HIBERNATING BEARS, ANTIBIOTICS AND THE EVOLVING RIBOSOME

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by

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ABSTRACT

High resolution structures of ribosomes, the cellular machines that translate the genetic code into proteins, revealed the decoding mechanism, detected the mRNA path, identified the sites of the tRNA molecules in the ribosome, elucidated the position and the nature of the nascent proteins' exit tunnel, illuminated the interactions of the ribosome with non-ribosomal factors, such as the initiation, release and recycling factors, and provided valuable information on ribosomal antibiotics, their binding sites, modes of action, principles of selectivity and the mechanisms leading to their resistance. Notably, these structures proved that the ribosome is a ribozyme whose active site, namely where the peptide bonds are being formed, is situated within a universal symmetrical region that is embedded in the otherwise asymmetric ribosome structure. As this symmetrical region is highly conserved and provides the machinery required for peptide bond formation and for the ribosome polymerase activity, it may be the remnant of the proto-ribosome, which was a dimeric prebiotic machine that formed peptide bonds and non-coded polypeptide chains. Structures of complexes of ribosomes with antibiotics targeting them, revealed the principles allowing for the clinical use of antibiotics, identified resistance mechanisms and showed the structural bases for discriminating pathogenic bacteria from hosts, hence providing valuable structural information for antibiotics improvement and for the design of novel compounds that can serve as antibiotics.

INTRODUCTION

An adult human body contains approximately 100 trillion (10^{14}) cells. There is a major disparity between the numbers of proteins in various mammalian cells. There are over 7,000 different types of proteins in typical eukaryotic cells; the total number depends on the cell class and function. Liver cells contain up to 10,000 different proteins, the abundance of which varies widely, from 20,000 molecules for the rather rare proteins that bind the hormone insulin, to the plentiful structural protein actin, with a number molecules that can reach over 5 billions. Proteins (also known as polypeptides)

are made of amino acids arranged in a linear chain that folds into globular or fibrillar forms, depending on the sequence of their amino acids, which is defined by the sequence of a gene that encoded in the genetic code.

Proteins are constantly being degraded. Therefore simultaneous production of proteins is required. The translation of the genetic code into proteins is performed by a complex apparatus comprising the ribosome, messenger RNA (mRNA), transfer RNAs (tRNAs) and accessory protein factors. The ribosome, a universal dynamic cellular ribonucleoprotein complex, is the key player in this process, and typical mammalian cells can contain over a million ribosomes (the 'factories' that translate the genetic code into proteins). Even bacterial cells contain ~100,000 ribosomes. Many ribosomes act simultaneously along the mRNA, forming superstructures called polysomes. They act as polymerases synthesizing proteins by one-at-a-time addition of amino acids to a growing peptide chain, while translocating along the mRNA template.

Ribosomes act fast and efficiently, producing proteins on a continuous basis at an incredible speed, of ~20 peptide bonds per second. 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, in which almost half of the bacterial rRNA is replaced by r-proteins. All ribosomes are constituted by two unequal subunits. 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 3000 nucleotides in total, and 31–35 different proteins. In all organisms the two subunits exist independently and associate to form functionally active ribosomes. In each, the ribosomal proteins are entangled within the complex rRNA conformation, thus maintaining a striking dynamic architecture that is ingeniously designed for ribosome functions: precise decoding, substrate mediated peptide-bond formation and efficient polymerase activity.

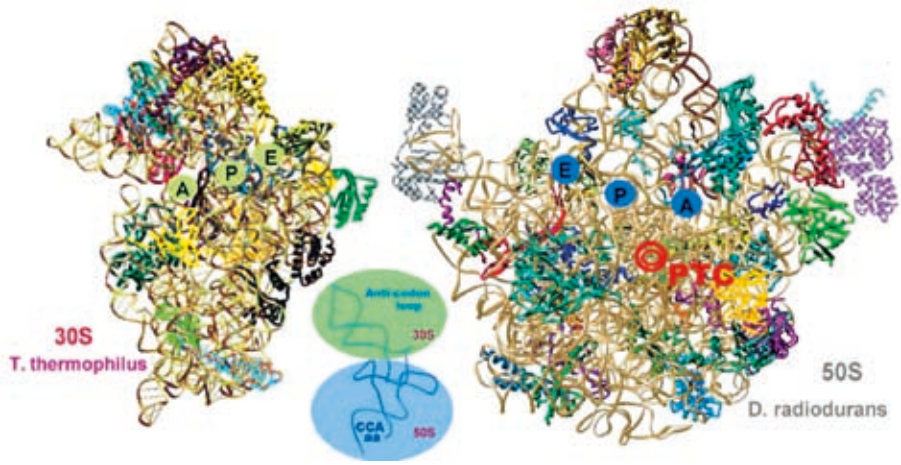

Figure 1. The structures of the ribosomal subunits: The three dimensional structures of the two ribosomal subunits from eubacteria, with a tRNA molecule, their substrate, placed between them. The interface surfaces are shown, as seen in the 3 structures of the two ribosomal subunits of the eubacterium *D. radiodurans* and *T. thermophilus*. The r-RNA is shown in brownish colors, and each of the r-proteins is painted in a different color. Note that these interfaces are rich in RNA. Insert: the backbone of a tRNA molecule. The circles designate the regions interacting with each of the ribosomal subunits. The approximate site of the PTC is marked in red.

Other players in the process are messenger RNA (mRNA), which carries the genetic code and transfer RNA molecules (tRNA) that bring the cognate amino acids to the ribosome. The three-dimensional structures of all tRNA molecules from all living cells across evolution are alike, although each of them is specific to its amino acid (Figure 1). They are built mainly of double helical L-shape molecules in a stem-elbow-stem organization, and contain a loop complementing the three-nucleotide codes on the mRNA. About 70 away, at their 3'end, they 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 (Figure 1). 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.

