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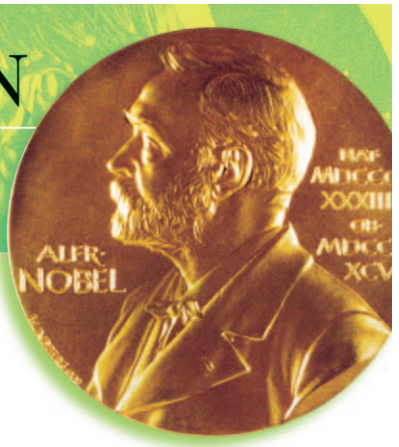

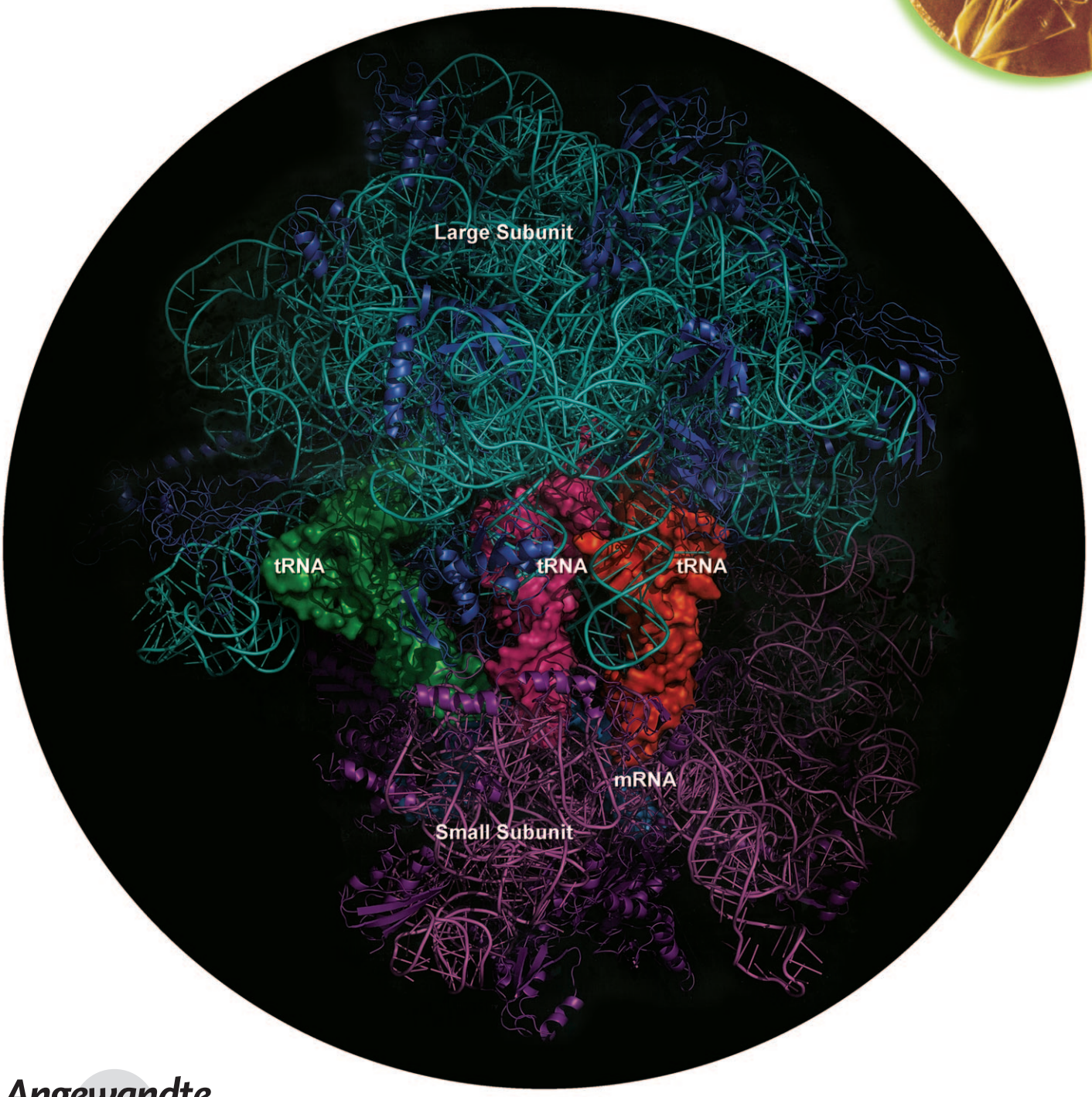

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Hibernating Bears, Antibiotics, and the Evolving Ribosome (Nobel Lecture)**

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antibiotics · Nobel lecture · protein synthesis · ribosomes

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. 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 ribosome polymerase activity, it may be the remnant of the proto-ribosome, 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 their clinical use, 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.

From the Contents

1. Introduction

An adult human body contains approximately 10^{14} cells. There is a major disparity between the numbers of proteins in various mammalian cells. Eukaryotic cells contain over 7000 different types of proteins, the total number depending on the cell class and function. For example, liver cells contain up to 10000 different proteins, the abundance of which varies widely, from 20000 molecules for the rather rare proteins that bind the hormone insulin, to the plentiful structural protein actin, with a number of molecules that can reach over 5 billions. Proteins (polypeptides) are made of amino acids arranged in a linear chain that folds into globular or fibrilar forms, depending on the sequence of the amino acids. The sequence of amino acids in a protein is defined by the sequence of a gene, which is encoded in the genetic code.

