Elongation factor G initiates translocation through a power stroke

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During the translocation step of prokaryotic protein synthesis, elongation factor G (EF-G), a guanosine triphosphatase (GTPase), binds to the ribosomal PRE-translocation (PRE) complex and facilitates movement of transfer RNAs (tRNAs) and messenger RNA (mRNA) by one codon. Energy liberated by EF-G’s GTPase activity is necessary for EF-G to catalyze rapid and precise translocation. Whether this energy is used mainly to drive movements of the tRNAs and mRNA or to foster EF-G dissociation from the ribosome after translocation has been a long-lasting debate. Free EF-G, not bound to the ribosome, adopts quite different structures in its GTP and GDP forms. Structures of EF-G on the ribosome have been visualized at various intermediate steps along the translocation pathway, using antibiotics and nonhydrolyzable GTP analogs to block translocation and to prolong the dwell time of EF-G on the ribosome. However, the structural dynamics of EF-G bound to the ribosome have not yet been described during normal, uninhibited translocation. Here, we report the rotational motions of EF-G domains during normal translocation detected by single-molecule polarized total internal reflection fluorescence (polarized TIRF) microscopy. Our study shows that EF-G has a small (~10°) global rotational motion relative to the ribosome after GTP hydrolysis that exerts a force to unlock the ribosome. This is followed by a larger rotation within domain III of EF-G before its dissociation from the ribosome.

Coupled translocation of transfer RNA (tRNA) and messenger RNA (mRNA) within the ribosome, catalyzed by the guanosine triphosphatase (GTPase) elongation factor G (EF-G), is one of the major steps in the elongation cycle of protein synthesis, allowing the next codon to enter into the ribosomal A site, in preparation for the next decoding step. Upon binding to the PRE-translocation (PRE) ribosomal complex, EF-G rapidly hydrolyzes GTP. Translocation follows, and EF-G-GDP dissociates from the resulting POST-translocation complex (1). Recent studies (2–4) have clearly demonstrated that EF-G-GTP promotes translocation from either the classic or hybrid PRE complex, which contain tRNAs in the A/A and P/P or A/P and P/E sites, respectively (first letter 30S position, second letter 50S position).

EF-G has five domains (5, 6). Its structures in the GTP and GDP forms free of the ribosome indicate a significant hinge-like joint motion of the C-terminal domains (III–V) with respect to N terminus (domains I and II) (7, 8). Structural and single-molecule studies have captured different EF-G structures and states on the ribosome (9–17), using antibodies and/or nonhydrolyzable GTP analogs to prevent rapid translocation and increase the lifetime of ribosome-bound EF-G. These studies have provided valuable insights into how EF-G interacts with the ribosome. However, studies of the EF-G:ribosome complexes obtained in these conditions do not necessarily capture all of the relevant intermediates and may sometimes represent off-pathway or rarely visited states. Rapid reaction kinetics studies have examined the structural changes of the ribosome and tRNAs during normal translocation (4, 18–21), whereas the corresponding kinetic and structural information for EF-G is mostly unknown. Moreover, none of these earlier studies have described the kinetics of conformational changes of ribosome-bound EF-G during normal, rapid translocation.

Here we address this gap by comparing rotational motions that individual EF-G subdomains, labeled with an appropriate fluorescent probe, undergo on the ribosome during both normal and antibiotic-inhibited translocation or with an empty ribosomal A site. We detect these motions with millisecond time resolution, using single-molecule polarized total internal reflection fluorescence (polarized TIRF) microscopy (22, 23). By monitoring the emission polarization of single fluorescent probes under different polarizations of the excitation light, polarized TIRF microscopy allows for the detection of rotational fluctuations in single molecules. Previous studies (24) have described the kinetics of conformational changes of ribosome-bound EF-G during rapid translocation. Our observations strongly imply a hybrid model, in which the initial steps of translocation are ribosome unlocking driven by a force generated via EF-G-dependent GTP hydrolysis, and further steps of translocation are mainly driven by the energetics of the ribosome itself. These results demonstrate that the ribosome and EF-G make use of power-stroke and Brownian-ratchet mechanisms to ensure efficiency and accuracy of translocation.

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Supporting Information

Significance

Elongation factor G (EF-G) uses energy stored in GTP to catalyze movement of transfer RNAs and messenger RNA in the ribosome during the translocation step of prokaryotic protein synthesis. Using single-molecule polarized fluorescence microscopy, three-dimensional rotational motions of individual domains of EF-G were directly captured, for the first time to our knowledge, during normal translocation. Our observations strongly imply a hybrid model, in which the initial steps of translocation are ribosome unlocking driven by a force generated via EF-G-dependent GTP hydrolysis, and further steps of translocation are mainly driven by the energetics of the ribosome itself. These results demonstrate that the ribosome and EF-G make use of power-stroke and Brownian-ratchet mechanisms to ensure efficiency and accuracy of translocation.

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translocation may follow either a power-stroke or a Brownian ratchet mechanism, the latter driven by stochastic thermal fluctuations.

We labeled EF-G using bifunctional rhodamine (BR), a fluorescent probe that cross-links two engineered cysteine (Cys) residues located seven residues apart in an α-helix (Fig. 1A). Rotation of BR relative to the protein is highly restricted, as documented by the polTIRF signals, and thus rotations of the labeled sites can be inferred from the probe angle changes. We prepared several EF-G mutants, labeled them with BR, and demonstrated that BR cross-links the two engineered Cys residues, using site-specific cleavage and mass spectrometry (Fig. S1). After testing their activities in translocation (Table S1), we selected the following BR-labeled EF-Gs for further polTIRF experiments: EF-GR630-637 (domain V), EF-GR555-562 (domain III), EF-GR429-436 (domain II), and EF-GR692-699 (formally domain V, but actually in between IV and V) (Fig. 1B).

