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Structure of the GTP form of elongation factor 4 (EF4) bound to the ribosome

Veerendra Kumar1,2, Rya Ero2, Tofayel Ahmed2, Kwok Jian Goh2, Yin Zhan2, Shashi Bhushan2,3, and Yong-Gui Gao1,2,3

1 Institute of Molecular and Cell Biology, A*STAR, 61 Biopolis Drive, 138673, Singapore
2 School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, 637551, Singapore
3 NTU Institute of Structural Biology, Nanyang Technological University, Singapore

Running title: Structure of ribosome-EF4-GDPCP complex

To whom correspondence should be addressed: Prof. Yong-Gui Gao, Telephone: (65) 69082211; Fax: (65) 67913856; E-mail: ygao@ntu.edu.sg

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ABSTRACT

Elongation factor 4 (EF4) is a member of the family of ribosome-dependent translational GTPase (trGTPase) factors, along with elongation factor G (EF-G) and BPI-inducible protein A (BipA). Although EF4 is highly conserved in bacterial, mitochondrial, and chloroplast genomes, its exact biological function remains controversial. Here, we present the cryo-EM reconstitution of the GTP form of EF4 bound to the ribosome with P- and E-site tRNAs at 3.8 Å resolution. Interestingly, our structure reveals an unrotated ribosome rather than a clockwise-rotated ribosome, as observed in the presence of EF4-GDP and P-site tRNA. In addition, we also observed a counterclockwise rotated form of the above complex at 5.7 Å resolution. Taken together, our results shed light on the interactions formed between EF4, the ribosome, and the P-site tRNA and illuminate the GTPase activation mechanism at previously unresolved detail.

In all cells, proteins are synthesized based on messenger RNA (mRNA) templates via charged transfer RNAs (tRNAs) by large macromolecular RNA-protein assemblies called ribosomes. Ribosomes harbor three tRNA binding sites, A (aminoacyl-tRNA), P (peptidyl-tRNA), and E (exit tRNA) sites, and oscillate between two main states during the peptide chain elongation process, namely, the pre-translocation (PRE) and post-translocation (POST) states, with tRNAs bound to either the A- and P- or P- and E-sites, respectively. The translocation of tRNAs along with mRNA at the end of each round of polypeptide elongation is catalyzed by elongation factor G (EF-G) (1). Since it was reported a decade ago that the EF-G paralog LepA (Leader peptidase A, known more commonly thereafter as elongation factor 4, EF4) can induce back-translocation (the movement of tRNAs from the E- and P- to the P- and A-sites, respectively) in vitro (2), the biological function and modus operandi of EF4 have become a matter of dispute.

The EF4 gene is universally conserved in the genomes of bacteria and bacteria-derived organelles (2, 3), yet it seems dispensable under favorable growth conditions (2, 4-7). In 2006, EF4 was reported to catalyze back-translocation in vitro by binding to the POST complex (2). Subsequently, the crystal structure of the isolated apo form of EF4 was determined (8). EF4 consists of five domains, four of which (I, II, III, and V) are topologically equivalent to EF-G. The N-terminal domain I, also named the G domain, is the GTP/GDP binding site and is universally conserved among the trGTPase factors (3). Although EF4 lacks the G’ sub-domain insertion as well as domain IV of EF-G, it has an additional C-terminal domain (CTD). The additional CTD is a feature that is only observed in two trGTPase families, EF4 and BipA; however, their structures are not only different from one another but also represent entirely novel folds (8, 9).
The ∼11 Å resolution single particle cryo-electron microscopy (cryo-EM) reconstitution of the ribosome complex resulting from prolonged incubation of the POST complex with EF4 and a non-hydrolyzable GTP analog (GDPNP) suggested that EF4 directly interacts with the tRNA in the A-site thereby stabilizing it in a position distinct from the classical A/A tRNA (named A/L-tRNA, L for LepA binding), with the acceptor arm shifted away from the peptidyl transferase center (PTC) (10). The tRNA was predicted to fall back into the classical A-site upon EF4 release from the ribosome. Using a centrifugal binding assay, the authors showed that EF4 preferentially binds to the POST complex rather than the PRE complex and concluded that the resulting complex represents a back-translocation intermediate state (10). While an ensemble of in vitro kinetic assays showed that ribosome back-translocation proceeded through at least three intermediate states, they also highlighted the relative slowness of the proposed EF4-mediated back-translocation process, thereby raising the possibility that POST complex is not the main substrate of EF4 (11). In support of this view, single-turnover experiments and single-molecule Förster resonance energy transfer (smFRET) measurements reveal that EF4 prefers to bind to the PRE state ribosomes, and this interaction occurs in a competitive fashion with EF-G (12), suggesting that the PRE complex is the main substrate of EF4 during translation. The elucidation of structures of EF4-ribosome complexes in various states, such as in the GTP form, and additional biochemical assays would greatly boost our understanding of its precise mechanism and function.