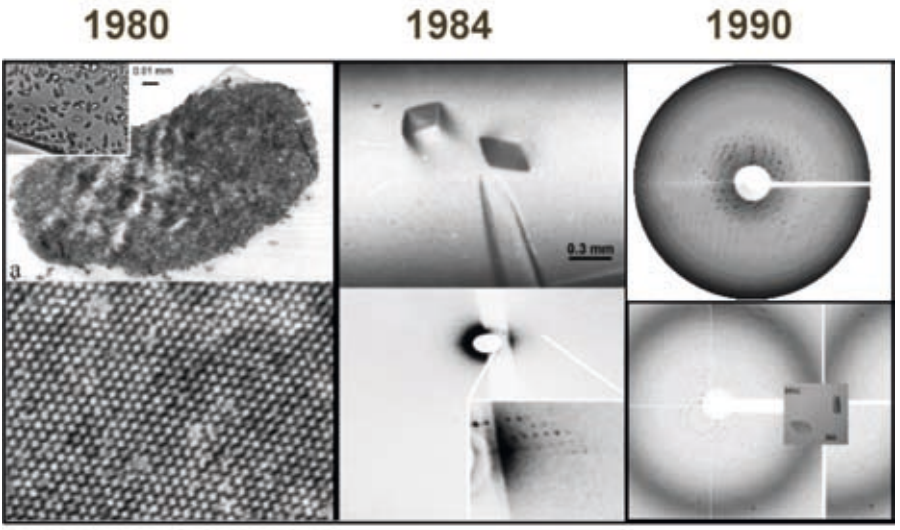

Figure 2. From poor micro crystal to three dimensional crystals yielding useful diffraction of ribosomal crystals: Left: Microcrystals of B50S, obtained in 1980 and a negatively stained section of them, view by electron microscopy. Middle: the tip of a ~2 long crystal of B50S and its diffraction patter, obtained in 1984 at the EMBL beam line at DESY/Hamburg at 40˚C. Diffraction patterns, from crystals of H50S obtained at ID13 ESRF at –1800 C. Note that the diffraction extends to 2.8Å (top right), and the decay it underwent (bottom), even at cryo temperature, after collecting about 3% of the data.

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 catalytic function, polymerization of the amino acids and the protein exit tunnel (Figure 3). To increase 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.

Ribosomes act by providing the framework for proper positioning of all participants in this fundamental process, thus enabling decoding, successive peptide bond formation and the protection of the nascent proteins chains. Since the turn of the third millennium, several three dimensional structures of ribosomes were determined (for details and references see below). Consequently, currently many of the mechanisms involved in ribosome's functions are rather well understood. A partial list includes the decoding mechanism (reviewed in Ogle *et al.,* 2003), the mRNA progression mode (Yusupova *et al.,* 2006), the relative positions of the A-P- and E- tRNAs (Yusupov *et al.,* 2001), the way the initiation, and the termination of the elongation cycle, is being modulated by initiation (Carter *et al.,* 2001; Pioletti *et al.,* 2001), release (Laurberg *et al.,* 2008; Weixlbaumer *et al.,* 2008) and recycling factors (Wilson *et al.,* 2005; Borovinskaya *et al.,* 2007), peptide bond formation and the provision of the architectural and dynamic elements required for amino acid polymerization (Bashan *et al.,* 2003; Bashan & Yonath, 2008b).

The involvement of RNA rich particles in genetic expression was suggested over five 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), in accordance with the idea that ribosome's ancestor was made exclusively of RNA (Crick, 1968). The localization of the cellular translation site and the extensive biochemical research that followed yielded illuminating findings about the overall nature of ribosome function, but detailed functional information was not available because of the lack of three dimensional structures and hence led to several common wisdom hypotheses, which underwent significant alterations once the structures became available. Striking examples for conceptual revolutions in the understandings of ribosomal function (reviewed in Wekselman *et al.,* 2008) are related to the functional contribution of the different ribosomal components and the path taken by nascent chains. Originally it was assumed that decoding of the genetic code and peptide bond formation are performed by r-proteins while rRNA provides the ribosome scaffold (Garrett & Wittmann 1973). Challenging this assumption (Noller *et al.,* 1992) met with skepticism, although major roles played by RNA molecules in various life processes became evident around this period. Shifting the focus from the r-proteins to the rRNA was proven to be right a decade later, when high resolution structures showed that both the decoding center and the site of peptide bond formation (called peptidyl-transferase-center or PTC) reside in rRNA predominant environments.

Another assumption was that nascent proteins reside and grow on the surface of the ribosome until their maturation. Even after biochemical experiments indicating nascent chains masked (hence protected from degradation) by the ribosome (Malkin & Rich 1967; Sabatini & Blobel 1970) and visualizing this tunnel in EM reconstructions from two-dimensional sheets at rather low resolution [namely 60 and 25 resolution (Milligan & Unwin 1986; Yonath *et al.,* 1987 respectively), doubt was publicly expressed (*e.g.* Moore 1988). Furthermore, experiments aimed to indicate that the nascent proteins are not degraded while growing because all adopt the conformation of an alpha helix since the very instant that the first peptide bond is being formed (Ryabova *et al.,* 1988) have been carried out. In fact, doubts as to the mere existence of the ribosomal tunnel were commonly expressed for additional long period (almost a decade since the first visualization), until verified by cryo electron microscopy (Frank *et al.,* 1995, Stark *et al.,* 1995). Remarkably, when a tunnel of dimensions matching those predicted in the 1960s (Malkin & Rich 1967) was first observed in high resolution crystal structures, it was suggested to be of a teflon-like character, with no obvious chemical properties allowing its interactions with progressing nascent chains (Ban *et al.,* 2000, Nissen *et al.,* 2000), although such description was in disagreement with previous observations [*e.g.* (Crowley *et al.,* 1993, Walter & Johnson 1994, Nagano *et al.,* 1991)] (Figure 3). Later on, further results of biochemical, microscopic and computational experiments, showed clearly that this tunnel participate actively in nascent chain progression, arrest

and cellular signaling [*e.g.* (Gabashvili *et al.,* 2001, Nakatogawa & Ito 2002, Gong & Yanofsky 2002, Berisio *et al.,* 2003, 2006, Woolhead *et al.,* 2004, 2006, Gilbert *et al.,* 2004, Johnson & Jensen 2004, Ziv *et al.,* 2005, Amit *et al.,* 2005, Mankin 2006, Tenson & Mankin 2006, Cruz-Vera *et al.,* 2006, Kaiser *et al.,* 2006, Deane *et al.,* 2007, Petrone *et al.,* 2008, Mitra *et al.,* 2006, Voss *et al.,* 2006, Schaffitzel & Ban 2007)], Furthermore crystal structure indicated that the tunnel can be hindred in trafficking the nascent proteins progress along this tunnel until they emerge into a shelter formed by chaperones preventing aggregation and misfolding (Baram *et al.,* 2005, Schluenzen *et al.,* 2005).