Proteins are constantly being degraded, and simultaneous production of proteins is therefore 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. Even bacterial cells contain ca. 100000 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 produce proteins on a contin-

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uous basis at an incredible speed of 15 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 (ca. 2:1) is maintained throughout evolution, with the exception of the 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 30S, contains an RNA chain (16S) of about 1500 nucleotides and 20 or 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.

Born in Jerusalem (Israel), Ada Yonath studied at the Hebrew University, earned her Ph.D. degree from the Weizmann Institute of Science (WIS), and completed her postdoctoral studies at the Massachusetts Institute of Technology (USA). In her research she attempts to determine the structural bases for processes involved in protein biosynthesis and their inhibition by antibiotics or similar compounds, including elucidation of mechanisms used for acquiring resistance, and the properties utilized for the discrimination between pathogens and eukaryotes, namely those allowing for clinical usefulness. She uses X-ray crystallography supported by molecular biology, mutagenesis, and other biophysical methods and focuses on ribosomes, which translate the genetic code into proteins. For this aim, she established in the 1970s the first laboratory for protein crystallography in Israel, which was the only laboratory of this kind in the country for almost a decade.

Currently she is a professor of structural biology at the WIS, holds the Kimmel Professorial Chair, and is the director of the Kimmelman Center for Biomolecular Structure and Assembly. From 1986 to 2004 she also headed the Max-Planck Research Unit in Hamburg, Germany.

She is a member of the US National Academy of Sciences (NAS), the American Academy of Arts and Sciences, the Israel Academy of Sciences and Humanities, the European Academy of Sciences and Art, the European Molecular Biology Organization, and the International Academy of Astronautics. Additionally, she has honorary doctorates from Tel Aviv, Ben Gurion, and Oxford Universities.

Her awards include the 1st European Crystallography Prize (2000); the Israel Prize, which is the most prestigious prize of the State of Israel (2002); the Harvey Prize (2002); the Cotton Medal of the American Chemical Society (2002); the Anfinsen Prize of the Protein Society, USA (2003); the Massry Award, USA (2004); The Paul Karrer Gold Medal, Zurich, Switzerland (2004); the Louisa Gross Horwitz Prize of Columbia Uni, NYC (2005); the Israeli Prime minister EMET prize (2006); the Paul Ehrlich–Ludwig Medal, Germany (2007); the Wolf Prize (2007); the UNESCO Award for Women in Science Prize, representing Europe (2008); the Palade Gold Medal (2008); the Albert Einstein World Award of Science (2008); the Erice Prize for Peace, Rome, the Vatican (2009); the Nobel Prize for Chemistry, Stockholm (2009).

Other players in the process are mRNA, which carries the genetic code, and tRNA molecules, which bring the cognate amino acids to the ribosome. The three-dimensional structures of all tRNA molecules from all living cells across evolution are similar, 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 Å away, at their 3′ end, they contain a single strand with the universal sequence CCA (C cytosine, A adenine), 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 E- (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 is located on the large subunit.

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. The large subunit contains the site for the main ribosomal catalytic function, that is, polymerization of the amino acids, and the protein exit tunnel (Figure 2). For increasing the 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. By the turn of the third millennium several three-dimensional structures of ribosomes were determined. Consequently, many of the mechanisms involved in ribosome function are rather well understood today, owing to the crystal structures of ribosomes and their complexes. Among those are the decoding mechanism (reviewed in Ref. [1]), the mRNA progression mode,[2] the relative positions of the A-, P- and E-tRNAs,[3] the way the initiation and the termination of the elongation cycle are being modulated by initiation factors,[4,5] release[6,7] and recycling factors,[8,9] peptide bond formation, and the provision of the architectural and dynamic elements required for amino acid polymerization.[10,11]

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,[12,13] in accordance with the idea that the ribosome ancestor was made exclusively of RNA.[14] The localization of the cellular translation site and the extensive biochemical research that followed yielded illuminating findings about the overall nature of the 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 that underwent significant alterations once the structures became available. Striking examples of conceptual revolutions in the understanding of

