Review

Protein Elongation, Co-translational Folding and Targeting

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Abstract

The elongation phase of protein synthesis defines the overall speed and fidelity of protein synthesis and affects protein folding and targeting. The mechanisms of reactions taking place during translation elongation remain important questions in understanding ribosome function. The ribosome—guided by signals in the mRNA—can recode the genetic information, resulting in alternative protein products. Co-translational protein folding and interaction of ribosomes and emerging polypeptides with associated protein biogenesis factors determine the quality and localization of proteins. In this review, we summarize recent findings on mechanisms of translation elongation in bacteria, including decoding and recoding, peptide bond formation, tRNA–mRNA translocation, co-translational protein folding, interaction with protein biogenesis factors and targeting of ribosomes synthesizing membrane proteins to the plasma membrane. The data provide insights into how the ribosome shapes composition and quality of the cellular proteome.

Introduction

Translation elongation is the central phase of the protein synthesis which maintains protein production and affects protein folding, processing and—for some predestined proteins—selection for targeting to cellular compartments. Elongation entails three major steps: decoding, peptide bond formation and tRNA–mRNA translocation. Elongation proceeds rapidly, with an average of 10–25 aa incorporated into the nascent peptide per second in *Escherichia coli* [1]. Despite the high overall rate of protein production, elongation is not a uniform process, as periods of rapid synthesis are interrupted by pauses. For rapidly translated stretches, the overall rate is mostly limited by the codon-specific delivery of cognate aminoacyl-tRNA (aa-tRNA) into the A site of the ribosome. The abundance of the respective tRNAs and other factors, such as secondary structure elements in the mRNA, codon context, ribosome pausing and stalling, collisions between ribosomes in polysomes, or cooperation between translating ribosomes and the RNA polymerase machinery may contribute to local pauses and thus to the variation of translation rates.

The relation of speed and accuracy of translation and the mechanisms of peptide bond formation and translocation remain important issues in understanding the processivity of translation. Furthermore, the ribosome can alter the meaning of individual codons, alleviate the co-linearity of the sequences of the mRNA and the protein, or change the reading frame on particular mRNAs. These recording phenomena suggest how mRNA signals can redirect the ribosome to the synthesis of an alternative protein, thereby enriching the proteome.

Proteins may start to fold during translation elongation. Translational pauses were long suggested to affect folding. A peptide emerging from the exit tunnel encounters a number of proteins, such as protein biogenesis factors and chaperones. Interactions of the nascent protein with these factors ensure correct processing of the N terminus, help folding or keep unfolded and ensure that the proteins will find their destination in the cell, for example, are inserted into the plasma membrane. We present a view of

translation elongation as a complex network of reactions that ensure the composition and quality of the cellular proteome.

Elongation

Decoding

Speed and accuracy of protein synthesis are fundamental parameters that affect the fitness of the cell, the quality of the proteome and the evolution of ribosomes. At each round of elongation, the ribosome selects an aa-tRNA corresponding to the codon in the A site among other aa-tRNAs. Aa-tRNAs are delivered to the ribosome in a ternary complex with elongation factor (EF)-Tu and GTP. On the ribosome, the fidelity of translation is controlled at two basic selection stages (Fig. 1): (i) during initial selection, which allows for the rejection of incorrect ternary complexes prior to GTP hydrolysis, and (ii) at the proofreading stage after GTP hydrolysis, in which incorrect aa-tRNAs with high frequency dissociate from the ribosome before the amino acid is incorporated into peptide. In addition, aa-tRNA release from EF-Tu on the ribosome may provide a selection checkpoint, for example, when non-natural amino acids are used [2]; the contribution of this potential selection mechanism to the discrimination of natural aa-tRNAs is not known. These pre-transfer selection mechanisms ensure that even if a non-cognate ternary complex is delivered to the ribosome, the chance that an incorrect amino acid is incorporated is very small and the synthesis of the nascent chain can continue after the incorrect aa-tRNA was rejected. If an erroneous amino acid is nevertheless incorporated, the peptide can be released from the ribosome by termination factors, which provides a post-transfer editing step controlling the fidelity of translation [3,4]. Notably, in this case, the synthesis of the polypeptide chain is terminated and the portion of the protein that has been produced has to be discarded.

Kinetic studies have identified which elemental reactions during initial selection and proofreading differ for cognate and near-cognate aa-tRNAs [5–7]. The selectivity is due to higher rates of the forward reactions of GTP hydrolysis and peptide bond formation for the cognate, as compared to a near-cognate, tRNA, and different stabilities of cognate and near-cognate codon–anticodon complexes (Fig. 1). One long-known, but poorly understood phenomenon is the trade-off between the speed and fidelity, which is particularly relevant for the initial

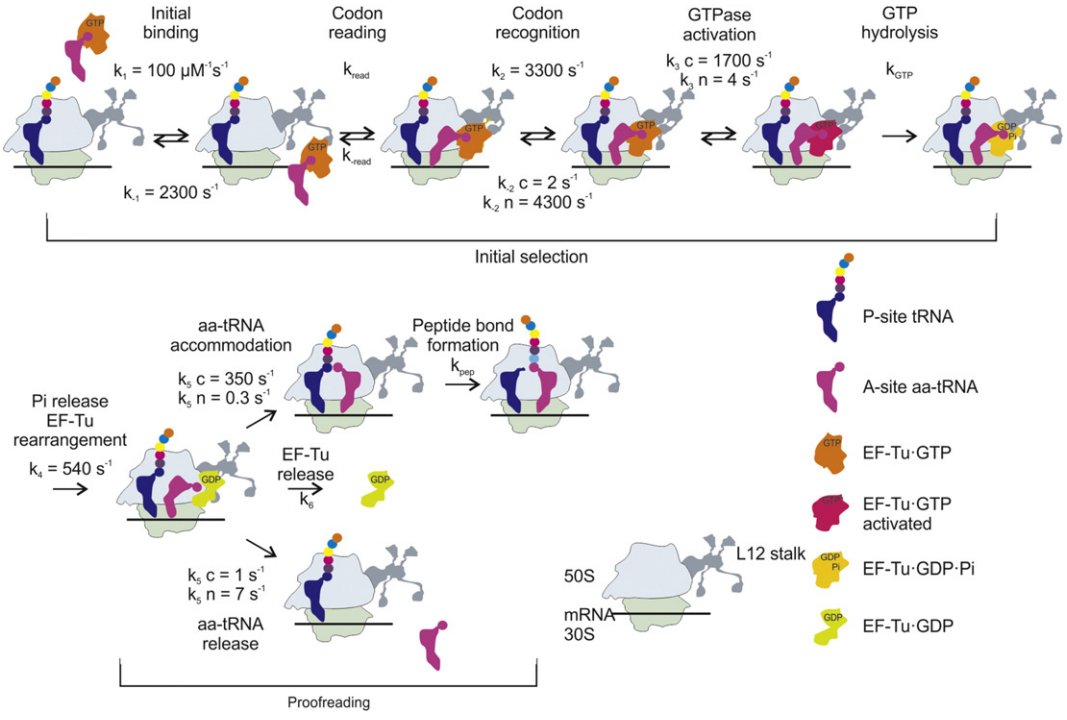

Fig. 1. Rates of the elemental steps of decoding extrapolated for the conditions *in vivo*. The values for the rate constants are from Ref. [9]. Rate constants specific for the cognate aa-tRNA have a subscript "c"; for the near-cognates, the subscriot is "n." Rate constants without subscript are similar for cognate and near-cognate ternary complexes. The steps of codon reading, GTP hydrolysis and peptide bond formation are intrinsically rapid but rate-limited by the preceding rearrangements [6,83]; the rate of EF-Tu release *in vivo* is not known but can be assumed to be high as well.

selection step. Because translation is rapid, the reversible steps of decoding do not equilibrate due to the rapid transition toward the irreversible GTP hydrolysis step, and thus the stability differences are not utilized in full [6]. The extent of the speed-fidelity trade-off *in vivo* is not known; at conditions *in vitro*, where the error rate is about 10^{-3} and the rate of decoding about 1 s^{-1} (at 20 °C) [8,9], initial selection is achieved predominantly by different GTP hydrolysis rates for the cognate, near- and non-cognate ternary complexes [6,8]. *In vitro* experiments performed at decreasing Mg^{2+} concentrations to modulate decoding suggested a linear dependence between speed and fidelity [10]. That is, if the speed of translation is maximum, then the accuracy is low, whereas when the speed approaches zero, the fidelity reaches its theoretical maximum [11]. It is not known whether the extrapolations based on Mg^{2+} titrations provide values comparable to those *in vivo*, as the complexes used for these experiments may be unstable at very low Mg^{2+} concentrations [8]. Moreover, because the elemental rates of different reaction were not measured [11], the mechanistic description of the effects is lacking. Nevertheless, regardless of the exact trade-off value *in vivo*, the ribosome appears to be optimized toward high speed of translation at the cost of fidelity [8,12,13].

