Time-resolved cryo-EM visualizes ribosomal translocation with EF-G and GTP

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During translation, a conserved GTPase elongation factor—EF-G in bacteria or eEF2 in eukaryotes—translocates tRNA and mRNA through the ribosome. EF-G has been proposed to act as a flexible motor that propels tRNA and mRNA movement, as a rigid pawl that biases unidirectional translocation resulting from ribosome rearrangements, or by various combinations of motor- and pawl-like mechanisms. Using time-resolved cryo-EM, we visualized GTP-catalyzed translocation without inhibitors, capturing elusive structures of ribosome•EF-G intermediates at near-atomic resolution. Prior to translocation, EF-G binds near peptidyl-tRNA, while the rotated 30S subunit stabilizes the EF-G GTPase center. Reverse 30S rotation releases Pi and translocates peptidyl-tRNA and EF-G by ~20 Å. An additional 4-Å translocation initiates EF-G dissociation from a transient ribosome state with highly swiveled 30S head. The structures visualize how nearly rigid EF-G rectifies inherent and spontaneous ribosomal dynamics into tRNA-mRNA translocation, whereas GTP hydrolysis and Pi release drive EF-G dissociation.

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Continuous protein synthesis depends on the synchronous translocation of mRNAs and tRNAs through the ribosome (reviewed in refs. 1–3). After peptide bond formation, the pre-translocation ribosome contains peptidyl-tRNA in the A (aminoacyl-tRNA) site and deacyl-tRNA in the P (peptidyl-tRNA) site, which must be translocated with their mRNA codons to the P and E (exit) sites, respectively (Fig. 1a). The pre-translocation ribosome samples two globally different conformations, which interconvert spontaneously. These are the non-rotated and rotated conformation, in which the small subunit is rotated by up to 10°. In the rotated ribosome, the tRNA anticodon stem loops (ASLs) remain bound to the mRNA codons in the A and P sites on the small subunit, while the acceptor arms of tRNAs are shifted into the P and E sites of the large subunit6–9, thus adopting hybrid states denoted as A/P peptidyl-tRNA and P/E deacyl-tRNA10. In the next translocation step, the ASLs and mRNA shift along the small subunit, forming a post-translocation ribosome—where P-site peptidyl-tRNA and E-site deacyl-tRNA—prepared to accept the next aminoacyl-tRNA and continue the elongation cycle11.

Translocation of the ASLs and mRNA along the small ribosomal subunit is catalyzed by a conserved GTPase, elongation factor G (EF-G) in bacteria or EF-2 in archaea and eukaryotes (Fig. 1a). The structural mechanism of translocation has not been visualized because the rapid GTP hydrolysis step has prevented the capture of authentic EF-G-bound structural intermediates. Prior studies relied on stalling EF-G on the ribosome by antibiotics12–15, EF-G mutations16,17, or non-hydrolyzable GTP analogs18,19, which might capture off-pathway states20. Structural studies captured ribosome-EF-G conformations ranging from rotated pre-translocation-like12 through mid-rotated13,14,19 to non-rotated post-translocation-like15 or non-rotated pre-translocation-like states17. The structural relationship between GTP hydrolysis, EF-G rearrangements, and translocation, however, remains uncharacterized, as some stalled structures may be inconsistent with the biochemical progression of translocation. For example, a crystallographic pre-translocation-like ribosome structure captured mutant EF-G with GDP15, whereas in solution, pre-translocation ribosomes bind EF-G•GTP16,21. Furthermore, post-translocation ribosomes were reported with GTP-bound-like conformations of mutant EF-G or of EF-G with GTP analogs16,18,19, whereas authentic post-translocation states must feature post-GTP-hydrolysis states of EF-G.

Two groups of mechanistic models, as well as their combinations, have been suggested to explain EF-G•GTP-catalyzed translocation. In the first group of mechanisms, the energy of GTP hydrolysis is proposed to directly contribute to translocation23 by causing a large-scale conformational change of EF-G17,24 to exert force25,26 and/or by inducing ribosome rearrangements that propel tRNA movement29,28. A ribosome crystal structure with a compact EF-G mutant fused with I9 suggested a nearly 100-Å inter-domain movement17 toward an extended EF-G conformation captured in most structural studies, in keeping with EF-G acting as a flexible motor. The second group of mechanistic models argues that EF-G acts as a steric hindrance, or pawl, that rectifies the inherent thermal motions of the ribosome, including spontaneous interconversion between non-rotated and rotated conformations, into tRNA translocation29. These models are

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**Fig. 1 Time-resolved cryo-EM of translocation with EF-G and GTP.** a Scheme of the translocation reaction of the 70S•mRNA•fMet•tRNA•fMet•Pro•tRNA Pro complex with EF-G•GTP. b Segmented cryo-EM maps of 8 states of the translocation reaction, and their assignment as substrates, EF-G-bound intermediates, or products of the reaction. The maps are colored to show the 50S ribosomal subunit (light blue), 30S ribosomal subunit body (yellow) and head (tan), tRNAfMet (dark blue), tRNA Pro (green), mRNA (magenta) and EF-G (red). c Relative abundance of substrates (blue), EF-G intermediates (red), and translocation products (green) over time, obtained from particle distributions in cryo-EM datasets. d Domain organization of EF-G; Arabic numerals denote the five conserved domains of the elongation factor. e Cryo-EM density of the EF-G GTase center in the transient pre-translocation and pre-Pi-release state (III). For additional density views, see Fig. 4 and Supplementary Figs. 3, 4, and 5.
consistent with the ribosome’s ability to slowly translocate tRNA in the absence of EF-G \(^{30-33}\), indicating that translocation is an inherent property of the ribosome. Because non-hydrolyzable GTP analogs efficiently catalyze translocation, accelerating it by more than 10\(^3\)-fold \(^{11,23,27,34}\), the translocation stage was proposed to be independent of GTP hydrolysis and large-scale interdomain rearrangements of EF-G \(^{12}\). Nevertheless, the rate of translocation in the presence of EF-G and GTP is up to 50-fold higher than with GTP analogs or catalytically inactive EF-G \(^{11,16,23,27,35}\). Thus, neither group of models fully explains the structural roles of EF-G and GTP hydrolysis \(^\text{1,3}\).

