REVIEW

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Ribosomal Translocation: One Step Closer to the Molecular Mechanism

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ABSTRACT Protein synthesis occurs in ribosomes, the targets of numerous antibiotics. How these large and complex machines read and move along mRNA have proven to be challenging questions. In this Review, we focus on translocation, the last step of the elongation cycle in which movement of tRNA and mRNA is catalyzed by elongation factor G. Translocation entails large-scale movements of the tRNAs and conformational changes in the ribosome that require numerous tertiary contacts to be disrupted and reformed. We highlight recent progress toward elucidating the molecular basis of translocation and how various antibiotics influence tRNA−mRNA movement.

In all cells, proteins are synthesized by ribosomes, megadalton RNA-protein machines that use aminoacyl-tRNA (aa-tRNA) molecules to translate messenger RNA (mRNA). Each ribosome is composed of a large and small subunit. The *Escherichia coli* large (50S) subunit consists of 2 RNA molecules (23S, 2904 nt; 5S, 120 nt) and 34 proteins (L1−L7 and L9−L36), whereas the small (30S) subunit consists of 1 RNA molecule (16S, 1542 nt) and 21 proteins (S1−S21). During translation, incorporation of each amino acid into the nascent polypeptide chain involves three sequential steps: decoding, peptidyl transfer, and translocation (Figure 1). Decoding is facilitated by elongation factor Tu (EF-Tu), which delivers aa-tRNA to the A (aminoacyl) site as part of a ternary complex with GTP (*1*, *2*). When codon recognition occurs in the 30S A site, EF-Tu is activated to hydrolyze GTP, which promotes release of the acceptor end of aa-tRNA and its movement into the 50S A site. Once aa-tRNA is in the A site of both subunits, the ribosome catalyzes transfer of the peptidyl group of P-site tRNA to A-site aa-tRNA. This leaves a complex containing peptidyl-tRNA in the A site and deacylated tRNA in the P site, termed the pretranslocation (PRE) complex. Translocation of the tRNAs to their adjacent sites is then catalyzed by elongation factor G (EF-G), which hydrolyzes GTP in the process. Translocation is believed to occur in a stepwise manner. First, the tRNA acceptor stems move within the 50S subunit to form the hybrid-state complex, where peptidyl-tRNA and deacylated tRNA occupy the A/P and P/E sites, respectively. Then, the codon−anticodon helices move within the 30S subunit to form the post-translocation (POST) complex, in which peptidyl-tRNA and deacylated tRNA occupy the P and E sites of both subunits (P/P and E/E sites). This leaves the A site vacant, ready for the next round of elongation.

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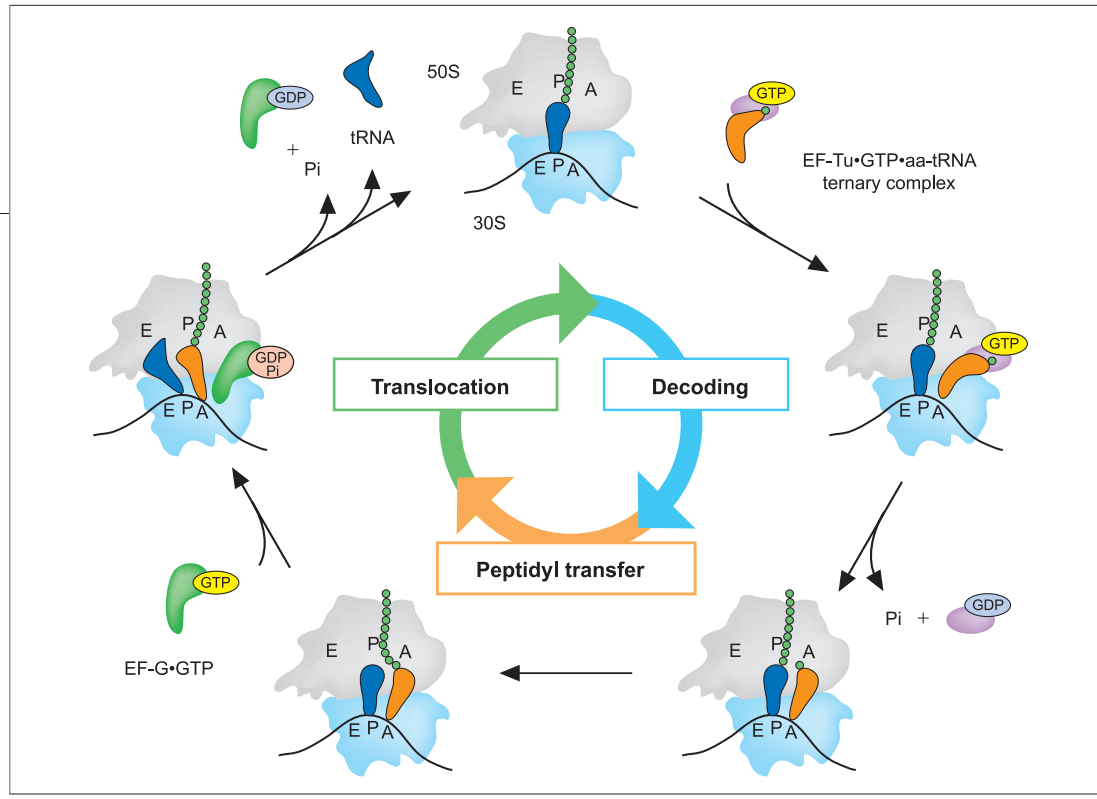

Figure 1. Scheme for a translation elongation cycle. Each cycle of translation elongation is composed of three major steps: decoding, peptidyl transfer, and translocation. In decoding, aminoacyl-tRNA (aa-tRNA) is delivered to the A site as part of a ternary complex with EF-Tu and GTP. This is followed by rapid and functionally irreversible transfer of the peptidyl group from P-tRNA to A-tRNA. EF-G-GTP then catalyzes translocation, the coupled movement of tRNA and mRNA in the ribosome. Deacylated tRNA dissociates from the E site before or during the next round of elongation.

Structural Insights into Translocation. With a diameter of ~200 Å, the ribosome is one of the largest molecular particles in the cell. Its basic architecture of two asymmetric subunits has been known since 1955, when it was first visualized by electron microscopy (*3*). However, a clear view of the ribosome has come only recently, when atomic resolution structures of each subunit were solved in 2000 (*4–9*). These data, along with structures of functional 70S complexes (*10*), set a milestone for the ribosome field and RNA research in general. These and more recent structures have provided a wealth of information relevant to translation and have increased our knowledge of RNA–RNA and RNA–protein interactions tremendously (*11*).

The ribosome forms multiple interactions with tRNA in each of the A, P, and E sites (Figure 2). Most contacts involve the sugar–phosphate backbone of tRNA, explaining how ribosomes deal with a diverse set of tRNA species. There are, however, several examples where base-specific contacts play significant roles in ribosome function. Because detailed intra-/intermolecular interactions in ribosomal complexes have been summarized elsewhere (*10–14*), our focus in this Review will be on interactions that may hold particular relevance for translocation.