As the kinetic analysis of decoding was performed *in vitro* [5,6,8], an important question is whether the rate constants determined *in vitro* can explain the kinetics and fidelity of translation in the *E. coli* cell. To answer this question, Rudorf et al. have developed a theoretical approach that allows deducing the *in vivo* rates using the values measured *in vitro* as initial guesses [9]. The resulting set of rate constants (Fig. 1) not only accounts for the overall rate and accuracy of protein synthesis but also provides an excellent fit for the experimentally measured time courses of translation. For all rates of the elongation cycle, the deviations between the deduced *in vivo* rates and those measured or estimated *in vitro* correspond to relatively small ($<2k_BT$) shifts of the corresponding free energy barriers. Because the cytosol is a rather complex environment, small changes in the free energy barriers can be easily envisaged, arising, for example, from changes in the hydrogen bond networks around the ribosome or from changes in the flexibility of some parts of this complex. On the other hand, these results also show that the high-fidelity buffer used to determine elemental rate constants [6] represents a good approximation to the cytosol as far as kinetics of translation is concerned.

Although the theoretical work [9] accounts quite well for the overall kinetics of protein synthesis *in vivo* [14,15], the current model assumes that all cognate aa-tRNAs have very similar kinetic properties, although there might be differences due to different sequences, strength of codon–anticodon interaction and in the reactivity of the amino acid the tRNAs carry. While this simplification appears to be a good starting approximation for the cognate aa-tRNAs [16], the range of intrinsic differences between near-cognate tRNAs is not negligible [5] and will have to be included in future calculations. Further attenuation in overall translational kinetics may come from the isoacceptor tRNAs that normally read different codons in a given codon family. As they deliver the same amino acid, these tRNAs are usually simply considered as cognate. However, the codon–anticodon interaction may contain a mismatch in a first or third codon position. For example, the Leu-$tRNA_5^{Leu}$ isoacceptor that decodes UUA/G codons is a very inefficient decoder of the CUX family of Leu codons. On the other hand, Ala-$tRNA_{1B}^{Ala}$ with the anticodon $C_{36}G_{35}cmo^5U_{34}$, carrying the modification at the wobble position, permits fairly efficient reading of non-Watson-Crick and non-wobble bases in the third codon position, such that, for example, the GCC codon incorporates Ala only about 10 times slower than a fully matching Ala-tRNA [7]. The ribosome accepts the C_3-cmo^5U_{34} codon–anticodon pair as an almost-correct base pair, unlike third-position mismatches, which would lead to the incorporation of incorrect amino acids and are efficiently rejected [7]. Taking into account such decoding phenomena, as well as variations in the rates of peptide bond formation, for example, the slow incorporation of Pro and Gly in certain contexts, it would be important to refine the model of translation, as it may provide a powerful tool to better understand not only the fidelity of translation, but also the different decoding rates for the 61 sense codons as well as translational pausing *in vivo* [17].

GTP hydrolysis by EF-Tu is a major checkpoint that controls both rate and fidelity of decoding [12]. The intrinsic GTPase activity of unbound EF-Tu (or ternary complex) is very low and is accelerated when the ternary complex EF-Tu–GTP–aa-tRNA binds to the ribosome. The extent of acceleration depends on whether the aa-tRNA in the ternary complex is cognate, near-cognate or non-cognate to the codon in the A site, but the exact mechanism of the GTPase activation remains uncertain. In the cognate ribosome–EF-Tu–GTP–aa-tRNA complex stalled at the stage after codon–anticodon recognition on the small ribosomal subunit (SSU) but preceding GTP hydrolysis, the nucleotide binding pocket docks onto the sarcin-ricin loop (SRL) of 23S rRNA in the large ribosomal subunit (LSU) [18]. Genetic, structural and kinetic data suggest that a distortion of the tRNA molecule during codon reading is necessary to trigger rapid GTP hydrolysis in EF-Tu in response to cognate codon–anticodon recognition [19–21]. On the other hand, a distorted tRNA intermediate appears to form also upon reading a near-cognate codon [22,23]. Thus, the tRNA distortion alone is not sufficient to discriminate between

cognate and near-cognate tRNAs, implying that other regulators of signaling may be involved [24]. Notably, only a small re-organization at the nucleotide binding site is necessary to bring all the residues that take part in the reaction into their catalytic positions [20]. This would imply that the key step in GTPase activation may be the correct docking of EF-Tu on the SRL. In this case, the distortion of the tRNA molecule and codon-induced orientation of the tRNA molecule with respect to the SSU and LSU would promote the accurate EF-Tu docking on the SRL, rather than initiate conformational changes at the nucleotide binding site of the factor.

GTP cleavage proceeds through a nucleophilic attach of the hydrolytic water molecule on the γ phosphate of GTP (Fig. 2). The reaction on the ribosome depends on the presence of His84 and Asp21 of EF-Tu and the phosphate group of A2662 in the SRL [25–27]. A2662 is likely responsible for the stabilization of the conformation where His84 is rotated toward the nucleotide that is observed in the activated state [28]. Asp21 may further stabilize the transition state; high-resolution structures of ribosome-bound EF-G, which has a GTP-binding pocket very similar to EF-Tu, show the side chain of the respective Asp residue coordinating a magnesium atom close to the crucial A2662, indicating that the formation of the GTPase-activated state might also involve a rearrangement of Asp21 [29,30]. Molecular dynamics and free energy calculations suggested that the most favorable reaction path requires that His84 be doubly protonated and involves stabilization of negative charge development on the water nucleophile as a major part of the catalytic effect. This effect is achieved both by the His84 side chain and by the backbone of the universally conserved PGH motif. Asp21 favors movement of one negative charge toward His84 [31] (Fig. 2). Thus, the ribosome appears to accelerate GTP hydrolysis by EF-Tu by arranging the catalytic site in a productive way, consistent with the exceptionally high entropic contribution to catalysis [13]. How LSU protein bL7/12, which accelerates GTP hydrolysis by EF-Tu by 2 orders of magnitude [32], contributes to catalysis is unknown; recent evidence obtained for the archaeal homolog of bL7/12, aP1, suggested that these proteins play a crucial role for the recruitment of the translation factors to the SRL [33]. The essential accessory function of the translational GTPases provides yet another example of how the ribosome, an ancient RNA-based catalyst, solves problems posed by the limited catalytic power of RNA.

The ribosome is evolutionarily optimized for the incorporation of the 20 standard amino acids and for the rejection of incorrect substrates. Thus, the mechanisms that ensure the high fidelity of protein synthesis with natural amino acids presumably limit the incorporation of unnatural substrates, that is, the production of designer proteins containing unnatural amino acids. Two independent control mechanisms related to EF-Tu function appear to impair the incorporation of unnatural amino acids into peptides [2,34,35]. A tRNA carrying an unnatural amino acid may be excluded from translation because it is impaired either in binding to EF-Tu or in the release from EF-Tu after GTP hydrolysis on the ribosome. EF-Tu has evolved to bind all natural aa-tRNA uniformly by idiosyncratic tuning of the individual contributions of the tRNA and amino acid moieties to an average binding affinity, which balances between the tight binding needed to ensure delivery to the ribosome and the necessity to rapidly release aa-tRNA for accommodation in the ribosome [36].

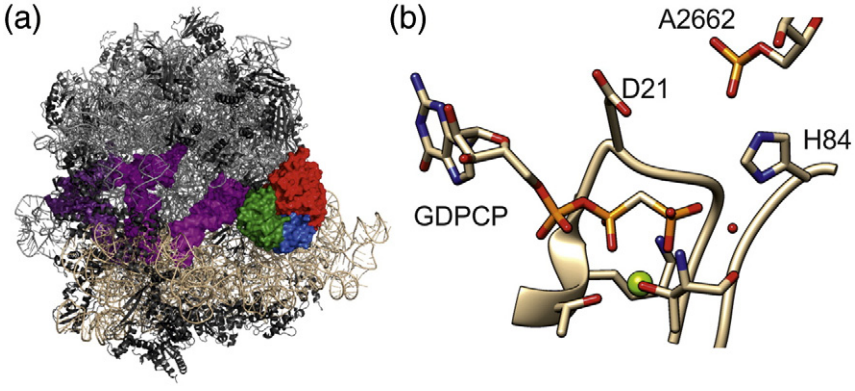

Fig. 2. Schematic of the GTPase of EF-Tu. (a) Structure of the complex EF-Tu–GDPCP–aa-tRNA trapped on the programmed ribosome before GTP hydrolysis [18]. The color code for EF-Tu shows domains 1 (red), 2 (green) and 3 (blue); the A/T aa-tRNA is shown in magenta, the P-site tRNA in purple and the *E*-site tRNA in dark purple. (b) Close-up view of the nucleotide binding pocket of EF-Tu. Residues important for the catalysis of GTP hydrolysis are shown as sticks; the catalytic water and the Mg^{2+} ion are shown in red and green, respectively (PDB: 2XQD and 2XQE). Modified from Maracci et al., in press.

Circumventing this potential quality control checkpoint that specifically prevents incorporation of unnatural amino acids into proteins may provide a new strategy to increase yields of designer proteins.