To understand how EF-G and GTP catalyze translocation, we performed time-resolved cryogenic electron microscopy to visualize authentic translocation intermediates without inhibitors (Fig. 1 and Supplementary Figs. 1–2). We report three ribosome intermediates with EF-G (Fig. 1a–b), resolving EF-G’s GTPase center at ~3.5 Å local resolution (Fig. 1h and Supplementary Fig. 3). Together with the pre-translocation and post-translocation states observed without EF-G, our data allow reconstruction of the structural pathway of translocation, elucidating the structural roles of EF-G (Figs. 2 and 3) and GTP hydrolysis (Fig. 3). In addition to inhibitor-free complexes, we report a 3.2 Å structure of a pre-translocation complex formed with EF-G•GTP and stalled with viomycin, which supports our previous structural studies with different tRNAs. Our data also contain ribosomes non-rotated Structure I and rotated Structures II-A and II-B (Fig. 1b and Supplementary Fig. 4). Structure I contains tRNAs in the classical A/A and P/P states, and a low-occupancy E-site (Supplementary Fig. 4a), similar to those in previous studies of elongation (e.g. Structure V-B in ref. \(^{36}\)). Structure II-A features a rotated ribosome with an A/A-like tRNA\(^{\text{Pro}}\)and P/E tRNA\(^{\text{Met}}\) (Supplementary Figs. 4b–c and 5a–b). This state reveals that upon intersubunit rotation, dipeptidyl-tRNA can remain in the 50S A site while the acceptor arm of the deacyl-tRNA shifts to the E site, as suggested by studies using Fluorescence/Förster Resonance Energy Transfer (FRET) \(^{37}\) and mutant bacterial ribosomes \(^{38}\).

Structure II-B features a rotated ribosome with an A/P* and P/E hybrid-state tRNAs, whose acceptor arms are shifted to the P and E sites on the large subunit, respectively (Supplementary Figs. 4d and 5a–b) \(^{6,9}\). The elbow of A/P* tRNA is shifted by ~25 Å toward the P site relative to the canonical A/P tRNA observed in most studies with different tRNAs. Our data also contain ribosomes with weak density for A/P tRNA, although we could not unambiguously separate them into a high-resolution class with A/P tRNA. Overall, conformations of tRNA\(^{\text{Pro}}\) are similar to those sampled by other tRNA species (e.g. tRNA\(^{\text{Phe}}\)) on
Fig. 3 Positions and interactions of EF-G in translocation intermediates. a–b Positions of EF-G and tRNAs relative to the decoding center (yellow) in Structures III and IV. c Superposition of EF-G in Structures III and IV demonstrates an overall similar extended conformation. d Movement of EF-G relative to the 3OS subunit from Structure III (gray) to IV (colored). Structures are aligned on 16S rRNA and colored ribosomal elements are from Structure III. e Movement of EF-G relative to the 3OS subunit from Structure IV (colored) to V (blue-gray). Structures are aligned on 16S rRNA and colored ribosomal elements are from Structure IV. f Buried surface area (contact area) showing the extent of interactions of EF-G domains with the ribosome, mRNA, and/or tRNA in Structures III to V.

Fig. 4 The GTPase center of EF-G in translocation Structures III, III-vio and IV. a–c Cryo-EM densities are consistent with GDP•Pi in Structures III and III-vio, and with GDP in Structure IV. Grey model shows GTP for reference (1WDT). d Positions of sw-I in Structure III (ordered) and IV (gray, disordered) between the SRL and h14 of the 3OS subunit. e The catalytic conformation of His92 in Structure III (colored) differs from position of this side chain in the off-ribosome EF-G homolog (archaeal EF-2, gray) bound with GTP analog. f Pretranslocation 70S•EF-G•GDP•Pi structure captured with viomycin (III-vio; gray) is nearly identical to Structure III. Box shows density for EF-G GTPase center in Structure III-vio (His92 density is shown in Supplementary Fig. 2c).
pre-translocation ribosomes from bacteria\textsuperscript{36} and eukaryotes\textsuperscript{39,40}, in keeping with the conservation of ribosomal and tRNA rearrangements during the elongation cycle. Our Structures I through II-B confirm that prior to translocation, the ASL of peptidyl-tRNA remains in the 30S A site, whereas the acceptor arm and elbow spontaneously sample different conformations, including the A/P\textsuperscript{P*} state, which is most advanced toward the P site.

Three structures of transient translocation intermediates (III, IV, and V) include long-sought EF-G-bound states resolved at ~3.5 Å average resolution. These structures represent distinct stages of tRNA advancement along the translocation trajectory (Fig. 2). Structure III features a pre-translocation 70S-EF-G complex with A/P\textsuperscript{P} and P/E tRNAs (Fig. 2a). In Structure IV, EF-G is shifted ~20 Å along the 30S subunit, with partially translocated “chimeric” ap/P and pe/E tRNAs (Fig. 2b; tRNA nomenclature as in\textsuperscript{14}). In Structure V, tRNA ASLs are further along the translocation pathway, reaching the P and E sites of the 30S body domain (we term the tRNAs ap*/P and pe*/E; Fig. 2c). EF-G translocase domain shifts with the tRNAs by another 4 Å whereas the N-terminal domains 1 and 2 are characterized by scattered low-resolution density (Fig. 2c; Supplementary Figs. 3h and 4g), indicating that they are dynamic in the intersubunit space.

