CHAPTER SIX

GLIMPSE INTO THE ORIGIN OF LIFE: WHAT WAS FIRST, THE GENETIC CODE OR ITS PRODUCTS, THE PROTEINS?

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The ribosome is an essential universal cellular apparatus common to all life forms. This molecular assembly decodes the genetic information and catalyzes peptide bond formation. Analyses of all of the known three-dimensional structures of ribosomes revealed that the major ribosomal functions are performed by the ribosomal RNA, in accordance with the suggestion that RNA existed before proteins. Careful examination of the conformations of the ribosomal functional sites revealed that the peptide bonds are being formed within a highly conserved pocket-like region, which seems to be a vestige of a prebiotic bonding entity, termed by us "the proto-ribosome".

Based on the suggested existence of an RNA world, and on the findings that RNA chains can replicate and elongate themselves, as well as possess catalytic capabilities, we proposed that the proto-ribosome is the entity around which life has evolved. We also discuss the consequences of the plausible chemical capabilities of the proto-ribosome in producing random oligopeptides. Based on the "survival of the fittest" evolutionary pathways, we propose that the genetic code co-evolved together with its products, the proteins, as well as the machinery creating them, the ribosomes.

The fundamental role played by the ribosome

Polymerization of amino acids into nascent proteins according to the genetic code is a complex cellular process performed by ribosomes. The

contemporary ribosomes are a multi-component universal cellular assemblies, built of two unequal subunits, each comprising of very long RNA chains (called ribosomal RNA or r-RNA) and a large number of proteins (called ribosomal proteins or r-proteins). For example, bacterial ribosomes, which are the smallest known, are composed of about 54 different proteins and three rRNA chains of a total of 4500 nucleotides and have a molecular weight of ~2.5 mega Da. The more evolved ribosomes (e.g. of mammalians) contain many more rRNA and r-proteins components, and their molecular weights can reach 4 mega Da. Nevertheless, throughout evolution, the actual translation process is performed almost identically by the ribosomes, together with messenger RNA (mRNA) that carries the genetic information, and aminoacylated tRNA molecules that carry the amino acids.

All ribosomes possess three tRNA binding sites, termed A-(aminoacylated), P-(peptidyl) and E-(Exit) sites, each of which resides on both subunits (Fig 1). The amino acylated tRNA resides at the A-site and the peptidylated tRNA at the P-site. The decoding of the genetic information by codon-anticodon base-pairing between the mRNA and the tRNA occurs in the small ribosomal subunit. Peptide bonds are being formed within the large subunit, between the amino acid of the A-site tRNA and the peptidyl of the P site tRNA at the Peptidyl Transferase Center (PTC), and the newly born protein emerges from the ribosomes through a long (~100A) internal tunnel.

We have determined the high resolution crystal structures of the two ribosomal subunits from eubacteria: the small subunit from *Thermus thermophilus*, T30S (Schluenzen et al., 2000) and the large one from *Deinococcus radiodurans*, D50S (Harms et al., 2001). Additionally, we determined the structures of their complexes with various substrate analogues (Bashan et al., 2003), inhibitors, non-ribosomal factors, and over two dozen of clinically-useful, as well as of antibiotics not yet in clinical use, but with the potential to become lead compounds (Auerbach et al., 2009, 2010; Baram, et al., 2005; Belousoff et al., 2011; Berisio et al, 2003a & b, Davidovich et al., 2007,2008; Pioletti, et al., 2001; Pyetan et al., 2007; Schluenzen, et al.,2001, 2004; Yonath 2005).

These studies led to an atomic portrait of the various functional stages of the bacterial translation apparatus, which provides in-depth understanding of the fundamental process of protein biosynthesis. Particularly, analyses of all of the high resolution structures showed that the main catalytic activity of the ribosome is substrate positioning in stereochemistry,

allowing for peptide bond formation. Importantly, in all ribosomes, RNA is the major component (RNA: proteins=2:1), except for in mitochondria, where ½ of the RNA is replaced by proteins (RNA: proteins=1:1). Yet, even in mitochondrial ribosomes the ribosomal active regions are composed of RNA. Remarkably, an RNA machine for peptide bond formation had already been suggested by F. Crick, in 1968. However, as protein enzymes were known to produce almost all cellular tasks, this idea was "hidden" for over two decades, and even when experimental results led to a similar suggestion (Noller et al., 1992).), it was hardly accepted.

