Communication

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Origin of Life: Chiral Short RNA Chains Capable of Non-Enzymatic Peptide Bond Formation

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Abstract: A semi-symmetric vestige of an RNA apparatus with stereochemically controlled ribozyme capabilities is embedded and functions as the site of peptide bond formation within all contemporary ribosomes, a finding that is in line with Lahav and Leiserowitz earlier discoveries on stereochemically controlled chemical reactions. As the structure of this semi-symmetrical self-folded RNA entity is almost fully conserved in all known ribosomes, it seems to be resilient to evolution, thus hinting at its pre-biotic origin and hence, suggested by us, to be the protoribosome. Recent studies, described shortly below, demonstrated peptide bond formation by the laboratory-designed protoribosomes, which supports the notion that a pre-biotic bonding entity is still functioning in all contemporary ribosomes.

Introduction

Stereochemistry and Protein Biosynthesis

Meir Lahav and Leslie Leiserowitz (LL) have been working together for many years on the topology of crystals and the stereochemical control of crystal formation and growth.[1] Among their eminent contributions are their milestone pioneering experiments relating crystal shape to molecular structure.[2,3] These, alongside their later studies, cracked the 140 years enigma of the relationship between crystal morphology and molecular chirality, illuminated macromolecules' self-assembly, set the bases for engineering 3-dimensional crystals, explained crystal growth dynamics, and demonstrated the existence of self, non-catalytic reactions. These renowned achievements were obtained in the mid-eighties, when we initiated our structural studies on ribosomes, the universal cellular multicomponent RNA-protein complexes that translate the genetic code to proteins in all living cells.

Almost two decades later, we determined the structure of the large ribosomal subunit,[4] where peptide bonds are being formed. Consequently we could critically examine its mode of biosynthetic function, which was found to be tangential to LL non-enzymatic reactions. Thus, we showed that in all known contemporary ribosomes the peptide bonds are formed within an almost fully conserved RNA-made semi-symmetrical pocket that provides the proper stereochemistry for peptide bond formation.[5–7] This reaction is totally dependent on the positioning of the amino acid substrates and does not require any enzymatic activity, in contrast to a suggestion made elsewhere, in accordance with the previous common belief of ribosomal enzymatic catalysis.[8]

Ribosomal Functional Centers

The concerted universal process of the translation of the genetic code to proteins is performed by a complex apparatus composed of the ribosome, messenger RNA (mRNA), transfer RNAs (tRNAs), and several protein factors. The origin of this fundamental process and its evolving pathway have been intensively studied.[9] The ribosome, a universal dynamic cellular riboprotein apparatus, was found to be the key player in this process as it provides the required sites and machinery. All ribosomes are assemblies of very long RNA (rRNA) chains and many ribosomal proteins (RPs), arranged within two unequal subunits in a precise fine-tuned interwoven dynamic structure. Both ribosomal duties, decoding (performed at the small subunit) and peptide bond formation (performed at the large one), are highly conserved, hinting at the ribosomes pre-biotic origin.

In the nineties, it was shown that despite the common assumption that ribosomal proteins perform the ribosome's activities, both ribosomal duties are actually performed by their RNA.[10–13] Nevertheless, the actual mode of ribosomal function remained elusive, as non-enzymatic stereochemical bonding was not readily employed in biochemistry, and no three-dimensional structure of the ribosome was available. Only after the ribosome structure became available was it proved that the ribosome is a ribozyme, and biochemical studies aiming at connecting the ribosome structure to the nature of its active sites started to emerge.[14,15]

In the ribosome, peptide bonds are being formed between the amino acids that are brought to it when bound to the fully cobserved CCA ends of the tRNA molecules in a specific manner.[16–19] The actual reaction occurs within a semi-symmetrical molecular pocket that hosts the A- and P-tRNAs, situated within the peptidyl transferase center (PTC),), which

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provides the sites for the two CCA-tRNA ends. This semi-symmetrical entity provides the framework for optimal positioning of the ribosomal substrates in a favored stereo-chemistry for peptide bond formation[20] and it confines the void required for the motions associated with protein elongation.[21–23] The amazingly high conservation of this semi-symmetrical site structure, which seems to be preserved throughout the entire living kingdom,[6,24,25] indicates that it is resistant to evolution. Hence, suggesting that it could have existed as a self-folded active entity in the pre-biotic world. Therefore, called by us the protoribosome[5] *i. e.* the ancestor of the contemporary ribosome.

