CAN STRUCTURES LEAD TO BETTER DRUGS?
LESSONS FROM RIBOSOME RESEARCH

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J.L. Sussman and P. Spadon (eds.), From Molecules to Medicines, 229-250.

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Abstract. Ribosome research has undergone astonishing progress in recent years. Crystal structures have shed light on 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. New findings include the two concerted elements of tRNA translocation: sideways shift and a ribosomal-navigated rotatory motion; the dynamics of the nascent chain exit tunnel and the shelter formed by the ribosome-bound trigger-factor, which acts as a chaperone to prevent nascent chain aggregation and misfolding.

These 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, namely the conversion of two weakly acting compounds to a powerful antibiotic agent; indicated correlations between antibiotics susceptibility and fitness cost and revealed an novel induced-fit mechanism exploiting ribosomal inherent flexibility for reshape the antibiotic binding pocket by remote interactions.

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1. Introduction

An adult human body contains approximately 10^{14} cells, each containing about a billion proteins. 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 (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. In bacteria, ribosomes produce proteins on a continuous basis at an incredible speed of >15 peptide bonds per second.

Ribosomes are composed of two subunits (Table 1); comprising long chains of ribosomal RNA (rRNA) in which many ribosomal proteins (r-proteins) are entangled. The ratio of 2:1 for rRNA:r-proteins 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. Despite the size difference (Table 1), ribosomes from all kingdoms of life are functionally conserved; with the highest level of sequence conservation in the functional domains. Comparisons of rRNA sequences of widely diverged species and extrapolation of structures from eubacteria via archaea to eukaryotes indicate that the largest structural differences are at the periphery, away from the central core.

TABLE 1. Biophysical and chemical characterization of ribosomes.

	Prokaryotic ribosome	Eukaryotic ribosomes
Sedimentation coefficient	70S (~2.4 MDa)	80S (~4 MDa)
small subunit	30S - One rRNA molecule (16S with ~1500 nucleotides) ~ 21 different proteins (S1-S21)	40S - One rRNA molecule (18S with 1,900 nucleotides) ~ 33 different proteins (S1-S33)
large subunit	50S - Two rRNA molecules (5S and 23S, with ~120 and ~2900 nucleotides, respectively) ~ 31 different proteins (L1-L31), among which only L12 is present in more than a single copy	60S - Three rRNA molecules (5S, 5.8S and 28S, with 120, 156 and 4,700 nucleotides, respectively) ~ 50 different proteins (L1-L50)

2. Recent progress in ribosomal crystallography

Remarkable accomplishments in characterizing the machinery of protein biosynthesis have been made at the turn of the millennium. Following two decades of preparative efforts [1], structures of ribosomal particles have been determined. These include the large ribosomal subunit of the archaeon *Haloarcula marismortui*, H50S [2] and the eubacterium *Deinococcus radiodurans*, D50S [5], the small subunit from the eubacteria *Thermus thermophilus*, T30S [4,5] and the entire ribosome from the same source, T70S [6]. The earlier studies are reviewed extensively (e.g. [7–9]). More recent structures include vacant ribosome [10], functional complexes of ribosomes with mRNA and tRNAs [11–15] and/or with recycling [16,17] and release factors [18]. Additional crystal structures are of functional complexes of small subunits with mRNA [19] and modified tRNAs [20,21]; large subunits with substrate analogs extending from the initial (e.g. [22]) to more sophisticated complexes [23,24]; large subunit with non-ribosomal auxiliary factors: the first chaperone to encounter the emerging nascent protein, the trigger factor [25–27] and the ribosomal recycling factor [28]. Most of the currently available structures are of ribosomes from organisms that have adapted to extreme environments, as these are more suitable for crystallization. Yet, owing to the high level of conservation of the ribosomal functionally relevant domains, the extremophile ribosomes and

their genetically modified phenotypes can represent ribosomes from non-extremophile species [29].

Stimulated by the emerging structures, ribosome research has undergone a quantum jump, yielding exciting findings concerning various aspects of protein biosynthesis in prokaryotes (e.g. [30–53]), which could be extended and/or paralleled with corresponding events in eukaryotes (e.g [54, 55]). Likewise, the structural basis for clinical relevance of antibiotics targeting ribosomes despite their high conservation has progressed significantly. Crystal structures of complexes of ribosomal particles with their antibiotics obtained until 2005 have been reviewed elsewhere (e.g. [56–60]. More recent findings are reported in [61, 67] or presented here. Still emerging are elaborate analyses of results that have led to plausible [68] or controversial biological implications. An example for the latter is the finding that mutation of the nucleotide determining macrolide antibiotic binding to eubacterial ribosomes (2058) form guanine, as in eukaryote, to adenine, as in pathogen [70] results in antibiotic binding, but does not confer antibiotics sensitivity [71] as originally expected [70].