Here, we present the cryo-EM reconstitution of the GTP form of Thermus thermophilus EF4 in its GTP form bound to the ribosome with tRNAs in the P- and E-sites. Our structure reveals that the position of the tRNA in the P-site and its interactions with the CTD of the GTP form of EF4, are similar to that observed in the GDP form of EF4 bound to the ribosome. Although a clockwise ribosome rotation was observed in the presence of the EF4-GDP complex, our ribosome-EF4-GDPCP (non-hydrolyzable GTP analog) structure is more similar to that of an unrotated state. As a result, the positions of EF4 and a number of contacts formed between EF4 and the ribosome vary in these two complexes. Furthermore, the well-defined electron density for EF4, including the functionally important switch 1 region, allows us to model the nucleotide binding and GTPase activation center and to shed light on the GTP hydrolysis mechanism catalyzed by EF4.

While our manuscript was in preparation, cryo-EM reconstitutions of Escherichia coli EF4 in complex with the PRE and POST state ribosomes were reported (19). The similarities and differences between our structure and these complexes will be discussed.

**EXPERIMENTAL PROCEDURES**

Preparation of ribosomes and EF4 – T. thermophilus ribosome with a C-terminal truncation of the L9 protein was purified as previously described (20, 21). The T. thermophilus EF4 gene was cloned into the PET26M vector (Novagen, WI, USA) and transformed into the E.
coli BL21(DE3) Rosetta T1R (Novagen, WI, USA) cell line. EF4 protein was over-expressed with an N-terminal His6-tag. The protein was purified using three chromatographic steps. Briefly, EF4 was first purified by affinity chromatography using a Ni-NTA column. The His-tag was then removed by TEV protease cleavage, after which the untagged protein was collected and further purified by gel filtration and ion exchange chromatography. The protein was finally concentrated to 236 µM and stored in buffer (25 mM Tris-HCl, pH 8.0, 50 mM NaCl, 10 mM MgOAc, and 5 mM 2-mercaptoethanol) at −80°C.

Cryo-EM grid preparation, image processing, and modeling – The 70S ribosome and GDPCP state EF4 complex was prepared in buffer G (5 mM HEPES, pH 7.5, 10 mM MgAc, 50 mM KCl, 10 mM NH4Cl, and 6 mM 2-mercaptoethanol). Briefly, the EF4-ribosome complex was reconstituted in the following order: 360 nM ribosomes were incubated with 720 nM mRNA Z4C at 55°C for 6 min. The Z4C mRNA was chemically synthesized (Dharmacon, Lafayette, CO, USA) with the sequence 5′ GGCAAGGAGGUAAAA AUGUUC AAAA 3′. The fMet and Phe codons at the P- and A-sites are shown in bold, and bold and underlined, respectively. Ribosome-mRNA incubation was followed by incubation with 720 nM tRNA fMet and 720 nM tRNA Phe at 55°C for 30 min. In parallel, 9.0 µM EF4 was incubated with 100 µM of GDPCP at room temperature. Finally, the ribosome and EF4 reactions mixtures were combined and allowed to form a complex at 37°C for 30 min.

Four microliter aliquots of the reconstituted EF4-ribosome complex were incubated on a glow discharged 2-nm carbon-coated holey grid (Quantifoil, Großlöbichau, Germany) for 30 sec. The grids were blotted for 3 sec in 100% humidity at 4°C and flash frozen in liquid N2-cooled liquid ethane using Vitrobot (FEI, Hillsboro, OR, USA). The grids were loaded into an Arctica electron microscope (FEI, Hillsboro, OR, USA) operated at 200 kV. The data were acquired using FEI’s automated data acquisition software (EPU). A total of 2,251 micrographs were recorded in movie mode as a set of 7 frames (total dose of 22 electrons per square Å) on a back-thinned FEI Falcon II direct electron detector at a calibrated magnification of 73,684, resulting in a pixel size of 1.9 Å at defocus values ranging from 1.5-4.0 µM.

