Biochimica et Biophysica Acta xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagrm

Review

A structural view on the mechanism of the ribosome-catalyzed peptide bond formation

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ARTICLE INFO

Article history:
Received 20 April 2009
Received in revised form 23 June 2009
Accepted 25 June 2009
Available online xxxx

Keywords:
50S subunit
70S ribosome
Peptidyl-transferase center
Peptide bond formation
Peptide release
Ribozyme

ABSTRACT

The ribosome is a large ribonucleoprotein particle that translates genetic information encoded in mRNA into specific proteins. Its highly conserved active site, the peptidyl-transferase center (PTC), is located on the large (50S) ribosomal subunit and is comprised solely of rRNA, which makes the ribosome the only natural ribozyme with polymerase activity. The last decade witnessed a rapid accumulation of atomic-resolution structural data on both ribosomal subunits as well as on the entire ribosome. This has allowed studies on the mechanism of peptide bond formation at a level of detail that surpasses that for the classical protein enzymes. A current understanding of the mechanism of the ribosome-catalyzed peptide bond formation is the focus of this review. Implications on the mechanism of peptide release are discussed as well.

The ribosome is a large RNA–protein machine that catalyzes protein synthesis in all living organisms. It is composed of two subunits in all kingdoms of life. In eubacteria and archaea the large subunit comprises ~3000 nucleotides of ribosomal RNA (rRNA), >30 proteins and sediments at 50S, whereas the ~1500 nucleotides and >20 proteins of the smaller subunit sediment at 30S. These conveniently different sedimentation coefficients are used as descriptors of the large (50S) and the small (30S) ribosomal subunits, while the entire ribosomal particle is referred to as the 70S ribosome (in eukaryotes these are 60S, 40S and 80S, respectively). Not only do the two subunits differ in their size and morphology, as evidenced by the early micrographs of the entire *E. coli* 70S particle [1], but they also perform distinct roles during protein synthesis [2]. The 50S subunit contains the peptidyl-transferase center (PTC) that catalyzes the synthesis of peptide bond, whereas the 30S subunit contains the decoding center that ensures that the tRNA with the correct anticodon is bound to the ribosome and paired with the mRNA codon.

The early studies of the ribosome and the mechanism it utilizes to make proteins were confined within the realm of basic biochemistry and the low-resolution electron microscopy. However, by the early 1990s both ribosomal subunits and the entire 70S ribosome from several organisms were crystallized [3–5]. The major challenge to determining their structures, however, was to correctly solve a heavy atom derivative. This was finally accomplished in 1998 using heavy atom cluster compounds at very low resolution (15 Å), which allowed Steitz and colleagues to phase the crystals of the 50S ribosomal subunit from the archaeon *Haloarcula marismortui* (Hma50) and produce the first map, at 9 Å resolution, showing continuous RNA helices [6]. Ramakrishnan and colleagues rapidly took this approach and obtained an electron density map of the 30S ribosomal subunit from *T. thermophilus* at 5.5 Å [7], while that of the Hma 50S subunit was extended to 5.0 Å resolution [8]. In only another year the resolution of the large ribosomal subunit map was extended to 2.4 Å [9] and that of the small subunit to 3.0 Å resolution [10]. These important successes were followed by a crystal structure of the 50S subunit from *Deinococcus radiodurans* at 3.3 Å resolution in 2001 [11], and a 5.5 Å electron density map obtained for the 70S ribosome from *T. thermophilus* (Tth70) complexed with three tRNA molecules and mRNA [12] that allowed fitting of the atomic structures of the 30S and 50S subunits to make a 70S ribosome model. Then, the 3.5 Å resolution apo-structure of the *E. coli* 70S ribosome was determined [13]. Finally, Ramakrishnan and coworkers were able to determine the crystal structure of the entire *T. thermophilus* 70S ribosome complexed with mRNA, partially ordered A-site tRNA, the P-site tRNA and the E-site tRNA at 2.8 Å resolution [14], while Noller and colleagues determined the crystal structure of a similar complex at 3.7 Å resolution [15].

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doi:10.1016/j.bbagrm.2009.06.006

A rapid accumulation of structural data was paralleled by a series of elegant mutational, kinetic and computational experiments that had a common goal of deciphering the mechanism of the ribosome-catalyzed peptide bond formation. Upon binding of suitable substrates, the 70S ribosome catalyzes the peptide bond formation rapidly with the rate of $>300\ s^{-1}$ [16]. The 50S subunit alone is also able of catalyzing the peptidyl transfer reaction at a similar rate when fragments of the tRNA substrates are used [17]. This is the essence of the fragment assay that was used in the biochemical studies of the PTC for decades. The advantage of the fragment assay is that it is not dependent on the complete tRNA substrates and translation factors, which are otherwise necessary if the 70S ribosome is used. An approach analogous to the classical fragment assay was employed in the detailed structural studies of the PTC in which the 50S subunit of *H. marismortui* was used as a model system. A series of small mimics of the tRNA substrates were soaked into the Hma50 crystals with the goal to capture the heart of the ribosome in distinct stages of the catalytic cycle. Indeed, the approach produced a series of high-resolution snapshots of the PTC in various stages of the peptidyl transfer reaction. The current detail of structural data acquired for this large RNA–protein machine outshines that of the many classical protein enzyme systems. However, one should be aware that this method has its natural limitations. For instance, all the processes where the communication between the tRNA molecule, the translational factor(s) and the two subunits is essential for ribosomal function, such as accommodation or translocation, could not be addressed using this approach.

After a brief description of the elongation phase of the ribosome-catalyzed translation, the detailed description of the structure of the ribosomal PTC in complex with substrates, products and intermediates along the reaction pathway of peptide elongation is presented. Also, the current consensus on the mechanism of the ribosome-catalyzed peptide bond formation is summarized.

1. Translation

The ribosome "reads" mRNA in a 5′–3′ direction and synthesizes the corresponding protein from its N-terminus. Both ribosomal subunits contain three binding sites for tRNA molecules that are in distinct functional states: (*i*) the A site binds the aminoacyl-tRNA, which brings a new amino acid that is to be incorporated into the growing polypeptide, (*ii*) the P site binds the peptidyl-tRNA (as well as the initiator fMet-tRNA^{fmet}), and (*iii*) the E site binds the deacylated tRNA that is to be soon dissociated from the ribosome (Fig. 1). The 30S subunit binds mRNA and it ensures the fidelity of translation through close monitoring of the anticodon–codon interactions [18]. The 50S subunit, on the other hand, binds the acceptor ends of substrate tRNAs and it catalyzes the peptide bond formation in which an α-amino group of the aminoacyl-tRNA attacks the carbonyl carbon of the peptidyl-tRNA. From a chemical standpoint the reaction is an aminolysis of an acyl-ester link formed between the carbonyl carbon of the peptidyl moiety and the O3′ atom of the P-site A76. Upon peptide bond synthesis, the lengthened peptidyl-tRNA is bound to the A site, whereas the deacylated tRNA is in the P site. Peptide elongation is further promoted by the GTP-dependent protein elongation factors EF-G and EF-Tu. EF-G promotes translocation of the A-site bound peptidyl-tRNA into the P-site and of the P-site bound deacylated tRNA into the E site. Consequently, the ribosome moves down the mRNA filament with an active site that is ready for a new reaction cycle. Then, the elongation factor EF-Tu delivers the aminoacyl-tRNA to the A-site. The proper codon–anticodon interactions stimulate the GTP-ase activity of EF-Tu, leading to its dissociation from the complex. The acceptor end of the A-site aminoacyl-tRNA reorients and positions an incoming amino acid for the reaction with the peptidyl moiety attached to the P-site peptidyl-tRNA in a process known as accommodation. The rate of accommodation is significantly slower than the rate of peptide bond formation and is measured to be