Account of the currently available crystallographic data and highlights of some of the issues that remain unresolved, alongside a brief summary of the functional implications of the recent structures of the bacterial ribosomes are presented in this review. The bacterial ribosomes are of immense contributions to the understanding the universality of protein biosynthesis and the divergence from it. Thus, although the translation apparatus in eukaryotes is larger and more complicated than in bacteria, the research on the bacterial ribosome has led to imperative insights into key issues concerning ribosomes of the eukaryotic kingdom as well as opened new routes for the development and improvement of ribosomal antibiotics . These are accompanied by several (out of many) of the recently published numerous biochemical, genetic and cryo-EM studies that expand ribosome research beyond the crystal structures.

3. Ribosome mode of action

Ribosomes comprise two ribonucleoprotein subunits (Figure 1a) that associate to form the functional ribosome. While elongation proceeds, each subunit operates cooperatively. The small subunit provides the mRNA binding machinery (Figure 1b) and the path along which the mRNA progresses, the decoding center and the major component controlling translation fidelity. The large subunit performs the main ribosomal catalytic function, namely amino acid polymerization, and provides the protein exit tunnel. tRNAs, the molecules decoding the genetic information and carrying the amino acids to be incorporated in the growing protein, are the

non-ribosomal entities that join the two subunits, as each of their three binding sites: A-(aminoacyl), P-(peptidyl), and E-(exit) reside on both subunits (Figure 1a). The initial tRNA binds to the first codon of the mRNA at the P-site and the next tRNA, which enters the ribosome via the dynamic L7/12 stalk (Figure 1a), attaches to the next codon at the A-site. While a peptide bond is formed, the A-site tRNA is translocated to the P-site and the deacylated tRNA moves from the P-site to the exit (E)-site on its way out from the ribosome, through the mobile L1 stalk (Figure 1a). At each elongation cycle both subunits participate in translocating the mRNA and the tRNA molecules by a single codon.

The surface of the intersubunit interface is composed predominantly of ribosomal RNA (rRNA), and in the assembled ribosome all functional sites are located close to this interface. Hence, unlike typical polymerases, which are protein enzymes, RNA is the major player in ribosome activities. The site of peptide bond formation, the peptidyl transferase center (PTC), is positioned within a universal pseudo 2-fold symmetrical region (Figure 1c), composed of highly conserved nucleotides and called 'the symmetrical region'. This means that each point on the fold 90 nucleotides comprising one half of the symmetrical region, is related by a rotation of 180 degrees around an imaginary axis, located at the middle of the PTC, to its mate on the other half, which is also composed of 90 nucleotides. In addition to the rRNA fold, this internal symmetry relates the nucleotide orientations (Figure 1d and figure 2a-c), but not nucleotides sequences. The entire symmetrical region is highly conserved [39, 40] in which 98% of the nucleotides are ‘frequent’ (found in >95% of sequences from 930 different species from the three domains of life), whereas only 36% of all *E. coli* nucleotides, excluding the symmetrical region, can be categorized as such. Importantly, 75% of the 27 nucleotides lying within 10Å distance from the symmetry axis are highly conserved. Among them seven are completely conserved [40].

The high level of conservation of the symmetrical region, its central location and its link to all ribosomal features involved in amino acid polymerization (Figure 1c) [7, 23, 39, 40] indicates that it can serve as the element signaling between remote ribosomal locations (up to 200Å away from each other) and thus can coordinate translation processes. This is consistent with the observed relationship between PTC occupation and mRNA binding to the small subunit [48].