The contrast transfer function (CTF) parameters were estimated using CTFFIND3 (22). The particles were picked using EMAN2 (23) and processed with Relion (24). A total of 164,338 particles were used for reference free 2D classification to discard the bad particles. Then, the 147,720 particles selected after 2D classification were used for further 3D classification using a 60 Å-filtered empty 70S ribosome (free of tRNAs and factors) as a reference to sort out the heterogeneity in the dataset. The 110,981 particles with strong densities for EF4 and the P-site tRNA were used for the final reconstruction with statistical movie processing to correct the beam-induced particle movements using Relion (24). The final reconstruction yielded a 3.8 Å resolution map, as determined with the gold standard Fourier cell correlation (FCS) criteria in Relion (Supplementary Fig. S1). Initial docking of 30S and 50S subunits (PDB ID: 5AA0) and EF4 (PDB ID 4W2E) X-ray crystal structures into the cryo-EM maps was performed in Chimera (25). We also reconstructed the structure of the same ribosome complex with a counterclockwise rotation at 5.7 Å resolution in a similar fashion.

**RESULTS**

Overall structure of the ribosome-EF4-GDPCP complex – Here, we report the structure of *T. thermophilus* EF4-GDPCP bound to the ribosome with tRNA in the P- and E-sites at 3.8 Å resolution, which was reconstructed by single particle cryo-EM (Fig. 1A and Supplementary Fig. S1). A comparison with the structure of an unrotated ribosome harboring mRNA and tRNAs (26) revealed that the present structure is also unrotated (Fig. 1B), representing a ribosome complex in the POST state, despite the presence of a non-cognate tRNA in the E-site, which adopts a virtually identical position as that of a cognate tRNA in the ribosome (27). Upon closer inspection, we noticed a small ribosome population that was refined to 5.7 Å resolution and displayed an counterclockwise 30S body rotation with respect to the 50S subunit, as well as 30S head swiveling (Fig. 1C). The unrotated and slightly counterclockwise rotated states of our
structures are different from the notable clockwise rotation (30S body rotation by 5°) that was recently reported in the crystal structure of EF4-GDP (14) and the cryo-EM reconstitution of the minor population of EF4-GDPNP (non-hydrolyzable GTP analog) bound to the ribosome (19). On the other hand, the major population of EF4-GDPNP in the recent cryo-EM study and the previous low-resolution reconstitution of the GTP form of EF4 bound to the ribosome with tRNAs both in P- and A-sites (10) also display unrotated states (10), similar to the major ribosome population in present structure. The structural parameters of the various EF4-ribosome complexes reported to date are summarized in Supplementary Table 1.

In addition to the variations in rotation, both the L11 region and the L1 stalk have different orientations in the structures of the GDP and GTP forms. The tip of H23 (the helices of the 50S and 30S subunits are labeled with H and h, respectively) in the L11 region and H78 in the L1 stalk shift away from the A-site finger (H38 in 23S rRNA) and the E-site tRNA by approximately 18 Å and 6 Å, respectively, compared to the structure of EF4-GDP bound to the ribosome (Fig. 1D).

The electron density map clearly shows both the P-site tRNA and EF4 (Supplementary Fig. S1B). We were able to build a complete model for EF4, including the regions that are crucial for its function: switch 1 (SW1, residues 38-60), switch 2 (SW2, residues 81-104), and the unique C-terminal domain (CTD, residues 490-610). Although the two switch regions SW1 and SW2 had been visualized at low resolution based on homology modeling using EF-G and EF-G-2 as templates (10), SW1 was partially traced in EF4-GDPNP bound to the ribosome (19). Therefore, our structure provides a more detailed model for these regions. In addition, we could also fit the nucleotide GDPCP in the nucleotide-binding center. Taken together, our results provide the most complete model of EF4 bound to ribosome to date.

Interactions between EF4 and the ribosome – Translational GTPase factors share a common binding pocket in the ribosome, but the detailed interactions with the ribosome vary among different factors and within the same factor in diverse ribosome states. In agreement with the universal trGTPase-binding mode, the EF4-GDPCP in the present structure is held in position by extensive contacts with both the 50S and 30S subunits. The close interaction of the EF4 G domain with the sarcin-ricin loop (SRL) in the 50S subunit (Fig. 2A) occurs in a way that is universally conserved in trGTPase factors. The ribosomal protein L6 in the 50S subunit is also involved in the interaction with the G domain (Fig. 2A). Domain II (more specifically residues Tyr208, Gln209, and Asp270) contacts h5 of the 16S rRNA in the 30S shoulder (Fig. 2B). In contrast, in the recent cryo-EM structure of the E. coli EF4-GDPNP bound to the PRE state ribosomes, Tyr203 (E. coli numbering, equivalent to Tyr208 in T. thermophilus LepA) is observed to stack with the A55 residue of the 16S rRNA (19).