The primordial ribosome (the proto-ribosome)

Among our novel findings is the identification of an internal ribosomal region that seems to be a vestige of a prebiotic bonding molecular machine (Agmon et al., 2005; Belousoff et al., 2010; Krupkin, et al., 2011). Thus, analysis of all known three-dimensional structures of ribosomes from prokaryotes, archæa and eukaryotes (Ban et al., 2000; Bashan et al., 2003; Ben-Shem et al., 2011; Harms et al., 2001; Korostelev et al., 2006; Krupkin et al., 2011; Schuwirth et al., 2005; Selmer et al., 2006) revealed that despite the size difference (prokaryotes: 2.5 MDa, eukaryotes: 4 MDa) all known ribosomes function in a similar (almost identical) manner, and that the PTC is situated at the centre of a region of an exceptionally high sequence and structure conservation (Fig 2). This pocket-like structural element accounts for 3–4% of the total ribosomal RNA (depending on the source). It is made of 180 rRNA nucleotides, and is arranged in a semi-symmetrical manner (Bashan et al., 2003 Agmon et al., 2005; Yonath, 2009; Harms et al., 2001; Bashan et al., 2003; Krupkin et al., 2011). This is an extremely unusual feature within the otherwise asymmetric contemporary ribosome. In this pocket-shape element each half binds the 3' ends of the amino acylated and the peptidylated tRNA, at a stereochemistry required for peptide bond formation, hence called A- and P- regions, respectively. In addition, we showed that this region provides not only the scaffold for peptide bond formation, but also for the elongation of the nascent proteins, which involves the translocation of the aminoacylated 3' end of tRNA within the PTC (Bashan et al., 2003).

Importantly, only the backbone and the orientation of the nucleotides of the RNA composing the symmetrical region obey the pseudo two-fold symmetry. There is no sequence identity between its two halves. It is conceivable that the lack of sequence symmetry in the contemporary ribosomes reflects that small, albeit significant differences in the chemical

environments required for each of the PTC parts to perform its specific tasks. Thus, although basically the aminoacylated and the peptidylated tRNA 3' ends are almost identical, each has to support a different action. Whereas the A-site should encourage rotatory translocation to the P-site (Bashan et al., 2003) the P-site tRNA should support stable positioning until peptide bond is formed, and then exit sideways.

The universality of this region implies that it may be a vestige of a prebiotic entity that could have functioned as a bonding apparatus in the RNA world during the prebiotic era. Once amino acids appeared, these turned into forming peptide bonds molecular machines, hence termed by us the proto-ribosomes (Bashan et al., 2003; Harms et al., 2001; Krupkin et al., 2011; Yonath, 2009).

The Proto-ribosome hypothesis

Our hypothesis is consistent with the proposition that the contemporary nucleic acids and protein dominated life emerged from RNA based world, namely the RNA world. This proposition is consistent with observations made by analyzing structures of ribosomes from phylogenetic disparate regions (Bokov &. Steinberg, 2009). It is based on RNA dual functionality, as it can act as a replicase capable of storing and expressing genetic information and, in parallel, as an enzyme with some inherent functions that could have been useful in the RNA world (e.g. splicing, self-splicing and replication, namely synthesis of complementary RNA strands by template-directed assembly of oligonucleotides, where the template could have been one of its own strands, etc.). Notably, the ability of RNA to form peptide bonds was shown by *in vitro* selection experiments (Zhang &Cech. 1997), albeit in a fashion that may allow chain elongation in the reverse fashion compared to natural nascent protein synthesis.

The prebiotic creation of the proto-ribosome

It is conceivable that the proto-ribosome evolved from a molecular entity that performed RNA needs in the RNA world, termed the pre-proto-ribosome, which was made solely of RNA with still uncertain structural properties. It could have been an enzymatically active (Doudna et al., 1991; Zhang, & Cech. 1997) entity reconstituted by a spontaneous self-assembly of two oligonucleotides, each of a rather unstructured RNA chain. It could have had a stable or semi rigid conformation containing a

chemically active pocket, which was "hijacked" by the amino acids, once they invaded the RNA world. Interestingly, the main structural motif of the symmetrical region, namely stem-elbow-stem motif (SES, figure 2) (Belousoff et al., 2010; Davidovich et al., 2009; Krupkin et al., 2011) has been detected frequently in many "ancient" RNA molecules, including tRNA (Fig 2). It is also likely that this prebiotic entity has been rather flexible and possessed an inducible conformation, which could obtain its functional fold upon its substrates binding. The latter options bypasses the seemingly formidable challenge for two relatively large RNA chains to form a pocket with its active site situated at the interface between them.

