Published in final edited form as:

J Mol Biol. 2007 December 14; 374(5): 1345–1358.

Specific interaction between EF-G and RRF and its implication for GTP-dependent ribosome splitting into subunits

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Summary

After termination of protein synthesis, the bacterial ribosome is split into its 30S and 50S subunits by the action of ribosome recycling factor (RRF) and elongation factor G (EF-G) in a GTP-hydrolysis dependent manner. Based on a previous cryo-electron microscopy (cryo-EM) study of ribosomal complexes, we have proposed that the binding of EF-G to an RRF containing post-termination ribosome triggers an inter-domain rotation of RRF, which destabilizes two strong intersubunit bridges (B2a and B3) and, ultimately, separates the two subunits. Here, we present a 9 Å (FSC at 0.5 cutoff) cryo-EM map of a 50S EFG GDPNP RRF complex and a quasi-atomic model derived from it, showing the interaction between EF-G and RRF on the 50S subunit in the presence of the non-cleavable GTP analogue GDPNP. The detailed information in this model and a comparative analysis of EF-G structures in various nucleotide- and ribosome-bound states show how rotation of the RRF head domain may be triggered by various domains of EF-G. For validation of our structural model, all known mutations in EF-G and RRF that relate to ribosome recycling have been taken into account. More importantly, our results indicate a substantial conformational change in the Switch I region of EF-G, suggesting that a conformational signal transduction mechanism, similar to that employed in tRNA translocation on the ribosome by EF-G, translates a large-scale movement of EF-G's domain IV, induced by GTP hydrolysis, into the domain rotation of RRF that eventually splits the ribosome into subunits.

Keywords

Ribosome recycling; cryo-EM; conformational signal transduction; real-space refinement; intersubunit bridge

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Accession Numbers

Atomic model for the 50S-EF-G-GDPNP-RRF complex has been deposited in the Protein Data Bank under the ID code 2RDO. Cryo-EM density map for the 50S-EF-G-GDPNP-RRF complex has been deposited in EmDep at the EBI under the ID code EMD-1430.

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Introduction

Protein synthesis starts with the formation of a 70S initiation complex from free ribosomal subunits^{1–3} and ends with the recycling of the post-termination ribosome back to free subunits.^{4, 5} Apart from ribosome splitting, the recycling process includes the release of transfer RNA (tRNA) as well as messenger RNA (mRNA) from the 30S subunit and requires elongation factor G (EF-G), ribosome recycling factor (RRF), and initiation factor 3 (IF3). Subunit splitting is induced by EF-G and RRF in a GTP hydrolysis-dependent manner.^{4, 6–9} Rapid association of IF3 with the small (30S) ribosomal subunit accelerates the dissociation of deacylated tRNA and mRNA^{4, 8} and prevents re-association of the 30S with the large (50S) ribosomal subunit, until a new mRNA and an initiator tRNA are in place in the 30S pre-initiation complex.^{2, 3} It has been proposed that IF3, in addition to its primary role as an anti-association factor,^{2–4, 6, 8} takes an active part also in subunit dissociation.^{6, 10}

The interaction of RRF with the ribosome has been previously shown by various structural approaches.^{11–13} However, the dynamic process of the disassembly of the post-termination complex still remains elusive. The difficulties in visualizing the ribosome bound with both EF-G and RRF are mainly twofold: (1) In the presence of GDPNP or other non-hydrolyzable analogs of GTP, EF-G and RRF are incompatible on the 70S ribosome;^{9, 14–16} (2) in the presence of GTP, EF-G and RRF quickly split the ribosome into subunits.^{6, 8, 9} At the same time, biochemical studies have shown RRF and EF-G-GDPNP to bind with positive cooperativity to the isolated 50S subunit.^{4, 9, 15} This cooperativity and its functional meaning are intriguing, since ribosome splitting requires not only GTP but also GTP hydrolysis.^{4, 6, 8, 9} However, these biochemical observations suggest that either EF-G, RRF or both might adopt an alternative conformation on the 50S complex direct relevant for the ribosome splitting. It is therefore expected that valuable structural information, regarding the mechanism by which the post-termination ribosome is split by the action of EF-G, GTP, and RRF, can be extracted from this 50S complex.

Accordingly, in our previous cryo-EM work,¹⁷ we identified two conformations of ribosome-bound RRF; one in the 70S post-termination complex (70S-mRNA-P/E-tRNA-RRF), and the other in a complex with the 50S subunit (50S-RRF-EF-G-GDPNP). Comparison of the two conformations indicated that the head domain of RRF undergoes a ~60° rotation from the 70S conformation (pre-rotational state) to the 50S conformation (post-rotational state), whereas in the 50S complex EF-G adopts a very similar conformation as previously observed in the 70S ribosome,^{18, 19} which indicates that the rotation of the RRF head domain is apparently an effect due to the interaction of RRF with EF-G. This rotation, when mapped onto the 70S ribosome, is seen to directly disrupt two intersubunit bridges (B2a and B3), which could explain the mechanism of subunit separation in accordance with existing biochemical and mutational data.^{4, 6, 9, 20} Together with the structural data,¹⁷ the cooperative binding of EF-G and RRF on the 50S subunit indicates that GTP hydrolysis is not required for the rotation of the RRF head domain itself but for the subsequent separation of the subunits following the head domain rotation. Thus, from all these biochemical and structural observations, we believe that this 50S complex, although prepared from the isolated 50S subunit and locked by GDPNP, would have similarity with the state immediately after the two subunits have separated.¹⁷

