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Review

Peptide synthesis through evolution

K. Tamura [a,*] and R. W. Alexander [b,*]

[a] The Skaggs Institute for Chemical Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037 (USA), Fax: +1 858 784 8990, e-mail: tamura@scripps.edu
[b] Department of Chemistry, Wake Forest University, 108 Salem Hall, Winston-Salem, North Carolina 27109 (USA), Fax: +1 336 758 4656, e-mail: alexanr@wfu.edu

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Abstract. Ribosome-catalyzed peptide bond formation is a crucial function of all organisms. The ribosome is a ribonucleoprotein particle, with both RNA and protein components necessary for the various steps leading to protein biosynthesis. Evolutionary theory predicts an early environment devoid of complex biomolecules, and prebiotic peptide synthesis would have started in a simple way. A fundamental question regarding peptide synthesis is how the current ribosome-catalyzed reaction evolved from a primitive system. Here we look at both prebiotic and modern mechanisms of peptide bond formation and discuss recent experiments that aim to connect these activities. In particular, RNA can facilitate peptide bond formation by providing a template for activated amino acids to react and can catalyze a variety of functions that would have been necessary in a pre-protein world. Therefore, RNA may have facilitated the emergence of the current protein world from an RNA or even prebiotic world.

Key words. Peptide bond formation; ribosome; tRNA; minihelix; RNA world; oligonucleotides; template; evolution; prebiotic.

Introduction

Proteins are ubiquitous, essential components of biological systems, contributing structural, signalling and catalytic activities to all organisms [1]. Despite the great variety of functions accomplished by proteins, they are assembled from only 20 amino acids (with the exception of selenocysteine, the '21st amino acid' [2, 3], and pyrrolysine, the '22nd amino acid' [4]) linked by stable peptide bonds. The sequence of amino acids assembling to make a functional protein is dictated by gene sequence according to the genetic code. All organisms translate messenger RNAs (mRNAs) to proteins at the ribosome, the ribonucleoprotein particle responsible both for decoding and peptide bond synthesis [5, 6]. This process is protein dependent, requiring dozens of proteins to assemble even the simplest polypeptide. The question raised here is, How did peptide bond formation come about?

The modern ribosome is a sophisticated macromolecular machine even in the simplest organism. In contrast, evolutionary theory predicts an early environment devoid of complex biomolecules. We would like to know the origin of protein synthesis and how it evolved into the present form. The discovery of catalytic RNA provided a new perspective regarding the origins of biomolecules, with an RNA world thought now to precede the current protein world [7–9]. If protein synthesis evolved in an RNA world, it was probably preceded by simpler processes in which interaction with amino acids conferred selective advantage on replicating RNA molecules [10]. It is suggested that at first, the simple attachment of amino acids to the 2′(3′)-termini of RNA templates favored initiation

* Corresponding author.

of replication at the end of the template rather than at internal positions. The second stage in the evolution of protein synthesis would probably have been the association of aminoacylated RNA adaptor pairs in such a way as to favor noncoded formation of peptides. Only after nontemplated polymerization had become efficient could coded synthesis have emerged.

In this review, we will focus on two aspects: (i) amino acid formation and peptide synthesis under prebiotic conditions and (ii) peptide synthesis by biomolecules. Mechanisms of both should be closely related, but these typically have not been handled together because they are separated in time scale and complexity. The beginning of peptide bond formation would have been simple, but ribosome-catalyzed peptide synthesis looks complicated. This brief review will present a perspective for the evolution of peptide bond formation in biological systems. We will summarize the mechanism of peptide bond synthesis on the ribosome, then discuss experimental approaches that try to connect the proposed prebiotic and modern mechanisms.

Peptide bond formation on the ribosome

Overview of protein biosynthesis: protein and RNA components

Protein biosynthesis is catalyzed by ribosomes in all organisms, and while details vary somewhat, we expect the general mechanism to be conserved. Two ribonucleoprotein subunits of ribosomes contribute unique functions to protein synthesis. In the case of *Escherichia coli*, the large subunit contains 5S RNA, 23S RNA and at least 34 proteins. The small subunit contains 16S RNA and 21 proteins [11, 12]. Catalysis occurs on the large subunit, which is thought to be the ancient part, while the decoding function of the small subunit is thought to have come later [13].

The key step in ribosome-catalyzed peptide bond formation is the peptidyl transferase reaction that occurs on the large subunit. With an initiator transfer RNA (tRNA) or peptidyl-tRNA in the P-site (peptidyl or donor site) of the ribosome and an aminoacyl-tRNA in the A-site (aminoacyl or acceptor site) of the ribosome, the peptidyl transferase center of the ribosome promotes the formation of new peptide bonds, thereby lengthening the growing polypeptide with each cycle.

Early models were protein-centric

The fundamental question regarding the peptidyl transfer reaction has long been which component(s) of the ribosome participate in catalysis. Certainly numerous protein factors are essential for the various stages of initiation, elongation and termination of protein biosynthesis, particularly in facilitating the dynamic movement of ribosome, tRNAs and mRNA [14]. Before the discovery of catalytic RNA in the early 1980s, the prevailing perspective was that ribosomal protein(s) would play the catalytic role in peptide bond formation. Nierhaus et al. [15] suggested that the mechanism of peptidyl transfer was analogous to the serine protease reaction, in which three amino acid residues (Ser, His and Asp) participate directly [16] (fig. 1). The analogous peptidyl transferase model replaces the intermediate seryl-ester with peptidyl tRNA (fig. 2). This mechanism involves activation (deprotonation) of the nucleophilic α-amino group of the aminoacyl-tRNA by the His-carboxyl system (general base catalysis); stabilization of the tetrahedral intermediate resulting from nucleophilic attack of aminoacyl-tRNA on the ester linkage of peptidyl tRNA; and activation of tetrahedral intermediate breakdown by proton donation from the His-carboxyl system (general acid catalysis) [15]. (Nierhaus also proposed the alternative model that the ribosome is primarily a template that enables the necessary proximity of two aminoacyl-tRNAs. As we will discuss later, these arguments are now fundamental in considering the mechanism of peptide synthesis on the ribosome.)

With the working assumption that ribosomal proteins participate in the catalytic step of peptide bond formation, the minimum set of ribosomal proteins for peptidyl transferase activity was determined [17–20]. Of these proteins, L2 was most strongly implicated as being important for peptidyl transferase function. By analogy to the catalytic His residue of serine proteases, a single His residue (His-229) of L2 is important for peptidyl-transferase activity [21]. Reconstituted 50S subunits containing His-229 mutants are severely impaired in peptidyl transferase activity, suggesting an important role for this residue in peptide bond formation [22].

RNA involvement in protein biosynthesis

While the prevailing opinion still looked to ribosomal proteins for peptidyl transferase activity, momentum was gaining for a catalytic role for ribosomal RNA (rRNA), especially in the context of catalytic RNAs characterized by Cech and Altman [7–9]. Noller and co-workers sought to remove proteins from the ribosome by treating with phenol, SDS and proteinase K. This depleted ribosomal proteins by ~95%, but the ribosome remained active in peptidyl transferase activity [23]. This result foreshadowed the critical role that rRNA is now known to play in ribosome-catalyzed peptide bond formation, despite the later determination that the protein-depleted ribosome core still contained about eight stoichiometrically bound proteins [24].