Evidence for the pathway of the creation of the proto-ribosome is scarce. However, chemical synthetic biology experiments aimed at examination of some logical suggestions are being carried out (Belousoff et al., 2010; Davidovich et al., 2009; Krupkin et al., 2011). One of our hypotheses suggests that the catalytic proto-ribosome is a product of dimerization, and requires the existence of self-replicating, self-folding and self-dimerizing RNA molecules capable of self- and/or substrate-induced pocket formation. Ongoing experiments showed a non-uniform tendency to dimerize. Thus, when examining the dimerization tendency of chains resembling the P-region, the A-region (Fig. 3), and mixtures of A- and P-regions, only P-region homodimers could be detected. The preference of selected sequences over very similar ones (albeit not identical) seems to indicate that natural selection, which is commonly related to the evolution of species, could have played a major role in the prebiotic world.

Furthermore, the observed significant tendency to form homodimers composed of two P-region chains, as opposed to A-site homodimers or A/P heterodimers, may indicate that the proto-ribosome was originally a symmetrical dimer of an RNA chain of sequence resembling the P-side of the contemporary PTC, which later underwent optimization from homo to hetero dimers alongside the evolution of the entire translational machinery. Such homodimers could also be produced from a single chain obtained by "RNA-gene" duplication or fusion.

Proto-ribosome confinement

The proto-ribosome could have evolved in any environment, independent of any kind of compartization. However, confinement within vesicles that could act as protocells may have occurred. Such environment may have been advantageous for the contingency in proto-ribosome formation, as

compartization ensures proximity, hence higher local concentrations of the proto-ribosome components, their substrates and the major synthetic intermediates. It was shown that the combination of stability and dynamics of the cell boundaries is critical for building functional and replicating proto-cells, and that membranes made from simple amphiphiles can form stable vesicles capable of retaining encapsulated functional RNAs in the presence of divalent cations.

Such circumstances could have played a major role in the proto-ribosome stabilization. In support of this scenario is the finding that RNA can bind to vesicles made of ordered phospholipid with a high affinity (Janas & Yarus, 2006), thus may facilitate localization of the RNA apparatuses and enhance the interactions between their products. It is noteworthy that our approach, namely a bottom-up attempt at constructing an autonomous molecular bonding machine, is different from the top-down approach performed elsewhere, aimed at creating a minimal ribosome-like entity by detaching selected components from the contemporary ribosome (e.g. (Bokov, & Steinberg, 2009; Hsiao et al., 2009; Noller et al., 1992).

Modified nucleotides

The contemporary symmetrical region contains several post-translational modified RNA bases (Green & Noller, 1996). Interestingly, compared to the overall fold and sequence detected in this region (e.g. Woese et al., 1978), their number and positions are less conserved. In the contemporary world, modified nucleosides, which exist in all organisms, are formed during processing of nascent precursor RNA transcripts, and seem to provide additional functions since the variety of complex tertiary structures that can be formed of the four canonical bases are not sufficient to fulfill all functions required in the contemporary world (Doudna & Cech, 2002). Basically, such modifications could have occurred in the prebiotic RNA world by spontaneous chemistry (e.g. of adding an amine or an amino group to adenine and guanine under prebiotic conditions (Levy & Miller,1998; Maurel & Ninio, 1987). However, we have no evidence that implies that the proto-ribosome could not function without them.

The proto-ribosome within the contemporary ribosome

The preservation of RNA activity in performing the extremely important process of genetic code translation indicates that RNA is capable of

handling the complexity of the current cellular life, which requires a highly controlled sophisticated regulatory mechanism. Obviously, translation is much more complicated than accidental peptide bond formation. We propose that the kernel of the ribosome function has been transferred from the RNA world, and that most of the additional ribosome components were added while entering the protein-DNA-RNA-life era, which, in addition to peptide bond formation, requires performing the task of translation. Remarkably, within the contemporary ribosomes the distances between the regions involved in ribosome's function are far beyond the possibility of any direct "chemical talk" (70-140 A). The symmetrical region is located at the heart of the ribosome, and chemically connects to all of the ribosome functional centres involved in translation (fig 3). Hence, it can transmit signals between them.