However, the limited resolution of the 50S-RRF-EF-G-GDPNP complex (15.5 Å at 0.5 FSC cutoff)¹⁷ rendered difficult a more refined analysis of the specific interactions between the two factors and the role of GTP hydrolysis in ribosome splitting. In the present work, therefore, a larger data set was accumulated to further refine this 50S complex to a resolution of 9.1 Å, which allowed for the derivation of a quasi-atomic model by real-space refinement techniques.²¹ To this end, the high-resolution structures of the 50S subunit²², RRF²³ and EF-G (see Materials and Methods) were docked into the new cryo-EM density map, and further analysis

revealed different roles for the three interacting domains (III – V) of EF-G in effecting the rotation of RRF's head domain. Subsequent comparison with another 50S complex, containing EF-G-GDP and fusidic acid (50S-EF-G-FUS), revealed significant conformational changes on EF-G both in the global domain arrangement and in the local Switch I region. These observations suggest that a conformational signal transduction mechanism within EF-G, similar to that employed in tRNA translocation,²⁴ is responsible for the linkage between GTP hydrolysis, the domain rotation of RRF, and ribosome splitting into its subunits.

Results

A Quasi-atomic Model for the 50S-RRF-EF-G-GDPNP Complex from Real-space Refinement

We obtained a cryo-EM map of the 50S-RRF-EF-G-GDPNP complex with a resolution of 9.1 Å (using the FSC=0.5 cutoff criterion; 5.7 Å at 3σ) from over 120,000 particles. We employed a flexible fitting technique based on real-space refinement (RSRef module of TNT package)²¹ to dock the high-resolution crystal structures into this cryo-EM map. All the atomic structures used in the refinement are from *E. coli*; i.e., the 50S subunit is from an *E. coli* 70S ribosome crystal structure,²² the structure of EF-G from *E. coli* was obtained by comparative modeling using as template an X-ray crystal structure of EF-G from *T. thermophilus* (see Materials and Methods), and the X-ray structure of RRF is from *E. coli*.²³

The quality of the fitting was evaluated from the correlation coefficients (CC) and R-factors of the docked components (Table 1), which are comparable with those of previous real-space refinements of other ribosomal complexes.^{25, 26} It is likely that the slightly lower CC of the EF-G fitting is due to the missing residues 44–63 in the X-ray²⁷ and hence in the modeled structure (Figure 1). As shown in Figure 1, there are two major regions with unfilled densities. One is at the L7/L12 stalk, which is partially disordered in the crystallography data.²² The other is the arc-like density, which reflects the interaction between EF-G and the C-terminal domain of the L7/L12 stalk.^{19, 28} Except for these components and the missing coordinates of a small number of other ribosomal proteins in the crystal structure,²² rRNA helices, ribosomal proteins, EF-G, and RRF are all well accommodated in their respective densities. According to our previous work,²⁶ the real-space refinement technique is sufficient to give semi-quantitative information even for cryo-EM maps in the medium-resolution range (12–15 Å). In that work, the fitting precision, determined by the reproducibility of the fitting models for two independently obtained experimental cryo-EM maps, was shown to be around 1 Å for the 23S rRNA. The fitting accuracy, however, is difficult to evaluate, because the resolution of most cryo-EM density maps does not allow *ab initio* atomic modeling. By comparing our quasi-atomic model with three relevant X-ray structures^{13, 29, 30}, we conclude that in the present work, with a resolution of 9 Å, the fitting accuracy is equal to or better than 2.5 Å for the backbone conformation (Supplementary Materials). Since the typical length of a hydrogen bond is around 2.5 Å,³¹ the Cα atoms from EF-G and RRF that are in close proximity to each other could potentially form hydrogen bond or other types of interactions. Thus, we are able to analyze the specific interactions between EF-G and RRF based on the quasi-atomic model.

In this quasi-atomic model, factor interactions are established mainly between RRF and domains III and IV of EF-G (Figure 1D). In addition, the C-terminal helix of EF-G domain V interacts with residues in and near the C-terminus of RRF. The functional relevance of all these interactions and, in particular, their putative roles in effecting the inter-domain rotation of RRF were probed by comparison with mutational and phylogenetic data. In the following sections, the interactions of EF-G with RRF are presented in a domain-by-domain order. In addition, all known mutations in EF-G and RRF that relate to ribosome recycling are analyzed in the context of our three-dimensional structural framework in the Supplementary Materials.

EF-G Domain III Directly Interacts with RRF Hinges

The sites of interaction between RRF and domain III of EF-G are all located in or nearby the inter-domain hinges of RRF, and are mediated by two loops of domain III of EF-G (Figure 2). The first loop (residues Lys422 - Lys424) interacts with several residues (Thr106 - Glu108) from one hinge of RRF. The closest contact is between Lys422, Thr423 of EF-G and Glu107 of RRF (Figure 2). Residues Glu450 – Asn453 in the second EF-G loop interact with residues in both hinges (Arg31 - Ala32 and Pro103 - Pro104) and in the head domain (Ser62 - Arg63) of RRF (Figure 2). Among the second loop interactions, the most significant contacts are between Glu450 and Ser62, and between Asn453 of EF-G and its three targets Ser62, Arg31-Ala32 and Pro104 (Figure 2).