This article describes selected events in the chronological progress of ribosomal crystallography, as a semi historical report. It includes crystallization alongside the introduction of innovations in the procedures required for the determination of the ribosomal structures, such as cryo bio-crystallography and the use of heavy atom clusters [reviewed in (Gluehmann *et al.,* 2001)]. It focuses on the structural and dynamic properties of the ribosome that enable it to function as an efficient machine, mentions how antibiotics can hamper its function and addresses issues relating to the origin of ribosome.

THE INITIAL STEP: HIBERNATING BEARS STIMULATED RIBOSOME CRYSTALLIZATION

Because of the major significance of the ribosomes for cell vitality, attempts at the crystallization of ribosomal particles have been made worldwide for over two decades, all of which were found to be unproductive. Consequently, the crystallization of ribosomes has been considered formidable owing to repeated failures worldwide. The difficulties in ribosome crystallization stemmed from the marked tendency of ribosomes to deteriorate, their high degree of internal mobility, flexibility, functional heterogeneity, chemical complexity, large size and asymmetric nature. Nevertheless, the findings that in hibernating bears, large amounts of ribosomes are packed in an orderly way on the inner side of their cell membranes indicated that ribosomes can produce periodical arrangements *in vivo*. Similar observations were made on shock cooled fertilized eggs [*e.g.* (Milligan and Unwin, 1986)]. These phenomena were associated with cold or similar shocks, rationalizing them as the strategy taken by organisms under stress for storing pools of functionally active ribosomes that will be needed when the stressful conditions are removed. Indeed, structural studies, performed on samples obtained from shock cooled fertilized eggs led later to the visualization of ribosomal internal features [see below and in (Milligan and Unwin, 1986)].

The way to extend the level of order from two dimensional mono layers grown *in vivo* and supported by the membranes on which they are produced, to three dimensional crystals grown *in vitro* was not trivial, but doable. This was based on the interpretation of the life cycle of the hibernating bears, which are performing ribosomes packing/unpacking processes each year, as part of their healthy well being. The fact that these processes are associated

with living organisms which necessitate functionally active ribosomes immediately when awakening from their winter sleep, stimulated the notion that highly active ribosomes from any source, which can be maintained without undergoing deterioration for relatively long period, could also be crystallized in three dimensions.

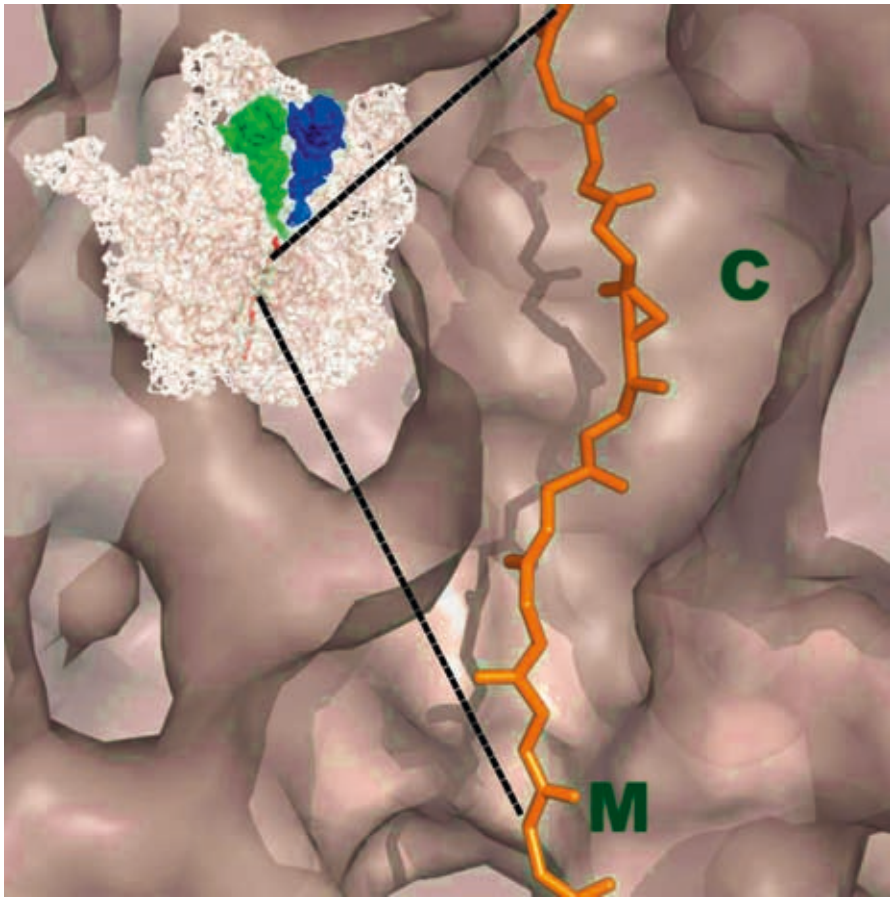

Figure 3. The ribosomal tunnel in eubacteria: Zoom into the upper end of ribosome tunnel, with "poly alanine" (orange) modeled in it. C denote a crevice where co translational initial folding may occur, and M shows the tunnel constriction, which provides the binding pocket for macrolide antibiotics. Insert in top left: the entire large subunit, viewed form its interface surface, with the A- and P- sites tRNA molecules (in blue and green respectively). The same modeled poly alanine indicates the tunnel's path.