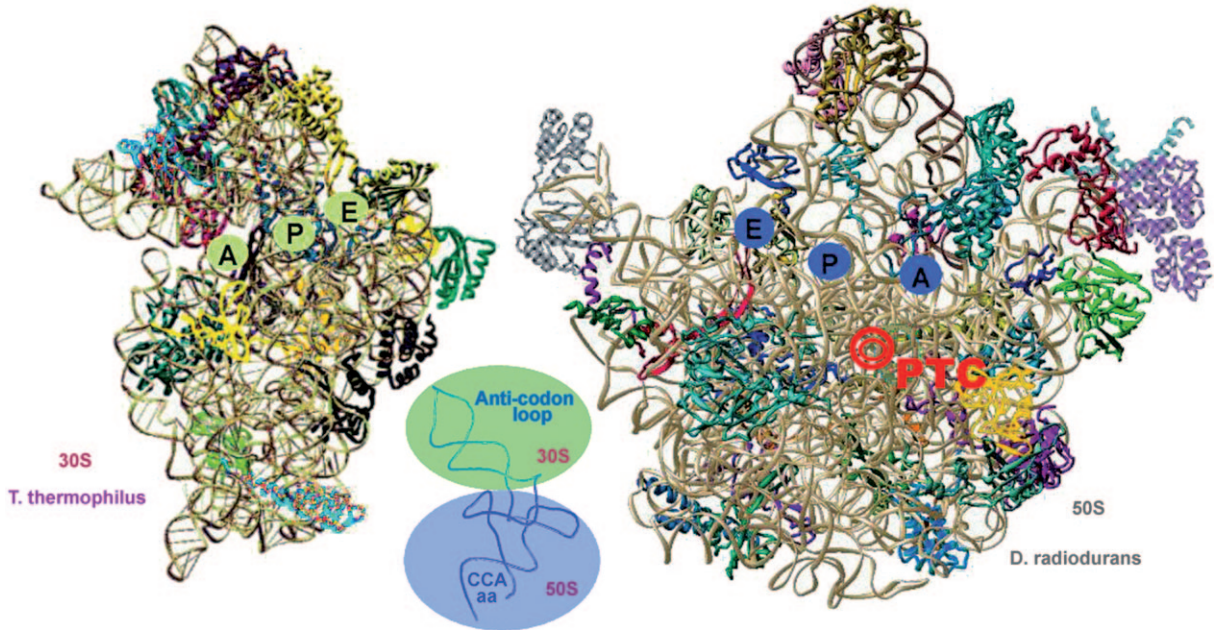

Figure 1. Three-dimensional structures of the two ribosomal subunits from eubacteria with a tRNA molecule—their substrate—placed between them. The interfaces are shown as observed in the 3 Å structures of the two ribosomal subunits of the eubacterium *D. radiodurans* (right) and *T. thermophilus* (left). The r-RNA is shown in brownish colors, and each of the r-proteins is shown in a different color. Note that the interfaces are rich in RNA. The approximate site of the peptidyl transferase center is marked in red. Insert: Backbone of a tRNA molecule; circles designate the regions interacting with each of the ribosomal subunits.

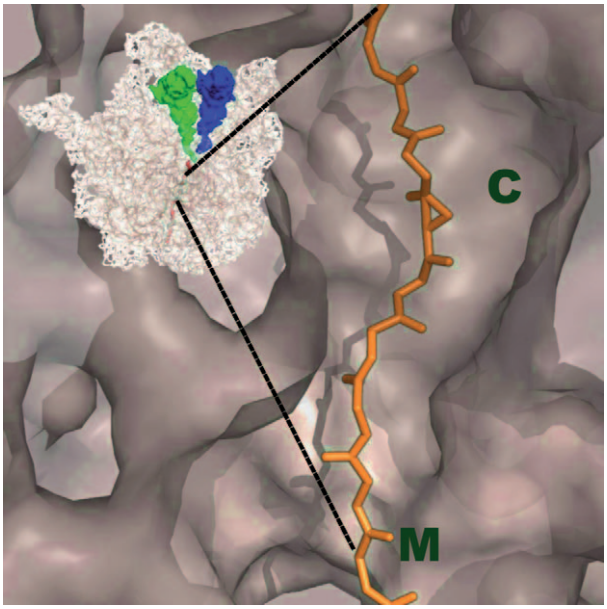

Figure 2. Zoom into the upper end of the ribosome tunnel with polyalanine (orange) modeled in. C denotes a crevice where cotranslational initial folding may occur. M denotes the tunnel constriction, which provides the binding pocket for macrolide antibiotics. Shown top left is the entire large subunit viewed from its interface surface, with the A- and P- site tRNA molecules in blue and green, respectively. A modeled polyalanine indicates the tunnel path.

the ribosomal function (reviewed in Ref. [15]) relate 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 r-RNA provides the ribosome scaffold.[16] This assumption was challenged,[17] and met with skepticism, although the 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 the 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 ribosome surface until maturation. Even after biochemical experiments indicating nascent chain protection by the ribosome[18,19] and visualizing this tunnel in electron microscopic reconstructions from two-dimensional sheets at rather low resolution (namely 60 and 25 Å resolution),[20,21] doubt was publicly expressed.[22] Furthermore, experiments aimed at verifying the assumption that nascent proteins are not degraded while growing because all adopt the conformation of an α helix since the very instance that the first peptide bond is being formed,[23] have been carried out. In fact, doubts on the mere existence of the ribosomal tunnel were commonly expressed for an additional long period (almost a decade since the first visualization), until verified by cryo-electron microscopy.[24,25] Remarkably, once a tunnel of dimensions matching those predicted in the 1960s[18] was observed in high-resolution crystal structures, it was suggested to be of Teflon-like character, with no chemical properties allowing interactions with progressing nascent chains,[26,27] although this description conflicted with previous observations (e.g. Ref. [28–30]) (Figure 2). However, further results

of biochemical, microscopical, and computational experiments clearly showed that this tunnel participates actively in nascent chain progression, arrest and cellular signaling (e.g. Refs. [31–50]), and that in eubacteria, nascent proteins progress along this tunnel and emerge into a shelter formed by chaperones, preventing aggregation and misfolding.[51,52]

This Review describes selected events in the chronological progress of ribosomal crystallography as a semi-historical report. It includes advances in crystallization as well as the introduction of innovations in the procedures required for the determination of the ribosomal structures, such as cryo-biocrystallography and the use of heavy-atom clusters (reviewed in Ref. [53]). It focuses on the structural and dynamic properties of the ribosome that enable its function as an efficient machine, mentions how antibiotics can hamper its function and addresses issues relating to the origin of ribosomes.