The multiple sequence alignments (Figure 3) for both EF-G and RRF show that, overall, the sequences of the two EF-G loops and their target residues on RRF are highly conserved. Interestingly, an exception is *T. maritima*, where the sequence at these interacting pairs has a co-variational pattern, for example, lysine to valine alteration at position 422 for EF-G, and glutamic acid to threonine alteration at position 107 for RRF (Figure 3).

These sequence data and the structural contacts identified in the present work (Figure 2) suggest highly specific inter-molecular interactions in the EF-G domain III: RRF interface. The outcome of a genetic search³² for alterations in EF-G that would allow RRF from *T. thermophilus* to complement RRF deficiency in an *E. coli* strain may therefore appear surprising. The search revealed gain-of-function mutations in domain IV, but not III, of EF-G,³² which led to the conclusion that domain IV, rather than domain III, interacts with RRF. With support from the present data, it can be concluded that the high specificity of the inter-molecular interactions in the EF-G domain III: RRF interface makes the probability to find viable gain-of-function mutations in domain III very small. This explains why such mutations in EF-G domain III were not found by Ito and coworkers.

It is well known that the flexibility of two intra-molecular hinges of RRF is crucial for its function.^{33–38} In addition, we have proposed, based on our cryo-EM data, that the inter-domain rotation of RRF around these two hinges is responsible for the separation of the two subunits in ribosome recycling.¹⁷ In this interpretation, the primary role of EF-G domain III would be to alter the inter-domain orientation of RRF by directly interfering with its hinges.

EF-G Domain IV Interacts with RRF Head Domain

Unlike the interactions between RRF and EF-G domain III, which involve a small number of residues with very specific contacts, the interactions between RRF head domain and EF-G domain IV involve a large number of surface residues from both factors along the interface (Figure 4). In EF-G, these residues belong to two α -helices of domain IV. In contrast, on the RRF head, the relevant residues belong to two β -strands. In addition, the loop of Ser508-Gly509-Gly510 of EF-G domain IV, which interacts with the intersubunit bridge B2a (H69:h44) in the 70S ribosome, is in close contact with three residues from RRF, *i.e.* Tyr44 - Tyr45 and Asp71 (Figure 4). Interestingly, we again observe EF-G's interaction with the hinge residue of RRF: a residue from a loop of EF-G domain IV, Gly567, is seen to closely contact with Arg28, very near one hinge of RRF (Figure 4), a result which further supports our proposal that EF-G acts on the hinge region of RRF.

Some of the residues in the EF-G domain IV:RRF head interface are also conserved across species (Figure 3). However, the overall sequence conservation is lower than the one observed for the interaction between domain III of EF-G and the RRF hinges, and no simple pattern of sequence co-variation is apparent.

The structural detail of the interface between EF-G domain IV and RRF correlates well with previous mutation data, in that a large number of RRF and EF-G mutations locate to this contact region or to neighboring regions (Figures 3 and 4; Supplementary Materials). There are two types of mutations in the head domain of RRF. The first type is non-functional mutations of RRF from *E. coli*³⁹ (Supplementary Materials), which mainly includes proline substitutions with apparent effects on the structural integrity of the head domain. The second type is gain-of-function mutations of defective or heterologous RRF that activates its function in the *E. coli* system,^{20, 38, 40} and this type of mutations often involves substitutions that significantly change the surface potential (Supplementary Materials). On EF-G, previously reported gain-of-function mutations³² also correlate very well with our structural data (Figure 4B; Supplementary Materials). Among the eight mutations found on EF-G domain IV, five (His504-->Tyr and Ser508-->Phe for *E. coli* EF-G; Glu548-->Gly, Gly553-->Arg, and Ser552-->Pro for a chimerical EF-G [EcoI-III-T.thIV-V])³² are located within this interface (Figures 3 and 4). For the remaining three mutations, one is at the hinge between domain III and domain IV (Glu485), apparently affecting the inter-domain mobility of EF-G, and the other two are situated on a loop far from the interface, probably with global effects on the structural integrity of domain IV (Supplementary Materials).

Therefore, judging from the relative orientation of EF-G domain IV to the head of RRF, along with the above mutation data, the function of EF-G domain IV in ribosome recycling appears to be to confine the RRF head domain in a conformation that clashes with the 30S subunit. We propose that this action of EF-G is due to physical clash and electrostatic repulsion between EF-G domain IV and the head domain of RRF. Accordingly, the existence of a large number of functional mutations in the interface between EF-G domain IV and RRF would mean that the repulsive effect can be cumulatively generated at many sites, in contrast to the strictly residue-specific recognition task of EF-G domain III. In the 70S ribosome, the movement of EF-G domain IV, initiated by GTP hydrolysis, would force the movement of RRF's head domain and finally result in the ejection of the 30S subunit (see Discussion).