Peptide bond formation

The active site of the ribosome, the peptidyl transferase center, catalyzes two reactions, peptide bond formation between peptidyl-tRNA and aminoacyl-tRNA as well as the release factor (RF)-dependent hydrolysis of peptidyl-tRNA. Peptide bonds are formed as a result of the nucleophilic attack of the α-amino group of aa-tRNA in the A site on the carbonyl carbon of the pept-tRNA in the P site. The peptidyl transferase center is composed of rRNA and thus catalysis must rely on the limited repertoire of the active groups provided by the RNA [37]. Extensive mutational studies and the analysis of pH/rate profiles of peptide bond formation suggested that the ribosome does not provide ionizing groups that contribute to catalysis of peptide bond formation (reviewed in Refs. [38,39]). Crystal structures as well as kinetic and computational work suggested a substrate-assisted reaction mechanism that involves the 2′OH group of A76 of the P-site tRNA (Fig. 3) [40–43]. A comprehensive analysis of heavy-atom kinetic isotope effects indicated that the formation of the tetrahedral intermediate and proton transfer from the attacking nitrogen to the leaving oxygen take place during the rate-limiting step, whereas the breakdown of the tetrahedral intermediate occurs in a separate rapid step, consistent with an early transition state (TS) [44]. Further evidence for an early TS comes from charge effects of the C-terminal amino acid of pept-tRNA in the P site, which are consistent with a negative charge accumulating in the TS [45], and a near-zero Brønsted coefficient for the α-amino nucleophile [46]. The analysis of kinetic solvent isotope effects showed that in the rate-limiting TS three protons move in a fully concerted manner [47]. The simplest model that explains these findings is that, in the rate-limiting TS, the attack of the α-amino group on the ester carbonyl carbon results in an eight-membered transition state in which a proton from the α-amino group is received by the 2′OH group of A76, which at the same time donates its proton to the carbonyl oxygen *via* an adjacent water molecule [47] (Fig. 3). Protonation of the 3′OH then would be an independent rapid step [44], possibly again involving a water molecule [43]. Alternatively, proton transfer can follow the proton wire model [43], which is also consistent with all findings described above [47], but suggests a different path of protons, with the N-terminal amino group of ribosomal protein bL27 restricting the access of water molecules by closing the reaction pocket. The latter suggestion is somewhat inconsistent with the experimental evidence that the ribosomes lacking bL27 are not affected in the rate or pH-dependence of peptide bond formation [48]. However, it is difficult to exclude that in the absence of bL27, some other group (protein, rRNA or water) takes over the functions of bL27 and closes the reaction pocket. Alternatively, it is also possible that although the wire model may be correct as to the path for proton transfer, bL27 does not play the proposed role even though it is located in the vicinity.

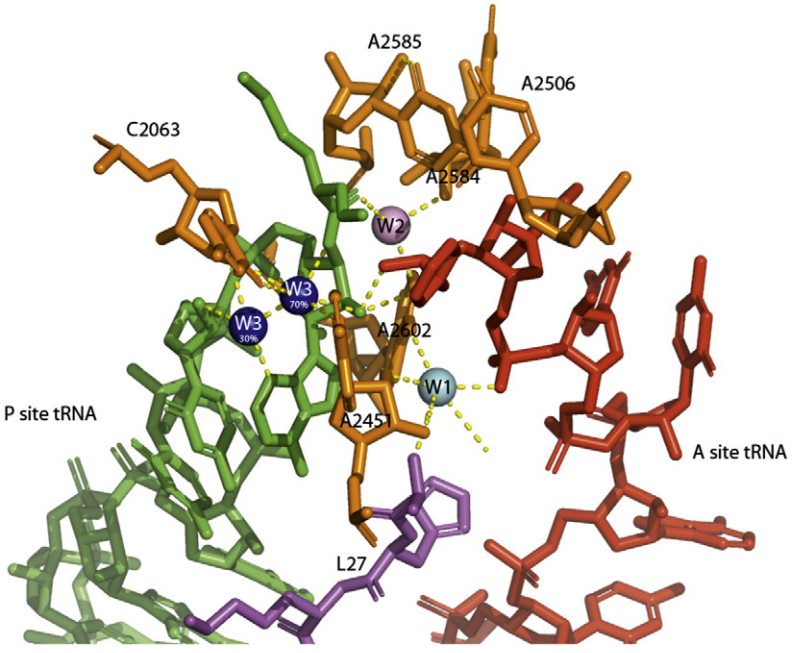

Fig. 3. Close-up on the peptidyl transferase center prior to peptide bond formation [43]. The P-site tRNA is shown in green, the A-site tRNA in red, protein bL27 in magenta and rRNA elements at the peptidyl transferase core in orange; 23S rRNA nucleotides are labeled using *E. coli* numbering. Potential catalytic water molecules are shown as spheres.

Finally, both pathways—the shuttle and the wire—may turn out to be isoenergetic; in the absence of bL27, the ribosome may favor the shuttle mechanism that does not involve bL27. Regardless of the detailed mechanism, the catalytic role of the ribosome is to provide a network of interactions that change the rate-limiting TS and lower the activation entropy [41,49,50].

In comparison to other amino acids, peptide bond formation with Pro is surprisingly slow [45,51,52] and leads to ribosome stalling when several consecutive Pro residues have to be incorporated [53,54]. The reasons for the low reactivity of Pro are not entirely clear and there is no simple explanation in terms of electrophilicity or preference for a particular configuration (*cis* or *trans*) or a particular ring pucker [55]. The entropic character of substituent effects suggests that the positioning of Pro-tRNA in the peptidyl transferase center of the ribosome is mainly responsible for the slow reaction [55]. Stalling occurs as soon as two Pro residues have been incorporated and another Pro-tRNA enters the A site; it also can occur with two Pro residues in some context with respect to the preceding and following amino acid [56–58]. Rapid translation of such mRNA sequences requires an auxiliary EF, EF-P [53,54] (Fig. 4). EF-P binds to the E site of the ribosome and interacts with the P site-bound tRNA [59]. Most likely, EF-P improves the positioning of Pro-tRNA^{Pro} in the peptidyl transferase center, thereby accelerating the reaction [55]. Given that the synthesis of a peptide bond usually does not require an auxiliary factor, EF-P appears to be a recent evolutionary addition to the repertoire of translation factors.

EF-G-catalyzed tRNA–mRNA translocation

Following peptide bond formation, the newly formed peptidyl-tRNA in the A site and the deacylated tRNA in the P site move to their respective post-translocation positions, P and E sites. The movement requires another EF, EF-G and GTP hydrolysis to proceed at a physiologically relevant rate. Translocation arguably is the step in elongation where ribosome dynamics matter the most. In the state after peptide bond formation, but prior to tRNA–mRNA movement, the ribosome fluctuates between the pre-translocation (PRE) state with the peptidyl- and deacylated tRNAs in the A and P sites, respectively, on both SSU and LSU (PRE(C)) and the state in which the SSU and LSU rotate relative to one another and the tRNAs assume hybrid position with the anticodons in the A and P sites on the SSU and the 3′ ends of the two tRNAs shifted toward the P and E sites, respectively, on the LSU (PRE(H)) [60–64] (Fig. 5). In addition to the SSU body rotation relative to the LSU, the head of the SSU undergoes a swiveling motion which is essential for tRNA translocation [65,66]. EF-G can bind both to the PRE(C) and PRE(H) states [67–69] and stabilizes the PRE(H) state. Upon transition to the post-translocation (POST) state, the rotation and head swivel of the SSU are reversed to the classical state, and the ribosome contains one tRNA in the P site, as the *E*-site tRNA dissociates rapidly and spontaneously [70–73]. The bL12 and uL1 stalks move together with tRNAs and EF-G [74–77]. The joint movement of the two tRNA and mRNA is assisted by the moving parts of the ribosome. One of the key questions is how the movement is induced and what the moving parts are.

Although in the cell translocation is promoted by EF-G, *in vitro* it can spontaneously, albeit slowly, proceed in forward and backward direction, depending on the thermodynamic preferences of the tRNAs for binding to the A and P sites [78–80]. Understanding the mechanism of spontaneous translocation is important even though the reaction does not play a functional role *in vivo*, because it provides an insight into the fundamental principles of movement on the ribosome. Cryo-electron microscopy (cryo-EM) and molecular dynamics simulations showed the exact pathway of the tRNAs when moving through the ribosome and provide an unprecedented insight into the mechanics of tRNA translocation [77,81,82]. They show that the

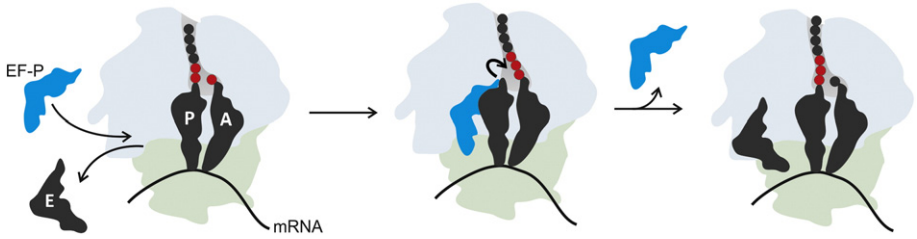

Fig 4. Mechanism of EF-P action on the ribosome. The ribosome stalls after the incorporation of two consecutive Pro residues with Pro or Gly as the next incoming amino acid. The dissociation of the E-site tRNA allows for the binding of EF-P. EF-P binding promotes rapid peptide bond formation by *re*-positioning the peptidyl-Pro-Pro-tRNA in the peptidyl transferase center. Following the dissociation of EF-P, translocation can take place and further translation resume.