Similar to the interaction between the EF-G domain III and the ribosome (17), domain III of EF4 in the current structure makes bilateral contacts with both the 30S and 50S subunits, namely, the S12 protein in the 30S shoulder, and the SRL in the 50S subunit, respectively (Fig. 2C). Domain V wedges into a cleft formed by the SRL and protein L6 on one side, and the L11 region (L11 protein and L11 RNA) on the other side, thereby establishing extensive contacts with the ribosome (Fig. 2D). Finally, the CTD of EF4 deeply projects into the PTC (Fig. 2E) in a very similar way to that observed in EF4-GDP bound to the ribosome (14). Note that our structure (POST) is similar to EF4 bound to the ribosome with both P- and A-tRNAs (PRE) (19).

The superposition of our structure with that of the crystal structure of EF4-GDP bound to the ribosome by aligning on the 23S rRNA clearly shows the different orientations of EF4 in the ribosome (Fig. 3A). Note that the 70S ribosome, regardless of the species, prefers to crystallize in a form where the ribosomal protein L9 from one ribosome extends to engage its C-terminal domain to contact the 16S rRNA of the neighboring ribosome. Although this interaction facilitates crystallization, it is incompatible with the binding of the trGTPase factor to the ribosome (20, 21). The crystal structure of the GDP form of EF4 bound to the ribosome was obtained by covalently linking the N-terminal domain of L9 to EF4 (14) (Supplementary Fig. S2). This engineered crystal contact involving EF4 may introduce interference with the conformation of EF4 and its interaction with the ribosome.
The β1 strand at the N-terminus, to which the N-terminal domain of L9 is attached, is shifted by approximately 8 Å toward the 30S subunit compared to our structure. With the engineered EF4, the entire G domain is re-orientated by 14°, and the β-barrel of domain II is displaced by approximately 15 Å (Asp226 position) toward h3 and h4 of the 16S rRNA in the 30S shoulder, whereas domain III approaches H71 (a component of the intersubunit bridge B2b) of the 23S rRNA in the 50S subunit by approximately 7 Å. The change in the overall orientation of the GDP form of EF4 toward the 30S subunit within the ribosome (Fig. 3A) is in agreement with its being attached to the N-terminal domain of L9, which is shown to bind to the neighboring ribosome in close proximity to the 30S shoulder (14) (Supplementary Fig. S2). Although it may cause a re-orientation within the ribosome, it appears that the engineered linker does not affect the overall conformation of domain G, given the good superposition of the individual G domains of the two ribosome-bound EF4 proteins (Fig. 3B).

Regardless of the diverse orientations of EF4 in the two structures, the deeply buried CTDs, particularly the two helices extending toward the PTC, exhibit virtually identical positions in the ribosome (Fig. 3C), indicating a strong interaction between these two helices and the ribosome. Accordingly, biochemical data have highlighted the importance of these helices for EF4 function (28). Furthermore, the orientation of the P-site tRNA is virtually identical in the previously reported EF4-GDP structure (14) and the present EF4-GTP structure (Fig. 3C).

**GTPase activation site of the ribosome bound EF4** – EF4 and BipA are both paralogs of EF-G, and these three proteins share significant structural similarity (9). Interestingly, large conformational changes have been shown to occur in both EF-G (20) and BipA (9) upon binding to the ribosome. In contrast, the overall conformation of ribosome-bound EF4, regardless of whether it is in the GDP (14) or GTP form (present results), is similar to that of the nucleotide-free isolated EF4 protein (8). In this study, the bound nucleotide GDPCP is clearly observed in the electron density map (Fig. 4A). Furthermore, both SW regions in EF4 have been stabilized by binding to the ribosome, resulting in a well-modeled structure of the nucleotide-binding pocket (Fig. 4B).

The G-nucleotide binding pocket includes five G motifs (G1-G5) that are conserved in trGTase factors (3). In the ribosome-bound EF4, the G1 motif (residues 18-24) establishes extensive contacts with the triphosphate moiety and ribose sugar of GDPCP (Fig. 4C). The side chains of Lys137 in G4 and Lys169 in G5 sandwich the guanine of GDPCP from both sides through hydrophobic interactions, and the hydroxyl group of Ser167 in G5 forms hydrogen-bonds with the amino group (NH2) of GDPCP. Notably, G2 is part of a switch comprised of a hydrophobic residue, which is proposed to regulate the “hydrophobic-gate”, and G3 is located in switch II, which contains the catalytic residue for GTase activity in EF-G (17). In the present structure, Thr58 in G2 interacts with the γ-phosphate oxygen of GDPCP (Fig. 4C). More importantly, the catalytic His86 residue is within hydrogen-bonding distance to GDPCP, indicating that EF4 is in an active conformation (Fig. 4C). In agreement with a previous report (8), our efforts to crystallize EF4 with the nucleotide in the absence of ribosome have not been successful. The G-nucleotide binding pocket in our structure is very similar to that of the GTP form of EF-G bound to the ribosome (17) and is consistent with recent biochemical data indicating that both factors have comparable affinity for GTP (28).