2. The Initial Step: Hibernating Bears Stimulated Ribosome Crystallization

Given the significance of ribosomes for cell vitality, attempts at the crystallization of ribosomal particles had been made worldwide for over two decades, all of which were found to be unproductive. Consequently, the crystallization of ribosomes was considered a formidable challenge. The difficulties in ribosome crystallization stemmed from their marked tendency to deteriorate, high degree of internal mobility, flexibility, functional heterogeneity, chemical complexity, large size, and asymmetric nature. Nevertheless, the findings that large amounts of ribosomes of hibernating polar bears are orderly packed 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. Ref. [20]). These phenomena were associated with cold or similar shocks, rationalizing them as a strategy taken by organisms under stress for storing pools of functionally active ribosomes which will be needed when the stressful conditions are removed. Indeed, structural studies performed on samples obtained from shock-cooled fertilized eggs later led to the visualization of ribosomal internal features (see below and Ref. [20]).

Extending the degree of order from two-dimensional monolayers grown in vivo, and which are supported by the membranes on which they are produced, to three-dimensional crystals grown in vitro was not a trivial task, but doable. The progess made was based on the interpretation of the life cycle of hibernating bears, which are performing such ribosome packing and unpacking processes each year. The fact that these processes are associated with living organisms who necessitate functionally active ribosomes immediately after awaking from winter sleep, stimulated the notion that highly active ribosomes from any source which can be maintained without undergoing deterioration for relatively long period could be crystallized also in three dimensions.

The first three-dimensional micro-crystals (Figure 3) of ribosomal particles, diffracting to relatively high resolution (3.5 Å) were obtained at the beginning of the 1980s.[54] 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

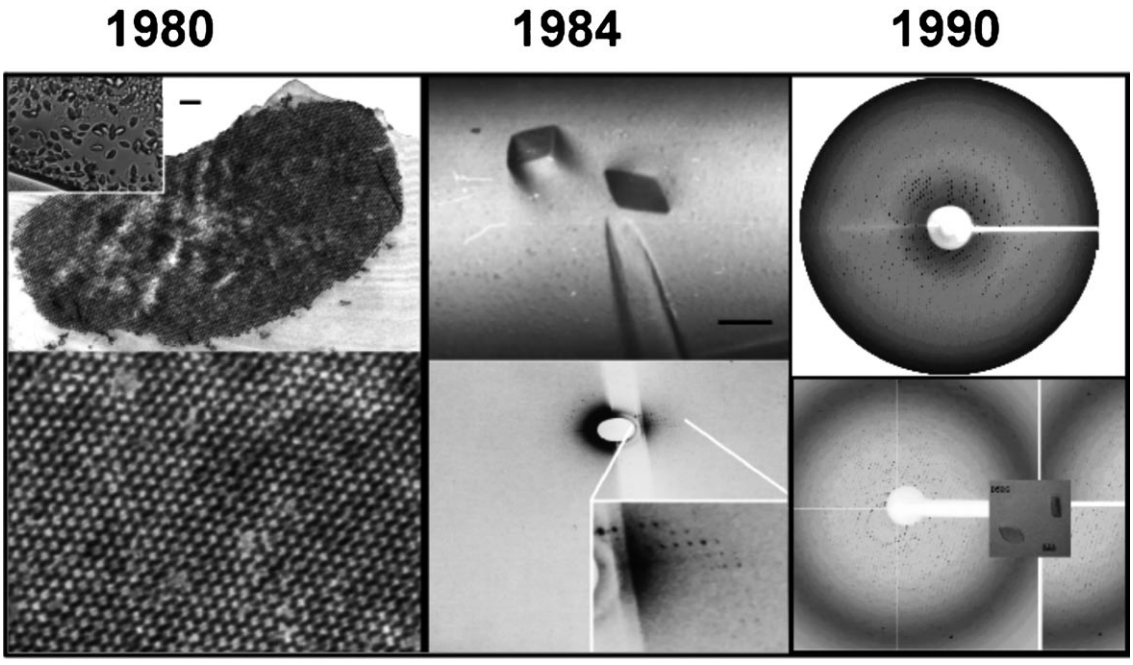

Figure 3. From poor microcrystals to three-dimensional crystals yielding useful diffraction data of ribosomes. Left: Microcrystals of B50S obtained in 1980 and a negatively stained section of them viewed by electron microscopy. Scale bar 0.01 mm. Middle: The tip of an approximately 2 mm long crystal of B50S and its diffraction pattern obtained in 1984 at the EMBL beam line at DESY/Hamburg at 4 °C. Scale bar 0.3 mm. Right: Diffraction patterns from crystals of H50S obtained at ID13 ESRF at −180 °C. Note that the diffraction extends to 2.8 Å (top), and the decay the crystal has undergone (bottom) even at cryo-temperature after collecting about 3% of the data.

ribosomes. Consequently, highly active ribosomes of bacteria species that grow under robust conditions were selected and conditions for optimization and maintenance of their activity[55,56] were sustained throughout the purification and crystallization process. In parallel, the nucleation of the crystals was carefully monitored,[57] and a systematic search for parameters supporting crystallization was performed.[58] The first crystals were obtained from 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 bacterium *H. marismortui*, which lives in the Dead Sea.[59] In 1987, seven years after the first crystallization of ribosomal particles, parallel efforts led to the growth of crystals of the small ribosomal subunit[60] and of the entire ribosome[61] 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 because 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[62] even when irradiating extremely large crystals (ca. 2 mm in length) with X-ray beams (Figure 3).