In polTIRF experiments, the ribosome was attached to a microscope slide through both the 3′ and 5′ ends of an mRNA to constrain its rotational mobility (Fig. S2A). A 40-nt length mRNA and 2-kDa PEG linkers provided an optimized attachment that immobilized the ribosomes with the least rotational mobility (Fig. S2B). Ribosomes immobilized under these conditions successfully completed approximately six elongation cycles (Fig. S3), as detected by single-molecule fluorescence resonance energy transfer (FRET) patterns using Cy3- and Cy5-labeled tRNAs (25). PolTIRF recordings, including 16 different simultaneous polarized fluorescence intensities (PFIs) as shown in Fig. 1C, began after injecting BR-labeled EF-G (EF-GRBR) and other necessary components into the flow chamber to start multiple elongation cycles. When an EF-GRBR transiently bound to an immobilized ribosome, a pulse of fluorescence was detected (Fig. 1C and D). During this binding event, simultaneous discrete changes of the individual PFIs signaled a sudden rotation of the EF-GRBR, whereas the total intensity remained almost constant (Fig. 1C–E). A maximum-likelihood, multitrace change-point algorithm identifies statistically valid change points caused by sudden rotations and localizes their timing within several milliseconds (26). For each instance of EF-GRBR binding to the ribosome, the orientation and extent of wobble of EF-GRBR before and after any structural changes were determined from the PFIs (details in Supporting Information).

For all of the EF-GRBR constructs tested, the observed fluorescence pulses under normal elongation conditions indicated two types of binding events. About half of the events had short dwell times of occupancy on the ribosome (42–71 ms; Fig. 2A, Fig. S4, and Table S2) and no detectable rotations. These events were assigned to sampling of the ribosome by EF-G and dissociation, without completion of translocation. The other half of the events showed rotational motions and had longer dwell times (130–183 ms; Fig. 2A and Table S2 and sample traces in Fig. 1C and D and Figs. S5–S9). These were considered to be successful translocation events. The assignments and durations of these two different types of binding events agree very well with a recent study using Cy5-labeled EF-G (4), in which EF-G binding events without ribosomal 30S–50S subunit rotations were considered to be sampling events and EF-G binding events that were correlated with subunit counter-rotations were considered to be translocations. For these events, all of the EF-GRBRs exhibited similar dwell times on the ribosome before and after the rotational change points (60–80 ms and 72–104 ms, respectively, at 18 °C; Table S2).

Rotational motions of individual EF-G binding events were defined by the orientation of the BR probe before and after motions. All EF-GRBR exhibited similar microsecond wobble amplitudes, demonstrating that the probe dipoles follow the labeled helix quite faithfully and that none of the labeled helices were artifactual to each other, indicating that the motions we detected are not artifacts caused by fluorophore labeling or changes of secondary structure. We speculate that the wide distribution of the domain III rotational angles results from flexibility of domain III in the EF-G-GDP form (6) that might give rise to multiple parallel translocation and/or dissociation pathways. Strikingly, EF-GR630-637 (domain IV), EF-GR692-699 (domain V), and EF-GR429-436 (domain III) all showed much smaller angle changes than domain III.

Fig. 1. Structures of BR and EF-G and polTIRF recordings of a single EF-GRBR binding event. (A and B) Structures of (A) BR and (B) EF-G. BR is shown cross-linking two Cys side chains on an α-helix. The α-helices of EF-G labeled with BR are marked in green, (C and D) Sixteen PFIs (C) and the sum of all PFIs (D) of a single EF-GRBR binding event during translocation. Intensities are plotted as photon counts per ms averaged every 8 ms. (E) Three-dimensional orientations, rotational motion, and wobble (δ) of BR-labeled EF-G reconstructed from PFIs of this binding event. In C, the labels, such as s1x, s2x, and s1y, represent 16 combinations of excitation path (1 in the x–y plane, 2 in the z–y plane), excitation polarization (s, perpendicular to and p, parallel to the plane containing the incident and reflected beams; and L, 45° to the left of p and R, 45° to the right of p), and emission polarization (x or y), which were collected and plotted as PFIs. The binding of EF-GRBR to the immobilized ribosome at time 0.02 s and the dissociation of BR-EF-G at time 0.12 s are determined by sudden increase and decrease of the total intensity, respectively. The rotational motion of EF-GRBR is clearly indicated by changes of many individual PFIs at time 0.08 s, whereas the total intensity remains almost constant. Three-dimensional orientations of the probe dipole before and after rotational motions were resolved from 16 PFIs and plotted as red and blue arrows in E, respectively. Wobble (δ) of the probe on the microsecond scale, reconstructed from PFIs, was plotted in E as a cone around the dipole.
The large and variable motions of the EF-G domain III we observe during normal translocation have not generally been detected among the X-ray and cryo-EM structures obtained using antibiotics and/or nonhydrolyzable GTP analogs that inhibit translocation (with the sole exception of EF-GBR429-436, GTP, and Figure S11), strongly suggesting the internal conformational change and for dissociation of EF-G. It is possible that time-resolved cryo-EM with single-particle sorting into different classes, by resolving structural heterogeneities in the sample, could reveal the motions of domain III detected by polTIRF. An exception to the structures lacking large motions within ribosome-bound EF-G is provided by a crystal structure in which the antibiotic dityromycin arrests the ribosome in a PRE-translocation structure (32). This structure shows domain III rotated about 90° from the position seen in isolated EF-G complexed with either GNPPNP or GPPNP, consistent with the large rotations of domain III that we observed. However, Lin et al. (32) assume that domains III–V rotate together as a rigid body, which is inconsistent with our polTIRF data showing domain IV rotates less than domain III. Molecular dynamic simulations (27) have revealed relative rotational motion between domains IV and III. Therefore, a model, in which domain III moves first to apply the force and then domain IV translates to complete translocation just before EF-G dissociates (see Fig. 4), would reconcile the structure presented by Lin et al. (32) and the rotational motions captured by us.