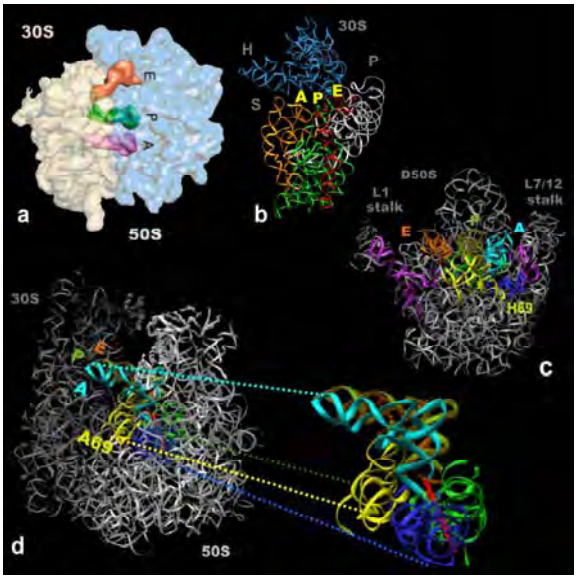

Figure 1. The ribosome functional centers. (a) The two ribosomal subunits. Left: the small ribosomal subunit (T30S) [4]. The approximate positions of codon-anticodon interactions of A-, P- and E- tRNAs are shown and the main functional domains are indicated. H, head; S, shoulder; P, platform; L, latch. The arrows designate the approximate directions of the coordinated motions associated with mRNA binding and translocation. The left arrow indicates the creation of the mRNA pore, i.e. the latch motion [4]. Right: The large ribosomal subunit (D50S) [2]. Regions that are involved in amino acid polymerization are indicated. These include the two stalks controlling the A-site tRNA entrance (L7/L12) and the E-site tRNA exit (L1), which are known to undergo a coordinated lateral movement during elongation; the positions where the acceptor stems of the three tRNA molecules (A-, P- and E-) interact with this subunit. Insert: a tRNA molecule on which its two functional domains (the anticodon loop and CCA 3'end, which binds the incoming amino acid or the newly born protein) are marked. The brown circle indicates the portion of the tRNA molecule interacting with the small subunit, and the blue circle shows the portion bound to the large subunit. (b) The positions of initiation factor 3 (IF3) and Shine Dalgarno (SD) region on the small subunit. The small ribosomal subunit is shown in grey. The arrow indicates the possible motion of IF3 C-terminal domain (IF3C). Top: a space-filled view similar to that shown in Figure 1a. Bottom: a more detailed representation of the opposite view. Marked are the IF3 domains (C-terminal, N-terminal and the linker between them); the SD region; the anticodon loops of the three tRNAs (A, P, E), and the proteins involved in IF3 binding. c) The central location of the symmetrical region in the large ribosomal subunit from D50S, shown in grey, with A- and P-site tRNAs (docked according to [6]) and the symmetrical region (colored blue and green) with its extensions (shown in gold). The symmetrical region is shown by blue and green (for A- and P- sites, respectively) with the pseudo 2-fold imaginary axis in red. Note that it connects directly or through its extensions (shown in gold) all the large subunit functional regions, including the bridge, connecting it to the decoding site on the small subunit [39,40].

The ribosome is a dynamic molecular machine that involves structural rearrangements as an integral part of the translation machinery. Various motions have been detected by investigating the reasons for disorder in functionally relevant regions in crystals grown under far from physiological conditions [2, 22] or by cryo electron microscopy (e.g. [72]) and single particles methods [48]. In addition, interpolation between the structure of the unbound large subunit, D50S (e.g. [3]) and that of the entire ribosome, T70S, with 3 tRNAs [6] identified fundamental motions, like the coordinated movement of the two large subunit stalks (Figure 1a) [3, 49, 34] involved in the entrance and release of the A- and E-tRNAs. Also detected in the 30S structure are the head-shoulder movement upon A-site occupation [8] and the 30S head-platform correlated motions (Figure 1a) enabling guidance to mRNA progression [4, 10, 13] together with elongation factor EF-G [73] as part of the ratchet-like intersubunit reorganization [74]. Additional motions were correlated with tunnel gating [68], possible trafficking of nascent chain progression [25], rearrangements caused by elongation factor EF-Tu ternary complex binding that are linked to fidelity control [8], motions within the PTC correlated with activation/deactivation [30], inhibitory action of antibiotics [63] and the rotatory component of the substrate's translocation [7, 23, 39].

4. On the functional contribution of ribosomal proteins

Over the years, the views on the contribution of the ribosomal proteins (r-proteins) to ribosome function have changed dramatically. Originally, r-proteins were thought to carry out the ribosomal catalytic tasks [75], but later it was shown that rRNA performs most of the ribosome functions. The high resolution crystal structures show that in addition to their peripheral globular domains, almost all r-proteins possess elongated loops or terminus extensions, penetrating into the rRNA core, thus seem to serve as entities stabilizing the rRNA conformation. However, alongside their stabilization roles, some r-proteins can facilitate functions requiring mobility (reviewed in [7]). For example, protein L22 appears to cause transient tunnel blockage [68] and L1 and L12, the main protein component of the dynamic L1 and the L7/L12 stalks of the large subunit (Figure 1a) seem to be involved in tRNA translocation (reviewed in [7–9]). Additionally, proteins situated in proximity to functional regions were proposed to support specific activities. Thus, proteins S5, S6 and S12 assist mRNA binding fidelity [8], and proteins L27 [11, 35] (which does not exist in the archaeon H50S) and L2 [76] were suggested to affect peptidyl transferase activity. S12 and L2 are among the few proteins that reside partially on the intersubunit interface and can support the biosynthetic process. Importantly, computational

methods found that S12 and L2 are among the most ancient ribosomal proteins [77].