We assume that the protoribosome could have functioned as an apparatus catalyzing various reactions involved in RNA metabolism. Once amino acids appeared, it was found suitable to provide the stereochemistry required for connecting two amino acids, namely for peptide bond formation and later on for non-coded oligopeptides elongation. Notably, the fold of each of the protoribosome's halves, namely those hosting the A- and P-substrates, resemble folds that were identified as primary building blocks of "ancient" as well as "modern" functional RNA molecules of comparable size, including tRNA, gene regulators, riboswitches, ribozymes catalyzing the phosphodiester cleavage, and RNA processors.

Our protoribosome concept, which relies on the existence of self-replicating, self-folding and self- dimerizing RNA molecules, is also partiallyrelated, partially, to studies performed elsewhere. These include for example, the ability to synthesize ribonucleotides under so called "pre-biotic conditions", self- sustained replication of an RNA enzyme;[26] the synthesis of activated pyrimidine ribonucleotides under pre-biotic plausible conditions[27] and systems chemistry on the early earth, which is a new way of looking at the synthesis of RNA.[28] Additionally, a very recent report indicated that even Mg dependent RNA dimers, of a sequence of ribosomal RNA chains that includes the protoribosome and a part of its immediate neighborhood, were found suitable for allowing the synthesis of a 9-mer oligo-lysine.[29]

Here we suggest that individual RNA chains can self-fold and iteract with each other to form dimeric apparatuses that could have evolved into the protoribosome. As only the structure of the backbone of the symmetrical region obeys the pseudo two-fold symmetry, with no sequence symmetry, we investigated the terms under which several sequences of this apparatus, found in various existing ribosomes, can produce RNA dimers capable of facilitating peptide bond formation by virtue of the amino acids positioning. Thus, our studies mimic the early steps in the transition from the probiotic era into the present form of life. Furthermore, we suggest that subsequently, once a code for selected oligopeptides was created, the proto-ribosome evolved into the multicomponent molecular machine, the ribosome.

After constructing a few representative protoribosome sequences, by using the "fragment reaction" and MALDI-TOF mass-spectrometry, we showed that some of our suggested protoribosome constructs are indeed capable of mediating autonomous peptide-bond formation. These findings present strong evidence supporting our hypothesis on the origin of life and ribosome's construction, thus suggesting that the proto-ribosome may be the missing link between the RNA-dominated world and the contemporary nucleic-acids/proteins life.

Furthermore, analysis of the progression of this process enabled visualization of a continuous path from the primordial world to contemporary genetic translation.[7] It also indicates that the ribosome is a naturally occurring ribozyme that outlived the transition from the pre-biotic era to modern life, where proteins have immense involvement.

Results and Discussion

As mentioned, the PTC seems to evolve from a vestige of a semi-symmetrical RNA apparatus with ribozyme capabilities, namely the protoribosome, which is shown in (Figure 1).

One of our hypotheses suggests that the protoribosome was formed by dimerization of two RNA chains, a process that required the existence of self-replicating, self-folding and self-dimerizing RNA molecules. As only the backbone and the orientation of the nucleotides of the RNA composing the symmetrical region obey the pseudo-two-fold symmetry, namely, there is no sequence symmetry as well as no sequence conservation, we attempted dimerization of RNA chains of the sequences observed in various ribosomes. We focused on sequences that were expected to mimic the PTC's stem-elbow-stem (SES) fold (Figure 2), which were connected by various combinations of ~4 nucleotides (Figure 3), and observed a non-uniform tendency to dimerize.

We found that constructs mimicking the P-region, called here P-reg (P construct in Figure 3 and its minimal variant, called min-P), derived from Thermus thermophilus (tt) sequences of various lengths, tend to form dimers, whereas those derived from the minimal A-region (namely A-reg), do not, even at higher concentrations. Formation of dimers was also observed with min-P analogs, derived from the PTC sequences of *D. radiodurans S. aureus* and *E. facium.* In addition, in some cases introducing slight modifications into the RNA sequence had a significant effect on the dimerization capabilities.[30] These observations, and the findings that A-reg constructs did not form dimers, led us to suggest that the minimal P-reg is actually the driving force for dimerization of the entire symmetrical region constructs. Hence, the observed preference of selected sequences over very similar, albeit not identical ones, may indicate that survival of the fittest and natural selection could have played a major role in the pre-biotic world, although these properties are commonly related to the evolution of species. Hence, it may indicate that we discovered Pre Darwinian Darwinism, namely the existence of natural selection and the selection of the fittest before living species existed.