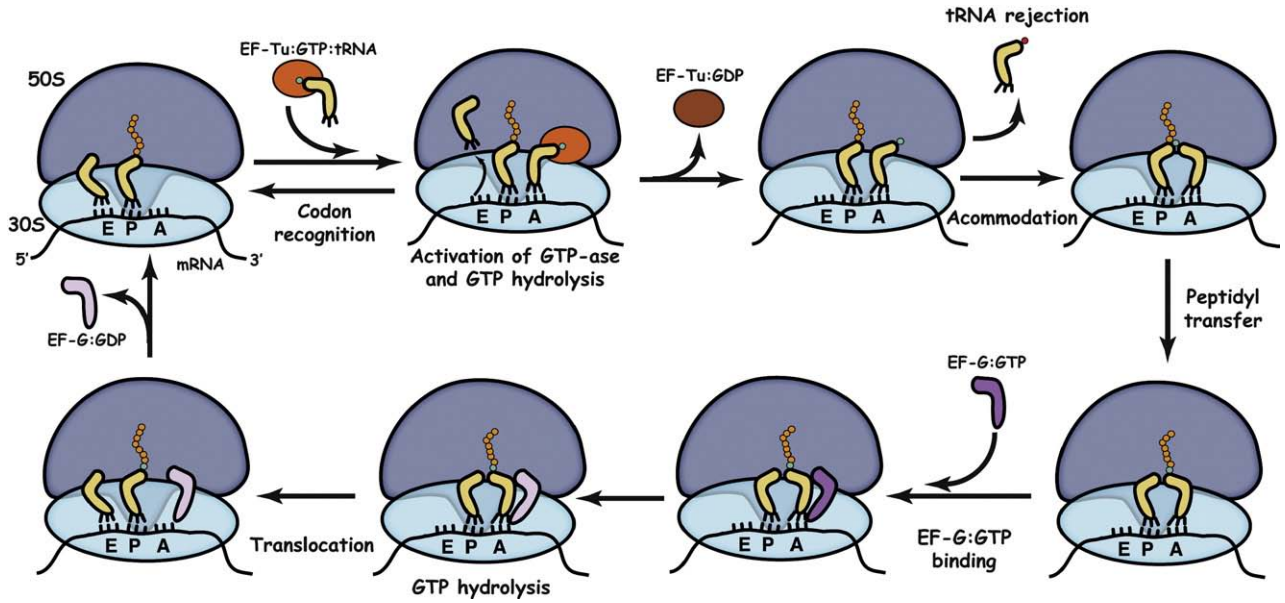

Fig. 1. A schematic diagram of the elongation phase of the ribosome-catalyzed translation. A peptidyl-tRNA is bound to the P site and the deacylated tRNA is in the ribosomal E site. The elongation factor EF-Tu complexed with GTP (orange) delivers an aminoacyl-tRNA to the A site. The deacylated tRNA dissociates from the E site on binding of the aminoacyl-tRNA to the A site. Upon codon recognition the GTP-ase activity of EF-Tu is stimulated and this causes a conformational change in EF-Tu upon which the factor dissociates from the ribosome. If the appropriate codon–anticodon interaction is established the CCA-end of the A-site aminoacyl-tRNA undergoes conformational change in a process known as accommodation, whereas the non-cognate tRNA is rejected at this point. After accommodation a free α-amino group of the aminoacyl-tRNA is oriented properly for the nucleophilic attack onto the acyl-ester link of the peptidyl-tRNA in the P site. The peptidyl transfer reaction occurs rapidly yielding a lengthened peptidyl-tRNA bound to the A site and the deacylated tRNA in the P site. The translocation of the reaction products and mRNA is promoted by the elongation factor EF-G in a GTP-dependent manner. The peptidyl-tRNA moves from the A site into the P site, whereas the deacylated tRNA moves from the P site into the E site. Also, the ribosome has now shifted in the 3′ direction of the mRNA and a new codon occupies the A site on the 30S subunit. After dissociation of the EF-G:GDP complex from the ribosome a new round of peptide synthesis ensues. Once the ribosome encounters the translational stop codon the termination phase of protein synthesis is initiated (not shown).

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~10 s^{-1} [19]. This rate is, however, much higher when small analogs are used and is estimated to be ~50 s^{-1} allowing detailed kinetic studies using this system [20,21].

Once the ribosome encounters a stop codon, protein synthesis stalls, one of the two class I protein release factors (RFs), RF1 or RF2, bind to the A site and promote hydrolysis of the acyl-ester link between the nascent peptide and the P-site bound tRNA. In this way the nascent polypeptide is released from the ribosome. The class II protein release factor (RF3) promotes the dissociation of the RF1/2 from the ribosome and, finally, the two ribosomal subunits dissociate with the help of a recycling factor.

2. The peptidyl-transferase center and its complexes with substrate analogs

The PTC is located in domain V of the 50S subunit. Although there are 15 proteins that interact with the domain V in the 50S subunit of *H. marismortui*, not a single protein was found within ~18 Å of the PTC confirming that the ribosome is indeed a ribozyme [22]. Even though subsequent structures of the *T. thermophilus* 70S ribosome with three bound tRNA molecules show that L27 protein (not present in *H. marismortui*) interacts with the CCA of the P-site tRNA, it is not close enough to directly participate in catalysis of peptide bond formation [14,23]. The walls of the PTC are composed of the A and P loops, whereas its floor opens up into the exit tunnel through which the nascent polypeptide chain protrudes (Fig. 2A). A number of crystal structures of Hma50 complexed with the appropriate substrate mimics revealed how ribosome recognizes its substrates.

The crystal structure of Hma50 in complex with a mimic of the aminoacyl-tRNA, CC-puromycin (CC-Pmn), bound to the A site revealed interactions between the A-loop and the CCA-end of the A-site substrate analog (Fig. 2B) [24] and confirmed the earlier proposal by Noller and colleagues [25] that the CCA-end of the A-site substrate is interacting with the residues of the A loop. More precisely, C74 forms a stacking interaction with U2590 (U2555), C75 forms a Watson–Crick base-pairing with G2588 (G2553), whereas A76 makes a type I A-minor interaction with U2590 (U2556) and G2618 (G2583). The numbering of the 23S rRNA residues based on the *H. marismortui* sequence is used throughout the text, while the corresponding position in the *E. coli* ribosome is shown in the brackets. The same interactions have been observed when other analogs were used [26–29]. Likewise, the P loop binds CCA of the P-site substrate [22,24,26–29]. For instance, when the peptidyl-tRNA analog, CCA-Phe-caproic acid-biotin (CCA-Pcb), was soaked into the Hma50 crystals it was found that C74 and C75 form Watson–Crick base-pairing with G2284 (G2251) and G2285 (G2252), respectively, whereas A76 stacks onto the ribose of A2486 (A2451) and makes a hydrogen bond with the O2′ hydroxyl of A2485 (A2450) (Fig. 2C) [24]. The CCA ends of the analogs bound to the A and the P sites are related by 180° rotation and it was proposed that this may contribute to the translocation of the peptidyl-tRNA from the A site to the P site once the peptide bond is formed [24] as well as being necessary for the appropriate positioning of the peptidyl-group and the attacking α-amino group. Examination of the crystal structure of Tth70 with the deacylated tRNA bound to the P site revealed that CCA of the complete tRNA interacts with the P loop in the same fashion as the small P-site substrate analogs do with Hma50 (see below: The PTC is the same in the 50S subunit as in the 70S ribosome) [14]. Other interactions have been observed in the PTC of Tth70. For instance, the N-terminal tail of the protein L27 interacts with the CCA-end of the P site tRNA, thereby stabilizing its conformation [14,23]. Although the deletion of the three N-terminal residues from L27 affects the rate of translation [30], this protein is not involved chemically in catalysis. The archaeal protein L10e, which is a homologue of the bacterial protein L16, may play a similar role in the *H. marismortui* 70S ribosome. However, the corresponding region is disordered in the Hma50 crystals [9].

The peptidyl moiety was oriented towards the exit tunnel in all the mimics of the peptidyl-tRNA that are bound to the P site (Fig. 2C). Specific interactions between the peptide mimic and the ribosome, however, have not been observed [22,24,26–29]. This is not surprising because tight interactions between the newly synthesized polypeptide and the exit tunnel would almost surely lead to inhibition of translation. Interestingly, CCA-Pcb binds equally well to both the A and P sites in the PTC, thus, to prevent the binding of the analog to the A site, the antibiotic sparsomycin was employed [24]. Perhaps the non-selective binding of CCA-Pcb should not be surprising since this analog is also a mimic of the A-site product and, therefore, should have substantial affinity for the A site as well. In addition, the footprinting experiments using small fragments of tRNA substrates revealed that the fragments would bind to the P-site only in the presence of sparsomycin [31]. A somewhat different P-site substrate analog, CCA-