Two post-translocation products (VI and VII) lack EF-G. The ribosome with a highly swiveled 30S head and ap*/P and pe*/E tRNAs in Structure VI is nearly identical to the EF-G-bound Structure V. Structure VI is found exclusively in the 25-s data set, indicating that it is a transient state formed after EF-G dissociation. By contrast, the terminal post-translocation Structure VII with fully translocated dipeptidyl-tRNA\textsuperscript{Pro} in the P site is present in both the 25- and 3600-s data sets. Here, the non-rotated ribosome with a non-swiveled head is nearly identical to the ribosome in Structure I (Fig. 2h) and previous structures of post-translocation complexes, and the ribosome is ready for the next round of elongation.

Extended EF-G binds rotated pre-translocation ribosomes with A/P\textsuperscript{P*} tRNA. During translocation, the ASL of peptidyl-tRNA with its mRNA codon must traverse a ~25 Å distance from the A/P\textsuperscript{P} and pe/E tRNAs (Fig. 2a). In Structure IV, EF-G is shifted ~20 Å along the 30S subunit, with partially translocated “chimeric” ap/P and pe/E tRNAs (Fig. 2b). In Structure V, EF-G domain 4 occupies the ribosomal A site during reverse 30S body rotation. In the mid-translocation Structure IV, EF-G retains an extended conformation, similar to that in Structure III, with EF-G domain 5 anchored to the 30S head domain (we term the tRNAs ap*/P and pe*/E; Fig. 2c). EF-G translocase domain shifts with the tRNAs by another 4 Å whereas the N-terminal domains 1 and 2 are characterized by scattered low-resolution density (Fig. 2c; Supplementary Figs. 3h and 4g), indicating that they are dynamic in the intersubunit space.

To accommodate into the A site, EF-G undergoes small-scale rearrangements (Fig. 3c), as domain 4 shifts relative to the GTPase domain by ~7 Å (RMSD, root-mean-square distance between superimposed EF-G from Structures III and IV). The range of interdomain rearrangements is similar to or less than interdomain fluctuations of free EF-G in solution\textsuperscript{49} and in crystal structures of free EF-G homologs\textsuperscript{15,50,51} (up to ~20 Å; Supplementary Fig. 7), suggesting that EF-G undergoes local stochastic rearrangements to accommodate into the A site during translocation.

Structure V represents a heretofore unseen EF-G-bound ribosome state with a highly swiveled head and further translocated tRNAs (Fig. 2c, g). 30S body is less rotated (1.1°) than that in Structure IV, whereas head swivel (18.1°) is slightly increased (Fig. 2h). EF-G domain 4 and tRNAs have advanced 3–5 Å along the 30S subunit. The ASL of dipeptidyl-tRNA\textsuperscript{Pro} is placed deeper into the P site of the 30S body, forming a late translocation state ap*/P (Fig. 2g and Supplementary Fig. 6h). Strong density shows EF-G domain 4 occupying the ribosomal A site and domain 5 attached to the L11 stalk. Density for domain
3 is weaker, and densities for domains 1 and 2 are non-
continuous and low-resolution (Fig. 3e and Supplementary
Figs. 3d, h and 4g). Thus, Structure V is consistent with a late
translocation intermediate, in which EF-G releases its hold on
the ribosome, as the GTPase domain leaves the SRL and domain 2
leaves the 30S subunit. Dissociation of EF-G domains 1 and 2
 correlates with steric hindrance presented by ribosomal protein
uS12 (bacterial S12; Fig. 3e) and loss of interactions between
domain 1 and the back-rotating 30S subunit, as discussed below.
Step-wise dissociation of EF-G resembles that of EF-Tu, whose
GTPase domain is released from the ribosome before other
domains during tRNA decoding.

Structural analyses of EF-G-bound intermediates highlight that
progression from Structure III to V is correlated with a stepwise
loss of EF-G contact with the ribosome (Fig. 3f). Extensive
interactions of the GTPase with the SRL and the small subunit in
Structure III (GTPase-domain buried surface area of ~960 Å²)
are halved in Structure IV (493 Å²) on the path to dissociation of
the GTPase domain in Structure V (~0 Å²). By contrast, the translocase superdomain retains its interactions with the
ribosome. The invariant interaction of EF-G Domain 5 with the
L11 stalk of the 50S subunit in all three structures (~900 Å²)
holds EF-G in place to allow the entry into the A site during
reverse 30S rotation. Interactions of domain 4 with the 30S
subunit gradually expand from 800 Å² in Structure III through
1190 Å² in Structure IV to 1440 Å² in Structure V (Fig. 3f).
Nevertheless, the overall contact area of EF-G during transloca-
tion reduces from 3730 Å² (whole EF-G buried surface area in
Structure IV) to 1440 Å² in Structure V (Fig. 3f). Extensive
progression from Structure III to V is correlated with a stepwise
dissociation of EF-G is driven by different affinities of EF-G to
the ribosome in different 30S rotation/swivel states.

Structure VI lacks EF-G, but the tRNA positions and 30S
conformation only marginally differ from those in Structure V
(Fig. 1b and Supplementary Fig. 4h). With a slightly more
swiveled head (18.9°), Structure VI represents a transient
translocation intermediate following EF-G dissociation.

The completion of tRNA and mRNA translocation along the
head requires an ~20° reversal of head swivel, to the post-
translocation state captured in Structure VII (Fig. 1b and
Supplementary Fig. 4i). The non-rotated/non-swiveled Structure
VII features an empty A site and tRNAPro with the associated
proline codon clearly resolved in the P site (Fig. 2d–e). Very low
density suggests that the bulk of deacyl-tRNA has dissociated
from the ə site (Fig. 1b and Supplementary Fig. 4i). Extensive
classification of cryo-EM data did not detect EF-G on non-
rotated, post-translocation ribosomes that would resemble EF-G-
bound structures stalled by fusidic acid, GTPase-defective EF-G
mutant, or non-hydrolyzable GTP analog. Our structures therefore
suggest that in the absence of inhibitors, EF-G dissociates before or during reversal of head swivel.