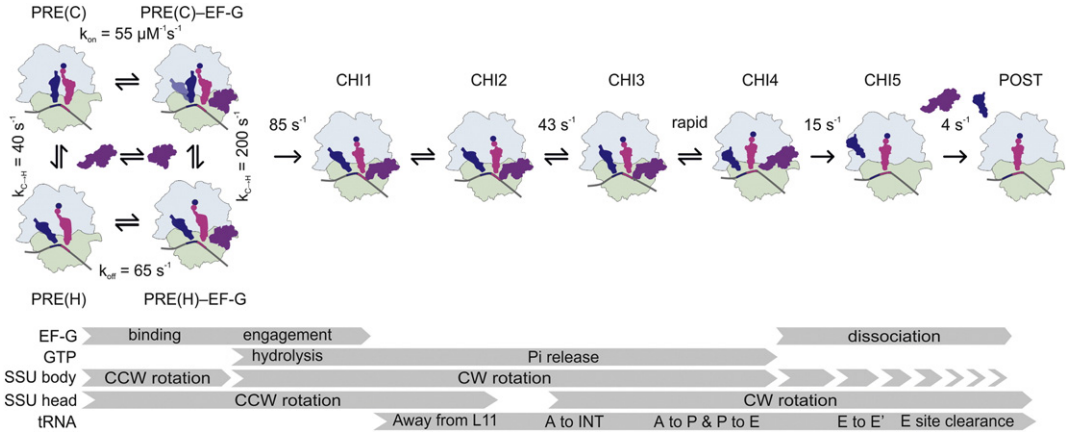

Fig. 5. Mechanism of tRNA–mRNA translocation. PRE(C) and PRE(H) are classical and hybrid/rotated PRE states, respectively. PRE(H) forms spontaneously upon peptide bond formation. EF-G (magenta) in the complex with GTP can sample an extended and compact state. Binding of the compact form to the ribosome avoids the steric clash with the A-site tRNA [68]. Binding of EF-G favors and accelerates the PRE(H) state of the ribosome. GTP hydrolysis and EF-G engagement interrupt fluctuations to the PRE(C) state [91], resulting in a CHI1 state. The A-site tRNA moves away from protein uL11 into a structurally distinct CHI2 state [91]. In the next step, the 3′ end of the A-site peptidyl-tRNA moves toward the P site on the LSU (CHI3) [69,91]; on the SSU, the two tRNAs move into positions between the hybrid A–P and P–E states, respectively [66]. CHI3 can be isolated when GTP hydrolysis is blocked [69]. When there is GTP hydrolysis, the reaction rapidly proceeds toward CHI4 in which the tRNAs have moved into the P and E sites [69]. Pi is released from states CHI3 and CHI4 [93]. In the next step, the E-site tRNA moves away from the P-site peptidyl-tRNA and then dissociates. EF-G is released from the ribosome in a stepwise fashion, first losing the contact with protein bL12, forming CHI5 and dissociating in the following step. The directions of SSU head and body motions are indicated [98]. The rate constants $k_{C\to H}$, describing the rotation of the SSU body relative to the LSU (Sharma et al., unpublished) and the other rate constants [98] were measured at 37 °C. Structurally, the PRE(H)–EF-G or CHI states may be represented by the viomycin-stalled complex [94], CHI3 by the neomycin-stalled ap/ap–pe/E complex [97] and CHI4 by the intermediate blocked by fusidic acid [66].

spontaneous movements of parts of the ribosome are rapid and that the translocation step is rate-limited by the slow movement of the tRNAs [77].

EF-G is a large GTPase that consists of five domains. Recent crystal structures and single-molecule fluorescence resonance energy transfer (smFRET) measurements suggested that EF-G can exist in two grossly different conformations, in a closed GTP conformation (which is presumably favored in the GTP-bound state) and an open, extended conformation that can be found in the structures of EF-G in the GDP-bound and apo forms [68,83] (and, possibly, with GDPNP). The current model (Fig. 5) suggests that the closed form of EF-G is recruited by the ribosome, because docking of the open form of the factor results in a steric clash of domain 4 of EF-G with the peptidyl-tRNAin the SSU A site [68]. It appears that the initial recruitment may occur to the ribosome in either PRE-C or PRE-H state, possibly *via* interactions with the L12 stalk [69,84]. The initial recruitment is followed by a tightening of the complex, presumably due to interactions of the G-domain of EF-G with the LSU [29,30,85–87]; we denote this step as EF-G engagement to distinguish it from the preceding recruitment step which is readily reversible [64,88–90]. At the same time, the rotated state of the ribosome is stabilized in the PRE-H state and the fluctuations toward the PRE-C state are abolished [91] and EF-G hydrolyses GTP [89,92]. The engagement of EF-G alters the conformation of the ribosome in a way that promotes rapid tRNA translocation [93], which then occurs by gradually moving on both SSU and LSU from the PRE to POST states. So far, several of such intermediate states were identified [66,91,94–97]; in the following, we call them chimeric, CHI, states. Structural data [66] and a recent kinetic analysis [98] suggest that early in translocation, the body of the SSU starts to move toward its non-rotated conformation, whereas the SSU head remains in a swiveled state. This motion may open the A site on the SSU and destabilize the binding of the A-site tRNA to the SSU. The 3′ end of the A-site tRNA is further shifted toward the P-site on the LSU [69]. It is not known when EF-G changes its overall conformation from compact to elongated [68]; at the time when the tRNAs move, EF-G is likely to be in the elongated conformation with domain 4 following the movement of the tRNA and blocking its backward rotation. The reverse movements of EF-G itself are blocked by the dissociation of Pi which occurs at the same time as tRNA translocation [93]. After the tRNAs have been displaced to the P and E sites, the E site tRNA moves further through at least one more

intermediate state and then dissociates from the ribosome [99,100]. The head of the SSU now also moves backwards and EF-G dissociates from the ribosome (Fig. 5).

To understand how EF-G works, it is informative to compare the pathways and intermediates in spontaneous and EF-G-catalyzed translocation. The ribosome undergoes similar rotational and swiveling motions, regardless of whether EF-G is present of absent or GTP is hydrolyzed [63,76,82,101,102]. During spontaneous translocation, the motions of the SSU head and body are coupled, albeit loosely [82]. EF-G uncouples the motions of the SSU head and body, which may coincide with EF-G engagement [64,91,95], the inhibition of the backward rotation to the classical states [91] and the unlocking of the tRNA movement [93]. A conformation of the ribosome in which the SSU head is in a highly swiveled state, whereas the body already moves backwards [66,97], which may open the decoding site, allowing the mRNA-bound anticodon arms of the two tRNAs to move. Although various conformations within the PRE or POST complexes rapidly interconvert spontaneously, in the absence of EF-G, the PRE and POST pools are separated by a high activation energy barrier [77,78,82]. EF-G alters the conformational energy landscape by creating a new pool of CHI conformations that readily interconvert and are connected to the exit and entry conformations of the PRE and POST states, respectively [91].

One of the controversial issues is the role of GTP hydrolysis. While everybody agrees that the lack of GTP hydrolysis inhibits EF-G release from the ribosome at the end of translocation, some groups report that in addition the lack of GTP, hydrolysis slows down tRNA displacement by a factor of 10–50 [88,89,92,102], whereas others have seen little or no effect. We observed that the lack of effect on translocation is often due to a minor contamination of GTP in GDPNP, a broadly used non-hydrolyzable analog. Removal of the GTP contamination from GDPNP, using a different non-hydrolyzable nucleotide (e.g., GTPγS), or an EF-G mutant (H84A) that is inactive in GTP hydrolysis restores the effect [91,96,99]. Another observation is that the magnitude of the effect depends on spermine in the buffer: in the presence of spermine, the rate of translocation is severely decreased and the contribution of GTP hydrolysis becomes small, in contrast to conditions that favor rapid translocation. In that context, it should be noted that spermine is not a constituent of *E. coli* cells.

The detailed analysis of the effect of GTP hydrolysis on tRNA displacement suggests that it is particularly important for the formation of a key CHI state in which the head and body of the SSU have moved in opposite directions. This facilitates rapid movement on the SSU and synchronizes of the movements on the SSU and LSU [96,98]. EF-G combines an energy regime that is characteristic for motor proteins, in that it accelerates movement by a conformational change induced by GTP hydrolysis, with that of a switch GTPase, which upon Pi release switches the conformations of EF-G and the ribosome to low affinity, allowing the dissociation of the factor [96,99].

Recoding

While the fidelity of translation is generally high, some mRNAs contain signals which alter the rules for reading the information in the mRNA and lead to recoding. One example of recoding is provided by the selenocysteine (Sec) insertion system, which ensures the incorporation of Sec, the 21st natural proteinogenic amino acid, by reading a UAG stop codon with the help of a specialized tRNA, Sec-tRNA^{Sec}, guided by a downstream stem/loop (SECIS, selenocysteine incorporation sequence) in the mRNA. Sec-tRNA^{Sec} is delivered to the ribosome by a specialized translation factor, SelB, which is related to EF-Tu, but contains one more domain, domain 4, which binds to the SECIS. The efficiency of UGA recoding by the Sec machinery *in vivo* is 30%–40%, independently of the growth rate of the cells [103]. Surprisingly, RF2, the termination factor that usually reads the UGA stop codon, does not compete with the Sec incorporation machinery, probably because early recruitment of Sec-tRNA–SelB–GTP to the mRNA by binding of domain 4 to the SECIS outside the mRNA channel on the SSU blocks the access of RF2 to the stop codon, thereby prioritizing recoding over termination at Sec-dedicated stop codons [103].