From proto-ribosome to the contemporary ribosome

The ribosome has been, and still is, an RNA machine. RNA enzymes are known to be inefficient and hence earned nicknames such as lousy and lazy. Nevertheless, in the contemporary world, the ribosome is an amazing chemical apparatus, indicating that nature devised means to create an efficient apparatus even from RNA.

We propose that the turn from the inefficient prebiotic RNA enzymes to the amazingly efficient contemporary ribosome occurred by the addition of the ribosomal proteins. Thus, in the contemporary world, the ribosomes' architecture is stabilized by proteins, and although the actual catalytic and decoding events are performed by rRNA, the ribosomal proteins contribute significantly to the maintenance of the ribosome accurate structure and function. The ribosomal proteins also perform various functions: particularly the interactions with cellular components utilize r-proteins.

How did the ribosomal proteins appear? It is conceivable that the initial di-amino acids were the substrates for the following peptide bond formation reaction, and so on. Molecular selection maintained and multiplied those oligopeptides that stabilized the proto-ribosome, or fulfilled tasks needed in the RNA world. It is conceivable, albeit not obligatory, that the initial oligopeptides were rather small, contained a high proportion of basic amino acids, and had a rather simple tertiary structure. As they were performing needed tasks, their existence could have triggered the creation of the initial genetic code. With the appearance of life, including the initial

genetic code and the evolution of cellular entities, there was a need to increase the complexity of the machinery that produces proteins.

We suggest that the contemporary ribosomes expanded around the proto-ribosome. Although no species carrying a ribosome that is smaller or simpler than that of the bacterial ribosome is known, a careful analysis of the internal ribosome RNA interactions confirmed the above hypothesis (Bokov, & Steinberg, 2009; Hsiao et al., 2009).

The number and complexity of the proteins that interacted with the proto-ribosome grew simultaneously with the expansion of the ribosomal RNA. Some of those became the current ribosomal proteins. All bacterial ribosomal proteins acquired a three-dimensional structure tailored for their main function—stabilizing and maintaining the ribosomal accurate structure (Fig 4). In all species several proteins interact with the symmetrical region. Some of those that are in close proximity to the symmetrical region may contribute to its functionality. An example is the bacterial protein L2 that facilitates protein elongation although it is not needed for single peptide bond formation (Cooperman et al., 1995). It has direct interaction with the symmetrical region of the contemporary prokaryotic and eukaryotic ribosomes (Fig 5) and has been suggested to be one of the most ancient ribosomal protein appeared (Sobolevsky and Trifonov, 2005).

A few bacterial ribosomal proteins evolved to withstand extreme conditions. Examples are S17 and CTC (Schlunzen et al., 2000; Harms et al., 2001, respectively), the structures of which reveal possible pathways from mesophiles to thermophiles to extremely robust radiophiles. Thus, in thermophiles, both S17 and CTC possess at least one additional domain (compared to mesophiles), which is positioned at a location that can minimize the harm that can be caused by the increased motions of the proteins owing to the increased available energy at high temperature (Fig 6). Further adaptation to the environment is observed in protein CTC, which contains an additional domain, connected to the rest of the protein by highly flexible hinge, which allows swinging into the active site and preventing tRNA binding under starvation (Bashan et al, 2003; Yonath, 2002).

A Similar trend is observed in the evolution from prokaryotes to eukaryotes, as the growth in ribosome size is related mainly to additional ribosomal proteins. Furthermore, a large fraction of them have more sophisticated chemistry, obtained mainly by post-translational modifications

(e.g. phosphorylation), which seem to be needed for interactions and signal transmission with higher organisms cellular components, in order to function at much higher life complexity.

The emergence of the genetic code

As mentioned, we propose that the genetic code was developed and optimized alongside the evolution of the ribosome and the appearance of the proteins. We envisage this process as progressing from accidental formation of peptide bonds. Activated amino acids are suitable substrates for the modern as well as the ancient bonding machine. These could be formed by their attachment to nucleotides, exploiting rather common reactions that were shown to occur by diverse processes under prebiotic conditions. Structural analysis of the contemporary ribosomes indicated that the suggested pocket-like entity can accommodate substrates such as amino acids bound to up to three nucleotides. Hence mono, di- and tri-nucleotide carrying natural or modified amino acids could serve as substrates of the proto-ribosome. In the RNA world such compounds could have been obtained by self aminoacylation (Illangasekare & Yarus, 1999).