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,[63] 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 3). Additionally, multi-heavy-atom clusters suitable for phasing were identified.[64] 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 significantly increased the resolution from the initial 7–9 Å to 3 Å,[65] presumably by minimizing the internal flexibility required for facilitating mRNA binding and progression through the ribosome.[66]

Continuous efforts aimed at improving crystals included the assessment of the influence of the relative concentrations of mono- and divalent ions[67] on crystal properties, which led to significant improvements in the quality of the crystals from the large ribosomal subunits from *H. marismortui* (H50S). Also, constant refinements of bacterial growth[68] alongside a thorough investigation on crystallization conditions[69] 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[70] whereas the same regions were highly disordered in crystals obtained under far from their physiological environment.[26] 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.[71] The advances of the brightness and collimation of synchrotron radiation X-ray beams, the installation of advanced detectors, and the introduction of cryo-biocrystallographic techniques[72] yielded impressive advances in resolution from crystals of functional complexes of the whole ribosome.[2,3,73–75] Also, these techniques enabled structure determination of ribosomes trapped at a specific, albeit not necessarily functional, conformation.[76]

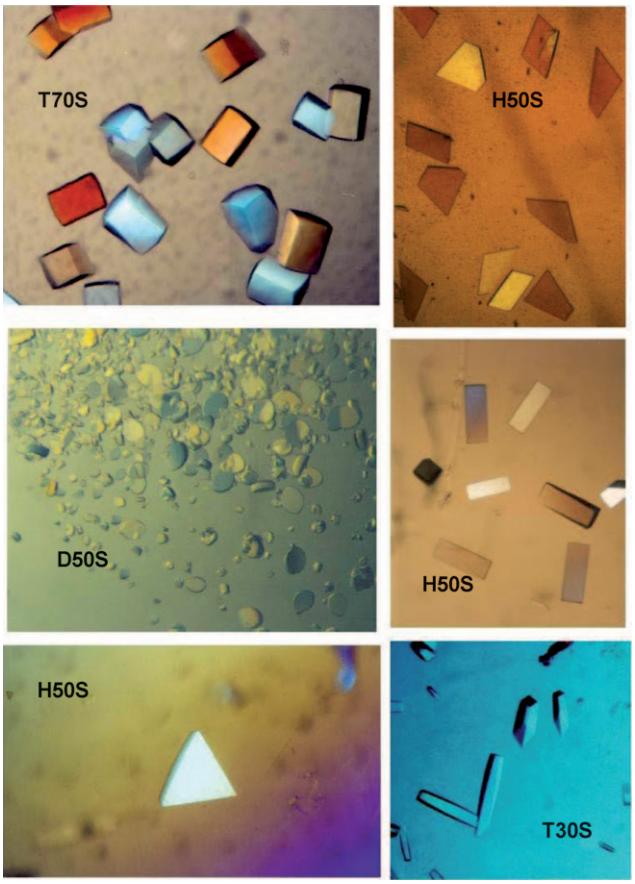

Figure 4. Several crystal forms of ribosomal particles suitable for X-ray analyses. Average sizes are 0.15–0.4 mm.

3. Strategies Taken by Antibiotics Targeting Ribosomes

Despite the high ribosomal conservation, many of the antibiotics targeting ribosomes are clinically relevant (see, for example, Refs. [43, 77–80]). Since there are no crystals available of ribosomes from pathogenic organisms, structural information is currently obtained from the crystallizable

eubacterial ribosomes which have shown to be relevant for determining directly (see below) or indirectly (e.g. Refs. [81–84]) the antibiotic modes of action on pathogens.

The crystallographic analyses revealed 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, interfering with ribosomal dynamics, minimizing ribosomal mobility, facilitating miscoding, hampering the progression of the mRNA chain, and blocking the nascent 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 differences within the antibiotic-binding pockets and that fine-tuning of the binding pocket can alter the binding mode.[77,79,85] These subtle sequence and/or conformational variations enable drug selectivity, thus facilitating clinical usage. Furthermore, the available structures have illuminated factors which 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 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, from *Deinococcus radiodurans*, and from 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 stereochemical configuration governs the antibiotic orientation within the binding pocket[77,79] 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*.[86] 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.