We next monitored the rotational dynamics of EF-GBR8 in the presence of the antibiotics viomycin (Vio) (5 μM) and spectinomycin (Spc) (2 mM), which do not affect the rate of EF-G–dependent GTP hydrolysis (1, 33) but block translocation at subsequent steps along the pathway (19, 34, 35). Vio exhibits a bimodal behavior in its effects on ribosome dynamics (36). At the low concentration we use, Vio stabilizes tRNAs in the hybrid states and the ribosome in the rotated conformation and it halts translocation at an early stage (36–38). Spc disrupts translocation later in the process by blocking swiveling of the head domain of the small ribosomal subunit (39). Both Vio and Spc considerably reduced the extent of rotations in domain III (EF-GBR429-436 and EF-GBR467-474, Fig. 3 and Fig. S12) toward the amplitude of the global motion reported by the other domains. The average angle change of EF-GBR429-436 decreased from 46° ± 1° to 27° ± 1° and 37° ± 1° (SEM) in the presence of Vio and Spc, respectively.

In contrast, Vio and Spc had little or even no effect on rotations of domains I (EF-GBR232–239), IV (EF-GBR555–562), and IV-V (EF-GBR692–699) (Figs. 2B and 3A and Fig. S12). Moreover, the antibiotics had no significant effect on the proportion between rotating and nonrotating EF-GBR binding events and their occupancy times on the ribosome (Table S3). Most strikingly, neither Vio nor Spc had perceptible effects on rotation of EF-GBR429-436 domain III, when the A site was empty (Fig. S11), strongly suggesting...
that tRNA must be present in the A site for Vio and Spc to hinder the large rotational motions of EF-G domain III. The simplest explanation of this result is that when the tRNAs cannot move due to the action of the antibiotics, they become a physical barrier that blocks domain III's local motion as well, consistent with the notion introduced above that such local motion is directly correlated with tRNA translational movement.

Whether EF-G functions as a force-generating power-stroke motor or the pawl of a Brownian ratchet that blocks reversal of ribosome and tRNA/mRNA thermal fluctuations has long been
We illustrate these two ideas by the simplified schematic energy landscapes in Fig. 3B. The reaction coordinate here is conceptually the translocation distance of the mRNA and tRNAs between their PRE- and POST-translocation positions. In the power-stroke model (Fig. 3B, Upper), EF-G converts biochemical energy of GTP hydrolysis and product release to mechanical work. The force generated by EF-G along the reaction coordinate tilts the energy landscape by slope ΔG/Δx = force (blue line), which decreases the energy barrier (red curve before force generation vs. green curve after application of force) and actively promotes movements of tRNAs and mRNA from the PRE-translocation state. In the Brownian-ratchet model, the energy barrier between initial and final states is lower, and thermal energy drives fluctuations between initial and final states of translocation. In this model, conformational changes in EF-G and/or the ribosome, triggered by the transiently formed POST-translocation state, cause domain IV of EF-G to act as a pawl (blue line), suppressing the backward reaction and thereby stabilizing the POST state (2). In the Brownian-ratchet model of translocation, the role of GTP hydrolysis is mainly to facilitate EF-G dissociation after translocation.

Vio and Spc at the low concentrations used here stabilize the ribosome in PRE conformational states, with the Vio-stabilized state occurring earlier in the translocation process than the Spc-stabilized state (14, 19, 33–37, 43, 44). Such stabilizations correspond to large energy barriers (shown in magenta for Vio in Fig. 3) between the initial and final states (33, 45). According to the Brownian-ratchet model, the induced barrier blocks thermally driven fluctuations of the ribosomal complex between the PRE- and POST-translocation states without affecting the position of the energy minimum in the initial state. EF-G conformational changes, required for blocking the back reaction, would not take place in the presence of Vio or Spc. In contrast, we find that EF-G domains, including domain III, undergo similar rotations in the presence of Vio and Spc (Fig. 3D and Fig. S12). In the power-stroke model, the force generated by EF-G-catalyzed GTPase can still tilt the energy landscape of the initial state before the antibiotic-generated energy barrier is encountered, thus shifting the position of the energy minimum in the PRE state (black arrow in Fig. 3B). The EF-G–bound ribosome complex moving along the reaction coordinate to this new energy minimum promotes small global conformational changes, including in EF-G domain III, as we detect in the presence of Vio and Spc. Therefore, our findings strongly suggest that, at least for the initial portion of translocation, EF-G tilts the energy landscape, by generating a force (Figs. 3B and 4). In the presence of Vio and Spc, A-site–bound peptidyl-tRNA presents a physical barrier that suppresses large motions of EF-G.

Disrupting the interactions between the mRNA–tRNA duplex and ribosomal RNA in the decoding center (bases A1492 and A1493) by the tip of EF-G domain IV is thought to be a critical step that reduces the energy barrier for tRNA and mRNA movement (29, 46). Binding of Vio to the 30S subunit affects the positioning of A1492 and A1493, which presumably strengthens interactions of the mRNA–tRNA duplex with the decoding center (43). If the effect of EF-G’s initial stroke is to accelerate disruption of mRNA–tRNA interactions, then Vio would be expected to slow EF-G’s motions. Our observation that rates of EF-G rotational motions on the ribosome are unaffected by Vio implies that the initial power-stroke motion of EF-G does not act directly to disrupt interactions between mRNA, tRNA, A1492, and A1493. Instead, the power-stroke force is likely to foster structural rearrangements in the ribosome before tRNA and mRNA movements. Therefore, force applied by the ribosome on mRNA to unwind downstream secondary structures (47–49) is unlikely to be directly generated by the initial power stroke of EF-G. This is consistent with earlier suggestions that GTP hydrolysis triggers an “unlocking process” in the ribosome that enables further translocation steps, including ribosomal subunit rotation, tRNA and mRNA motion, and 30S head movement (18).