It is conceivable that some of the initial accidental dipeptides produced by the proto-ribosome could have been the substrates for following reactions of peptide bond formation, which could have been elongated into oligopeptides. The well performing oligopeptides may have survived, and consequently led to the emergence of the genetic code. Examples for well performing oligopeptides are those catalyzing fundamental reactions. A hypothetical non-coded “enzyme” that can be useful for RNA metabolism could have been formed from histidine-rich oligopeptides, which could be useful as metal carriers (Belousoff et al., 2010). In contrast to the common view, histidine may been among the first amino acids, since its imidazole ring could be snatched by the amino acids, owing to its availability in the RNA world as a left-over of damaged nucleotides.

Another task that could have been needed in the RNA world, and that could have been performed by the oligopeptides, is stabilizing the machines producing them (Fig 5), namely the proto-ribosome. Such a sequence of events suggests that the genetic code was created by, or according to, its products, which were found fit and useful—therefore survived. They could have led to the creation of a primitive genetic code, which co-evolved together with its products and the ribosomes.

Indeed, the high-resolution structures of the ribosomes show that almost all ribosomal proteins possess extended termini and/or elongated internal loops that are injected into the ribosomal RNA in a fashion that can stabilize its structure, hinting that they are presumably partially responsible for the transition from a poorly operating proto- ribosome to an efficient contemporary apparatus. This is in accord with the general description of proteins as essential features in the transition of the RNA world into the protein-nucleic acids era (Szathmary, 1999).

Conclusions and open questions

We suggest that the origin of the contemporary ribosome is a functionally active proto-ribosome, which can be defined as an RNA molecular entity capable of binding substrates and catalyzing the formation of chemical bonds that functioned in the prebiotic era as a molecular machine. We further propose that the proto-ribosome was the "molecular kernel" around which the modern ribosome evolved, although still open is the question: was the sequence of the proto-ribosome identical, similar, or different to the sequence observed in the symmetrical region within contemporary ribosomes?

The preservation of RNA activity in performing ribosomal functions shows that RNA, which is commonly known to be a rather inefficient enzyme, could become an efficient biological machine for producing proteins. Our hypothesis also suggests that the incorporation of ribosomal proteins facilitated the alteration of the ribosomes from an inefficient to a highly efficient molecular machine. This notion is in line with the suggestion that the genetic code co-evolved together with the ribosome as well as its products, the proteins. Hence, from this point of view we provide a plausible answer to the chicken-or-the-egg conundrum.

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Chapter Six Figures

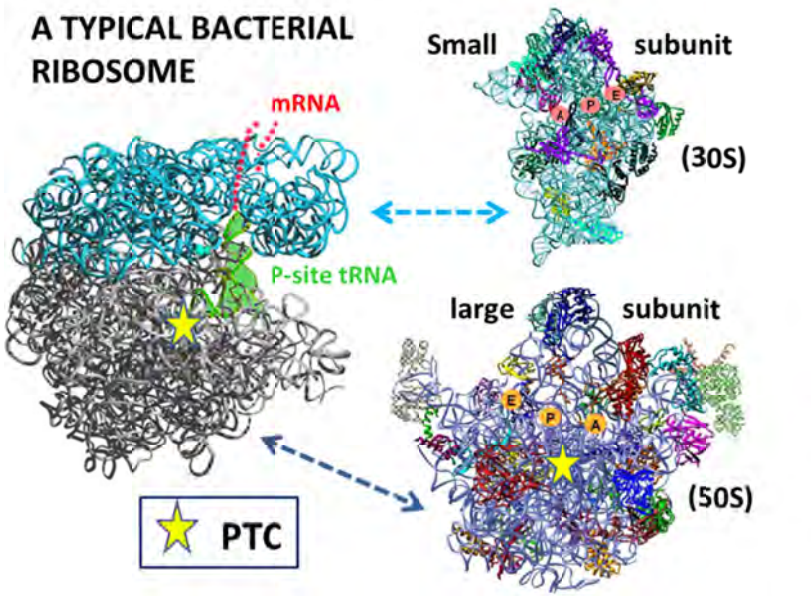

Figure 1. The assembled bacterial ribosome (left), and its two subunits (right), the three tRNA binding sites are marked. The approximate mRNA path and a P- site tRNA is shown on the left. The yellow star shows the approximate PTC position.