In the A site, C75 of the universally conserved 3′ CCA end of tRNA forms a Watson–Crick base pair with G2553 of 23S rRNA, which helps position the aminoacyl group of A-tRNA for peptidyl transfer (Figure 2, panel b) (*6, 15, 16*). The elbow region of tRNA interacts with helix 38 of 23S rRNA (H38, also called the A-site finger), which additionally contacts the N terminus of ribosomal protein S13 to form bridge B1a. Shortening of the A-site finger can stimulate translocation, suggesting that this element normally acts to negatively control tRNA movement (*17*). The anticodon stem loop (ASL) of tRNA binds the 30S A site, which is located between the shoulder and head domains of the 30S subunit. The first two base pairs of the codon–anticodon helix interact with G530, A1492, and A1493, three universally conserved 16S rRNA nucleotides (Figure 2, panel c). Binding of cognate tRNA to the A site causes A1492 and A1493 to flip out of helix 44 (h44) and dock into the minor groove of the codon–anticodon helix, forming type II and I base triples, respectively. This rearrangement has been implicated in discrimination of cognate from near-cognate aa-tRNA in decoding (*2*). EF-G is unable to promote translocation in complexes with P-tRNA and a vacant A site, whereas the additional presence of an ASL in the 30S A site allows translocation (*18*). Thus, the ribosomal conformational changes induced by EF-G are insufficient to move P-tRNA to the E site, unless A-tRNA is engaged in the 30S decoding center. This idea is corroborated by a recent study showing that mutations at

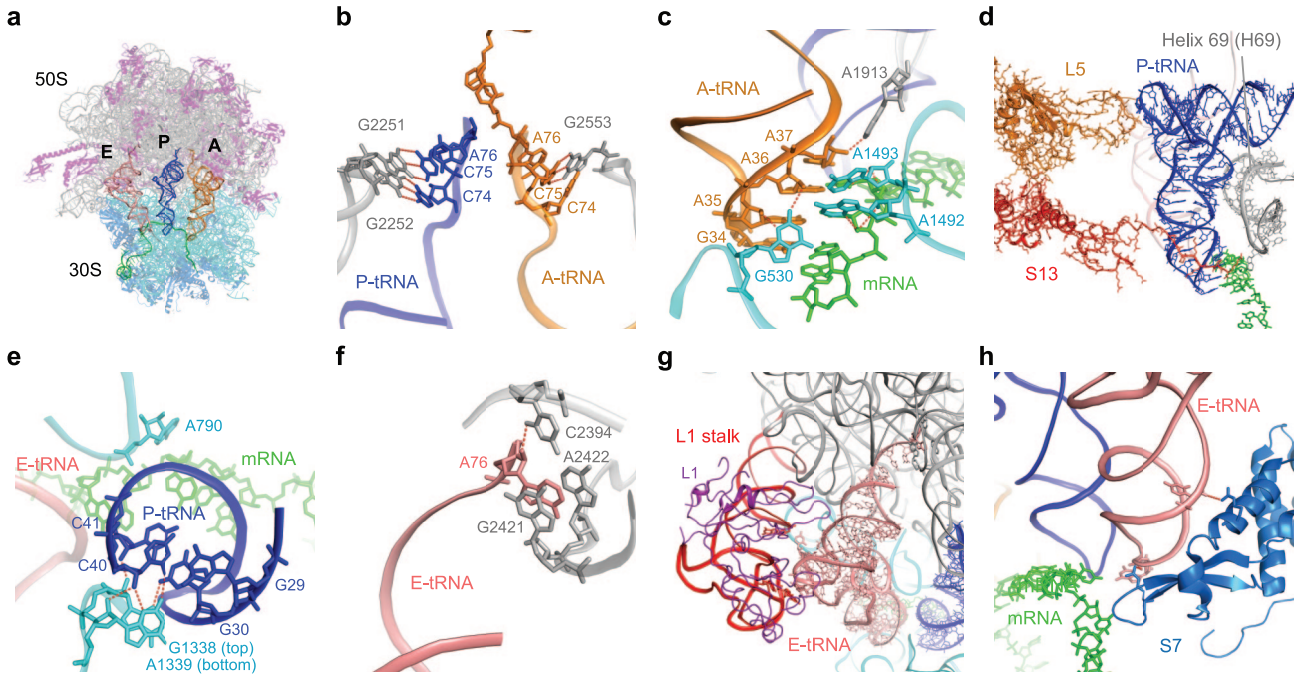

Figure 2. tRNA−ribosome interactions in A, P, and E sites. a) A model of a ribosomal complex containing mRNA and tRNAs in all three sites. b) Interactions at the 3′ CCA ends of A-tRNA and P-tRNA. c) Interactions at the 30S A site. d) Interactions of P-tRNA with H69 of 23S rRNA and ribosomal proteins L5 and S13. e) Type I/II base triples at A1339 and G1338 of 16S rRNA in the 30S P site and the "gate" formed by the A790 loop and the 1338−1339 loop. f) Interactions between A76 of tRNA and 23S rRNA. g) Interactions between the L1 stalk and E-tRNA. h) Interactions between E-tRNA and ribosomal protein S7. Images in panels a and c−h were generated from crystal structures of *Thermus thermophilus* ribosomes containing A-, P-, E-tRNA, and mRNA (PDB IDs: 2HGP, 2HGQ, 2J00, and 2J01) (*14, 125*). An image in panel b was generated from a structure of the *Haloarcula marismortui* 50S subunit with A- and P-site substrates (PDB ID: 1KQS) (*126*). 23S rRNA and 16S rRNA are shown in gray and cyan, respectively. *E. coli* numbering of rRNA nucleotides is used throughout.

G530, A1492, and A1493 increase puromycin reactivity of A-tRNA and inhibit EF-G-dependent translocation but do not affect EF-G-independent tRNA movement (*19*). These data suggest that these nucleotides ensure correct positioning of tRNA in the A site, which is important for EF-G-catalyzed translocation.

In the P site, C74 and C75 of tRNA form Watson−Crick base pairs with G2252 and G2251 of 23S rRNA, respectively, orienting the 3′ end of peptidyl-tRNA for peptidyl transfer (Figure 2, panel b) (*6, 15, 16*). The tRNA elbow interacts with the C terminus of L5, whose N terminus binds S13 to form bridge B1b (Figure 2, panel d). The D stem of P-tRNA interacts with the minor groove of H69 of 23S rRNA, which also contributes to bridge B2a (Figure 2, panel d). The ASL of P-tRNA is contacted by ribosomal proteins S9 and S13 and several residues of 16S rRNA, including A1339 and G1338. These nucleotides interact with base pairs 30−40 and 29−41 in the acceptor stem of P-tRNA to form type I/II base triples, respectively (Figure 2, panel e) (*12–14*). Mutational studies have shown that these 16S rRNA nucleotides, especially A1339, are critical for translation *in vivo* and for binding of tRNA to the P site *in vitro* (*20–23*). The loop containing A1339 and G1338 forms a "gate" with the 790 loop between the P and E sites (Figure 2, panel e). This gate appears to block movement of tRNA from the P to the E site and thus may act to control tRNA movement during translocation (*14, 24*).