After the initial power stroke, EF-G undergoes further steps to complete translocation. During these steps, domain IV is likely to escort the A-site tRNA into the P site in the 30S subunit or follow it after spontaneous tRNA arrival. Domain IV does not exhibit the large rotations of domain III, indicating that they are not rigidly coupled during the rotation of domain III. These later motions of EF-G could proceed by either the power-stroke or Brownian-ratchet mechanism (Fig. 4). Recent evidence obtained from the force dependence of translational velocity in an optical trap favors the latter (48), consistent with the proposal that these later steps are mainly driven by the energetics of the ribosome itself (20). Holtkamp et al. (24) also suggested a hybrid model combining power-stroke and Brownian-ratchet mechanisms. Using Vio and Spc, we were able to trap EF-G motions at early and later intermediate states, respectively. The dissociation rates of EF-G from the states trapped by Vio and Spc are almost the same as the ones during normal translocation, implying that EF-G can naturally dissociate before translocation is completed. The variability of domain III rotational motions indicates that EF-G may adopt various structures in different ribosomes after its abrupt conformational change (Fig. 1C), which is in line with various translocation intermediates that have been described (9, 15, 29). A hybrid model (Fig. 4), in which EF-G functions as a Brownian-ratchet pawl after its initial force generation, could rationalize the multiple structures and reaction pathways of EF-G.

Summary
Our studies directly capture previously undetected rotational motions of EF-G on the ribosome during normal translocation. Neither Vio, nor Spc, nor an empty A site completely abolishes motions of EF-G on the ribosome. Vio, which prevents translocation by increasing the affinity of tRNA to the A site ~1,000 fold, barely affects kinetic rates of EF-G conformational changes, but markedly reduces the magnitude of EF-G domain III rotation. These findings suggest that conformational changes of EF-G on the ribosome that follow GTP hydrolysis early in translocation generate a mechanical force that either moves the mRNA and tRNAs directly or facilitates an “unlocking” of the ribosome that enables movement. Combining our results with those of others, we conclude that the ribosome and EF-G make use of both power-stroke and Brownian-ratchet mechanisms to ensure the efficiency and accuracy of translocation.

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Supporting Information

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mRNA Sequences

40-nt mRNA: G UUA AUA AGG AUA CAU AUG UUC GUG UUC GUG UUC GUG.

65-nt mRNA: AUU UAA AAG AUA AUG AAG AGA CAU ACU AUG UUC GUG UUC GUG UUC GUG UUC GUG.

80-nt mRNA: AUU UCA ACAA AUU UAA AAG UUA AUG AUA AUG AUA CAU ACU AUG UUC GUG UUC GUG UUC GUG UUC GUG.

The underlined sequences are start codons. All mRNAs were purchased from Dharmacon and had both 5' and 3' biotinylated.

Ribosome and tRNA Preparation

The 70S ribosomes were prepared according to published procedures (3, 25, 50, 51). Initiation complexes were formed by incubation of 70S ribosomes with mRNA, initiation factors I,–3, GTP, and fMet-tRNA^Met in TAM (25 mM MgAc, 50 mM Tris-HCl, pH 7.5, 30 mM NH₄Cl, 70 mM KCl, and 1 mM DTT), at 37 °C for 25 min, followed by centrifugation through a sucrose cushion (3).

Amino acid-specific tRNAs, Escherichia coli tRNA^Met, yeast tRNA^phe, and E. coli tRNA^val were purchased from Chemical Block and charged and labeled as described (52, 53). Neither the charging nor the labeling reactions were stoichiometric. Partial separation of charged from uncharged tRNAs was achieved by reverse-phase HPLC, using a LiChrospher WP-300 RP-18 column (5-μm beads, 250–4 mm; Merck KGaA-Darmstadt). The tRNA mixture was applied to the column equilibrated with buffer E (20 mM NH₄Ac, pH 5.0, 10 mM MgAc₂, and 400 mM NaCl) and the aminoclayed tRNAs were eluted with a linear gradient of buffer E containing 30% (vol/vol) ethanol. Cy3/Cy5-labeled tRNAs were fully resolved from unlabeled tRNAs using a gradient of buffer E containing 30% (vol/vol) ethanol. Ch stomica of Cy5 imaging were collected, allowing unambiguous 3D resolution of tRNA translocation were collected, allowing unambiguous 3D resolution of tRNA translocation. All probes reported here had microsecond wobbles via two ends of the mRNA as shown in Fig. S2. On the other hand, the remaining ambiguity is due to the dipolar nature of the fluorescent individual probe dipole orientations within a hemisphere. The polarization and simultaneously measured emission polarization were collected, allowing unambiguous 3D resolution of tRNA translocation polarization and simultaneously measured emission polarization were collected, allowing unambiguous 3D resolution of individual probe dipole orientations within a hemisphere. The remaining ambiguity is due to the dipolar nature of the fluorescent probe. The ribosomes were unlabeled and their absolute orientations during experiments were unknown. Therefore, we do not obtain absolute angles between EF-G and the ribosomes. The rotational motions of the ribosomes were restricted by attachment via two ends of the mRNA as shown in Fig. S2. On the other hand, rotational motions of the labeled EF-G domains could be clearly quantified as the angle change between different probe dipole orientations during each individual EF-G binding event that led to translocation. All probes reported here had microsecond wobbles with conical half-angles of ~45°. This wobble is the aggregate of individual probe dipole orientations within a hemisphere.

The Principle of polTIRF

Details of the polTIRF method, including the instrumentation, data acquisition, and analysis, have been described elsewhere (22, 23, 26, 60).