SelB–GTP binds Sec-tRNA^{Sec} with extraordinary high affinity, about 0.2 pM, compared to nM to μM affinities of aminoacyl-tRNA binding to EF-Tu–GTP [104]. The tight binding of SelB–GTP to Sec-tRNA^{Sec} is driven enthalpically and involves the net formation of four ion pairs, three of which involve the Sec residue. Accordingly, the dissociation of the tRNA from the ternary complex SelB–GTP–Sec-tRNA^{Sec} is extremely slow, and GTP hydrolysis on the ribosome accelerates the release by more than a million-fold. As estimated by isothermal titration calorimentry, GTP hydrolysis dramatically decreases the number of amino acids buried by the nucleotide from 43 to 15, indicating a rearrangement at the nucleotide binding site [105]. Thermodynamic coupling in binding of Sec-tRNA^{Sec} and GTP to SelB ensures the selectivity for Sec- versus Ser-tRNA^{Sec} (the latter is a precursor on the Sec-tRNA biogenesis pathway in *E. coli*) and rapid release of Sec-tRNA^{Sec} from SelB after GTP cleavage on the ribosome. SelB provides an example for the evolution of a highly specialized protein–RNA complex toward recognition of a unique set of

identity elements. The mode of tRNA recognition by SelB is reminiscent of another specialized factor, eIF2, rather than of EF-Tu, the common delivery factor for all other aminoacyl-tRNAs, in line with a common evolutionary ancestry of SelB and eIF2 [106].

Other exciting examples of translational recoding include frameshifting and bypassing of parts of mRNA [107]. Programmed – 1 ribosomal frameshifting (– 1PRF) is an mRNA recoding event utilized by cells to enhance the information content of the genome and to regulate gene expression, and there are numerous new examples of how –1PRF can be used for the regulation of translation in eukaryotic cells (reviewed in Ref. [108]). Programmed frameshifting usually requires two signals in the mRNA, a slippery sequence of the type X XXY YYZ, which can be decoded by the same pair of tRNAs in the 0- and – 1-frame, and a downstream secondary structure element, such as a stem–loop or a pseudoknot. Additional signals, such as Shine–Dalgarno-like sequences upstream of the slippery site, can increase the efficiency of frameshifting [109].

While there are numerous examples of –1PRF and a wealth of genetic studies *in vivo*, the molecular mechanism has been investigated for two examples, the avian infectious bronchitis virus (IBV) [100] (Fig. 6) and bacterial dnaX [110–112]. We have used the IBV frameshifting sequence as a model system, because it directs efficient frameshifting in organisms from *E. coli* to mammals and can thus be considered a universal example of frameshifting [100]. The original frameshifting sequence of IBV contains a sequence U UUA AAC followed by a downstream pseudoknot [113]. To produce efficient frameshifting in *E. coli*, the original slippery sequence was replaced with U UUA AAG, which is decoded by $tRNA^{Leu}$ (anticodon 3′AAU5′) and $tRNA^{Lys}$ (anticodon 3′UUU5′) U UUA AAG sequence with the codons for Leu-$tRNA^{Leu}_5$ (UUA) and Lys-$tRNA^{Lys}$ (AAG) [114]. Rapid kinetic assays (the “codon walk” that monitors the kinetics of incorporation of each consecutive amino acid into the peptide by quench-flow and is combined with fluorescence measurements) allowed us to determine the exact timing of ribosome

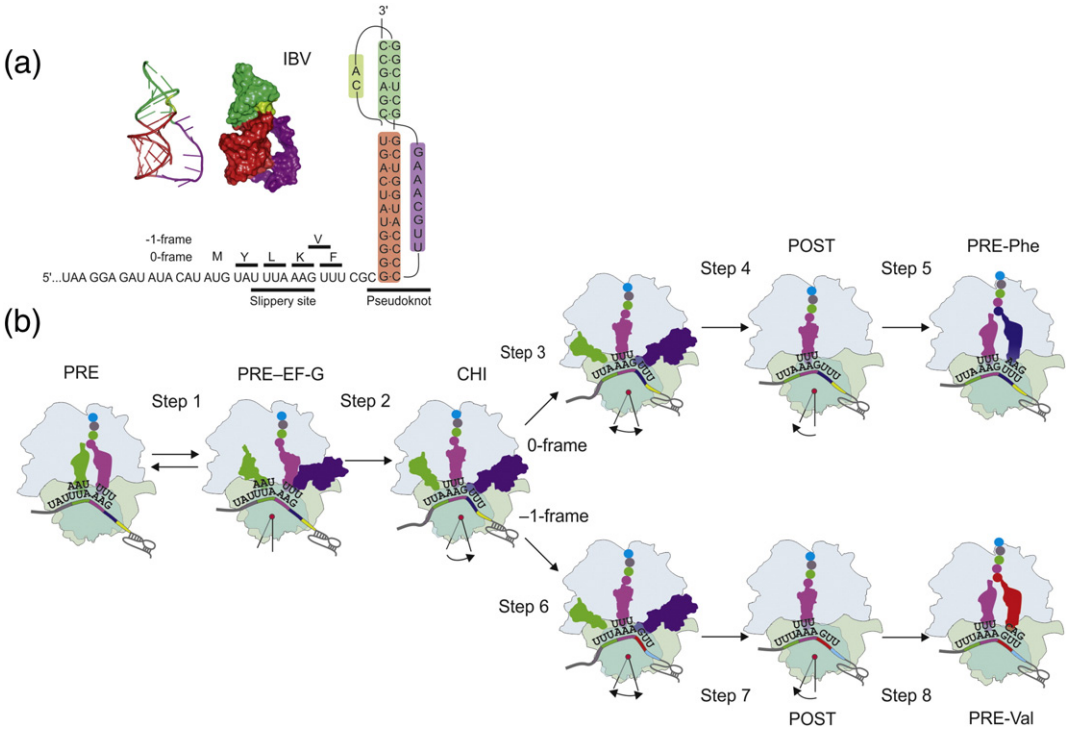

Fig. 6. Kinetic model of –1PRF. (a) Sequence of the IBV frameshifting element and the presumed structure of the IBV pseudoknot (reproduced from Ref. [189] with permission). (b) The slippage occurs during ongoing translocation of the two tRNAs bound to the slippery sequence. Recruitment of EF-G (step 1) to the PRE complex facilitates rapid tRNA movement (step 2) into a chimeric state (CHI); the following steps are slowed down by the pseudoknot. The SSU head attempts to move into the CW direction but is sterically hindered by the pseudoknot (steps 3 and 6). Slippage occurs at this step, resulting in kinetic partitioning between 0-frame (steps 3–5) and – 1-frame (steps 6–8). Steps 3 and 4 are particularly slow for the tRNA that remains in the 0-frame, which limits the rate of the following Phe-$tRNA^{Phe}$ binding (step 5). In contrast, movement on those ribosomes which switched to the – 1-frame is faster (step 6), followed by $tRNA^{Leu}$ dissociation from the SSU, SSU head rotation, dissociation of EF-G (step 7) and binding of Val-$tRNA^{Val}$ (step 8) [100].

slippage. It turned out that frameshifting takes place during tRNA–mRNA translocation when the YYZ codon (AAG in the case of IBV) moves from the A to the P site [100]. The slippage occurs when the SSU head attempts to move backward (see section EF-G-catalyzed tRNA-mRNA translocation), which is apparently prevented by the pseudoknot and results in ribosome stalling at the pseudoknot base (Fig. 6). The slippery sequence allows the ribosome to move backward into the −1 frame. Those ribosomes that changed the frame can resume rapid translation, possibly because −1 slippage places the pseudoknot base at the active helicase site of the ribosome, resulting in more efficient unwinding. The ribosomes that remained in the 0-frame continue translation after a considerable delay [100], possibly because in this case the disruption of the mRNA secondary structure is achieved by spontaneous thermal fluctuations only [115].

For the dnaX system, the mechanism of −1PRF is controversial. Based on smFRET and optical tweezer experiments, Kim et al. [111] and Yan et al. [110] suggest a mechanism for −1PRF that is fundamentally similar to that on IBV [100], although the conditions and approaches these groups have used [110,111] are quite different from those our group has used [100]. The main features are that the slippage occurs during tRNA–mRNA translocation at the second codon of the slippery sequence, which is located at a defined distance from the stem–loop element, and that there is stalling during −1PRF [110,111]. However, another smFRET study of −1PRF on dnaX by Chen et al. [112] suggested a different mechanism which entails slippage on the 1st slippery codon, competition between EF-Tu–GTP–aa-tRNA ternary complexes for binding in the 0- and −1-frame, and a strong dependence on spontaneous structural fluctuations in the pseudoknot. The discrepancy between the two models can in part be explained by differences in the mRNA sequences of the model constructs. Our experiments, which utilized an unaltered sequence of the dnaX frameshifting site, suggested that the major pathway for −1PRF is slippage during translocation at the second slippery codon (Caliskan et al., unpublished), consistent with the model of Kim et al. [111] and our model for −1PRF on IBV sequence [100]. However, we also found a potential for alternative slippage events, which might explain the results of Chen et al. [110]. The results show how the ribosome deals with structural elements of the mRNA, how ribosome dynamics facilitate the movement along the mRNA, and how kinetics define decoding and recoding of genetic information.