The first three-dimensional micro-crystals (Figure 2) of ribosomal particles, diffracting to relatively high resolution, 3.5, were obtained in the early 1980s (Yonath *et al.,* 1980). This breakthrough was based on the presumptions that the higher the sample homogeneity the better the crystals, and that the preferred conformation is that of the functionally active ribosomes. Consequently, highly active ribosomes of bacteria species that grow under robust conditions were selected, and conditions for optimization and main-

tenance of their activity (Vogel *et al.,* 1971; Zamir *et al.,* 1971) were sustained throughout the purification and crystallization process. In parallel, the nucleation of the crystals were carefully monitored (Yonath *et al.,* 1982a), and a systematic search for parameters supporting crystallization was performed (Yonath *et al.,* 1982b). The first micro crystals that were obtained were of the large ribosomal subunits from *Bacillus stearothermophilus* (B50S), a source considered to be almost an extremophile at the beginning of the 1980s. A few years later, crystals were obtained from the large ribosomal subunits of the extreme halophilic bacteria, *H. marismortui* that live in the Dead Sea (Shevack 1985). In 1987, seven years after the first crystallization of ribosomal particles, parallel efforts led to the growth of crystals of the small ribosomal subunit (Yusupov *et al.,* 1987) and of the entire ribosome (Trakhanov *et al.,* 1987) from the extreme thermophilic bacterium *Thermus thermophilus*.

At that time it was widely assumed that even if there are crystals, ribosome structure may never be determined, since it was clear that alongside the improvement of the crystals, ribosome crystallography required the development of innovative methodologies. Thus, because of the weak diffraction power of the ribosome crystals, even the most advanced rotating anode generators were not sufficiently powerful to yield suitable diffraction patterns, and synchrotron radiation was at its embryonic stages. Hence, only a few diffraction spots could be recorded (Yonath *et al.,* 1984) even when irradiating extremely large crystals (~2 mm in length) by X-ray beam (Figure 2).

Figure 4. Suitable crystal forms: Several crystal forms of ribosomal particles, suitable for structural analyses with X-rays. Avarage sizes are 0.15–0.4 mm.

When more suitable synchrotron facilities became available, and several crystal forms were grown (Figure 4), the radiation sensitivity of the ribosomal crystals caused extremely fast crystal decay. Hence, pioneering data collection at cryo temperature became crucial (Hope *et al.*, 1989), and once established it yielded interpretable diffraction patterns at high resolution even from extremely thin crystals, although decay was observed even at

cryo temperature (Figure 2). Additionally, multi-heavy atom clusters suitable for phasing were identified (Thygesen *et al.,* 1996). One of these clusters, originally used for providing anomalous phasing power, 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, and increased dramatically the resolution from the initial 7–9Å to 3Å (Schluenzen *et al.,* 2000) presumably by minimizing the internal flexibility required for facilitating mRNA binding and progression through the ribosome (Bashan & Yonath, 2008a).

Continued efforts aimed at improving crystals included the assessment of the influence of the relative concentrations of mono- and divalent ions (von Bohlen *et al.,* 1991) on crystal properties, which led to dramatic improvements in the quality of the crystals from the large ribosomal subunit form *H. marismortui* (H50S). Also, constant refinements of bacterial growth (Auerbach-Nevo *et al.,* 2005), alongside a thorough investigation on crystallization conditions (Zimmerman & Yonath, 2009), indicated a noteworthy correlation between the conditions under which these ribosomes function and the quality of the resulting crystals. Along these lines, it is worth mentioning that flexible regions were detected in electron-density maps obtained from ribosomal crystals grown under close to physiological conditions (Harms *et al.,* 2001), whereas the same regions were highly disordered in crystals obtained far from their physiological environment (Ban *et al.,* 2000). An alternative strategy for crystal refinement was to crystallize complexes of ribosomes with substrates, inhibitors and/or factors that can trap them at preferred orientations. Indeed, the initial diffracting crystals of the whole ribosome from *T. thermophilus* (T70S) with mRNA and tRNA molecules diffracted to rather low resolution (Hansen *et al.,* 1990). With advances in the brightness and collimation of synchrotron radiation X-ray beam, the installation of advanced detectors and the introduction of cryo-bio-crystallographic techniques (Hope *et al.,* 1987), an impressive improvement in resolution from crystals of functional complexes of the whole was achieved (Yusupov 2001; Yusupova *et al.,* 2006; Selmer *et al.,* 2006; Korostelev *et al.,* 2006; Voorhees *et al.,* 2009). Also, these techniques enabled structure determination of snapshots of ribosomes trapped at a specific, albeit not necessarily functional, conformation (Schuwirth *et al.,* 2005).

STRATEGIES EMPLOYED BY ANTIBIOTICS TARGETING RIBOSOMES

Despite ribosome conservation, many of the antibiotics targeting ribosomes are clinically relevant [*e.g.* reviewed in the following a partial list (Yonath and Bashan, 2004; Polacek and Mankin, 2005; Yonath, 2005; Tenson and Mankin, 2006; Boettger, 2007)]. Since so far there are no crystals of ribosomes from pathogenic organisms, structural information is currently obtained from the crystallizable eubacterial ribosomes that have shown to be relevant for determining directly (see below) or indirectly *e.g* Pfister,

et al., 2005; Tu, *et al.,* 2005; Hobbie, *et al.,* 2008; Bommakanti *et al.,* 2008) the antibiotic modes of action on pathogens.

Crystallographic analyses have shown that antibiotics targeting ribosomes exploit diverse strategies with common denominators. Thus, it was found that antibiotics target ribosomes at distinct locations within functionally relevant sites, mostly composed solely of rRNA. They exert their inhibitory action by diverse modes, including competing with substrate binding, inter-fering with ribosomal dynamics, minimizing ribosomal mobility, facilitating miscoding, hampering the progression of the mRNA chain, and blocking the nascent protein exit tunnel. In more detail, all antibiotics bind to functionally relevant regions, and each prevents a crucial step in the biosynthetic cycle, including causing miscoding, minimizing essential functional mobility, inhibiting translation initiation, interfering with tRNA substrate binding at the decoding center, hindering tRNA substrate accommodations at the peptidyl transferase center (PTC), preventing interactions of the ribosomal recycling factor (RRF) and blocking the protein exit tunnel.