Biochemical experiments elucidated particular regions of rRNA critical for catalysis of peptide bond formation. The tRNA binding sites were composed of conserved 23S

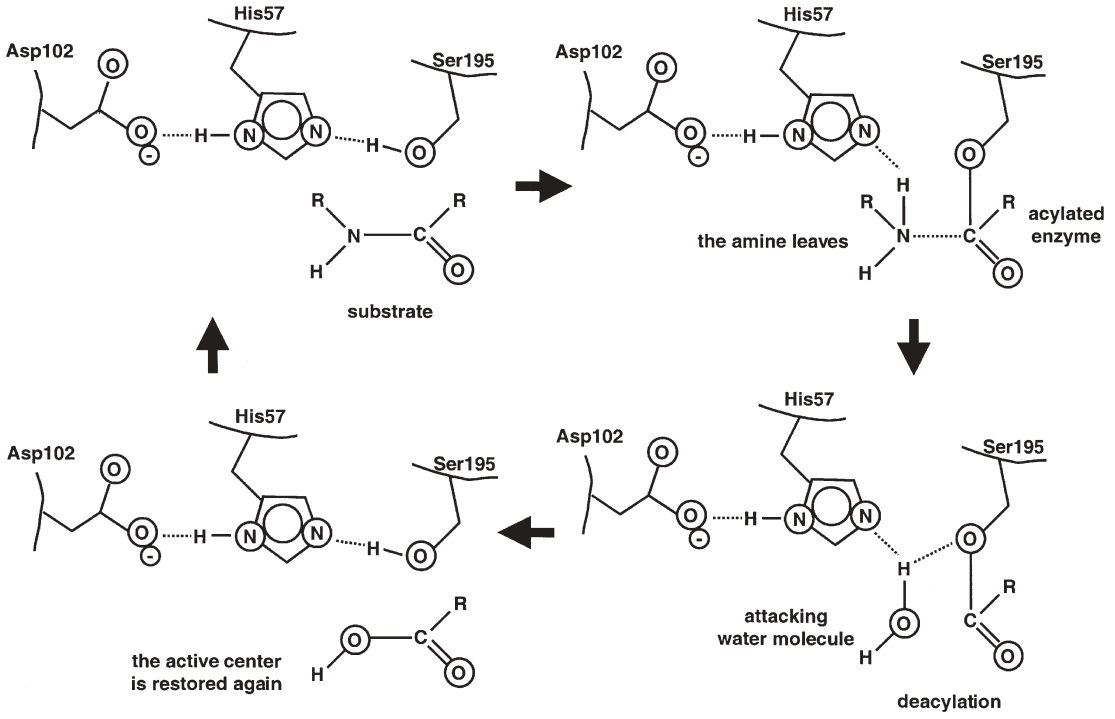

Figure 1. Mechanism of serine proteases. The serine hydroxyl group attacks the substrate, forming an acyl-enzyme intermediate. The serine hydroxyl is then displaced via nucleophilic attack by H_2O. The histidine/apartate charge relay system activates serine as a nucleophile in acyl-enzyme formation [16].

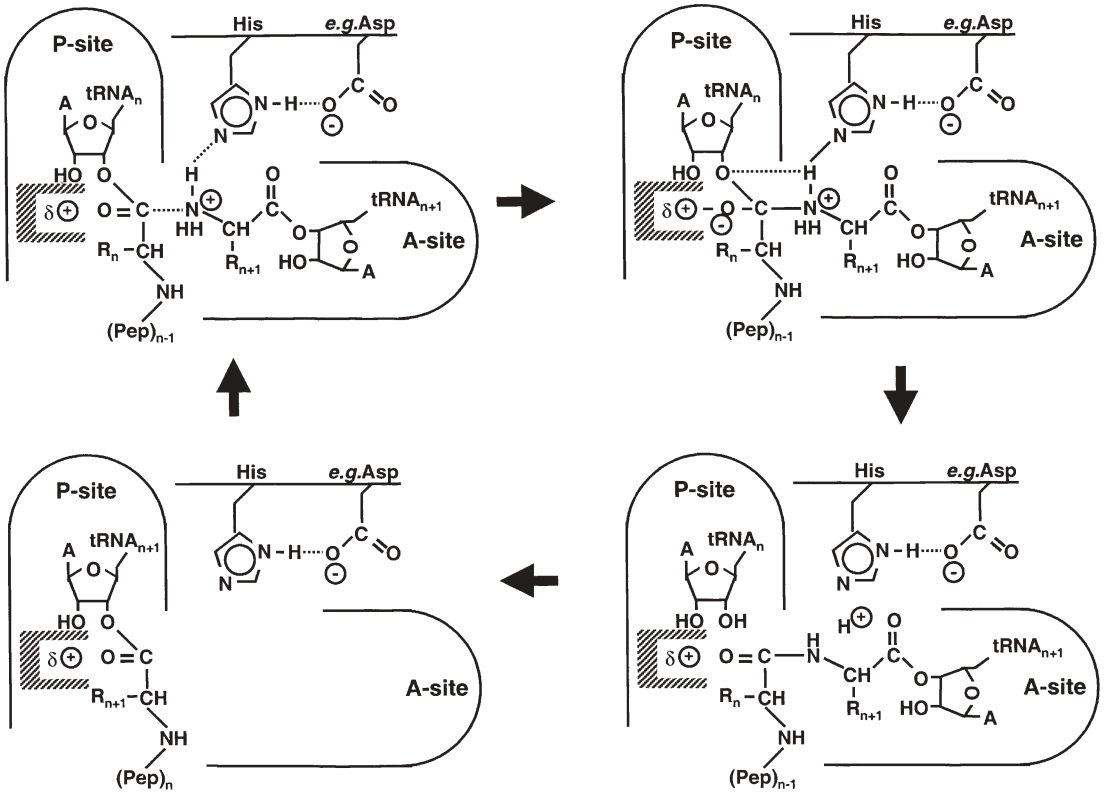

Figure 2. Possible mechanism of the catalysis of peptide bond formation on the ribosome. By analogy with the serine protease mechanism, the A-site tRNA's amino group is deprotonated by a general base mechanism involving ribosomal protein side chains [15].

nucleotides located almost exclusively in domain V, primarily in or adjacent to the so-called 'central loop'. This region was previously identified with the peptidyl transferase function, because several antibiotics known to interfere with peptide bond formation bind at the loop and mutations in it confer antibiotic resistance [25, 26].

Once tRNA binding sites were identified, more detailed interactions were characterized. The 3′-terminal CCA sequence is conserved among all tRNAs [27], which suggested the importance of base-pairing interactions between the CCA trinucleotide and 23S rRNA in the peptidyl transfer reaction. Indeed, certain tRNA-dependent protections of 23S rRNA were abolished when the CCA terminus of tRNA was altered or removed [28]. The mutation of any one base in the CCA in of *E. coli* Val-tRNA^{Val} transcripts caused the decrease of peptidyl transferase activity [29] in the 'fragment assay' [30].

