RIBOSOME: AN ANCIENT CELLULAR NANO-MACHINE FOR GENETIC CODE TRANSLATION

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Abstract   The ribosome is a ribozyme whose active site, the peptidyl transferase center (PTC), is situated within a highly conserved universal symmetrical region that connects all ribosomal functional centers involved in amino-acid polymerization. The linkage between this elaborate architecture and A-site tRNA position revealed that the A- > P-site passage of the tRNA terminus in the peptidyl-transferase center is performed by a rotatory motion, synchronized with the overall tRNA/mRNA sideways movement. Guided by the PTC the rotatory motion leads to stereochemistry suitable for peptide bond formation as well as for substrate mediated catalysis, consistent with quantum mechanical calculations illuminating the transition state mechanism for peptide bond formation and indicating that the peptide bond is being formed during the rotatory motion.

Analysis of substrate binding modes to inactive and active ribosomes illuminated the significant of PTC mobility and supported the hypothesis that the ancient ribosome produced single peptides bonds and non-coded chains, utilizing nucleotide conjugated amino acids. Genetic control of the reaction evolved after polypeptides capable of enzymatic function were created, and an ancient stable RNA fold was converted into tRNA molecules. As the symmetry relates only the backbone fold and nucleotides orientations, but not nucleotide sequence, it emphasizes the superiority of functional requirement over sequence conservation, and indicates that the PTC has evolved by gene fusion, presumably by taking advantage of similar RNA fold structures.

The increase in antibiotic resistance among pathogenic bacterial strains poses a significant health threat. Therefore, improvement of existing antibiotics and the design of advance drugs are urgently needed. Ribosomes provide binding sited for many antibiotic families, utilizing their inherent functional flexibility, which triggers induced fit mechanism by remote interactions, and facilitates antibiotics synergism as well as reshaping less suitable binding pockets,

leading to clinical usefulness even for antibiotics that bind to conserved functional regions. Exploitation of the diverse properties of antibiotics binding and benefiting from the detailed structural information that keeps emerging, should result in significant antibiotics improvement.

Introduction

Ribosomes, the universal cellular riboprotein assemblies, are the nano-machines which translate the genetic code into proteins. The translation process requires a complex apparatus composed of many components, among them the ribosome is the key player, as it provides the framework for the proper positioning of all other participants and is actively involved in the translation process. Ribosomes operate is each living cell continuously since the constant programmed cell death, which implies constant proteins degradation, requires simultaneous production of proteins. For example, hundreds of thousands of ribosomes are present in typical mammalian cells. Fast replicating cells, e.g. liver cells, may contain a few millions ribosomes. Even bacterial cells may contain to 100,000 ribosomes during their log period.

Within the framework of living cells, ribosomes are giant assemblies, composed of many different proteins (r-proteins) and long ribosomal RNA (rRNA) chains. The ratio of rRNA to r-proteins (~2:1) is maintained throughout evolution, with the exception of mammalian mitochondrial ribosome (mitoribosome) in which almost half of the bacterial rRNA is replaced by r-proteins, and consequently in mitoribosome the ratio of RNA to proteins is ~1/1. All ribosomes are constituted by two unequal subunits (Table 1). In prokaryotes, the small subunit, denoted as 30S, contains an RNA chain (16S) of about 1500 nucleotides and 20–21 different proteins, whereas the large subunit (called 50S in prokaryotes) has two RNA chains (23S and 5S RNA) of about 3,000 nucleotides in total, and different 31–35 proteins. In all organisms the two subunits exist independently and associate to form functionally active ribosomes.

The process of mRNA-encoded protein synthesis requires a complex apparatus composed of the ribosome, transfer RNA molecules (tRNA) and accessory protein factors. The mRNA chains are produced by the transcription of the segments of the DNA that should be translated. The mRNA chains carry the genetic information to the ribosomes, and tRNA molecules bring the cognate amino acids to the ribosome. For increasing efficiency, a large number of ribosomes act simultaneously as polymerases synthesizing proteins by one-at-a-time addition of amino acids to a growing peptide chain, while translocating along the mRNA template, producing proteins on

a continuous basis in an incredible speed (namely >20 new peptide bonds per second). While the elongation of the nascent chain proceeds, the two subunits perform cooperatively. The small subunit provides the path along which the mRNA progresses, the decoding center and the mechanism controlling translation fidelity, and the large subunit contains the site for the main ribosomal

TABLE 1. Ribosome composition.

Prokaryotic ribosome: Sedimentation coefficient: 70S
- **Small subunit: 30S**
  - One rRNA molecule (16S with 1500 nucleotides)
  - ~21 different proteins, called S1–S21
- **Large subunit: 50S**
  - Two rRNA molecules (5S and 23S, with 120 and 2,900 nucleotides, respectively)
  - ~31 different proteins, called L1–L31, among which only L12 is present in more than a single copy

Eukaryotic ribosomes: Sedimentation coefficient: 80S
- **Small subunit: 40S**
  - One rRNA molecule (18S with 1,900 nucleotides)
  - ~33 different proteins, called S1–S33
- **Large subunit: 60S**
  - Three rRNA molecules (5S, 5.8S and 28S, with 120, 156 and 4,700 nucleotides, respectively)
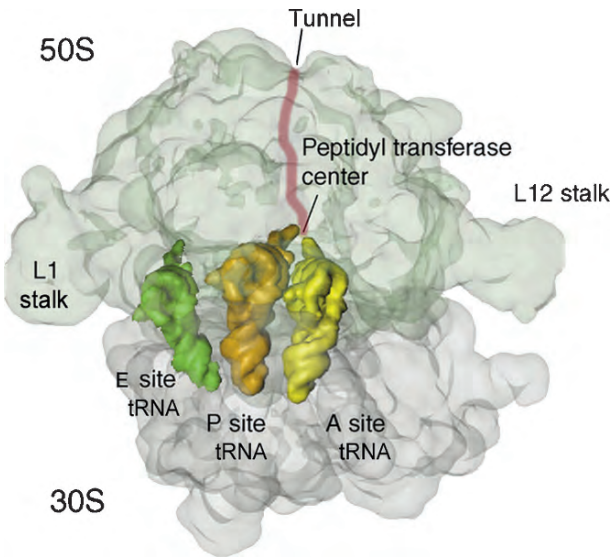 - ~50 different proteins, called L1–L50

Fig. 1. Structure and functional sites of bacterial ribosomes.

catalytic function, polymerization of the amino acids and provides the protein exit tunnel (Fig. 1).

The three-dimensional structures and secondary diagrams of all tRNA molecules from all living cells across evolution are alike, although each of them is specific to its amino acid. They are all built mainly of double helical L-shape molecules, made by a stem–elbow–stem organization, and contain an anticodon loop that matches its complementary three-nucleotide codes on the mRNA on one of their edges. About 70 Å away, at their 3′ end, the tRNA molecules contain a single strand with the universal sequence CCA, to which the cognate amino acid is bound by an ester bond. The tRNA molecules are the non-ribosomal entities combining the two subunits, as each of their three binding sites, A-(aminoacyl), P-(peptidyl), and (exit), resides on both subunits. At the A- and P-sites the tRNA anticodon loops interact with the mRNA on the small subunit, and the acceptor stem with the aminoacylated or peptidylated 3′ end are located on the large subunit. However, so far the involvement of the existing tRNA in codon-anticodon interactions remains unclear. Peptide bond is being formed during A- to P-site tRNA translocation, comprised of sideways shift and a ribosomal navigated rotatory motion. Nascent proteins progress along a dynamic tunnel and emerge into a shelter formed by ribosome-bound trigger-factor, acting as a chaperone preventing aggregation and misfolding.

The recent availability of over two dozens of crystal structures of bacterial ribosome and their complexes (see below), enabled a quantum jump in the understanding of the machinery of protein biosynthesis. These structures showed that in each of the two subunits the ribosomal proteins are entangled within the complex rRNA conformation, thus maintaining a striking dynamic architecture that is ingeniously designed for their functions: precise decoding; substrate mediated peptide-bond formation and efficient polymerase activity. This chapter focuses on the structural and dynamic properties of the ribosome that facilitate its function as an efficient nano-machine, addresses issues relating to the ribosome origin and attempt to answer the question: "Can structures lead to improved antibiotics?"