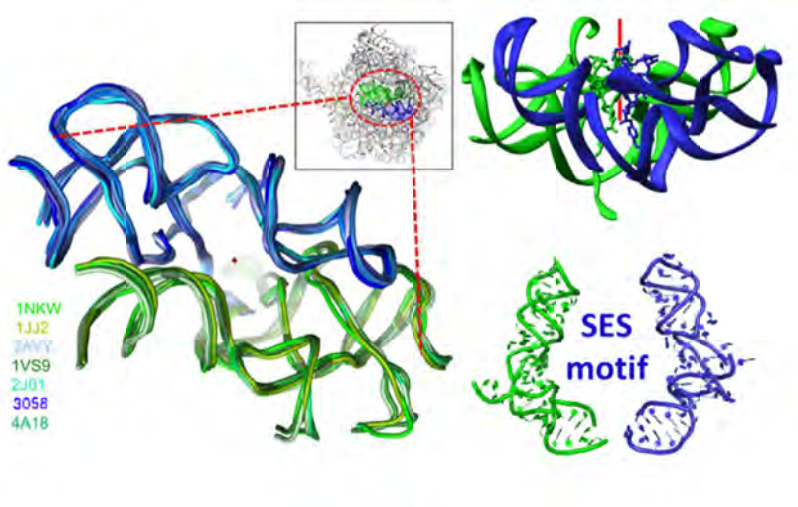

Figure 2. In all figures the A-region is shown in blue and the P-region in green. Left Top: zoom at the symmetrical region within the RNA scaffold of the large subunit. Left bottom: view into the overlay of the symmetrical region in all known ribosome structures. The red dot represents the position of the symmetry axis, which is perpendicular to the plane. Top right: the pocket-like structure of the symmetrical region with its substrates. Bottom right: the main structural element of the two halves of the symmetrical region (SES: Stem-Elbow-Stem), indicating the similarities and differences between them.

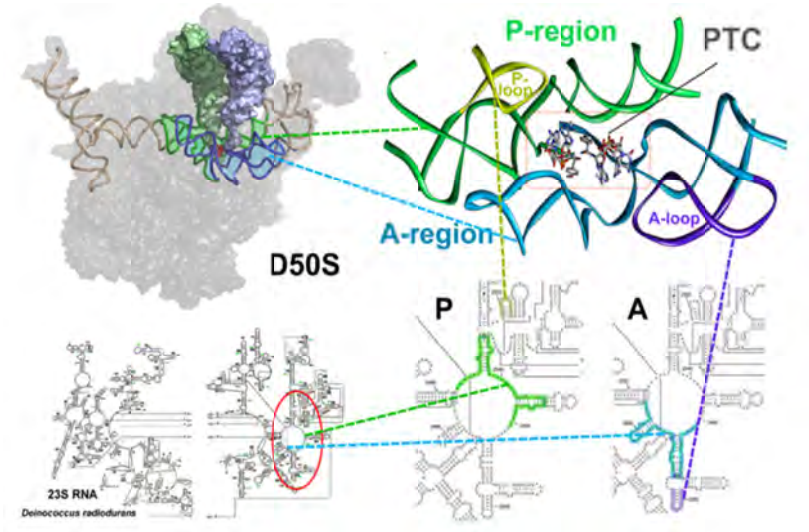

Figure 3. Top left: The location of the symmetrical region within the contemporary large subunit (grey background). Right: a view into the symmetrical region and its substrates CCAaa and CCpuromycin (in the middle). Bottom: left: the two-dimensional diagram of the 23S RNA in D50S. The symmetrical region is encircled in red. Its two components, namely the A and the P-regions are shown in more detail on the right.

