation of the actions of antibiotics in-situ (Brodersen et al., 2000; Carter et al., 2000; Nissen et al., 2000).
We will discuss in this work the structure of the 30S·edeine and 30S·tetracycline complexes.
The antibiotic edeine is a peptide-like compound produced by a strain of *Bacillus brevis*. Edeine contains a spermidine-type moiety at its C-terminal end and a beta-tyrosine residue at its N-terminal end (Kurylo-Borowska, 1975). Edeine acts on ribosomes of all kingdoms by affecting translation initiation (Odon et al., 1978). Tetracycline is a product of the aromatic polyketide biosynthetic pathways and belongs to a family of bacteriostatic antibiotics that act against a wide variety of bacteria. Tetracyclines inhibit protein synthesis by interfering with the binding of aminoacylated tRNA to the A-site of the 30S subunit (reviewed in Spahn and Prescott, 1996). However, ribosome-dependent GTP hydrolysis by elongation factor Tu (EF-Tu) is unaffected by tetracyclines. Hence, tetracyclines seem to have no effect on the initial binding of the ternary complex of EF-Tu·tRNA to the A-site (Gordon, 1969).

1.3 X-ray crystallography

X-ray crystallography is the most powerful technique currently available for studying the three dimensional structure of large molecules. X-ray crystallography studies on crystals of very large proteins or nucleic acid-protein complexes have yielded the complete structure at a level of resolution better than 3 Å.
A fundamental requirement for X-ray crystallographic studies is to obtain good diffracting crystals. A crystal is a three-dimensional periodic array that consists of a unit cell defined by the vectors **a**, **b**, **c**, replicated in space. The vertices of the cell define a crystal lattice. The periodicity of the array restricts observable scattering to a very limited set of geometry, which forms the reciprocal lattice of the crystal.
The X-ray scattering from an atom depends on its position in space and on the number of electrons it contains. The X-ray scattering can be described as the Fourier transform of the electron density of the object that generated it. Thus, if one could measure both the phase and the intensity of the scattered radiation, one could directly perform an inverse

Fourier transfer and reproduce the structure. Unfortunately, all one can measure is the intensity. In principle, the pattern of scattered intensities still contains sufficient information to reconstruct the array that generated it. However, this information is not as easy to use or interpret. The inverse Fourier transform of the scattered intensity is called the Patterson function, which is a map of all interatomic vectors. Thus, if the structure contains n atoms per unit cell, there will be n^2 vectors per unit cell of the Patterson function. For this reason the phase of the scattered radiation should be calculate independently using some special methods.

Phasing. The most commonly used method to determine the phases of unknown structures of biological macromolecules is the isomorphous replacement. This method is based on the changes in the structure factor amplitudes caused by the addition of heavy atoms to the native crystals. Successful derivatisation requires attachment of heavy atoms at a limited number of sites within the unit cell while keeping the crystal structure isomorphous to that of the native molecule. When only one derivative is available, the single isomorphous replacement procedure, which yields ambiguous phasing, is used. When more than one such derivatives can be obtained, multiple isomorphous replacement is used.
Since the differences in the intensities of the reflections of the native and derivatised crystals are used for phasing, the added compounds are chosen according to their potential ability to induce measurable signals. Useful heavy atom derivatives for proteins of average size consist of one or two heavy atoms. Because of the large size of the ribosome, ideal compounds for derivatisation are compact dense materials of a proportionally larger amount of electron, such as multi-metal salts or dense metal clusters.
The derivatisation of macromolecular crystals may be performed in two ways: by soaking crystals in solutions containing the heavy-atoms or by covalent specific binding of the heavy atom before crystallisation. Soaking is the more common way since the experimental requirements are rather simple and the results can be assessed in a relatively short time.
In case the derivatised crystal is truly isomorphous to the native one it may lead to the localisation of the heavy atoms. Thus the problem of determining the phases is reduced to the elucidation of several locations. Once the position of these atoms has been accurately determined, they are used to calculate a set of phases for data measured from the native crystals and for the construction of electron density maps.

An alternative method to determine the phases is the multi wavelength anomalous dispersion (MAD). Non isomorphism and high radiation sensitivity of the ribosomal crystals make MAD phasing advantageous, because it frees the measurements from comparison between crystals. MAD exploits the scattering effects of anomalous scatters (e.g. selenium), introduced into the molecule. These scatters may also be used as a reliable marker after phasing. However, the MAD experimental requirements are highly demanding, as the anomalous signals are significantly lower than those obtained per heavy atom with MIR.

Another method to determine the phases is the molecular replacement. In this case is necessary to know the structure of a similar macromolecule or part of the structure of interest in order to obtain a difference map that can help to solve the phase. This method can be used to locate substrate-binding or ligand-binding sites on a given macromolecule.