Historical Comments

Owing to the vast unpredictable expansion of studies on the various aspects of ribosome function and their significant clinical impact, time has come to shed light on selected points in the history of ribosome structural research. The involvement of RNA rich particles in genetic expression was suggested over 5 decades ago, when the so-called 'Palade particles' were located within RNA rich regions, in close association with the membrane of the endoplasmic

reticulum (Palade, 1955; Watson, 1963). The localization of the cellular translation site and the extensive biochemical, biophysical and genetic research that followed, led to illuminating findings about the overall nature of ribosome function. Nevertheless, because the structure of the ribosome was not available, detailed functional information could not be obtained. Moreover, owing to the huge size and the complexity of the ribosome, it was widely assumed that it would never be crystallized, and even if it was its structure would be extremely hard to be elucidated. Consequently, a series of hypothesis was accumulated, based on common wisdom combined with results of biochemical experiments. For example, originally, it was assumed that the decoding of the genetic information as well as the enzymatic activity of the ribosome is performed mainly (or solely) by the ribosomal proteins and that the r-RNA provides the scaffold holding the numerous ribosomal proteins in the stereochemistry allowing for performing their tasks (Garrett and Wittmann, 1973a, b).

Challenging the assumption that the r-proteins catalyze peptide bond formation by a series of biochemical experiments (Noller et al., 1992) was first met with some skepticism. However, as the major role played by RNA in various life processes (not only protein biosynthesis) became evident at approximately the same time, the notion that the main actor in ribosome function is rRNA was accepted within a relatively short period. In contrast, the latter example, namely that the nascent proteins progress on the ribosome surface until its maturation, was well implanted in researcher's mind. Hence, even after biochemical experiments indicated clearly protection of nascent chains by the ribosome (Malkin and Rich, 1967; Sabatini and Blobel, 1970) and three dimensional image reconstructions of electron microscopical data obtained from ordered array of ribosomes visualized the tunnel (Milligan and Unwin, 1986) or their large subunits (Yonath et al., 1987) doubt was publicly expressed (Moore, 1988; Ryabova et al., 1988). The notion that the ribosome indeed contains a long tunnel along which the nascent proteins progress on their way out became more acceptable only after verification by cryo electron microscopy (Frank et al., 1995; Stark et al., 1995), which was performed almost a decade after the tunnel first visualization (Milligan and Unwin 1986; Yonath et al., 1987) and 3 decades after the initial suggestion. Remarkably, even once a tunnel with dimensions matching accurately those predicted in the 1960s, namely about 100 Å in length (Malkin and Rich, 1967; Sabatini and Blobel, 1970) and observed experimentally and detected in a high resolution crystal structure, it was assigned a Teflon-like passive character, with no dynamic and/or chemical properties that allow its interactions with the progressing nascent chains (Ban et al., 2000; Nissen et al., 2000). This idea was conflicting previous observation (e.g. Crowley et al., 1993; Walter and Johnson, 1994; Nagano et al., 1991), and hence was soon challenged by various experimental and computational methods, including biochemistry,

electron microscopy and crystallography, as can be seen in the following partial ensemble of papers (e.g. Gabashvili et al., 2001; Nakatogawa and Ito, 2002; Gong and Yanofsky, 2002; Berisio et al., 2003a; Woolhead et al., 2004; Gilbert et al., 2004; Johnson, 2004; Ziv et al., 2005; Amit et al., 2005; Baram and Yonath, 2005; Mankin, 2006; Deane et al., 2007; Petrone et al., 2008; Mitra et al., 2006; Voss et al., 2006; Schaffitzel and Ban, 2007).

Twenty years passed from the first indications for potential organization of ribosomes by the growth of microcrystals diffracting to relatively high resolution, namely 3.5 Å (Yonath et al., 1980) to the vast expansion in the availability of ribosome structures. The shift from poorly diffracting microcrystals to high-resolution structures was achieved gradually, based on the presumption that the higher the sample conformational homogeneity the better the crystals, and that the preferred conformation is that of functionally active ribosomes. Assuming that the ribosomes of bacteria that grow under robust conditions are less sensitive to external conditions, we focused on such sources, and, indeed, the first three dimensional microcrystals were obtained from the large ribosomal subunits from *Bacillus stearothermophilus* (Yonath et al., 1980), a source considered to be almost an extremophile at the beginning of the 1980s. This hypothesis was verified thoroughwort, as by extensive systematic explorations for suitable bacterial sources it was found that the key for obtaining crystals suitable for crystallographic studies is to use ribosomes from relatively robust bacteria (Gluehmann et al., 2001) or ribosomes mutated in a fashion that minimizes their mobility, so that they can be trapped at a specific, albeit not necessarily functional, conformation (Schuwirth et al., 2005). An alternative strategy is to crystallize complexes of ribosomes with substrates, inhibitors and/or factors that can trap them at preferred orientations. Among such complexes are the initial crystals of the whole ribosome from *Thermus thermophilus* (T70S) with mRNA and tRNA molecules (Hansen et al., 1990) and of the large subunit from *Deinococcus radiodurans* (D50S) with the antibiotic clindamycin (Schluenzen et al., 2001).

Efforts aimed at the improvement of existing crystals included a through examination of the influence of the relative concentrations of mono- and di-valent ions (von Bohlen et al., 1991), on the growth and properties of the crystals from the large ribosomal subunits form *Haloarcula marismortui* led to dramatic improvements in the quality of these crystals. Furthermore, a constant refinements of bacterial growth pathways and its linkage to crystal growth (Auerbach-Nevo et al., 2005) alongside a thorough investigation on crystallization conditions in relation to those required for ribosome functional activity, indicated a noteworthy correlation between the conditions under which these ribosomes function and the quality of the resulting crystals. Remarkably, reasonable conformational rearrangements were observed for

flexible functional regions in ribosome crystals grown under conditions mimicking their physiological environment (Harms et al., 2001), whereas in ribosome crystals obtained under far from their physiological environment such regions may be highly disordered (Ban et al., 2000).

Alongside the improvement of the ribosome crystals, ribosome crystallography required the development of innovative methodologies. Examples are the bio-crystallography at cryogenic temperatures, which was introduced because of the extreme radiation sensitivity of the ribosomal crystals (Hope et al., 1989), and an unconventional use of multi-heavy atom cluster, which was found to play a dual role in the determination of the structure of the small ribosomal subunit from *Thermus thermophilus* (T30S). Thus, post crystallization treatment with these clusters, originally used for providing anomalous phasing power (Thygesen et al., 1996) increased dramatically the resolution of the X-ray diffraction from the initial 7–9 to 3 Å (Schluenzen et al., 2000) presumably by minimizing the internal flexibility involved naturally in mRNA binding to the ribosome and its progression through the ribosome (See below and in Bashan and Yonath, 2008a; Zimmerman and Yonath, 2009).

The currently available high resolution crystallographic structures of ribosomal particles and their complexes with substrate analogs, factors, inhibitors and antibiotics that target the ribosome (Ban et al., 2000; Nissen et al., 2000; Schluenzen et al., 2000, 2001, 2003, 2004, 2005; Wimberly et al., 2000; Carter et al., 2000; Brodersen et al., 2000; Pioletti et al., 2001; Carter et al., 2001; Yusupov et al., 2001; Harms et al., 2001, 2004; Clemons et al., 2001; Ogle et al., 2001, 2002; Brodersen et al., 2002; Hansen et al., 2002a, b, 2003; Bashan et al., 2003; Berisio et al., 2003a, b; Murphy et al., 2004, Murphy and Ramakrishnan, 2004; Schuwirth et al., 2005; Ferbitz et al., 2004; Petry et al., 2005; Yusupova et al., 2006; Korostelev et al., 2006; Dunham et al., 2007; Selmer et al., 2006; Wilson et al., 2005a, b; Baram et al, 2005; Baram and Yonath, 2005; Schmeing et al., 2005a, b; Davidovich et al., 2007, 2008; Pyetan et al., 2007; Weixlbaumer et al., 2007, 2008; Blaha et al, 2008; Ippolito et al., 2008; Tu et al, 2005; Schroeder et al., 2007; Evans et al., 2008; Simonovic and Steitz, 2008; Auerbach et al., 2009).