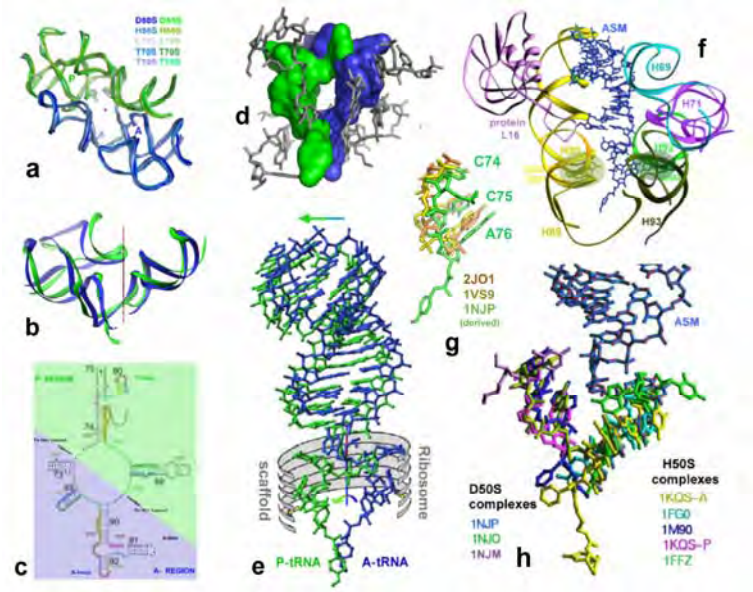

Figure 2. The symmetrical region and peptide bond formation. (a–c). The universal symmetrical region backbone fold. In all structures, the A- and P- sub-regions are shown in blue and green, respectively. The imaginary symmetry axis is shown in red. (a) Superposition of fold of the 180 nucleotides comprising the symmetry region in all known structures, shown as ribbons. The two pseudo-symmetrical sub-regions, containing the A- and the P-sites, are shown in blue and green respectively. The imaginary axis relating the two halves of the symmetrical region is shown as a red rod (or its cross-section). The center of the PTC lies roughly on this axis. (b) Superposition of the backbones of the rRNA comprising the A- and P- sub-regions of the symmetrical region, as obtained by a 180 degrees rotation around the imaginary symmetrical axis, indicating the level of the ribosomal internal symmetry. (c) Two-dimensional representation of the 23S rRNA segment that belongs to the symmetrical region. Symmetrical features are shown in identical colors. (d) Superposition of the locations of short substrate analogs used in crystallographic studies together with H50S and D50S. The PDB accession codes are indicated. (e) The tRNA translocation motion, comprising a synchronized sideways shift, performed as part of the overall mRNA/tRNA sideways translocation (in the direction of the horizontal arrow), and the rotatory motion of the A-tRNA 3'end along a path confined by the PTC grey walls (shown here as ribs). The A-site tRNA and the derived 3'end of the P-site tRNA are shown in blue and green (respectively). The direction of the rotatory motions is indicated by a blue-green curved arrow, the imaginary two-fold symmetry axis is red, and the approximated positions of the symmetrical basepairs [23, 32, 39, 40] are shown in yellow. (f) Superposition of the derived P-site CCA (from ASM 3'end by the rotatory motion) on the crystallographically determined locations of the P-site CCA in crystals of 70S complexes [11, 12]. The PDB accession codes are indicated.

5. Non ribosomal compounds involved in initiation and elongation

tRNA molecules decode the genetic information by matching the complementary bases of their anticodon loop with the codon on the mRNA. All tRNAs are double helical L-shape molecules, except for their anticodon loop and the single stranded 3'end (almost universally CCA) to which the cognate amino acid or the growing peptidyl chain is bound (Figure 1a).

Three non-ribosomal protein factors are involved in the initiation. Initiation factor 2 (IF2) is a GTPase that binds preferentially to initiator tRNA. It acts in a cooperative manner with initiation factor 1 (IF1), which occludes the ribosomal A-site at the small subunit (Figure 1b) and flips out two functionally important bases (A1492 and A1493). These localized changes lead to global alterations in the 30S conformation [8], which seem to be essential for the next steps in translation. Initiation factor 3 (IF3) interferes with subunit association and promotes the ribosome fidelity at the initial phase, by assisting the selection of the initial P-site codon–anticodon interactions. The crystal structure of the C-terminal domain (IF3-c) in complex with T30S indicates its binding to a region proximal to the mRNA channel [8], in a mode suggestive of exploiting its inherent flexibility for an over-the-platform swing to a location suitable for facilitating subunit dissociation (Figure 1b) [56]. Interestingly, IF1 and IF2 (a/eIF1A and a/eIF5B in eukaryotes) are conserved across all three kingdoms of life and cryo-EM studies suggest that they interact with the 30S in a similar manner, although initiation in eukaryotes and archaea requires additional factors.

