Structural Basis of the Translational Elongation Cycle*

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Abstract

The sequential addition of amino acids to a growing polypeptide chain is carried out by the ribosome in a complicated multistep process called the elongation cycle. It involves accurate selection of each aminoacyl tRNA as dictated by the mRNA codon, catalysis of peptide bond formation, and movement of the tRNAs and mRNA through the ribosome. The process requires the GTPase factors elongation factor Tu (EF-Tu) and EF-G. Not surprisingly, large conformational changes in both the ribosome and its tRNA substrates occur throughout protein elongation. Major advances in our understanding of the elongation cycle have been made in the past few years as a result of high-resolution crystal structures that capture various states of the process, as well as biochemical and computational studies.

Contents

50S subunit: the large subunit of the ribosome; binds the 3′ ends of the tRNA and catalyzes peptide bond formation

INTRODUCTION

In all living organisms, protein synthesis is carried out by the large macromolecular machine known as the ribosome. Both bacterial and eukaryotic ribosomes, 2.5 and 4 MDa in size, respectively, are composed of approximately two-thirds RNA and one-third protein (1). All ribosomes contain two subunits: a large subunit, known as the 50S in bacteria (60S in eukaryotes), which contains the active site of the enzyme, and a small subunit, known as the 30S (40S in eukaryotes), which is responsible for ensuring fidelity. These subunits can reversibly associate into the complete 70S or 80S ribosomes. Protein synthesis occurs through binding of transfer RNAs (tRNAs) and their associated amino acids to the ribosome in an order determined by a messenger RNA (mRNA) template. During this process, known as translation, tRNAs move sequentially through three ribosomal binding sites: the aminoacyl (A), peptidyl (P), and exit (E) sites.

Translation can be divided into roughly four stages: initiation, elongation, release, and recycling (for a recent review, see Reference 2). During initiation, the ribosome is positioned over the mRNA start codon, which is recognized by a unique initiator tRNA. Initiation is followed by elongation, which involves the sequential addition of amino acids to the growing polypeptide chain. When the ribosome reaches a stop codon, protein synthesis is terminated by a release factor (RF) that recognizes the mRNA stop codon and catalyzes hydrolysis of the polypeptide chain from the peptidyl tRNA. Finally, in recycling, the ribosome recycling factor (RRF) and elongation factor G (EF-G) dissociate the ribosome into its subunits, preparing for a new round of protein synthesis. Thus, the actual linking of amino acids into proteins occurs during the elongation cycle, which accordingly lies at the heart of translation, and is the subject of this review. The elongation cycle is highly conserved, unlike initiation and termination, which differ significantly between bacteria and eukaryotes. Therefore, although this review focuses on insights derived from studies on the bacterial ribosome, the majority of these observations will hold true across all kingdoms of life.

An overview of the elongation cycle is shown in **Figure 1** (adapted from Reference 2).

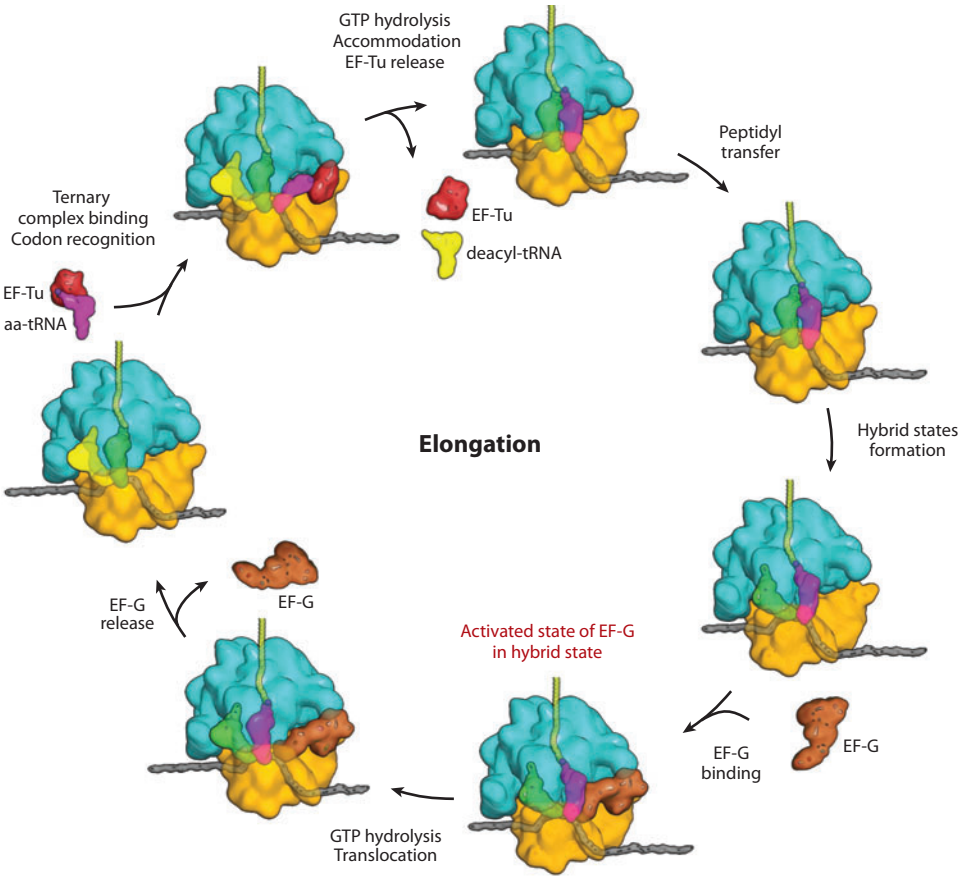


Figure 1

Schematic of the bacterial elongation cycle. At the heart of protein synthesis is the elongation cycle, which involves the sequential addition of amino acids to the growing peptide chain, facilitated by the GTPase factors elongation factor G (EF-G) and elongation factor Tu (EF-Tu). Due in part to its high degree of conservation, the bacterial elongation cycle has been studied extensively both structurally and biochemically. A high-resolution crystal structure has been determined for each state depicted here with the exception of the ribosome with hybrid transfer RNA (tRNA) states before and (labeled in *red*) just after EF-G is bound. It should also be noted that the precise timing of exit-site tRNA (E-site tRNA) dissociation from the ribosome is not well defined and could therefore occur either immediately after translocation or upon binding and/or accommodation of aminoacyl tRNA. Its depiction here should therefore not be considered definitive.

Following initiation, the ribosomal P site is occupied by fMet-tRNA^{fMet}, while the A site remains empty. Aminoacyl tRNAs are delivered to the A site of the ribosome by the GTPase elongation factor Tu (EF-Tu). At this step, known as decoding, the appropriate or cognate tRNA must be selected from the pool of cellular tRNAs. The binding of a ternary complex with a cognate tRNA to the ribosome triggers the hydrolysis of GTP by EF-Tu and the dissociation of the factor. In the absence of EF-Tu, the aminoacyl end of the tRNA swings into the peptidyl transferase center in a process termed accommodation. Accommodation leads to rapid peptide bond formation, which transfers the protein chain from the P- to the A-site tRNA and results in addition of the new amino acid to the growing peptide chain. After peptidyl transfer, the tRNAs must shift from the A and P sites to the P and E sites,

30S subunit: the small subunit of the ribosome; binds mRNA and the anticodons of tRNA

A site: the tRNA and mRNA binding site in the ribosome that binds the new aminoacyl tRNA (aminoacyl or A)

respectively, and the mRNA must advance by one codon. This process, termed translocation, occurs in two main steps, the first involving the movement of the tRNAs with respect to the 50S subunit and the second, catalyzed by EF-G, involving the movement of the mRNA and the anticodon ends of the tRNA relative to the 30S subunit. Translocation brings a new mRNA codon into the A site and prepares the ribosome for another round of the elongation cycle.

A major breakthrough in our understanding of bacterial translation was made possible by several high-resolution structures, initially of the ribosomal subunits and subsequently of the entire ribosome trapped in various functional states (2). Analysis of these structures has been greatly enhanced by concurrent biochemical and genetic studies and by lower-resolution structures of the ribosome determined by cryoelectron microscopy (cryo-EM), which together have greatly improved our understanding of the various steps of the elongation cycle including decoding, peptidyl transfer, and translocation. More recently the structures of the 40S and 60S eukaryotic ribosomal subunits (3, 4) and the entire 80S ribosome (5) have revealed differences between the bacterial and much larger eukaryotic ribosomes. However, given the high conservation of the ribosomal core, the details of the elongation cycle are virtually unchanged across the kingdoms. In this review, we summarize our current understanding of the various steps of this process.

P site: the tRNA and mRNA binding site in the ribosome that binds the tRNA attached to the growing nascent peptide chain (peptidyl or P)

E site: the tRNA and mRNA binding site in the ribosome that holds the deacylated tRNA just prior to ejection from the ribosome (exit or E)

Decoding: the process of selecting a new aminoacyl tRNA in the A site of the ribosome based on the mRNA codon

Cognate tRNAs: tRNAs whose anticodons base-pair with the mRNA codon in the A site of the ribosome, as specified by the genetic code

Peptidyl transferase center: the pocket in the 50S subunit that catalyzes peptide bond formation between the nascent polypeptide chain and the new amino acid

DECODING

During decoding, the ribosome selects tRNA based on the ability of its anticodon to base-pair with the mRNA codon. This selection occurs rapidly, with a rate of up to ~20 amino acids s^{-1} (6), and requires the GTPase EF-Tu, which delivers aminoacyl tRNAs to the ribosome as part of a ternary complex along with GTP. For accurate tRNA selection, binding of a cognate, but not a noncognate, tRNA to the ribosome must trigger GTP hydrolysis by EF-Tu, which requires communication between the decoding center of the 30S subunit and EF-Tu, which is more than 80 Å away. This complex process can be broken down into a series of necessary steps (7) including (*a*) rapid and reversible binding and recruitment of ternary complexes to the decoding center; (*b*) stabilization of cognate tRNA, but not near-cognate or noncognate tRNA, on the ribosome; (*c*) communication of cognate-tRNA binding to EF-Tu to (*d*) trigger GTP hydrolysis; and (*e*) dissociation of EF-Tu from the ribosome so that the tRNA is free to enter the peptidyl transferase center. These steps, which require active contributions from the tRNA, ribosome, and EF-Tu, function in concert to ensure fidelity in tRNA selection and thereby accurate protein synthesis on the ribosome.