The best-studied example for recoding through translational bypassing is the gene product 60 (gp60) of bacteriophage T4. Gp60 is synthesized as a single polypeptide from a discontinuous reading frame as a result of the ribosome bypassing a non-coding mRNA region of 50 nt [116] (Fig. 7). Systematic analysis of the gp60 mRNA suggests unexpected contributions of mRNA sequences upstream and downstream of the non-coding gap region and of interactions of the nascent peptide within the polypeptide exit tunnel of the ribosome [117]. Ribosomes rapidly translate the gp60 mRNA until they reach the take-off Gly codon where they pause in a non-canonical rotated state. During bypassing, ribosomes slide forward on the mRNA track in a processive way. The non-coding gap of the mRNA is most likely unstructured [118]. The forward direction of sliding is maintained by the formation of secondary structures in the mRNA upstream of the take-off site and the non-coding gap [117–119], whereas the processivity (which depends on the retention of the peptidyl-tRNA in the P site) may be ensured by the interactions of the nascent polypeptide chain with the exit tunnel [117]. Close to the landing site, the ribosome scans the mRNA in search of optimal base-pairing interactions with the anticodon of the P-site peptidyl-tRNA^{Gly} [119]. The choice of the cognate landing codon is affected by an mRNA element 3′ of the gap which may hinder further progression of the ribosome and promote tRNA landing (Fig. 7) [117]. Sliding may play a role not only for gp60 synthesis but also in regular mRNA translation, for example, in reading frame selection during initiation, or for tRNA translocation during elongation. Recent evidence suggests that bypassing phenomena may be more common than thought so far; for example, a high frequency of ribosome bypassing was demonstrated for the mitochondria of the yeast *Magnusiomyces capitatus*, and there are other interesting candidates [120].

Co-translational Protein Folding

Folding of many proteins begins when the nascent peptide is still attached to the synthesizing ribosome (for recent reviews, see Refs. [121–129]). The nascent peptide travels through a polypeptide exit tunnel (Fig. 8). The tunnel covers about 30–40 aa of the nascent peptide, assuming an unfolded, fully stretched conformation. The width of the tunnel constrains the folding of the nascent peptide and does not permit formation of extended tertiary structure elements. However, some structures can form within the ribosome, for example, compacted non-native states [130], α-helices [131–133], hairpins [126,134,135] or even a small α-helical domain [136]. These local folding events can start as soon as the N-terminal part of the nascent peptide has been synthesized and proceed in a vectorial fashion. Formation of larger tertiary structure elements, such as domain folding, occurs when the protein emerges from the peptide exit tunnel of the ribosome [130,137–141]. The vectorial folding and the interactions of

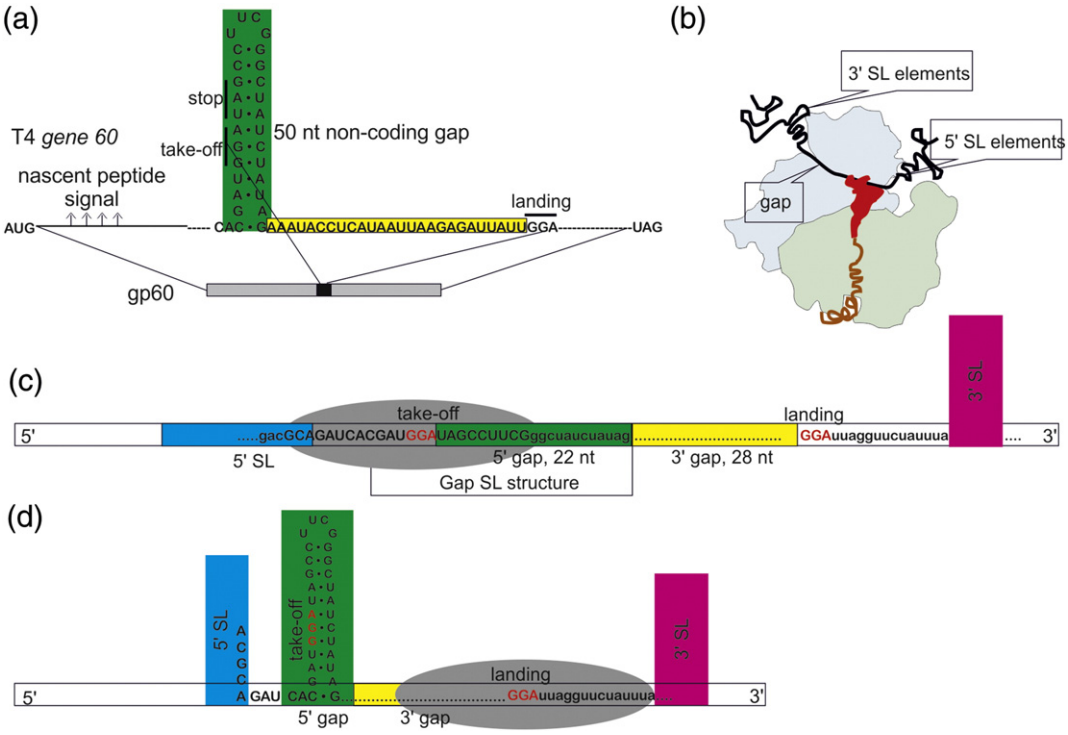

Fig. 7. Ribosome bypassing on gp60 mRNA. (a) Schematic of the gp60 mRNA and the corresponding protein (gray). Depicted are mRNA elements that promote bypassing, for example, the sequence that codes for the nascent peptide, the take-off helix (green), take-off and landing codons and the stop-codon following the take-off site. (b) Other putative secondary structure elements of the mRNA, for example, stem–loop upstream (5′SL) and downstream (3′SL) of the gap region. The bypassing peptidyl-$tRNA_2^{Gly}$ is depicted in red. (c) The position of the ribosome (gray oval) at the onset of bypassing. 5′SL and 3′SL structures are shown in blue and red, respectively, and the 5′ and 3′ parts of the gap sequence are green and yellow, respectively. A large part of the 5′SL and the SL part of the gap are unfolded (drawn horizontally) and reside in the mRNA channel of the ribosome. The 3′-part of the gap region is predominantly unfolded as well (horizontal), whereas the 3′SL element is structured (drawn vertically). (d) Upon movement of the ribosome onto the landing site, the 5′ SL upstream of the gap and the 5′SL in the gap refold (vertical bar), whereas the ribosome covers most of the unstructured 3′ part of the gap up to the presumably structured 3′SL (vertical bar) (modified from [117]).

nascent chains with the ribosome change the folding landscape, resulting in the formation of length-dependent folding intermediates that may not form during protein folding in solution [130,139,142,143].

The ribosome may either induce an alternative folding pathway or stabilize intermediates that are only transiently formed in solution. Biophysical characterization of protein folding during ongoing translation indicated that nascent polypeptides may attain a compact, non-native structure within the exit tunnel [130]. This finding is in line with the observation of a “collapsed” non-native intermediate

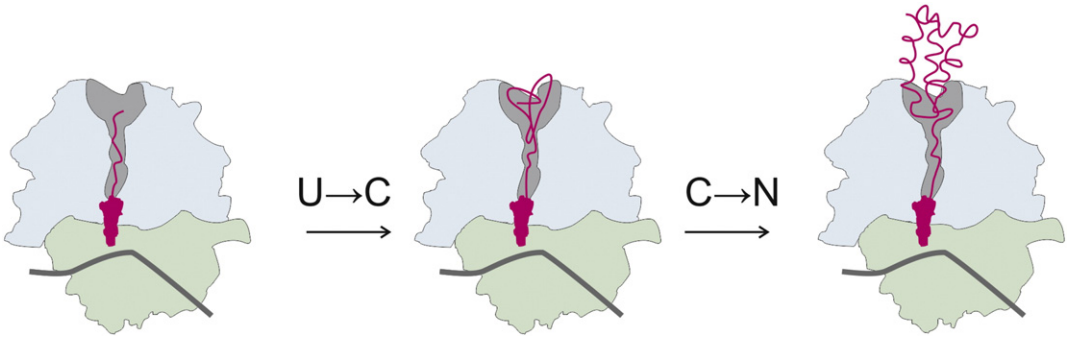

Fig. 8. Schematic of co-translational folding of a small α-helical domain. Step 1 (unfolded to compact), formation of a compact folding state within the ribosome exit tunnel when about 50 aa have been incorporated. Step 2 (compact to native), folding into the native-like structure upon emergence of the entire domain from the peptide exit tunnel.

identified by pulse-chase experiments in intact cells that rearranged into the native conformation late in the folding process [144]. Similarly, the ribosome can slow down the formation of stable tertiary interactions in a protein that has fully emerged from the exit tunnel [143]. Retention of compact or intermediate states may represent a fundamental feature of co-translational folding acting to prevent the chain from falling into kinetic traps, such as stably misfolded non-native conformations that may form when only a part of a protein has been synthesized. The final, stable tertiary structure forms only when all elements of the peptide that are required for folding are leaving the exit tunnel of the ribosome [5,64,70–72]. Similarly, profiling experiments showed that in mammalian cells, protein domains acquire their native state shortly after the emergence of the entire domain from the exit tunnel [138]. The transition to a stable domain arrangement may proceed in a concerted way or by a series of adjustments that gradually bring all elements of the protein into their native orientations [145].