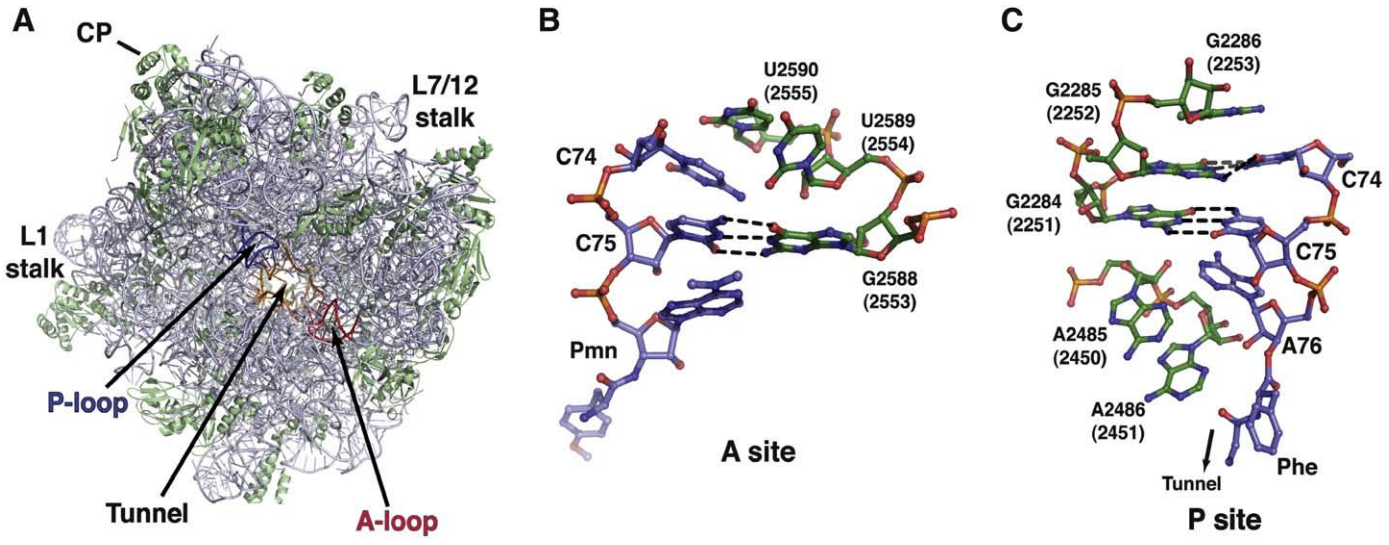

Fig. 2. The structure of the ribosomal PTC and the mode of tRNA recognition. (A) A cartoon diagram of the 50S subunit from *H. marismortui* showing the exact location of the PTC [116]. The two-fold symmetry of the active site is evident. The P loop is blue, the A loop is red, whereas the floor and the walls of the PTC are orange. The arrow points to the tunnel entrance. rRNA is light blue, while the proteins are colored in light green. (B) The A loop (green) binds CCA of the A-site substrate analog CC-Pmn (blue) [26]. C75 makes Watson–Crick interactions with G2588 (G2553) and C75 base stacks with U2590 (U2555). (C) The P-loop (green) bases G2285 (G2252) and G2284 (G2251) form Watson–Crick base pairs with C74 and C75 of the peptidyl-tRNA mimic CCA-Pcb (blue), respectively [26]. In A and B hydrogen bonds are shown with dashed lines.

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puromycin-Phe-caproic acid-biotin (CCA-Pmn-Pcb), binds only to the A site in the absence of sparsomycin, presumably because one or more methyl groups on the puromycin moiety decreased the affinity of the ligand for the P site [24]. In the context of the 70S ribosome, however, the peptidyl-tRNA has a higher affinity for the P site than for the A site suggesting that the entire tRNA is needed for appropriate substrate selection [32,33]. Also, a number of binding assays revealed that the peptidyl-CCA has higher affinity for the P site on the 50S subunit [34–36]. Thus, two determinants may influence the stronger binding of the CCA-Pcb, and presumably that of the peptidyl-CCA, to the P site. The first is an additional Watson–Crick interaction formed between C74 and the P-loop base G2284 (G2251), whereas the second is a rotation of CCA by ~180° relative to the acceptor arm, which presumably allows a more favored conformation of the peptidyl-CCA to be adopted. The higher affinity of the peptidyl-CCA for the P site might drive the formation of the so-called hybrid state intermediate during the elongation phase of translation.

3. Products translocate after peptidyl transfer

A number of footprinting, kinetic and single-particle cryo-electron microscopy (cryo-EM) studies have shown that upon peptide bond formation the acceptor end of a tRNA in the A site, which carries now a longer polypeptide chain, rapidly reorients and moves into the P site of the 50S subunit, whereas its anticodon stem is still bound to the A site of the decoding center in the 30S subunit [25,32,33,37]. Likewise, the deacylated tRNA in the P site reorients and binds to the vacant E site on the 50S subunit, while its anticodon loop remains attached to the P site on the 30S subunit [25,32,33,37]. This is the basis of the widely accepted hybrid states model of protein synthesis, which postulates that the anticodon and the acceptor ends of a tRNA can simultaneously bind different tRNA-binding sites on the 30S and 50S subunits.

Over 40 years ago Watson hypothesized that translocation takes place after peptidyl transfer, thus, explaining how the ribosome might move along mRNA [38]. Also, Noller and coworkers have proposed that the peptidyl transfer can occur uncoupled from movement of the A-site tRNA [25]. That this is indeed the case was confirmed by using Hma50 crystals and appropriate reaction substrates. The peptidyl-tRNA mimic CCA-Pcb was used as the P-site substrate, while the aminoacyl-tRNA analog C-puromycin (C-Pmn) was used as the A site substrate (Fig. 3A) [28]. Although reaction substrates were soaked into the crystals, the resulting electron density maps revealed that only the products of the reaction were bound to the PTC of the 50S subunit: (*i*) C-puromycin-Phe-caproic acid-biotin (C-Pmn-Pcb) was bound to the A site, (*ii*) the CCA oligonucleotide was attached to the P site, albeit with much lower occupancy (Fig. 3B) and (*iii*) the E site was empty. The observed low occupancy of the CCA oligonucleotide in the P site suggests that the affinity of the P site for the reaction product is significantly lower than that for the reaction substrate. The Hma50 crystals were catalytically active with a slower rate when compared with the sample in solution [28]. More importantly, CCA ends of the resulting products remained attached to the substrate binding sites suggesting that the translocation of products can only take place after peptidyl transfer leading to the revised hybrid states model of protein synthesis (Fig. 4) [28].

4. Product translocation in the context of the 70S ribosome

Because spontaneous formation of hybrid states, also known as A/P and P/E states, is not sufficient for completion of translocation [32], it has been proposed that, upon GTP hydrolysis, the domain IV of EF-G displaces the anticodon loop of the A-site tRNA as the 30S subunit rotates clockwise back to the classical state [39,40]. Kinetic studies have shown that the rate of translocation increases by 50-fold upon EF-G-catalyzed GTP hydrolysis [41], and it was proposed that both the GTP hydrolysis and the release of inorganic phosphate may trigger structural rearrangements in the ribosome that further promote the translocation [42,43]. Indeed, Frank and colleagues have shown that binding of EF-G induces the counter-clockwise rotation of the 30S subunit, which is accompanied by formation of the P/E hybrid state [44,45].

The hybrid states, however, can form spontaneously on the 70S ribosome and they have recently been visualized by cryo-EM on the 70S ribosome particles that were free of translational factors [46,47]. The ratcheting movement of the 30S subunit thus promotes the tRNA repositioning from the A/A-P/P state into the A/P-P/E state. In addition, the single-molecule fluorescence energy transfer experiments have shown that the pretranslocation ribosomes spontaneously fluctuate between the classical and hybrid conformations, whereas the posttranslocation ribosomes are locked in the classical, non-rotated state [48,49]. Kinetic and FRET studies have also suggested that the binding of the elongation factor EF-G to the 70S ribosome and the movement of the acceptor stem of the deacylated tRNA into the E site of the 50S subunit both stabilize the hybrid state of the ribosome [48,50–53]. Moreover, Noller and colleagues have shown that the hybrid state intermediate is stabilized in presence of GTP, GDPNP or GDPNP·fusidic acid but not in presence of GDP [52]. Also, only EF-G·GTP and EF-G·GDPNP are capable of promoting the tRNA translocation [52].

Further, it has been shown that GTP hydrolysis by EF-G and/or EF-G dissociation from the ribosome promotes the dissociation of the E site bound deacylated tRNA from the ribosome [52,54], which is in agreement with the earlier proposal that the binding of the deacylated tRNA to the E site involves a stable codon–anticodon interaction and that there is an anticooperative allostery between the A and E sites on the ribosome [55]. In contrast, Wintermeyer, Rodnina and colleagues have shown that the binding of the deacylated tRNA to the E site is transient and that its dissociation from the ribosome is spontaneous [56].