The crystal structures were soon accompanied by impressive advances in cryo electron microscopy (cryo EM) of ribosomal particles, which revealed additional elements of ribosomes functional dynamics (e.g. Agrawal et al., 1999; Frank et al., 2000; Stark et al., 2000; Gao et al., 2003; 2005; Valle et al., 2003a, b; Passmore et al., 2007) and shed more light on many issues concerning ribosomal mode of function. These include the initiation of the translation, which is among the most complex and the most divergent, tRNA selection, decoding, translational fidelity, peptide bond formation, polymerase activity, the energetic of the elongation cycle, translation termination etc. These, in turn, led to numerous studies, documented

in large number of original publication as well in many summarizing reviews, some of which are cited below (Thompson et al., 2001; Barta et al., 2001; Xiong et al., 2001; Bayfield et al., 2001; Polacek et al., 2001, 2003; Ramakrishnan and Moore, 2001; Ramakrishnan and Moore, 2002; Harms et al., 2002; Katunin et al., 2002; Ramakrishnan, 2002; Moore and Steitz, 2002, 2003, 2005; Yonath, 2002, 2003a, b, 2005a, b; 2006; Steitz and Moore, 2003; Rodnina and Wintermeyer, 2003; Beringer et al., 2003, 2005, 2007; Yonath and Bashan 2004; Thompson and Dahlberg, 2004; Auerbach et al., 2004; Zarivach et al., 2004; Agmon et al., 2005; Bashan and Yonath, 2005, 2008a, b; Ogle et al., 2003; Marintchev and Wagner, 2004; Nakatogawa and Ito, 2004; Fujiwara et al., 2004; Sievers et al., 2004; Cochella and Green, 2004; Blanchard et al., 2004; Gromadski and Rodnina, 2004; Weinger et al., 2004; Weingner and Strobel, 2006; Maier et al., 2005; Ogle and Ramakrishnan, 2005; Ogle and Ramakrishnan, 2005; Saguy et al., 2005; Nilsson and Nissen, 2005; Polacek and Mankin 2005; Sharma et al., 2005; Diaconu et al., 2005; Bieling et al., 2006; Kaiser et al., 2006; Sato et al., 2006; Gindulyte et al., 2006; Trobro and Aqvist, 2006; Wohlgemuth et al., 2006; Brunelle et al., 2006, 2008; Rodnina et al., 2007; Anderson et al., 2007; Shaw and Green, 2007; Fabbretti, et al., 2007; Uemura et al., 2007; Konevega et al., 2007; Wekselman et al., 2008; Petry et al., 2008; Steitz, 2008; Lang et al., 2008; Johansson et al., 2008; Beringer and Rodnina, 2007; Youngman et al., 2006, 2007, 2008; Lang et al., 2008; Zimmerman and Yonath 2009).

Architectural and Dynamic Aspects of the Initiation Step

The initiation of protein biosynthesis is an incredibly intricate and a highly divergent process (Marintchev and Wagner, 2004) This dynamic step is triggered by initiation factors and matures in constructing the initiation complex composed of the small ribosomal subunit, mRNA and an initiator tRNA. The performance of the initiation complex hinges on accurate positioning of messenger RNA on the small ribosomal subunit, which is a prerequisite for correct translation. Specific ribosome architectural elements allows for accurate tRNA selection by the ribosome in addition to codon-anticodon pairing that leads to a closure of the domains of the small subunit around cognate tRNA, believed to trigger subsequent steps in tRNA recognition and selection (Ogle et al., 2003). Similarly, mRNA positioning is navigated and controlled by the elaborate ribosomal architectural-design, a purine-rich sequence made of a few (up to ten) nucleotides, called "Shine–Dalgarno sequence" in prokaryote. This sticky sequence anchors the mRNA 5′-end onto the small subunit by forming numerous interactions including base pairing (Yusupova et al., 2006) mRNA binding involves a latch-like closing-opening mechanism (Schluenzen et al., 2000; Schuwirth et al., 2005), performed within the small subunit by

coordinated motions, which seem to be minimized by the entrance of the mRNA to its groove on the small subunit.

As mentioned above and in (Schluenzen et al., 2000; Bashan and Yonath, 2008a) the motions involved in mRNA attachment and progression could also be minimized chemically by post crystallization treatment using minute amounts of the heteropolytungstate $[(NH_4)_6(P_2W_{18}O_{62})14H_2O]$, which led to a increased dramatically the resolution of the X-ray diffraction (from 7–9 to 3 Å). Analysis of the tungstenated structure T30S structure indicating firm and quantitative attachment of 14 clusters to well defined locations on each subunit, within crystals containing more than two different conformations of the small subunit. This binding was later found to stabilize the selected functional conformation, called "open latch" (Schluenzen et al., 2000) that enables the attachment of the mRNA chain to the small subunit. Thus, four cluster that bind to the r-protein (S2), which is exposed on the ribosome surface, across from the flexible region, by making numerous interactions with the positively charged side chains of its extremely flexible C- and N-positively charged termini, hampered the motions of the entire region in a manner that consequently influenced the rigidity of the subunit and controlled the internal flexibility of the region surrounding the mRNA chain. Local ribosome mobility plays a major role also the selection of the following incoming aminoacylated tRNAs (aa-tRNAS) and in proofreading in translation (Blanchard et al., 2004; Murphy et al., 2004; Murphy and Ramakrishnan, 2004; Cochella and Green, 2004), exploiting the inherent flexibility of the decoding center for monitoring base pairing at the first two positions of each codon, but tolerating non-canonical base pairs at the third position.

Once the initiator tRNA binds to the start codon (AUG) at the P-site, downstream of the sticky sequence that anchors and direct the mRNA binding, the construction of the initiation complex is completed. The two subunits associate and intersubunit bridges are being formed by their conformational rearrangements (Bashan and Yonath, 2008b; Harms et al., 2001; Selmer et al., 2006; Korostelev et al., 2006; Yusupov et al., 2001). Among the newly formed intersubunit bridges, B1a, which is located near the entrance region of A-site tRNA, hence called the A-finger, and bridge B2a, the connecting element between the environs of the ribosomal active site, the peptidyl transferase center (PTC), in the large subunit with the decoding center in the small subunit, play key roles in substrate accommodation and positioning. Bridge B2a (helix H69 of the large subunit) which is located at the heart of the ribosome, is a highly flexible multi-task feature. In the assembled ribosome, regardless of its source, T70S or E70S (namely the *E. coli* ribosome) and/or and of the functional state, it stretches out towards the small subunit and interacts with both the A-site and the P-site tRNA molecules (Schuwirth et al, 2005; Selmer et al., 2006; Korostelev et al., 2006; Yusupov et al., 2001) whereas in

the unbound large subunit of, D50S, it is positioned at the subunit interface with a distinctly different conformation (Harms et al., 2001). The significant flexibility of the bridges that allows their conformational rearrangements seems also to facilitate deactivation of ribosomes under unfavorable condition. Thus, these bridges can become readily disordered under conditions far from physiological, as observed in crystal structures of the large ribosomal subunit from *Haloarcula marismortui*, H50S (Ban et al., 2000).

It is likely that the ribosome benefits from the flexibility of bridge B2a (helix H69) are beyond its participation in intersubunit bridging. Helix H69 and its extension, Helix H70, originate near the peptidyl transferase center (PTC) in the large subunit, and reach the vicinity of the decoding site in the small subunit. Connecting the two ribosomal active sites, this bridge seems to be designed for transmitting signals between them. Its proximity to both the A- and the P-site tRNAs in the large subunit suggests that it may also participate in translocation by assisting the shift of the A-site tRNA acceptor stem into the P-site. Helix H69 is also one of the constituents of the PTC upper side making crucial contributions to the remote interactions that govern the precise positioning and accurate orientation of the tRNA substrates (Bashan et al., 2003).