The identification of the various modes of action of antibiotics targeting ribosomes and a careful analysis of the ribosomal components comprising the binding pockets confirm that the imperative distinction between eubacterial pathogens and mammalian ribosomes hinges on subtle structural difference within the antibiotic binding pockets and that fine tuning of the binding pocket can alter the binding mode (Yonath and Bashan, 2004; Yonath, 2005; Pyetan. *et al.,* 2007). These subtle sequence and/or conformational variations enable drug selectivity, thus facilitating clinical usage. Furthermore, the available structures have also illuminated factors that discriminate between pathogenic bacteria and non-pathogenic eukaryotes, which are of crucial clinical importance, since most ribosomal antibiotics target highly conserved functional sites.

Noteworthy are the results of comparisons between the different crystal structures of ribosomal particles in complexes with the same antibiotics. Indeed, important implications were deciphered by comparisons of high-resolution structures of complexes of antibiotics with ribosomal particles from eubacteria resembling pathogens, *D. radiodurans* and of an archaeon that shares properties with eukaryotes. These comparisons highlighted the distinction between binding and inhibitory activity. Specifically, it indicated that the identity of a single nucleotide determines antibiotic binding, whereas proximal stereochemistry governs the antibiotic orientation within the binding pocket (Bashan and Yonath, 2004; Yonath 2005) and consequently its therapeutic effectiveness. This is in accord with recent mutagenesis studies showing that mutation from guanine to adenine in 25S rRNA at the position equivalent to *E. coli* A2058 does not confer erythromycin sensitivity in *Saccharomyces cerevisae* (Bommakanti *et al.,* 2005). Thus, it was clearly demonstrated that minute variations in the chemical entities of the antibiotics can lead to significantly different binding modes, and that the mere binding of an antibiotic is not sufficient for therapeutic effectiveness.

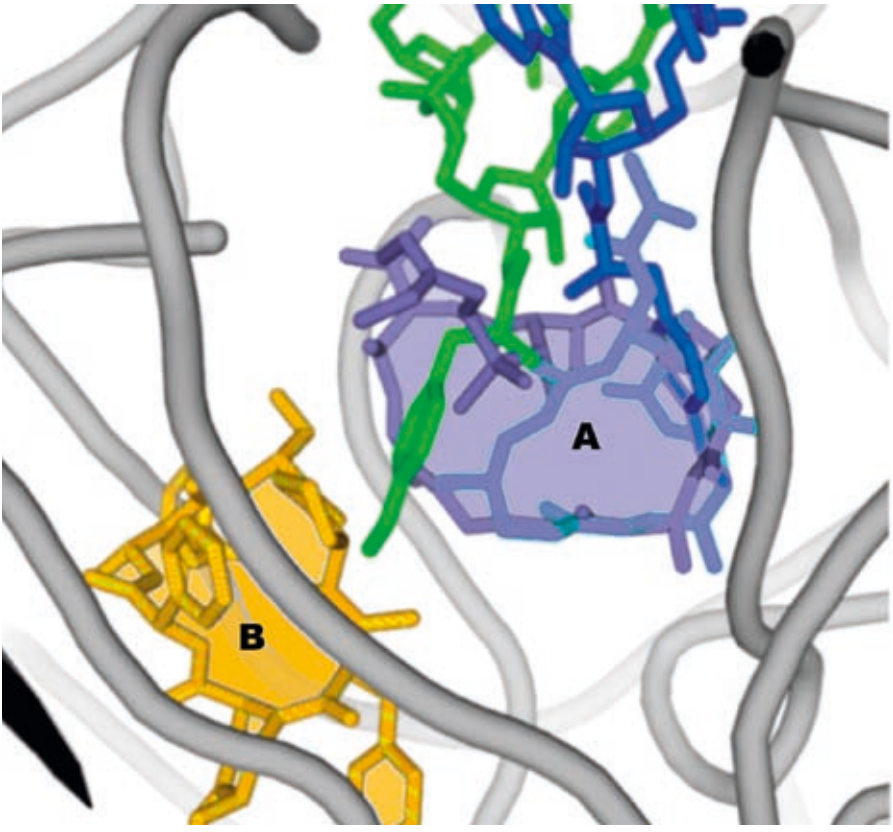

Figure 5. An example of antibiotics synergism: Synercid, a member of the streptogramin family that acts on the ribosomal PTC and exit tunnel. For orientation, the ribosomal RNA backbone is shown in silver and the amino acylated 3Å ends of A- and P- sites tRNAs in blue and green, respectively. The SA compound dalfopristin is shown in blue and its SB mate, quinupristin, is shown in gold.

Alongside rationalizing many genetic, biochemical and medical observations, the available structures have revealed unexpected inhibitory modes. Examples are the exploitation of the ribosomal inherent flexibility for antibiotic synergism (Figure 5) (Harms *et al.,* 2004; Yonath, 2005; Auerbach *et al.,* 2009) and for triggering an induced-fit mechanism by remote interactions that reshape the antibiotic binding pocket (Davidovich, *et al.,* 2007). Among the ribosomal antibiotics, the pleuromutilins are of special interest since they bind to the almost fully conserved PTC, yet they discriminate between eubacterial and mammalian ribosomes. To circumvent the high conservation of the PTC, the pleuromutilins exploit the inherent functional mobility of the PTC and trigger a novel induced-fit mechanism that involves a network of remote interactions between flexible PTC nucleotides and less conserved nucleotides residing in the PTC-vicinity. These interactions reshape the PTC contour and trigger its closure on the bound drug (Davidovich, *et al.,* 2007). The uniqueness of the pleuromutilins' mode of binding led to new insights into ribosomal functional flexibility, as it indicated the existence of an

allosteric network around the ribosomal active site. Indeed, the value of these findings is far beyond their perspective clinical usage, as they highlight basic issues, such as the possibility of remote reshaping of binding pockets and the ability of ribosome inhibitors to benefit from the ribosome's functional flexibility.

Similar to the variability of binding modes despite the overall resemblance, the nature of seemingly identical mechanisms of drug resistance is dominated, directly or via cellular effects, by the antibiotics' chemical properties (Davidovich *et al.,* 2007, 2008). The observed variability in antibiotic binding and inhibitory modes justifies expectations for structurally based improved properties of existing compounds as well as for the discovery of novel drug classes. Detailed accounts can be found in several reviews [*e.g.* (Auerbach *et al.,* 2004, Yonath & Bashan 2004, Yonath 2005, Poehlsgaard & Douthwaite 2005, Tenson & Mankin 2006, Boettger 2006, 2007)].