EF-G Domain V Interacts with the C-terminus of RRF

In the quasi-atomic model, the α -helically structured C-terminus in domain V of EF-G interacts with two regions of RRF. The first region is one of the hinges of RRF, where the strongest contact is between Arg701 - Lys703 of EF-G and Arg28 - Thr29 of RRF (Figure 5A). The second region is the C-terminus from the α 1-helix of RRF's tail domain (Figure 5B), and the interaction of the two C-termini is established between Lys703 of EF-G and two residues, Lys178 and Glu181, of RRF (Figure 5B). These observations once more corroborate our proposed action of EF-G on the inter-domain hinges of RRF, and indicate that the inter-domain rotation of RRF is accomplished by the joint action of three domains of EF-G.

The C-terminus of EF-G is less conserved, with respect to sequence and length (Figure 5C), than the C-terminus of RRF (Figure 3). In the latter, the above-identified residues (Lys178 and Glu181) are in fact universally conserved (Figure 3). According to alignments of sequence and structure (Figure 5), the orientation of the C-terminal helix of EF-G in relation to RRF is very different across species, suggesting the existence of a species-specific interaction between the C-termini of RRF and EF-G. This view is supported by several previous genetic studies. First, it is known that the C-terminal residues of RRF from *E. coli* are important for its function: deletion of the last three to ten residues produces a temperature-sensitive phenotype, and further deletion totally abolishes RRF function.^{5, 40, 41} Secondly, in contrast, RRF from *T. thermophilus* is nonfunctional in *E. coli* strain, but an amber mutation with deletion of its last five C-terminal residues activates its function in the *E. coli* system.⁴¹ Conversely, an amber mutation at *E. coli* EF-G Q695, with a deletion of the last nine C-terminal residues, activates the function of the full-length RRF from *T. thermophilus* in the *E. coli* system.³² Taken

together, these genetic observations indicate that the required interaction between the two C-termini of these two factors in the *E. coli* system could not be established when using a heterologous EF-G:RRF pair. This indicates that the interaction between the two C-termini is structurally different across species.

The contribution of EF-G domain V to the inter-domain rotation of RRF is less pronounced than the contributions of EF-G domains III and IV. It is, however, known that the C-terminal residues of RRF are important for its binding to the ribosome.⁴² Thus, it is likely that the interaction between the two C-termini has a stabilizing effect on RRF's binding, because an efficient rotation of the head domain will need the tail domain to be tightly anchored. It is also possible that the interaction between the two C-termini directly contributes to the rotation of RRF's head domain, since the C-terminus of RRF is in close proximity to the hinges.

EF-G Domain Rearrangement after GTP Hydrolysis

Splitting of the ribosome into subunits requires GTP hydrolysis on EF-G,^{4, 6, 8, 9} suggesting a causal link between a conformational change in EF-G induced by GTP hydrolysis and the inter-domain rotation of RRF. To study this effect, we applied real-space refinement to one of the previously obtained 50S subunit complexes (50S-EF-G-GDP-FUS).¹⁷ In the two resulting quasi-atomic models, EF-G binds at roughly the same position in the 70S ribosome as we previously reported.^{19, 43, 44} Comparison of the two models (50S-EF-G-GDPNP-RRF and 50S-EF-G-GDP-FUS) shows that upon GTP hydrolysis domains III, IV and V of EF-G jointly move toward the 30S subunit, whereas domains I and II of EF-G move in the opposite direction, toward the L11-region (Figure 6B). Along with the conformational changes in EF-G, there are also significant conformational changes in the 50S subunit: in the more compact 50S-EF-G-GDP-FUS complex, the L1 and L7/L12 stalks are closer to the central protuberance than in the 50S-EF-G-GDPNP-RRF complex (Figure 6A). Importantly, this pattern of EF-G domain rearrangement following GTP hydrolysis is in agreement with the results of our recent cryo-EM study of the 80S ribosome (*Thermomyces lanuginosus*), bound with eEF2 before (GDPNP-bound) and after (GDP-sordarin-bound) GTP hydrolysis.²⁴ In particular, the movement of domain IV toward the 30S subunit is in accordance with the present structural model that requires a movement of EF-G domain IV, in a concerted action with domains III and V, to promote the inter-domain rotation of RRF.

Conformational Change of EF-G Switch I after GTP Hydrolysis

The intracellular functions of many GTPases depend on a pair of conserved structural switches, I and II, located on their surface.⁴⁵ It has been suggested that the two switches of EF-G, which are spatially proximal to the nucleotide- and Mg^{2+} -binding sites, respond to the signal of γ -phosphate, and convert this signal into conformational changes of the entire molecule, on account of the fact that the two switches are also close to the domain interface of EF-G.^{27, 46–48} The backbone of Switch I, consisting of residues 40–65 of domain G (*T. thermophilus* numbering), is disordered in all known crystal structures of EF-G. However, in our cryo-EM map of the 50S-EF-G-GDPNP-RRF complex, there are some residual densities in the region of Switch I (Figure 6). These densities can be readily filled by superimposing the Switch I structure of the ternary complex of Phe-tRNA^{Phe}, EF-Tu, and GDPNP,⁴⁹ or of a *T. thermophilus* EF-G homolog, EF-G-2.⁵⁰ This result indicates that Switch I is more ordered in the GDPNP-bound form than in the GDP-FUS-bound form. More importantly, this finding is again consistent with our recent work on the 80S ribosome (*Thermomyces lanuginosus*) bound with eEF2 and GDPNP,²⁴ which showed that Switch I of eEF2 is more ordered in the ribosome-bound GDPNP form. Thus, this observation clearly shows that similar conformational signal transmission mechanisms are used by EF-G in tRNA-mRNA translocation on the ribosome and in ribosomal recycling (see Discussion).