The ability to form peptide bonds by our lab constructed representations of the protoribosome, was determined by the

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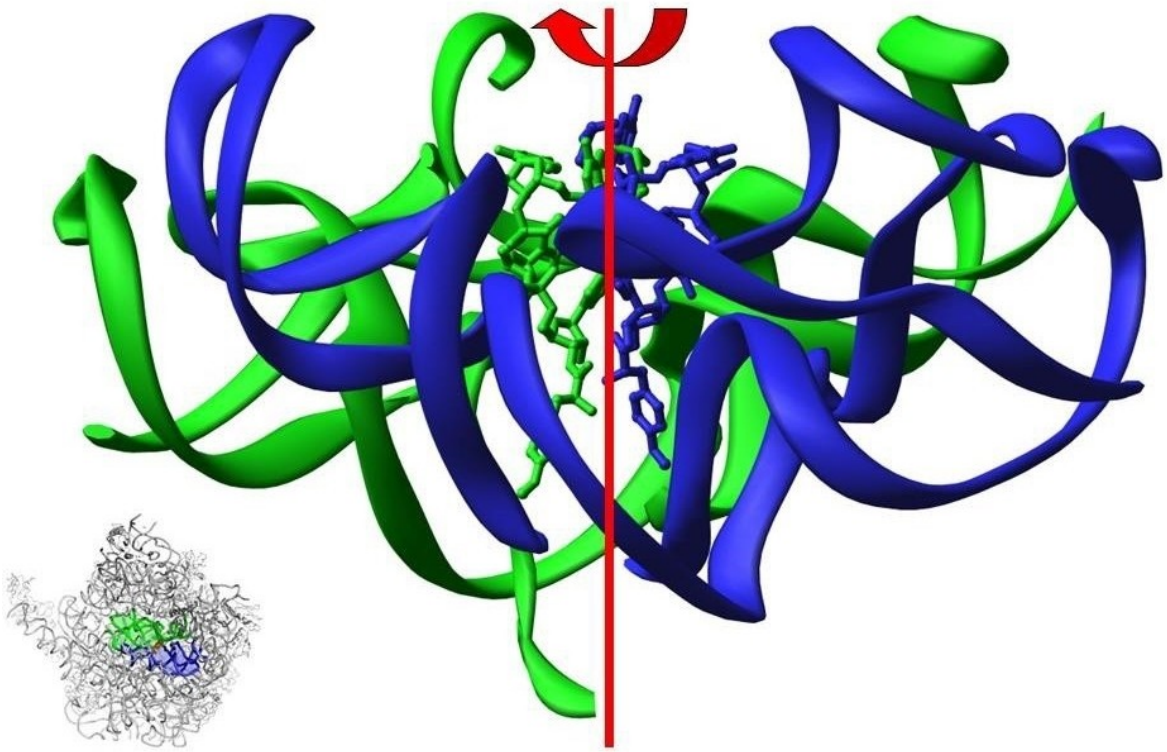

Figure 1. Side view of the PTC semi symmetric region with its tRNA CCA substrates. Its two halves are shown in blue and green, for the P and A sub-sites respectively. The red line indicates the semi-symmetry axis. The position of the symmetrical region within the large subunit of the prokaryotic ribosome is shown in the lower left corner.

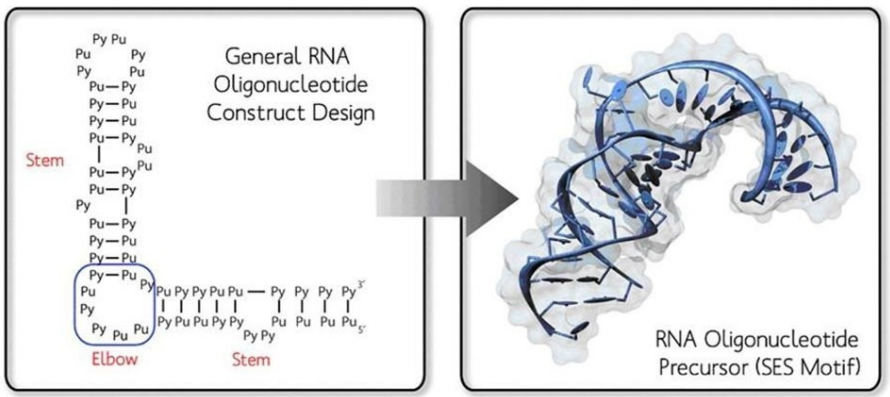

Figure 2. Left - A two-dimensional representation of a general SES construct design. Right – its expected fold.

"fragment reaction" using CCA-pcb and C-Pmn as substrates (Figure 4). MALDI-TOF mass-spectrometry analysis showed clearly that a few of our protoribosome constructs mediated peptide-bond formation (Table 1), hence strongly supporting our hypothesis on the existence of a chemically active protoribosome.