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)[79,87,88] and for triggering an induced-fit mechanism by remote interactions that reshape the antibiotic-binding pocket.[89] Among the ribosomal antibiotics, the pleuromutilins are of special interest, since they bind to the almost fully conserved PTC, yet discriminate between eubacterial and mammalian ribosomes. To circumvent the high conservation of the PTC the pleuromutilins exploit its inherent functional

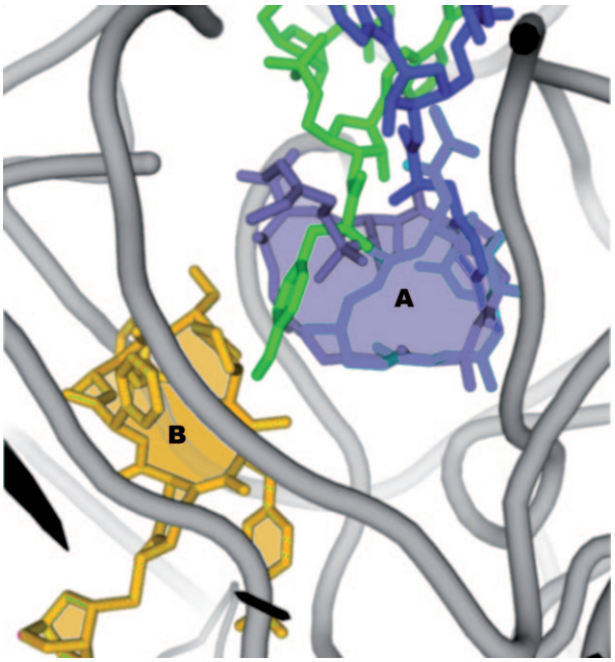

Figure 5. An example for antibiotics synergism: synercid, a member of the streptogramin family, acts on the ribosomal PTC and exit tunnel. The ribosomal RNA backbone is shown in silver, and the amino-acylated 3′ ends of A- and P-site tRNAs are shown in blue and green, respectively. The SA compound dalfopristin is shown in blue and its SB mate, quinupristin, is shown in yellow. A and B are representing SA and SB, respectively.

mobility 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.[89] The uniqueness of 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 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 through cellular effects, by the antibiotics' chemical properties.[89,90] 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., Refs. [43,77,79,80,91–93]).

In short: Over two dozens of 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.

4. The Ribosome is a Polymerase

The recent availability of crystal structures of bacterial ribosomes 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 aminoacylated or peptidylated tRNA molecules, accommodated in three sites (Figure 1). Translocation of tRNA molecules from the A- to the P-site is comprised of at least two highly correlated motions: sideways shift (which may contain internal rearrangements), and a ribosomal navigated rotatory motion[10,11,94–98] during which peptide bonds are being formed.[99] This process involves also the translocation of the tRNA 3′ end from A- to 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.

Although aminoacylated tRNA molecules are the natural substrates of ribosomes, "minimal substrates" or "fragment reaction substrates", which are capable of forming single peptide bonds, are the substrate analogues commonly used in biochemical experiments. 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 with those of full-size tRNA. The mystery of the increased duration of peptide bond formation by these single-bond substrate analogues was recently clarified, as it was shown that the excessive time is due to conformational rearrangement of the substrates, as well as of specific PTC components.[73,100]

Consistently, it was found that the peptidyl transfer reaction is modulated by conformational changes at the active site,[101–104] and this process consumes time. The fragment reaction substrate analogues 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,[27] which challenged almost instantaneously by a battery of biochemical and mutational studies (e.g., Ref. [78,105–108]), as well as by structural comparisons that showed that the H50S active site contains key PTC components in orientations that differ significantly from those observed in functional complexes of the T70S ribosome.[73,74] 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 the subsequent occurrence of peptidyl transfer by rRNA, has not been fully demonstrated,[109] and it is conceivable that the r-protein L2 is involved in the efficient elongation of the nascent chain.[110]

It appears that the choice of substrate analogues may be the reason for the misinterpretation. The structure of the large ribosomal subunit from *D. radiodurans* (D50S) in complex with a substrate analogue 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 a stereochemical configuration suitable for peptide bond formation, thus providing the machinery for peptide bond formation and tRNA translocation.[10,95] Furthermore, the ribosomal architecture that facilitates positional catalysis of peptide bond formation, promotes substrate-mediated chemical acceleration in accord with the requirement of full-length tRNAs for rapid and smooth peptide bond formation, observed by various methods, including the usage of chemical,[104,111,112] mutagenesis,[98] computational,[99,113,114] and kinetic procedures.[102,103,115,116] 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., Refs. [11,102,103]) even by those who originally suggested that the ribosome catalyzes peptide bond formation by acid/base mechanism.[117]

5. Mobility and Motions Within the Peptidyl Transferase Center

Both ribosomal main catalytic tasks—formation of peptide bonds and the processivity of this reaction, namely for amino acid polymerization—are governed by the ribosomal striking architecture, which contains a highly conserved region of 180 nucleotides, related by pseudo two-fold symmetry, the rRNA fold, but not 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.[10,94,118,119] Particularly, the same substructure was identified in the cores of ribosomes from mesophilic, thermophilic, radiophilic, and halophilic bacteria form eubacteria and archaea, in assembled

empty ribosomes 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.[95,96] Thus, despite size differences between ribosome 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.[120,121] Although there is no sequence symmetry, the sequences of the nucleotides constructing the symmetrical region are highly conserved throughout evolution,[96,97,122] indicating low or no sensitivity to environmental conditions. This symmetrical region includes the PTC and its environment, 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.[123]