Motions of bridge B1a, which appears to act also as a guide for eliminating the entrance of A-site tRNA to uncontrolled directions, are limited by a particular r-protein, called L25, TL5 and CTC in *E. coli, T. thermophilus* and *D. radiodurans*, respectively. This protein seems to have evolved according to environmental conditions. In the mesophile *E. coli*, the single domain ribosomal protein (r-protein) L25 is located on the solvent side of this bridge, in a position enabling preventing slippage of this bridge towards the solution. In the thermophile *T. thermophilus* one of the two domains of r-protein TL5 replaced L25 and its second domain interacts with B1a from its other solvent side, so that the two domains can hinder almost all uncontrolled motions of this bridge towards the ribosome periphery, thus providing additional stability at high temperature. In *D. radiodurans*, an extremely robust organism that can survive under extreme stress conditions, including starvation, irradiation, high as well as low temperatures, this protein (called CTC, after a general shock protein) replaces the *E. coli* r-proteins L25 and its homologue TL5 in T50S. Within the known members of the CTC protein-family, that from *D. radiodurans* is the longest. It contains 253 residues, about 150 residues longer than L25 and 60 residues longer than TL5 and has three domains. The CTC N-terminus is similar to the entire L25, the middle domain is similar to the C-terminal domain of TL5, and the third domain can reach the A-site and restricts the space available for the tRNA molecules. As it is built of three long alpha helices connected by a pointed slim end that can function as a flexible hinge, CTC can control or even eliminate A-site RNA binding under

unfavorable conditions, hence may be parts of the mechanisms that *D. radiodurans* developed for survival under extremely stressful conditions (Yonath, 2002).

In prokaryotes, three non-ribosomal protein factors are involved in initiation. Among them initiation factor 3 (IF3) binds to the small subunit in proximity to the mRNA progressing channel so that its C-terminal domain interacts extensively with the flexible termini of r-protein S18 that perturb into the solution. Interestingly, one of the heteropolytungstate molecules that bind to the small subunit stabilizes the conformation of the flexible termini of the r-protein S18 in a fashion mimicking this r-protein involvement in the binding of the C-terminal domain of initiation factor IF3 and competes with its binding (Pioletti et al., 2001; Yonath and Bashan, 2004). In this position the C-terminal domain of IF3 can interfere with subunits association and promote the fidelity of P-site codon–anticodon interactions, by exploiting its inherent flexibility alongside the formation of transient perturbations in the 30S subunit. Once the initial P-site tRNA binds the two subunits associate to form the functional ribosome concurrently with IF3 dissociation (Fabbretti et al., 2007).

The Ribosome is an RNA Nano-machine Acting as a Polymerase

Simultaneously with the advancement of the mRNA/tRNA along the path in the small subunit, peptide bonds are being formed in the large subunit. This process involves also the translocation of the tRNA 3′ end from the A- to the P-site, the detachment of the P-site tRNA from the growing polypeptide chain, the passage of the deacylated tRNA molecule to the E-site and its subsequent release. In all currently available crystal structures of assembled ribosomes (Yusupov et al., 2001; Schuwirth et al., 2005; Selmer et al., 2006; Korostelev et al., 2006) as well as in the unbound subunits (Ban et al., 2000; Schluenzen et al., 2000; Wimberly et al, 2000; Harms et al., 2001) the intersubunit interface surfaces are rich in rRNA, and based on the high sequence conservation of these ribosomal regions, it seems that the intersubunit interfaces of all ribosomes, throughout evolution, are rich in rRNA. Since both the decoding center and the site of peptide bond formation (called the peptidyl-transferase-center or PTC) reside in proximity to the intersubunit interface, they are located in rRNA predominant environments. Hence, unlike typical polymerases, which are protein enzymes, the major player in ribosomes activities is RNA. However, although in addition to the structural observations, protease resistance and structural data implicated rRNA as the catalytic entity for peptide bond formation, and although it is likely that single peptide bonds can be produced solely by RNA, the polymerase activity of the ribosome, namely subsequent occurrence of peptidyl transfer by rRNA has not been fully demonstrated (Anderson et al., 2007).

Peptide bond formation, the detachment of the P-site tRNA from the growing polypeptide chain, the passage of the deacylated tRNA molecule to the E-site, where it is engaged by codon–anticodon interactions, and its subsequent release, occur simultaneously with the advancement of the mRNA. Amino-acylated tRNA molecules are the natural substrates of ribosomes. However, 'minimal substrates' or 'fragment reaction substrates' were, and still are the substrate analogs commonly used biochemically, although the time required for peptide bond formation is much longer than the time required by the real substrates, namely tRNA molecules. Importantly, the mystery of the increased duration of peptide bond formation by these single-bond substrate analogs was recently partially clarified, as it was shown that the excessive time is due to conformational rearrangements of the substrates, as well as of specific PTC components (Selmer et al., 2006; Yonath, 2003a). Consistently, it was found that the peptidyl transfer reaction is modulated by conformational changes at the active site, and this process consumes time (Schmeing et al., 2005a and b; Beringer and Rodnina, 2007; Beringer et al., 2005; Brunelle et al., 2006).

The 'fragment reaction substrates' analogs are basically derivatives of puromycin, and although they are capable of producing single peptide bonds, they were overestimated to be suitable to mimic the natural ribosome function. They were, and still are, being used in a series of crystallographic studies on complexes of the large ribosomal subunit from *Haloarcula marismortui* (H50S) with such minimal substrates. These complexes were obtained under far from optimal functional conditions and contain key PTC components in orientations that differ significantly from those observed in functional complexes of T70S ribosome. Noteworthy, the initial suggestion, that three specific rRNA nucleotides catalyze peptide bond formation by the general acid/base mechanism that was based on the crystal structure of complexes of the H50S with such minimal substrates (Nissen et al., 2000) was challenged almost instantaneously by a battery of biochemical and mutational studies (e.g. Polacek et al., 2001; Barta et al., 2001; Thompson et al., 2001; Polacek et al., 2001).

The current consensus view is consistent with ribosomal positional catalysis that is assisted by its P-site tRNA substrate. The importance of the accurate positioning f the substrates within the ribosome frame, accompanied by the key role that the tRNA interactions with 23S rRNA play in peptide bond formation on the ribosome, are currently widely accepted (e.g. Beringer et al., 2007; Bashan and Yonath 2008b) even by those who suggested originally that the ribosome catalyze peptide bond formation by acid/base mechanism (Simonovic and Steitz 2008). Nevertheless, the detailed mechanism of peptide bond formation is still a mystery, waiting for availability of structures of ribosomes containing both the aminoacylated and the peptidyl tRNA molecules

in the PTC, trapped at the midst of this reaction. Furthermore, so far very few studies deal with the process allowing the ribosome to act not only as the frame for peptide bond formation, but also as the entity providing the machinery for amino-acid polymerization. In contrast, most biochemical studies focused on either peptide bond formation or P-site tRNA release and the termination of the process.