**Pi release during tRNA translocation.** Structures III and IV
reveal two functional EF-G GTPase states distinguished by dif-
fering interactions with the 30S subunit and the SRL, which is
essential for the hydrolysis of GTP. Structure III resembles a
7.4 Å cryo-EM structure of an EF-G-bound ribosome stalled by the addition of the antibiotics fusidic acid and viomycin. Viomycin stabilizes the pre-
translocation tRNA in the decoding center without inhibiting GTP hydrolysis. The low resolution prevented a detailed
structural analysis of the GTPase center in the antibiotic-bound
structure. To further resolve the GTPase center in the pre-
translocation EF-G state, we used cryo-EM to visualize a pre-
translocation 70S complex assembled with EF-G-GTP and
viomycin (Supplementary Fig. 2). Remarkably similar to
Structure III, the 3.2 Å resolution structure of the viomycin-
stalled ribosome (i.e., III-vio; Supplementary Fig. 2d) features a
better resolved GTPase center (Fig. 4b, f and Supplementary
Fig. 3i). Structure III-vio supports our finding that the pre-
translocation ribosome contains post-hydrolysis Pi and GDP
(Fig. 4b; Supplementary Table 3) stabilized by the switch loops
and ions, likely magnesium, which coordinate the phosphate
groups (Fig. 4f). These findings are similar to those in two cryo-
EM studies of ribosomal EF-G complexes with antibodies
published while our manuscript was under review. GDP and Pi
were reported in the cryo-EM structure of pretranslocation
ribosome stalled with the antibiotic apramycin, which locks the
coding center similarly to viomycin. EF-G-bound ribosome
with bacterial holo-translocon is also consistent with post-
hydrolysis GDP and Pi, although the structural model was
reported as GTP53 (Supplementary Fig. 6i; see Methods and
Supplementary Table 3). Thus, our structures III and III-vio
demonstrate that (a) GTP is hydrolyzed on pretranslocation
ribosome, and (b) after hydrolysis, the switch loops of the GTPase
center remain well ordered because they are stabilized by the
rotated 30S conformation.
Structure IV, by contrast, features a post-Pi-release conformation of EF-G. Here, movement of EF-G into the 30S A site coincides with separation of the GTPase domain from the SRL (Fig. 4d). The GTP-binding pocket is ~2 Å further from the catalytic SRL phosphate than in Structure III, consistent with an inactive post-reaction state (Supplementary Fig. 6k). The GTPase movement relative to the SRL is consistent with mutational studies showing that perturbing the conformation of the SRL abolishes translocation even if the GTPase activity is retained54. Whereas GDP is clearly resolved, densities for the switch loops and Pi are absent (Fig. 4c–d and Supplementary Fig. 3k), indicating that the switch loops become dynamic and thus release Pi from the GTPase center59,66. These rearrangements of EF-G coincide with a > 10-Å movement of h14 away from sw-I, as a result of reverse 30S rotation (Fig. 4d). Thus, disruption of the contact between the 30S and GTPase is correlated with Pi release.

Discussion

Structural mechanism of EF-G-GTP-catalyzed translocation.

Time-resolved cryo-EM of authentic translocation answers several long-standing questions; rationalizes previous structural, biochemical and biophysical observations; and suggests a parsimonious model for the translocation mechanism (Fig. 5 and Supplementary Movie 1). As Structures I through II-B report, pre-translocation ribosomes spontaneously interconvert between non-rotated and rotated conformations, in which the peptidyl-tRNA samples A/A and A/P* states (Fig. 5a–d). This is consistent with a large body of biochemical and biophysical data reporting fast tRNA fluctuations on the 50S subunit and intersubunit rotation prior to EF-G binding4,37,67,68. EF-G-GTP binds to a rotated pre-translocation ribosome22, where the relative position of the small subunit and the 50S SRL are complementary to the GTP-bound conformation of EF-G’s GTPase domain, as in Structure III (Fig. 5e, k). The EF-G translocase domain 4 binds near the ASL of the A/P* tRNA (Fig. 5e). Because EF-G appears sterically incompatible with A/A and A/P tRNA conformations (Supplementary Fig. 5e–f), this binding must shift the conformational equilibrium toward the "elbow-translocated" A/P* conformation. Indeed, in the 25-s dataset with EF-G, no classes of rotated ribosomes with the A/A tRNA (as in Structure II-A) are observed, indicating substantial depletion in comparison with the 0-s dataset (Fig. 1c and Supplementary Fig. 1). The binding of EF-G to the rotated pre-translocation ribosome is consistent with biochemical observations of transient stabilization of the rotated 70S by EF-G with GTP or GTP analogs and increased rates of forward 30S rotation28,60.

Structures of EF-G-bound intermediates (III through V) report the trajectory of translocation consistent with FRET solution studies showing that translocation on the 30S subunit occurs during the reverse 30S rotation11 and proceeds in at least two
Dissociation due to the inability to hydrolyze GTP or due to the swiveled ribosomes with EF-G were reported when EF-G cannot during authentic translocation. By contrast, non-rotated/non-swiveled data have not revealed EF-G bound to non-rotated/non-swiveled (Supplementary Fig. 4g) and without EF-G (Structure VI), suggesting that EF-G equilibrium between the ribosomes with EF-G (Structure V) retains the interactions between the 30S head and translocating tRNAs and most swiveled 30S head capture a transient tRNAs. Novel states in the 25-s dataset with the most translocated retain the interactions between the 30S head and translocating tRNAs and most swiveled 30S head capture a transient equilibrium between the ribosomes with EF-G (Structure V) and without EF-G (Structure VI), suggesting that EF-G-GDP can dissociate from ribosomes prior to the reversal of head swivel (Supplementary Fig. 4g–h). Despite extensive classification, our data have not revealed EF-G bound to non-rotated/non-swiveled ribosomes, suggesting that they are exceedingly rare if they exist during authentic translocation. By contrast, non-rotated/non-swiveled ribosomes with EF-G were reported when EF-G cannot dissociate due to the inability to hydrolyze GTP or due to the presence of an antibiotic. Our findings therefore illustrate that EF-G-GTP-catalyzed translocation of tRNAs occurs in two major steps: first, relative to the 30S body, coincident with the forward head swivel (with EF-G); second, relative to the head, upon reversal of the head swivel (without EF-G, or coincident with EF-G dissociation).