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.[10] 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,[100] in accord with the finding that the tRNA core region is functionally important for its dynamic interactions with the ribosome.[124] 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 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. In addition, two flexible nucleotides that seem to anchor and propel it. Hence, the ribosomal architecture and its mobility provides all structural elements enabling the ribosome to function as an amino acid polymerase, including the formation of two symmetrical universal base pairs between the tRNAs and the PTC,[10,95] a prerequisite for substrate-mediated acceleration[112] 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.[98] Furthermore, the rotatory motion positions the proximal 2′-hydroxy group 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,[73,74] and allows for chem-

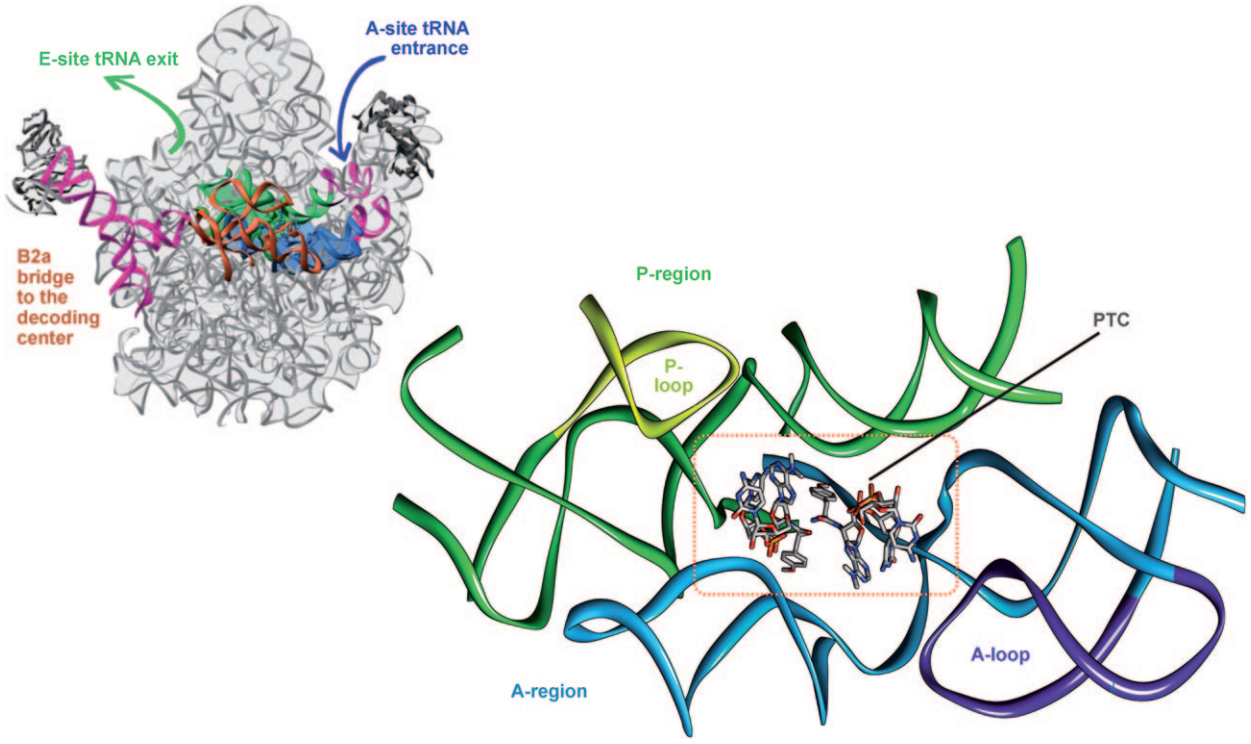

Figure 6. Top left: The symmetrical region within the ribosome. The A-region is shown in blue, the P-region in green, and the non-symmetrical extensions are shown in magenta. Bottom right: Zoom into the symmetrical region highlighting the basic structure that can form the active site pocket and the loops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

ical catalysis of peptide bond formation by A76 of the P-site tRNA.[112]

Simulation studies indicated that during this motion the rotating moiety interacts with ribosomal components confining the rotatory path along the PTC rear wall.[95,96] Consistently, quantum mechanical calculations, based on D50S structural data, indicated that the transition state (TS) of this reaction, namely peptide bond formation, is formed during the rotatory motion and is stabilized by hydrogen bonds with rRNA nucleotides[99] 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 analogue.[125] The correlation between the rotatory motion and amino acid polymerization rationalizes 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 analogues 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 analogues[125] near the P-site.

6. The Ribosomal Core is an Optimized Vestige of an Ancient Entity

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—indicates 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), which can be described as a pro-ribosome, by gene fusion or gene duplication.[119] 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 a stereochemical configuration 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).