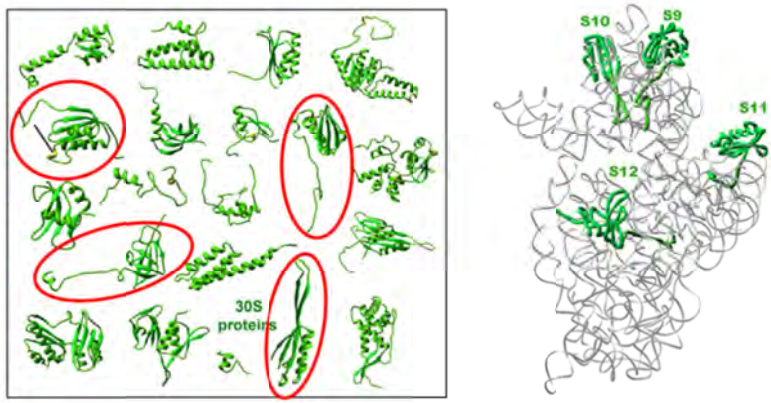

Figure 4. Left: the structures of all the small-subunit ribosomal proteins, those encircled in red are shown in the right within the rRNA scaffold of the small subunit.

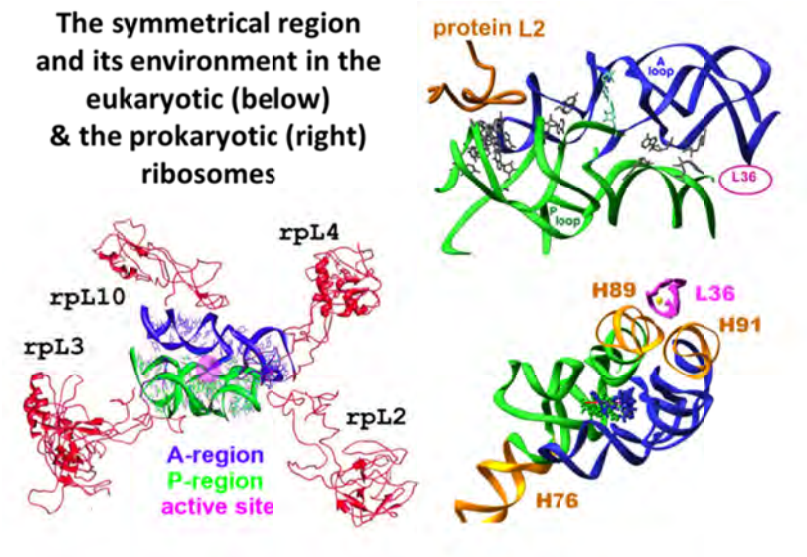

Figure 5: the proteins interacting with the symmetrical region in eukaryotic ribosomes (shown in red) and prokaryotic ribosomes (in gold).

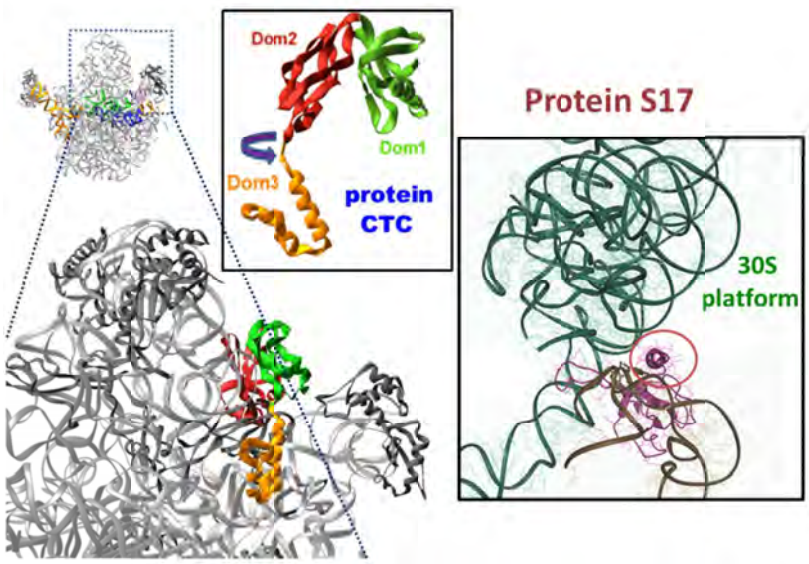

Figure 6. Left: protein CTC in its position within the large subunit. Its three domains are shown within the frame on the top right. Domain 1, called L25, exists in all mesophiles (e.g. E. coli) and seems to protect the bridge between the large and small subunit, in the proximity of A-site tRNA anti-codon loop. In the thermophile Thermus thermophilus this protein is built of domains 1+2 (collectively called TL5), is situated so that it should minimize additional motions that become possible by the increased available energy at high temperature. Domain 3 is connected to domain 2 by a highly flexible hinge, which allows swinging into the active site and filling it up, thus preventing A-site attachment under starvation.