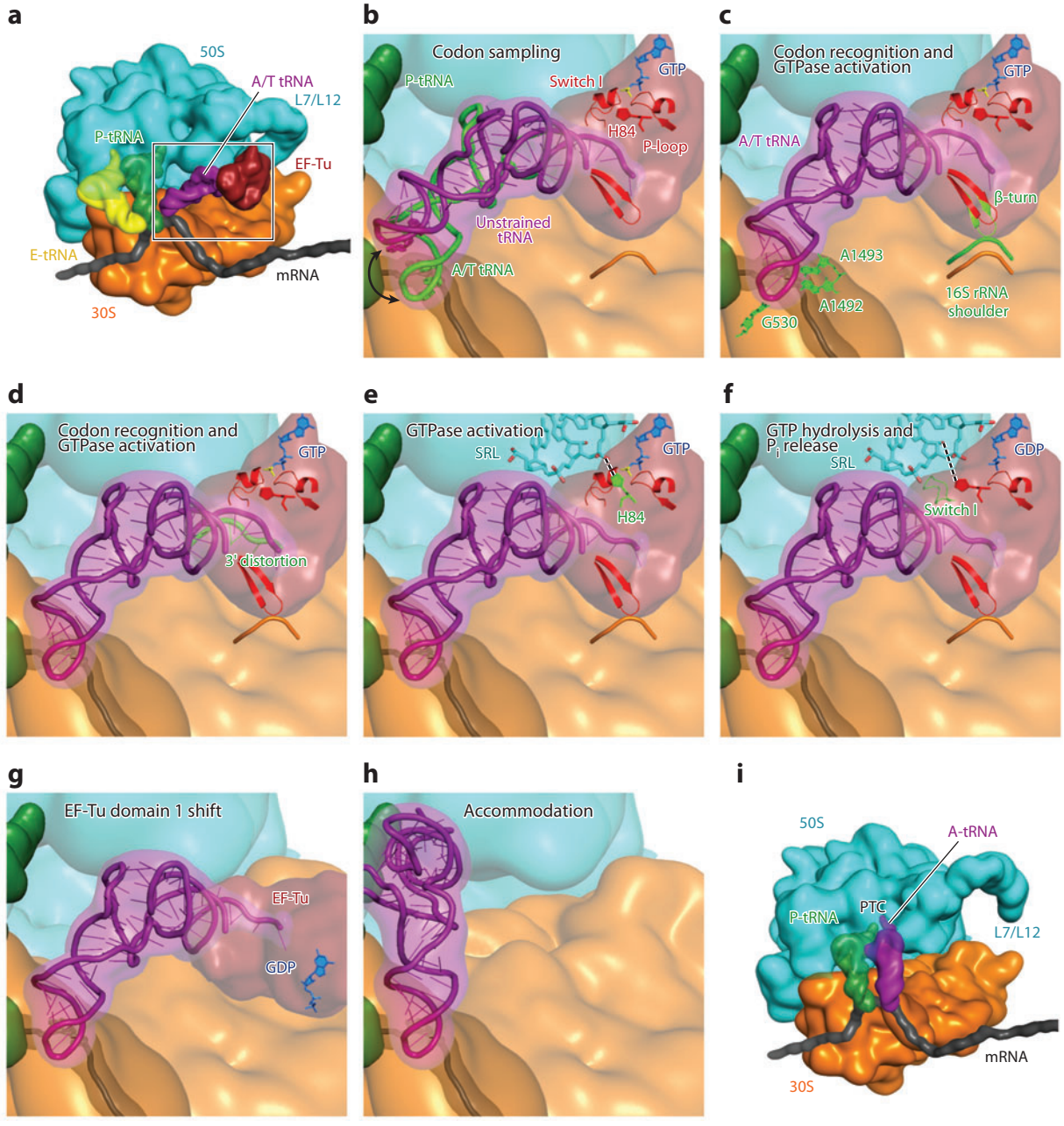
Initial Binding and mRNA Sampling

Initial binding of the ternary complex is rapid and mRNA independent and is assisted by

Figure 2

Schematic representation of the decoding pathway. (*a*) The L7/L12 stalk recruits the ternary complex to the ribosome. Deacylated transfer RNA (tRNA) may be bound in the exit (E) site (*yellow*) and peptidyl tRNA is in the peptidyl (P) site (*green*). The black rectangle represents the enlarged area in panels *b–h*. (*b*) The tRNA (*purple*) samples codon:anticodon pairing until a match (*c*) is sensed, by decoding center nucleotides G530 and A1492-A1493. Codon recognition triggers domain closure of the 30S subunit, bringing the shoulder domain into contact with elongation factor Tu (EF-Tu) (*red*), and shifting regions in domain 2 of the GTPase. (*d*) This results in a distortion of the acceptor arm of the aminoacyl tRNA. These conformational changes are all critical for properly positioning EF-Tu on the ribosome to allow GTPase activation. (*e*) GTPase activation does not require a large opening of the hydrophobic gate. Instead, residue A2662 of the sarcin-ricin loop (SRL) of the 23S ribosomal RNA (rRNA) positions His84 into the GTPase center, resulting in rapid GTP hydrolysis. (*f*) Release of P_i results in the disordering of the switch I loop and (*g*) a domain rearrangement of EF-Tu. (*h,i*) This leads to dissociation of EF-Tu from the ribosome, accommodation of aminoacyl tRNA, and peptidyl transfer. Abbreviations: A, aminoacyl; PTC, peptidyl transferase center.

interaction with the multimeric protein L7/L12 (**Figure 2*a***) (8, 9). Given that the rate of association of the ternary complex with the ribosome is faster than would be expected for a purely random interaction, one molecule of EF-Tu is predicted to interact with each of the flexible C-terminal domains of L7/L12 (four in *Escherichia coli*), thereby increasing its effective biological concentration (9, 10). Mutational studies suggest that L7/L12 interacts with helix D of EF-Tu in a manner similar to EF-Ts (11, 12).



Accommodation: the movement (during decoding) of the aminoacyl end of tRNA into the peptidyl transferase center after it has been released by EF-Tu

Translocation: the movement of tRNAs from the A and P sites to the P and E sites, respectively, while the mRNA advances by one codon

Noncognate tRNAs: tRNAs with anticodons that do not match the mRNA codon and thus are not accepted by the ribosome at a readily measurable frequency

Near-cognate tRNAs: certain tRNAs that are sufficiently similar to cognate tRNA (generally with a single base difference) to be accepted at a low frequency

Interaction with L7/L12 delivers the ternary complex to the A site of the ribosome. From here, the tRNA is positioned to sample the codon-anticodon interaction (**Figure 2*b***) (13). This is consistent with kinetic data in which an intermediate fluorescence change is observed for a fluorophore at residues 16 and 17 of the aminoacyl tRNA during sampling (14).

Codon Recognition

Sampling of the tRNA-mRNA interaction continues until a match is detected (**Figure 2*c***). This brings us to the heart of decoding, which relies on the complementary base-pairing between the tRNA anticodon and mRNA codon and is the single essential step of translation that connects the genetic code with the amino acid to be added to the polypeptide chain. However, the differences in base-pairing free energy for cognate versus near-cognate tRNA are too small to alone account for the observed accuracy of translation (reviewed in Reference 15).

Structural studies on the 30S subunit showed that codon recognition induces a conformational change in the universally conserved ribosomal RNA (rRNA) residues A1492, A1493, and G530 such that they interact with the minor groove of the codon-anticodon helix at the first and second but not the third positions (16). The tertiary interactions made by A1492 and A1493, termed A-minor motifs, are commonly found elsewhere in the ribosome as well as more generally in RNA structure, including the group I intron (17, 18). The interactions of these three residues depend on the Watson-Crick geometry of the codon-anticodon base pairs at the first two positions but allow wobble pairs (i.e., G•U) at the third position (16). This observation provides a structural explanation for how the ribosome accommodates the degeneracy of the genetic code and is consistent with the long-standing wobble hypothesis (19). Direct recognition of base-pairing geometry is also utilized by DNA and RNA polymerases (reviewed in Reference 20), suggesting a common mechanism of ensuring Watson-Crick complementarity.

Thermodynamic measurements show that the free energy difference of interaction of these residues with cognate compared with noncognate tRNA would more than account for the accuracy of translation (21, 22). However, this significant additional binding energy is only partially used to increase the relative affinity of cognate tRNA; it is mainly used to induce conformational changes in the ribosome and the ternary complex, as predicted by kinetic studies (7). These changes include a large-scale domain closure in the 30S subunit (22) and provide a structural explanation for the observed increase in rate of GTPase activation for cognate compared with noncognate tRNA (7, 23). The idea that the excess binding energy from recognition of cognate base pairs at the minor groove is used to induce conformational changes essential for GTPase activation can explain a large body of genetic and biochemical data. For example, the 30S domain closure involves disruption of the S4-S5 interface and additional contacts involving S12. Mutations that make the domain closure more difficult, such as those in S12, result in a hyperaccurate phenotype, whereas those that make it easier, such as mutations that disrupt the S4-S5 interface, result in an error-prone phenotype (15, 22).

However, some recent experiments are not easy to rationalize based on a simple 30S domain closure model. For example, some mutations at the S4-S5 interface produce hyperaccurate rather than the expected error-prone phenotypes (24). Moreover, mutations in S4 that affect S4-S5 binding in a two-hybrid assay do not correlate with fidelity (25). Mutations in 16S rRNA helices 8 and 14 (part of the ribosomal shoulder that moves toward EF-Tu) show that disruption of this interface results in a loss of fidelity, possibly by making an inward rotation of the 30S shoulder easier (26). The streptomycin resistance of three S12 mutations can be abolished by a mutation in EF-Tu more than 50 Å away, suggesting a complex interplay between different parts of the ribosome to facilitate the active form (27). Finally, some *ram*

mutations affect GTPase activation rates as predicted, but hyperaccurate S12 mutations affect mainly the accommodation rate, suggesting that some aspects of conformational changes in the 30S occur only after GTP hydrolysis (28). The structural basis for this is not clear because the interactions between the anticodon stem loop of tRNA and the decoding center (including S12) change very little before and after accommodation (e.g., compare References 13, 29). Clearly, each of these mutations may affect the ability of attaining a GTPase-active form in subtle ways that are not obvious from the structures determined to date. Furthermore, the transition to an active form involves not only changes in the 30S subunit but also distortions or altered interactions in the tRNA, EF-Tu, and the 50S subunit (15) (described in detail below), which could help to explain the unexpected phenotypes of these mutations.

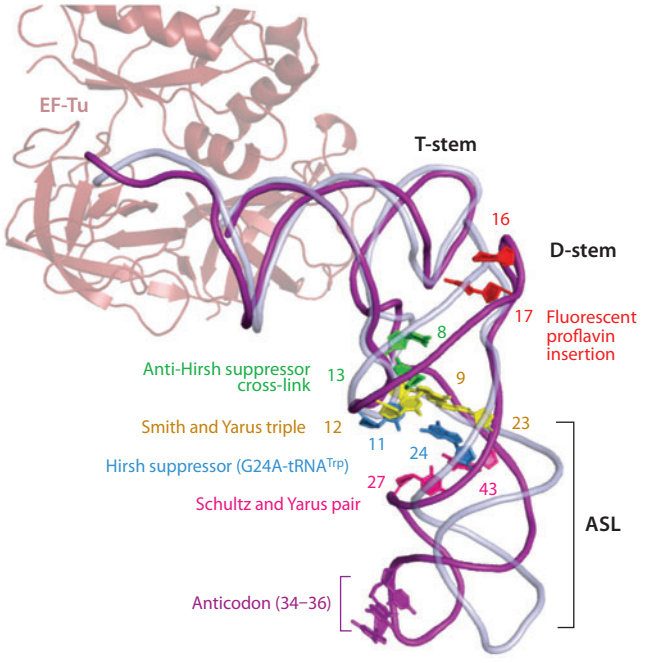


Figure 3

Mutations and insertions whose effects are the result of distortions in the A/T state. Shown here is the superposition using elongation factor Tu (EF-Tu) (*red*) of the ribosome-bound aminoacyl tRNA (*purple*) (13) with that from the isolated ternary complex (*gray*) (156). Adoption of the A/T state requires two distinct regions of distortion, the first in the anticodon stem loop (ASL) and the second a rotation of the D-stem away from the acceptor/T-stem stack. Mutations to the transfer RNA (tRNA) body that affect these conformational changes can result in profound defects in decoding on the ribosome, and several are highlighted here.