Five out of a total of 12 designed constructs mediated peptide bond formation at 37 °C. The remaining seven constructs, among them 4 that dimerized, exhibited incapability to form peptide bonds. Hence, it appears that dimerization by itself does not assure peptide bond formation. Also, comparisons of the peak intensities between the protoribosome constructs and 50S clearly show that the protoribosome is much less efficient in promoting the fragment reaction (figure 4).

Of particular interest is the finding that constructs that include A- or P-loops, do not lead to peptide bond formation. It is conceivable that the extended constructs may fold to

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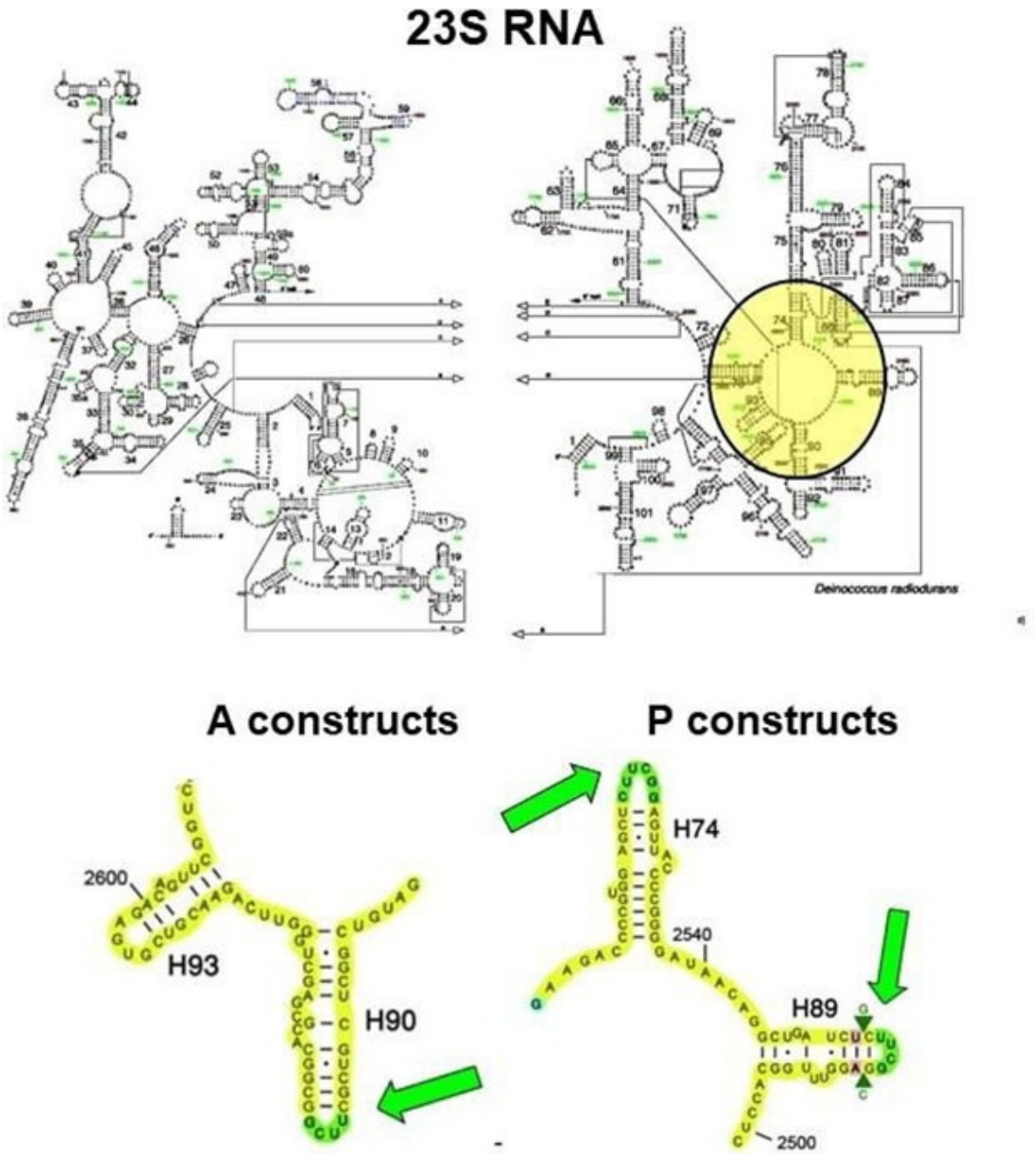