Discussion

Concerted Action of EF-G Domains III –V on Inter-domain Rotation of RRF

In the present work, we identify detailed interactions between EF-G and RRF on the 50S subunit based on the quasi-atomic model of the 50S·EF-G·GDPNP·RRF complex that we derived from the cryo-EM map. All three domains of EF-G interact with the hinge regions of RRF, in line with our previous proposal for the action of EF-G on the inter-domain rotation of RRF.¹⁷ Additionally, combination of our structural data with mutational and phylogenetic analyses suggests different roles for EF-G domains III, IV and V. Specifically, EF-G domain III directly interferes with the inter-domain hinges of RRF, thereby changing the inter-domain orientation of RRF. EF-G domain IV, in contrast, generates a repulsive force mediated by a large interfacial surface area that stabilizes the rotation of RRF's head domain. And last, EF-G domain V enhances the binding affinity of RRF to the ribosome, through the interaction between the C-termini of the two molecules, and therefore provides a strong anchor point for the rotation of RRF's tail domain.

The three domains of EF-G need to work in a concerted manner to facilitate the domain rotation of RRF, as indicated by genetic studies showing that mutational defects in one inter-molecular interface can be compensated by gain-of-function mutations in another inter-molecular interface.³⁸ For instance, a *T. thermophilus* RRF variant, containing a Pro104->Ala mutation at one hinge, can complement RRF deficiency in an *E. coli* strain by a gain-of-function mutation in the interface between the head domain and domain IV of EF-G³⁸ (Supplementary Materials). This observation suggests that the deleterious effects of the Pro104->Ala mutation in the hinge of the *T. thermophilus* RRF can be reversed by altered interactions in the EF-G domain IV: RRF interface through enhanced repulsion. To give another example, deletion of the last 9 residues from the C-terminus of RRF, which abolishes its interaction with domain V of EF-G and is lethal, can be compensated by gain-of-function mutations at various locations, including both the tail and head domains of RRF.⁴⁰

Comparison of the Actions of EF-G on the A-site tRNA and RRF

The primary role of EF-G in promoting translocation of the tRNA:mRNA complex is experimentally well documented. Biochemical studies have indicated that the two actions of EF-G, namely translocation of the complex of tRNA and mRNA and disassembly of the post-termination ribosomal complex, have similar dependence on GTP hydrolysis and respond similarly to various EF-G inhibitors.^{8, 9, 15, 51} The present observations are relevant for a detailed discussion of the structural basis for this similarity.

Overall, the EF-G domain arrangement in our 50S·EFG·GDPNP·RRF map is similar to that in the maps of the 70S ribosome bound with EF-G,^{18, 19} suggesting similar rearrangements in EF-G during translocation and recycling as the factor moves from its free to its ribosome bound state. Beside the EF-G domain reorganization, translocation and recycling might have another feature in common. That is, EF-G might adopt a similar, unstable transition conformation in the two processes, considering the fact that the binding site of domain IV of ribosome-bound EF-G overlaps with its two targets, the A-site bound tRNA in the pre-translocational complex and the head domain of RRF in the post-terminational complex.

Importantly, the present results show that in the ribosome-bound GDPNP form (Figure 6), the Switch I of EF-G is in a more ordered structure than in the GDP-bound form, an observation that is consistent with our recent result on the ribosome-bound eEF2.²⁴ Both observations are in line with the general mechanism of many cellular GTPases, in which a conformational signal is generated by the relaxation of two switch regions from their tensioned GTP form to the GDP form.⁵² Presumably, in the case of EF-G or eEF2, local changes in the chemical environment

of the nucleotide-binding pocket induced by GTP hydrolysis and γ -phosphate release are eventually amplified, through the action of two structural switches, and converted to a large-scale inter-domain rearrangement. Numerous experimental studies have shown that the integrity of the conformational signal transduction pathway and the maintenance of the proper configuration at the inter-domain interfaces of EF-G are crucial requirements for the role of the factor in translocation.^{20, 27, 53, 54} There is also direct evidence related to EF-G's action on RRF. Gain-of-function mutations on EF-G from *A. aeolicus* which activate the function of *A. aeolicus* RRF in the *E. coli* strain were found in different regions of domains I and II of EF-G (far from the EF-G/RRF interface), and most of these mutations are located in inter-domain hinge or interface regions, such as the I–II and II–III interfaces.²⁰ Taking all the above results together, the apparent similarity in the modes of action motivates a discussion of translocation along with recycling in a broader context.

Movement of EF-G Domain IV after GTP Hydrolysis

The very tip of EF-G domain IV is formed by the 580–588 and 508–511 loops,^{46, 47} and the former loop is essential for tRNA translocation on the ribosome.^{55, 56} The relative orientation of EF-G domain IV and the A-site bound aminoacyl-tRNA suggests facilitation of translocation by a direct contact between the tip of domain IV and the A-site tRNA in the pre-translocation ribosome.²⁴ At the same time, mutational³² and structural (Figure 4) data showed that two α -helices of EF-G domain IV, distinct from its tip, directly contact RRF, thereby inducing the inter-subunit rotation of RRF and subsequent ribosome splitting. This could allow for EF-G mutants with selectively perturbed translocase or recycling functions, perfectly in line with the finding that a translocase-deficient mutant of EF-G is fully active in recycling, as judged from an *in vitro* polysome breakdown assay.¹⁴ At the same time, the movement of EF-G domain IV after GTP hydrolysis is essential for both the translocase and recycling activities of EF-G, which motivates a closer look at the orientation of domain IV in previously characterized EF-G conformations off and on the ribosome.