Distortions in the tRNA Body

In addition to codon-anticodon base-pairing, the tRNA body, beyond the anticodon, also plays an essential role in decoding. This was first demonstrated by the discovery of the so-called Hirsh suppressor tRNA, which contained a single mutation in the D-stem of tRNA^{Trp} that allowed read-through of UGA stop codons (30–32). Subsequent work has demonstrated that many other mutations in the tRNA body can also affect accuracy (**Figure 3**) (33–36).

We now know that the structural properties of the tRNA body are essential for formation of a distorted conformation during decoding. The binding of aminoacyl tRNA to the ribosome along with EF-Tu requires adoption of what is known as the A/T state, which allows the simultaneous interaction of the anticodon with the mRNA in the decoding center and EF-Tu in the 50S factor binding site (37–39). The A/T conformation is composed of two regions of distortion: The first is in the anticodon stem, and the second is a movement of the D-stem away from the acceptor/T-stem stack (**Figure 3**) (13). Some mutations that lead to miscoding (33, 34) appear to reduce the energetic penalty required to reach the distorted A/T state. In contrast, a recent crystal structure of the Hirsh suppressor tRNA bound to the ribosome suggests that the D-stem mutation allows formation of an additional hydrogen bond that preferentially stabilizes the A/T conformation (40), providing a structural explanation for the observed increase in the rate of GTPase activation by the Hirsh suppressor tRNA on its near-cognate codon (41) and thus its suppressor phenotype. These results are consistent with experiments showing that the sequence of the tRNA body is precisely tuned to the identity of the amino acid as well as the strength of the codon-anticodon interaction (42–44). Each tRNA, within a given species, thus likely employs a unique strategy for binding the

ribosome, which must balance the energy gained from interactions with the decoding center with that lost in stabilizing the distorted A/T conformation to allow for accurate decoding.

Sarcin-ricin loop (SRL): a highly conserved region of the 23S rRNA in the 50S subunit that interacts with translational GTPase factors

Elongation Factor Tu

Along with the distortion of the tRNA body, codon recognition pulls EF-Tu into the factor binding site and is accompanied by a domain rearrangement. This conformational change is mimicked by binding of kirromycin to the isolated ternary complex (45) (Protein Data Bank code: 1OB2). EF-Tu is composed of three domains: domain 1, the nucleotide binding or GTPase domain, which is conserved in both structure and sequence across all translational GTPases; domain 2, which is localized adjacent to the 30S shoulder upon ribosome binding; and domain 3. Ribosome binding and codon recognition require movement of the GTPase domain to avoid a steric clash with the highly conserved sarcin-ricin loop (SRL) of 23S rRNA.

GTPase Activation

The conformational changes induced by codon recognition, which include the domain closure of the 30S subunit, the distortion of the tRNA, and conformational changes in EF-Tu, are all part of the process of GTPase activation. To ensure fidelity, GTPase activation must occur only upon association of cognate tRNA and thereby requires that the excess energy from cognate tRNA binding is used to reach the activated state for GTP hydrolysis by EF-Tu. Premature GTP hydrolysis was initially predicted to be prevented by a conserved hydrophobic gate composed of residues Val20 (P-loop) and Ile60 (switch I) (46). Upon GTPase activation, the catalytic His84 (switch II) (47) is localized into the active site, past the hydrophobic gate, to coordinate a water molecule for in-line attack on the γ-phosphate of GTP.

Recent crystal structures of the ternary complex bound to the ribosome suggest several conformational changes in EF-Tu that appear to be important for GTPase activation by the ribosome (13). Binding of EF-Tu induces a shift in regions of domain 2—including a highly conserved β-turn—toward the now closed shoulder of the 16S rRNA (**Figure 2*c***). The role of this β-turn in decoding is consistent with biochemical experiments in which mutation of residues in this region lead to specific defects in GTPase activation of EF-Tu (48). This interaction between EF-Tu and the 16S rRNA is facilitated by the conformational change to the 30S subunit that occurs upon codon recognition (22) and thus may be important for selectively activating GTP hydrolysis for cognate but not near- or noncognate tRNA.

Movement of this β-turn leads to a ∼5 Å distortion of the 3′ end of the tRNA, which disrupts interactions between the tRNA and the switch I loop (**Figure 2*d***). However, contrary to what was proposed based on an initial posthydrolysis structure of EF-Tu bound to the ribosome (13), the loss of these interactions does not lead to the disordering of the switch I loop. Indeed, based on a structure of EF-Tu bound in its GTP state to the ribosome (49), in which His84 has rotated into an active conformation in the GTPase center, only subtle rearrangements of the hydrophobic gate appear necessary for adoption of the GTPase-activated state, despite previous predictions (**Figure 4*a***) (13, 45, 50–52). Although we cannot exclude the possibility that a large conformational change occurs transiently during the reorientation of His84, this seems unlikely given that the activated conformation of His84 is compatible with the closed conformation of the hydrophobic gate. Therefore, whether a hydrophobic gate poses a true barrier to GTP hydrolysis prior to activation on the ribosome remains uncertain.

Finally, GTPase activation appears to involve the reordering of the conserved His84 into the GTPase center by the phosphate of residue A2662 of the SRL (**Figures 2*e*** and **4*b***) (49). The movements within EF-Tu (β-turn), the tRNA (3′ end), and the ribosome (30S domain closure) appear important for the precise

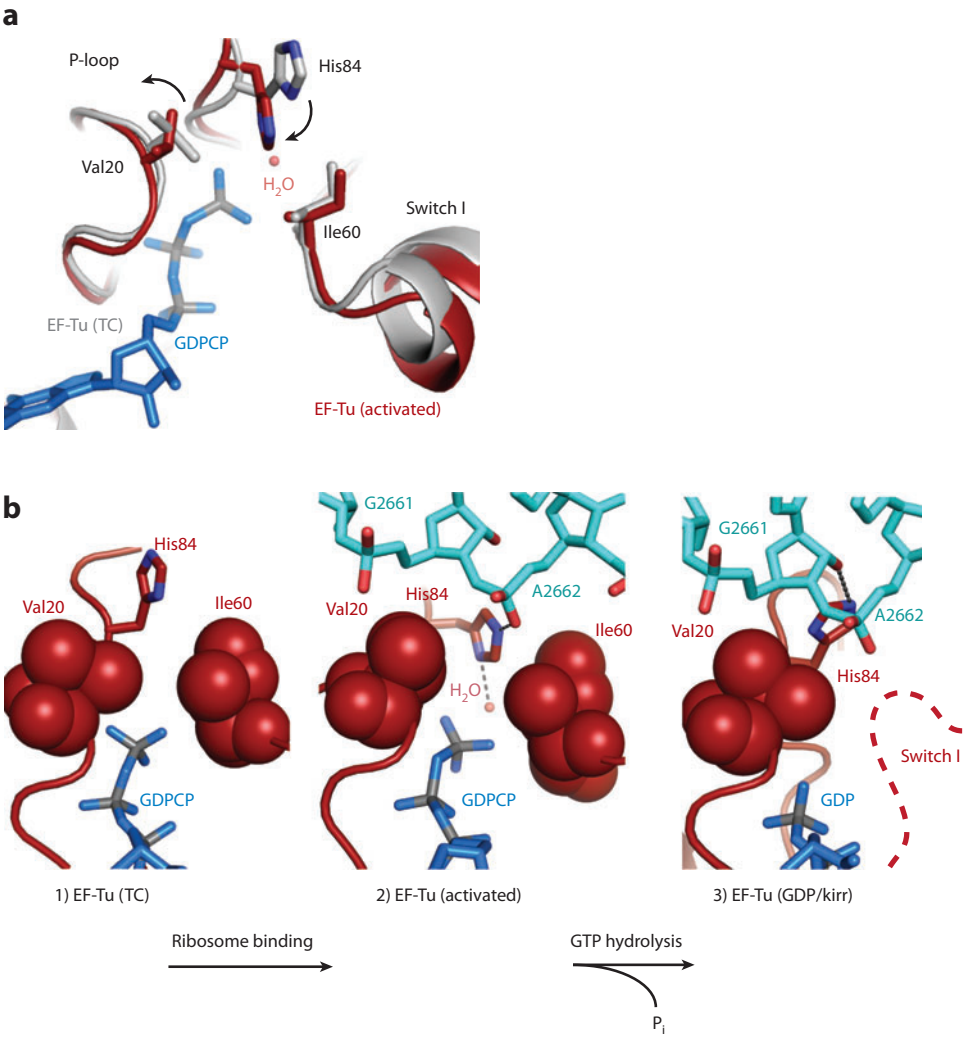


Figure 4

Organization of the active site of elongation factor Tu (EF-Tu) in different stages of decoding. (*a*) Superposition of the GTPase center from the crystal structure of the isolated ternary complex (TC) in solution (*gray*) (156) with that from the ribosome-bound structure stalled with a GTP analog (*red*) (49). In the activated complex, His84 coordinates a water molecule that is in position for an in-line attack of the γ-phosphate of GDPCP. A small movement away from the nucleotide is also observed in the hydrophobic gate residues Val20 and Ile60. (*b*) Prior to binding of the ternary complex to the ribosome, the catalytic histidine is in an inactive conformation, rotated away from GTP (156). Binding to the ribosome and GTPase activation allows the phosphate of residue A2662 of the sarcin-ricin loop (SRL) (*cyan*) to position His84 into the active site. Finally, after GTP hydrolysis (13) and P_i release, the switch I loop is disordered (*dashed line*) and His84 has returned to an inactive conformation, contacting residue G2661 of the SRL.

positioning of EF-Tu to allow this interaction to occur. This is consistent with the previously unexplained observation that the SRL plays a critical role in the binding and function of both EF-Tu and EF-G on the ribosome (53–55). Additionally, it is now clear how the ribosome, via the SRL, functions as a GTPase-activating protein for EF-Tu, stabilizing the transition state

for GTP hydrolysis, similar to cellular GTPases and their catalytic partner proteins.

However, a recent biochemical study, in which a conserved base pair in the SRL was deleted, observed only a modest effect on the rate of GTP hydrolysis by EF-Tu (56). The authors predicted that this mutation would move the phosphate of A2662 more than 8 Å away from His84 and therefore suggested that the interaction with the SRL is not critical for GTP hydrolysis by EF-Tu. However, in the absence of additional structural data, the precise effect of this deletion on the localization of A2662 is difficult to predict, and, as the authors pointed out, another functional group in the SRL may substitute for the phosphate of A2662. Further experiments, perhaps involving specific chemical substitutions within the SRL in conjunction with structural work, are required to assess the role of the phosphate of A2662 in GTPase activation. However, based on the crystal structure of EF-Tu in an active conformation (49), the universal conservation of the catalytic histidine in translational GTPases, and the large catalytic effect of mutation of His84 in EF-Tu (47), the reorientation of the conserved histidine into the active site, by either the phosphate A2662 or some other moiety, appears to be important for GTP hydrolysis.