In prokaryotes, the elongation cycle is driven by GTPase activity of elongation factors. Tu (EF-Tu) delivers the cognate aminoacylated-tRNA to the ribosomal A-site as a ternary complex with GTP, induces long- and short-range conformational alterations, and dissociates after GTP hydrolysis. EF-G contributes to bias the translocation in the forward direction [73]. It binds preferably to the ribosome at its ratcheted conformation, obtained by a rotation of the small subunit relative to the large subunit in the direction of the mRNA movement [74], thus facilitating GTP hydrolysis. Both EF-Tu and EF-G bind to the mobile L7/L12 entrance stalk (Figure 1a) via a conserved region of protein L12 C-terminal domain [49]. In concert with these motions, the deacylated tRNA at the E-site moves towards protein L1, on the other side of the ribosome (Figure 1a), and consequently this protein undergoes a significant conformational alteration in order to release it [2, 6, 10–12].

6. Initiation, subunit association, decoding, and translocation

A prerequisite for correct translation is accurate positioning of mRNA on the ribosome. This step is of utmost importance, hence any divergence can destabilize tRNA binding and inhibit canonical translation initiation [61,62]. In prokaryotes, mRNA placement is assisted by a target pyrimidine-rich region (‘anti Shine-Dalgarno’), located at the 3’end of the 16S RNA. This region anchors the complementary purine-rich sequence at the 5’-end of mRNA (‘Shine-Dalgarno’ or SD) by numerous interactions (Figure 1b) [13] and creates a chamber for transient stabilization of this otherwise labile double helix [19]. In eukaryotes, mRNA placement requires highly sophisticated machinery [54, 55], and throughout evolution it has involved various non-ribosomal factors.

Crystal structures of prokaryotic ribosomes imply that mRNA entrance to its groove on the small subunit involves a latch-like closing/opening mechanism [4,6,15]. These structures also suggest that the mRNA kinks between the A- and P-sites at the decoding region [4,6], and that this conformation seems to be stabilized by a metal ion, which delineates the border between the two sites and prevents uncontrolled mRNA sliding [11]. Once mRNA and initiator P-site tRNA bind to the small subunit the two subunits associate to form the functional ribosome. The surface complementarily is stabilized by over a dozen intersubunit bridges formed by conformational changes of the interface components [3, 6, 11, 12]. Several bridges seem to play roles beyond merely guaranteeing correct subunit interactions. Among them, bridge B2a is particularly important as it connects the immediate environments of the PTC with the decoding center and has the ability to adopt several conformations, depending on the ribosome functional state [2, 23].

The elongation cycle is composed of decoding, peptide bond formation, amino acid polymerization, detachment of the P-site tRNA from the growing polypeptide chain and release of the deacylated tRNA. These processes are facilitated by translocation, which is a successive coordinated movement of the mRNA and its associated tRNAs through the ribosome from A-site to the P-site and then to the E-site, by one codon at a time (in 3’' to 5’' direction). Decoding fidelity, namely avoiding disparity between the mRNA codons and the tRNA anticodons is vital for guaranteeing translation accuracy. The incoming aminoacylated-tRNAs are selected for forming the codon- anticodon base pairing with an error rate of 10^{-3} to 10^{-4} at the highly conserved RNA-rich decoding center of the small ribosomal subunit. The ribosome plays a major role in this selection, exploiting the inherent flexibility of the decoding center for strictly monitoring the base pairing at the first two positions of each codon, but tolerating non-canonical

base pairs at the third position [8]. Furthermore, it appears that normal triplet pairing is not an absolute constraint of the decoding center. For example, flexible expanded anticodon loops of frameshift promoting tRNAs can adopt conformations that allow three bases of the anticodon to span four mRNA bases [20].

The current integrated model for decoding proposes that tRNA selection hinges on discrimination based on the interactions between the ribosomal rRNA and the minor groove of the codon–anticodon duplexes, with a potential to lead to domain closure. Cognate tRNA binding induces global structural rearrangements by domain movements and these modify the conformation of the universally conserved decoding regions so that bases residing in it can interact with the first two base-pairs of the codon-anticodon helix.

7. Peptide bond formation and the polymerase activity of the ribosome

All ribosome crystals structures indicate that the major player in ribosomes activities is RNA [3, 22, 23, 57]. During the past three decades, the preferred substrate analogs used for determining ribosomal functional activity, were ‘minimal substrates’, namely puromycin derivatives capable of creating a single peptide bond. Using similar compounds, which were believed to act as substrate and transition state analogs for complexes with H50S, it was proposed that four universally conserved rRNA nucleotides catalyze peptide bond formation by a general acid/base mechanism [22]. This proposition was soon challenged by various biochemical and mutational studies (e.g. [30, 31, 43]) and additional crystallographic studies on complexes of H50S with similar, albeit more sophisticated, substrates analogs (e.g. [24]) illuminated several aspects of peptide bond formation, such as conformational rearrangements that the PTC can undergo, but did not lead to a feasible consensus mechanism This could be linked to the finding that in all structures of H50S and its complexes with substrate analogs, almost all regions involved in ribosome function are disordered (namely posses simultaneously multiple conformations) presumably owing hey were constructed under far from physiological conditions [2, 22]. Consequently although these structures did not yield the mechanism of peptide bond formation they illuminated an important aspect in cellular regulation of ribosome function, namely that disorder of functionally relevant ribosome regions might represent a common strategy for avoiding non-productive protein biosynthesis.