Based on this observation, we have proposed[96,122,126] 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

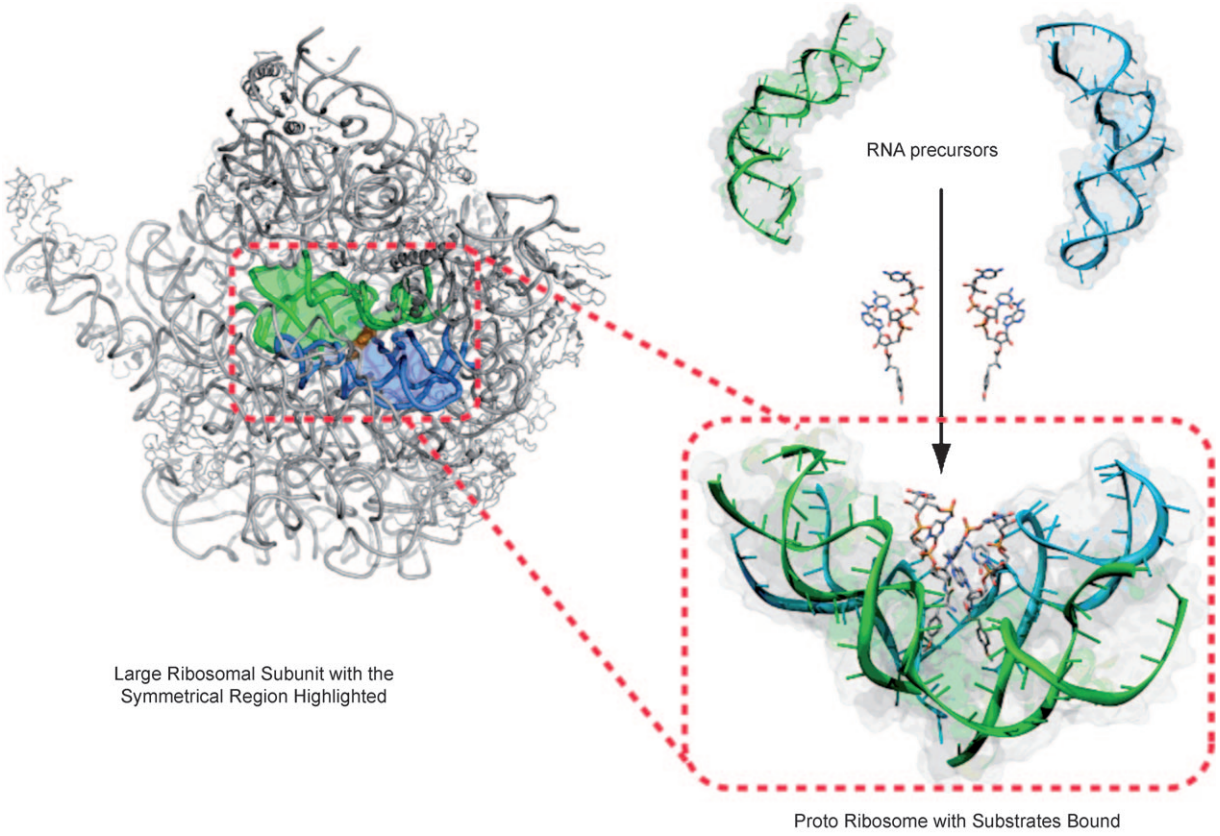

Figure 7. The suggested proto-ribosome: The regions hosting A- und P-site tRNA are shown in blue and green, respectively. The A-site tRNA mimic[10] is shown in blue, and the derived P-site tRNA (by the rotatory motion) is shown in green.

that could acquired stable conformations, which were sufficiently stable to survive changing evolution stresses. These surviving ancient RNA chains could fold spontaneously and then dimerize. The products of the dimerization yielded three-dimensional structures with a symmetrical pocket that could accommodate two small substrates (e.g. amino acids conjugated with mono- or oligo-RNA nucleotides in a stereochemical configuration 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. As RNA chains can act as gene-like molecules coding for their own reproduction,[127] the surviving ancient pockets became the templates for the ancient ribosomes. At a 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 have evolved gradually around these symmetrical region until it acquired its final shape.[128]

The substrates of the ancient ribosomes, which could be initially spontaneously produced amino acids conjugated with single or short oligonucleotides,[129,130] 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 could 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-stabilizing features, thus forming the ancient tRNA molecules 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 allowed some genetic control, presumably after polypeptides capable of enzymatic function were created. Analysis of substrate-binding modes to inactive and active ribosomes led to similar conclusions.[131]

In short, the ancient ribosome (called here the proto-ribosome) appears to be a dimeric ribozyme—produced by dimerization of self-folded RNA chains (Figure 6)—that formed a pocket that could be involved in RNA chemical reactions and produced peptide bonds sporadically. Since the products of this reaction may act as substrates, elongation of the dipeptides could occur. Once 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 (tRNA) evolved. The size and the complexity of the proto-ribosome were increased until it reached the size and shape for hosting the newly developed tRNA molecules and acquired the properties enabling smooth translation of genetic information into proteins.

7. Concluding Remarks and Future Prospects

Ribosome research has undergone astonishing progress in recent years. The 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 emerged from the structures are the intricate mode of decoding, the 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 rotatory motion.

The linkage between these findings and crystal structures of ribosomes with over two dozen antibiotics targeting the ribosome, most of which of a 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 the 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 in reshaping the antibiotic binding pocket by remote interactions. Thus, the high-resolution structures of the complexes of ribosomes with 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 the high-resolution structures has stimulated unpredictable expansion in ribosome research, which has resulted in new insights into the translation process. However, despite the extensive research and the 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 excitements.

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