Figure 3. An example for the two halves of a protoribosome construct, based on Deinococcus. radiodurans ribosome.[4] Top: the entire 23S RNA component of the ribosome in which the semi- transparent yellow circle overlaps the PTC. Bottom: A and P constructs denote the bases for the sequences of the designed proto ribosome, based on the A and P components of the PTC. The green arrows point at the green-colored nucleotides, which are needed to connect the fragmented chains that were selected from the contemporary ribosome structure, and consequently were designed and modified by us. We preferred GNRA tetraloop (Where N can be any nucleotide, and R is purine) or the A-minor[31] motifs, which may promote RNA dimerization[32] and even modify them in order to enhance or reduce their symmetrical tendency.

different conformations and accordingly form various dimers, which hinder the binding of the substrates. In contrast, we observed that in most cases the SES PTC motif promoted dimerization/pocket formation. In addition, complementarity [33] between the nucleotides that constitute the two halves of the PTC cavity was detected.

Our results highlight the P-reg as an effective dimer-forming region compared to the A-reg. Accordingly, we suggest that the P-reg served as the ancestral sequence, which

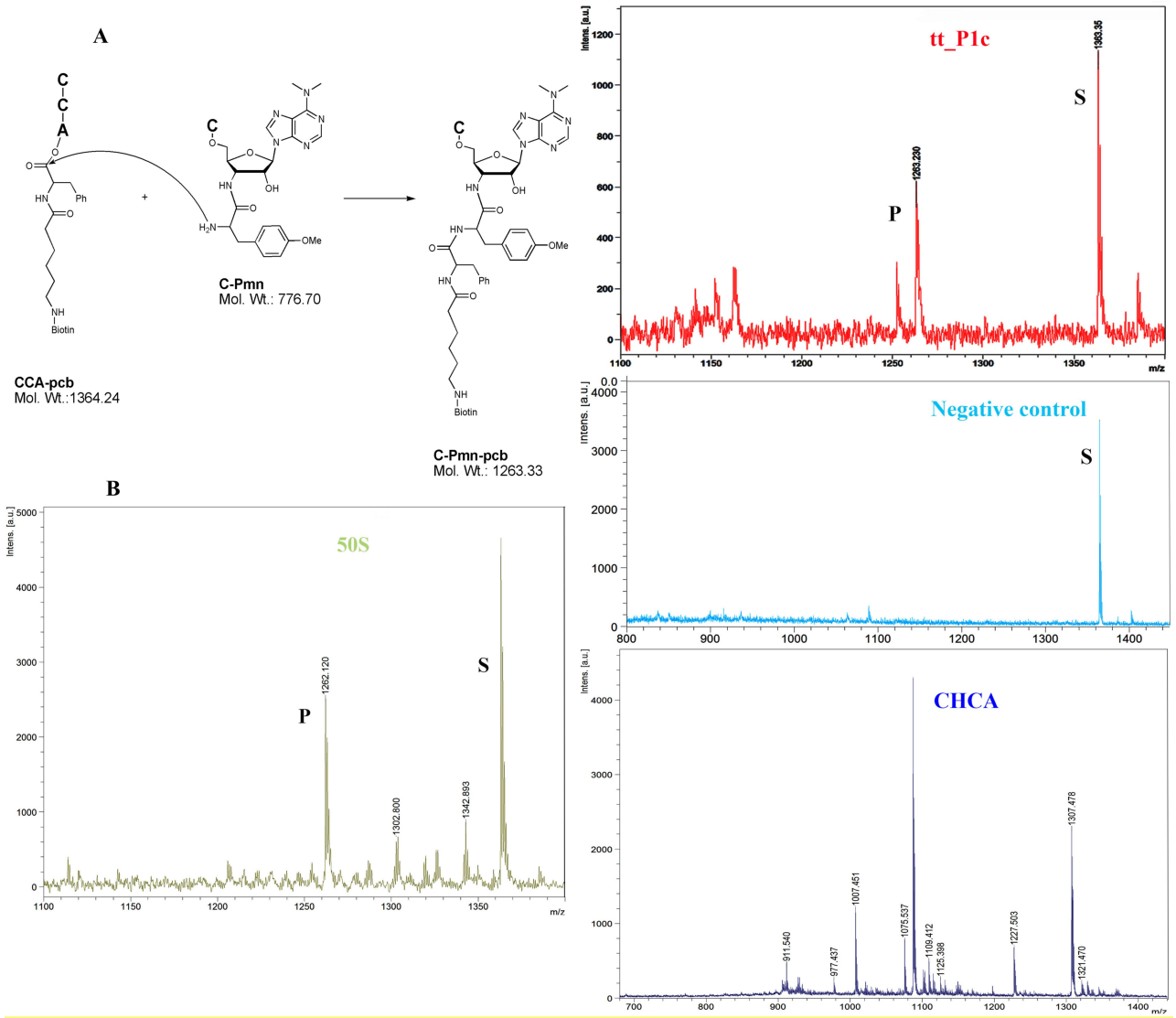

Figure 4. The "fragment reaction" assay and analyses of peptide bond formation using MALDI TOF mass spectroscopy. A. Schematic representation of a fruitful condensation between CCA- phenylalanine-biotin (CCA-pcb) and C-puromycin (C-Pmn) leading to the formation of the product C-Puromycin-phenylalanine-caproic acid-biotin (C-Pmn-pcb). **B**. Representative MALDI-TOF spectra of fragment reaction mixtures of 50S (positive control), tt_P1c, Negative Control (No RNA) and CHCA(matrix). The peak of CCA-pcb (starting material) and the C-Pmn-pcb (product peak) are marked with S and P, respectively.

formed an RNA machine from dimers capable of mediating RNA needed reactions. Later on, the formation of peptide bonds. This initial molecular machine did not possess the ability to support substrates motion from one site to the other, as required in contemporary protein biosynthesis.