In short: over two dozen three dimensional structures of complexes of ribosomes with the antibiotics targeting them revealed the principles allowing for clinical use, illuminated mechanisms for acquiring resistance and showed the bases for discrimination between pathogens and host cells. The elucidation of common principles of the mode of action of antibiotics targeting the ribosome, combined with variability in binding modes, the revelation of diverse mechanisms acquiring antibiotic resistance, and the discovery that remote interactions can govern induced-fit mechanisms enabling species discrimination even within highly conserved regions, justify expectations for structural based improved properties of existing antibiotics as well as for the development of novel drugs.

THE RIBOSOME IS A POLYMERASE

The recent availability of crystal structures of bacterial ribosome and their complexes, all obtained by advanced synchrotron radiation, enabled a quantum jump in the understanding of the machinery of protein biosynthesis. These structures showed that the interface surfaces of both ribosomal subunits are outstandingly rich in RNA, and its two active sites: the decoding region and the PTC are made exclusively of RNA components. Hence, the ribosome is a ribozyme. The PTC is situated within a highly conserved universal symmetrical region that is embedded in the otherwise asymmetric structure, and this region provides the machinery required for peptide bond formation and for the ribosome polymerase activity, the latter being of particular significance for smooth production of the nascent proteins. The substrates for this reaction are amino acylated or peptidylated tRNA molecules, accommodated at three sites (Figure 1). A- to P-site tRNA translocation is comprised of at least two highly correlated motions: sideways shift (which may contain internal rearrangements), and a ribosomal navigated rotary motion (Bashan *et al.,* 2003, Agmon *et al.,* 2003, 2005, 2006, 2009, Sato *et al.,* 2006, Bashan & Yonath 2008b), during which peptide bonds are being formed (Gindulyte *et al.,* 2006). This process also involves 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, hence enabling the ribosome's polymerase activity, as the ribosome is not a mere peptide bond former but the machine elongating the nascent proteins. Thus, single peptide bonds can be formed by 'minimal substrates' (see below) or by approximately placed longer substrates, as opposed to those accurately positioned benefiting from interactions with the cavity leading to the PTC, which thus can perform the rotatory motion into the P-site, which provides the mechanism for elongation.

Although amino-acylated tRNA molecules are the natural substrates of ribosomes, 'minimal substrates' or 'fragment reaction substrates' that are capable of forming single peptide bonds, are the substrate analogs commonly used biochemically. Despite being small and consequently presumed to be readily diffused into their locations within the ribosome, the reactions with these compounds are significantly slower, compared to those of full-size tRNA. The mystery of the increased duration of peptide bond formation by these single-bond substrate analogs was recently 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 2003), thus demonstrating that accurate substate positioning is the rate limiting step.

It was consistently found that the peptidyl transfer reaction is modulated by conformational changes at the active site (Schmeing *et al.,* 2005b, Beringer & Rodnina 2005, 2007, Brunelle *et al.,* 2006), and this process consumes time. The 'fragment reaction substrates' analogs are basically derivatives of puromycin. Although they are capable of producing only single peptide bonds, they were overestimated to be suitable to mimic the natural ribosome function. Complexes of H50S with minimal substrates obtained under far from optimal functional conditions led to 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). This 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 & Mankin 2005, Bieling *et al.,* 2006)], as well as by structural comparisons that showed that the H50S actives site contains key PTC components in orientations that differ significantly from those observed in functional complexes of T70S ribosome (Selmer *et al.,* 2006, Korostelev *et al.,* 2006). Notably, it should be kept in mind that although 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) and it is conceivable that in addition to accurate positioning, the r-protein L2 is involved in the efficient elongation of the nascent chain (Cooperman *et al.,* 1995).

It appears that the choice of substrate analogs may be the reason for

the misinterpretation. The structure of the large ribosomal subunit from *Deinococcus radiodurans* (D50S) in complex with a substrate analog mimicking the A-site tRNA part interacting with the large subunit, called ASM, advanced the comprehension of peptide bond formation by showing that ribosomes position their 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 facilitates positional catalysis of peptide bond formation, promotes substrate mediated chemical acceleration, in accordance with the requirement of full-length tRNAs for rapid and smooth peptide bond formation, observed by various methods, including the usage of chemical (Brunelle *et al.,* 2006, Weinger *et al.,* 2004, Weinger & Strobel 2006) mutagenesis (Sato *et al.,* 2006), computational (Trobro & Aqvist 2006, Sharma *et al.,* 2005, Gindulyte *et al.,* 2006) and kinetic procedures (Beringer *et al.,* 2005, Wohlgemuth *et al.,* 2006, Beringer & Rodnina 2007, Rodnina *et al.,* 2007). The current consensus view is consistent with ribosomal positional catalysis that allows for chemical catalysis by its P-site tRNA substrate. The importance of the accurate positioning of 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.,* 2005, Beringer & Rodnina 2007, Bashan & Yonath 2008b)] even by those who originally suggested that the ribosome catalyze peptide bond formation by an acid/base mechanism (Simonovic & Steitz 2008).

MOBILITY AND MOTIONS WITHIN THE PTC

Both main ribosomal catalytic tasks, the formation of peptide bonds and the processivity of this reaction, namely amino acid polymerization, are governed by the striking ribosomal architecture, which contains a highly conserved region of 180 nucleotides that are related by pseudo two-fold symmetry of the rRNA folds, but not of the sequences. This sizable intra-ribosomal symmetrical region is located within the otherwise asymmetric ribosome, and has been identified in all known ribosome structures, regardless of their source, their functional state, or their kingdom of life (Bashan *et al.,* 2003, Agmon *et al.,* 2003, Zarivach *et al.,* 2004, Baram and Yonath, 2005). In particular, 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 subunits, including complexes with ribosomal anti-biotics and non ribosomal factors involved in protein biosynthesis (Agmon *et al.,* 2005, 2006). Thus, despite size differences between ribosomes of the various kingdoms of life, the functional regions are well conserved, with the highest level of sequence conservation at their central core, and the largest structural differences at the periphery (Mears *et al.,* 2002, Thompson &

Dahlberg 2004). Although there is no sequence symmetry, all of the nucleotides constructing the symmetrical region are highly conserved throughout evolution (Agmon *et al.,* 2006, Agmon *et al.,* 2009, Davidovich *et al.,* 2009), indicating law or no sensitivity to environmental conditions. 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, the nascent protein exit tunnel and the bridge connecting the PTC cavity with the vicinity of the decoding center in the small subunit. 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 (Uemura *et al.,* 2007).