Finally, cryo-EM reconstructions have revealed that the L1 stalk moves inward upon EF-G binding to the ribosome [45]. The authors speculated that the movement may stabilize the binding of the deacylated tRNA to the E site. More recently, the FRET studies have shown that the L1 stalk moves independently of intersubunit rotation [53,57]. Both groups have found that the L1 stalk fluctuates between the open (ratcheted) and closed (nonratcheted) states in the pretranslocation ribosomes. In the closed (nonratcheted) state, the tRNAs adopt classical conformation and there are no interactions between the L1 stalk and the E-site tRNA. In the open (ratcheted) state, the tRNAs are in the hybrid state and the E-site tRNA interacts with the L1 stalk. Fei et al. [53] have also found the EF-G binding stabilizes the open (ratcheted) state, whereas Cornish et al. [57] have found that the L1 stalk is either in the fully open or the fully closed states in the ribosomes with the vacant E site. The two studies, however, describe the conformation of the L1 stalk in the posttranslocation ribosomes differently. While Fei et al. [53] have shown that the L1 stalk is in the closed (nonratcheted) state, Cornish et al. [57] have suggested that the L1 stalk adopts a newly identified half-closed conformation. More importantly, both groups proposed that the L1 stalk plays an important role in binding, movement and release of the deacylated tRNA.

5. The A-site substrate binding induces conformational change in the PTC

After completion of the peptidyl transfer and following translocation, the peptidyl-tRNA is bound to the P site, the deacylated tRNA has moved over to the E site, whereas the A site remains empty until the next aminoacyl-tRNA is delivered. An important question here is how the translating ribosome prevents an unwanted hydrolysis of the peptidyl-tRNA bound to the P site when the A site is empty? Crystal structures of Hma50 complexed with substrate and intermediate

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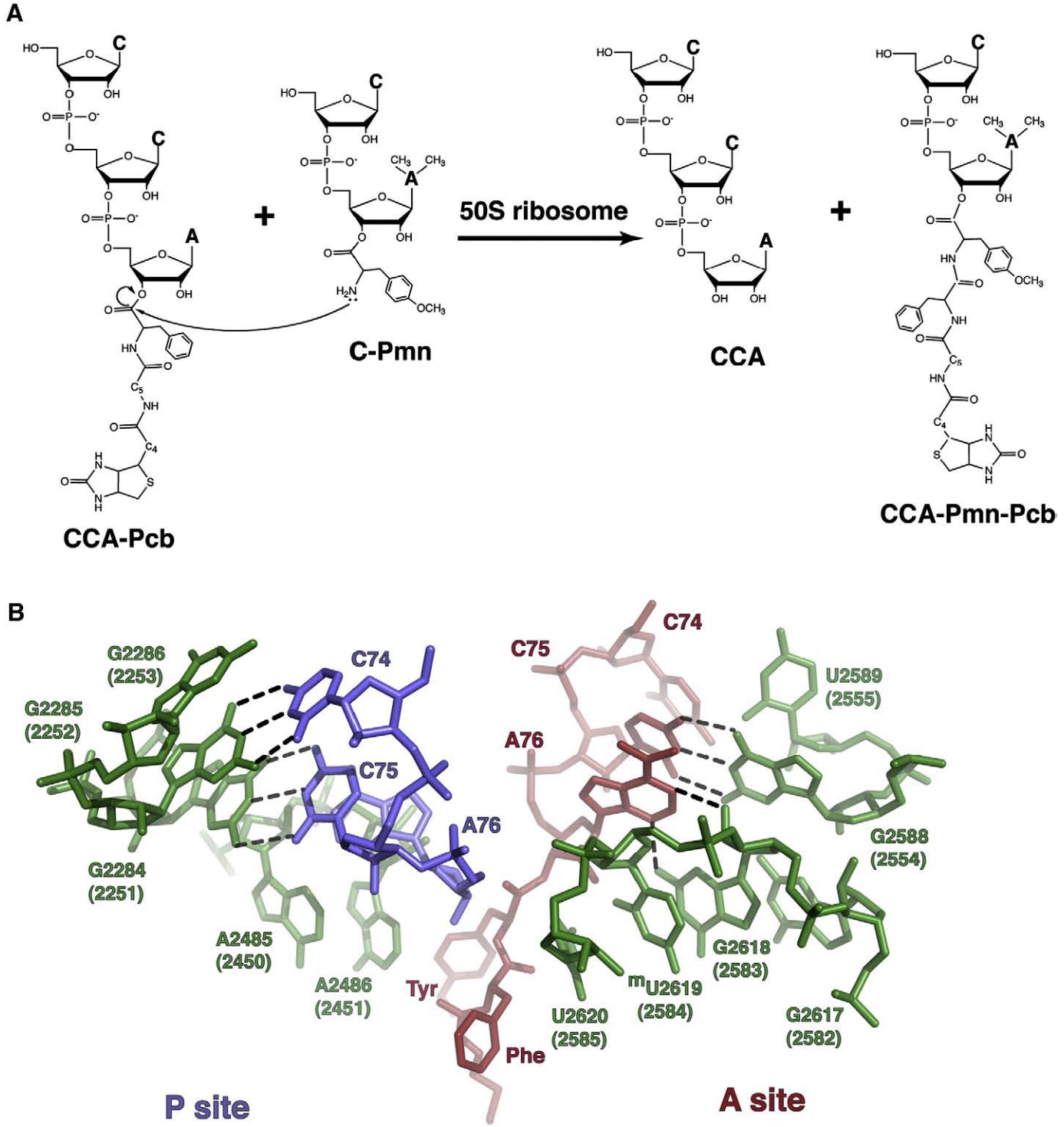

Fig. 3. Identification of the pretranslocation intermediate in catalytically active crystals of the 50S subunit from *H. marismortui*. (A) Crystals of Hma50 were soaked with a mixture of substrates shown on the left hand side with the expectation that the active 50S ribosome would yield products shown on the right hand side. (B) Examination of the resulting electron density map revealed that the lengthened peptidyl-tRNA analog, CCA-Pmn-Pcb (red), was produced and bound to the A site (dark green), whereas CCA (blue) was found in the P site (light green). The figure is adapted from the reference [24].

analogs provided evidence that the PTC undergoes a conformational change upon binding of the proper A-site substrate. The absence of this conformational change without the A-site tRNA precludes an unwanted hydrolysis of the peptidyl-tRNA [26,27].

In the posttranslocation complex in which the peptidyl-tRNA mimic (CCA-Pcb) occupies the P site and the A site is empty, the acyl-ester link is completely sequestered from water by 23S rRNA bases U2620 (U2585), A2486 (A2451) and C2104 (C2063) (Fig. 5A). Furthermore, the ester oxygen is oriented toward the position where the α-amino group of the incoming amino acid will eventually be positioned. This essentially means that the carbonyl carbon is not positioned properly for a possible nucleophilic attack in the absence of the A-site tRNA. However, the binding of the A-site substrate mimic, CC-hydroxy puromycin (CC-hPmn), to the same complex induces a conformational change in the PTC in which the base of U2620 (U2585) moves away from the ester link (Fig. 5B, C) [27]. This movement

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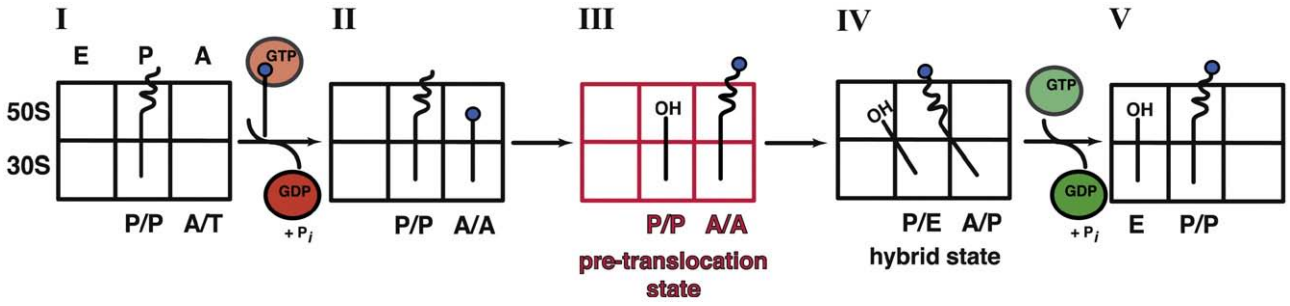