1.4 Crystallographic studies of the ribosome

Crystallographic studies on ribosome particles were started around 20 years ago. At the initial stages *E. coli* ribosomes were the main subject for crystallisation, because they are fully biochemically characterised. Unfortunately, these ribosomes were rather sensitive and unstable. Hence, only small two-dimensional arrays and micro three-dimensional crystals could be grown from them (Wittmann et al., 1982). The use of ribosomes from thermophilic and halophilic sources for crystallographic studies is favourable because of their relatively high stability. Originally it was not clear that phase information from such a large asymmetric unit could be obtained to high resolution, but the development of bright, tunable synchrotron radiation sources, large and accurate area detectors, vastly improved computing and the development of cryo-crystallography contributed to the success of structural studies of the ribosome.
The whole ribosome of *Thermus thermophilus* (T70S) in its complex with mRNA and aminoacyl-tRNA (Cate et al., 1999) and the large ribosomal subunit from *Haloarcula marismortui* (H50S) (Ban et al., 2000) crystallise well and the structures were solved at 7 and 2.4 Å respectively. The small ribosomal subunit exhibits the lowest level of stability and the highest level of flexibility among the ribosomal particles (Yonath et al., 1998; Harms et al. 1999). Nevertheless, crystals of the small ribosomal subunit from *Thermus thermophilus* (T30S) were obtained and the structure was solved at a resolution close to 3.0 Å (Schlünzen et al., 2000; Wimberly et al., 2000). The T30S subunit is composed of 19 proteins (S2-S20) (Tsiboli et al., 1994), a 26 amino acids peptide named Thx (Choli et al., 1993), and an rRNA chain (16S rRNA) of 1518 nucleotides for a total molecular weight of around 850 KDa. The electron density maps obtained for the T30S subunit reveal recognisable features, resembling those seen in the electron-microscopy (EM) reconstruction of the corresponding particles, obtained either by using averaging techniques of single particles or diffraction from ordered arrays (Stark et al., 1997).
Synchrotron radiation is required for all steps of the crystallographic analysis of the ribosomal particle, due to the extremely weak diffraction power of the ribosomal crystals. It was found that a moderate synchrotron radiation beam causes minor crystal damage but yields only medium resolution data. For collecting data at the higher resolution shells, beyond 5 Å, brighter synchrotron radiation is required. Under this conditions the 30S ribosomal crystals show severe radiation sensitivity even at helium stream temperatures, 15 to 25 K (Yonath et al., 1998). However, procedures overcoming part of the decay problem were developed for the crystals of the 30S subunit of the thermophilic ribosomes (Schlünzen et al., 1999).

Although currently the structure of the ribosomal subunits are known at a resolution of around 3 Å, questions concerning the efficiency, the fidelity and the control of the protein biosynthesis are still not fully answered. To better understand the mechanism of protein

synthesis, the structure of relevant functional complexes should be solved. Towards this goal, we focused on the crystallisation of the T30S subunit with oligonucleotides complementing specific regions of the 16S rRNA, with the initiation factor 3 and with different antibiotics.

1.5 *Thermus thermophilus* organism

The Thermus genera were first described in 1969 by von Brock and Freeze from microorganisms found in the sea-grass of thermal spring (*Thermus aquaticus*). *Thermus thermophilus* HB8 was isolated from Oshima and Imahori in 1971 from a thermal spring in Japan. It is a Gram-negative, not motile, not sporulating, strictly aerobe bacterium.
Thermus thermophilus cells grow at an optimal temperature of 75°C. The bacteria can grow at temperatures between 48°C and 85°C. The molecular mechanism of the heat-stability is very complicated since it affect the whole metabolism of the cells. All the studied biological macromolecules isolated from thermophilic organisms present higher stability compared with the homologous mesophilic molecules.
The high stability is an advantage for some industrial processes and can be also an important tool for checking catalytic reaction mechanisms of enzymes. Often the mesophilic enzymes lose their activity during the purification process or during long incubation times. This disadvantage can be reduced using the homologous thermophilic enzyme.
This special stability is used also for crystallisation of biological macromolecules. The progress done in ribosome structure using extremophilic bacteria is only one of the examples. The stability of the thermophilic or halophilic molecules reduces the risk to lose part of the protein during the isolation and purification of the ribosomes using for example high salt or sucrose gradient centrifugation. The use of thermophilic macromolecules for crystallographic studies is also an advantage since the crystals are less sensitive to radiation and the damage created from them is reduced. Indeed, in the last few years an increasing number of structures of macromolecules isolated from thermophilic organisms have been solved (Gilliland et al., 1994).