Structures of a complex of D50S with either an A-site tRNA acceptor stem mimic (composed of 35 nucleotides, including an aminoacylated

3’end, called ASM) [23] obtained under conditions close to those optimized for protein biosynthesis revealed that the acceptor stem of A-site tRNA interacts extensively with the cavity leading to the PTC, and the bond between it and the tRNA 3'end overlaps the symmetry axis (Figure 2d). The high conservation of the components of the symmetrical region, the linkage between the elaborate PTC architecture and the position of the A-site tRNA observed crystallographically [23] indicates that the translocation of the tRNA 3’end is performed by a combination of two independent, albeit synchronized motions: a sideways shift, performed by the overall mRNA/tRNA translocation, and a rotatory motion of the A-tRNA 3’end along a path confined by the PTC walls (Figure 2e). Navigated and guided by the ribosomal architecture, this rotatory motion provides all of the structural elements for ribosome function as an amino acid polymerase, including the formation of two symmetrical universal base pairs between the tRNAs and the PTC [23, 39, 40], a prerequisite for substrate mediated acceleration, rather than acid-base catalysis [32, 33, 43, 51], and for directing the nascent protein into the exit tunnel.

Remarkably, the position of the 3’end of P-site, derived by the rotatory motion that was suggested based on the mode of binding of a tRNA mimic to unbound large ribosomal subunit (D50S), overlaps the positions of full-size tRNAs bound to the entire 70S ribosome (Figure 2f) [11, 12]. Furthermore, all nucleotides involved in this rotatory motion of the tRNA 3’end have been shown to be essential by a comprehensive genetic selection analysis [45]. Consistently, quantum mechanical calculations, based on D50S structural data, indicated that the transition state (TS) for this reaction is being formed during the rotatory motion, and is stabilized by hydrogen bonds formed between the rotating moiety and the same rRNA nucleotides [46]. The location of the computed TS is similar to that observed crystallographically for a chemically designed TS analog in the large subunit from a different ribosome, H50S [24].

Differences between full-size tRNAs and ‘minimal substrates’ were also obtained by biochemical mutagenesis, kinetics and computational studies (e.g. [30-33, 36, 37, 42–44, 50, 51, 53]). These studies showed that the mechanism of peptide bond formation by full-size tRNAs involves substrate mediated catalysis [32], and require the stereochemistry obtained by the rotatory motion [39]. They also highlighted the importance of accurate positioning of the tRNAs, which can be achieved by full-size tRNA or its mimics containg the acceptor stem nucleotides that interact with proximal ribosomal nucleotides [7, 23]. It is important to note, however, that a symmetrical relationship between the reactants of peptide bond formation has been observed in all known structures of ribosomal complexes (Figure 2d), including ‘minimal substrates’ requiring additional rearrangements. In

principle, suitable systems for studying this machinery should include a full-length A-site tRNA bound to the ribosome. However, although 70S ribosomes complexed with full-length aminoacylated-tRNA were crystallized, A-site tRNA 3'ends could not be detected in any of the electron density maps [11–13]. Hence, the only relevant crystallographic information currently available originates from the structure of the complex of D50S with ASM [23].

The correlation between the rotatory motion and amino acid polymerization n rationalizes the apparent contradiction associated with the location of the growing protein chain, since the traditional biochemical methods for the detection of ribosome activity as well as most of the crystallographic studies were based on minimal substrate analogs designed for producing a single peptide bond. These analogs do not undergo A- to P-site translocation, whereas nascent protein elongation requires this motion. Furthermore, the difference between the formation of single peptide bond by minimal substrates and amino acid polymerization highlights the PTC ability to rearrange itself upon substrate binding [7, 30, 58].

The conservation of the symmetrical region is consistent with its vital functions in intra-ribosomal signaling, peptide bond formation and amino acid polymerization. The preservation of the three-dimensional structure of the two halves of the ribosomal frame regardless of the sequence demonstrates the rigorous requirements of accurate substrate positioning in stereochemistry supporting peptide bond formation. This, as well as the universality of the symmetrical region led to the assumption that the ancient ribosome was made of a pocket confined by RNA chains and that the ribosome evolved by gene fusion or duplication [40].