The appearance of the compact state and its maturation to the native structure are limited by the rate of translation. A slow pace of translation would allow for the equilibration of different folding micro-states that are accessible on the ribosome, thereby shaping the thermodynamically favorable folding pathway inside the ribosome [146]. Thus, co-translational folding of intrinsically rapidly-folding domains appears to exhibit equilibrium-like properties [147] with a restricted landscape of accessible conformations. Changes in translational velocity—for example, due to ribosome pausing—may alter the conformational space of the nascent polypeptide and affect folding [140,146,148–153]. In fact, synonymous codon substitutions can lead to folding defects and changes in protein function [86,154,155]. Analysis of synonymous codon usage across transcriptomes revealed systematic biases toward more slowly translating codons found at the boundaries of α-helical and β-strand structural motifs [123,156,157]. Recent computational work suggests how relative rates of translation and local folding events can either direct the protein toward its native structure or cause misfolding [158–160]. Thus, synonymous codon usage serves as a secondary code that guides *in vivo* protein folding and constitutes an additional source of conformational variability of proteins [140].

Interactions of Nascent Peptides with Protein Biogenesis Factors on the Ribosome

Depending on sequence, folding properties and final destination of newly synthesized proteins in the cell, nascent peptides emerging from the ribosome interact with a number of ribosome-associated protein biogenesis factors (RPBs). In bacteria these include, among others, the chaperone trigger factor (TF), which prevents misfolding of the nascent peptide, the signal recognition particle (SRP), which promotes co-translational targeting of ribosome-nascent-chain complexes (RNCs) to the membrane, and the enzymes peptide deformylase (PDF) and methionine aminopeptidase (MAP) that modify the N terminus of the nascent peptide chain by deformylation and subsequent cleavage of methionine. All four RPBs bind to the ribosome at or near the peptide exit where the nascent peptide emerges (Fig. 9). In this section, we address the interplay of RPBs on translating ribosomes, focusing on TF and SRP.

TF is the first chaperone that binds to nascent peptide chains emerging from the ribosome, preventing misfolding of proteins during synthesis [161]. The sequence specificity of TF is low, although there is a preference for nascent peptides encompassing hydrophobic stretches flanked by positive charges [162]; RNCs presenting those motives bind TF with high affinity (e.g., K_d = 2.5 nM for a RNC carrying 75 N-terminal amino acids of proOmpA) [163]. TF binding to RNCs exposing nascent peptides lacking TF-specific sequences is of much lower affinity (K_d values around 0.1 μM, as for TF binding to non-translating ribosomes), although—given its high concentration in the cell (50 μM)—TF can be bound to those RNCs as well. Qualitatively similar results were reported for a number of different RNCs [164,165].

TF binding to RNCs takes place in a single, diffusion-controlled step, with k_{on} ~ 200 μM^{-1} s^{-1} for RNCs presenting TF-specific nascent peptides and ~100 μM^{-1} s^{-1} for the others [163]. The large affinity differences are mainly reflected in the dissociation rate constants, k_{off}, which are up to 30-fold

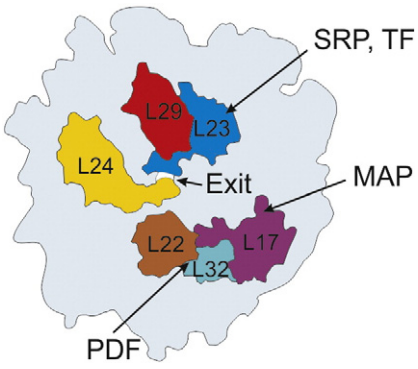

Fig. 9. Binding platform for RPBs at the peptide exit of the bacterial LSU. Ribosomal proteins at or near the peptide exit of the LSU (gray outline) are indicated along with the RPBs binding to protein L23 (SRP, TF), L22 (PDF) or L17 (MAP). Figure adopted from Ref. [163].

lower for TF-specific compared to non-specific RNCs (12 s^{-1} compared to 0.4 s^{-1}). Thus, the kinetic analysis indicates that, rather than staying bound to translating ribosomes unspecifically and "waiting" for the nascent peptide to emerge from the ribosome, as suggested by previously reported slow kinetics, the interaction of TF with ribosomes is highly dynamic. An off-rate around 12 s^{-1} (measured at 25 °C) of TF complexes with non-specific RNCs matches an average translation rate of 10 s^{-1} (at 37 °C) and, combined with an effective on-rate of about 1000 s^{-1} (assuming 10 μM free TF monomer in the monomer-dimer equilibrium [166]), allows TF to inspect many translating ribosomes at appropriate speed and settle on TF-specific sequences as soon as they emerge from the ribosome. This is probably important for the chaperone function of TF in preventing premature, potentially erroneous folding of nascent proteins without interfering with the function of other RPBs. The equilibrium binding data obtained *in vitro* show that TF binds strongly to RNCs presenting TF-specific sequences of chain lengths of 75 aa of which about 40 are exposed outside the peptide exit tunnel [163].

The situation seems somewhat different *in vivo*, as data obtained by selective ribosome profiling indicate a preference of TF binding to RNCs carrying nascent peptides of about 100 aa or longer [167]. While this observation may indicate a higher selectivity of TF at *in vivo* conditions, it is also possible that the difference results from a preferential loss of short-chain RNC–TF complexes during cell opening, crosslinking or complex isolation.

TF and SRP can be bound to one ribosome at the same time, although changes of crosslink patterns indicate that concurrent binding leads to changes of the arrangements of either factor on the ribosome [168,169]. Equilibrium titration experiments revealed that these rearrangements are accompanied by an about 10-fold increase of the apparent K_d of the complexes of TF or SRP with non-translating ribosomes or RNCs exposing nascent peptides containing sequences specific for the respective factor [163], that is, TF and SRP bind to the ribosome in an *anti*-cooperative fashion (Fig. 10). As a consequence, owing to its high concentration in the cell, TF in effect prevents SRP binding to the majority of ribosomes, except those presenting SRP-specific signal sequences, explaining how the small amount of SRP in the cell can be effective in targeting SRP-specific RNCs to the membrane. Similar results have been presented recently [170].

The modifying enzymes PDF and MAP do not compete with TF or SRP for binding to translating ribosomes [163], indicating that nascent-chain processing can take place before or in parallel with TF or SRP binding. On the other hand, PDF and MAP appear to compete for binding to translating ribosomes, indicating that their binding sites overlap and that MAP can bind and cleave off the N-terminal methionine only after deformylation and dissociation of PDF [171].

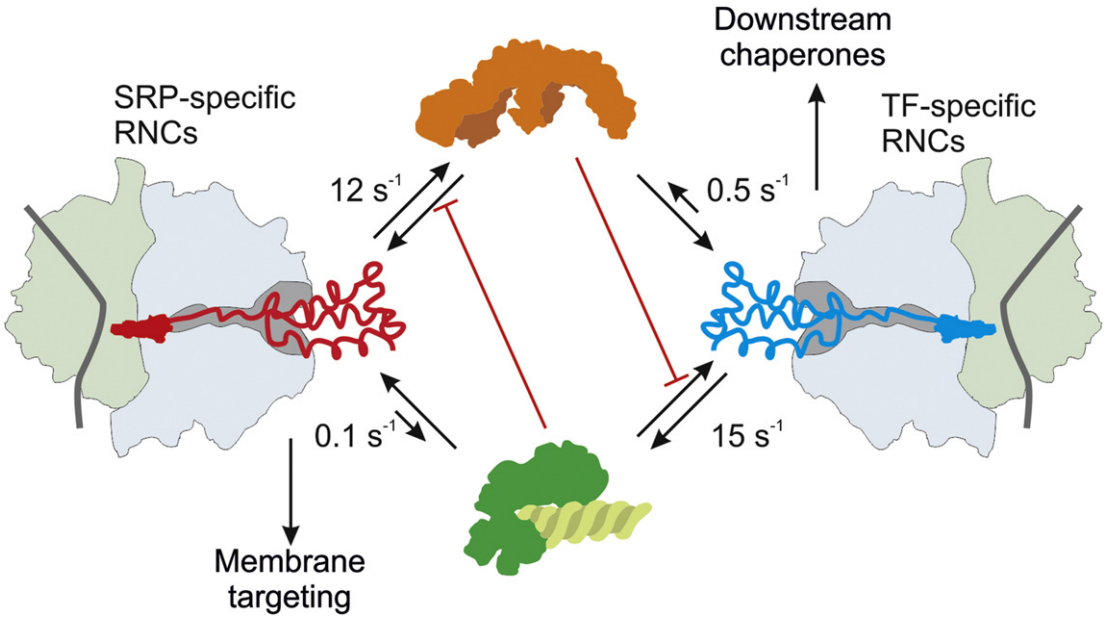

Fig. 10. Interplay of TF and SRP on translating ribosomes. Anti-cooperative binding of TF (orange) or SRP (green) to RNCs presenting the respective specific nascent chain (SRP-specific SAS, red; TF-specific sequence, blue) leads to about 10-fold weakening of the binding of the respective other ligand, as indicated by red lines. Dissociation rate constants for SRP and TF [163,180] are taken from the references. Figure modified from Ref. [163].

Co-translational Membrane Targeting of Ribosomes Synthesizing Membrane Proteins

About one quarter of the bacterial proteome consists of proteins that are integrated into the plasma membrane. To avoid misfolding and aggregation due to exposed hydrophobic patches, membrane proteins are inserted into the membrane co-translationally by way of a protein conducting channel (translocon) located in the membrane. Ribosomes synthesizing membrane proteins are targeted to the translocon by the SRP pathway (for a recent review, see Ref. [172]). *E. coli* SRP consists of an RNA, 4.5S RNA, and one protein, Ffh, which is composed of two domains, the NG domain and the M domain. The NG domain comprises the GTP-binding G domain, and the M domain, which forms a strong complex with 4.5S RNA and binds to RNCs exposing signal-anchor sequences (SAS) of nascent membrane proteins to form a high-affinity complex [173]. RNC binding is accompanied by a conformational change of SRP, which assumes an open structure in which the NG domain is exposed, as deduced from FRET and equilibrium binding studies [174] and a low-resolution cryo-EM structure [175]. Molecular details of the interaction of SRP with RNCs are provided by a recent high-resolution cryo-EM structure [176].