In the E site, the 3′ terminal adenosine (A76) of tRNA intercalates between G2421 and A2422 and interacts with C2394 of 23S rRNA in the 50S subunit (Figure 2, panel f) (*13, 14, 25*). Truncation of A76 decreased the affinity of $tRNA^{Phe}$ for the E site by 100-fold, suggesting that A76 is critical for the stability of E-tRNA (*26*). Either modification of A76 of P-tRNA or mutation of C2394 of 23S rRNA inhibited translocation, providing evidence

that movement of tRNA into the 50S E site is integral to the mechanism (*26–30*). The L1 stalk of the 50S subunit interacts with the tRNA elbow and is thought to facilitate the movement of tRNA from the P site to the E site (Figure 2, panel g) (*13, 31*). In the 30S subunit, the ASL of E-tRNA interacts predominantly with the β-hairpin and C-terminus of S7 and makes no direct contacts with 16S rRNA (Figure 2, panel h) (*13, 14*). These observations may explain why E-tRNA failed to protect any 16S rRNA nucleotides from chemical probes (*32–34*). It was recently shown that truncation of the β-hairpin of S7 decreases E-tRNA binding but does not inhibit translocation (*35*). Thus, the role of the E site in translocation is largely confined to the 50S subunit.

Spontaneous Movement of tRNA within the 50S Subunit. The first experimental evidence for the hybrid-state model came from chemical probing studies (*33*). It was observed that, following peptidyl transfer, the tRNAs moved spontaneously within the 50S subunit, that is, from the "classical" A/A and P/P sites to the "hybrid" P/E and A/P sites. These findings were corroborated by concurrent bulk FRET studies, which showed that the newly deacylated tRNA moved >20 Å toward L1 after peptidyl transfer, while the peptidyl group moved little (*36*). More recently, single-molecule FRET studies have shown spontaneous and rapid fluctuations of tRNA between the classical and hybrid sites in the PRE complex (*31, 37, 38*). Interestingly, an additional tRNA configuration was found in the PRE complex, consistent with one tRNA in the A/A site and the other in the P/E site (*38*) The stabilities of these distinct configurations (*i.e.*, P/P, A/A; P/E, A/A; P/E, A/P) were similar to one another, and the rate of tRNA fluctuation within the 50S subunit ($1-5\ s^{-1}$) was much higher than that of spontaneous forward translocation ($<5 \times 10^{-4}\ s^{-1}$) (*38*). These data indicate that movement of tRNA in the 30S subunit limits the overall rate of translocation.

Spontaneous Movement of tRNA and mRNA in the Ribosome. For many years it was believed that translocation is inherently exergonic, or thermodynamically favorable (*39–41*). This idea stemmed largely from the observation that ribosomes programmed with polyuridylic acid (poly-U) can synthesize polyphenylalanine in the absence of elongation factors and GTP (*42–44*). It was proposed that peptidyl transfer destabilizes the PRE state, making the subsequent step of translocation energetically favorable (*45*). Consistent with this "thermodynamic gradient" model (*40*), efficient factor-independent translocation of *N*-acetyl-$(Phe)_2$-$tRNA^{Phe}$ in ribosomes programmed with poly-U was observed (*46, 47*). However, recent studies have shown that spontaneous translocation can also proceed in the reverse direction at rates comparable to those measured for spontaneous forward translocation (*48, 49*). In several contexts, complexes in the POST state were completely converted to the PRE state, showing that tRNA−mRNA movement in the forward direction can be endergonic. The relative stability of the POST versus PRE state depended on the species of tRNAs bound (*48, 49*). For instance, complexes containing $tRNA^{fMet}$ and $tRNA^{Phe}$ in the E and P sites were less prone to undergo reverse translocation than those containing $tRNA^{fMet}$ and $tRNA^{Val}$. This context dependence may explain why spontaneous reverse translocation was observed only recently and why factor-independent translocation on heteropolymeric mRNA has not been reported.

EF-G Structure. Translocation is catalyzed by EF-G, which accelerates the rate of the reaction by 4−5 orders of magnitude (*50*). EF-G is a ~78 kDa protein composed of 5 domains, and its structure has been fittingly described as tadpole-shaped (Figure 3, panel a) (*51, 52*). The largest domain (domain I or G domain) is homologous to GTPases of the Ras superfamily (*53, 54*). Members of this family have in common the ability to couple GTP hydrolysis to a conformational change that alters the functional state of the protein (*55*). Domain II of EF-G is an all-β structure, classified as a twisted β-barrel or β-sandwich. Structural-based sequence alignments indicate that domains I and II of EF-G are homologous to the corresponding domains of EF-Tu and other translational GTPases (*e.g.*, LepA, SELB, IF2, and RF3) (*53*), suggesting that these two domains function together on the ribosome and coevolved from a common ancestral protein. A notable difference between the G domains of the elongation factors is the presence of an ~90-residue subdomain (G′) in EF-G. Domains III and V contain a common protein motif referred to as the ribonucleoprotein (RNP) or RNA recognition motif (RRM), observed in many RNA-binding proteins (*56*). Domain IV of EF-G has a unique α-β fold and extends out from the rest of the protein, like the tail from the tadpole body.

EF-G Interaction with the Ribosome. Cryo-electron microscopy (cryo-EM) and chemical probing studies have shown that EF-G binds the intersubunit space on the A-site side of the ribosome (*57–65*). The position and relative orientation of the domains of EF-G are most

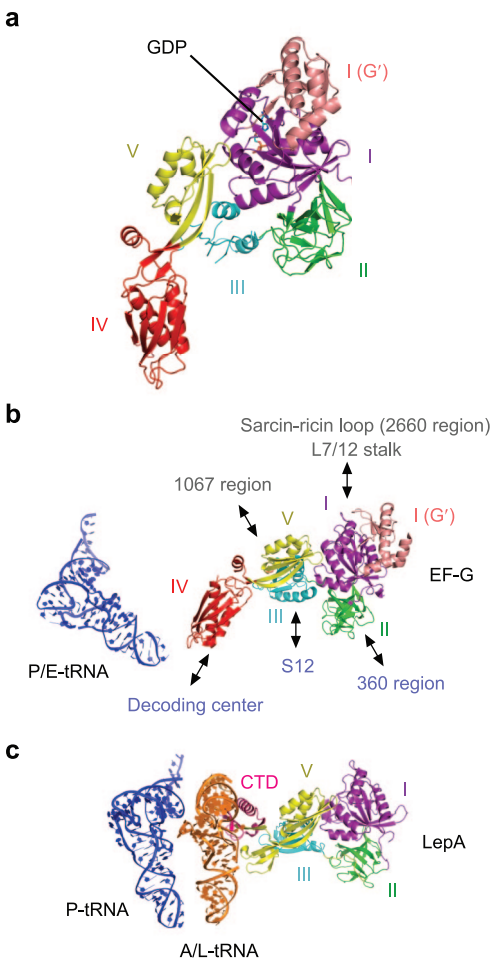

Figure 3. Structure of EF-G. a) A crystal structure of *T. thermophilus* EF-G·GDP (PDB ID: 2EFG) (*52*). Domain III is partially disordered and thus not fully visible. b) A structural model of EF-G based on cryo-EM reconstructions of complexes containing EF-G·GDPNP and P/E-tRNA (PDB ID: 2OM7) (*64*). Notable interactions of EF-G with ribosomes are shown by arrows. Ribosomal components in the 50S and 30S subunits are colored in gray and blue, respectively. c) A structural model of LepA based on cryo-EM reconstructions of complexes containing LepA·GDPNP, P-tRNA, and A/L-tRNA (PDB ID: 3DEG) (*116*).

clearly resolved in complexes lacking an A-tRNA (and hence resembling a POST-like state). In these complexes, domains I and V of EF-G interact primarily with the 50S subunit, while domains II, III, and IV interact primarily with the 30S subunit. On the 50S side, domain I contacts the universally conserved sarcin-ricin loop (2660 region of 23S rRNA) and the L7/12 stalk. This stalk, composed of multiple copies of L7/12 bound to L10, is thought to "catch" and "hand over" translation factors to the ribosome (*66, 67*). Domain V of EF-G interacts with the 1067 region of 23S rRNA, the site targeted by the antibiotic thiostrepton. On the 30S side, domain II of EF-G makes contact to 16S rRNA in the 360 region, while domain III is positioned to interact with S12. Domain IV, the extended "tadpole" domain, protrudes into the 30S decoding center, functionally occluding A-tRNA.