Additionally, the precise roles of other ribosomal components in GTPase activation, in particular L7/L12, remain unclear. This protein has long been implicated in the function of EF-Tu on the ribosome (57), and recent biochemical evidence in favor of this role has been reported (8, 58). However, the crystal structures of EF-Tu bound to the ribosome suggest that L7/L12 cannot be directly involved in catalysis as it is not sterically possible for the protein to interact directly with GTP (49). Furthermore, deletion of the eukaryotic homologs of L7/L12 is not lethal (59). These observations are therefore consistent with the proposed indirect role L7/L12 plays in GTPase activation (8, 11).

Given the high sequence conservation and shared binding site for all translational GTPases, the interaction with the SRL seen for EF-Tu may be an essential feature of the universal mechanism for GTPase activation on the ribosome. Apart from the mutational studies on the SRL discussed above, which argue against this idea (56), a recent crystal structure of RF3 bound in its GTP state to a ribosome in which the subunits were rotated (60) showed that the conserved nucleotide binding domain of RF3 was in a very different orientation relative to the SRL than that of EF-Tu (49). It is not clear whether this structure represents an activated complex of RF3 on the ribosome, in part owing to the absence of a P-site tRNA. Therefore, additional crystal structures of other translational GTPases bound in an activated conformation to the ribosome will help determine if the interactions of the SRL with the catalytic site are universally conserved.

Finally, GTPase activation has been identified as a kinetic step following codon recognition and preceding GTP hydrolysis. The various structural changes that characterize this step do not necessarily occur in a specified sequence. Rather, codon recognition induces conformational changes that may occur in some coordinated or stochastic manner to result in the activated form of EF-Tu.

GTP Hydrolysis and P_i Release

Following GTPase activation, His84 is in position to coordinate an H_2O molecule for in-line attack on the γ-phosphate of GTP (46, 47, 61). Based on the crystal structure of EF-Tu bound to the ribosome in an activated conformation, we proposed that His84 functioned as a general base to deprotonate the catalytic H_2O for GTP hydrolysis (49). This chemical mechanism is consistent with the observation that a histidine in this position is universally conserved in all translational GTPases and would explain the 10^6-fold decrease in the rate of GTP hydrolysis of EF-Tu upon mutation of this histidine to alanine (47). However, it has rightly been pointed out (62) that cellular GTPases such as Ras and Ran, whose active sites are architecturally very similar to that of EF-Tu, use a substrate-assisted mechanism for catalysis (63). Additionally, there is evidence that suggests

His84 in EF-Tu may not function as a general base (47, 63). This is consistent with recent molecular modeling studies (64) that suggest His84 instead plays an allosteric role in organizing the EF-Tu active site for GTP hydrolysis. It has therefore been predicted that translational GTPases utilize a substrate-assisted catalytic mechanism analogous to that of Ras/Ran (62). Rigorous biochemical and mutational studies, including Brønsted analysis of the GTP substrate, as well as higher-resolution structural experiments, perhaps utilizing transition state analogs, are required to conclusively determine the catalytic mechanism of EF-Tu and other translational GTPases.

Following GTP hydrolysis, release of P_i disrupts its interactions with the switch I loop, resulting in its disordering (**Figure 2*f***), consistent with previous kinetic studies (65) and as observed in the posthydrolysis structure of EF-Tu bound to the ribosome (**Figure 4*b***) (13). EF-Tu then undergoes a conformational change to its GDP form, which involves a ~100° rotation of the nucleotide binding domain relative to domains 2 and 3 (**Figure 2*g***) (46). This movement disrupts the interactions of the G domain with the SRL as well as those between switch II and the tRNA acceptor arm. The weakened interactions of EF-Tu with the ribosome and tRNA result in its dissociation.

Accommodation and Proofreading

Following the release of EF-Tu, the strained aminoacyl tRNA is held on the ribosome almost entirely via interactions with the decoding center, which are much stronger for cognate than near-cognate tRNA. Given the few remaining stabilizing interactions, the strain on the aminoacyl tRNA is relieved by either accommodation into the peptidyl transferase center (**Figure 2*b***) or dissociation from the ribosome. The rate of accommodation is accelerated for cognate compared with near-cognate tRNA (23), further contributing to accurate tRNA selection.

In general, noncognate and near-cognate tRNAs will be rejected during the initial, reversible selection step as described. However, they can also dissociate from the ribosome after GTP hydrolysis. As initial tRNA selection and proofreading are separated by irreversible GTP hydrolysis (66), it is their multiplicative effects that contribute to the overall accuracy of decoding in a process often termed kinetic proofreading (67, 68). The first evidence that the ribosome employs such a mechanism was the observation that incorporation of an amino acid at a near-cognate codon required a tenfold increase in GTP consumption compared to at a cognate codon (69). However, at noncognate codons, GTP hydrolysis was not observed. Later studies suggested that GTP consumption could be increased as much as 50-fold for amino acid incorporation at a near-cognate codon (70).

Following dissociation of EF-Tu, the few interactions between the distorted tRNA and the ribosome, as revealed by crystal structures (13), leave a largely clear path to the peptidyl transferase center. A near-cognate tRNA would likely have weaker interactions with the decoding center, as it cannot induce the closed 30S conformation, characteristic of cognate tRNA binding, and would more likely dissociate (22). Furthermore, cognate tRNA would be held by strong interactions at the decoding center and thus be conformationally restricted, thereby facilitating its accommodation into the peptidyl transferase center and rationalizing the observed enhancement in the rate of accommodation for cognate compared with noncognate tRNAs (23). The Hirsh suppressor tRNA, containing its single D-stem mutation, also exhibits an increased rate of accommodation compared with near-cognate tRNA (41). However, the structural explanation for this observation is not yet clear because, as mentioned previously, the Hirsh suppressor mutation appears to preferentially stabilize the A/T state, and other interactions, e.g., at the decoding center or with helix 69, seem similar to those of the wild-type tRNA (40). Accommodation of an aminoacyl tRNA into the peptidyl transferase center leads to rapid peptide bond formation, representing the completion of a successful decoding cycle

(**Figure 2*i***). However, there is increasing evidence that even after peptidyl transfer, if the incorrect amino acid is aberrantly incorporated, additional proofreading mechanisms can preferentially terminate translation (71).

Kinetic Studies on Decoding

The pre-steady state kinetic studies of Rodnina and coworkers (7) showed that GTP hydrolysis was faster for cognate, compared with near-cognate, tRNA. The investigators therefore proposed that cognate tRNA induces a productive form of the ribosome. These studies represented a major advance in our understanding of the biochemical steps in decoding. Although the observation that GTP hydrolysis is faster for cognate tRNA has been widely confirmed, differences have emerged in the details of specific steps in decoding.

In the original scheme, because the fluorescence observed for tRNA bound to EF-Tu containing the nonhydrolyzable analog GDPNP was much lower than the peak reached with GTP (14), it was reasoned that only the codon-recognition state was reached with the analog. The off-rate could then be measured by competition with unlabeled ternary complex containing GDPNP. This off-rate was very low for cognate tRNA (especially compared with the subsequent GTPase activation), leading to the conclusion that nearly all cognate tRNAs are accepted once codon recognition takes place.

Recent single-molecule FRET (Förster resonance energy transfer) experiments with donor and acceptor pairs placed on P- and A-site tRNAs show three FRET states, identified as a codon-recognition state (0.21 FRET), a GTPase-activated state (0.32), and an accommodated state (0.54) (10, 72). In these experiments, the codon-recognition state is short lived for both cognate and near-cognate tRNA (although to different extents). The ternary complex reaches a stable GTPase-activated state even with GDPNP, but only after passing through the codon-recognition state. The observation that the GTPase-activated state can be obtained in the presence of GDPNP is consistent with a crystal structure in which a GTP-stalled complex was determined in an apparently activated conformation (49). Moreover, this structure is globally very similar to the kirromycin-stalled state immediately after GTP hydrolysis (13), so it is not clear why the kirromycin-stalled complex should have a much higher proflavin fluorescence than the GDPNP state in the original pre-steady state kinetic experiments (14). Finally, in the single-molecule experiments, nearly half of cognate tRNAs that reach the low (codon-recognition) FRET state dissociate from the ribosome (72), which is incompatible with data from ensemble kinetic measurements (14). The correlation of transient steps observed in ensemble and single-molecule kinetic experiments to specific structural states is not well defined. It is also not clear from a visual inspection of the structure how codon recognition in the low FRET state could occur in the absence of the bent tRNA that is characteristic of the GTPase-activated intermediate FRET state.

There is also disagreement regarding the actual rates of particular steps during decoding as well as the overall selectivity of the process. Ehrenberg and colleagues (73) showed that at high rates of dipeptide formation, very high intrinsic selectivity was also observed. They suggested that accommodation was not rate limiting and thus the rate of peptidyl transfer could be measured directly (74). Subsequently, the Rodnina group (75) was able to reproduce this very high rate of peptidyl transfer by using similar buffer conditions in saturating concentrations of the ternary complex. In the same work, the rates for accommodation and peptidyl transfer using fluorescently labeled tRNA were equal (but lower than the aforementioned rates because of the reduced concentration of ternary complex needed for these experiments), suggesting that accommodation was rate limiting. The very high selectivity observed by Ehrenberg and colleagues was attributed to the reduction of free Mg^{2+} ions as a result of chelation by the phosphoenol pyruvate present in their reaction buffer. The Ehrenberg group (76) has now addressed the question of

selectivity as a function of Mg^{2+} and also conducted studies suggesting that the rate of peptidyl transfer depends on the identity of the amino acid in the A site (74). All sides agree that the maximum possible theoretical selectivity is not obtained but instead sacrificed for speed, but disagree on by precisely how much.

An important future goal is to conclusively determine whether or not accommodation is rate limiting for translation. Molecular dynamics calculations have tried to address this question and suggested that the 3′ end of tRNA moves in a corridor through a gate as it accommodates into the peptidyl transferase center (77). However, mutation of the residues that were proposed to compose this gate resulted in no change in the apparent rate of accommodation in vitro (78) as well as no apparent growth defects in yeast (79).

A general problem in interpreting and comparing these data, including those from multiple-turnover, single-turnover, and single-molecule studies, is that different reagents, reporters, and buffer conditions have been used in each experiment. It would be very informative if the same set of reagents and reporters were studied by multiple techniques to understand the source of these discrepancies.

A New View of Decoding?

Recently, some aspects of this general view of decoding, particularly the role of conformational changes in the 30S subunit, have been questioned on the basis of structures of near-cognate tRNAs bound to the ribosome (80). In these studies, it was found that near-cognate tRNA was bound to the 70S ribosome in the closed conformation, and that instead of a G·U wobble pair, a G-U Watson-Crick base pair was observed in the first or second position of the codon-anticodon helix, along with other small differences in the conformation of the near-cognate compared with cognate tRNA. On the basis of these studies, the authors suggested that both cognate and near-cognate tRNA induce a closed form of the 30S subunit, and the role of the ribosome is to force near-cognate tRNA to adopt Watson-Crick geometry. Discrimination was proposed to arise from the energetic penalty associated with stabilizing the unfavorable tautomer required for adoption of a G-U Watson-Crick base pair.