Samaha and co-workers further elucidated the specific base-pairing interaction between the tRNA's CCA terminus and P-site rRNA by in vitro genetics [31]. Efficient peptidyl transferase activity required a Watson-Crick G-C pair between G2252 of 23S rRNA and C74 of tRNA. In addition, the aminoacyl-tRNA analog 4-thio-dT-p-C-p-puromycin was photochemically crosslinked to G2553 of 23S rRNA [32]. The covalently linked substrate reacted with a peptidyl-tRNA analog to form a peptide bond, demonstrating the functional proximity of the A-loop (conserved 2555 loop) of 23S rRNA to the A-site tRNA's CCA end.

High-resolution structures implicate rRNA further

A clearer understanding of the peptidyl transfer reaction has come with the recent elucidation of the ribosome crystal structure (reviewed in [33, 34]). Atomic resolution structures of the *Haloarcula marismortui* large ribosomal subunit and its complexes with substrate analogs revealed an all-RNA active site [35, 36]. There are no proteins closer than about 18 Å to the reaction center [36], and the substrate analogs are contacted exclusively by conserved nucleotides of 23S rRNA domain V (fig. 3). Steitz and co-workers suggested that the mechanism of peptide bond synthesis is analogous to the serine protease reaction, as predicted earlier by Nierhaus. However, the N3 of A2486 nucleobase (A2451 in *E. coli*) rather than a histidine (of L2 or another ribosomal protein) is thought to play the general base role.

What is the catalytic role of rRNA?

While the conclusion that the ribosome is a ribozyme was not unexpected, the proposed role of A2451 (*E. coli* numbering) as a general base led to numerous experiments and rigorous debate. Muth and co-workers [37] screened for nucleotides in *E. coli* 23S rRNA that show altered dimethylsulfate (DMS) reactivity with varying pH. They concluded that only A2451 exhibits such a change in reactivity, with an apparent pK_a of 7.6, which is about the same as that reported earlier for the peptidyl transferase reaction [38, 39]. In addition, their in vivo mutational analysis of

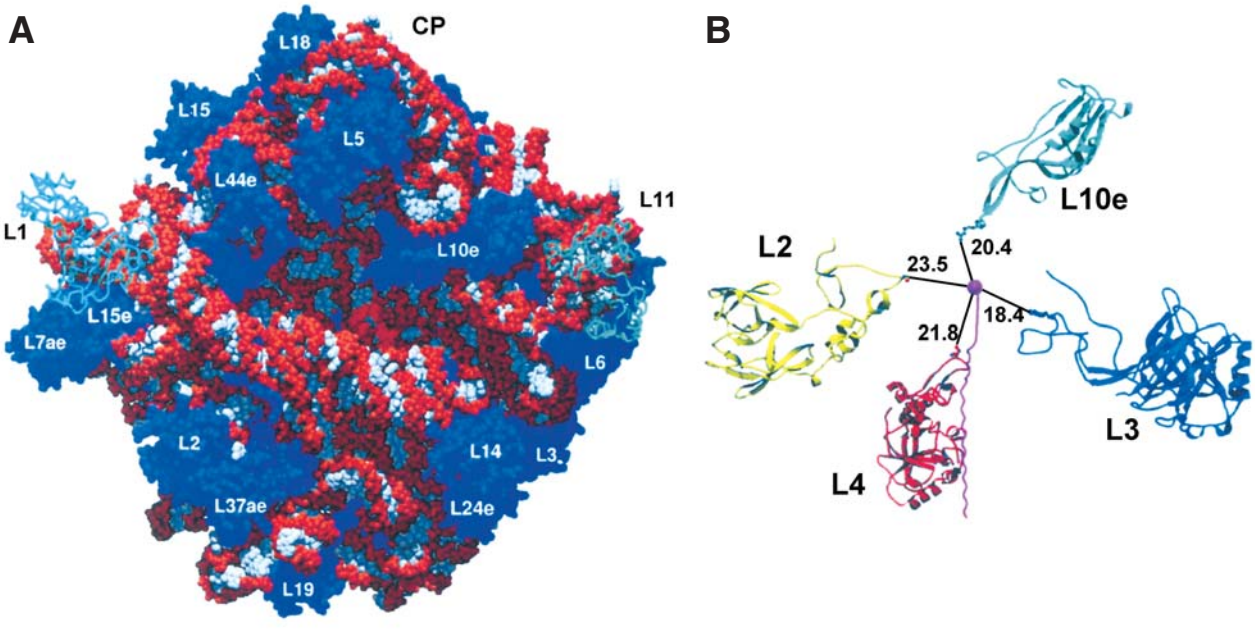

Figure 3. The structure of 50S ribosomal subunit from *Haloarcula marismortui*. (*A*) A space-filling model of the 23S and 5S rRNA, the proteins and the combined CCA models. (*B*) A view of the active site with RNA removed. Reprinted with permission from Nissen P., Hansen J., Ban N., Moore P. B. and Steitz T. A. (2000) The structural basis of ribosome activity in peptide bond synthesis. Science **289:** 920–930 [36].

this conserved adenine indicated that it has an essential role in ribosome-catalyzed peptide bond formation [37].
However, other assays indicated that large ribosomal subunits with mutated A2451 showed significant peptidyl transferase activity in vivo and in vitro, arguing against a critical catalytic role for A2451 [40, 41]. Experiments by Mankin and co-workers showed no pH dependence of A2451 DMS modification in *Thermus aquaticus* and *Mycobacterium smegmatis* ribosomes [42]. Barta and co-workers found that the pH-dependent DMS reactivity of A2451 occurs only in inactive 50S and 70S ribosomes [43]; heat activation causes a conformational change in the peptidyl transferase center. Strobel and co-workers also extended their earlier DMS study and found that A2451 in *Haloarcula marismortui* ribosomes displays an inverted pH profile [44]. Furthermore, the reaction intermediate analog CC-dA-p-puromycin, crystallized with the large subunit in the Steitz structure [36], was found to bind *E. coli* 50S ribosomes with nearly equal affinity at all pH values between 5.0 and 8.5 [45]. These results argue against the proposed hydrogen bond between a protonated N3 of A2451 and the CC-dA-p-puromycin nonbridging phosphoramidate oxygen [36].
While these studies seem to weaken the possibility of a general acid-base mechanism of peptide synthesis, this mechanistic question has not been fully resolved. Rapid kinetic analysis of the peptidyl transferase reaction with puromycin remains consistent with involvement of a ribosomal group with a pK_a of ~7.5. Protonation of this single ionizing group, whatever its identity, as well as protonation of the α-amino group of aminoacyl-tRNA, inhibits peptidyl transferase activity [46]. Interestingly, an inhibitory effect of the same magnitude is found to be caused by an A2451U mutation. These observations are consistent with general acid-base and/or conformational catalysis involving an ionizing group at the peptidyl transferase active site.