Much effort has been made to determine the position of EF-G on the ribosome before translocation takes place, although such experiments have proven more challenging. The available data regarding EF-G bound to ribosomes in the PRE state suggest that domains I and II occupy the same positions (as in the POST-like state) but the other domains are reoriented and/or mobile (*59, 61, 68*). It has been suggested that EF-G initially docks with domains I and II, and a hinge-like motion of the remainder of the protein (domains III, IV, and V) accompanies translocation (*41*). This general model is consistent with evidence that domains IV and V play a crucial role in catalysis of tRNA−mRNA movement. Deletion of these domains decreases the rate of single-turnover translocation by 3−4 orders of magnitude without affecting GTP hydrolysis (*69–71*). Furthermore, an engineered EF-G variant with a cross-link between domains I and V was inactive in translocation, consistent with the idea that catalysis involves movement of domains III−V relative to domains I and II (*72*).

Movement of tRNA into the P/E Site Is Accompanied by an Intersubunit Rotation. Cryo-EM studies have shown a correlation between occupation of the P/E hybrid site and a ratchet-like subunit rotation (RSR) (Figure 4, panel a) (*58, 62, 64, 73*). In complexes containing deacylated tRNA and EF-G·GDPNP, the acceptor stem of the P-tRNA was dis-

KEYWORDS

Ribosomes: Megadalton ribonucleoprotein particles that synthesize protein in all cells and bacterium-derived organelles. The ribosome is composed of three or four large RNA molecules and >50 distinct protein molecules. Each ribosome has two subunits and three tRNA binding sites, aminoacyl (A), peptidyl (P), and exit (E), which lie at the subunit interface. Codons on mRNA are iteratively decoded on the small subunit, while peptidyl transfer occurs on the large subunit.

Translocation: Coupled movement of tRNA and mRNA catalyzed by elongation factor G (EF-G) and GTP. After peptidyl transfer, the pretranslocation (PRE) complex contains peptidyl-tRNA and deacylated tRNA in the A and P sites of the ribosome, respectively. Translocation converts the PRE complex to the post-translocation (POST) complex, containing tRNAs in the P and E sites.

Sparsomycin: An antibiotic produced by *Streptomyces sparsogenes* that binds at the 3′ end of peptidyl-tRNA in the 50S P site and blocks peptidyl transfer. Sparsomycin has a unique activity in that it can promote translocation in the absence of EF-G and GTP.

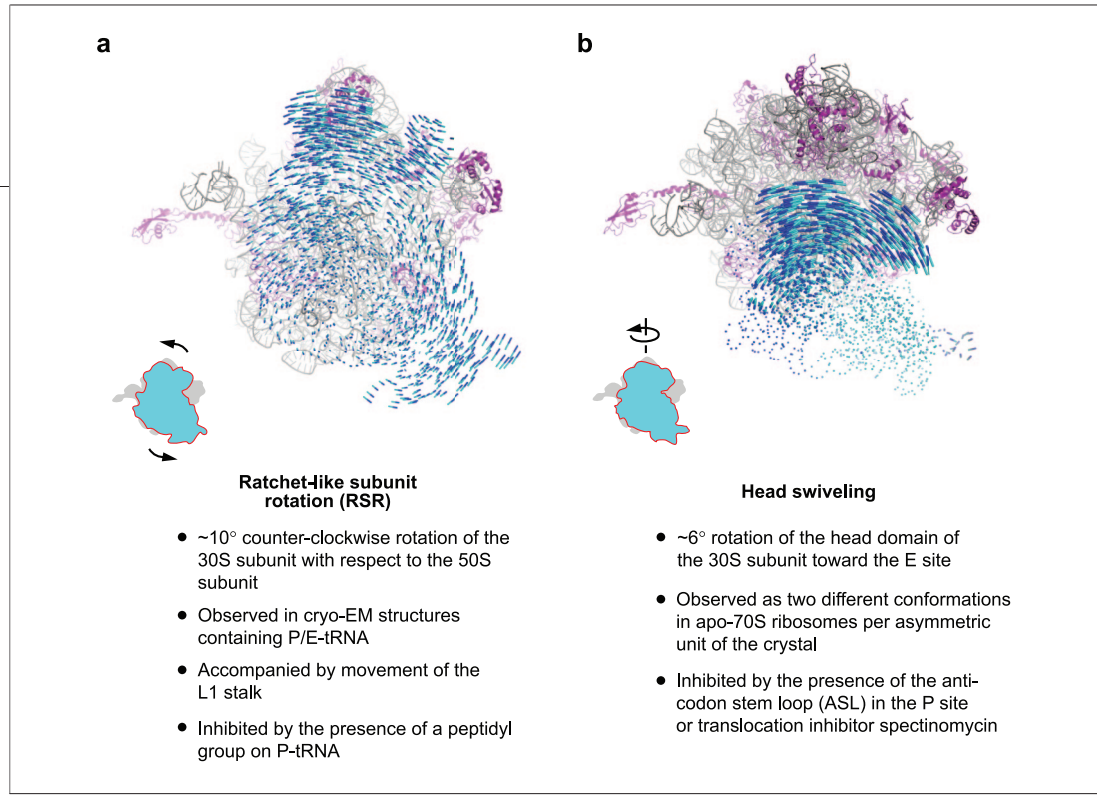

Figure 4. Structural rearrangements in the 30S subunit. Connected dots represent movement of phosphorus atoms in 16S rRNA during the RSR (panel a) or the head swiveling (panel b). Images were generated from available cryo-EM (PDB ID: 1P87, 1P86, 1P6G, and 1P85) (*127*) and crystal structures (PDB ID: 2AVY, 2AW7, 2AW4, and 2AWB) (*24*).

placed by ~35 Å into the 50S E site, while the L1 stalk was repositioned inward by ~20 Å. This movement of the L1 stalk may result in direct interaction with tRNA that is maintained throughout translocation (*31*). At the same time, the 30S subunit was observed to rotate by 7−10° with respect to the 50S subunit. As a result of this rotation, the A-site finger lost its contact with S13 and formed a new one with S19, consistent with biochemical evidence that the A-site finger participates in translocation (*17*). While deacylated tRNA can occupy the P/E site and allows the RSR, peptidyl-tRNA is restricted to the P/P site and prohibits the RSR, suggesting that the RSR is coupled to the movement of deacylated tRNA from the P/P to the P/E site. However, it was found that eEF2 (a eukaryotic homologue of EF-G) can induce the RSR on eukaryotic 80S ribosomes in the absence of tRNA, suggesting that the RSR and the P/P-to-P/E transition are not strictly coupled (*60*). According to a single-molecule FRET study, either the presence of EF-G or the ability of tRNA to occupy the P/E site is sufficient for intersubunit rotation (*74*). This study also provided evidence that the RSR is reversible, and fluctuation between the rotated and nonrotated states occurs at a rate of 0.2−2 s^{-1}, somewhat slower than that observed for the P/P ⇆ P/E fluctuation (1−5 s^{-1}) (*38*). Recently, cryo-EM reconstructions of the PRE complex in the absence of EF-G, containing deacylated tRNA and peptidyl-tRNA in the P/E and A/P sites, respectively, were reported (*75, 76*). The RSR was again observed, providing further evidence that the RSR is related to hybrid-state formation and can occur in the absence of EF-G.