The crystal structures of isolated EF-G in the GDPNP- and GDP-bound forms have very similar orientation of domain IV.^{27, 48} To date, all known conformations of ribosome-bound EF-G come from cryo-EM studies, and they can be grouped into two classes. The first class, the GTP-like state, is obtained in the presence of GTP analogs, including GDPNP^{17, 18} and GMPP (CH₂)P.¹⁹ The second class is obtained in the presence of antibiotics, such as fusidic acid (GDP-FUS state)^{17–19, 57} and thiostrepton.⁵⁷ The GDP-FUS state was derived in the presence of fusidic acid and either GDP^{17, 18} or GTP.^{19, 57} From previous cryo-EM studies in a medium resolution range (12–18 Å), it was concluded that EF-G in GDP-FUS state adopts a conformation similar to that in its GTP-like state. This, however, does not mean that the GTP and GDP conformations of the ribosome-bound EF-G are similar, since the impact of fusidic acid on the structure of the ribosome-bound EF-G-GDP is the likely cause of the inhibitory action of this drug. In contrast, at a much improved resolution (8–9 Å), very recent results on the domain rearrangement of eEF2²⁴ and our present result on EF-G (Figure 6) now show that GTP hydrolysis on the ribosome-bound eEF-2/EF-G moves domains III, IV and V toward the small and domains I and II of the factor toward the large ribosomal subunit. Although this conclusion is based on a comparison of two ribosomal states that could, in principle, be compromised by the presence of nucleotide analogs or antibiotics, normal mode analysis suggests this movement pattern to be an intrinsic property of the domain architecture of EF-G (N. Gao and J. Frank., data not shown), and thus to be similar during translocation or ribosome recycling. In fact, the observed orientation of EF-G on the ribosome suggests that EF-G domains III and V might act as anchors on the 30S and 50S subunits, respectively, whereas domain IV, without direct contact with the ribosome and connected with domains III and IV through long, flexible loops (489–494, 609–612), could move relatively freely in response to the conformational signal passed by the switches.

The remaining conceptual problem is the *initial* conformation of EF-G, just when it has bound to a pre-translocation or an RRF-containing post-termination ribosome. In the latter case, EF-G-GTP must adopt a conformation in which the space between domains III and IV is sufficiently open so that a clash with the ribosome-bound RRF is avoided. No such crystallographic conformation of prokaryotic EF-G has been reported (*E. coli* and *T. thermophilus*), but the crystal structure of eEF2 from yeast in complex with sordarin⁵⁸ has an extended conformation in which domain IV has undergone a large-scale (up to 75°) rotation (Figure 7). After minor adjustments, EF-G in this very conformation could bind to an RRF containing post-termination ribosomal complex with no clash between the factors. Accordingly, we propose that EF-G-GTP upon binding to the RRF containing post-termination ribosome rapidly adopts a conformation similar to the sordarin structure of eEF2.⁵⁸

From these experimental observations and structural analyses, we propose a model for the dynamic events of ribosomal recycling (Figure 7): When EF-G-GTP binds to the RRF-containing post-termination ribosome, EF-G domain IV adopts a high standard free energy conformation with increased space between EF-G domains III and IV, allowing for intermittent, simultaneous presence of both factors on the 70S ribosome (Figure 7A). Subsequently, rapid GTP hydrolysis makes EF-G domain IV movement toward the 30S subunit possible. This movement, together with the concerted actions of EF-G domains III and IV, induces head domain rotation of RRF and, eventually, subunit separation (Figure 7B). The energy released through GTP hydrolysis is used to break the intersubunit bridges. When, in contrast, a nonhydrolyzable GTP analog, such as GDPNP, is used in the ribosome recycling, the swing of domain IV cannot occur. Instead, the 70S-EF-G-GDPNP-RRF complex quickly disintegrates and is replaced by a ribosome in complex with either EF-G-GDPNP or RRF, in line with biochemical data demonstrating competitive binding of the two factors to the ribosome.^{9, 15}

While the present article was under review, a cryo-EM study published by Agrawal and coworkers⁵⁹ put forward a different mechanistic model for the separation of the two subunits by EF-G and RRF. Since, however, their reconstruction was mainly based on a very small fraction (~5700 particles, ~6% of the total number of particles) of a 70S ribosome sample containing only RRF, the statistical significance of this report is a concern. Therefore, the suggested model would benefit greatly from further biochemical verification and the inclusion of a larger experimental data set.

Very recently, the crystal structure of the 70S ribosome bound with RRF was solved independently by two groups,^{29, 30} providing information on the atomic interaction of RRF with the ribosome. Here, we must refrain from a discussion of their results, but note that the agreement with our results regarding the position of the RRF domain contacting the 50S subunit is within 2.5 Å (see Supplementary Materials.)