Further X-ray structures

Subsequent to elucidation of the large subunit structure, the *Thermus thermophilus* 70S ribosome crystal structure was solved to 5.5 Å resolution [47]. The 70S ribosome was complexed with mRNA and A-, P- and E-site tRNAs, and the resulting structure supported the earlier conclusion that ribosome function is rRNA based. As with the 50S subunit structure, no proteins were found in close enough proximity to directly catalyze the peptidyl transferase reaction. The three tRNAs are bound between the 30S subunit and 50S subunit interface, and the interface region (like the peptidyl transferase center) is composed of RNA, with proteins located mainly at the periphery [47].
The overall structure of the ribosome from different organisms is similar to that from *H. marismortui*, as is expected. However, detailed comparison between the *H. marismortui* and *Deinococcus radiodurans* 50S subunits showed notable differences [48]. For example, the 3.1 Å structure of *D. radiodurans* indicated a different orientation of nucleotides in the peptidyl transferase center and different structures for many ribosomal proteins [48]. Whether subtle or significant, structural variations among the subunits and ribosomes investigated remind us that the ribosome is a dynamic macromolecular machine. Mechanistic conclusions must therefore be drawn based on the combination of ongoing structural and biochemical studies.

Reconsideration of the peptidyl transfer reaction on the ribosome

Given the conformational flexibility that must occur in protein biosynthesis, a major question remains whether the 'crystal' of the ribosome really represents the active form. Using a version of the fragment assay, crystals of the *H. marismortui* large subunit have been shown to be enzymatically active [49]. Addition of these crystals to solutions containing the substrates CCA-Phe-caproic acid-biotin and C-puromycin results in formation of CC-puromycin peptide [49]. Further, structures of the large subunit with low molecular weight substrates and transition-state analogs have been solved [49, 50]. These structures indicated that the α-amino group of an aminoacylated fragment in the A-site forms one hydrogen bond with N3 of A2486 (*E. coli* A2451) and may form a second hydrogen bond either with the 2′-OH of the A76 ribose in the P site or with the 2′-OH of A2486 (A2451). Such interactions may position the α-amino group adjacent to the carbonyl carbon of the esterified P-site substrate, thereby orienting the α-amino group for nucleophilic attack. On the other hand, crystal structures of the *D. radiodurans* large subunit complexed with substrate analogs indicate that various orientations of the analogs are possible for peptide bond formation. Precise positioning of tRNA is thought to be crucial for catalysis, and remote interactions with 23S rRNA and protein L16 orient tRNA in the peptidyl-transferase center [51]. Therefore, although each structure represents a single snapshot, we begin to see how the active site can accommodate necessary ligands.
The view that the ribosome is a ribozyme because there are no proteins within 18 Å of the putative active center in isolated 50S subunits does not rule out a catalytic role for proteins [36]. Neutron-scattering analysis is useful for evaluating the structure of a biomolecule in its native state [52]. Using the neutron-scattering technique of spin-contrast variation [53–55], Nierhaus and co-workers determined the positions of L2 protein in both the isolated *E. coli* 50S subunit and the 70S ribosome [56]. They found that L2 changes its conformation when the 50S and 30S subunits reassociate to form a 70S ribosome, moving approximately 30 Å into the 50S matrix [56]. From the X-ray structures of the ribosome so far, it would be very

hard to imagine that L2 or another ribosomal protein plays a direct catalytic role in peptide bond formation (i.e. the histidine-catalyzed acid-base mechanism proposed by Nierhaus more than 20 years ago [15]). Yet again we must remember the dynamic nature of biomolecules and biomolecular interactions.

Despite several stunning high-resolution structures and sophisticated biochemical and genetic assays, the ribosomal component(s) responsible for catalyzing peptide bond formation remain elusive. The ribosome may accelerate peptide bond formation primarily through an entropic effect (proximity effect), or chemical catalysis of 23S rRNA might contribute directly (or assist the entropic effect). If the ribosome performs such dynamic structural changes as those shown by neutron scattering, chemical contributions of ribosomal proteins might still be significant in the peptidyl transfer reaction. In any case, we are clearly reminded that modern ribosomes are ribonucleoprotein particles, and both rRNAs and proteins likely play critical roles in translation.

With the assumption that ribosome-catalyzed peptide bond formation is the end product of an evolutionary path, we will next look at how the complexity described above may have evolved from simple components.

Prebiotic peptide bond formation

Amino acid content in the 'lightning chamber'

While we don't know the origin of the 20 standard amino acids, the classic work by Miller indicates that some amino acids can be formed prebiotically [57, 58]. Attempting to mimic conditions of the early atmosphere, Miller mixed CH_4, NH_3 and H_2O in a sealed chamber prior to introducing an electric spark. This 'lightning chamber' yielded several amino acids; the major products were Gly and Ala, followed by Asp and Val among the standard 20 amino acids. It is interesting to note that these four amino acids are located on the bottom row in the genetic code table and are coded by the GNC triplet. Eigen developed a model regarding the origin of the genetic code [59, 60], in which GNC is the most primitive code. He rationalized that Watson-Crick base pairing is favorable in the G-C pair, and that the coding frame can be specified in the case of GNC. (In contrast, the frame cannot be determined in the case of GGG or CCC.) Biosynthetic processes might have added the number of amino acids in the modern biological system and frozen at some point [61, 62]. The questions of why 20 amino acids and why these 20 will remain unanswered for now.

Models for prebiotic peptide bond formation

Working with the assumption of at least some prebiotic amino acids, how might peptide bond formation occur? The free energy of the dipeptide is higher than that of two amino acids; therefore, peptide bond formation does not occur spontaneously. However, if the amino acid is 'activated' such that its energy is higher than that of the peptide bond, the direction of peptide bond synthesis is preferable.

Interstellar space might be a good candidate for peptide bond formation, because there is no water molecule that would hydrolyze the activated amino acid. Some have suggested that prebiotic precursors might have occurred in interstellar space prior to transfer to Earth by comets, asteroids or meteorites [63–65]. The extreme example, known as 'directed panspermia' is hypothesized by Crick and Orgel [66]. They theorized that a basic system of life might have come from space, because the time scale required on Earth might be too short for the appearance of systematic life. (They did not deny the possibility that some forms of life occurred on Earth.) It is probable that some of the presumed prebiotic molecules may have been generated in space by gas-phase ion/molecule reactions. Wincel and co-workers showed experimentally that gaseous ion/molecule reactions between amino acids promote the synthesis of protonated dipeptides. These dipeptides could then be chemically extended, suggesting the possibility of peptide bond formation in space [67].

Reactions on clay also have been discussed as a model for the prebiotic environment [68]. In the absence of water, clay may facilitate the condensation of activated amino acids, forming peptide bonds. Paecht-Horowitz and coworkers indicated that moderate concentrations of aminoacyl-phosphate could produce peptide in the presence of clay, but without clay, much higher concentrations of aminoacyl-phosphate were necessary for peptide synthesis [68].

Dry mixtures of amino acids have been shown to yield protein-like material (protenoids) by thermal condensation at 170 °C [69]. In addition, electric discharge also produced polymers of amino acids [70–72]. It is also known that peptides are formed from nitriles. For example, by heating glycinamide in aqueous solution at 100 °C, polyglycine is formed [73]. These observations introduce the possibility that poly(oligo) peptide could have formed under abiotic contitions on the primitive Earth.