Our work provides structural insights into the role of GTP hydrolysis in translocation. EF-G accelerates translocation by more than 3 orders of magnitude with either GTP or non-hydrolyzable GTP analogs. Yet, translocation rates are 2- to 50-fold higher with GTP than with GTP analogs with inactivating His92 mutations. The structural basis for this difference has remained unclear. Our structures demonstrate that rather than being coupled with the chemical step of GTP hydrolysis, tRNA translocation is coupled with switch-loop rearrangements of EF-G and phosphate release (from Structure III to Structure IV). Structures III and III-vio are consistent with biochemical studies, showing that Pi release is slower than hydrolysis and may determine the rate of tRNA translocation. In the pre-translocation ribosome (Structure III), sw-1 bridges the SRL with the rotated 30S subunit, preserving an ordered GTP-bound-like conformation of the EF-G GTPase center. Reversal of the 30S subunit rearranges sw-1, allowing Pi diffusion from the GTPase center (Structure III to IV). By contrast, artificial prevention of Pi release — e.g., in the presence of non-hydrolyzable GTP analogs or catalytically defective EF-G mutants — stabilizes a GTP-bound-like conformation of EF-G until late translocation states. The inability of sw-1 to rearrange correlates with the reduced rates of 30S rotation with GTP analogs, at least in part explaining the slower translocation. Moreover, in the presence of non-hydrolyzable GTP analogs, the GTP-like conformation of EF-G prevents the dissociation of the GTPase domain from the SRL at latter stages of translocation, which coincides with the reversal of head swivel (Structure IV to V to VI). Our structural analyses of EF-G-bound intermediates (Structures III through V) highlight that GTP hydrolysis contributes to the directionality and completion of translocation by enabling a stepwise loss of EF-G contact with the ribosome (Fig. 3). Indeed, single-molecule FRET and biochemical studies showed that transitions between the late translocation states and dissociation of EF-G may determine the rates of EF-G-GTP-catalyzed translocation.

Due to the strict directionality, large-scale tRNA movements, and fast rates of GTP-catalyzed translocation, some mechanistic models proposed that translocation is driven by large-scale rearrangements of EF-G. Discussions considered that EF-G could act as a flexible GTPase motor, akin to classic ATP-driven motors, such as myosin and kinesin, whose conformational changes are commensurate with their molecular size. The structures captured in this work suggest that EF-G does not act as a highly flexible motor and that the proposed nearly 100-Å rearrangement of domain 4 is not required for translocation to occur (see Supplementary discussion). EF-G adopts similarly extended conformations in the pre-translocation state before Pi release (Structure III) and in the nearly post-translocated ribosome after Pi release (Structure IV). The ~7 Å displacement of domain 4 from the GTPase domain (Fig. 3c) cannot account for ~25-Å translocation of tRNA and mRNA. Rather, the EF-G interdomain movement is consistent with spontaneous thermal fluctuations of ~10 Å observed in solution studies. Thus, modest interdomain rearrangement of EF-G accounts for accommodation of domain 4 in the 30S A site during reverse 30S rotation. If some large-scale interdomain EF-G rearrangements occur on the ribosome, they must take place prior to formation of the pre-translocation Structure III and thus do not drive translocation. By contrast, the 30S body rotation and head swivel are the inherent and spontaneously sampled properties of the ribosome, which have been observed without EF-G. The rates of intersubunit rotation are directly coupled to the rates of translocation, indicating that ribosomal rearrangements are the driver of translocation. Thus, EF-G accelerates translocation by acting as a nearly-rigid sterical block (i.e., a pawl), that rectifies inherent ribosomal rearrangements into tRNA movement on the 30S subunit. The GTPase activity serves as a switch controlling the ability of EF-G to bind and leave the ribosome.

The translocation intermediates captured in this work also illustrate how the mRNA frame is preserved to prevent frameshifting events that could produce toxic proteins and premature termination. While the pre-translocation ribosome stabilizes the tRNA-mRNA helix in the decoding center and in the P site, the thermodynamically labile three-base pair codon-anticodon helix may be destabilized during the transition between these two sites, leading to tRNA slippage and frameshifting. Indeed, a recent crystal structure revealed that the tRNA-mRNA base-pairing can be destabilized in the absence of EF-G, if the 30S body and head are rotated similarly to those in our EF-G-bound Structure IV and without EF-G (Supplementary Fig. 8d). We recently reported cryo-EM structures of EF-G-bound complexes with a frameshifting-prone mRNA, which suggest that +1 frameshifting can occur before completion of the 30S head swivel. In the current work, domain 4 of EF-G interacts with both the codon and the anticodon in Structures IV and V (Supplementary Fig. 6f–h). They demonstrate that EF-G must remain bound to the ribosome until achieving the latest head-swiveled intermediate Structure V with the most translocated...
tRNAs, to support the tRNA-mRNA helix and prevent frameshifting.

Together with recent time-resolved cryo-EM studies of translation initiation, mRNA decoding, termination, and recycling, our work offers a more complete structural visualization of the ribosomal translation cycle. Consistent with biochemical studies, the structural studies revealed that similar inherent and spontaneous ribosomal dynamics (e.g., intersubunit rotation, 30S head swivel) are essential for each step of translation, and that translation factor protein domains contribute Checkpoints that promote accuracy and directionality. These structural dynamics are similar between bacterial systems (E. coli and Th. thermophilus) being the predominant model systems), yeast and mammalian cytosolic and mitochondrial ribosomes, in keeping with the central role of ribosomal RNA and universal conservation of the two-subunit and subunit-domain architecture of the ribosome.