In brief, BR probes attached to EF-G were excited by polarized evanescent waves generated in a prism-type total internal reflection microscope. The polarized fluorescent emission from single BR probes was used to determine its 3D orientation. A total of 16 combinations of temporally modulated excitation path and excitation polarization and simultaneously measured emission polarization were collected, allowing unambiguous 3D resolution of individual probe dipole orientations within a hemisphere. The remaining ambiguity is due to the dipolar nature of the fluorescent probe. The ribosomes were unlabeled and their absolute orientations during experiments were unknown. Therefore, we do not obtain absolute angles between EF-G and the ribosomes. The rotational motions of the ribosomes were restricted by attachment via two ends of the mRNA as shown in Fig. S2. On the other hand, rotational motions of the labeled EF-G domains could be clearly quantified as the angle change between different probe dipole orientations during each individual EF-G binding event that led to translocation. All probes reported here had microsecond wobbles with conical half-angles of ~45°. This wobble is the aggregate of individual probe dipole orientations within a hemisphere. The remaining ambiguity is due to the dipolar nature of the fluorescent probe. The ribosomes were unlabeled and their absolute orientations during experiments were unknown. Therefore, we do not obtain absolute angles between EF-G and the ribosomes. The rotational motions of the ribosomes were restricted by attachment via two ends of the mRNA as shown in Fig. S2. On the other hand, rotational motions of the labeled EF-G domains could be clearly quantified as the angle change between different probe dipole orientations during each individual EF-G binding event that led to translocation. All probes reported here had microsecond wobbles with conical half-angles of ~45°. This wobble is the aggregate of individual probe dipole orientations within a hemisphere.

EF-G^BR Preparation and Activity

Mutation sites of all EF-G^BRs are listed in Table S1. EF-G variants labeled with bifunctional rhodamine dye (BR-I, a generous gift from J. E. T. Corrie, National Institute for Medical Research, Mill Hill, London) were prepared and characterized according to published procedures (54, 55). EF-G was exchanged into the labeling buffer (50 mM Hepes, pH 7.4, 1 mM MgCl₂, 100 mM NaCl, 15 μM GDP). TCEP [Tris (2-chloroethyl) phosphatase] was then added to a final concentration of 1 mM, with incubation at room temperature for 50 min. BR-I dye was added in equal portions four times at 30-min intervals, bringing the final BR-I: EF-G ratio to 2:1. Following incubation for an additional 3 h at room temperature, the reaction was quenched with MESNA (sodium 2-sulfanylthiolethanesulfonate, final concentration 3 mM). Unreacted dye was removed using a Thermo 22858 column (Thermo Fisher Scientific). The sample was next dialyzed into 50 mM Tris-HCl (pH 7.5), 70 mM NH₄Cl, 30 mM KCl, 7 mM MgCl₂, 1 mM DTT, and 15 μM GDP overnight at 4 °C.

Purification of EF-G^BR was achieved by FPLC, using a TSK column. The EF-G^BR mixture was applied to the column with buffer A (3.5 M NaCl, 20 mM Hepes (pH 7.4), 15 μM GDP, 5 mM MgCl₂) and was eluted with a linear gradient of 100% buffer A to 100% buffer B [20 mM Hepes (pH 7.4), 15 μM GDP, 5 mM MgCl₂]. Eluted peaks with fluorescence were collected and dialyzed into 50 mM Tris-HCl (pH 7.5), 70 mM NH₄Cl, 30 mM KCl, 7 mM MgCl₂, 1 mM DTT, and 15 μM GDP for storage.

To verify that BR cross-linked the two engineered Cys residues, each EF-G^BR was digested by a site-specific protease (endo-protease Lys-C or trypsin, sequencing grade). Digested peptide fragments were separated by analytical HPLC (reverse-phase C18). The fluorescent fragments were collected and analyzed by electrospray time-of-flight mass spectrometry (Fig. S1) (54). No masses corresponding to fragments derived from monofunctional labeling were found, whereas the masses of the expected bifunctional labeled fragments were present, indicating that all of the EF-G^BR samples have the two engineered Cys residues cross-linked by BR.

Cysteine-free EF-G and its mutants have been used in several previous studies, including refs. 4, 16, 17, 56, and 57. These EF-G mutants were shown to have similar activities to those of wild-type EF-G (WT-EF-G) to promote translocation. Using single-molecule fluorescence resonance energy transfer (smFRET) between ribosomal protein L11 and A-site tRNA (3), we characterized the activities of EF-G mutants in promoting translocation by quantifying movements of tRNA from A site to P site (Table S1). Those EF-G^BRs, whose translocation activities were 70% or higher compared with the WT-EF-G, were used in further polTIRF experiments. The measured dwell times from all of our labeled mutants used in polTIRF experiments (Tables S2 and S3) are in the same range and consistent with other measurements in the literature (1, 4, 18, 19, 58, 59), indicating high activity.
Acquisition and Analysis of polTIRF Data

All polTIRF studies were carried out at 18 °C. Initiation complexes were specifically attached to microscope flow cells via biotinylated mRNA to PEG-passivated quartz slides (61), decorated with biotin-PEG and streptavidin. polTIRF experiments were performed in TAM buffer (15 mM MgAc, 50 mM Tris-HCl, pH 7.5, 30 mM NH₄Cl, 70 mM KCl) with 100 mM DTT to diminish photoblinking and bleaching of the BR probe. Two alternating beams from a 532-nm Nd:YAG laser were projected through a coupling prism at a glancing incident angle, producing an evanescent field at the quartz slide/aqueous interface. Switching between the two beams (termed path 1 and path 2), which were in perpendicular planes, was achieved by computer-controlled voltages applied to a Pockels cell and a polarizing beamsplitting cube. Similarly, the polarization of the laser in each beam was switched between four different linear polarizations (9°, 0°; p, 0°; L, +45°; and R, −45° relative to the plane defined by the incident and reflected beams) by an additional Pockels cell and a Berek compensator in each beam. These combinations result in a total of eight incident excitations. Each of the polarized excitations illuminated the sample for 100 μs.