Role of GTP Hydrolysis in Translocation. In the 1970s, it was shown that EF-G can promote single-turnover translocation in the presence of nonhydrolyzable analogs of GTP (*e.g.*, GDPNP) but GTP was necessary for multiple-turnover translocation (*77*). These data implied that GTP hydrolysis was important for release of EF-G after translocation rather than for tRNA−mRNA movement itself. For many years following this observation, it was believed that interaction of the GTP-bound form of EF-G with the PRE complex promoted tRNA−mRNA movement and that subsequent GTP hydrolysis facilitated release of the factor. However, this model was refuted in the 1990s, when it was shown that hydrolysis of GTP occurs much more rapidly than movement of tRNA−mRNA (*69*). Furthermore, GTP hydrolysis stimulated the rate of translocation and the rate of EF-G turnover by ~50-fold, indicating that the free energy released from GTP hydrolysis is coupled to both translocation and EF-G release (Table 1) (*50*). The molecular mechanism by which GTP hydrolysis promotes these subsequent events remains unclear but is thought to involve coordinated conformational rearrangements of the ribosome and EF-G.

TABLE 1. Rates of translocation

Direction	Antibiotic	Factor	Rate ($s^{-1} \times 10^3$)	refs
Reverse	None	None	0.7−2	*49, 85, 91*
Reverse	Par/Neo/Gen[a]	None	6	*49, 85*
Reverse	Streptomycin	None	30	*49*
Forward	None	None	0.007−0.5	*47, 49, 50, 96*
Forward	Sparsomycin	None	5	*96*
Forward	None	EF-G·GTP	10,000−30,000	*30, 69, 81, 86, 120*
Forward	None	EF-G·GDPNP[b]	500	*69*
Forward	None	EF-GΔ4,5[c]	8	*86*

[a]Par, paromomycin; Neo, neomycin; Gen, gentamicin. [b]GDPNP, guanosine 5′-[β,γ-imido]-triphosphate. [c]EF-GΔ4,5 contains deletions in domains IV and V of EF-G.

At least in certain ribosomal complexes, EF-G·GDPNP accelerated single-turnover translocation by >1000-fold compared to the uncatalyzed rate (Table 1) (*50*). An EF-G mutant (R29A) that lacks GTPase activity stimulated translocation similarly (*78*). On the other hand, EF-G containing GDP or lacking a guanosine nucleotide exhibited little or no translocase activity (*50, 69, 79–81*). Together, these data indicate that a large part of catalysis comes from the binding of EF-G, although productive binding requires an active conformation of the G domain.

Kinetic Model of EF-G-Dependent Translocation. Wintermeyer and colleagues (*50, 69, 70*) have employed rapid mixing methods to measure apparent rates for several observables during EF-G-catalyzed translocation, including EF-G binding, GTP hydrolysis, Pi release, tRNA movement, and mRNA movement. Based on these data, a kinetic model for translocation has been proposed (*70*). According to this model, EF-G·GTP binding is facilitated by L7/L12 (*66*) and is followed by rapid GTP hydrolysis (>150 s^{-1}). Then, a slower step occurs (~25 s^{-1}), which limits the observed rates of both tRNA−mRNA movement and Pi release. This slower step was attributed to a conformational change in the ribosome that has been termed *unlocking*. Deletion of domains IV and V of EF-G slowed tRNA−mRNA movement and Pi release identically, supporting the existence of a rate-limiting conformational change that precedes both events (*70*). Although both are limited by unlocking, tRNA−mRNA movement and Pi release are independent of one another and probably occur in random order. Evidence that these events are independent comes from antibiotics and mutations that block either tRNA−mRNA movement or Pi release. Paromomycin, hygromycin B, and viomycin strongly inhibited tRNA−mRNA movement but did not decrease the rate of Pi release (*70, 82*). Mutation K70A of ribosomal protein L7/12 conferred the opposite phenotype, a decreased rate of Pi release without a substantial decrease in the rate of tRNA−mRNA movement (*70*). Finally, ribosomal rearrangements must occur to *relock* the tRNAs in their new sites, followed by release of EF-G·GDP from the POST complex, although the kinetics of these events have yet to be fully characterized.

Recently, this kinetic model has been expanded to incorporate the movement of tRNAs with respect to both subunits. In the work of Pan *et al*. (*81*), which examined translocation of proflavin-labeled tRNAs, two intermediates were detected that are believed to correspond to individual movements of the P- and A-site tRNAs with respect to the large subunit. Addition of viomycin stabilized a state in which tRNAs were predicted to occupy the P/E and A/A sites, while spectinomycin stabilized a kinetically competent intermediate called the INT complex, thought to contain P/E- and A/P-tRNAs. The rate of formation of these intermediates was slower than GTP hydrolysis, suggesting that GTP hydrolysis precedes tRNA movement within the 50S subunit.

Support for the conclusions drawn by Pan *et al*. came from another study that investigated the movement of tRNAs within the large subunit (*30*). In these experiments, movement of tRNA into the P/E or A/P site was inhibited by rRNA and tRNA substitutions. When movement of tRNA into the P/E site was inhibited by mutation

of C2394 (of the 50S E site), both the maximal rate of translocation and the apparent affinity of EF-G for the PRE complex were decreased. In contrast, when movement of tRNA into the A/P site was inhibited by substitution of the peptidyl group on the A-site tRNA with an aminoacyl group, translocation was slowed, but no decrease in the apparent affinity of EF-G for these PRE complexes was seen. This difference suggests that movement into the P/E and A/P sites are kinetically separable events, and movement of tRNA into the P/E site contributes to high-affinity interaction of EF-G with the PRE complex. It was also shown that C2394A did not decrease the rate of single-turnover GTP hydrolysis. Together these data suggest that rapid and reversible GTP hydrolysis precedes P/E-state formation (*30*).

To explain how EF-G promotes translocation, a "Brownian motor" model has been proposed by Wintermeyer and colleagues (*83*). According to this model, EF-G acts as a Brownian motor that couples GTP hydrolysis to directional movement of tRNA. Conformational changes in a Brownian motor imposed by hydrolysis of NTP allows Brownian movement of the molecule along the reaction coordinate, followed by a second step that creates a biased thermodynamic gradient that produces forward movement (*84*). Likewise, it was proposed that tRNA freely fluctuates between their PRE and POST configurations in the unlocked ribosome, and the subsequent relocking step biases tRNA movement in the forward direction (*83*). Structural and biochemical studies have shown that translocation inhibitor spectinomycin inhibits swiveling of the head of the 30S subunit (Figure 4, panel b) (*85*) but does not inhibit tRNA movement with respect to the 50S subunit (*81*), implying that unlocking requires 30S head swiveling. In the unlocked ribosome, domain IV of EF-G would be inserted into the 30S A site to block the reverse movement of tRNA as part of relocking (*83, 86*). It is worth noting that domains IV and V of EF-G must also participate in unlocking, since deletion of these domains drastically decreases the rate of Pi release and tRNA−mRNA movement without affecting GTP hydrolysis (*69, 70, 86*). This Brownian motor model is consistent with the finding that spontaneous reverse translocation can occur (*48, 49*), which implies that EF-G must act to prevent the backward movement of tRNA in the unlocked ribosome.