**Methods**

**Preparation of EF-G and ribosomal subunits.** The gene encoding the full-length C-terminally His6-tagged E. coli EF-G was cloned into a pET24a (+) vector (NovaGen, kanamycin resistance), and the plasmid was transformed into E. coli BLR(D3). The cells were cultured in Luria-Bertani (LB) medium with 50 µg/mL kanamycin for 37 °C. OD600 of 0.7-0.8 was induced by 1 mM IPTG (Gold Biotechnology Inc., USA), followed by cell growth for 9 h at 16 °C. The cells were harvested, washed and resuspended in buffer A: 50 mM Tris-HCl (pH 7.5), 50 mM NH4Cl, 10 mM MgCl2, 5% glycerol, 10 mM imidazole, 6 mM β-mercaptoethanol (β-ME) and protease inhibitor (complete Mini, EDTA-free protease inhibitor tablets, Sigma-Aldrich, USA). The cells were disrupted by a microfluidizer (Microfluidics, USA), and the soluble fraction was collected by centrifugation using a JA-20 rotor at 39,200 × g for 50 minutes and filtered through a 0.22 µm pore size sterile filter (CELLTREAT Scientific Products, USA). EF-G was purified in three steps. The purity of the protein after each step was assessed by 12% SDS-PAGE stained with Coomassie Brilliant Blue R 250 (Sigma-Aldrich). First, affinity chromatography with Ni-NTA column (Nickel-nitrilotriacetic acid, 5 ml HisTrap, GE Healthcare) was performed using FPLC (Akta explorer, GE Healthcare) at 4 °C. The cytoplasmic fraction was loaded onto the column equilibrated with buffer A and washed with the same buffer. EF-G was eluted with a linear gradient of buffer B (buffer C with 0.7 M KCl). The fractions containing EF-G were pooled and dialyzed against buffer C (50 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM MgCl2, 0.5 mM EDTA, 6 mM β-ME and protease inhibitor). The second purification step involved ion-exchange chromatography using a 20-ml HiPrep FF Q column (GE Healthcare). The column was equilibrated and washed with buffer C. EF-G sample was loaded in buffer C and eluted with a linear gradient of buffer D (buffer C with 0.7 M KCl). Finally, the protein was dialyzed against 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 10 mM MgCl2, 0.5 mM EDTA, 6 mM β-ME and purified using size-exclusion chromatography (Hiload 16/60 Superdex 200 200 pg column, GE Healthcare). The fractions of the protein were pooled and dialyzed against 50 mM Tris-HCl (pH 7.5), 100 mM NH4Cl, 10 mM MgCl2, 0.5 mM EDTA and 6 mM β-ME, 5% glycerol and concentrated with an ultrafiltration unit using a 10-kDa cutoff membrane (Millipore). The concentrated protein was flash-frozen in liquid nitrogen and stored at −80 °C. 70S ribosomes were prepared from E. coli (MER6000), and stored in the ribosome-storage buffer (20 mM Tris-HCl (pH 7.0), 100 mM NH4Cl, 12.5 mM MgCl2, 0.5 mM EDTA, 6 mM β-ME) at −80 °C. Ribosomal 30S and 50S subunits were purified using sucrose gradient (10−35%) in a ribosome-dissociation buffer (20 mM Tris-HCl (pH 7.0), 500 mM NH4Cl, 1.5 mM MgCl2, 0.5 mM EDTA, 6 mM β-ME). The fractions containing 30S and 50S subunits were collected separately, concentrated and stored in the ribosome-storage buffer at −80 °C.

**Preparation of charged tRNAs and mRNA.** Native E. coli tRNA was prepared from Chemical Block and was aminoacylated as described (Lancaster and Noller, 2005). Native E. coli tRNA (UUG) was over-expressed in E. coli from an IPTG-inducible proM gene encoded by the pRK2233 plasmid. Total RNA was isolated using differential centrifugation and RNAaseA (UUG) was isolated using a complementary biotinylated oligonucleotide attached to streptavidin-sepharose, yielding approximately 40 nmoles RNAaseA (UUG) from 1 liter of culture. RNAaseA (UUG) (10 µM) was aminoacylated in the charging buffer (50 mM HEPES (pH 7.5), 50 mM KCl, 10 mM MgCl2, 10 µM tRNA, 10 mM DTT, 2 µM poly-ribi-trna synthetase, 0.625 mM ATP and 15 mM elution factor EF-Tu. EF-Tu was purified as described. The mixture was incubated for 10 minutes at 37 °C. To stabilize the charged Pro-tRNA in the form of Pro-tRNA-EF-Tu-GTP ternary complex, 0.25 mM GTP was added to the mixture. The mixture was incubated for 10 minutes at 37 °C.

Model tRNAs, containing the Shine-Dalgaro sequence and a linker to place the AUG start codon (underlined) in the P site and proline (bolded) in the A site (GGC AAG GAG GUA AAA AUG CCA AGU UCU AAA AAA AAA AAA) was synthesized by IDT.

**Preparation of the 70S translation complex with EF-G-GTP.** The 70S ribosome-Met-tRNA35-Pro-tRNA55-Pro-tRNA57+EF-G-GTP reactions were prepared as follows. First, a pre-translocation complex with Met-Pro-tRNA35 in the A site was assembled. 0.33 µM 30S subunit (all concentrations are specified for the final solution) was pre-activated at 42 °C for 5 minutes in the ribosome-reconstitution buffer (20 mM HEPES (pH 7.5), 120 mM NH4Cl, 20 mM MgCl2, 2 mM spermidine, 6 mM β-mercaptoethanol, 0.33 µM 50S subunit). Subsequently, 0.33 µM 50S subunit were added to the 30S solution and incubated for 10 minutes at 37 °C. To form the 70S initiation-like complex, 0.33 µM Met-tRNA35 was added, and the solution was incubated for 3 minutes at 37 °C. To deliver Pro-tRNA55 to the A site, the pre-incubated ternary complex (Pro-tRNA57+ at 0.33 mM; EF-Tu at 0.5 µM; GTP at 0.25 mM) was added to the solution and incubated for 10 minutes at 37 °C, as described.14 Translational complexes with EF-G were formed by addition of the mixture of ice-cooled 3.5 µM EF-G and 0.66 mM GTP to the ice-cooled pre-translocation complex, on ice. No EF-G and GTP were added to the 0-s time-point reaction, which was applied to a grid and blotting. This reaction with EF-G was mixed and an aliquot was immediately applied on the grid, blotted and plunged into a cryogen, as described below, resulting in the 25-s time-point sample. The 360-s sample was obtained by incubation of the pre-translocation complex with EF-G and GTP for 60 minutes on ice followed by grid blotting and plunging into a cryogen.