Activated metal might also have facilitated prebiotic peptide synthesis. Silica and alumina are widely found in the Earth-like planets' crust, and in particular, alumina and related mineral surfaces might have catalyzed peptide formation on the primitive Earth [74–76]. The catalytic effect of activated alumina on amino acid condensation was investigated, and peptide bonds formed under mild conditions with heating [74].

Related to the characteristic environment of the Earth, we also mention the possibility of peptide synthesis in a volcanic or hydrothermal environment. Iron sulfate is known to cause unusual reducing reactions, especially with H_2S.

The conditions seen in magmatic exhalation upon volcanic eruption typically produce an environment rich in iron sulfate and H_2S. Using a coprecipitated FeS and NiS system, Huber and Wächtershäuser indicated that amino acids were converted into simple peptides [77]. However, CO gas in the presence of H_2S (or CH_3SH) was needed as a catalyst and condensation agent, implying a nontrivial role for CO and Ni. In any case, these results demonstrate that peptide bond formation can occur under geochemically relevant conditions, suggesting a thermophilic origin of peptides.

Each of these model systems is provocative in considering very early mechanisms for peptide bond formation. However, we must remember that the Earth is a planet of water and that all biological systems function in an aqueous environment. We will therefore make a logical and chronological leap to consider aqueous peptide biosynthesis and how peptide bond formation may have emerged from biomolecules simpler than the ribosome.

A possible evolutionary path to the ribosome

Involvement of oligonucleotides

In considering evolutionary steps, the RNA world hypothesis is critical [9]. In the early 1980s, it was discovered that RNA not only carries genetic information but also functions as a catalyst in some reactions [7, 8]. Although extant polynucleotide ribozymes such as the group I self-splicing introns presumably appeared later, shorter oligonucleotides would have been present at an earlier evolutionary stage. So we will first take a look at experiments demonstrating peptide bond formation based on the proximity effect using nucleotides and oligonucleotides.

Proximity effect of peptide bond catalysis

In the contemporary protein biosynthesis pathway, amino acids are esterified to the 3′-teminal OH group of cognate tRNAs by aminoacyl-tRNA synthetases [78–80]. As an ester bond is higher in energy than the subsequent peptide (amide) bond, peptide bond formation should be spontaneous if two activated amino acids are perfectly positioned, whether in a prebiotic or an aqueous environment. Based on this strategy of catalysis by proximity effect, several attempts have been made using simplified systems to demonstrate that the role of the ribosome is to position and orient aminoacyl-tRNAs.

Weber and Orgel [81] attempted to mimic peptide bond formation using 3′(2′)-O-glycyl-adenosine-5′-O-methylphosphate, which is an analog of the 3′-terminus of aminoacylated tRNA (fig. 4A). They demonstrated that at high concentration (0.4 M), the methylphosphate reacts to

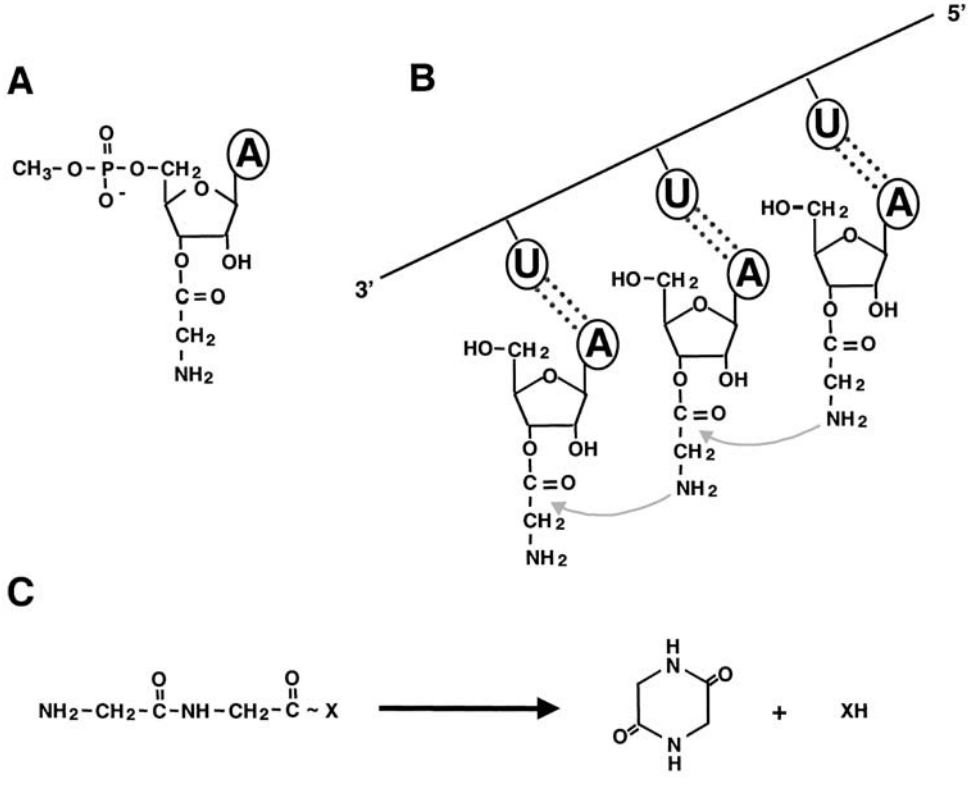

Figure 4. Analogs of the aminoacyl-tRNA 3′-terminus and corresponding models of peptide bond formation. (*A*) 3′-O-glycyl-adenosine-5′-O-methylphosphate. (*B*) Peptide bond formation using 3′-O-aminoacyl-adenosines on a poly(U) template. (*C*) Formation of diketopiperazine from activated Gly-Gly.

yield Gly-Gly (5.5%), although the cyclic Gly-Gly (diketopiperazine) is produced in greater abundance (11.5%) [82]. These researchers also tried catalyzing peptide formation with 3′(2′)-O-glycyl-adenosine-5′-O-methylphosphate mixed with different amino acids. Dipeptide products were detected in varying amounts, with Gly-Ser, Gly-Cys and Gly-Thr yields considerably greater than other dipeptides formed [81].

Poly(U) RNA has been used as a template for peptide bond formation using 3′(2′)-O-aminoacyl adenosine, in a strategy that mimics mRNA anchoring of aminoacyl-tRNAs on the ribosome. Poly(U) effectively catalyzes the reaction by the proximity effect, resulting in decreased substrate concentrations, such that 1–10 mM 3′(2′)-O-glycyl esters of adenosine yielded as much dipeptide as reactions with 0.4 M substrate but without poly(U) (fig. 4B) [83, 84]. In the case of glycyl esters, however, synthesis of longer peptides is less likely than that of the diketopiperazine side product (fig. 4C) [84].