However, these observations do not necessitate a revision of our understanding of decoding. Previous structural (22) and biochemical (81) studies suggest that near-cognate tRNA has essentially the same structure when it is accepted by the ribosome, which adopts a form capable of activating GTP hydrolysis by EF-Tu. However, the probability of reaching that state is much lower for near-cognate tRNA unless agents that increase the error rate, such as paromomycin, are present. Nonphysiologically high concentrations of magnesium have long been known to increase the error rate of translation (82, 83), and magnesium ions play a structural role in facilitating the tRNA-bound closed form of the 30S subunit (16). Given the high concentrations of Mg^{2+} in the cryoprotection buffers utilized, in addition to the high concentration of polyethylene glycol (which increases the effective concentration of both ribosomes and ions), near-cognate tRNA is likely able to bind 70S ribosomes in the closed form even without paromomycin. In previous studies on the 30S subunit (22), these factors were offset by the naturally low affinity of A-site tRNA for the 30S subunit as compared with the whole ribosome (84, 85), so that discrimination between near-cognate and cognate tRNAs was fortuitously maintained even within the crystal.

A G·U Watson-Crick pair was previously observed for a second-position mismatch between serine tRNA and a phenylalanine codon (22), and it was noted that the energetic consequences of either losing the minor groove interaction due to this mismatch geometry or stabilizing an unfavorable enol tautomer of U are similar (15). Thus, in either case, it is only for cognate tRNA that the minor groove interactions by the highly conserved 16S rRNA bases provide additional energy that is used to induce the conformational changes needed to reach a productive GTPase form of the ribosome. In the context of the 70S ribosome

containing an mRNA that is unbroken between the A and P sites, the data suggest that the ribosome similarly imposes Watson-Crick geometry through an enol tautomer at the first position (80). A possible role for A1913 in H69 from 23S rRNA in recognizing cognate tRNA binding was pointed out previously (29), and it appears to have a somewhat different conformation upon binding of cognate versus near-cognate tRNA (80).

Thus, these recent studies on the 70S ribosome augment our understanding of decoding from previous studies on the 30S subunit. However, as discussed above, these observations can be integrated in a straightforward manner into the general understanding of the mechanism of decoding that has developed over the past decade (15).

PEPTIDYL TRANSFER

The key catalytic step in protein synthesis is peptide bond formation, which occurs through the nucleophilic attack of the α-amino group of the aminoacyl tRNA on the aminoacyl ester of the peptidyl tRNA (**Figure 5*a***). This reaction leads to formation of a peptide bond and release of an alcohol product. The ribosome enhances the rate of aminolysis ~10^7-fold compared with the spontaneous rate in solution (86). Initial biochemical experiments suggested that the catalytic power of the ribosome was derived from its RNA components (87). Indeed, although proteins have been observed in the peptidyl transferase center of the bacterial ribosome (88), structural evidence suggests that no ribosomal proteins are directly involved in catalysis (29, 88–90).

Induced Fit

Upon binding of an A-site substrate, a series of conformational changes in the 23S rRNA exposes the peptidyl tRNA ester for nucleophilic attack by the α-amine (91). These movements were first observed in structures of the 50S subunit using minimal oligonucleotide substrates (91) and were later confirmed in the context of the whole ribosome with intact tRNAs (88). Indeed, no fundamental differences in the peptidyl transferase center, or the binding of substrates within it, were observed between the 50S subunit and the 70S ribosome. This conclusion is further supported by kinetic studies that show the rate of peptidyl transfer by the 50S subunit is comparable to that of the intact ribosome provided that full-length tRNA substrates are used (92). Therefore, questions regarding the validity of studies performed on the 50S subunit using minimal substrates (93, 94) are unfounded.

These conformational changes are induced by the binding of any A-site substrate containing at least residue C75 (92, 95–97), consistent with observations that binding of even a deacylated tRNA in the A site increases the rate of hydrolysis of the peptidyl tRNA. Crystal structures of termination complexes report similar changes to the 23S rRNA upon binding of RF1/2 to the 70S ribosome (98, 99). Thus, as suggested previously (91), conformational changes in 23S rRNA that expose the peptidyl tRNA ester for hydrolysis are a feature of both peptidyl transfer and peptide release.

Chemical Mechanism

Several catalytic mechanisms for peptidyl transfer have been proposed including general acid-base catalysis (89, 100), substrate-assisted catalysis (101, 102), and catalysis by entropic effects alone (86). Early structural studies ascribed a catalytic role to a highly conserved 23S rRNA residue, A2451, which was within hydrogen-bonding distance of the nucleophilic amino group (89, 100). However, mutation of this and several other active site rRNA bases had only modest effects on the rate of peptidyl transfer (103–105). These observations suggested one of three possibilities: that the ribosome does not employ general acid-base catalysis; that catalysis occurs through a functional group that is unchanged by mutation, such as a backbone phosphate or ribosyl group; or that catalysis is performed by a functional group located not on the rRNA but on the substrates themselves.

Biochemical data suggest that peptidyl transfer is catalyzed by entropic effects alone (86). Indeed, the enthalpy of activation for

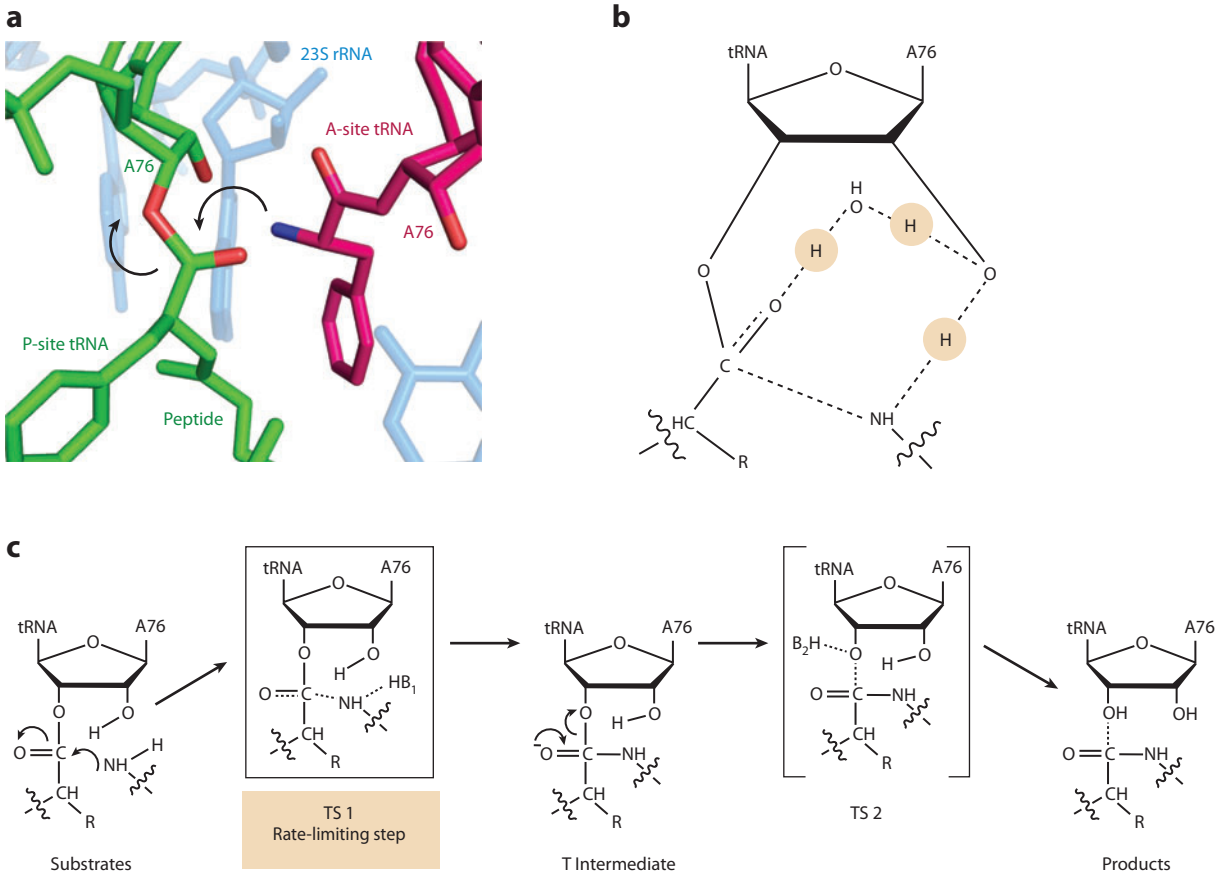


Figure 5

The chemical mechanism of peptide bond formation. (*a*) Peptidyl transfer involves the nucleophilic attack of the α-amino group on the aminoacyl transfer RNA (tRNA) (*magenta*) on the peptidyl-ester on the peptidyl tRNA (*green*). (*b*) Fully concerted mechanism of peptidyl transfer as proposed in Reference 118 involving an eight-membered proton shuttle in the transition state (TS). (*c*) Two-step mechanism proposed in Reference 119. Abbreviations: A, aminoacyl; P, peptidyl; rRNA, ribosomal RNA.

aminolysis is less favorable on the ribosome than in solution. This result was interpreted to indicate that peptide bond formation on the ribosome is catalyzed primarily by substrate positioning and water exclusion. This is consistent with molecular modeling studies that suggest that the catalytic effect of the ribosome is primarily a result of solvation entropy (106).

Consistent with this observation, only modest effects on the rate of peptide bond formation have been reported for mutation of functional groups on the rRNA backbone. For example, the removal of the 2′ OH of active site residue A2451 results in a 60-fold decrease in the rate of peptidyl transfer (107). Other structural and modeling studies have predicted that this functional group is involved in a hydrogen-bonding network with the 2′ OH of A76 of the peptidyl tRNA (108–110). However, the small effects of these rRNA groups on the rate of peptidyl transfer suggest they do not play a direct role in catalysis.

Role of the 2′ OH of A76 of the Peptidyl tRNA

By contrast, a primary candidate to perform substrate-assisted catalysis was the 2′ OH of

A76 of the peptidyl tRNA. Crystal structures indicated that the 2′ OH of A76 is one of the few functional groups properly positioned to function directly in peptide bond formation (111). Biochemical studies initially reported that substitution of this hydroxyl moiety with either a fluorine or hydrogen results in a $\sim 10^6$-fold reduction in the rate of peptidyl transfer (102). Owing to its high pK_a, the apparent lack of a strong acid nearby (e.g., Mg^{2+}), and the absence of a corresponding pK_a in the pH dependence of the peptidyl transferase reaction (112), the 2′ OH was hypothesized to be unlikely to act as a general base. Instead this moiety was widely accepted to function as part of a proton-shuttle mechanism (101) that catalyzed peptidyl transfer by facilitating proton exchange and orienting the substrates for reactivity, consistent with the ribosome functioning primarily as an entropy trap (86).

Recently, however, the role of the 2′ hydroxyl group in translation has been questioned. Using a cell-free translation extract, researchers showed that the ribosome could successfully utilize a single 2′ dA76 $tRNA^{Ser}$ suppressor tRNA during synthesis of a full-length protein, though less efficiently than the corresponding 2′ rA76 $tRNA^{Ser}$ (113). Although the quantitative effect of the 2′ OH on peptide bond formation could not be determined from these experiments, that translation can proceed in the absence of a 2′ OH appears to contradict the 10^6-fold rate effect that had been previously observed (102).