To form a viomycin-bound pre-translocation complex (Structure III-vio), 0.13 mM viomycin was added to the pre-translocation complex and incubated for 3 minutes at 37 °C. 5.3 µM EF-G and 0.66 mM GTP were added to the solution, incubated for 5 minutes at 37 °C, cooled down to room temperature, applied to a grid and plunged into a cryoogen.

**Cryo-EM grid preparation, data collection, and image processing.** QUANTI-FOIL R 2/1 grids with 2 nm carbon layer (Cu 200, Quantifoil Micro Tools) were glow-discharged with 25 mA with negative polarity for 60 s in a PELCO easyGlow discharge unit. 2.5 µl of each complex was separately applied to the grids. Grids were blotted at blotting force 10 for 4 s at 5 °C, 95% humidity, and plunged into liquid ethane using a Vitrobot M4 (FEI). Grids were stored in liquid nitrogen.

25-s dataset — Data collection and processing of all datasets were performed similarly to those for the 25-s data set (Supplementary Fig. 1 and Table 1), with differences outlined below. Cryo-EM data were collected at the Cryo-EM Center, University of Massachusetts Medical School. From a grid with the 70S ribosome-Met-tRNA35-Pro-tRNA57+EF-G-GTP complex, the 25-s sample was cryo-precipitated using 25% ethanol at −80 °C. The 25-s sample was deposited on a holey carbon film, being the cryo-precipitated 25% solution of the 70S ribosome-Met-tRNA35-Pro-tRNA57+EF-G-GTP reaction. EF-G was mixed and an aliquot was immediately applied on the grid, blotted and plunged into a cryogen, as described below, resulting in the 25-s time-point sample. The 360-s sample was obtained by incubation of the pre-translocation complex with EF-G and GTP for 60 minutes on ice followed by grid blotting and plunging into a cryogen.
using a spherical mask (40 Å radius) focused to cover most of the ribosomal A and P sites. Classification into 16 classes yielded two 70S maps, each of which contained densities that could be related to EF-G. To better model the EF-G, these maps were subject to additional classification. To this end, the particles assigned to these two classes were extracted from the 2× binned stack into two substacks (with 50% occupancy and scores > 0) using merge_classes.exe, resulting in stack-1 and stack-2 with 7173, and 12,327 particles, respectively. Prior to classification, a low-resolution, defocused model was built from the P-site density cutoff of 6 Å. Classification was performed for 100 cycles, using the same A site focused mask. Classification yielded into 2 classes yielded a 3.3 Å class (Structure IV) which contained both tRNAs and EF-G, and a class containing a rotated ribosome with EF-G bound (Structure V). A low-resolution classification of stack-1 using a separate masking strategy was performed (Supplementary Fig. 1b). Classification of stack-1 was performed for 100 cycles, using a focused spherical mask with the radius of 35 Å, covering the GTAPase domain of EF-G. Classification into two classes yielded a 3.3 Å class (Structure IV_p) which contained both tRNAs and EF-G, and a heterogeneous class requiring further classification. Subsequent classification of the second class into 2 classes using a focused mask of 30 Å around the 50S E site produced a 3.9 Å class with two tRNAs and EF-G (Structure V), and a heterogeneous class requiring additional classification. A final classification of the heterogeneous class for 100 cycles with a 55 Å spherical focused mask around the translocase domain of EF-G produced a 3.8 Å class that contained the 70S ribosome with a swiveled head, two tRNAs, and no EF-G (Structure VI). Stack-2 exhibited heterogeneity at the EF-G binding site and 305 domain conformation, so it was first classified into 2 classes for 100 cycles, using a 3D mask covering the shoulder domain, filtered to 30 Å and down-weighted to 0.1. The class containing EF-G was used to create a substack of 5,379 particles (stack-2a) which was subject to refinement using the original 40 Å mask. This classification produced a 3.8 Å class of 1,657 particles containing a pre-translocation 70S-tRNA-EF-G state (Structure III). An additional classification of stack-2a using a separate masking strategy was performed (Supplementary Fig. 1c). Classification of stack-2a into 2 classes, performed for 100 cycles, with a focused spherical mask with the radius of 35 Å, covering the GTAPase domain of EF-G. Classification into 3 classes yielded a 3.7 Å class of 1,884 particles containing a pre-translocation 70S-tRNA-EF-G state (Structure III_p).