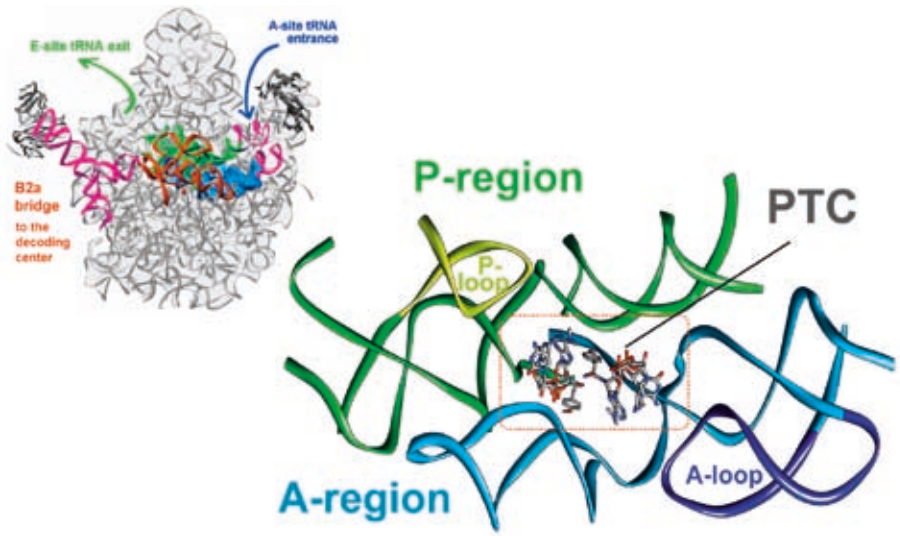

Figure 6. The ribosomal symmetrical region: Top left: The symmetrical region within the ribosome and its details. The A-region is shown in blue, the P-region in green, and the non-symmetrical extensions are shown in magenta. Bottom right: A zoom into the symmetrical region, highlighting the basic structure that can form the active site pocket and the loops that accommodate C74 of the 3' end of the A and the P site tRNAs. The inter subunit bridge to the small subunit is shown in light brown.

The PTC is located at the midst of this symmetrical region (Figure 6) in the bottom of a V-shaped cavity and is built as an arched void. The tRNA acceptor stem interacts extensively with the cavity's walls, as observed for the complex D50S-ASM (Bashan *et al.* 2003). Although the PTC has significant tolerance in the positioning of 'fragment reaction substrates', the interactions of the tRNA acceptor stem seem to be crucial for accurate substrate positioning in the PTC at the configuration allowing for peptide bond formation (Yonath 2003), in accordance with the finding that the tRNA core region is functionally important for its dynamic interactions with the ribosome (Pan *et al.,* 2006).

The linkage between the elaborate architecture of the symmetrical region and the position of the A-site tRNA indicates that the translocation of the tRNA 3'end is performed by a combination of independent, albeit synchronized motions: a sideways shift, performed as a part of the overall mRNA/tRNA translocation, and a rotary motion of the A-tRNA 3′end along a path confined by the PTC walls. This rotary motion is navigated and guided by the ribosomal architecture, mainly the PTC rear wall that confines the rotary path, and two flexible nucleotides seem to anchor and propel it. Hence, 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 & Strobel 2006) and for the direction of the nascent protein into the exit tunnel. Importantly, all nucleotides involved in this rotary motion have been classified as essential by a comprehensive genetic selection analysis (Sato *et al.,* 2006). Furthermore, the rotary 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 & Strobel 2006).

Simulation studies indicated that during this motion the rotating moiety interacts with ribosomal components confining the rotary 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 rotary motion. It is stabilized by hydrogen bonds with rRNA nucleotides (Gindulyte *et al.,* 2006) formed during the rotary motion 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.,* 2005a). The correlation between the rotary motion and amino acid polymerization rationalize the apparent contradiction associated with the 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.,* 2005a), near the P-site.

THE RIBOSOMAL CORE IS AN OPTIMIZED VESTIGE OF AN ANCIENT ENTITY

Remarkably, the high level of conservation of components of the symmetrical region 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. Hence, we suggest that the modern ribosome evolved from a simpler entity (Figure 7) that can be described as a proto-ribosome, by gene fusion or gene duplication (Baram & Yonath 2005). In particular, the preservation of the three-dimensional structure of the two halves of the ribosomal frame, regardless of the sequence, emphasizes the superiority of functional requirement over sequence conservation, and indicates that the PTC has evolved by gene fusion. In particular, it 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 was composed of a pocket confined by two RNA chains, which formed a dimer, and this pocket is still embedded in the modern ribosome and appears as its symmetrical region (Figure 6).