The universally conserved SRL of the 23S rRNAs does not form any direct contacts with GDPCP. However, the SRL inserts into a cleft formed by domains G, III, and V and is involved in the formation of the nucleotide binding pocket by partially occluding the entrance in a similar way as has been observed for EF-G (17) and BipA (9) bound to the ribosome. Our structure demonstrates that residue A2662 in the SRL appears to directly interact with His86, which in turn interacts with GDPCP. Note that the SRL plays an essential role in trGTase activation. Structural studies have revealed that nucleotide A2662 of the SRL places the catalytic His84 residue in EF-Tu and His87 in EF-G into their activated positions (16, 17, 29). Recently, a more detailed role for A2662 was revealed by the atomic mutagenesis approach, namely, that the non-bridging phosphate oxygen of A2662 participates in the activation of GTP hydrolysis on trGTases (30). Here, A2662 in the SRL is shown to interact with the catalytic His86 residue to place...
it into its activated position, further corroborating a universal mechanism of trGTPase activation through the SRL. In agreement with the catalytic role of His86, mutation of this residue in E. coli (His81 in E. coli numbering) impairs EF4 function in vivo (13).

Conformational change of EF4 upon ribosome binding and GTP hydrolysis – Recently, great advances have been made in understanding the structural basis of how the ribosome-dependent GTP hydrolysis by EF-G triggers the conformational changes in switch I and switch II that lead to the rearrangements of domains III-IV (16, 17, 20). Given that EF4 is a paralog of EF-G, it is of interest to know whether similar conformational changes also occur in EF4 in response to GTP hydrolysis. A comparison of the structures of EF4 and EF-G trapped in the ribosome by GDPCP (17) shows that domains G (including the two switch regions and the bound nucleotide, but excluding the additional G' sub-domain of EF-G), II, III, and V superpose well (Fig. 4D), consistent with the hypothesis that both factors share the same binding pocket in the ribosome. However, the unique domains (domain IV in EF-G and CTD in EF4) are positioned in completely different orientations relative to the shared domains. This observation is in agreement with their diverse positions in the ribosome, namely, in the DC (for domain IV of EF-G) and in the PTC (for CTD of EF4).

When aligning the isolated EF4 structure (8) onto the ribosome-bound structure of the GTP form of EF4 based on the G domain, a dramatic conformational change can be observed, although the G domains themselves fit relatively well (Fig. 5A). Compared with the present structure, in the structure of the isolated EF4, the switch I region was disordered and switch II and the subsequent helix were reoriented toward domain III by approximately 5 Å (Fig. 5B), resulting in a notable conformational change in the contacting helix in domain III. In particular, the catalytic residues in the two structures are oriented in almost opposite directions, either toward GDPCP or domain III in the ribosome-bound and isolated EF4 structures, respectively. The aforementioned conformational change would cause rearrangement of domains III, V, and the CTD, leading to an overall architecture that is incompatible with ribosome binding. As mentioned above, the overall orientations of EF4-GDPCP and EF4-GDP in the ribosome are rather different (Fig. 3A); the switch II region and domain III of EF4-GDP bound to the ribosome are more similar to the isolated EF4, which agrees with the proposed conformation change triggered by GTP hydrolysis. Note that the relatively stable binding of the GDP form of EF4 to the ribosome could be a result of the engineered fusion strategy (14). EF4 is capable of binding to the ribosome in the presence of both A- and P-site tRNAs (PRE) or the P-site tRNA only (POST) (2, 10, 12). The overall arrangement of the GTP form of EF4 bound to the POST state is virtually identical to that of the PRE state (Supplementary Fig. S3A), according to the recently reported cryo-EM study of E. coli EF4 bound to the ribosome (19). In addition, the position of the P-site tRNA and its interaction with the CTD of EF4 in the three structures (GDP or GTP form of EF4 bound to the POST state and GTP form of EF4 bound to the PRE state) are very similar (Supplementary Fig. S3B)(14, 19).

DISCUSSION

Structural studies have yielded insights into the translocation process catalyzed by EF-G by illustrating the numerous intermediate states of tRNAs moving from one site to the next, which are facilitated by ribosome rotation. In contrast, despite the fact that EF4 was reported a decade ago to catalyze the movement of tRNAs in the reverse direction (2), its precise mechanism remains elusive. In addition to the lack of structural evidence, some biochemical studies have called into question the proposed mechanism by which EF4 recognizes the POST complex and catalyzes its conversion to the PRE complex (12, 13).