The marked preference to dimerize of RNA chains of sequences resembling one side of the contemporary PTC, mainly the P-region, may indicate that the protoribosome was a symmetrical homo-dimer with an identical sequence on each of its sides. This result is in line with the assumption that originally the entire happening within the protoribosome dealt with RNA reactions alongside the occasional creation of peptide bonds. Later on, with the evolving preference of initial beneficial oligopeptides, e. g. those that seem to stabilize the proto ribosome, (Figure 5), alongside the evolving optimization of the protoribosome into an RNA machine, its two parts were independently mutated. In this way, the protoribosome adapted to the specific polypeptides formation requirement, a process through which it matured into the PTC contemporary form, so that the genetic code could drive the directionality of the ribosome function. Consequently, the so evolved A site accommodates the aminoacylated tRNA, whereas the P-side pushes out free tRNA. Thus, our results suggest that the homodimer, which was formed from the P-region based protoribosome, could have served as an ancestor of the evolving heterodimers. The transition from homo- to hetero-

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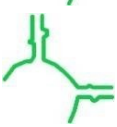
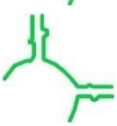
Table 1. Several RNA constructs used, their dimerization capability and activity in peptide bond formation.

Constructs type and source	2D PTC Location	Number of nucleotides	Dimer Construction	Peptide bond formation
min-A, TT		64	–	–
min-P, TT		71	**yes**	**yes**
min-A + min-P, TT		135	**yes**	**yes**
A-reg (includes A loop), TT		91	–	–
P-reg (includes P loop), TT		102	**yes**	–
A-reg + P-reg TT		193	**yes**	–
P-reg + GNRA, TT		98	**yes**	–
min-P + GNRA tetraloop, TT		67	**yes**	**yes**
min-P + GNRA tetraloop EC		67	–	–
min-P + GNRA tetraloop SA		67	**yes**	**yes**
min-P + GNRA tetraloop EF		67	**yes**	**yes**
min-P + UUAA loop TT		65	**yes**	–