Fig. 4. A schematic diagram of the revised hybrid state model of translation. (I) The peptidyl-tRNA is bound in the P site and the A site is empty. (II) The elongation factor EF-Tu (light red) delivers an aminoacyl-tRNA to the ribosomal A site in a GTP-dependent manner. Upon GTP hydrolysis EF-Tu (dark red) dissociates from the ribosome. The anticodon and the CCA-end of the aminoacyl-tRNA are bound to the A sites in the 50S and the 30S subunit, respectively. Likewise, the ends of the peptidyl-tRNA are attached to the P sites producing the so-called P/P and A/A states. (III) Upon peptidyl transfer the peptidyl-tRNA is now attached to the A site, whereas the deacylated tRNA is in the P site. Again, both the anticodon and the CCA ends of the product tRNAs are bound to the same ribosomal sites suggesting that the tRNAs are in their P/P and A/A states after peptidyl transfer. This state is observed in the crystals of Hma50 (see Fig. 3) and is termed as the pretranslocation state. (IV) CCA of the deacylated tRNA moves into the E site, while the CCA-end of the peptidyl-tRNA translocates into the P site. The corresponding anticodon loops, however, remain attached to the A and P sites giving a rise to the A/P and P/E hybrid state. (V) Finally, the elongation factor EF-G (green) promotes the translocation of the anticodon loops in a GTP-dependent fashion. The figure is adapted from reference [24].

allows repositioning of the acyl-ester so that its carbonyl carbon is now exposed to the nucleophilic attack by the properly positioned α-amino group of the aminoacyl-tRNA (Fig. 5B, C). These findings suggested that the ribosome promotes the reaction of the amino acid condensation by properly orienting the reaction substrates. Along these lines it has been suggested that the ribosome serves as an entropic trap that draws its catalytic power from the ability to orient and position substrates [58]. In this respect, however, the ribosome is not unlike other enzymes, all of which obtain a major portion of their catalytic power by correctly positioning their substrates [59].

6. Catalytic mechanism

The ribosome catalyzes an aminolysis of an acyl-ester bond in the P site. The reaction begins with a nucleophilic attack of the α-amino group of the aminoacyl-tRNA bound in the A site onto the carbonyl carbon of the peptidyl-tRNA positioned in the P site and it proceeds through a tetrahedral oxyanion intermediate. The intermediate collapses into the lengthened peptidyl-tRNA in the A site and the deacylated tRNA in the P site. The ribosome brings ~10^7-fold increase in the reaction rate compared to an uncatalyzed reaction in solution [58]. Early biochemical experiments in which substrate and product analogs were used revealed how ribosome specifically recognizes its ligands, but provided inadequate answers to more pertinent questions concerning the catalytic mechanism. The role of the ribosome in positioning the reactive groups involved in the reaction as well as the identity of any ribosomal group that contributes to the catalysis was not revealed. It was also not clear whether metal ions or water molecules bound to the PTC participate in catalysis. Furthermore, the stereochemistry of the reaction intermediate as well as the mechanism of the stabilization of its oxyanion remained unanswered.

The Yarus inhibitor, an analog of the tetrahedral intermediate, was employed in initial structural studies [22]. The binding of the Yarus inhibitor unambiguously identified the PTC on the 50S subunit [22], but the analog had a few structural shortcomings. The lack of C74 and C75 in the A-site component contributed to the flexibility of the

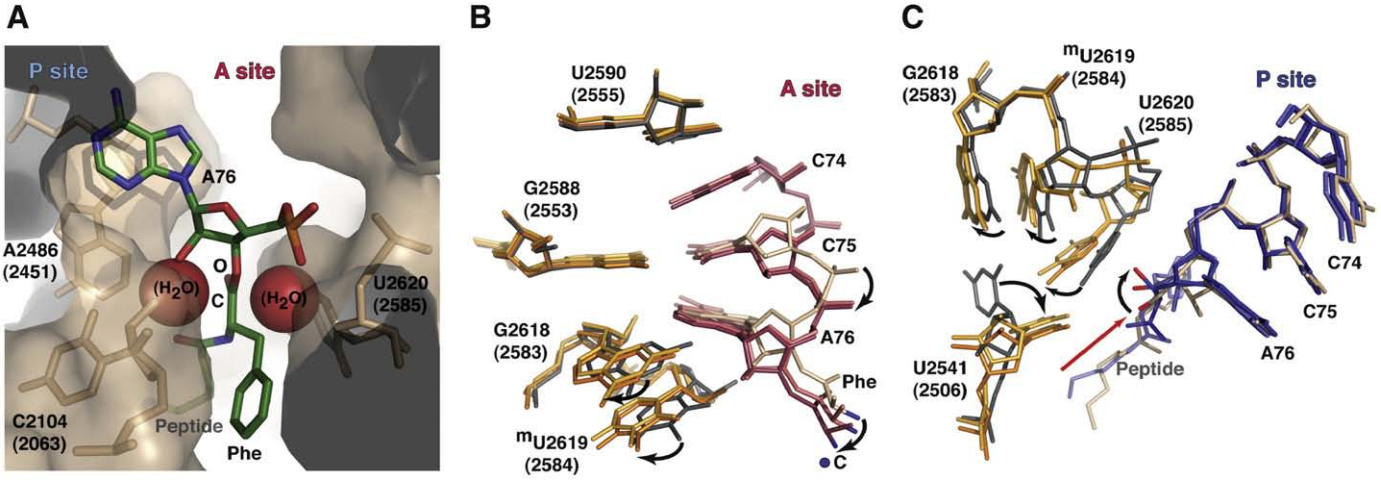

Fig. 5. The binding of the A-site substrate induces a conformational change in the PTC, which in turn promotes peptidyl transfer. (A) When the peptidyl-tRNA is in the P site and the A site is empty, the acyl-ester link is sequestered from water by the 23S rRNA. The peptidyl-tRNA mimic CCA-Pcb is colored green, the PTC is shown as a beige surface, the 23S rRNA bases C2104 (C2063), A2486 (A2541) and U2620 (U2585) are shown as beige sticks as well. Two water molecules (red spheres) are modeled at the putative attack locations and it is clear that due to steric clashes water cannot access the acyl-ester link when the A site is empty. (B) The binding of either the transition state analog (TSA) (red) or CC-hPmn (pink) to the A site induces the conformational change in the PTC, whereas C-hPmn (beige) does not promote the conformational change. C-hPmn binds somewhat higher in the A site when compared to both CC-hPmn and TSA, and its α-amino group is positioned far from the carbonyl carbon of the aminoacyl-tRNA (blue sphere). (C) The induced-fit conformational change in the residues G2618–U2620 (G2553–U2585) and U2541 (U2506) allows the rotation of the carbonyl oxygen. The rotation is the largest in the case of the transition analog soak (blue) allowing the nucleophilic attack by the free α-amino group onto the carbonyl carbon. The rotation occurs in the presence of CC-hPmn (light blue) as well but to a somewhat lesser degree. In the presence of C-hPmn (beige), however, the carbonyl oxygen prevents the nucleophilic attack. The direction of the nucleophilic attack is shown with a red arrow. In B and C, the PTC residues are colored as following: orange for the TSA soak, light orange for the CC-hPmn soak and grey for the C-hPmn soak. The figure is adapted from reference [23].

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puromycin moiety, which often ended up binding to the entrance of the tunnel. The somewhat lower resolution of the initial study (3.2 Å) significantly hampered the efforts to correctly position the phosphorous mimic of the tetrahedral carbon as well as to correctly assign the oxyanion oxygen. Initially, the oxyanion was proposed to form a hydrogen bond with the N3 atom of A2486 (A2451) and suggested that this nitrogen might play the role of a general base and that of an oxyanion hole [22]. However, Mankin and coworkers revealed that the catalytic power of the ribosome remained almost unscathed when A2486 (A2451) was replaced by any other nucleotide in the *E. coli* 70S ribosome [60] and that the chemical modification of A2486 (A2451) by dimethylsulfate, which was previously used to monitor pKa of this residue, was in fact not dependent on pH [61]. Concurrently, Dahlberg and colleagues found that neither G2482 (G2447) nor A2486 (A2451) are involved in catalysis by analyzing the mutants of both the *E. coli* 70S ribosome and the *Bacillus stearothermophilus* 50S subunit [62]. Further, Strobel and colleagues demonstrated that the binding of the Yarus inhibitor was not pH dependent suggesting that the oxyanion does not interact with a group with near-neutral pKa [63]. Finally, Green and coworkers provided the crucial evidence that A2486 (A2451) is not directly involved in catalysis by a study in which they showed that mutating A2486 (A2451) in the 70S ribosome did not affect the rate of peptidyl transfer when the intact aminoacyl-tRNA substrates were used [64]. Other mutational and kinetic studies converged to the conclusion that none of the other conserved 23S rRNA residues in the PTC are involved in catalysis either [21,64–66]. The rate of reaction was affected greatly only when the non-canonical A2487-C2104 (A2450-C2063) pair was mutated into the G–U pair [67], or when puromycin was used as the A-site substrate [21,64–66].