A comparison of the conformations of the POST state ribosomes bound to the GTP form of EF4 (reported here) and the GDP form of EF4 (14) revealed not only noticeable differences in 30S body rotation and head swiveling but also a different orientation of several domains, most notably the G domain (Fig. 3A). However, the CTD of EF4, the position of the tRNA in the P-site (Fig. 3C), and their interactions were nearly identical. The differences in ribosome rotation and the orientation of the G domain observed in the EF-4-GDP structure could reflect the functionally significant interaction of the GDP state of EF4.
with the ribosome. In other words, GTP hydrolysis could lead to the re-orientation of the G domain as well as domains II, III, and V, whereas the positions of the CTD and P-tRNA would remain unaffected. However, such a conformational change in response to GTP hydrolysis would remarkably deviate from the one observed in structurally related trGTPase factors, where the orientations of the G domain and domain II remain the same after GTP hydrolysis on the ribosome, but the orientations of domains III and V, but most notably domain IV of EF-G (15, 16, 18, 20) and CTD of BipA (9), undergo a significant conformational changes with respect to the G domain and the ribosome. Therefore, it is possible that the particular conformational attributes of EF4 in the crystal structure may be introduced by the engineered fusion protein (the N-terminal domain of L9 linked to EF4, L9NTD-EF4) that was employed to facilitate crystallization (Supplementary Fig. S2) (21). In support of this view, the ribosome contacts observed in the crystal packing with the L9-linker strategy (Supplementary Fig. S2) have not been observed in any of the previously published crystal forms. The chimeric fusion protein L9NTD-EF4 has been shown to result in a crystal contact between two neighboring ribosomes (14) and could stabilize the binding of the GDP form of EF4 to the ribosome and introduce conformational features that might otherwise not occur, such as EF4 G domain reorientation toward the 30S subunit and 30S subunit rotation away from the 50S subunit, which agrees with the direction of the engineered crystal contact (Supplementary Fig. 2).

However, it should be noted that clockwise rotation of the 30S body, albeit to a smaller extent, was also observed in the cryo-EM reconstitution of the small population of ribosome-EF4-GDPNP (Post state ribosome with tRNA in the P-site) complex, whereas the majority of the ribosomes (Pre complex with tRNA in P/P and A/4 sites) were in a state reminiscent of the unrotated ribosome (19). This observation could suggest that: (i) the clockwise rotation of the ribosome may occur without the linker between EF4 and L9 and, (ii) arguably, rotation and back-translocation do not require GTP hydrolysis. Consistent with this notion, Qin and co-workers showed that EF4-dependent back-translocation could occur slowly with GDPNP (19). However, it should be noted that only a minor fraction of EF4-ribosome complexes was in the POST state and exhibited sub-stoichiometric occupancy for EF4, resulting in a poorly resolved map, and only 26 CTD residues were modeled in the map that was contoured at low level by Qin and co-workers (19), thus challenging the biological relevance of the EF4-POST ribosome complex. The similarity of the structure of the minor population of the reported GDPNP-EF4-ribosome complex (19) to that of the GDP-EF4-ribosome complex (14) may reflect nucleotide contaminants (like GTP and its hydrolysis product GDP) that are often observed in commercially available GDPNP preparations (31), but such a contamination problem may not be the case for commercial GDPCP (used in the current study). Therefore, it could be possible that the minor ribosome population characterized by Qin and co-workers represents (19) the GDP form of EF4 bound to the ribosome. The interaction of the GDP form of EF4 with the ribosome would likely differ from the that of the GTP form, possibly explaining the poorly resolved map for the majority of the EF4 protein observed in the minor population (19). If indeed the minor population corresponds to the GDP-EF4-ribosome complex, the result would suggest that the interaction of the GDP form of EF4 with the ribosome is relatively stable, even without the L9 linker. In agreement, we observed that the GDP form of native EF4 interacts with the ribosome in the centrifugal binding assay, albeit less efficiently than the GDPCP form (data not shown). Therefore, the clockwise rotation of the ribosome could be required to “accommodate” the GDP form of EF4.