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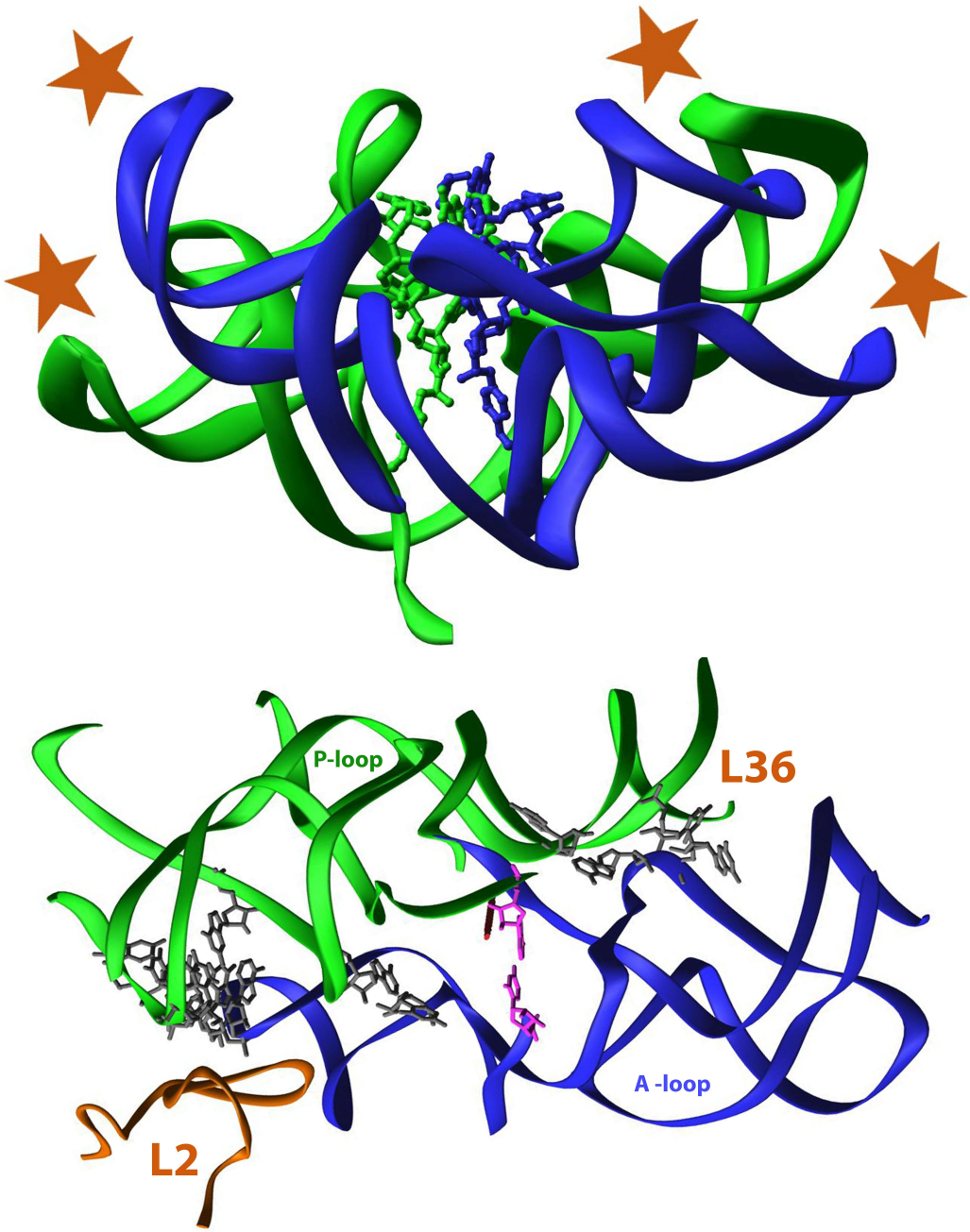

Figure 5. A side view of the protoribosome (as shown in Figure 1) with stars that mark the positions of oligopeptides that could stabilize it, based on the actual structure of the PTC of the bacterial ribosome and its two proximal ribosomal proteins, L2 and L36, that seem to stabilize it (as shown in the bottom).

dimers occurred by optimizing the functionality of the protoribosome towards its developed, actual molecular machinery, the ribosome. Notably, our concept implies protoribosome existence before tRNA or mRNA were available, in line with the biochemical model of the ancestral ribosome,[34] but in contrast to the suggestion that the PTC originated from a proto-tRNAs.[35]

In summary, our results demonstrate the necessity of RNA dimerization for stereochemical peptide bond formation. Furthermore, the finding that our designed protoribosome constructs, which vary in their sequences, could mediate peptide bond formation, strongly support our hypothesis that self-dimerized protoribosome entities that provided the framework for peptide bonds formation could have existed in a prebiotic era.

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Conclusions

It is important to mention that we came up with the protoribosome concept when the only structures of ribosomes from prokaryoteswere available. Later on, structures of ribosomes from additional domains of life and/or types were determined all containing the almost fully conserved PTC structures (Figure 6) despite the variations in the ribosome size (*e.g.*, prokaryotes: eukaryotes = 2 : 3), and composition (*e.g.*, in mitochondria ~ 1/2 of the rRNA is replaced by RPs). Hence indicating that a **pre-biotic bonding entity is still functioning in all contemporary ribosomes.**

In short, by using biochemical, computational, modeling and structural methods, we proved the feasibility of the assumption that a pre-biotic self-folded RNA entity evolved into a rather simple symmetrical pocket-like RNA apparatus capable of providing the stereochemistry for peptide bond formation. The preference of constructs mimicking the P-region of the PTC to form homodimers might relate to the earlier pre-biotic origin of that region, an era in which there was no need to differentiate between the two sides of the pocket. Importantly, a remnant of this pre-biotic molecular apparatus, within peptide bonds are being formed based on their stereochemical positioning, exists in all contemporary ribosomes, thus, supporting LL basic observation of the central contribution of stereochemistry to the creation of life.