Together, the data discussed above suggest the following model of EF-G-dependent translocation (Figure 5). After peptidyl transfer, EF-G·GTP binds to the PRE complex (with tRNAs in the classical A and P sites) and hydrolyzes GTP. P-tRNA then reversibly moves with respect to the 50S subunit to form the P/E state, which could be coupled to the intersubunit rotation of the ribosome (RSR). Then, A-tRNA is allowed to move with respect to the 50S subunit to form the A/P state, which appears to be a functionally irreversible step. Next, ribosomal unlocking occurs, which allows thermal fluctuation of tRNAs. Head swiveling of the 30S subunit may occur in this step. Movement of tRNAs and Pi release occur independently of each other in the unlocked ribosome, and tRNAs are then locked in the classical P and E sites by reverse structural rearrangements of the ribosome (relocking). Insertion of domain IV of EF-G into the 30S A site during this step can explain how a bias in directionality is created. Finally, dissociation of EF-G·GDP may be facilitated by structural changes in the complex such as retraction of domain IV or reverse RSR.

Effects of Antibiotics on Translocation. Aminoglycosides are well-known for their ability to cause miscoding (Figure 6) (*87–90*). The aminoglycoside paromomycin binds to h44 of 16S rRNA and causes A1492 and A1493 to flip out, positioning them to contact the A-site codon−anticodon helix (*5, 91, 92*). By doing so, paromomycin stabilizes a *closed* conformation of the 30S subunit, thereby perturbing the induced-fit mechanism that controls decoding (*1, 2, 5, 92*). Neomycin and gentamicin also bind to h44 and are thought to promote miscoding by a similar mechanism (*91, 93, 94*). Besides inducing miscoding, these aminoglycosides inhibit translocation (*82, 95–97*), presumably by stabilizing A-tRNA (*82*). The aminoglycoside streptomycin, another miscoding agent (*88–90*), binds a distinct site and induces a "closed-like" conformation of the 30S subunit without flipping A1492 and A1493 (*5*). While paromomycin stabilized A-tRNA by 210-fold and inhibited translocation by 160-fold, streptomycin stabilized A-tRNA by 45-fold but inhibited translocation by only 2-fold. On the basis of these data, it was suggested that the conformation induced by streptomycin may resemble the transition state of translocation (*82*). Consistent with this idea, streptomycin accelerated reverse translocation to a much higher degree than other aminoglycosides, such as paromomycin, neomycin, and gentamicin (*49, 82, 91*).

Hygromycin B is an aminoglycoside with unique chemical and structural properties (Figure 6). It binds h44, very close to where other aminoglycosides such

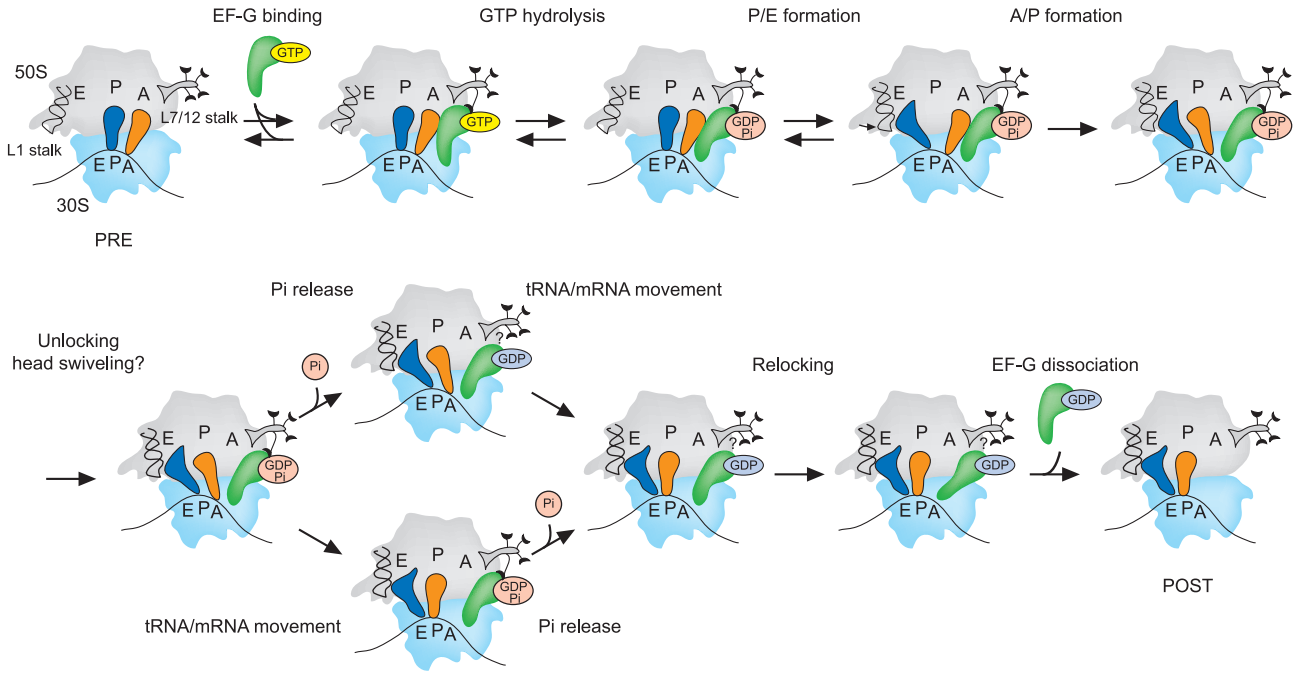

Figure 5. Kinetic model for EF-G-dependent translocation.

as paromomycin bind (*91, 98*). Like other aminoglycosides, hygromycin B induces miscoding, stabilizes A-tRNA, and inhibits EF-G-dependent translocation (*82, 88–90, 99, 100*), but unlike many typical h44-binding aminoglycosides, hygromycin B does not induce the closed conformation on the 30S subunit (*91, 98*). A recent structure of apo-70S ribosomes with hygromycin B showed that the antibiotic flips out A1493 but not