Materials and Methods

Preparation of 50S Complex and Cryo-electron Microscopy

The 50S complex (50S-EF-G-GDPNP-RRF) was prepared according to our previous work.¹⁷ The cryo-grid of the 50S complex was prepared following the standard protocols.⁶⁰ Micrographs were recorded on a Philips Tecnai F20 (200kV and 49,700X magnification) with low-dose kit (10–15 e⁻/Å²). Micrographs were scanned on a Zeiss/Imaging scanner (Z/I Imaging Corporation, Huntsville, AL) with a step size of 14 μm (2.82Å pixel size).

Image Processing

Image processing followed standard procedures.⁶⁰ A total of 259 micrographs (defocus range: 1.1–4.9 μm) were grouped in 60 defocus groups. Particles were selected by a program using a locally normalized cross-correlation procedure.⁶¹ Particles were first subjected to manual screening. The resulting 163,859 particles were then subjected to a supervised classification procedure⁶² using two 50S reference maps, with and without EF-G RRF bound, to select particles with the factors bound. Finally, 113,355 “good” particles were used for reconstruction. The angular refinement and computation of the 3D reconstruction was done using SPIDER, following the standard projection-matching technique.⁶³ An empirical procedure for amplitude-correction was incorporated in the refinement iterations,⁶⁴ where the Fourier amplitudes are multiplied with a spatial frequency-dependent scale factor to match those of low-angle x-ray solution scattering data. The final resolution was estimated using the Fourier Shell Correlation criterion with 0.5 cutoff ($\sim 9\text{\AA}$, Supplementary figure 2).

Homology Modeling of *E. coli* EF-G Structure

There is no crystal structure for *E. coli* EF-G to date. By virtue of the high sequence identity (59%) between EF-G from *E. coli* and *T. thermophilus*, an atomic model was built for *E. coli* EF-G using one of the *T. thermophilus* EF-G crystal structures (PDB id: 2BM0)²⁷ as a template. The comparative modeling was performed with program MODELLER.⁶⁵ The sequence of *E. coli* EF-G (swissprot: locus EFG_ECOLI, accession **P0A6M8**) was aligned against the template using a 2-D alignment module (align2d) of MODELLER, which takes into account the structural profile of the template in addition to the sequence information. 50 models were calculated with MODELLER, based on the alignment derived from align2d. Two of the initial models were selected for further assessment, based on the MODELLER objective function, calculated Discrete Optimized Protein Energy (DOPE) and the GA341 method.⁶⁶ The stereochemical quality of the two models was checked with program PROCHECK,⁶⁷ and the fold of the two models was checked with VERIFY3D⁶⁸ and ProSa2003.⁶⁹ The average VERIFY3D scores of both models are over 0.4, suggesting the overall validity of the models. The final model was chosen due to its slightly better scores. There are three loops in the final model that were modeled independent of the template, due to the *E. coli* insertions and disorder in the respective regions of the template. These loops, namely residues 44–63, 74–80, and 692–696, were not included in the subsequent analysis. The structure of C-terminal tail of *E. coli* EF-G (690–703) was separately modeled in the same way using as the template the crystal structure of an EF-G homolog, EF-G-2 from *T. Thermophilus*,⁵⁰ due to its completeness in the C-terminal tail.

Real-Space Refinement

A real-space refinement module, RSRef,21 for the TNT package⁷⁰ was used to flexibly dock the atomic structures into the cryo-EM density map. The atomic structures used in the refinement included the crystal structure of *E. coli* ribosome for the 50S subunit (PDB id: 2AW4),²² the crystal structure of *E. coli* RRF (PDB id: 1EK8),²³ and our atomic model, derived from comparative modeling for *E. coli* EF-G. For completeness in the fitting, the X-ray crystal structure of protein L1⁷¹ was also included, which is missing in the crystal structure of the *E. coli* 70S ribosome. The 23S rRNA was divided into rigid bodies based on its secondary structure. Most of the ribosomal proteins were treated as single rigid bodies, and in some cases, proteins were cut into more rigid bodies due to their significant multiple-domain architectures. A table for the complete rigid-body assignment is included in Supplementary Materials, comprising 63 rigid bodies for the 23S rRNA, one for 5S rRNA, 32 for ribosomal proteins, two for RRF, and five for EF-G. The initial positions of these structures were taken from our previous work.^{17, 72} Refinement parameters were listed in Supplementary Materials. Computations for the real-space refinement were done on a UNIX workstation (IRIX 6.5,

Silicon Graphics, Mountain View, CA). The quality of the fitting was evaluated by the sizes of the correlation coefficients (CC) and R-factors calculated for the docked components (Table 1). After real-space refinement, fitted coordinates were subjected to energy minimization (Discover, Accelrys Inc., San Diego, CA) to relieve unfavorable steric interactions. The subsequent structural analysis was mainly conducted using PyMol.⁷³

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Michael Watters for assistance with the illustrations. This work was supported by HHMI and NIH grant R01 GM29169 (to J.F.), and NIH grant R01 GM70768 and the Swedish Research Council (to M.E.).