Regardless of the differences discussed above, our structure of the GTP form of EF4 and the crystal structure of the GDP form of EF4 (14) are remarkably similar (Supplementary Fig. 3A). Furthermore, a comparison of our structure with the cryo-EM reconstitution of EF4-GDPNP in a complex with a ribosome harboring three tRNAs (19) reveals that the overall arrangements of EF4 are virtually identical (Supplementary Fig. 3B). The tRNA in the A-site, named A/4-tRNA by Qin and co-workers, displayed a conformational change in the acceptor arm, similar to the tRNA in the low-resolution cryo-EM reconstitution (named A/L tRNA by Spahn and co-workers) (10). Most importantly, in all three structures (Supplementary Table 1), the tRNA is observed in the classical P-
site. In contrast to EF-G-catalyzed translocation, no structural information is available for back-translocation intermediate complexes with tRNAs in hybrid sites that could shed further light on the function of EF4 on the ribosome.

In a very recently published study (19), it was proposed that the Post-EF4 and Pre-EF4 complexes represent ribosomes prior to and after back-translocation, respectively. Furthermore, based on the structural and biochemical data, it was concluded that EF4 facilitates back-translocation via its CTD interfering with the P-site tRNA 3’-CCA interaction with the PTC on one hand and stabilizing of A/4-tRNA binding on the other hand. Curiously, after the POST state ribosomes (formed by incubating the PRE state ribosomes with EF-G, followed by purification through sucrose cushion centrifugation) were incubated with EF4 and GDPNP, the majority of the ribosomes were in the PRE state, and only a minor fraction was in the POST state. Based on the puromycin reactivity assay, the authors concluded that GDPNP still enables slow back-translocation activity of EF4. Alternatively, it could be that the Pre-EF4 complex arises from EF4 binding to the PRE state ribosomes, rather than binding to and back-translocating of the POST state ribosomes in the presence of the non-hydrolyzable GTP analog. This observation would be in agreement with the kinetic assays performed by Cooperman and co-workers (12), suggesting that EF4 preferentially binds to the PRE state ribosomes, whereas, according to Spahn and co-workers, the POST state is a better substrate for EF4 (10). From where could the PRE state ribosomes come? The efficiency of EF-G-mediated POST complex formation reported by Qin and co-workers was approximately 80%, based on the puromycin reactivity assay (19), which is in agreement with the previously reported toeprinting results (2). Thus, there could be a notable fraction of ribosomes that remain in the PRE state and could act as substrates for EF4 in the subsequent assays.

Nevertheless, our findings of the GTP form of EF4 bound to the POST state ribosome in unrotated and counterclockwise rotated conformations (major and minor populations, respectively) provide new structural information about the interaction of EF4 with the ribosome. Our findings also raise questions regarding the functional significance of the EF4-dependent back-translocation that is accompanied or facilitated by the clockwise rotation of the ribosome (2, 19) and “counteracts” the EF-G catalyzed translocation facilitated by the counterclockwise rotation of the ribosome. Indeed, EF4 has been implicated in other functions, such as remobilizing “stuck” ribosomes; facilitating protein folding, ribosome assembly and biogenesis; and bacterial stress responses (4, 13, 32-36). Furthermore, Frederick and co-workers recently proposed that its primary function lies in the translation initiation rather than the elongation phase (13). Although the mechanisms of GTP hydrolysis and the interactions of EF4 with the ribosome and tRNAs are starting to emerge at atomic resolution, the biological relevance of the interaction of EF4 with the translation machinery is still ambiguous. Further biochemical assays are needed to fully understand the structure-function relationship of EF4.

Note: While this paper was under revision, a crystal structure of the EF4 in its GTP form bound to the PRE state ribosome was published (PDB not released) (37), which is very similar to that in reference 19.

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The cryo-EM density maps have been deposited in the Electron Microscopy Data Bank with accession numbers EMD-6584 and EMD-6585, and the atomic coordinates of structure models are deposited in Protein Data Bank with accession numbers 5IMQ and 5IMR for the 3.8 Å and 5.7 Å resolution structures, respectively.

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.
Author contributions: YGG designed the project; VK performed most of the experiments; RE, TA, KJG, and YZ participated in some of the experiments; VK, SB, RE, and YGG analyzed the data; and YGG, RE, and VK wrote the manuscript.

REFERENCES

15. Pulk, A. and Cate, J. H. (2013) Control of ribosomal subunit rotation by elongation factor G. Science 10.1126/science.1235970
ribosome complexes trapped in intermediate states of translocation. *Science* 10.1126/science.1236086


**FIGURE LEGENDS**

**FIGURE 1.** Structure of the ribosome-EF4 complex. (A) Overall view of the GTP form of the EF4-ribosome complex. The EF4 protein, 50S, and 30S subunits are shown as cryo-EM densities in red, orange, and cyan, respectively. The P- and E-site tRNAs are shown as cryo-EM densities in lemon and lilac, respectively. The tRNAs are barely visible from this angle. Structural landmarks of the 50S subunit are indicated for clarity. (B and C) Ribosome ratcheting shown as rotation of the 16S rRNA in the 30S subunit relative to the 50S subunit (viewed from the solvent side of the 30S subunit). The 16S rRNAs of the present complexes of major (B) and minor (C) ribosome populations are shown in cyan and green, respectively. For comparison, the 16S rRNA (gray) in the classical unrotated ribosome with an mRNA and tRNA (26) is shown. The structures are aligned to the 23S rRNA. (D) Comparison of the 23S rRNAs in the current GTP form of the EF4-ribosome structure (orange) and in the crystal structure of GDP form of the EF4-ribosome complex (14) (gray). The ribosomal L1 and L11 stalks are labeled, and the arrows indicate the direction of the conformational change.