Studies that followed proved to be influential in identifying the elusive group responsible for the ribosome-catalyzed peptide bond formation. Barta and colleagues provided the first evidence for the role of the P-site O2′ hydroxyl group by using a nucleotide analog in the P site and proposed proton shuttling mechanism [68]. Subsequently, Weinger et al. [72] found that the removal as well as the replacement of the O2′ hydroxyl by the fluoro group in the P-site tRNA can account for an almost 10^6-fold reduction in the rate of peptide bond formation. Involvement of the O2′ hydroxyl group in peptide bond formation was proposed before [68–71], but the critical difference was that Strobel, Green and colleagues measured the rate of the peptidyl transfer in the context of the 70S ribosome, complete tRNA substrates, and under the conditions where the chemical step is rate-limiting [72]. However, the effect of the O2′ hydroxyl group should be taken with caution, since the absence of O2′ hydroxyl in A76 leads to the reduction of the rate of the non-catalyzed reaction by almost 100-fold. Finally, Steitz and colleagues determined a series of Hma50 structures complexed with a variety of transition state analogs and they reached the following conclusions: (*i*) the peptidyl transfer reaction proceeds through a tetrahedral intermediate with S chirality, (*ii*) metal ions (mono- or divalent) observed in the PTC do not promote catalysis, (*iii*) the oxyanion hole is formed by a water molecule positioned by the 23S rRNA bases A2637 (A2602) and ^{methyl}U2619 (U2584), and (*iv*) the oxyanion oxygen points away from A2486 (A2451) (Fig. 6A, B) [26]. Because of the proximity of the O2′ hydroxyl to the O3′ atom of A76 that is linked to the peptide chain, it was proposed that O2′ hydroxyl is critical for proton shuttling from the attacking α-amino group to the leaving O3′ hydroxyl. Indeed, it has been shown that the O2′ hydroxyl group remains neutral in the transition state confirming its role in the proton shuttle [73]. More recently, Strobel colleagues have concluded that at the transition state the α-amino nucleophile is neutral, which is in a stark contrast to the development of the positively charged amino nucleophile during an uncatalyzed aminolysis reaction in solution [74]. The formation of the N–C bond is, therefore, proportionate to the disruption of the N–H bond suggesting that peptide bond formation and the breakage of the acyl-ester link occur in concert (Fig. 6B).

In addition, Polacek, Micura and colleagues developed an *in vitro* system in which they were able to replace specific moieties and individual atoms within the PTC of the *Thermus aquaticus* 70S ribosomes, and they have shown that the O2′ hydroxyl of A2486 (A2451) is critical for transpeptidation reaction, but not for peptide release regardless of the substrate utilized [75,76]. It appears that the hydrogen bonding capability of the O2′ hydroxyl of A2486 (A2451) is critical for its role [77]. These findings are in agreement with structural studies that used Hma50 as a model system. The O2′ hydroxyl of A2486 (A2451) forms a hydrogen bond with the O2′-hydroxyl group of the P-site A76 when both the A and P sites of Hma50 were occupied with appropriate substrate mimics and transition state analogs [24,26–28]. In a structural study on the CCA-promoted peptide release, however, it was found that the O2′ hydroxyl of the P-site A76 faces away from the A site and that it forms a hydrogen bond with the O2′ hydroxyl of C2104 (C2063) instead. This may explain why replacing the O2′ hydroxyl of A2486 (A2451) is found not to impair the peptide release reaction. Polacek and coworkers suggested that the O2′ hydroxyl of A2486 (A2451) plays a role in stabilizing the 2′-endo conformation of the P-site A76, which in turn may prevent the transesterification reaction between the O2′ and O3′ hydroxyls of the P-site substrate that is bound to the ribosome [77]. Thus, all the studies suggested that the ribosome does not contribute groups that are directly involved in the chemistry of catalysis, but it serves to position and orient substrates properly for reaction.

In conclusion, the ribosome utilizes a combination of the entropic, general acid–base and electrostatic shielding mechanisms to promote peptide bond formation. The two binding interfaces in the PTC, the A loop and the P loop, specifically recognize the acceptor ends of the aminoacyl- and the peptidyl-tRNA, respectively. The binding of the A-site substrate induces conformational change in the PTC, which allows reactive groups to be oriented properly for the peptidyl transfer reaction (entropic component). Peptide bond formation is further promoted by a substrate-assisted mechanism in which the O2′ hydroxyl group of the P-site A76 plays a crucial role in the proton shuttling (general acid–base component), whereas a water molecule coordinated by the ribosomal bases stabilizes the oxyanion of the tetrahedral intermediate (electrostatic shielding component).

7. Implications for the mechanism of peptide release

When the ribosomal A site encounters a stop codon, protein synthesis stalls and this signals that the nascent polypeptide should be released from the ribosome. Thus, the ribosome is not only capable of promoting peptide bond formation, but it is also able to catalyze hydrolysis of the acyl-ester link. In contrast to the reaction of peptide bond formation where proteins are not involved in catalysis, the release of newly synthesized peptide is directly promoted by protein release factors, RF1 and RF2. A number of mutational studies have identified key residues both in the PTC [64,78,79] and in RFs [80–85] that are important for the release reaction. Also, it was found that a preferred nucleophile for the RF-promoted peptide release is water and that the O2′ hydroxyl group of the peptidyl-tRNA substrate might play the same role in the RF-promoted peptide release as in the peptide bond formation [86,87].

The cryo-EM studies have revealed that RF2 is in open conformation when bound to the ribosome and that it emulates both structurally and functionally the A-site tRNA substrate [88,89]. Subsequently, several crystal structures of the 70S ribosome complexed with RFs have been determined. Initially, Ramakrishnan and coworkers determined the crystal structure of the 70S-RF2 complex at 6 Å resolution [90]. In agreement with the cryo-EM studies, it was found that the domain 3 of RF2 swings away from domains 2 and 4 placing the GGQ motif in the PTC, whereas domains 2 and 4 interact with the decoding center [90]. The domain 1 was found to interact

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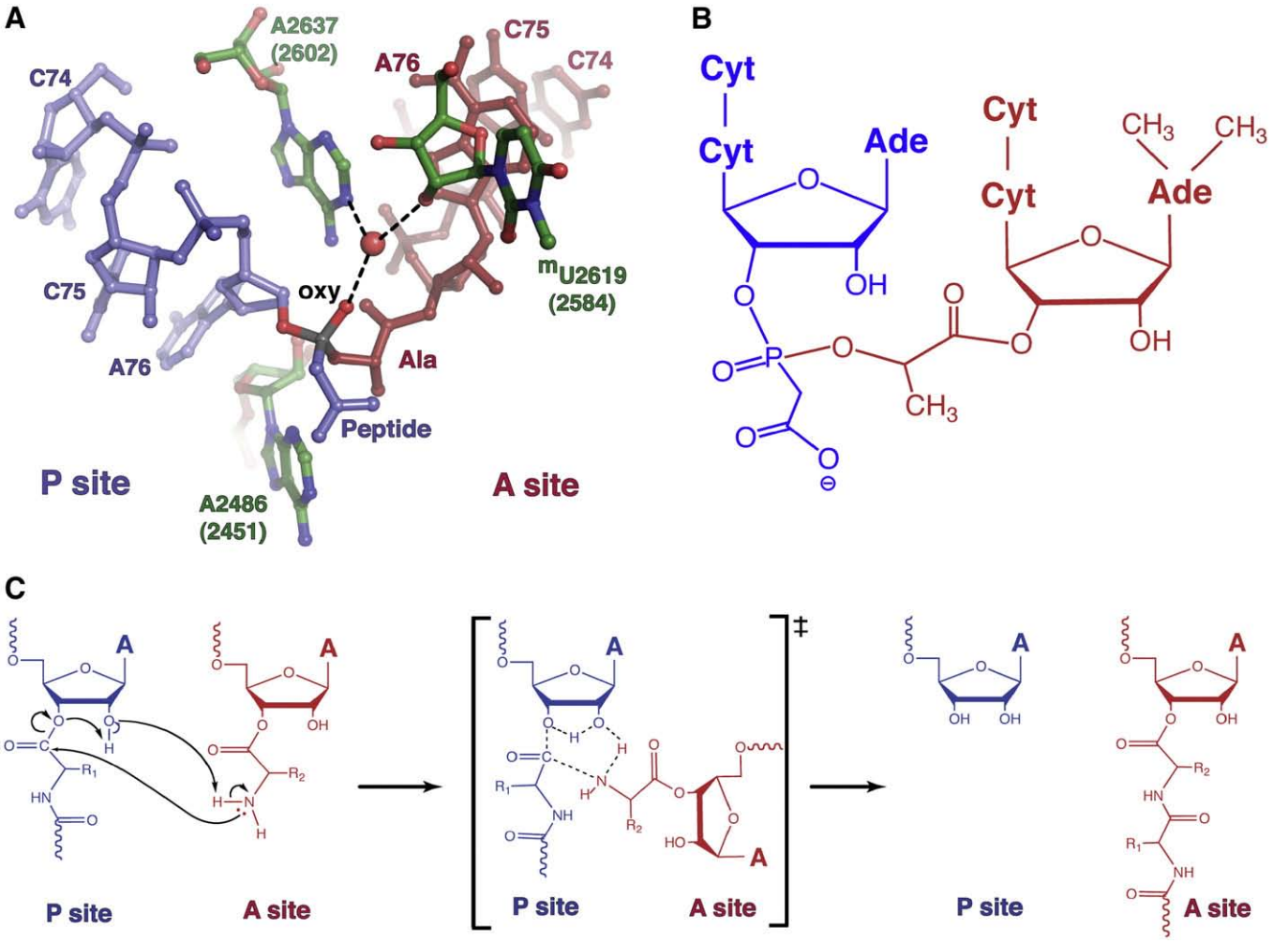