Materials and Methods

Preparation of the Suggested Protoribosome

RNA constructs mimicking the suggested protoribosome were prepared according to the sequences of several ribosomes. The dimerization capabilities of the RNA constructs were determined using the Electrophoretic Mobility Shift Assay (EMSA).[36] The binding capability of the dimers was examined under conditions similar to those used in the activity assay reaction. This was sought, as we envisaged that constructs under this condition might imitate the "pocket-like" structure surrounding the PTC such to provide activity.

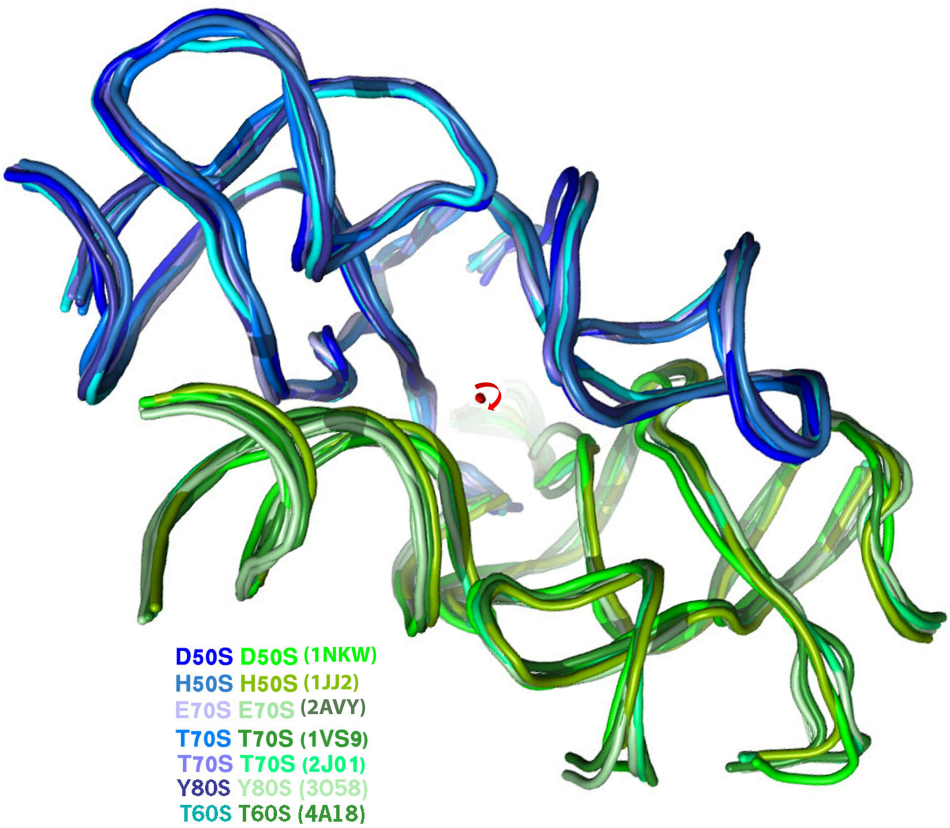

Figure 6. Top view of the superposition of the almost identical main-chain semi-symmetrical region detected in the known ribosome structures from eubacteria, archaea, eukaryotes, and even mitochondria. The PDB accession codes are provided in the box on the lower left. A red dot marks the assumed position of the semi-symmetric axis. The location of the entire region within the ribosome is indicated by the red circle on the top right.

Facilitating the Fragment Reaction by the RNA Constructs

To test the ability of the protoribosome RNA constructs to facilitate the fragment reaction, i.e., mediate peptide bond formation, 2.8 μM of RNA constructs were incubated in 50 mM Sodium phosphate pH = 7.5, 20 mM KCl, and 10 mM $MgCl_2$ with 49 μM CCA-pcb and 19 μM C-Pmn as the P and A-site analogs, respectively (Figure 4) for 24 h at 37 °C.

Matrix-Assisted Laser Desorption-Time of Flight (MALDI-TOF) mass spectrometry analysis[37] was used to verify the success of these reactions. A successful condensation between these counterparts provides the product C-Puromycin-phenylalanine-caproic acid-biotin (C-Pmn-pcb, of molecular weight 1263.33), which could be detected using MALDI-TOF, in a negative mode, as observed from the analysis of the reaction mixture of these substrates with *E. coli* large ribosomal subunit positive control (Figure 4B).

A similar protocol with milli-Q water as a replacement of the RNA constructs was tested as a negative control. A sequence complementary to tt_P1c region, which was constructed according to base-paring rules, and did not catalyze the reaction, served as an additional negative control. Analyses of the MALDI data were based on their respective spectra. The presence of the related product peak in the MALDI spectra indicated the ability of the constructs to mediate peptide bond formation.

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Conflict of Interest

The authors declare no conflict of interest.

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