**FIGURE 2.** Interaction of EF4 with the ribosome. (A) G domain interaction interface with the SRL of the 23S rRNA and the ribosomal protein L6. GDPCP is shown as sticks, with the carbon, oxygen, nitrogen and phosphate atoms colored cyan, red, blue and orange, respectively. (B) Domain II interaction interface with helix 5 (h5) of the 16S rRNA. The EF4 residues involved in the interaction are shown as sticks and labeled. (C) Domain III interaction interface with h5 of the 16S rRNA, ribosomal protein S12, and the SRL of the 23S rRNA. (D) Domain V interaction interface with 23S rRNA helix 44 (H44) and the L11 protein of the 50S subunit. (E) CTD interaction interface with 23S rRNA helix 89 (H89) and the P-site tRNA. The same color scheme is used for EF4, the P-site tRNA, and 23S rRNA in all figures unless otherwise stated. The 16S rRNA is shown in cyan, and the ribosomal proteins are shown in dark green.

**FIGURE 3.** Comparison of the ribosome-bound GTP and GDP form of the EF4 structures. (A) The present ribosome-EF4-GDPCP structure is aligned with the ribosome-EF4-GDP structure (14) based on the 23S rRNA. The 30S structure is shown as a surface representation in cyan, and the 23S rRNA of the 50S subunit is shown in orange. EF4 is represented as a cartoon, with the α-helices shown as cylinders for clarity. GTP form of the EF4 domains G, II, III, and V and the CTD are labeled and colored green, violet, yellow, blue, and red, respectively. The GDP form of EF4 is shown in gray. The engineered link to L9 protein is also indicated. (B) The same two EF4 structures are aligned based on the G domain. EF4 is represented as a cartoon, with the α-helices shown as ribbons. GDPCP is shown as a surface representation. (C) The same alignment is shown as panel (A), highlighting the reorientation of the G domains, whereas the positions of the CTD of EF4 and the P-site tRNAs are nearly identical. The P-site tRNAs in current ribosome-EF4-GDPCP and in ribosome-EF4-GDP (14) structures are shown in purple and gray, respectively.

**FIGURE 4.** GTPase activation center. (A) Unbiased difference Fourier electron density map with refined maps for SRL, GDPCP, and the G3 region of EF4. GDPCP, SRL residue A2662, and the EF4 catalytic residue (His86, *T. thermophilus* numbering) are shown as sticks, with the oxygen, nitrogen and phosphate atoms colored red, blue and orange, respectively. (B) Unbiased difference Fourier electron density map, with refined maps for the switch 1 region of EF4 and GDPCP (shown in sticks). (C) GTPase activation center of EF4. The G1, G2, G3, G4, and G5 motifs of EF4 involved in nucleotide binding and GTPase activation are shown. The non-hydrolyzable GTP analog GDPCP and the crucial EF4 residues are shown as sticks. The interactions between the Thr58 and His86 residues of EF4 and the γ-phosphate of the bound nucleotide are shown with dashed lines. (D) The current EF4 structure was compared with the ribosome-bound GTP form of the EF-G structure (17) by aligning the G domains. EF4 is colored as in Fig. 2, and EF-G is colored gray. The EF-G G’ sub-domain and domain IV are indicated. DC and PTC indicate the decoding center and the peptidyl transferase center in the ribosome, respectively.
FIGURE 5. EF4 GTPase activation upon ribosome binding. (A) Comparison of the present structure of the GTP form of EF4 bound to the ribosome (colored as previously) with the structure of the isolated apo form of EF4 (8) (gray), which were aligned based on the G domain. (B) Close-up view of the GTPase center. GDPCP and the catalytic residue (His86 and His81 in *T. thermophilus* and *E. coli* EF4, respectively) are shown as sticks, with the oxygen, nitrogen and phosphate atoms colored red, blue and orange, respectively. The reorientation of the catalytic regions (SWII and its subsequent helix) observed in the comparison of the structures is indicated with an arrow.