This discrepancy was proposed to arise from several differences in experimental design (114). Unexpectedly, however, it appears that the primary role of the 2′ OH of A76 of the peptidyl tRNA is to induce an active conformation of the peptidyl transferase center, which slowly rearranges when bound to a 2′ deoxy substrate (114). Therefore, when the ribosome–nascent chain complexes (containing dA76 fMet-Lys-$tRNA^{Lys}$ in the P site) were pelleted prior to reaction with A-site substrate in the initial biochemical study (102), the peptidyl transferase center was given sufficient time to revert to an inactive conformation, hence the observed 10^6-fold decrease in the reaction rate. However, for an actively translating ribosome (113) or uninterrupted tripeptide formation (114), the effect is far more subtle. Improved estimates from the same groups as the initial study (102) suggest that mutation of the 2′ OH of A76 is responsible for only a ~100-fold decrease in the rate of peptide bond formation (114). The magnitude of this effect raises the question of whether the 2′ OH of A76 is playing the central catalytic role once predicted, but would still be consistent with a role in proton transfer or substrate orientation. Additional experiments are required to conclusively determine its precise function during peptide bond formation by the ribosome.

Where the Protons Go

A comprehensive understanding of the mechanism of peptide bond formation requires detailed knowledge of the chemical composition of the transition state of the reaction. If the ribosome catalyzes peptidyl transfer through substrate positioning alone, the chemical mechanism, and thus the transition state, of the catalyzed and uncatalyzed reactions should be identical. Classic studies of the Brønsted coefficient of solution aminolysis reactions suggest that the nucleophilic amine becomes positively charged at the transition state of the reaction (115). In contrast, similar studies of aminolysis on the ribosome observed a Brønsted coefficient near zero, consistent with an essentially uncharged transition state for the peptidyl transferase reaction (116, 117) and suggesting that the reaction mechanism on the ribosome differed from that in solution.

To better understand the nature of these differences, two isotope effect studies were simultaneously reported, one involving kinetic solvent isotope effects (KSIEs) (118) and the other involving kinetic isotope effects (KIEs) (119). KSIEs are determined by measuring the rate of reaction at increasing concentrations of deuterated water (D_2O). The relationship between the reaction rate and the concentration

of deuterium reflects the number of hydrogen bonds that are broken and formed during the rate-limiting step of the reaction (reviewed in Reference 120). KSIEs for peptidyl transfer have been interpreted to indicate three protons in flight at the transition state of the reaction (118). Based on this and other studies, peptide bond formation was hypothesized to be catalyzed by a fully concerted eight-membered proton-shuttle mechanism involving protons originating from the α-amine, a crystallographic water molecule, and the 3′ OH of A76 of the peptidyl tRNA (**Figure 5*b***). Importantly, this model differs from the previously accepted proton-shuttle mechanism (101), as it is now clear that ester-bond cleavage does not occur at the rate-limiting step.

KIE analysis involves specific isotopic substitution of atoms directly involved in the reaction. The effects of these substitutions on the rate of peptidyl transfer reflect the chemical nature, and more specifically the change in bond order, of the rate-limiting step of the reaction. To summarize, the KIE analysis of five such substituted substrates suggests that (*a*) there is partial peptide bond character present at the transition state, suggesting that formation of the tetrahedral intermediate is rate limiting, consistent with previous KIE studies (116); (*b*) nucleophilic attack and deprotonation of the α-amine are concerted, consistent with Brønsted analysis indicating a neutral amine at the transition state (117); (*c*) there is no change in bond order between the 3′ O leaving group and carbonyl carbon in the rate-limiting step, suggesting the ester bond remains intact in the rate-limiting step; and (*d*) the transition state is tetrahedral in character (119).

Thus, the KIE data as a whole are best explained by a two-step mechanism with an early transition state in which peptide bond formation and deprotonation of the nucleophile are concerted and occur in the rate-limiting step, in agreement with that predicted by the KSIE study (118) and followed by the rapid breakdown of the tetrahedral intermediate into products (**Figure 5*c***). The KIE data cannot exclude the possibility of a proton shuttle; however, the fully concerted mechanism predicted by Reference 118 (**Figure 5*c***) is not consistent with the tetrahedral transition state predicted by isotopic substitution of the α-hydrogen (119). Further experiments to elucidate both the destination of the proton from the α-amine and the precise role of the 2′ OH of A76 are required for a comprehensive definition of the chemical mechanism of peptidyl transfer.

What is clear, however, is that the mechanism of ribosome-catalyzed peptide bond formation differs significantly from that of the uncatalyzed reaction, in which nucleophilic attack and deprotonation are not concerted. Therefore, the ribosome not only increases the rate of peptide bond formation by more than six orders of magnitude but also significantly perturbs the energetic landscape of the reaction. This observation cannot be explained by catalysis through substrate positioning alone, and it is increasingly apparent that the ribosome must also play a role in orchestrating the chemical mechanism of peptide bond formation.

TRANSLOCATION

After peptide bond formation, the ribosome is left with a deacylated tRNA in the P site and a peptidyl tRNA, elongated by one residue, in the A site (**Figure 6**). In the next step, known as translocation, the mRNA and tRNA move with respect to the ribosome to allow a new round of elongation to occur. More specifically, the A- and P-site tRNAs move to the P and E sites, respectively, and the mRNA moves by precisely one codon, bringing its next codon into the now empty A site for recruitment of a new cognate tRNA. Translocation involves the movements of the tRNAs and mRNA by distances of ∼50 Å and thus involves large-scale conformational changes. Because of this, translocation remains the most poorly understood aspect of the elongation cycle. However, given its importance for maintaining the reading frame and more generally for translation to proceed, understanding the directionality and precision of translocation is of great interest.

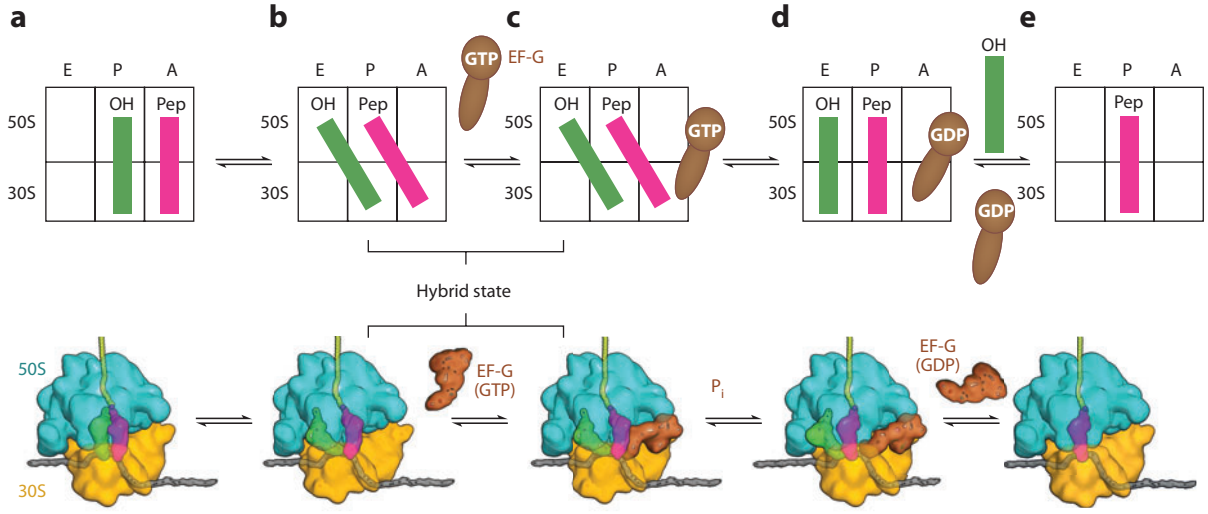


Figure 6

Schematic of translocation. Translocation can be divided into two steps: the first in which the transfer RNAs (tRNAs) move from their canonical conformations (*panel a*) relative to the 50S subunit (*panel b*), and the second, catalyzed by the GTPase elongation factor G (EF-G) (*brown*), in which the messenger RNA (mRNA) and tRNAs move relative to the 30S subunit (*panels c and d*). Although a high-resolution crystal structure of EF-G bound to the ribosome in the post-GTP hydrolysis state (*panel d*) was recently determined (172), the detailed structures of A/P tRNA and EF-G bound in its GTP state to the ribosome are not known (*panels b and c*). Abbreviations: A, aminoacyl; E, exit; P, peptidyl.

Intermediate (Hybrid) States During Translocation

The idea that translocation proceeds via an intermediate state in which movement of the tRNAs has occurred with respect to one subunit but not the other was proposed as early as 1968 (121), but more than 20 years passed before the existence of a hybrid state of the ribosome was demonstrated using chemical footprinting methods (122). That landmark study showed that after peptide bond formation, the tRNAs form A/P and P/E hybrid states in which the 3′ ends of the A- and P-site tRNAs have moved to the P and E sites of the 50S subunit, whereas their anticodon ends and the mRNA remain anchored in the 30S subunit (**Figure 6**). Following the binding of EF-G and GTP hydrolysis, the mRNA and anticodon ends of the tRNA move into the P and E sites of the 30S subunit to restore the canonical state of the ribosome, leaving the A site empty and ready to accept the next aminoacyl tRNA. Recent structural and biochemical studies have shed light on the details of the various steps of this process.

Hybrid state: intermediate translocation state in which the 3′ ends of A- and P-site tRNAs have moved to occupy the P and E sites in the 50S subunit

Initial cryo-EM studies with both A- and P-site tRNAs did not show evidence for a hybrid state unless EF-G, in its GTP form, was also bound (123), in apparent contrast to earlier chemical footprinting data. However, investigators later showed that an aminoacyl tRNA favored the canonical A/A state, whereas a peptidyl tRNA favored the A/P conformation, demonstrating that peptidyl transfer drives the formation of hybrid states (124). This was confirmed by the observation that authentic transpeptidation resulting in a dipeptide on the A-site tRNA also favored the hybrid state (125). With the use of sorting techniques to refine subpopulations in a sample, more recent cryo-EM studies using authentic transpeptidation have unambiguously shown the existence of hybrid states even in the absence of EF-G (126, 127).

Crystal structures have demonstrated why only a deacylated tRNA can sterically bind

to the E site of the 50S subunit (128), thus ensuring that a hybrid P/E state can form only after peptidyl transfer. Several experiments have shown that any mutations or modifications in either tRNA or the 50S subunit that interfere with E-site binding in the 50S result in reduction or loss of translocation, presumably by preventing the formation of the P/E state (reviewed in Reference 2). Using one such mutation, the formation of the P/E state was shown to precede the formation of the A/P state in kinetically distinct events (129, 130).

Ratcheting of Subunits During Hybrid State Formation

Cryo-EM studies showed that the binding of EF-G with a nonhydrolyzable analog of GTP resulted in a ribosome in which the 30S subunit was rotated (ratcheted) with respect to the 50S subunit as compared with the factor-free ribosome. This observation suggests that the formation of tRNA hybrid states during translocation was coupled to a rotation of the subunits relative to one another (131). Subsequent cryo-EM studies observed a rotated ribosome in which the L1 arm had moved inward to stabilize a P/E tRNA (123), but these studies lacked an A-site tRNA.