It appears that the choice of substrate analogs is partially responsible to the misinterpretation. Thus, analysis of the structure of a complex of the large ribosomal subunit form D50S with a substrate analog mimicking the whole tRNA acceptor stem and its aminoacylated 3′ end, called ASM, which is the entire fraction of A-site tRNA that binds to the large subunit. This D50S-ASM complex was obtained under conditions similar to those found to be suitable for optimal ribosome function, advanced the comprehension of peptide bond formation by showing that rather than participating chemically in peptide bond formation, the ribosomal architecture positions the substrates in stereochemistry suitable for peptide bond formation, thus providing the machinery for peptide bond formation and tRNA translocation. (Bashan et al., 2003; Agmon et al., 2005). Furthermore, the ribosomal architecture that dictates "positional catalysis" to peptide bond formation revealed by this complex, also promotes substrate mediated chemical acceleration of peptide bond formation, in accord with the finding that rapid and smooth peptide bond formation requires full-length tRNA in both A and P sites, observed by various methods, including the usage of chemical (Brunelle et al., 2006; Weinger et al., 2004; Weinger and Strobel, 2006) mutagenesis (Youngman et al., 2006; Sato et al., 2006), computational (Trobro and Aqvist, 2006; Sharma et al., 2005; Gindulyte et al., 2006) and kinetic procedures (Beringer et al., 2005; Wohlgemuth et al., 2006; Beringer and Rodnina, 2007). Notably, the D50S-ASM complex (Bashan et al., 2003) is so far the only complex with A-site tRNA mimic extending beyond the tip of the tRNA 3′ end, although crystals supposed to contain assembled ribosomes with two and/or three tRNA molecules have been subjected to crystallographic analysis (Selmer et al., 2006; Korostelev et al., 2006).

Thus, it is clear that the ribosome provided the suitable stereochemistry and the geometrical means for substrate mediated catalysis. Nevertheless, despite intensive efforts to elucidate the details of peptide bond formation by various non-crystallographic methods, the elucidation of the detailed mechanisms that connect peptide bond formation to the polymerase function of the ribosome should benefit significantly from the availability of structures of complexes of ribosomes containing its two substrates at the A- and the P-sites (namely both the aminoacylated and the peptidyl tRNAs in the PTC) and a nascent protein trapped in the exit tunnel.

Is the Ribosomal Core an Optimized Ancient Entity?

Different from enzymes catalyzing a single chemical reaction and similar to polymerases, the ribosome provides the means not only for the mere formation of peptide bonds, but also, actually mainly, for the processivity of the reaction, namely for amino acid polymerization. Both tasks are governed by the ribosomal striking architecture, as it contains a highly conserved region of 180 nucleotides, relating by pseudo two-fold symmetry the rRNA fold and the orientations of its nucleotides, but not their sequences. This sizable intra-ribosomal pseudo symmetrical region is located within the otherwise asymmetric ribosome, and has been identified in all known structures of the whole ribosome or the large ribosomal subunits, regardless of their source, their functional state, or their kingdom of life. Particularity, the same sub-structure was identified in the cores of ribosomes from mesophilic, thermophilic, radiophilic and halophilic bacteria form eubacteria and archaea; in assembled empty or in complexes of them with substrates; in unbound and complexed large subunit, including complexes with ribosomal antibiotics and non ribosomal factors involved in protein biosynthesis (Agmon et al., 2005, 2006). Thus, despite size differences between the various kingdoms of life, the functional regions of the ribosomes are rather well conserved, with the highest level of sequence conservation at their central core, and the largest structural differences are located at the periphery. Remarkably, this core contains the symmetrical region in which 98% of the nucleotides are found in >95% of sequences (from 930 different species from the three domains of life), whereas only 36% of all *E. coli* nucleotides, excluding the symmetrical region, can be categorized as such. Importantly, 75% of the 27 nucleotides lying within 10 Å from the symmetry axis are highly conserved. Among them, seven are completely conserved (Agmon et al., 2006). This exceptional high conservation of the ribosome core, namely the symmetrical region, throughout revolution indicates that its structure is not sensitive environmental conditions, contrary to the large differences detected at the periphery of the ribosome, far away from the central core (Mears et al., 2002).

This symmetrical region includes the PTC and its environs, and connects all ribosomal functional regions involved in amino-acid polymerization, namely the tRNA entrance/exit dynamic stalks, the PTC, and the bridge connecting the PTC cavity with the vicinity of the decoding center in the small subunit and the nascent protein exit tunnel. Hence it appears that the internal symmetrical region is a universal feature facilitating peptide bond formation. Furthermore, as it is located at the heart of the ribosome, it can serve as the central feature for signaling between all the functional regions involved in protein biosynthesis, that are located remotely from each other (up to 200 Å away), but must "talk" to each other during elongation (Agmon et al., 2005; 2006). A suitable

example is the direct connection between peptide bond formation in the large subunit and the formation of the Shine–Dalgarno interactions on the small one (Uemura et al., 2007).

The PTC is located at the midst of this symmetrical region in the bottom of a V-shaped cavity and is built as an arched void. The complex D50S-ASM demonstrates the significance of the interactions between the tRNA acceptor stem and the cavity's walls. These key interactions seem to govern the accurate substrate positioning in the PTC, which has dimensions suitable for accommodating the 3′ ends of the A- and P-site tRNA molecules (Bashan et al., 2003) with significant tolerance (Yonath, 2003a) and a shape apt for accommodating the acceptor stems of both A- and P-tRNAs in configuration allowing for peptide bond formation. The linkage between the elaborate architecture of the symmetrical region and the position of the A-site tRNA, as observed crystallographically (Bashan et al., 2003) indicates that the translocation of the tRNA 3′ end is performed by a combination of two independent, albeit synchronized motions: a sideways shift, performed as a part of the overall mRNA/tRNA translocation, and a rotatory motion of the A-tRNA 3′ end along a path confined by the PTC walls.

This rotatory motion is navigated and guided by the ribosomal architecture, mainly the PTC rear wall that confines the rotatory path and the flexible nucleotides 2602 and 2585 (*E. coli* nomenclature) that anchor and seem to propel it. Hence, it appears that the ribosomal architecture and its mobility provides all structural elements enabling ribosome function as an amino acid polymerase, including the formation of two symmetrical universal base pairs between the tRNAs and the PTC (Bashan et al., 2003; Agmon et al., 2005), a prerequisite for substrate mediated acceleration (Weinger and Strobel, 2006) and for the direction of the nascent protein into the exit tunnel. Importantly, all nucleotides involved in this rotatory motion have been classified as essential by a comprehensive genetic selection analysis (Sato et al., 2006). Furthermore, the rotatory motion positions the proximal 2′-hydroxyl of P-site tRNA A76 in the same position and orientation found in crystals of the entire ribosome with mRNA and tRNAs, as determined independently in two laboratories (Selmer et al., 2006; Korostelev et al., 2006), and allows for chemical catalysis of peptide bond formation by A76 of the P-site tRNA (Weinger and Strobel, 2006).

Simulation studies indicated that during this motion the rotating moiety interacts with ribosomal components confining the rotatory path, along the 'PTC rear wall' (Agmon et al., 2005, 2006). Consistently, quantum mechanical calculations, based on D50S structural data, indicated that transition state (TS) of this reaction, namely peptide bond formation, is being formed during the rotatory motion and is stabilized by hydrogen bonds with rRNA nucleotides (Gindulyte et al., 2006) and is located between the A- and the P-sites at a position similar

to that found experimentally in the crystal structure of a complex made of the large subunit from a ribosome from a different source, H50S, with a chemically designed TS analog (Schmeing et al., 2005). The correlation between the rotatory motion and amino acid polymerization rationalize the apparent contradiction associated with location of the growing protein chain. Thus, the traditional biochemical methods for the detection of ribosome activity were based on the reaction between substrate analogs designed for producing a single peptide bond and do not involve A- to P-site translocation, whereas nascent protein elongation by substrates suitable to perform the A- to P-site passage occurs close to the P-site in a position close to that of properly designed TS analogs (Schmeing et al., 2005), near the P-site. Consistently, the difference between the formation of single peptide bond by minimal substrates and possessive amino acid polymerization highlights the PTC remarkable ability to rearrange itself upon substrate binding (Selmer et al., 2006; Yonath, 2003a) and with the finding that the peptidyl transfer reaction is modulated by conformational changes at the active site (Schmeing et al., 2005; Beringer et al., 2005; Beringer and Rodnina, 2007; Brunelle et al., 2006).