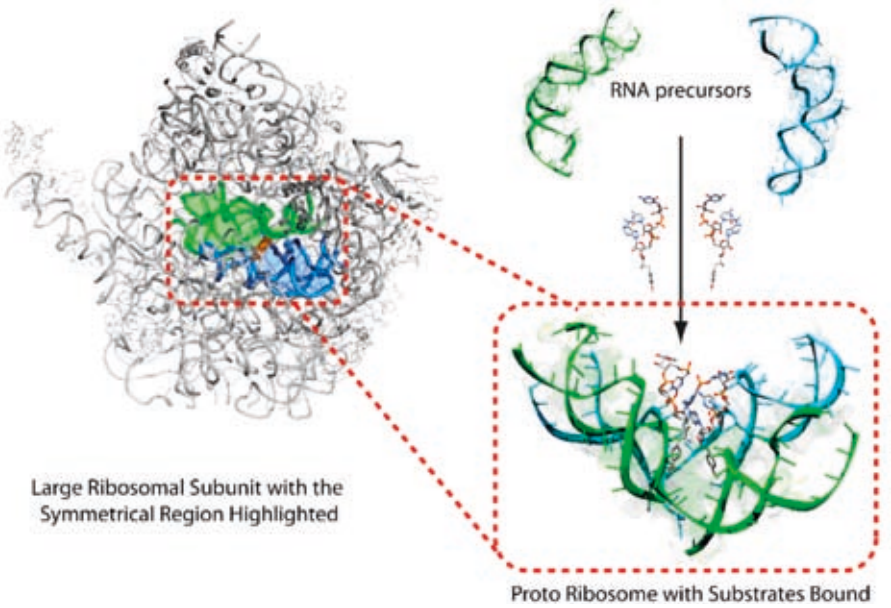

Figure 7. The suggested proto-ribosome: The region hosting A-site tRNA is shown in blue and that hosting the p-site tRNA in green. The A-site tRNA mimic (Bashan *et al.,* 2003) is shown in blue, and the derived P-site tRNA (by the rotary motion) is shown in green.

Based on this observation, we have proposed (Agmon *et al.,* 2006, Davidovich *et al.,* 2009, Belousoff *et al.,* 2010) that the ancient machinery that could form peptide bonds was made exclusively from RNA molecules, utilizing substituents available in the primordial soup, such as short RNA chains that could acquire stable conformations, sufficiently stable to survive changing evolution stresses. These surviving ancient RNA chains could fold spontaneously and then be dimerized. The products of the dimerization yielded three-dimensional structures with a symmetrical pocket that could accom-

modate two small substrates (*e.g.* amino acids conjugated with mono or oligo RNA nucleotides in a stereochemistry suitable for spontaneous reaction of peptide bond formation). Hence, they could become the ancestors of the RNA chains that construct the symmetrical region in the contemporary ribosome. The most appropriate pockets for accommodating this reaction survived. Since RNA chains can act as gene-like molecules coding for their own reproduction (Lincoln and Joyce, Costanzo *et al.,* 2009), the surviving ancient pockets became the templates for the ancient ribosomes. In later stage these initial RNA genes underwent optimization to produce more defined, relatively stable pockets, and when the correlation between the amino acid and the growing peptidyl sites was established, each of the two halves was further optimized for its task so that their sequences evolved differently. The entire ribosome could evolve gradually around these symmetrical regions until it acquired its final shape (Bokov and Steinberg, 2009).

The substrates of the ancient ribosomes, which were initially spontaneously produced amino acids conjugated with single or short oligonucleotides (Ilangasekare *et al.,* 1995, Lehmann *et al.,* 2007), could have evolved in parallel to allow accurate binding, as occurs for aminoacylated CCA 3'end. Later on, these were converted into longer and more compounds with a contour that can complement the inner surface of the reaction pocket. For increasing specificity, these short RNA segments were extended to larger structures by their fusion with RNA stable features to form the ancient tRNA, presumably capable of storing, selecting and transferring instructions for producing useful proteins. Subsequently, the decoding process was combined with peptide bond formation. Adding a feature similar to the modern anticodon loop could have allowed some genetic control, presumably after polypeptides capable of enzymatic function were created. Analysis of substrate binding modes to inactive and active ribosomes have led to similar conclusions (Johansson *et al.,* 2008).

In short, it appears that the ancient ribosome (here called the proto-ribosome) was a dimeric ribozyme, produced by dimerization of self-folded RNA chains (Figure 6) that formed a pocket involved in RNA chemical reactions and produced peptide bonds sporadically. Since the products of this reaction may act as substrates for it, elongation of the dipeptides could occur. Once these polypeptides acquired the capacity to perform enzymatic tasks, the information about their desired structure was stored in genes. Consequently, molecules capable of decoding this information while transporting the cognate substrates (tRNA) evolved. The size and the complexity of the proto-ribosome increased until it reached the size and shape for hosting the newly developed tRNA molecules and acquired properties enabling smooth translation of genetic information into proteins.

CONCLUDING REMARKS AND FUTURE PROSPECTS

Ribosome research has undergone astonishing progress in recent years. High resolution structures have shed light on many of the functional properties

of the translation machinery and revealed how the ribosome's striking architecture is ingeniously designed as the framework for its unique capabilities: precise decoding, substrate mediated peptide-bond formation and efficient polymerase activity. These structures have clearly shown that all ribosomal tasks are performed by the ribosomal RNA and supported by the ribosomal proteins.

Among the new findings that have emerged from the structures are the intricate mode of decoding, the inherent mobility of most of the ribosomal functional features, the symmetrical region at the core of the ribosome, the dynamic properties of the ribosomal tunnel, the interactions of the ribosome with the progressing nascent chains, the possible signaling between the ribosome and cellular components and the shelter formed by the first chaperone that encounters the nascent chains (trigger factor) for preventing nascent chain aggregation and misfolding. Novel insights from these new findings include the suggestion that the translocation of the tRNA involves at least two concerted elements: sideways shift (which may be performed in a hybrid mode) and a ribosomal-navigated rotary motion.

The linkage between these findings and crystal structures of ribosomes with over two dozen antibiotics targeting the ribosome, most of which have high therapeutical relevance, illuminated various modes of binding and action of these antibiotics; deciphered mechanisms leading to resistance; identified the principles allowing for the discrimination between pathogens and eukaryotes despite high ribosome conservation; enlightened the basis for antibiotics synergism (Figure 5), namely the conversion of two weakly acting compounds to a powerful antibiotic agent; indicated correlations between antibiotics susceptibility and fitness cost and revealed a novel induced-fit mechanism exploiting ribosomal inherent flexibility for reshaping the antibiotic binding pocket by remote interactions. Thus, the high resolution structures of the complexes of the ribosomes with the antibiotics bound to them address key issues associated with the structural bases for antibiotics resistance, synergism, and selectivity and provide unique structural tools for improving antibiotic targets.

The availability of high resolution structures has stimulated unpredictable expansion in ribosome research, which has resulted in new insights into the translation process. However, despite extensive research and immense progress, several key issues are still unresolved, some of which are described above. Thus, it is clear that the future of ribosome research and its applicative aspects hold more scientific excitement.

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Portrait photo of Professor Yonath by photographer Ulla Montan.