In vitro selection of catalytic RNAs

Based on the RNA world hypothesis, RNAs would have catalyzed a majority of the reactions now performed by proteins. Once the naturally occurring ribozymes were identified, the search began for other catalytic RNAs, and the most powerful tool in this effort has been in vitro selection or SELEX (systematic evolution of ligands by exponential enrichment) [85–88]. In this approach, RNAs possessing a desired function are isolated from a large, randomized RNA population (typically produced enzymatically from a corresponding DNA pool). Selected RNAs that have catalytic properties are ribozymes, while those that bind tightly to the target molecule are typically termed aptamers [89]. Numerous ribozymes have been isolated so far, including those which catalyze RNA replication, a critical function in the envisioned RNA world [90–93].

In addition to the artificially selected ribozymes and aptamers that are not known to occur in extant biological systems, several naturally occurring RNA aptamers have recently been identified. These ‘riboswitches’, found in the 5′-untranslated regions of mRNA, bind a metabolite and regulate expression of genes involved in the metabolite’s biosynthesis [94–98]. The discovery of riboswitches makes the specific interaction of RNA with an amino acid and ATP, such as in a primitive system of peptide bond formation, more plausible than ever.

The demonstration that peptide bond formation could be catalyzed by RNA alone would both provide evidence for the RNA world and indicate how the RNA world might have led to the contemporary DNA-protein world. Several laboratories have tried without success to catalyze the ribosome peptidyl transfer reaction using only 23S rRNA (or portions thereof) [99–101]. In contrast, in vitro selection methods have isolated ribozymes able to catalyze each of the critical steps resulting in protein synthesis [102]: aminoacyl-AMP formation [103], amino acid transesterification to tRNA [104–110] and peptide bond formation [111, 112].

Of particular note are the small (196 nucleotides) in vitro-selected ribozymes with peptidyl transferase activity [111]. A randomized RNA pool presenting Phe at the 5′-end was able to react with an N-blocked Met-AMP analog to produce a tethered depeptide product. The resulting ribozyme was capable of utilizing Leu- and Phe- as well as Met-derived substrates for peptide bond formation. Remarkably, two regions of the active ribozyme resemble the peptidyl transferase region of 23S rRNA in sequence and structural context [112]. Furthermore, when the Phe-containing substrate is presented on a short oligonucleotide rather than on the ribozyme itself, the ribozyme is able to catalyze multiple turnovers [112]. Thus, demonstration that these selected ribozymes can carry out all the critical steps of protein biosynthesis argue for an RNA-mediated emergence from an RNA world to the current DNA-protein world.

Minihelices are the historical domains of tRNA

The RNA world hypothesis predicts that the translational apparatus must have developed from a more primitive structure. Just as tRNAs are key components of ribosome-catalyzed protein biosynthesis, we presume they have played a critical role in the development of the translational machinery. We will therefore outline a possible evolutionary scenario for peptide bond formation using tRNA-derived minihelices and minihelix-like hairpin RNAs as starting molecules.

Prior to peptide bond formation, each amino acid is attached specifically to the terminal adenosine of its corresponding tRNA by a cognate aminoacyl-tRNA synthetase (AARS) via the formation of aminoacyl-AMP [113, 114]. The L-shaped three-dimensional structure of tRNA emphasizes the different functions of its two helical arms (fig. 5). These two arms interact both with the AARS domains and with ribosomal subunits in distinct ways. The acceptor arm (consisting of the coaxially stacked acceptor stem and TΨC stem/loop) is aminoacylated by its cognate AARS and participates in the ribosome-catalyzed peptidyl transferase reaction. The anticodon arm (the coaxially stacked D stem/loop and anticodon stem/loop) contains the anticodon trinucleotide that defines the rules of the genetic code and decodes the incoming mRNA in protein biosynthesis. The arms can be physically dissected to investigate possible evolutionary origins of these individual functions.

RNA oligonucleotide helices that recapitulate the acceptor stems of tRNAs retain their native functions in aminoacylation and peptide bond-forming assays, suggest-

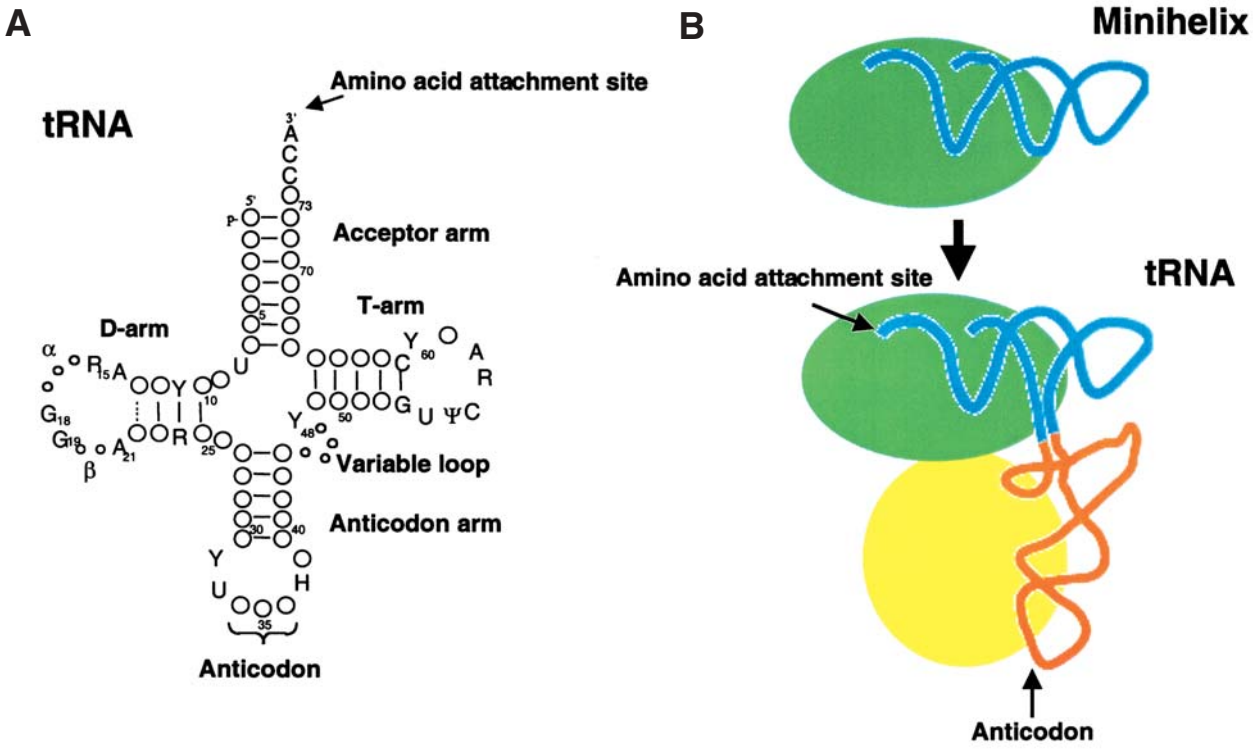

Figure 5. Structure of transfer RNA. (*A*) Secondary structure of tRNA, indicating invariant nucleotides and conserved secondary structure elements. (*B*) Synthetic minihelix constructs recapitulate the sequence and function of the tRNA acceptor arm, and may represent the ancestral core of tRNA.