The rotatory motion machinery explains the remarkable difference between the formation of single peptide bond by minimal substrates and by full size tRNA, which can undergo subsequent amino acid polymerization. It is also connects the fragment reaction with the PTC remarkable ability to rearrange itself upon substrate binding (Selmer et al., 2006) by indicating that the peptidyl transfer reaction is modulated by conformational changes at the active site (Yonath, 2003a, Schmeing et al., 2005). Additionally, this mechanism indicates that dipeptides can be detected in the A-site simply because minimal substrates do not posses the structural element that can undergo the rotational motion, as they are too short. In addition, by their nature, the minimal substrate reaction are capable of a single peptide bond formation, hence, have cannot elongate. Thus, these finding contradict the notion that actual nascent chains are elongated at the A-site, and supports the finding that peptide bond formation that can lead to nascent chain elongation occurs during the rotatory motion (Gindulyte et al., 2006).

Remarkably, the high level of conservation of components of the symmetrical region that was detected even in mitochondrial ribosomes, in which half the ribosomal RNA is replaced by proteins and the ability of the symmetrical region to provide all structural elements required for performing polypeptide elongation, suggest that the modern ribosome evolved from a simpler entity that can be described as a pro-ribosome, by gene fusion or gene duplication. In particular, the preservation of the three-dimensional structure of the two halves of the ribosomal frame regardless of the sequence demonstrates the rigorous requirements of accurate substrate positioning in stereochemistry supporting peptide bond formation. This, as well as the universality of the

symmetrical region led to the assumption that the ancient ribosome contained a pocket confined by two RNA chains, which formed a dimer.

A feasible sequence of events, which is based on the hypothesis that the two halves of a structure resembling the symmetrical region were the core of the ancient ribosome, may shed light on the evolving complexity of the ribosome. The main assumption for this hypothesis is that the ancient machinery that could form peptide bonds was made exclusively from RNA chains that dimerize. It is conceivable that among other substituents, the primordial soup contained single nucleotides, short RNA segments, and RNA chains of significant size, of around 50–60 nucleotides, which survived since they acquired a stable conformation. These surviving ancient RNA chains were the ancestors of the RNA chains that construct the symmetrical region, and underwent dimerization as they posses the tendency to dimerize, similar to small RNA molecules that form dimers (Agmon et al., 2006).

The products of the dimerization yielded three-dimensional structures with a symmetrical pocket that could accommodate two amino acids facing each other, hence could trap the amino acids once they became available. The spontaneous reaction of peptide bond formation followed, and the structures that include the appropriate pockets for accommodating this reaction survived. As RNA chains can act as gene-like molecules coding for their own reproduction, it is conceivable that the surviving ancient pockets became the templates for the ancient ribosomes. In later stage these primitive RNA genes underwent initial optimization to produce a more defined, relatively stable pocket, and when a clear distinction was made between the amino acid and the growing peptidyl sites, each of the two halves was further optimized for its task so that their sequences evolved differently.

In parallel, the substrates of the ancient ribosomes, which were initially single amino acids, dipeptides or short polypeptides, evolved to allow accurate binding. Compounds mimicking the termini of the modern tRNA molecules (part or the entire amino acylated CCA 3′ end) provide more control on the reaction, its reactant and its products. Consequently, these were converted into compounds with a contour that can complement the inner surface of the reaction pocket. The binding affinity could be increased by enlarging the nucleic acid component, exploiting double or triple nucleotides, similar to the universal CCA end of the modern tRNA molecules. Later, for increasing specificity, these short RNA segments were extended to larger structures by their fusion with RNA stable features, to form the ancient tRNA. Later, RNA chains capable of storing, selecting and transferring instructions for producing useful proteins became available. Subsequently, the decoding process was combined with peptide bond formation. A single molecule evolved, capable of not only carrying the amino acids while bound to them, but also translating the genomic instructions, by adding a feature similar to the modern anticodon arm to the ancient tRNA structure.

To conclude: the ancient ribosome appears to be a simple ribozyme that produces peptide bonds, utilizing nucleotide conjugated or otherwise activated amino acids. The formation of single peptide bonds seems to be accidental or sporadic, thus not controlled by genetic instructions. Since the products of this reaction may be substrates for it, elongation of the dipeptides could occur. Only when these polypeptides acquired capacity to perform enzymatic tasks, the information about their desired structure was stored in genes. Consequently, molecules capable of decoding this information simultaneously with transporting the cognate substrates evolved and the pro-ribosome acquired properties enabling the smooth translation of genetic information into proteins.

Chaperoning Events Within the Tunnel and at Its Opening

As mentioned above, the nascent proteins progress towards their emerging site through their exit tunnel, a universal feature of the large ribosomal subunit that lies adjacent to the PTC (Harms et al., 2001; Nissen et al., 2000) and has the dimensions of (~120 Å in length and varying diameter, 10–25 Å). Lined primarily by rRNA with a few r-proteins reaching its walls from its exterior, this tunnel may provide a crevice adjacent to the tunnel wall that may provide space for cotranslational transient folding (Amit et al., 2005), which is predominantly suitable for hydrophobic regions of the translated proteins (e.g. Woolhead et al., 2004). Additionally, this tunnel possesses the dynamics required for gating and discriminating events and plays an active role in sequence-specific arrest of nascent chains and in responding to cellular signals. Examples include: (i) The secretion monitor (SecM) system, which contains a 17 amino acids sequence that under specific cellular conditions can interact with the tunnel gating components, thereby interfering with their own translation elongation (Nakatogawa and Ito, 2004; Woolhead et al., 2006); (ii) the 24 amino acid sequence of TnaC that appears to be involved in gene expression regulation by influencing the RF2-mediated cleavage, or by introducing PTC rearrangements (Cruz-Vera et al., 2006); (iii) hydrophobic trans membrane sequences that seem to controls the operational mode of the translocon at the ER membrane while being synthesized (Woolhead et al., 2004). Nevertheless, despite recent intensive research, performed by several groups (e.g. Petrone et al., 2008; Voss et al., 2006) the mechanisms facilitating tunnel's recognition are still to be revealed. Generation of homogenous and stable complexes of ribosome with nascent chains suitable for crystallographic studies (Schaffitzel and Ban, 2007) should provide some of the answers. So far the tunnel wall elements that appear to sense nascent-peptide specific sequences include the r-proteins L22 (Berisio et al., 2003a) that forms the tunnel's constriction together with protein L4 and L23 (Baram et al.,

2005; Baram and Yonath, 2005), within a cascade of rRNA features (Mitra et al., 2006).

At the end of their way, before gaining sufficient length to acquire the final fold, nascent proteins emerge from their protective exit tunnel into the crowded cellular environment. To assist correct folding and prevent aggregation under the cellular unfavorable conditions, all cells contain molecular chaperones, exerting various effects. In eubacteria, the first chaperone uncounted by the emerging nascent chain, called trigger factor (TF), binds to the translating ribosome and prevents aggregation and misfolding at 1:1 stoichiometry, by interacting with ribosomal proteins L23 and L29 (Baram et al., 2005; Schluenzen et al., 2005). Protein L23 belongs to the small group of ribosomal proteins that display a significant divergence from conservation and only in eubacteria it possesses a sizable elongated loop, which extends from ribosome exterior all the way into the tunnel walls. At this position the L23 extended loop can undergo allosteric conformational changes that, in turn, can modulate the shape of the tunnel, in a fashion implying a trafficking of the nascent protein (Baram and Yonath, 2005; Baram et al., 2005).

Studies aimed at revealing the mode of function of TF were carried out using a chimeric (Ferbitz et al., 2004; Maier et al., 2005; Rutkowska et al., 2008), as well as a physiological (Baram et al., 2005; Schluenzen et al., 2005) complexes of TF binding domain and large ribosomal subunits, alongside a complex of the entire TF with full length short proteins (Martinez-Hackert and Hendrickson, 2007). The crystal structures of physiologically meaningful complexes of D50S with TF binding domain (called TFa) (Baram et al., 2005; Schluenzen et al., 2005) revealed that TFa provides a shelter for the emerging nascent protein and undergoes conformational rearrangements. As a result of these rearrangements a sizable hydrophobic region becomes exposed thus acquiring a configuration suitable to adhere to hydrophobic patches on the nascent chain. Consistent with dynamic studies (Kaiser et al., 2006), it appears that TFa prevents the aggregation of the emerging nascent chain by transiently masking exposed hydrophobic surfaces on the elongating polypeptide chains until they can be buried in the interior of the mature protein (Yonath, 2006).