Fig. 6. The stereochemistry and mechanism of the peptidyl transfer reaction. (A) The complex with the transition state analog revealed that the oxyanion oxygen points away from A2486 (A2451). The structure further suggested that the reaction proceeds through an intermediate with S chirality. The base of U2637 (U2602) and the ribose of U2619 (U2584) coordinate a water molecule that forms an oxyanion hole. (B) A schematic diagram of the transition state analog shown in A. (C) The mechanism of the peptidyl transfer based on the structural and kinetic data. The attack of the α-amino group of the aminoacyl-tRNA (red) onto the carbonyl carbon of the peptidyl-tRNA (blue) occurs in concert with the proton shuttle. The O3′ oxygen and the O2′ hydroxyls of the P-site A76 as well as the α-amino group of the aminoacyl-tRNA are involved in the proton shuttle. The reaction proceeds through a six-membered intermediate state (in brackets) in which the formation of the new C–N bond is commensurate to the dissolution of the N–H bond. Finally, the intermediate collapses into the reaction products shown on far right. Panels A and B are adapted from reference [22], whereas panel C is adapted from reference [52].

with L11 of the 50S subunit [90]. However, the low resolution of the study hindered the efforts of identifying critical interactions between the conserved residues of RFs and those in the ribosome. Subsequently, Noller and colleagues obtained crystals of the 70S ribosome complexed with RF1 [91] and RF2 [92] that diffracted to 3.2 Å and 3 Å, repectively. Also, Ramakrishnan and colleagues improved considerably the diffraction limit of their 70S-RF2 complex crystal form to 3.5 Å [93]. These crystal structures revealed that the binding of the PxT/SPV motif of RF1/2 induces a conformational change in the A site of the 30S subunit, which in turn stabilizes the stop codon recognition. This further promotes the conformation of RF in which the interaction between its highly conserved GGQ motif and the PTC occurs [91]. Laurberg et al. [91] proposed that the backbone amide of Q230 in the RF1 is responsible for orienting the hydrolytic water, whereas Weixlbaumer et al. [93] suggested that the O2′ hydroxyl of the P site A76 might be involved in catalysis of the RF2-promoted peptide release along with the backbone and the side-chain amides of Q240. More recently, Noller and colleagues have replaced glutamine 240 with proline and showed that the removal of the backbone amide in Q240 abolishes the RF2-promoted peptide release [92], whereas the side-chain replacement alone did not affect the release activity.

In an alternative approach the mechanism of the peptidyl-tRNA hydrolysis was studied using Hma50 as a model system. The basis for this study was an observation that the deacylated tRNA [94] and CCA, but neither CA nor A [95], were able to promote peptide release. Thus, the oligonucleotides CCA and CA were used as the A-site substrate mimics, whereas a non-hydrolyzable analog of the peptidyl-tRNA, CCA-nPcb, was used as the P-site substrate [29]. It was found that only CCA, through its O3′ hydroxyl, is able to coordinate the hydrolytic water molecule, whereas CA was bound too remotely from the P site to be involved in catalysis of the peptide release (Fig. 7A, B). Despite the differences in their binding, both CCA and CA induced a conformational change in the PTC similar to the one observed in earlier studies on the peptide bond formation (Fig. 7C). Considering that CC-Pmn was able to induce conformational change and C-Pmn was not, it was proposed that the stacking interaction between C74 of the A-site substrate and the A-loop base U2590 (U2555) is responsible for the induced-fit conformational change in the PTC. In the same study it was suggested that release factor binding might induce similar changes in the PTC as observed in the 50S subunit and that its GGQ motif might serve to coordinate the attacking water molecule. Indeed, Weixlbaumer et al. [93] observed that RF2 binding induces a similar conformational change in the PTC

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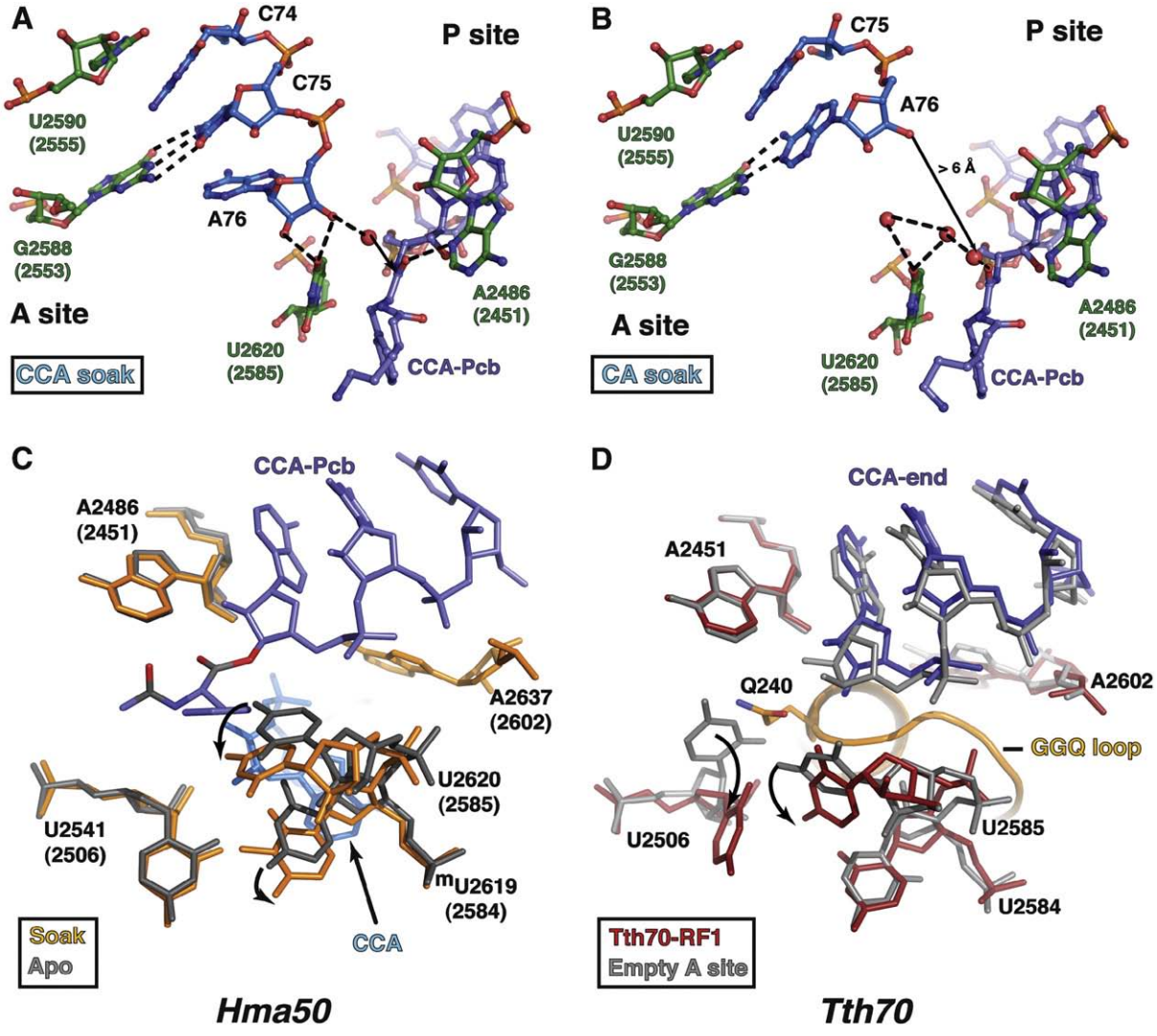