The rearrangement of SRP enhances the binding of SRP with the SRP receptor, FtsY, which involves the GTP-dependent interaction of the homologous NG domains of the two proteins, forming a pseudo-symmetric complex [177,178]. A high-affinity SRP–FtsY complex is also formed on RNCs that carry a short nascent chain of about 30 amino acids in the peptide exit tunnel [173]. Signaling the presence of the short nascent chain within the tunnel to the SRP binding site involves ribosomal protein uL23 [173] which is part of the binding site of SRP [179] and reaches into the peptide exit tunnel by a β-hairpin. The high-resolution cryo-EM structure suggests that a loop of the M domain of SRP protein Ffh reaches into the tunnel as well and binds to the nascent peptide [176]. SRP recruitment to short-chain RNCs that do not expose a signal sequence leads to a complex of low kinetic stability, compared to the complex with exposed signal sequence [180]. Low complex stability may be one reason why early SRP recruitment to short-chain RNCs was not observed in smFRET experiments conducted in a co-translational fashion [181], due to limited time resolution of the smFRET technique; another could be a different behavior of stalled and actively translating RNCs, as has been proposed [181].

The rapid kinetic analysis, monitoring FRET, shows that the high affinity of SRP binding to RNCs exposing an SAS is mainly due to a stabilization of the complex by rearrangements following the formation of the encounter complex, that is, by lowering the dissociation rate constants [180]. The stabilization of the RNC–SRP complex is accompanied by the acceleration of a rearrangement following the formation of the encounter complex between RNC–SRP and FtsY [180], thereby increasing the affinity of FtsY binding to SRP.

The protein-conducting pore in the membrane, the translocon, ensures the sequential membrane insertion of trans-membrane (TM) segments together with the necessary topological arrangements of TM segments and the connecting intra- or extracellular loops. The bacterial translocon consists of a ternary complex of proteins SecY, SecE and SecG (SecYEG). The crystal structure of the homologous archaeal SecYEβ [182] and crystal structures of bacterial SecYEG [183–185] show the translocon as a pseudosymmetrical structure with TM segments 1–5 and 6–10 of SecY forming a central pore that in the resting state is closed toward the periplasm by a small plug helix. The pore can open in two ways [186]. For protein translocation through the translocon into the periplasm, the plug domain is moved to the side. Alternatively, to allow for TM segments of membrane proteins to enter the lipid phase by laterally exiting the translocon, the two halves of the translocon move apart and open laterally. Once the lateral gate is opened, TM segments of nascent peptides can partition between the hydrophilic inner pore of the translocon and the hydrophobic lipid phase according to their hydrophobicity [187].

Opening of the lateral gate could be induced by ribosome binding to the cytosolic part of SecYEG or to the insertion into the translocon pore of a hydrophobic TM segment, or both. We have examined lateral gating of the translocon by using *E. coli* SecYEG integrated into nanodisks and employing a fluorescence approach, that is photo-induced electron transfer (PET) between two groups inserted into SecY helices forming the rim of the lateral gate of the translocon [188] (Fig. 11). We monitored the extent of PET in complexes of the double-labeled translocon with either vacant *E. coli* ribosomes or RNCs exposing nascent peptide chains of varying hydrophobicity.

The results suggest that ribosome binding alone causes partial opening of the lateral gate. The effect is reinforced to full opening with translating ribosomes exposing hydrophobic TM segments, suggesting that ribosome binding and hydrophobic peptide interactions within the translocon pore both contribute to lateral gate opening (Fig. 12). On the other hand, a peptide encompassing a TM segment added *in trans* together with non-translating ribosomes did not effect further gate opening, indicating that the RNC context is required to elicit the fully open conformation. We also observed that gate opening strongly depends on the temperature, in that

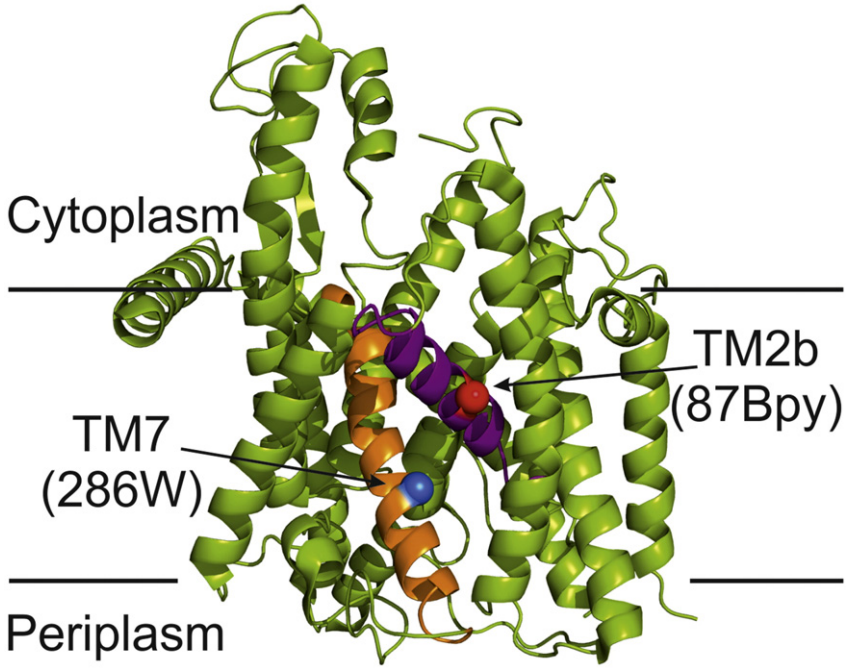

Fig. 11. Labels at the lateral gate of the translocon used for PET measurements. In the crystal structure of SecYEβ from *Methanocaldococcus jannaschii* [182], TM segments 2b (purple) and 7 (orange) with positions 87 and 286 for BODIPY (Bpy) labeling (red sphere) and insertion of Trp as a PET quencher (blue sphere) are indicated. The remaining parts of SecYEβ are colored green. Figure reprinted from Ref. [188].

the closed conformation of the translocon is strongly favored at low temperature, even in the presence of ribosomes or RNCs [188]. The translocon appears as a dynamic structure whose conformation changes between closed and open lateral gate and is modulated by interactions with various ligands, including ribosomes, SecA and hydrophobic TM segments of nascent peptides exposed on translating ribosomes. Many outstanding questions remain, among them the dynamics of the translocon and the ribosome–translocon interface during synthesis and membrane insertion of polytopic membrane proteins, including the topological arrangement of multiple TM segments and their connecting loops.

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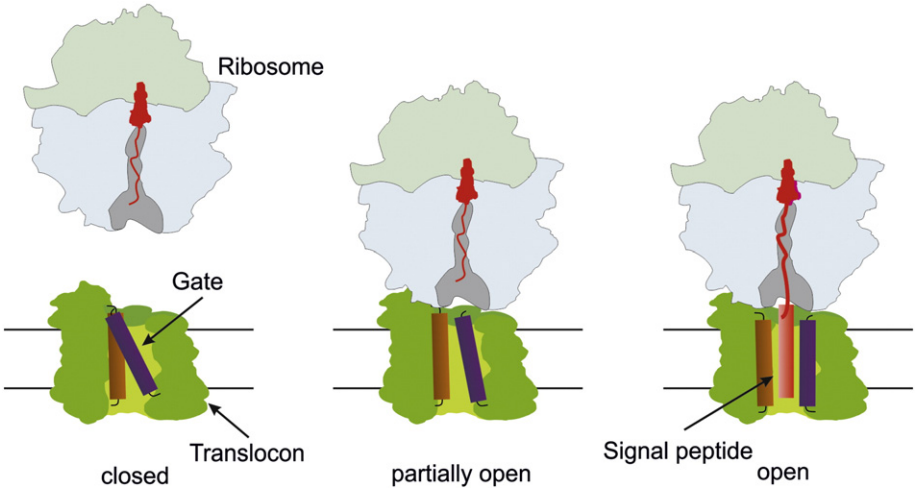

Fig. 12. Lateral opening of the translocon by ribosome binding and hydrophobic TM segment insertion. Figure modified from Ref. [188].

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Abbreviations used:
EF, elongation factor; aa-tRNA, aminoacyl-tRNA; rRNA, ribosomal RNA; SRL, sarcin-ricin loop; SSU, small subunit of the ribosome; LSU, large subunit of the ribosome; TS, transition state; cryo-EM, cryo-electron microscopy; PRF, programmed ribosome frameshifting; RPB, ribosome-associated protein biogenesis factor; TF, trigger factor; SRP, signal recognition particle; PDF, peptide deformylase; MAP, methionine aminopeptidase; RNC, ribosome-nascent-chain complex; SAS, signal-anchor sequence; TM, trans-membrane; FRET, fluorescence resonance energy transfer; smFRET, single-molecule FRET; PET, photo-induced electron transfer.

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