The Dual Contributions of Ribosomal Proteins

The significance of the contribution of the ribosomal proteins to ribosome function and to the integrity of the ribosomal particles has undergone dramatic alterations. As described above, originally the proteins were thought to carry out the catalytic tasks of the ribosome, but when it became clear that the ribosome is a ribozyme, the proteins were suggested to be mainly the

entities stabilizing the rRNA structure (Brodersen et al., 2002; Klein et al., 2004). Previous and recent biochemical experiments, combined with careful analysis of the ribosome crystal structures, indicated that r-proteins, which are situated in proximity to ribosomal functional regions, may support these functions by controlling their fidelity and/or facilitating specific activities, such as influencing ribosomal accurate function. Proteins S5, S6, S12 that affect mRNA binding accuracy reside in the small subunit (Cochella and Green, 2004), whereas proteins L2 and L27 seem to be involved in the ribosomal polymerase function (Cooperman et al., 1995; Maguire et al., 2005).

Proteins S12 and L2 are among the few proteins that are located partially on the rRNA rich intersubunit interface. Remarkably, computational methods showed that the most ancient ribosomal proteins are these two proteins (Sobolevsky and Trifonov, 2005), consistent with the hypothesis that the first proteins were incorporated into the ribosome in order to increase the fidelity and efficiency of the ancient RNA-only ribosome. These two proteins belong to the group of proteins that were found to be involved in the catalytic reaction of the ribosome. This group includes also L16 and L27, which seem to assist efficient peptide bond formation. Protein L16 contribute to correct positioning of the A-site tRNA acceptor stem (Harms et al., 2001; Bashan et al., 2003) and hence plays an important role in acquiring the right orientation of its aminoacylated CCA end within the PTC. Notably, similar to the symmetrical region, L16 displays conserved tertiary structure but diverged primary sequence. In addition to L16, the finding that the N-terminal tail of L27 can be cross-linked to A76 of P-site tRNA, the observation that deletion of L27 gene results in severe defects in cell growth (Maguire et al., 2005), and the likelihood that this L27 resides close to the PTC (Selmer et al., 2006) indicate its possible involvement in peptide bond formation. Protein L2 it is not required for peptide bond formation, but seems to play a key role in the processivity of the amino acid polymerization (Cooperman et al., 1995). As L2 is the only protein interacting with both the A- and the P-regions (Agmon et al., 2005), it appears that its main function is related to its interactions with the symmetrical region.

Several r-proteins facilitate ribosomal dynamic functions. Proteins L22 and L4 that line the tunnel walls and appear to be involved in tunnel gating (Berisio et al., 2003a), and protein L23 that may control nascent protein trafficking (Baram et al., 2005). Large scale rearrangements in the tRNA configuration, as well as lateral movements of the two large subunit stalks, called L7/L12 (the entrance door) and L1 (the exiting gate) have been detected, based on comparisons of crystals structures of ribosomes trapped at various functional states and results of various methods, including cryo electron microscopy and single-molecule fluorescence spectroscopy. Proteins L1 and L12, the main protein component of the L1 and the L7/L12 stalks, are dynamically

involved in tRNA translocation. L12, which is the only r-protein that appears in more than a single copy in the ribosome, binds directly to several elongation factors (Kavran and Steitz, 2007; Helgstrand et al., 2007; Zavialov et al., 2005; Wilden et al., 2006; Diaconu et al., 2005).

The task of L11, another component of this stalk, seems to be associated with rejection of unfavorable compounds. Thus it was found to interact with the antibiotic thiostrepton (Bowen et al., 2005), but it is not vital for cell life and its depletion causes thiostrepton resistance. Additional examples for proteins that bind to non-ribosomal factors or inhibitors are proteins S18 that interacts with initiation factor IF3 (Pioletti et al., 2001). Notably, the translational factors functioning at the subunit–subunit interactions are conserved in phylogenetically distant species despite highly divergent environments to which these species have adapted (Thompson and Dahlberg, 2004). Of interest is protein L25 (called also CTC) that, as described above, seems to control the creation of one of the intersubunit bridges (called the A-finger or H38) as well as to regulate A-site tRNA incorporation (Harms et al., 2001; Yonath, 2002).

Can Structures Lead to Improved Antibiotics?

The intensive research on ribosomes has some practical aspects; one of them has clinical relevance since many antibiotics target the ribosome. The increasing incidence of antibiotic resistance and toxicity creates serious problems in modern medicine, combating resistance to antibiotics has been a major concern in recent years. The vast amount of structural data on ribosomal antibiotics accumulated recently may be useful for this aim.

Antibiotics are compounds used in clinical medicine for treating bacterial infections inhibit selectively bacterial ribosomes and not the eukaryotic ones. More that 40% of the useful antibiotics interfere with the biosynthetic machinery and most of them target the ribosomes at distinct locations within functionally relevant sites. These act by diverse mechanism, many of them were revealed by analysis of crystallographic results (for review see Auerbach et al., 2004; Yonath and Bashan 2004; Yonath, 2005a; Tenson and Mankin, 2006; Poehlsgaard and Douthwaite, 2005; Bottger, 2006; 2007; Mankin, 2001, 2006, 2008). These analyses showed that the ribosomal antibiotics hamper protein synthesis in bacterial pathogens by diverse mechanism, including causing miscoding, minimizing essential mobility, interfering with substrate binding at the decoding center and at the PTC, or blocking the protein exit tunnel. Common to ribosomal antibiotic is that their binding pockets coincide with functionally critical centers of the ribosome, and as most of the ribosomal functions are performed by the rRNA, the antibiotics binding

sites are composed primarily of rRNA. Numerous structural, biochemical and genetic studies provided indispensable information that illustrated the basic mechanisms of ribosomal antibiotics activity and synergism; provided the structural basis for antibiotic resistance and enlightened the principles of antibiotics selectivity, namely the discrimination between pathogens and humans, the key for therapeutical usefulness.

By its nature, X-ray crystallography should be the choice method for investigating ribosome–antibiotics interactions. However, since X-ray crystallography requires diffracting crystals, and since so far no ribosomes from pathogenic bacteria could be crystallized, currently the crystallographic studies are confined to the currently available crystals of suitable pathogen models. Currently available are high-resolution structures of complexes of antibiotics with ribosomal particles from the eubacterium *Deinococcus radiodurans*, a species suitable to serve as a pathogen model; as well as complexes obtained form antibiotics bound to ribosomes from the Dead Sea archaeon *Haloarcula marismortui* that resembles eukaryotes in respect to antibiotics binding site. Importantly, enormously high concentrations were needed for obtaining these complexes, consistent with their high similarity to eukaryotic ribosomes. As seen below, comparisons between the two types of complexes proved indispensable for increasing our understanding on antibiotics action.

The structures of its large ribosomal subunit D50S complexed with various antibiotics determined so far (Schluenzen et al., 2001, 2004; Berisio et al., 2003a, b; Auerbach et al., 2004, 2009; Yonath and Bashan 2004; Harms et al., 2004; Pyetan et al., 2007; Yonath, 2005a and b; Davidovich et al., 2007, 2008; Vazquez-Laslop et al., 2008) revealed that among the modes of action of ribosomal antibiotics are causing miscoding, minimizing essential mobility, interfering with substrate binding at the decoding center and at the PTC, or blocking the protein exit tunnel. Common to ribosomal antibiotic is that their binding sites are composed primarily of rRNA and coincide with functionally critical centers of the ribosome. Furthermore, comparisons between these structures demonstrated that members of antibiotic families possessing common chemical elements with minute differences might bind to ribosomal pockets in significantly different modes, and that the nature of seemingly identical mechanisms of drug resistance may be dominated by the antibiotics' chemical properties.