Fig. 7. Peptide release is promoted by the induced-fit conformational change in the PTC. (A) The A site CCA (light blue) promotes peptide release by coordinating the hydrolytic water molecule (red sphere) through its O3′ hydroxyl group. Also, the carbonyl oxygen of the peptidyl-tRNA analog, CCA-Pcb (blue), forms a hydrogen bond with the base of A2486 (A2451), which allows the nucleophilic attack by the water. (B) On the other hand, CA (light blue) binds higher in the A site and its O3′ hydroxyl is positioned too remotely from the P-site acyl-ester bond to be able to catalyze peptide release. Also, the carbonyl oxygen forms a hydrogen bond with a water molecule and its not oriented properly for the nucleophilic attack. In A and B, the 23S rRNA residues are colored in green. (C) The binding of both CA and CCA to the A site induces the conformational change in the PTC. The P site is oriented toward the reader, whereas the A site is behind the U2619–U2620 (U2584–U2585). CCA-Pcb is in blue and the CCA oligonucleotide in the A site is cyan. The induced-fit conformation of the PTC is colored orange, whereas the apo-PTC is grey. (D) A conformational change is observed in the PTC of the *T. thermophilus* 70 S ribosome when in complex with RF2. The induced-fit change is similar to that observed in the 50S subunit complexed with the appropriate A site substrate. The 70S-tRNA complex is grey, whereas the ternary 70S-tRNA-RF2 complex is in red. The GGQ motif of RF2 is orange with the side-chain of Q240 shown as sticks. Panels A and B were adapted from reference [25], while panel D was adapted from reference [65].

of the 70S ribosome to that found in analogous complexes of Hma50 (Fig. 7C, D) [93]. However, to complete our understanding of the mechanism of peptide release, further structural studies on the 70S-RF complexes and in the presence of a mimic of the amino-acylated tRNA substrate are necessary.

8. The PTC is the same in the 50S subunit as in the 70S ribosome

The most detailed structural studies on the mechanism of peptide bond formation have been done using the 50S subunit from *H. marismortui* as a model system. On several occasions it was suggested that the PTC in the 70S ribosome might adopt a different conformation than that observed in the isolated 50S subunit [13]. Most recently, this proposal was reiterated with a crystal structure of the 70S ribosome from *T. thermophilus* complexed with both the P- and E-site tRNAs [15]. Upon examination of their 3.7 Å resolution model, Korostelev et al. [15] suggested a different position for A2486 (A2451), and closer orientation of the P-site A76 to the non-canonical A2485-C2104 (A2451-C2063) base pair than what was found in any of the crystal structures of Hma50 complexed with substrates, intermediates or products. The proposed differences in the PTC structure were, however, inconsistent with the proton shuttle mechanism of peptide bond formation. Concurrently, Selmer et al. [14] have obtained a crystal form of the *T. thermophilus* 70S ribosome complexed with the P-site tRNA in which the PTC adopted a conformation identical to the one found in Hma50 when in complex with the analogs of the P-site substrate. Apart from differences in the identity of the P-site tRNA as well as in the length of mRNA, the two Tth70 crystal forms were in the same functional state. Because of the high level of sequence conservation in the PTC, it is reasonable to expect that the structure of the active site would be conserved as well. Indeed, cross-crystal averaging of the data sets derived from both crystals of Tth70 has shown that the PTC adopts the same conformation in both crystal forms as in the 50S subunit of *H. marismortui* [96]. Furthermore, kinetic studies have shown that the 50S subunit catalyzes the reaction of peptide bond formation at the same rate as the 70S ribosome in the presence of

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appropriate substrates [17]. Also, a hypothesis was put forward that the PTC was the first element of the proto-ribosome that was present over 4 billion years ago in the Last Universal Ancestor and that its structure is likely to be strictly conserved in all domains of life [97]. Finally, while this manuscript was under preparation Ramakrishnan and colleagues published two crystal structures that represent distinct functional states of the intact *T. thermophilus* 70S ribosome [23]. In a structure that represents a state before peptidyl transfer reaction, a mimic of the amino-acylated tRNA was bound to the A site and an unacylated tRNA occupied the P site. On the other hand, in a structure that mimics the post-peptidyl transfer state the amino-acylated tRNA occupied both the A and the P sites. The authors found that binding of the aminoacyl-tRNA to the A site induces the conformational change in the PTC of the 70S ribosome that is very similar to that observed in structures of the *H. marismortui* 50S subunit when complexed with the mimics of the A-site substrate [27]. More importantly, the orientation of both the A-site α-amino group and the O2′ hydroxyl of the P-site A76 is the same as in the context of the 50S subunit and it is consistent with the proposed substrate-assisted mechanism of catalysis [72]. Taken together, these observations confirm that the structure of the PTC is the same in the 50S subunit and the 70S ribosome and that all conclusions on the mechanism of peptide bond synthesis derived from the structures of the 50S subunit are true for the entire ribosome.

9. Conclusions and future directions

Structural studies on the ribosome have undergone a dramatic expansion in the last decade. A number of crystal structures of the complete ribosomal particle complexed with mRNA, substrate tRNAs and with protein release factors are now available. Concurrently, cryo-EM maps of high quality have been obtained for the complexes between ribosomes and various elongation factors that represent distinct stages in the process of the ribosome-catalyzed translation. While this manuscript was in preparation a model of the ribosomal polysomes was obtained by cryo-electron tomography [98]. Here, "beads on a string" are shown in action at higher resolution than ever before providing an explanation as to how multiple ribosomes can simultaneously work on the same mRNA molecule. Further, while the mechanism of discrimination of the cognate tRNAs from the non-cognate or near-cognate tRNAs by the ribosome is understood [18,99–106], it is still unclear how the cognate codon–anticodon interaction is signaled to EF-Tu and then to the PTC. High-resolution structural studies on the 70S ribosome complexed with EF-Tu and either the cognate or the non-cognate tRNA are thus warranted. These structures would reveal how the cognate codon–anticodon interactions promote the catalytic activity of the PTC and would explain why the non-cognate interactions fail to do so. In addition, the exact molecular mechanism of product translocation that is promoted by EF-G is not clear. A number of cryo-EM studies revealed ribosomal sites in the hybrid state and proposed the role of EF-G and the L1 stalk in the translocation of the product tRNAs [48,49,53,57]. However, a high-resolution structure of the ternary complex between the 70S ribosome, EF-G and tRNA in the appropriate state remains to be determined. Perhaps the most challenging problem is the one concerning the mechanism of the co-translational translocation of proteins across biological membranes as well as the mechanism of the early events of protein sorting. A number of cryo-EM studies that have already been published offered conflicting answers [107–111]. Therefore, crystal structures of the ribosome that is stalled in the act of translation and is bound to either the translocon channel or to the signal recognition particle are necessary. Studies on co-translational translocation may not only be useful for deciphering the mechanism of protein sorting, but they may prove to be invaluable for a set of questions related to ribosomal stalling that occurs *in vivo*. A number of peptide sequences such as TnaC, SecM and ErmC were found to have the ability to regulate gene expression by stalling the ribosome-catalyzed mRNA translation (reviewed in [112–115]) and these exact peptides are being used in preparations of stalled ribosomes for structural studies on co-translational translocation. Beyond the identification of the sequence requirements necessary for stalling, little is known about specific interactions between the stalling peptide and the exit tunnel of the ribosome. More importantly, it is not clear how interactions between the nascent peptide and the exit tunnel may affect the PTC structure. Finally, a detailed atomic model of the eukaryotic ribosome is still missing. The monumental advances in the field of ribosome structure in the last decade bring these very important biological questions within grasp.

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Please cite this article as: M. Simonović, T.A. Steitz, A structural view on the mechanism of the ribosome-catalyzed peptide bond formation, Biochim. Biophys. Acta (2009), doi:10.1016/j.bbagrm.2009.06.006

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Please cite this article as: M. Simonović, T.A. Steitz, A structural view on the mechanism of the ribosome-catalyzed peptide bond formation, Biochim. Biophys. Acta (2009), doi:10.1016/j.bbagrm.2009.06.006

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Please cite this article as: M. Simonović, T.A. Steitz, A structural view on the mechanism of the ribosome-catalyzed peptide bond formation, Biochim. Biophys. Acta (2009), doi:10.1016/j.bbagrm.2009.06.006