FRET measurements using donor-acceptor pairs placed on the 30S and 50S subunits have shown that after peptide bond formation, the ribosome exists in an equilibrium between the rotated and canonical states (132). By combining chemical footprinting to measure hybrid state formation and FRET measurements to measure intersubunit rotation, viomycin, an antibiotic that prevents translocation, was shown to induce both the formation of hybrid tRNA states and intersubunit rotation, thus directly linking the two as part of the same process (133). Single-molecule studies have also explored the question of whether other changes are coupled to the movement of tRNAs into the hybrid state. Studies from one group suggest a coupling of the movement of the L1 stalk to tRNA movement (134, 135), whereas others suggest that the movements are loosely coupled and independent (136, 137).

To date, there is no high-resolution structure of the ribosome in the hybrid state during translocation. However, structures of the ribosome in a rotated conformation bound to a P/E tRNA in the presence of RRF (138) or RF3 (139) as well as that of a ribosome without tRNA but with RF3 have been determined (60), and are likely to be similar to this translocation intermediate. These structures reveal details of the conformational changes in each subunit, as well as the differences in the interactions between subunits, during ratcheting.

Rotated state: sometimes also known as ratcheted state, the rotation of the two ribosomal subunits relative to each other during translocation

Movement of mRNA and tRNA in the 30S Subunit

The second step of translocation is the movement of mRNA and tRNA with respect to the 30S subunit, which is catalyzed by EF-G. Recent FRET and biochemical studies show that the reverse rotation from the hybrid state to the classical state of the posttranslocated ribosome is coupled to the translocation of tRNA and mRNA (140). An important question is whether the mRNA moves strictly with the tRNAs, as would be the case if the base-pairing between them were maintained throughout translocation. Measurement of the rates of mRNA and tRNA translocation shows that they are essentially the same (141, 142) and occur at the same point in the cycle as the movements of tRNA in the 30S subunit (140), supporting this idea.

In the canonical state, a constriction or gate between the head and platform of the 30S subunit would inhibit the translocation of the anticodon stem of tRNA from the P to the E site (29, 143), such that the head would have to swivel out to allow translocation. Some recent studies suggest that the ribosome proceeds through a series of partially rotated intermediate states that involve varying degrees of head swivel (144, 145). Cryo-EM has been used to identify subpopulations corresponding to different states during translocation. In one of these, a novel pe/E state of tRNA made contact with both the P and E sites of a 30S

subunit with a swiveled head (145). There is some question about whether the head and tRNAs move together in translocation, as proposed in this work, or whether the head swivel opens the gate prior to the movement of P-site tRNA, as previously proposed. A recent kinetic study establishes that the ratcheting of the body occurs prior to head swiveling, followed by reversal of the head swivel, before the final reversal of the ratcheting (146).

In another cryo-EM study, a sample undergoing back-translocation, which is relatively slow, was utilized so that electron microscopy could be used to determine approximate rates and establish a sequence of events in structural terms (147). A second study used a mutation to enhance the population of some underrepresented states and elucidated the role of the L1 stalk in hybrid state formation (148). Both studies characterized a series of intermediates while also confirming earlier biochemical studies that hybrid states of the two tRNAs are separable (129, 130). Taken together, the cryo-EM studies have shed considerable light on the nature of the various intermediates formed during translocation and provide an important basis for understanding the detailed mechanism of the process.

Another long-standing question is when the E-site tRNA leaves the ribosome because its presence would inhibit movement of the P-site tRNA into the E site during translocation. In the allosteric three-site model, the E-site tRNA affinity is decreased when the A site is occupied and vice versa, suggesting that the presence of E-site tRNA increases the fidelity of A-site tRNA selection and dissociates only when the next cognate aminoacyl tRNA is brought to the ribosome by EF-Tu (recently reviewed in Reference 149). However, a recent single-molecule study shows that the dissociation of the E-site tRNA is uncoupled to the arrival of an A-site tRNA (150). Another study that is hard to rationalize either in favor of or against the model suggests that some coupling occurs in the early rounds of elongation but not later in translation (151). Furthermore, recent biochemical experiments show no increase in fidelity in the A site as a result of bound E-site tRNA (152), although these experiments have been criticized (153). Thus, some recent studies fail to support the allosteric three-site model, either for coupling of occupancy or for fidelity. However, this raises the question of why there should be a 30S E site at all, given that the 50S E site alone would be sufficient to stabilize the hybrid state during translocation. We may gain some insight from observations that release factors sense codon-anticodon mismatches in the E site as part of quality control by a yet unknown mechanism (71) and that mutations in the 30S E site do not affect miscoding but do affect frameshifting (154).

Elongation Factor G and the Kinetics of Translocation

Under some circumstances, elongation can proceed slowly even in the absence of exogenous protein factors (155), in keeping with the idea that a primordial ribosome was largely an RNA machine. However, in all kingdoms, EF-G catalyzes the translocation of tRNAs and mRNA in the 30S subunit. The factor mimics the ternary complex, as its GTPase domain resembles that of EF-Tu, and its extended domain IV mimics the anticodon arm of A-site tRNA (**Figure 7**) (156, 157).

EF-G in its GTP form stabilizes the rotated state of the ribosome, as shown by both biochemical methods (158, 159) and FRET measurements of intersubunit rotation (132, 160). A more recent FRET study suggests that EF-G can bind to the ribosome in either the classical or hybrid state, but translocation passes through the hybrid state in both cases (161). Structures using cryo-EM observed the binding of EF-G to the ribosome in the rotated state (123, 131). A more recent cryo-EM structure shows that EF-G in the GTP form binds to the rotated state with its switch helices ordered (162), as has been observed at high resolution with EF-Tu (49).

Kinetic studies show that GTP hydrolysis by EF-G precedes and greatly accelerates translocation (163). As a result, the currently

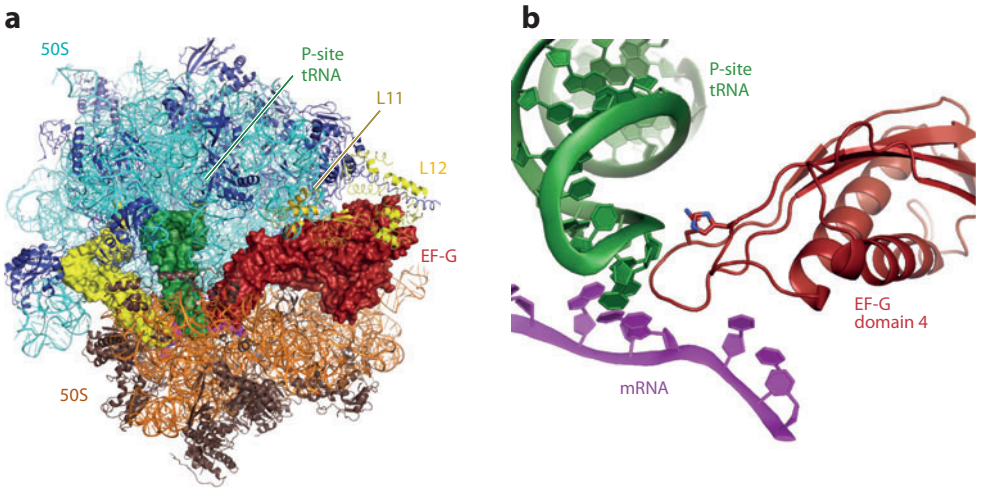


Figure 7

Crystal structure of elongation factor G (EF-G) bound in the posttranslocation state. (*a*) Overview of EF-G (*red*) bound to the ribosome in its GDP form (172). Ribosomal proteins L12, which was observed to interact with EF-G in this structure, and L11, which has been implicated in the activation of GTPases on the ribosome, are highlighted in yellow. (*b*) Extensive interactions of domain IV of EF-G with messenger RNA (mRNA) and the peptidyl tRNA were observed in the decoding center, although EF-G does not appear to interact with the aminoacyl (A)-site codon. These observed interactions may be important for maintaining the mRNA reading frame during translocation. Abbreviations: P, peptidyl.

accepted model predicts that the binding of EF-G and subsequent GTP hydrolysis lead to a rate-limiting unlocking step, which is followed by translocation and relocking (142). This view is supported by single-molecule studies (164, 165). In contrast, recent FRET measurements showed that GTP hydrolysis accelerated mRNA translocation by a factor of only two to three over the nonhydrolyzable analog GDPNP (140). The question of whether the free energy of GTP hydrolysis is used in a power stroke to drive translocation directly, or whether it is used to provide a free-energy gradient for a Brownian ratchet, was discussed extensively in a recent review (166). A direct measurement of the forces generated during translocation would be very useful to settle these questions.

In this regard, an important advance came from experiments that directly measured displacement in response to forces applied to the ends of an mRNA hairpin that impedes translocation (167). The study showed displacements that corresponded to a codon, thus directly measuring the movement as a result of translocation. However, what was particularly striking is that each step consisted of three smaller steps of roughly equal duration, suggesting that translocation may actually proceed one nucleotide at a time. This does not preclude the coupled movement of tRNA and mRNA but does suggest the existence of more intermediate states during mRNA translocation.

Several antibiotics affect various aspects of translocation. Spectinomycin binds to a hinge point between the head and neck of the 30S subunit and was proposed to inhibit translocation by inhibiting head movement (168, 169), an idea supported by recent kinetic studies (129). In higher-resolution hybrid state structures (60, 138, 139), the altered conformation of the head would apparently clash with spectinomycin. Thiostrepton, originally thought to inhibit GTP hydrolysis, has now been shown to slow a rearrangement of EF-G that follows GTP hydrolysis (129, 170). Viomycin

does not affect GTP hydrolysis but instead inhibits mRNA and tRNA movement (142) and has been shown to trap the ribosome in the intermediate or hybrid state (133), although some data suggest viomycin more specifically stabilizes a partial hybrid state with P/E and A/A tRNAs (129). It was therefore surprising to see crystal structures of both viomycin and the related capreomycin stably bound to the ribosome in the canonical state (171), in a location identical to that in the rotated state (60). The structures do not indicate why viomycin should preferentially stabilize the hybrid state.

The Posttranslocational State

Movement of the tRNAs and mRNA with respect to the 30S subunit resets the ratchet by reversing the rotation between the ribosomal subunits. This leaves the ribosome in the canonical state with tRNAs in the P and E sites. In the presence of fusidic acid, EF-G hydrolyzes GTP and catalyzes translocation but is not released from the ribosome. A crystal structure of EF-G bound with fusidic acid on the ribosome (**Figure 7*a***) reveals the interaction of the tip of domain IV of EF-G with the codon and anticodon in the P site (**Figure 7*b***) (172). Presumably this interaction initially formed in the A site and persisted throughout translocation. The structure also illustrated the binding site of fusidic acid and attempted to rationalize how the antibiotic would lock EF-G in a conformation that prevents its dissociation from the ribosome. Finally, the structure provided evidence for the interaction of GTPase factors with regions of the 50S such as the L11 region and the L12 stalk (**Figure 7*a***). The crystal structure was of modest resolution and was formed by binding EF-G to a ribosome with an empty A site. Thus the tRNA in the E site was not cognate. A higher-resolution structure of a proper posttranslocational state has the potential to settle the question of codon-anticodon pairing in the E site after translocation as well as yield the detailed interactions of fusidic acid with EF-G.

Despite the considerable progress outlined above, translocation remains the most poorly understood stage of the elongation cycle. There is still no high-resolution structure of a true translocation intermediate or even of a true posttranslocational state. No structure of the GTPase-activated form of the ribosome bound to EF-G has been determined, and thus whether the mechanism of GTP hydrolysis is identical to that of EF-Tu is unknown. Given the large conformational changes and the number of possible intermediate states, it will require a concerted effort using complementary techniques to make further progress toward understanding translocation.