ing that the acceptor arm is the more ancient portion of tRNA [115, 116]. Minihelices (acceptor stem and TΨC stem/loop) and microhelices (acceptor stem closed by TΨC loop) can be aminoacylated by several AARSs despite their lack of genetic code-defining anticodon nucleotides [117–120]. The specificity of aminoacylation is sequence dependent, and both specificity and efficiency are generally determined by only a few nucleotides proximal to the amino acid attachment site [114]. This sequence/structure-dependent aminoacylation of RNA oligonucleotides constitutes an operational RNA code for amino acids [115]. To a rough approximation, AARSs are organized into two major polypeptide domains [116]. The conserved domain contains the active site and incorporates determinants for recognition of RNA minihelix substrates. This domain may reflect the primordial synthetase, which was needed for expression of the operational RNA code. The second synthetase domain, which generally is less or not conserved, typically provides for interactions with the second domain of tRNA, which incorporates the anticodon. The emergence of the genetic code from the operational RNA code could have occurred when the second domain of synthetases was added to the conserved domain and the anticodon-containing domain of tRNAs was added to the minihelix domain (fig. 5B).

Following AARS-catalyzed aminoacylation, the tRNA acceptor arm interacts with the peptidyl-transferase center of the large ribosomal subunit. Sardesai et al. [121] showed that an enzymatically alanylated RNA minihelix derived from $tRNA^{Ala}$ was an efficient substrate for 50S-catalyzed transfer of alanine to puromycin. The aminoacyl minihelix supported alanine-puromycin synthesis just as effectively as did the aminoacyl tRNA, indicating that the aminoacylated acceptor arm interacts strongly with the large ribosomal subunit even in the absence of the anticodon arm. This demonstration is consistent with an evolutionary model in which the tRNA acceptor arm and the peptidyl transferase function preceded anticodon-mediated, template-directed peptide bond formation.

Model based on the interaction of CCA and 23S rRNA

Ribosome-catalyzed peptidyl tranferase activity depends on base pairing between 23S rRNA nucleotides and the universal CCA of tRNA's acceptor arm, in order to properly position the activated amino acids [29, 31, 32, 36]. Tamura and Schimmel sought to duplicate this interaction in a simplified system using minimal RNAs to represent both the acceptor arm and rRNA. An aminoacylated minihelix (N-acetylalanyl-minihelix) was combined with a puromycin-containing oligoRNA, and formation of N-acetylalanyl-Pm product was monitored. Peptide formation depended on base complementarity between the

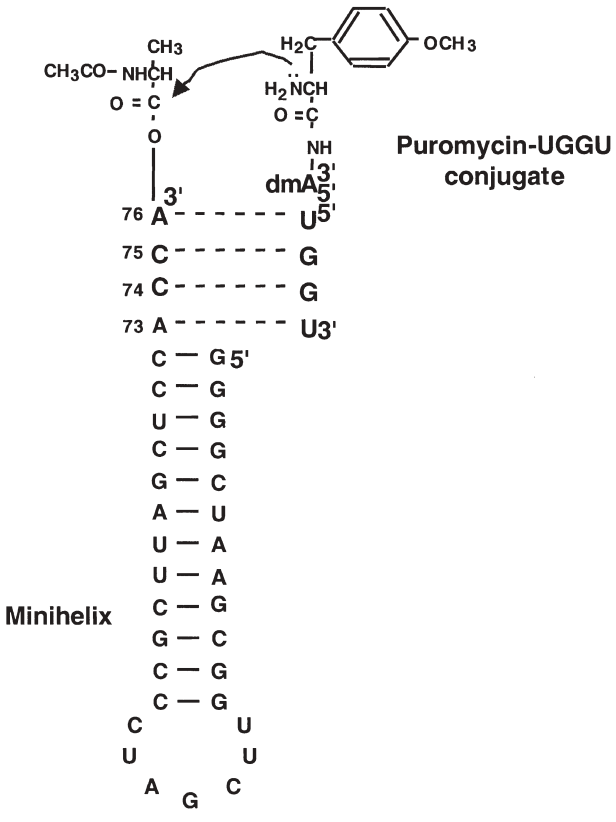

Figure 6. Puromycin-containing oligonucleotide substrates and minihelix^{Ala} for peptide bond formation. Peptide bond formation depends on sequence complementarity between the oligonucleotides. dmA, *N,N*-dimethyladenosine. According to [122].

Pm-oligo and the minihelix 3′-CCA (fig. 6), indicating that the minimized system effectively mimicked the ribosome active site in anchoring the acceptor arm [122]. This demonstration that very small complementary RNAs can promote peptide bond formation takes us closer to the RNA world model of peptide catalysis prior to introduction of the genetic code. Interestingly, imidazole (known to be synthesized in prebiotic conditions [123]) enhanced peptide bond formation significantly. This observation is consistent with the proposal that a component of the ribosome functions as a catalyst, and that the ribosome does more than hold the reacting species in close proximity [15, 37, 46]. However, it may also be that while the minimized system requires imidazole, the full ribosome constrains the aminoacyl-tRNAs more effectively such that the proximity effect contributes more than chemical catalysis.

Model reaction with a template-like RNA guide and aminoacyl phosphate adaptors

Template-directed polymerization is a key feature of synthetic enzymes in the transfer of genetic information. Although a polymerization ribozyme that incorporates up to 14 nucleotides has been reported by Bartel and co-workers [93], it is still a mystery how very long ribozymes could be produced by RNA molecules alone. Perhaps peptide bond formation developed in concert with a highly evolved RNA world, such that small peptides might have facilitated synthesis of sophisticated ribozymes. We then must consider how template-directed peptide bond formation might occur with only simple RNAs.

In the present system, AARSs synthesize a high-energy aminoacyl adenylate as the first step in aminoacylation of tRNA [79]. Notably, aminoacyl adenylate is formed under prebiotic conditions [124] and by ribozyme catalysis [103]. As a simplified model for template-directed peptide bond formation, Tamura and Schimmel used aminoacyl-p-oligonucleotide instead of aminoacyl adenylate as the amino acid donor [125]. Just as aminoacyl adenylate synthesis has been demonstrated using prebiotic conditions, we would expect formation of aminoacyl-p-oligonucleotide to be possible in an RNA world with simple ribozymes.

The minimal template-directed system consisted of an Ala-minihelix, a Phe-p-oligonucleotide and a template-like guide RNA (fig. 7A). Incubating the three components in equal concentrations resulted in the Phe-Ala dipeptide [125]. In this system, the oligonucleotide was DNA rather than RNA for technical convenience, but the resulting RNA-DNA hybrid would maintain RNA's A-form helix. The concentration of oligonucleotides used (10 μM) is up to 2000-fold lower than previous self-condensation studies with aminoacyl mononucleoside esters [83, 84].