In short, the intricate ribosomal architecture positions its substrates in an orientation that promotes peptide bond formation [23,39,40] and provides the machinery required for the processivity of this reaction, i.e. for enabling the repetition of peptide bond formation, which results in amino acid polymerization. The current consensus view is that the ribosome contributes positional catalysis to peptide bond formation and provides the path along which A- to P-site translocation occurs, whereas the proximal 2'-hydroxyl of P-site tRNA A76 provides the catalysis [32, 51]. This view answers most of the issues associated with this function, nevertheless further studies are clearly required in order to shed more light on the still unresolved issues, such as the possible involvement of protein L27 in this step [35].

8. The termination step

The hydrolytic cleavage of the ester bond in peptidyl-tRNA during the termination step is also catalyzed by the ribosome. In addition to the

participation of ribosomal components, e.g. A2602 ribose [57], peptide release requires auxiliary release factors that recognize the termination codons and promote the P-site peptidyl-tRNA hydrolysis and appear to induce ribosome conformational changes [18]. The disassembly of the ribosome at the end of translation is facilitated in bacteria by the ribosome recycling factor (RRF), in a manner yet to be elucidated. Thus, motions in intersubunit bridges that have been suggested based on a crystal structure of RRF bound to the large ribosomal subunit [28] and to the vacant ribosome [10], were not seen in the crystal structure of T70S in a complex containing a stop codon, a tRNA anticodon in the P-site, tRNAfMet in the E-site and RRF [16]. The mode of E-site tRNA release, its possible involvement in codon–anticodon interactions, and the biological meanings of the different conformations of vacant ribosomes, remain open questions.

9. Nascent protein voyage within the ribosome and its emergence into the cellular environment

Nascent polypeptides progress through their exit tunnel (Figure 3); a universal feature of the large ribosomal subunit that lies adjacent to the PTC [2, 3], and is lined primarily by rRNA with a few r-proteins reaching its walls from its exterior (Figure 3a). This tunnel (~120 Å in length and varying diameter, 10–25Å) possesses the dynamics required for interacting with the nascent protein. Thus, it seems to play an active role in sequence-specific arrest of nascent chains and in response to cellular signals [68], namely in gating and discriminating, as well as in controlling the operational mode of the translocon at the ER membrane [47]. Tunnel wall elements that appear to sense nascent-peptide specific sequences include, in addition to the rRNA, r-proteins L22 [7, 68] and L4 that form the tunnel's constriction, L23 that in eubacteria extends into the tunnel [25], and a crevice adjacent to the tunnel-wall that can provide space for cotranslational transient folding that was suggested by results obtained by non-crystallographic methods, including FRET measurements [41] and computational analyses [38].

While being translated nascent proteins emerge from their protective exit tunnel into the crowded cellular environment before gaining sufficient length to acquire the final fold. Molecular chaperones support correct folding within the crowded cells. In eubacteria, the first chaperone encountered by the emerging nascent chain, called trigger factor (TF), binds to the translating ribosome at ~1:1 stoichiometry by interacting with ribosomal proteins L23 and L29 [25-27]. Protein L23 belongs to the small group of ribosomal proteins that display significant evolutionary divergence. Whereas its globular domain is conserved [25], only in

eubacteria does it possess a sizable elongated loop, which extends from the ribosome exterior all the way into the tunnel walls (Figure 3). At this position, the L23 extended loop can undergo allosteric conformational changes that, in turn, can modulate the shape of the tunnel, which implies trafficking of the nascent protein [25, 26]. Modeling of full-length TF and the signal-recognition particle (SRP) onto the TFa-50S complex suggests simultaneous cohabitation [26] in a fashion that presumably allows screening for hydrophobic signal sequences on the emerging nascent chains [78]. Hence, an interplay between TF, SRP and the trafficked nascent chain while progressing through the tunnel, is plausible.