A major issue concerning the clinical usefulness of ribosomal antibiotics is their selectivity, namely their capabilities in the discrimination between the ribosomes of the eubacterial pathogens and those of eukaryotes. As described above, although prokaryotic and eukaryotic ribosomes differ in size (~2.4 and 4 Mega Dalton, respectively), their functional regions, which are the targets for the antibiotics, are highly conserved. Therefore the imperative distinction between eubacterial pathogens and mammals, the key for antibiotics

usefulness, is achieved generally, albeit not exclusively, by subtle structural difference within the antibiotics binding pockets of the prokaryotic and eukaryotic ribosomes. A striking example for discrimination between pathogens and humans is the immense influence of the minute difference between adenine and guanine in position 2058. This small difference was found to govern the binding of macrolides, a prominent antibiotics family (Lai and Weisblum, 1971) that obstructs the progression of the nascent proteins within the tunnel. However, although 2058 identity determines the antibiotic affinity, this analysis showed that the mere binding of the antibiotics is not sufficient for obtaining efficient therapeutical effectiveness. Comparisons between crystal structures of antibiotics bound to the eubacterial large ribosomal subunit, D50S to structures of complexes of the large ribosomal subunit from H50S, an archaeon sharing properties with eukaryotes, which required either extremely high antibiotics concentrations (Hansen et al., 2002a, 2003) or G2058A mutations, which facilitates macrolides/ketolides binding (Tu et al., 2005), indicated the significance of additional structural elements (Yonath and Bashan, 2004) of the binding pocket, which dictate inhibitory activity. Similar observations were made by mutagenesis in the yeast *Saccharomyces cerevisiae* at a position equivalent to *E. coli* A2058, which allows erythromycin binding but does not confer erythromycin susceptibility (Bommakanti et al., 2008).

The fine details of binding, resistance and selectivity of the members of the macrolide family and its off springs, namely the azalides and ketolides, presents additional issues, as the sequence specificity that determines the susceptibility and the fitness cost of the ketolides (Schlunzen et al., 2003; Pfister et al., 2005). Another intriguing issue, which led to continuous expansion of research and consequently to new insights, relates to the nature of the contributions of two ribosomal proteins, namely L4 and L22. The two proteins line the exit tunnel at its constriction, and do not interact directly with most of the members of the macrolides family, yet several types of mutations at their tip acquire resistance to them (Gregory and Dahlberg, 1999; Davydova et al., 2002; Lawrence et al., 2008; Berisio et al., 2006; Zaman et al., 2007; Moore and Sauer, 2008), presumably by perturbing the rRNA structure at the tunnel walls (Gregory and Dahlberg, 1999; Lawrence et al., 2008).

Attempts aimed at alleviating the resistance problem include the developments of synergetic antibiotics. An example is the very potent family of the streptogramins, a two-component antibiotics drug family, each of which is a rather weak drug. The impressive synergetic effect of this family can be understood by examining the mechanism exploited by the rather recent antibiotic drug, synercid. This mechanism is based on the binding of one of the components to the PTC that causes a dramatic alteration in the orientation of the very flexible nucleotide, U2585, which plays a principal role in the

A- to P-site rotatory motion, and the fixation of the altered orientation by the second compound that binds at the tunnel entrance (Harms et al., 2004; Yonath and Bashan, 2004).

Even subtle differences, such as the identity of nucleotide 2058 (A in eubacteria, G elsewhere) hardly exist in the PTC. Therefore, obtaining selectivity in antibiotics binding to the PTC, the core catalytic center of the ribosome, is more complex. Nevertheless, some of the PTC antibiotics bind to the PTC of eubacterial ribosomes with high affinity and great specificity, without significant effect on the eukaryotic hosts. The crystal structures of ribosomal complexes with antibiotics indicated that the PTC provides binding sites to several clinically useful antibiotics, called below the super family of PTC antibiotics, shed light on general as well as specific properties of the interactions of the members of this family with their binding pockets in the PTC. This family includes chloramphenicol, clindamycin, pleuromutilins, streptogramins$_A$ and oxazolidinones and lankacidins. Although basically all PTC antibiotics act by blocking part or the entire PTC, they utilize different binding modes and consequently they possess various inhibitory mechanisms. Thus, chloramphenicol was found to hamper the binding of the A-site tRNA, like (Schluenzen et al., 2001), the pleuromutilins, linezolid and streptogramins$_A$ occupy both the A- and the P-site tRNAs (Harms et al., 2004; Hansen et al., 2003; Ippolito et al., 2008), and clindamycin interfere with the peptide bond formation (Schluenzen et al., 2001).

Contribution of several PTC flexible nucleotides to productive binding was also observed by investigating the mode of action of the pleuromutilin family, which revealed a unique inhibitory mechanism alongside a novel selectivity and resistance strategies. In particular, the elaborate pleuromutilins binding mode demonstrates how selectivity and resistance are acquired despite almost full conservation (Davidovich et al., 2007, 2008; Schluenzen et al., 2004). As all nucleotides in the immediate vicinity of the binding site are highly conserved, pleuromutilins selectivity is determined by nucleotides that are not located in the immediate vicinity of the antibiotic binding site, hence are less conserved. Thus, pleuromutilins binding triggers an induced-fit mechanism by exploiting the flexibility of the rRNA nucleotides residing in and around the PTC, as well as a network of interactions with contacts with less conserved remote nucleotides, hence allowing for drug selectivity (Davidovich et al., 2007, 2008). In particular, this family exploits the remote interactions that effect the positioning of the extremely flexible nucleotide U2506, as well as of U2585 that participate in navigating and anchoring the rotatory motion. These interactions evacuate the binding region and at the same time tighten the binding pocket on the bound antibiotic molecule, without interacting with it. As mutations within the PTC should be lethal,

resistance to pleuromutilins requires mutations or modifications of nucleotides residing either in PTC components with identity that is less crucial for ribosome function, or in the PTC environs rather than within the core of the binding pocket, therefore should occur in a relatively slow pace. Remarkably, these crystallographic studies led the way to attempts to produce advanced compounds (Lolk et al., 2008). Indeed, cross resistance was detected between all PTC antibiotics, regardless of their mode of binding, and the nucleotides mediating it are residing only on one side of the PTC, similar or in close proximity to those acquiring resistance to the pleuromutilins.

Current attempts to overcome antibiotics resistance and increase their selectivity are being made (e.g. Yassin et al., 2005; Wilson et al., 2005a; Bottger, 2007). Those include several strategies, including the insertions of moieties that should compensate for the lost interactions of the resistant strains, benefiting from synergism of known or novel compounds possessing inhibitory properties of various levels of potency, and reviving "forgotten" antibiotics families, such as the lankacidins. Furthermore, as for each antibiotic family most eubacteria belonging to it utilize similar structural principles for selectivity and resistance, comprehending the factors allowing for selectivity should provide powerful tools to understand many of the mechanisms exploited for acquiring resistance. Therefore, the lessons learned from ribosome crystallography concerning combating resistance to antibiotics targeting the ribosome, are rather optimistic, as these studies opened new paths for antibiotics improvement. Thus, the elucidation of common principles of antibiotics action, combined with the variability in binding modes; with the revelation of the common as well as unique structural bases to antibiotics resistance mechanism; with the discovery that remote interactions can govern induced fit mechanisms enabling species discrimination even in highly conserved regions, and with the identification of deleterious mutations in the ribosomal RNA that can become potential targets for antibiotics, justify expectations for structural based improved properties of existing compounds as well as for the development of novel drugs.

The currently available crystal structures are of native and complexed ribosomal subunits from two eubacteria and one archaeon with substrate-analogs or inhibitors (including antibiotics). These are the small ribosomal subunit from *Thermus thermophilus* (called T30S), the large subunit from *Deinococcus radiodurans* (called D50S), and the large ribosomal subunit of the archaeon *Haloarcula marismortui* (called H50S). Also available are two structures of assembled ribosome complexed with their substrates from *Thermus thermophilus* (called T70S) and of empty ribosomes from *E. coli* (called E70S).

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