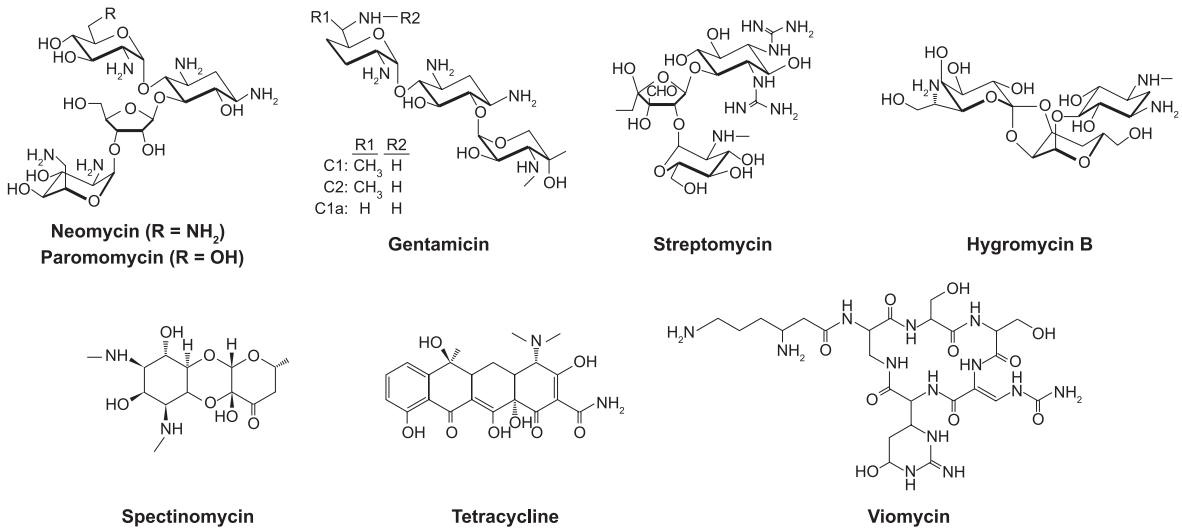

Figure 6. Chemical structures of antibiotics that affect tRNA—mRNA movement.

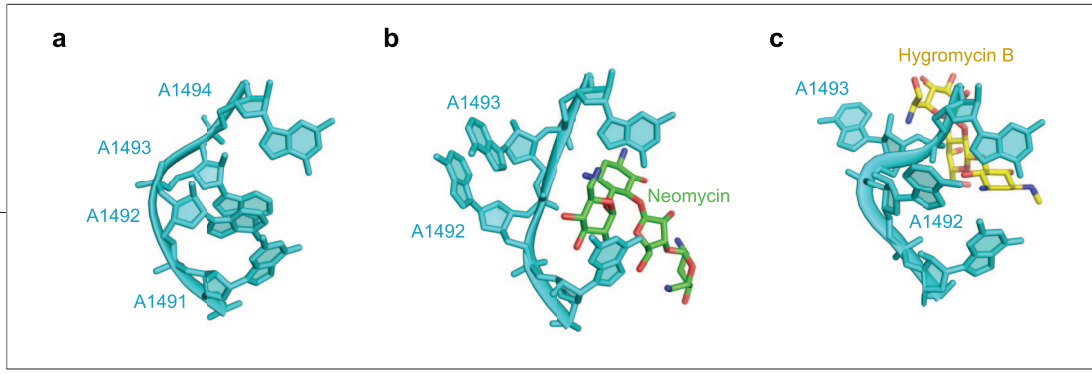

Figure 7. Structural rearrangements in h44 of 16S rRNA induced by aminoglycosides. Conformations of 16S rRNA nucleotides 1491–1494 in *E. coli* 70S ribosomes in the absence (panel a) or presence of neomycin (panel b) or hygromycin B (panel c) are shown. Paromomycin and gentamicin induce the same h44 conformation as neomycin (*91*). Structures were adopted from crystal structures of *E. coli* 70S ribosomes in complex with various antibiotics (PDB ID: 2AVY, 2QAL, and 3DF1) (*24, 91*).

A1492 (Figure 7) (*91*). The unique orientation of A1493 appears to sterically block tRNA movement between the A and P sites. Although stabilization of A-tRNA may play a major role in inhibition of EF-G-dependent translocation (*82*), the unique structural changes induced by hygromycin B potentially explain how hygromycin B can also inhibit spontaneous reverse translocation (*91*).

Spectinomycin is an aminocyclitol antibiotic that blocks translocation without affecting the fidelity of decoding (Figure 6) (*82, 96, 101, 102*). Spectinomycin binds to h34 at the "neck" of the 30S subunit (*5, 85*). In a crystallographic study, apo-70S ribosomes exhibited two distinct conformations of the 30S head domain, the swiveled and less-swiveled states, per asymmetric unit in the crystal (Figure 3, panel b) (*24*). Soaking spectinomycin into these crystals caused rotation of the head, such that all ribosomes assumed the less-swiveled state (*85*). Biochemical studies have shown that spectinomycin inhibits codon–anticodon movement in both forward and reverse directions (*82, 85*). However, spectinomycin does not inhibit tRNA movement within the 50S subunit during EF-G-dependent translocation (*81*), implying that head swiveling is related to the unlocking rearrangement that permits codon–anticodon movement in the 30S subunit.

Viomycin is a peptide antibiotic and a strong inhibitor of translocation (Figure 6) (*69, 82, 96, 97, 103*). Viomycin is believed to bind at the subunit interface (*104*), consistent with its ability to enhance subunit association and inhibit ribosome recycling (*105–107*), but the details of its interaction with the ribosome are not yet known. Viomycin shuts down EF-G-dependent forward translocation (>10,000-fold inhibition) (*82*), but it has little effect on spontaneous reverse translocation (*49*). A recent kinetic study suggested that viomycin stabilizes an intermediate state of translocation in which tRNAs occupy the P/E and A/A sites (*81*), whereas a chemical probing study suggested that viomycin traps complexes with tRNAs in the P/E and A/P sites (*108*). While both of these studies conclude that viomycin stabilizes an intermediate containing P/E-tRNA and thereby inhibits translocation, further work will be needed to resolve the question of how viomycin influences A-tRNA.

LepA: A Brownian Motor in Reverse Gear? In 2006, Nierhaus and colleagues found that LepA, or elongation factor 4 (EF4), can catalyze reverse translocation *in vitro* (*109*). The *lepA* gene was initially identified as the ORF upstream of the leader peptidase gene (*lepB*) in *E. coli* (*110, 111*). LepA bears a considerable similarity to EF-G but localizes in cytoplasmic membranes and periplasmic fractions (*110, 112*). Although LepA is widely conserved among bacteria and bacterium-derived organelles (*53, 54, 109*), deletion of *lepA* only affects growth under certain stress conditions (*113*). Guf1, a mitochondrial homologue of LepA, appears to be important for assembly of cytochrome oxidase (*114*), but the physiological role of LepA remains unclear.

Sequence and structural data show that protein domains of LepA are homologous to domains I, II, III, and V of EF-G (*109*). LepA does not have regions corresponding to EF-G domains G′ or IV but has a unique C-terminal domain that lacks similarity to any known proteins (*115*). Cryo-EM reconstructions of LepA-bound complexes revealed that LepA binds to ribosomes in the same orientation as EF-G (Figure 3, panel c) (*116*). The lack of domain IV on LepA allows tRNA to occupy the A site simultaneously with LepA, an observation made directly in complexes containing P-tRNA, A-tRNA, and LepA·GDPNP. Interestingly, tRNA in the A site of these complexes appeared to be displaced from the classical A/A site, adopting a previously unrecognized binding configuration, designated as A/L, where the tRNA is rotated around the codon–anticodon helix toward the A-site finger. Several contacts between LepA and A/L-tRNA were found, which may account for the unique conformation of A/L-tRNA. Direct interactions between LepA and tRNA and the strong positive charge in the C-terminal domain of the factor suggest that electrostatic interactions between LepA and tRNA facilitate the reverse movement of tRNA (*115, 116*). Analogous to

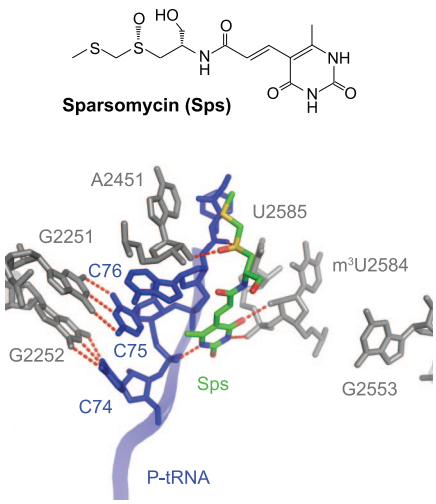

Figure 8. Sparsomycin interacts with the 3′ end of peptidyl-tRNA in the 50S P site. Interactions at the peptidyl transferase center of the *H. marismortui* 50S subunit containing P-site substrate and sparsomycin (PDB ID: 1VQ8) (*117*). 23S rRNA is colored in gray. *E. coli* numbering is used throughout.