THE PATH OF THE NASCENT CHAIN

A Tunnel in the 50S Subunit

During each step of the elongation cycle, the elongated polypeptide has to snake its way from the peptidyl transferase center through a tunnel in the 50S subunit, where it emerges on the solvent side of the ribosome (**Figure 8*a***). The existence of this tunnel was first inferred from experiments that localized antibodies to the nascent peptide on the far (cytosolic) side of the 50S subunit from the peptidyl transferase center (173). The exit tunnel was directly visualized first in reconstructions of 2D crystals from lizard ribosomes (174) and subsequently in that of 50S subunits from *Bacillus stearothermophilus* (175). However, these early reconstructions were of limited resolution, and more definitive evidence for the tunnel came from single-particle cryo-EM reconstructions of the ribosome (176).

The molecular details of the tunnel were first observed in the crystal structure of the 50S subunit (89). An analysis of the structure of the tunnel showed that it is effectively an unbranched tube that allows the passage of a nascent peptide (177). However, the authors also concluded that there were several points at which the tunnel would be permeable to ions and water molecules, so it could not act as a seal. It has therefore become important to understand how transmembrane ion flow is

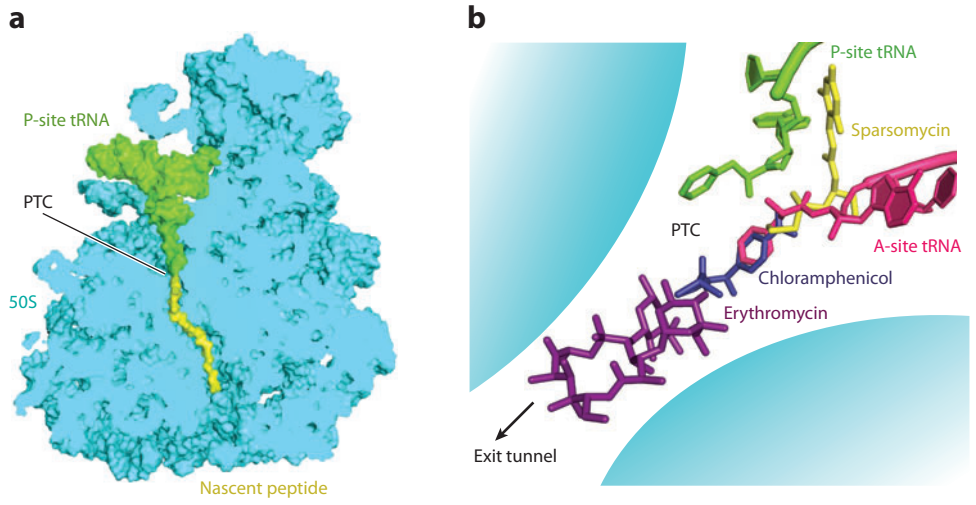


Figure 8

The ribosomal exit tunnel and antibiotic function. (*a*) Cutaway of the 50S subunit to reveal the exit tunnel containing a nascent polypeptide chain. As evidenced by a recent cryoelectron microscopy (cryo-EM) reconstruction (181), the exit tunnel provides a conduit through the 50S subunit (*cyan*) to connect the peptidyl transferase center (PTC) with the cytosol. Approximately 40 amino acids can be contained within the tunnel (*yellow*), while connected to the peptidyl transfer RNA (tRNA) (*green*). (*b*) Accepted binding sites of erythromycin (*purple*), chloramphenicol (*dark blue*), and sparsomycin (*yellow*) in the PTC and exit tunnel of the 50S subunit. Abbreviations: A, aminoacyl; P, peptidyl.

restricted when nascent peptides are inserted into a membrane via the translocon as they emerge from the ribosome. A molecular dynamics simulation suggested that the solvent in the tunnel has different properties from bulk water (178).

Conformation of the Nascent Peptide in the Tunnel

The tunnel must allow the passage of the vast number of peptide sequences synthesized by the ribosome and thus not interact too strongly with any particular sequence. However, some nascent peptide sequences are known to stall the ribosome, possibly by interacting with the tunnel and preventing progression of the peptide at subsequent steps of the elongation cycle (reviewed in Reference 179). Even more interestingly, ligands can interact with the nascent peptide to cause arrest, suggesting a general form of regulation. A particularly well-characterized example of this is the TnaC leader peptide, which requires the presence of tryptophan at the peptidyl transferase center to arrest translation (180). There is no high-resolution structure of this or any other nascent peptide in the ribosomal tunnel. However, in a cryo-EM at a resolution of 5.8 Å, a stalled TnaC leader peptide was directly visualized in the tunnel in the extended conformation (181).

The tunnel is not uniform in diameter. Near its entrance is a constriction that can accommodate only an extended polypeptide chain. Most of the tunnel has a width that can accommodate at most α-helices but not larger tertiary structures (89, 177). However, molecular dynamics simulations suggested that the last 20 Å at the mouth of the tunnel could accommodate a folded domain (182). On the basis of NMR experiments, we know that peptides can fold while being synthesized (183). However, given the space constraints within the tunnel and that several accessory proteins presumably aid the folding of the nascent peptide as it emerges

from the tunnel (184), this cotranslational folding is likely to occur after its emergence from the ribosome. Depending on whether it is secreted into or through membranes, the nascent peptide may assume specific conformations in the tunnel (185, 186).

Antibiotics in the Peptidyl Transferase Center and Entrance to the Tunnel

Many antibiotics bind at the peptidyl transferase center or at the entrance to the tunnel (reviewed in Reference 187) (**Figure 8*b***). For example, chloramphenicol binds at the A site of the peptidyl transferase center and thus directly inhibits peptide bond formation by preventing binding of the A-site substrate, whereas the macrolide antibiotics bind at the entrance to the tunnel and prevent access of the nascent peptide to the tunnel. This is in keeping with the finding that only antibiotics that interfere with the peptidyl transferase center prevent peptide bond formation (188). Resistance mutations to these antibiotics occur mainly at their binding sites and can be explained by crystal structures as a loss of affinity. However, mutations to proteins L4 and L22, portions of which line the exit tunnel, confer erythromycin resistance even though they are quite far from its binding site. Recent observations suggest that the antibiotic enters the ribosome through the tunnel and that these mutations block its passage (189). Although there are species-specific differences in drug action, these antibiotics are largely broad-spectrum, consistent with the high conservation of their binding sites in the ribosome.

The structures of several antibiotics bound to the 50S subunit were initially determined using the eubacterium *Deinococcus radiodurans* (190). However, several of these initial structures, including those of chloramphenicol, erythromycin, and sparsomycin, differ significantly from those subsequently determined by other laboratories using archaeal 50S subunits (191, 192), ribosomes from other bacterial species (193, 194), and even structures reported by a subsequent study using the original species of *D. radiodurans* (195). All these subsequent structures in both archaeal and several species of bacterial ribosomes are in good agreement with one another, and their interactions with the ribosome are chemically reasonable. Their differences with the original structures include an almost 90° rotation of the macrolide ring in antibiotics such as erythromycin, a flipped orientation for chloramphenicol, and a completely different binding site for sparsomycin. The original structures of these antibiotics (190) are therefore incompatible with subsequent studies by several other groups.

CONCLUSIONS

The past fifteen years have seen a dramatic advance in our understanding of ribosome structure and function, and in particular of the elongation cycle. A striking aspect of ribosome function is how dynamic both the ribosome and tRNA are during translation. The distortions of the tRNA as it goes through the elongation cycle are remarkable and provide insights into the constraints on its evolution (**Figure 9**). Similarly, large-scale conformational changes in the ribosome both within and between

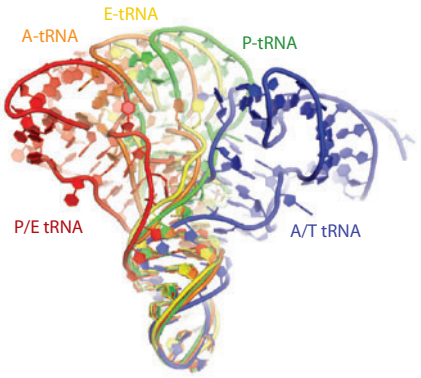


Figure 9

Dynamic movements of transfer RNA (tRNA) during translation. Recent high-resolution crystal structures have illustrated the range of conformations required of tRNA throughout translation. This was further demonstrated by cryoelectron microscopy reconstructions of several intermediate states in tRNA movement through the ribosome (147). Abbreviations: A, aminoacyl; E, exit; P, peptidyl.

subunits are a feature of virtually every step of the process. Biochemistry can inform us of rates for each step, key residues, and chemical mechanisms, whereas structural studies can provide snapshots of various states of the ribosome. However, there may be several short-lived intermediate states that are not accessible to these techniques. Moreover, how the ribosome progresses from one state to another will require new techniques, of which molecular dynamics is likely to play an increasingly important role. However, this will require advances in both computation and the quality of the structural data because the current timescales of these simulations do not generally match the timescales of the actual processes being simulated, and even the best structures of the whole ribosome are at approximately 3 Å resolution, so that the accuracy of the starting models is less than optimal.

The focus on the elongation cycle will gradually shift to other aspects of ribosome function including the interaction of the nascent chain with factors that aid its folding or transport and cellular factors that are involved in quality control or stress. We also expect future studies to focus on the mechanism and regulation of initiation in both bacteria and eukaryotes.

SUMMARY POINTS

1. The translational elongation cycle is the process by which the ribosome adds amino acids to a growing polypeptide chain during protein synthesis.
2. It is the most central and likely the oldest aspect of translation and is thus the most highly conserved.
3. Many of the stable intermediates in the elongation cycle have been crystallized and their structures determined to high resolution. Others have been characterized to lower resolution by cryoelectron microscopy.
4. New methods such as single-molecule biophysical measurements and molecular dynamics are playing an increasingly important role in understanding the mechanisms of translation.
5. The combination of structural information and biochemical studies has led to a far more detailed understanding of many aspects of the elongation cycle.

FUTURE ISSUES

1. There is no high-resolution structure of a true translocation intermediate in the hybrid state or of EF-G prior to GTP hydrolysis. These structures will aid our understanding of translocation and the mechanism of activation of translational GTPases on the ribosome.
2. Does translocation involve a force, and what is the role of GTP hydrolysis by EF-G during translocation?
3. What is the role of the protein L7/L12?
4. What is the function of the E site, and how can we address discrepancies about its role?

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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RELATED RESOURCES

1. Overall movie of translation in .wmv format (**http://www.mrc-lmb.cam.ac.uk/ribo/homepage/movies/translation_bacterial.wmv**) or Quicktime format (**http://www.mrc-lmb.cam.ac.uk/ribo/homepage/movies/translation_bacterial.mov**)

2. Movie of decoding in .wmv format (**http://www.mrc-lmb.cam.ac.uk/ribo/homepage/movies/New_decoding_minor_musical_opt2_title.wmv**) or Quicktime format (**http://www.mrc-lmb.cam.ac.uk/ribo/homepage/movies/New_decoding_minor_musical_opt2_title-comp1.mov**)
3. Movie of the peptidyl transferase reaction: **http://www.youtube.com/watch?v=OaQan4O0K_Q**

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