Fluorescence emission was collected by a Leica 100 × 1.2 NA water immersion objective lens, passed through a long-pass blocking filter, and directed onto two avalanche photodiodes (APDx and APDy) through a polarizing beam splitter that resolved its x and y components. Thus, a total of 16 combinations of excitation path, excitation polarization, and emission polarization were collected as a cycle each 800 μs and plotted as PFIs. They were termed sx, sy, sx, sy, px, py, px, py, R1, R1, R2, R2, L1, L1, L2, L2, and L2 (s, p, R, and L indicate incident polarization; the number indicates path; and x and y indicate the detector). A maximum-likelihood, mutitrace change-point algorithm was used to identify statistically valid change points caused by sudden rotations and to localize their timing within several milliseconds (26). For visualization purposes, single-photon counts were converted to and plotted as 16 PFIs by counting the number of photons detected during successive constant 8-ms time bins (Fig. 1 C and D and Figs. S4–S9). An analytical model of the PFIs expected from adjustable angles was fitted by maximum likelihood to averages of the 16 PFIs over the intervals between the sudden change points to determine the orientation of the probe absorption and emission dipole and the rotational motion.

The spot detection nature of the avalanche photodiodes necessitates observing repeated events of EF-G binding within the 2 × 2-μm detection area to obtain valid, reproducible data. EF-G binding and dissociation from normally translating ribosomes or repeated EF-G binding to Vio- or Spc-blocked ribosomes thus enable reliable events to be collected. Trapping EF-G on the ribosome, though, using nonhydrolyzable GTP analogs or antibiotic fusidic acid does not provide reliable data. Due to the stochastic nature of the normal reactions, each individual single-molecule polTIRF event has unique features that contain real thermal motions of the sample and stochastic fluctuations in photon counting, dwell times, and tilting. Hundreds of single-molecule polTIRF traces, such as the one shown in Fig. 1 C and D and Figs. S4–S9, were collected under each experimental condition to compile distributions of dwell times and rotation as shown in Figs. 2 and 3 and Figs. S11 and S12 and to extract thermal motions of the labeled helix.

Fig. S1. Characterization of EF-GBR232-239, EF-GBR232-239 was digested by endoproteinase Lys-C. Digested peptide fragments were separated by analytical HPLC (reverse-phase C18). The fluorescent fragments were collected and analyzed by electrospray time-of-flight mass spectrometry, which confirmed that BR cross-links the two engineered Cys residues (S4). As indicated, the expected fragment for the fragment containing the BR cross-link between the two Cys residues, mass 7397.1 Da, is present in the mass spectrum, whereas no mass corresponding to either of the two fragments expected for monofunctional labeling was observed.
Fig. S2. (A) Schematic representation of the immobilization linkage of the ribosome. (B) Flexibility of the ribosome characterized by microsecond wobble angles of BR under various attachment conditions. The values 80-nt, 65-nt, and 40-nt are the numbers of bases in the doubly biotinylated mRNA. The values 5k, 2k, and 0.5k are molecular weights of biotin-PEG. (C) At the shortest linker length (40 nt, 0.5 kDa PEG), the wobble was higher than at 2 kDa PEG, presumably because the linkers were too short for both ends to reliably form continuous linker chains to the substrate. We thus used the 40-nt mRNA and 2 kDa PEG for the remaining studies reported herein, limiting aggregate wobble of the ribosome, EF-G, and local attachment helix to less than ∼45°.

Fig. S3. Single-molecule FRET experiments indicate that most of doubly tethered ribosomes can complete about six elongation cycles using the 40-nt doubly biotinylated mRNA coded for MFVFVFV. (A) A single-molecule FRET trajectory shows alternative Cy3-F (green curve) and Cy5-V (red curve) binding and five FRET events (pulses of sensitized emission, blue curve) between simultaneously bound tRNAs. The ribosome completes six elongation cycles before the end of the mRNA. (B) Distribution of FRET events per ribosome. The decreasing number of the ribosome showing multiple FRET events is caused by imperfect labeling efficiency of tRNAs. In our previous studies, we estimated that the effective labeling efficiency of tRNAs is 40–50%. Estimates of the distribution of FRET events per ribosome are shown in green, black, and blue curves, corresponding to 40%, 45%, and 50% effective labeling efficiencies, respectively, assuming that the ribosomes can complete six elongation cycles. The estimated curves agree with experimental measurement quite well, indicating that most of the ribosomes can complete six elongation cycles. Each polTIRF binding event containing rotational motions corresponds to a translocation step in one of these cycles.
Fig. S4. Two EF-G<sup>BR429-436</sup> binding events during translocation without a detectable rotational motion. The noisy traces are the 16 polarized fluorescence intensities binned at 8-ms intervals.

Fig. S5. EF-G<sup>BR232-239</sup> binding events during translocation with rotational motions detected by the change-point algorithm with 95% confidence indicated by a dashed line. Polarized fluorescence intensities are shown binned at 8 ms.
**Fig. S6.** EF-G$^{BR467-474}$ binding events during translocation with rotational motions detected by the change-point algorithm with 95% confidence indicated by a dashed line. Polarized fluorescence intensities are shown binned at 8 ms.

**Fig. S7.** EF-G$^{BR555-562}$ binding events during translocation with rotational motions detected by the change-point algorithm with 95% confidence indicated by a dashed line. Polarized fluorescence intensities are shown binned at 8 ms.
Fig. S8.  
EF-G<sup>BR630-637</sup> binding events during translocation with rotational motions detected by the change-point algorithm with 95% confidence indicated by a dashed line. Polarized fluorescence intensities are shown binned at 8 ms.