0-s dataset — Data collection and processing for the 0-s time point pre-translocation 70S mRNA+Met-tRNA^Met+Pro-tRNA^Pro complex were performed as follows. Two 25-minute exposure series of 146 and 166 images were collected with nearly identical parameters on the Titan Krios microscope described above, with the −0.5 to −1.5 μm defocus range. Multi-shot multiple data acquisition was performed using SerialEM as described above. Each exposure was acquired with continuous frame streaming at 25 frames per ~1 s yielding a total dose of ~40 e-/Å². The movies were binned to pixel size 0.87 Å (termed unbinned or 1× binned). The initial alignment, refinement and 3D classification of both stacks of 157,421 (stack-1) and 686,850 (stack-2) particles into 16 classes for 100 cycles was performed, as described for the 25-s dataset above, with the exception of an ab initio model generated from 50% of the particles of stack-1 was used for initial alignment. After excluding the low-resolution (junk) classes and classes representing the 50S subunit, the extracted particles were combined into substack-1 (191,638 particles, respectively) and classified into 16 classes using a 40 Å focused spherical mask placed between the A and P sites (as in the 25-s data set) to resolve the pre-translocation 70S-A site densities. Seven classes from substack-1 containing non-rotated pre-translocation ribosomes were combined (37,257 particles) and refined resulting in Structure I. 5 classes of rotated ribosomes from substack-1 and 2 classes of rotated translocation ribosomes were combined (37,252 particles) and reclassified into 2×-binned substacks (91,638 particles) and substack-2 (346,334 particles). Additional classiﬁcation of this stack was performed for 50 cycles using a focused spherical mask with the 30-Å radius, covering most of the A and P sites. Classification into 16 classes yielded 5 high-resolution classes, each of which contained two tRNAs and EF-G. The particles assigned to the 3 high-resolution classes were extracted from the 2× binned stack (with >50% occupancy and scores > 0) using merge_classes.exe (part of the FREALIGN distribution), and merged into a stack containing 322,549 particles. Classification of this stack was performed for 50 cycles using a focused spherical mask with the 30-Å radius, yielding a total of 30 classes (50% occupancy and scores > 0) using merge_classes.exe (part of the FREALIGN distribution), and merged into a stack containing 48,345 particles. Classification of this stack was performed for 50 cycles using a focused spherical mask at the A site (30 Å radius, as implemented in FREALIGN). Classification into 3 classes yielded a single high-resolution class, which contained two tRNAs and EF-G. Additional classiﬁcation of each class into more classes did not yield other unique high-resolution structures with EF-G. For the class of interest (Structure III−vi, 20,167 particles), particles with >50% occupancy and scores > 0 were extracted from the 2× binned stack. Refinement to a 6 Å resolution using motion correction and very thin ice were excluded from further analysis after inspection of the averages and the power spectra computed by CTFIND4 of cistEM. The stack of 517,847 particles and the particle parameter files were assembled in cisTEM with the binnings of 2×, 4×, 8×, at 0.87 Å/pixel. Particles from the 2× dataset with the resolution of 6.8 Å were also used to interpret high-resolution details or lower-resolution features. SSc2 curves were calculated by FREELIGN v2 even and odd particle half-classes (Supplementary Figs. 1–2).

Map ﬁltering and resolution. Local-resolution filtering was applied to the resulting cryo-EM maps by a previously optimized procedure36, using blocks and block averaging from the Bsoo (vs. 1.9.1) package34 followed by sharpening the block-averaged maps with bfactor.exe using a constant B-factor of −50 Å² to the average resolution determined by FSC_part. These maps were used for model building and structure reﬁnements. Maps sharpened or softened with different B-factors (from −125 to +50 Å²) were also used to interpret high-resolution details or lower-resolution features. SSc2 curves were calculated by FREELIGN v2 even and odd particle half-classes (Supplementary Figs. 1–2).

Model building and reﬁnement. Cryo-EM structure of E. coli 70S-mRNA−Met-tRNA^Met−Pro−tRNA^Pro−EF−Tu−GDP−PCP structure III47, excluding EF-Tu and tRNAs, was used as a starting model for structure reﬁnements. The structures of EF-G were created by homology modeling and map ﬁtting, using ribosome-bound EF-G structures including PDB 4V7D (Brilot et al. 2013, PNAS), PDB 6WZI, and the crystal structure of EF-G (PDB 1WDT) as references. Initial protein and ribosome domain ﬁtting into cryo-EM maps was performed using Chimera35, followed by manual modeling using Pymol (vs. 1.7.x)36. The linkers between the domains and parts of the domains that were not well deﬁned in the cryo-EM maps (e.g. ribosomal proteins or loops of EF-G) were not modeled. The EF-G and tRNAs were reﬁned using FRODO by real-space simulated-anneling reﬁnement using atomic electron scattering factors in RSRef (2000)37. Secondary-structure restraints, comprising hydrogen-bonding restraints
Assessment of GTP and GDP-Pi models fit into cryo-EM maps. To differentiate between the stages of GTP hydrolysis in the EF-G GTAPase center, we quantitatively assessed the local fit of the alternative structural models (pre-hydrolysis GTP or post-hydrolysis GDP-Pi) into density maps for pre-translocation states in this work (III and III-vio) and in the recent study that captured ribosome-GFP with spectinomycin and reported a structural model with GTP69. The cryo-EM map from the latter study has a visually better fit for the γ-phosphate separated from the β-phosphate (Supplementary Figs. 6l–n), and the structural model for GTAP contained stereochemical outliers indicating overfitting (e.g. RMS deviation from ideal bond lengths of ~0.03 Å and RMS deviation from ideal bond angles of ~6°, Supplementary Table 3). To this end, the original nucleotide models were used as starting models. In addition, high-resolution reference models of GTP and GDP-Pi from a 1.5 Å crystal structure of αlF2 (PDB 4RD1; termed αlF2 nucleotide) were set to a uniform value (80) for all structural models prior to refinement. The corresponding maps were carved around the modeled nucleotide using phenix.map_box.114-3260. The nucleotides were refined using phenix.real_space_refine at an optimal weight resulting in a good stereochemical fit of the nucleotide/Pi into the map and gave stereochemical parameters, as indicated by the low MolProbity scores of ~2, low deviations from ideal bond lengths and angles, low number of protein and RNA outliers and other structure-quality statistics (Supplementary Table 1). Structure quality was validated using MolProbity.

Structure superpositions and distance calculations were performed in Pymol. To differentiate at an optimal weight resulting in a good stereochemical fit of the nucleotide/Pi into the map and gave stereochemical parameters, as indicated by the low MolProbity scores of ~2, low deviations from ideal bond lengths and angles, low number of protein and RNA outliers and other structure-quality statistics (Supplementary Table 1). Structure quality was validated using MolProbity.

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Competing interests

The authors declare no competing interests.

Additional information

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