The order of amino acid addition was not random, as the reversed system (Phe-tRNA, Ala-p-oligonucleotide and guide RNA) produced Ala-Phe instead of Phe-Ala [125]. The system therefore has distinct aminoacyl donor and acceptor molecules, as the ribosome does. Peptide bond formation was also dependent upon sequence complementarity between the template and aminoacyl-containing oligonucleotides. Tripeptide (Phe-Phe-Phe) synthesis was also demonstrated, using two Phe-p-oligonucleotides of different lengths as aminoacyl donors in addition to the oligoRNA guide and Phe-tRNA as the acceptor (fig. 7B). As with dipeptide synthesis, product formation depended on the presence of the guide sequence.

The formation of di- and tripeptides in this simplified system demonstrates the efficacy of aminoacyl phosphate oligonucleotide adaptors in template-directed peptide synthesis. One can imagine extending the reaction to higher-order peptides, especially if the catalytic environment is shielded from aqueous solvent, limiting aminoacyl phosphate hydrolysis. In the contemporary system, the AARS-synthesized aminoacyl adenylate remains tightly bound in the enzyme active site and thereby protected from solvent [126]. We envision aminoacyl phosphate oligonucleotides as intermediates in the development of protein syn-

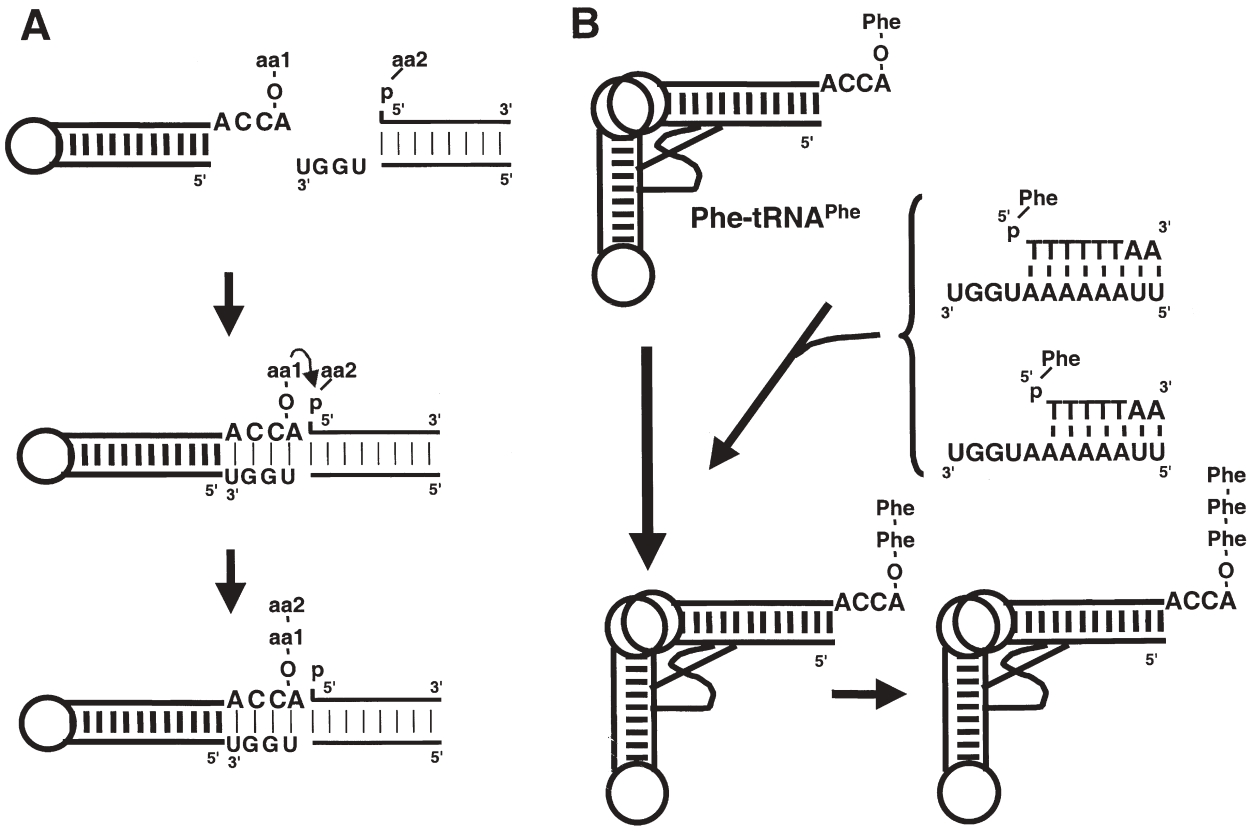

Figure 7. Peptide bond formation with a template-like RNA guide and aminoacyl-phosphate adaptor. (*A*) The reaction scheme for dipeptide synthesis. Peptide bond formation depends on sequence complementarity, and is directional. (*B*) Tripeptide formation using Phe-tRNA^{Phe}, template oligonucleotide, and a mixture of 5′-Phe-p-oligonucleotides (5′-Phe-pdT$_6$dA$_2$ and 5′-Phe-pdT$_5$dA$_2$). Adapted from [125].

thesis, subsequently replaced by the aminoacyl mononucleotide.

This peptide synthesis construct also has elements of the modern ribosome, in that the guide sequence interacts with the universal CCA end of tRNA in a sequence-dependent manner [125]. Previous studies demonstrated that ribosome-catalyzed peptide bond formation similarly depends on contacts between the tRNA CCA and specific nucleotides in 23S rRNA [29, 31, 32, 36].

Conclusion

With high-resolution crystal structures of the ribosome and a variety of biochemical and synthetic tools in hand, the molecular mechanism of peptide bond formation can be investigated as never before. Ongoing experiments may soon answer the question whether rRNA is a chemical catalyst in the peptidyl transferase reaction. Even if RNA does not act catalytically, it certainly facilitates peptide bond formation by providing the template for activated (tRNA-bound) amino acids to react, as evidenced by model systems using oligonucleotide templates linked to activated amino acids. In addition to the sequence complementarity offered by RNA, it is clear that RNA can catalyze an increasing variety of functions that would have been necessary in a pre-protein world. Indeed, ribozymes have been identified that perform each of the functions necessary for peptide bond formation: aminoacyl adenylate formation, aminoacylation of tRNA and transpeptidation [102]. Therefore emergence from the RNA world into the protein world could have been mediated by catalytic RNAs, first in a nontemplated fashion, then according to a developing genetic code.

As we have described, numerous experiments demonstrated the possibility of peptide bond formation, principally by bringing amino acids in close proximity and minimizing water. There remains a mechanistic and temporal gap between the RNA world and the mixtures of simple molecules that mimic prebiotic conditions. We have tried to narrow that gap by describing model systems that both extend the repertoire of prebiotic reactions and simplify the RNA world. It seems clear that in order to enter the RNA world from the prebiotic one, complex RNA molecules would have to be assembled from nucleotide monomers. Just as amino acid monomers could have been joined to form simple peptides, extended oligonucleotides would be necessary to achieve the range of

RNA functionality now demonstrated. It is also likely that assembly of amino acids and polymerization of nucleotides occurred in parallel, with amino acids (like imidazole) participating in catalytic reactions even prior to the protein world and with nucleic acids catalyzing peptide bond formation both chemically and by providing a template for reactions to occur. Further experiments will no doubt narrow these gaps and provide new mechanistic scenarios for the evolution of peptide bond formation.

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