Fig. S9.  
EF-G<sup>BR692-699</sup> binding events during translocation with rotational motions detected by the change-point algorithm with 95% confidence indicated by a dashed line. Polarized fluorescence intensities are shown binned at 8 ms.
Fig. S10. Kinetics of EF-G on the ribosome measured by single-molecule FRET between EF-G<sub>BR467-474</sub> and L11 labeled with Cy5 at residue 87 at 21 °C. (A) Total dwell time of EF-G on the ribosome averages 105 ± 1 ms from the single exponential decay. (B) Transition density plot of the initial FRET value after EF-G binds vs. the final FRET value before EF-G dissociates. The final FRET efficiency is 0.03 ± 0.01 higher than the initial FRET, which indicates a small decrease in distance when EF-G tilts on the ribosome. Crystal structures of the ribosome–EF-G complexes (Protein Data Bank IDs 4W29 and 4V9k represent early and late translocation intermediates, respectively) (12, 15) show a small distance decrease (~0.06 nm) between EF-G and the L11 labeling site during translocation, which corresponds to an ~0.015 increase in FRET efficiency. Our smFRET observation is in line with the prediction from these structures, which indicates that the FRET increase we captured is attributable to translocation. (C) Postsynchronization analysis (62) was used to extract kinetic information from the recordings at relatively low signal-to-noise ratio. The black points are the average of all FRET traces aligned at the moment of EF-G binding, which gives the lifetime of the state before EF-G’s conformational change (60 ± 7 ms). The red points are the average of all FRET traces aligned at the last time point before EF-G dissociates, which gives the lifetime of the state after EF-G’s conformational change (52 ± 5 ms). These times are somewhat shorter than those determined in the polTIRF experiments, which is attributable to the higher temperature (21 °C vs. 18 °C) at which they were performed.

Fig. S11. Distribution of rotational angles of EF-G<sup>BR</sup> binding to the ribosomes (initiation complexes) containing only P-site initiator fMet-tRNA<sup>fMet</sup>. In some cases, 5 μM Vio or 2 mM Spc was added. Both A-site tRNA and one of the antibiotics must be present to block rotation of EF-G domain III.
Fig. S12. Distributions of rotational angles of EF-G<sup>BR</sup> domains in the presence 2 mM Spc.

Table S1. Translocation activities of EF-G<sup>BR</sup>s

<table>
<thead>
<tr>
<th>Labeling sites and EF-G domains</th>
<th>Mutation sites</th>
<th>Labeling</th>
<th>Relative translocation activity compared with WT-EF-G*</th>
</tr>
</thead>
<tbody>
<tr>
<td>232-239/I</td>
<td>E232C, G239C</td>
<td>BR labeled</td>
<td>1.03 ± 0.03</td>
</tr>
<tr>
<td>429-436/III</td>
<td>E429C, G436C</td>
<td>BR labeled</td>
<td>1.04 ± 0.05</td>
</tr>
<tr>
<td>467-474/III</td>
<td>D467C, K474</td>
<td>Unlabeled</td>
<td>1.01 ± 0.07</td>
</tr>
<tr>
<td>555-562/IV</td>
<td>K555C, K562C</td>
<td>BR labeled</td>
<td>0.92 ± 0.09</td>
</tr>
<tr>
<td>599-606/IV</td>
<td>I599C, K606C</td>
<td>Unlabeled</td>
<td>0.92 ± 0.02</td>
</tr>
<tr>
<td>629-636/IV</td>
<td>G629C, S636C</td>
<td>Unlabeled</td>
<td>0.55 ± 0.05</td>
</tr>
<tr>
<td>630-637/IV</td>
<td>D630C, R637C</td>
<td>BR labeled</td>
<td>0.91 ± 0.04</td>
</tr>
<tr>
<td>668-675/IV</td>
<td>G629C, S636C</td>
<td>Unlabeled</td>
<td>0.61 ± 0.10</td>
</tr>
<tr>
<td>692-699/IV</td>
<td>S692C, I699C</td>
<td>BR labeled</td>
<td>0.73 ± 0.10</td>
</tr>
</tbody>
</table>

All Cys residues were introduced into the Cys-less triple variant C114D/C266A/C397S of EF-G (56).

*Relative translocation activity was measured by the disappearance of high FRET intensity between Cy5-labeled L11 and Cy3-labeled A-site tRNA in a PRE-translocation complex after incubation with 100 nM of the various EF-G constructs for 5 min. Active EF-G promotes translocation from PRE-translocation to POST-translocation complex, which leads to a larger distance between L11 and tRNA and thereby lowers the FRET efficiency.

†The mass spectrometry assay indicated that BR-labeled EF-G(599-606) contains monofunctional labeled product, in which BR is attached to EF-G through only one Cys residue. Therefore, it was not used for further polTIRF experiments.