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Figure 1. Overview of the Quasi-atomic Model of the 50S-EF-G-GDPNP-RRF Complex

(A) Stereo view of the quasi-atomic structure of the 50S-EF-G-GDPNP-RRF complex superimposed with the cryo-EM density map. (B) The same stereo view of the quasi-atomic structure only. (C) Surface representation of the cryo-EM density map. (D) Overview of the interactions between EF-G and RRF. Ribosomal proteins, rRNAs, EF-G and RRF are painted in green, grey, red and blue, respectively. The domains of EF-G are labeled as I-IV. The two domains of RRF are labeled as "tail" (domain I) and "head" (domain II). Landmarks: L7L12, L7/12 stalk; L1, L1 stalk; CP, central protuberance; arc, arc-like connection between EF-G and L7/L12 stalk base.

Figure 2. Interactions between EF-G Domain III and the Inter-domain Hinges of RRF

(A) The interactions between one loop of EF-G domain III (Lys422 - Lys424) and one of the hinges (Thr106 - Glu108) of RRF. (B) Interactions between the other loop of EF-G domain III (Glu450 - Asn453) and the two hinges and their neighboring residues of RRF. The residue stretches participating in the interactions are painted in green and yellow for RRF and EF-G, respectively. Residues that involve very strong contacts (less than 3 Å) are displayed as a stick model.

Figure 3. Multiple Sequence Alignments of EF-G and RRF
(A) Multiple sequence alignment of RRF. (B) Multiple sequence alignment of EF-G (Domains III and IV). The alignments were derived using a web-based program, MultAlin⁷⁴. Residues involved in the inter-molecule interactions are shadowed in grey color (see text for details). The positions of mutations previously reported are marked by open circles. Both alignments are labeled according to *E. coli* numbering.

Figure 4. Interactions between EF-G Domain IV and the Head Domain of RRF

(A) Structural details of the interface between EF-G domain IV and the head domain of RRF. The interface is composed of a large number of residues from both sides. Residues are painted in yellow for EF-G and green for RRF. Their corresponding side-chains are displayed as a stick model. H583, a residue essential in EF-G's translocase activity situated far from the EF-G:RRF interface, is also labeled. (B) Stick representations of the residues in which mutations have been reported, showing the high consistency of our structural model with the previous mutational data.

Figure 5. Interactions between the C-terminus of EF-G and RRF, and the Diversity of EF-G C-terminal Helical Tail

(A) Interactions of the very C-terminal residues (R701-K703) with one of the inter-domain hinges of RRF (R28 – T29), confirming EF-G's action on the inter-domain orientation of RRF. The residue stretches participating in the interactions are painted in green and yellow for RRF and EF-G, respectively, and their corresponding side-chains are displayed in stick representation. (B) Interactions of the C-terminal helix of EF-G with the C-terminal helix of RRF. Q695, a position in which an amber mutation has been reported to activate the function of a heterologous RRF (*T. thermophilus*) in *E. coli*, is also labeled. (C) Diversity of the C-terminal helical tail of EF-G, as shown by multiple sequence alignment. Residues interacting with RRF are shadowed in grey color. (D) Superimposition of the C-terminal helices from *E. coli* EF-G (red), *T. thermophilus* EF-G (pale cyan), *T. thermophilus* EF-G-2 (pink). Note: the last two residues of *T. thermophilus* EF-G are absent in the structure due to the disorder in the crystal structure.

Figure 6. Switch I Structures of EF-G and the Domain Re-organization of EF-G after GTP Hydrolysis

(A) EF-G structures in GDPNP (green) and GTP (cyan) states displayed with its relevant density maps, showing overviews for panel (B). Landmarks: CP, central protuberance; L1, L1 stalk; L7/L12, L7/L12 stalk. (B) Domain re-organization of EF-G after GTP hydrolysis. The cartoon structures of EF-G are painted in green and cyan for GTP and GDPNP states, respectively. The relative directions of the domain movement are indicated by arrows. Domains of EF-G are labeled as I–V. (C) The cryo-EM density map of the 50S-EF-G-GDPNP-RRF complex is displayed in mesh representation with fitted EF-G structure in GDPNP (GTP) state (green), and zoomed in the Switch I region. Two crystal structures of Switch I, from GTP-state EF-Tu

ternary complex (yellow) and EF-G-2·GTP (purple), are superimposed with the fitted EF-G structure. The structural alignment was done using as a reference a conserved helix upstream of Switch I. (D) The cryo-EM density map of the 50S·EF-G·GDP·Fusidic-acid complex is displayed in mesh representation with fitted EF-G structure in GDP state (cyan), and zoomed into the Switch I. The same two crystal structures of Switch I are aligned and superimposed with the fitted EF-G·GDP structure.

Figure 7. Proposed Model for the Movement of EF-G Domain IV in Ribosome Recycling
 (A) Crystal structure of eEF2-Sordarin (dark blue), aligned with respect to domains I-II of *E. coli* EF-G, and superimposed with the structure of 70S-bound RRF (red). (B) Structures of EF-G (cyan) and RRF (green) in the 50S-EF-G-GDPNP-RRF complex. (C) Structure of EF-G (pale green) in the 50S-EF-G-GDP-Fusidic-acid complex, superimposed with the structure of 50S-bound RRF (green). (D) Superimposition of all above structures. See text for details.

Table 1

Correlation coefficients and R-factors of the quasi-atomic model derived from real-space refinement.

Components	Correlation Coefficient		R-factor	
	Initial	Final	Initial	Final
Overall	0.473	0.674	0.294	0.242
EF-G	0.368	0.586	0.358	0.294
RRF	0.499	0.609	0.315	0.265