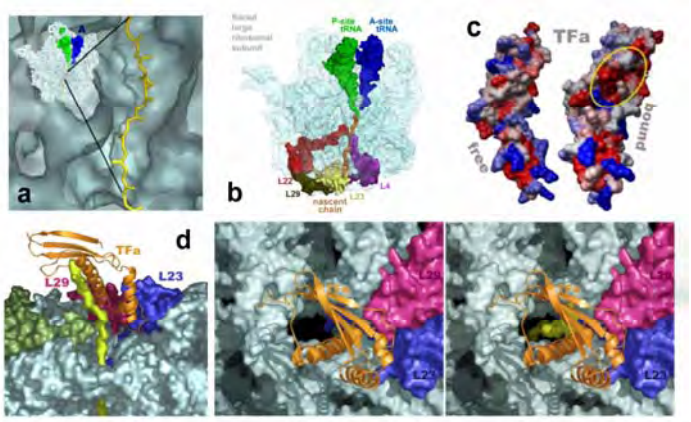

Figure 3. The nascent protein exit tunnel and chaperoning the emerging proteins. (a) The position, the curvature, and the varying diameter of the protein exit tunnel within the large ribosomal subunit are indicated by a modeled polyalanine (yellow). (b) Proteins reaching the tunnel's walls from the large subunit exterior. The tunnel interior is marked by a modeled nascent chain (orange). The large subunit is shown in blue-grey. (c) Conformational differences between free and ribosome bound TFa, based the structure of the homologous complex of TFa and the large ribosomal subunit from D. radiodurans [25] and on the very high level of homology between TF molecules in E. coli and in D. radiodurans. The yellow ellipse delineates the sizable hydrophobic region that becomes exposed upon its binding to the ribosome. The coordinates of E. coli free TFa were taken from [27]. (d) Spacefilling representation of ribosomal RNA (in grey) and r-proteins (in blue, dark red and dark green) at the tunnel opening. TFa is shown as gold ribbons, and a modeled nascent chain as yellow ribbons. Left: the emerging protein (modeled polyalanine) enters the shelter provided by the trigger factor binding domain (TFa). The proteins associated with the trigger factor, L23 and L29, are shown. Note L23 extension reaching the tunnel wall (as shown also in (b)). Middle and Right: a view perpendicular to the view shown in the left, of the tunnel opening. Middle: empty tunnel. Right: A modeled polyalanine chain is emerging from the tunnel. Note that in this crystal structure the tunnel was empty.

Based on the structure of unbound TF from *E. coli* [10], the homology between trigger factor from *E. coli* and *D. radiodurans* and analyses of crystal structures of physiologically meaningful complexes of D50S with

TF binding domain (called TFa) from the same source [25, 26], it was found that TFa undergoes conformational rearrangements that expose a sizable hydrophobic region (Figure 3), thus acquiring a configuration that is suitable for adherence to hydrophobic patches on the nascent chain. Consistent with dynamic studies [41], it appears that TFa prevents the aggregation of the emerging nascent chain by providing a hydrophobic surface that can transiently mask exposed hydrophobic regions of the elongating polypeptide chains until they become buried in the interior of the mature protein.

10. Strategies taken by antibiotics targeting ribosomes

Despite ribosome conservation many of the antibiotics targeting ribosomes are clinically relevant (reviewed in [56–61], [67–71]). As so far there are no crystals of ribosomes from a pathogenic organisms, structural information is currently obtained from the crystallizable eubacterial ribosomes that have shown to be relevant for determining antibiotic targets of pathogens. These structures have shown that antibiotics targeting ribosomes exploit diverse strategies with common denominators. All antibiotics bind to functionally relevant regions, and each prevents a crucial step in the biosynthetic cycle. These include 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.

Alongside rationalizing many genetic, biochemical and medical observations, the available structures have revealed unexpected inhibitory modes. An example is the exploitation of the ribosomal inherent flexibility for antibiotic synergism [56] and for triggering an induced-fit mechanism by remote interactions that reshape the antibiotic binding pocket [63] and consequently led to therapeutical usefulness of an antibiotic family that binds to conserved functional regions, hence not expected to be clinically relevant.

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 [63]. 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.

The identification of the various modes of action of antibiotics targeting ribosomes and a careful analysis of the ribosomal components comprising the binding pockets confirms that the imperative distinction between eubacterial pathogens and mammalian ribosomes hinges on subtle structural difference within the antibiotic binding pockets [56, 58]. Furthermore, comparisons of the different crystal structures of ribosomal particles in complexes with antibiotics indicate 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. Thus, the available structures have also helped to identify 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. Thus, comparisons between the antibiotic binding sites in ribosomes from eubacteria (e.g. from *D. Radiodurans*) and those from the archaeon *H. marismortui*, which shares properties with eukaryotes, highlighted the distinction between binding and inhibitory activity. Specifically, this comparison indicated that the identity of a single nucleotide determines antibiotic binding, whereas proximal stereochemistry governs the antibiotic orientation within the binding pocket [56, 58] 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* [71].

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.

11. Concluding remarks

The high resolution structures have shown that all ribosomal tasks are governed by the ribosome architecture and simulated unpredictable expansion in ribosome research, which has resulted in new insights into the

translation process. Among the new, less expected, findings are the intricate mode of decoding, the mobility of most of the ribosomal functional features, the symmetrical region, the dynamic properties of the ribosomal tunnel, its interactions with the progressing nascent chains, the possible signaling between the ribosome and cellular components and the way the trigger factor prevents misfolding. In addition, unique structural tools for improving antibiotic targets are now available and key issues associated with the structural bases for antibiotics resistance, synergism, and selectivity can now be addressed. 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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