EF-G, LepA could catalyze an unlocking of the ribosome and then bias Brownian movement of tRNA, but in the reverse direction.

Sparsomycin: A Simpler Brownian Ratchet? Sparsomycin interacts with peptidyl-tRNA in the 50S P site and blocks peptidyl transfer by preventing nucleophilic attack of the amino group of A-tRNA (Figure 8) (*117, 118*). A biochemical study showed that sparsomycin can promote efficient translocation in the absence of EF-G and GTP (*96*). At the time, the prevailing view was that translocation was intrinsically exergonic. Accordingly, it was proposed that sparsomycin acted as a "hair trigger", lowering the energy barrier to allow tRNA—mRNA movement (*96, 119, 120*). However, more recent studies have shown that, in analogous ribosomal complexes, the PRE state is more stable than the POST state (*48, 49*). These data raise the possibility that sparsomycin promotes forward translocation by shifting the PRE ⇆ POST equilibrium to the right. Generally consistent with this idea, sparsomycin stabilizes peptidyl-tRNA in the P site of the 50S subunit and in the P/P site of the 70S ribosome (*117*).

Although ample evidence exists that sparsomycin stabilizes the POST state, how does this stabilization accelerate translocation? The rates of sparsomycin-dependent forward translocation and spontaneous reverse translocation are highly comparable (Table 1) (*48, 49, 96*). This would be expected if the rate-limiting step of both reactions is governed by a common mechanism. A potential mechanism is unlocking of the ribosome, defined as rate-limiting for EF-G-dependent translocation (*70*). The molecular basis of unlocking is thought to involve reversible structural rearrangements of the ribosome, with the reverse rearrangements termed *relocking*. The Brownian motor model (see above) suggests that tRNAs in the unlocked ribosome undergo thermal fluctuation, where the Brownian movement of tRNA between their PRE and POST configurations takes place (*83*). Here, sparsomycin may strongly stabilize P/P-tRNA in the "unlocked POST" state (POST*), thus preventing the reverse movement of tRNA (Figure 9). Binding of sparsomycin depends on the presence of peptidyl-tRNA in the P site, and the affinity of this antibiotic is much higher than many other antibiotics that bind the peptidyl transferase center (*121*). Thus, binding of sparsomycin should provide a substantial stabilization of P-tRNA in POST* relative to the A/P-tRNA in the "unlocked hybrid PRE" state (PRE(HS)*). This stabilization of POST* would bias the microscopic equilibrium in the forward direction, thereby promoting efficient forward translocation.

In an analogous way, aminoglycoside antibiotics that stabilize A-tRNA such as paromomycin, neomycin, and gentamicin may stabilize PRE(HS)*, thereby enhancing reverse translocation (*49*). In the complexes used by Shoji *et al.*, PRE(HS)* is likely to be favored over POST* even in the absence of antibiotics, so that the magnitude of enhancement is marginal compared to acceleration of forward translocation by sparsomycin. Streptomycin accelerates spontaneous reverse translocation more substantially than other aminoglycosides but may additionally decrease the activation barrier for unlocking, as suggested previously (*49, 82*).

The idea of a microscopic equilibrium between PRE(HS)* and POST* is supported by the finding that tetracycline inhibits spontaneous reverse translocation. Tetracycline inhibits protein synthesis by inhibiting binding of aa-tRNA to the A site (Figure 6) (*122, 123*). Although multiple binding sites in a ribosome have been found by X-ray crystallography, the primary binding site is on h34 of 16S rRNA near the decoding center, where tetracycline sterically occludes the 30S A site (*93, 98,*

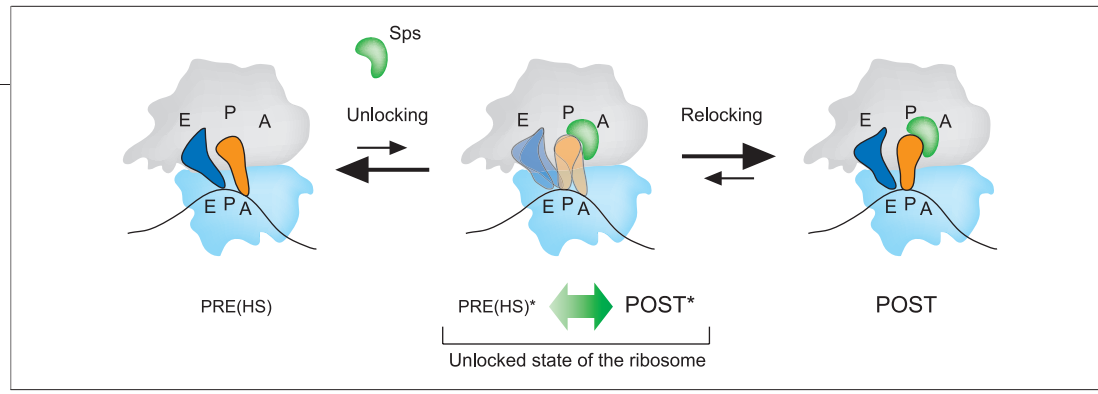

Figure 9. A possible mechanism for sparsomycin-dependent translocation. Sparsomycin (Sps) may promote efficient translocation by stabilizing the unlocked POST state (POST*) relative to the unlocked hybrid PRE state (PRE(HS)*). This model postulates that during translocation unlocking structural rearrangements would allow Brownian movement of tRNA in a complex that rapidly fluctuates between PRE(HS)* and POST*. Complexes in POST* would then be locked in the POST state by reverse structural rearrangements (relocking). Size of the letters underneath each complex infers relative stability of the complex.

124). While tetracycline does not seem to affect forward translocation, it inhibits spontaneous reverse translocation (*49, 82*). Because tetracycline does not induce a global conformational change in the ribosome (*98, 124*), tetracycline may interrupt formation of a transient intermediate of reverse translocation (*i.e.*, PRE(HS)*) and thereby reduce the rate of the reaction.

On the basis of these observations, we hypothesize that sparsomycin acts as a Brownian ratchet by preventing reverse tRNA−mRNA movement in the unlocked ribosome but does not promote ribosomal unlocking. Hence, the rate of sparsomycin-dependent translocation (5×10^{-3} s^{-1}) reflects that of ribosomal unlocking. An identical rate is seen for neomycin-promoted reverse translocation (6×10^{-3} s^{-1}) because an equivalent molecular event (*i.e.*, reverse relocking) limits that reaction. Testing these ideas may lead us another step toward understanding translocation.

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