Table S2. Dwell times of EF-G<sup>BR</sup> on the ribosome during translocation

<table>
<thead>
<tr>
<th>Labeling domain</th>
<th>Labeling site</th>
<th>No. events</th>
<th>% events with rotations</th>
<th>Nonrotating events</th>
<th>Rotating events</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Total</td>
<td>Before rotation</td>
</tr>
<tr>
<td>I</td>
<td>232-239</td>
<td>327</td>
<td>46.2</td>
<td>71 ± 6</td>
<td>160 ± 30</td>
</tr>
<tr>
<td>III</td>
<td>429-436</td>
<td>437</td>
<td>65.9</td>
<td>63 ± 4</td>
<td>140 ± 20</td>
</tr>
<tr>
<td>III</td>
<td>467-474</td>
<td>522</td>
<td>49.4</td>
<td>42 ± 1</td>
<td>152 ± 5</td>
</tr>
<tr>
<td>IV</td>
<td>555-562</td>
<td>567</td>
<td>52.9</td>
<td>53 ± 2</td>
<td>165 ± 11</td>
</tr>
<tr>
<td>V</td>
<td>630-637</td>
<td>394</td>
<td>53.0</td>
<td>53 ± 5</td>
<td>183 ± 13</td>
</tr>
<tr>
<td>V</td>
<td>692-699</td>
<td>470</td>
<td>58.7</td>
<td>46 ± 4</td>
<td>130 ± 12</td>
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</table>

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Table S3. Dwell times of EF-G<sup>BR</sup> on the ribosome under various conditions

<table>
<thead>
<tr>
<th>Labeling site</th>
<th>Experimental condition</th>
<th>No. events</th>
<th>% rotational events</th>
<th>Nonrotating events</th>
<th>Total</th>
<th>Before rotation</th>
<th>After rotation</th>
</tr>
</thead>
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<tr>
<td>429-436</td>
<td>Translocation</td>
<td>437</td>
<td>65.9</td>
<td>63 ± 4</td>
<td>137 ± 21</td>
<td>60 ± 3</td>
<td>80 ± 5</td>
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<tr>
<td></td>
<td>+ Vio</td>
<td>770</td>
<td>48.4</td>
<td>79 ± 4</td>
<td>191 ± 22</td>
<td>94 ± 7</td>
<td>96 ± 5</td>
</tr>
<tr>
<td></td>
<td>+ Spc</td>
<td>585</td>
<td>64.6</td>
<td>69 ± 7</td>
<td>143 ± 10</td>
<td>75 ± 6</td>
<td>75 ± 4</td>
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<tr>
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<td>1,080</td>
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<td>70 ± 2</td>
<td>35 ± 2</td>
<td>30 ± 2</td>
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<td></td>
<td>IC, + Vio</td>
<td>571</td>
<td>53.4</td>
<td>26 ± 2</td>
<td>81 ± 3</td>
<td>42 ± 2</td>
<td>31 ± 2</td>
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<tr>
<td></td>
<td>IC, + Spc</td>
<td>276</td>
<td>51.5</td>
<td>25 ± 1</td>
<td>58 ± 4</td>
<td>42 ± 3</td>
<td>24 ± 2</td>
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<tr>
<td>232-239</td>
<td>Translocation</td>
<td>327</td>
<td>46.2</td>
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<td>62 ± 5</td>
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<tr>
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<td>+ Vio</td>
<td>496</td>
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<td>164 ± 26</td>
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<td>+ Spc</td>
<td>476</td>
<td>50.2</td>
<td>58 ± 2</td>
<td>179 ± 14</td>
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<td>64 ± 6</td>
<td>25 ± 4</td>
<td>27 ± 2</td>
</tr>
<tr>
<td>467-474</td>
<td>Translocation</td>
<td>522</td>
<td>49.4</td>
<td>42 ± 1</td>
<td>152 ± 5</td>
<td>60 ± 3</td>
<td>92 ± 6</td>
</tr>
<tr>
<td></td>
<td>+ Vio</td>
<td>599</td>
<td>41.2</td>
<td>60 ± 4</td>
<td>158 ± 9</td>
<td>88 ± 6</td>
<td>89 ± 6</td>
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<tr>
<td></td>
<td>+ Spc</td>
<td>592</td>
<td>46.6</td>
<td>40 ± 4</td>
<td>131 ± 8</td>
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<td>69 ± 4</td>
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<tr>
<td></td>
<td>IC</td>
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<td>26 ± 1</td>
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<tr>
<td>555-562</td>
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<td>74 ± 5</td>
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<tr>
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<td>178 ± 14</td>
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</tr>
<tr>
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<td>IC</td>
<td>437</td>
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<td>25 ± 1</td>
<td>40 ± 2</td>
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<td>21 ± 1</td>
</tr>
<tr>
<td>630-637</td>
<td>Translocation</td>
<td>394</td>
<td>53.0</td>
<td>53 ± 5</td>
<td>183 ± 13</td>
<td>80 ± 3</td>
<td>104 ± 10</td>
</tr>
<tr>
<td></td>
<td>+ Vio</td>
<td>282</td>
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<td>49 ± 4</td>
<td>185 ± 19</td>
<td>111 ± 17</td>
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</tr>
<tr>
<td></td>
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<td>252 ± 17</td>
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<tr>
<td></td>
<td>IC</td>
<td>790</td>
<td>49.6</td>
<td>31 ± 2</td>
<td>75 ± 6</td>
<td>38 ± 1</td>
<td>37 ± 1</td>
</tr>
<tr>
<td>692-699</td>
<td>Translocation</td>
<td>470</td>
<td>58.7</td>
<td>46 ± 4</td>
<td>130 ± 12</td>
<td>62 ± 3</td>
<td>72 ± 6</td>
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<tr>
<td></td>
<td>+ Vio</td>
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<td>54 ± 3</td>
<td>150 ± 13</td>
<td>70 ± 5</td>
<td>80 ± 3</td>
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<td></td>
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<td>899</td>
<td>50.4</td>
<td>63 ± 3</td>
<td>174 ± 13</td>
<td>70 ± 3</td>
<td>94 ± 3</td>
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<tr>
<td></td>
<td>IC</td>
<td>363</td>
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<td>22 ± 1</td>
<td>59 ± 7</td>
<td>27 ± 2</td>
<td>25 ± 1</td>
</tr>
</tbody>
</table>

IC stands for initiation complex containing fMet-tRNA<sup>fMet</sup> in the P site and an empty A site whose codon is UUC. A total of 5 μM Vio or 2 mM Spc was included where indicated.