

Elongation factor G-induced structural change in helix 34 of 16S rRNA related to translocation on the ribosome

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ABSTRACT

During the translocation step of the elongation cycle, two tRNAs together with the mRNA move synchronously and rapidly on the ribosome. The movement is catalyzed by the binding of elongation factor G (EF-G) and driven by GTP hydrolysis. Here we study structural changes of the ribosome related to EF-G binding and translocation by monitoring the accessibility of ribosomal RNA (rRNA) for chemical modification by dimethyl sulfate or cleavage by hydroxyl radicals generated by Fe(II)-EDTA. In the state of the ribosome that is formed upon binding of EF-G but before the movement of the tRNAs takes place, residues 1054, 1196, and 1201 in helix 34 in 16S rRNA are strongly protected. The protections depend on EF-G binding, but do not require GTP hydrolysis, and are lost upon translocation. Mutants of EF-G, which are active in ribosome binding and GTP hydrolysis but impaired in translocation, do not bring about the protections. According to cryo-electron microscopy (Stark et al., *Cell*, 2000, 100:301–309), there is no contact of EF-G with the protected residues of helix 34 in the pretranslocation state, suggesting that the observed protections are due to an induced conformational change. Thus, the present results indicate that EF-G binding to the pretranslocation ribosome induces a structural change of the head of the 30S subunit that is essential for subsequent tRNA-mRNA movement in translocation.

Keywords: chemical modification; EF-G; ribosome structure; translocation

INTRODUCTION

The elongation cycle of protein synthesis entails three major steps. It begins with a ribosome that contains peptidyl-tRNA (or initiator tRNA) in the P-site and a free codon in the A-site. During the first step, a complex of elongation factor Tu (EF-Tu), GTP, and aminoacyl-tRNA (aa-tRNA) binds to the A-site, and, following codon recognition and GTP hydrolysis, aa-tRNA is released from EF-Tu and accommodates in the peptidyltransferase center. Once the aa-tRNA is accommodated, the peptide bond is formed rapidly, yielding deacylated tRNA in the P-site and peptidyl-tRNA in the A-site. The cycle is completed by translocation, that is the movement of the tRNA·mRNA complex on the ribosome that is catalyzed by elongation factor G (EF-G) with GTP. During

translocation, the 3' end of peptidyl-tRNA moves into its puromycin-reactive position on the 50S subunit, and the anticodon end, along with the mRNA, moves on the 30S subunit. Thereby, the peptidyl-tRNA is displaced from the A-site to the P-site, while the deacylated tRNA is moved to the E-site and released from the ribosome. As the result of translocation, the initial state of the ribosome is restored, which then enters the next round of elongation or, when a stop codon is encountered, proceeds to termination.

EF-G is a large, five-domain GTPase that catalyzes the translocation of tRNAs on the bacterial ribosome at the expense of GTP. In the crystal structure of GDP-bound EF-G, domain 1 (G domain) makes direct contacts with domains 2 and 5, whereas domain 4 protrudes from the body of the molecule (Ævarsson et al., 1994; Czworkowski et al., 1994; Al-Karadaghi et al., 1996; Laurberg et al., 2000). Recent results from pre-steady-state kinetic analysis suggest that translocation is a multistep process (Rodnina et al., 1997), and models derived from low-resolution cryo-electron microscopy

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(cryo-EM) suggest that EF-G assumes different overall conformations at different stages of translocation, mainly by a movement of domains 3/4/5 relative to domains 1/2 (Agrawal et al., 1998, 1999; Stark et al., 2000). Conformational changes are essential for the function of EF-G in translocation, presumably for coupling GTP hydrolysis and inorganic phosphate (P_i) release to rearrangements of the ribosome leading to translocation (Rodnina et al., 1997; Savelsbergh et al., 2000).

Several studies by cryo-EM have indicated that the interaction of EF-G with the ribosome induces structural changes of the ribosome that may be related to translocation (Agrawal et al., 1999; Frank & Agrawal, 2000; Stark et al., 2000). Cryo-EM reconstructions of ribosome·EF-G complexes stabilized by a nonhydrolyzable GTP analog have been interpreted as to show a six-degree, ratchet-like rotation of the small 30S subunit relative to the large 50S subunit as well as an opening and closing of a putative mRNA channel (Agrawal et al., 1999; Frank & Agrawal, 2000). In the pretranslocation ribosome·EF-G complex stalled by binding the antibiotic thiostrepton to the 50S subunit, the relative orientation of the ribosomal subunits is altered and the structure of the 30S subunit is changed dramatically such that the cleft between the head and the body of the subunit is opened up, and there are other substantial structural changes in the body of the subunit (Stark et al., 2000). Although conformational changes in the ribosome·EF-G complex obtained by cryo-EM were modeled in terms of different structures of rRNA (van Loock et al., 2000), it is to be noted that the resolution of cryo-EM reconstructions of ribosome·EF-G complexes available so far is within 15–20 Å, too low to allow a detailed interpretation of structural alterations of the ribosome in different complexes.

In the present article, we use chemical probing by dimethyl sulfate, hydroxyl radicals generated from Fe(II)-EDTA and H_2O_2 , and, to a limited extent, kethoxal, to identify residues in ribosomal RNA that exhibit changed reactivities due to EF-G binding and/or translocation. EF-G-catalyzed translocation is quite rapid, 25 s^{-1} with GTP and up to about 0.5 s^{-1} with nonhydrolyzable GTP analogs or GDP (Rodnina et al., 1997), much faster than the time scale of chemical modification reactions (minutes). Therefore, to use chemical modification for studying structural changes related to translocation, intermediate ribosome·EF-G complexes have to be stabilized. The antibiotic thiostrepton, which binds to the 1060 region of 23S rRNA, is particularly useful, because it does not affect EF-G binding to the ribosome and GTP hydrolysis, whereas it slows down translocation and stabilizes ribosome·EF-G complexes both before and after translocation (Rodnina et al., 1999). Because the antibiotic does not block tRNA movement altogether, the same ribosome complexes could be compared before and after translocation, the only difference being an incubation period.

RESULTS

Structural changes of the ribosome in the complex with EF-G

In the absence of EF-G, DMS modification patterns in 16S and 23S rRNA were identical in pretranslocation and posttranslocation ribosomes, except for the protections characteristic for A- and P-site-bound tRNAs reported earlier (Moazed & Noller, 1989, 1990), and were not influenced by thiostrepton, except for the footprints in the 1051–1108 region of 23S rRNA (Egebjerg et al., 1989; data not shown). In the pretranslocation (pre) complex with EF-G, residues C1054, A1201, and C1203 in helix 34 of 16S rRNA were strongly protected, whereas after translocation, with EF-G still bound to the complex, the initial modification pattern was re-established (Fig. 1A). The extent of protection in the pre complex was dependent on the concentration of EF-G (Fig. 1B). About 50% protection of A1201 was found at $0.3\text{ }\mu\text{M}$ EF-G, consistent with the binding af-

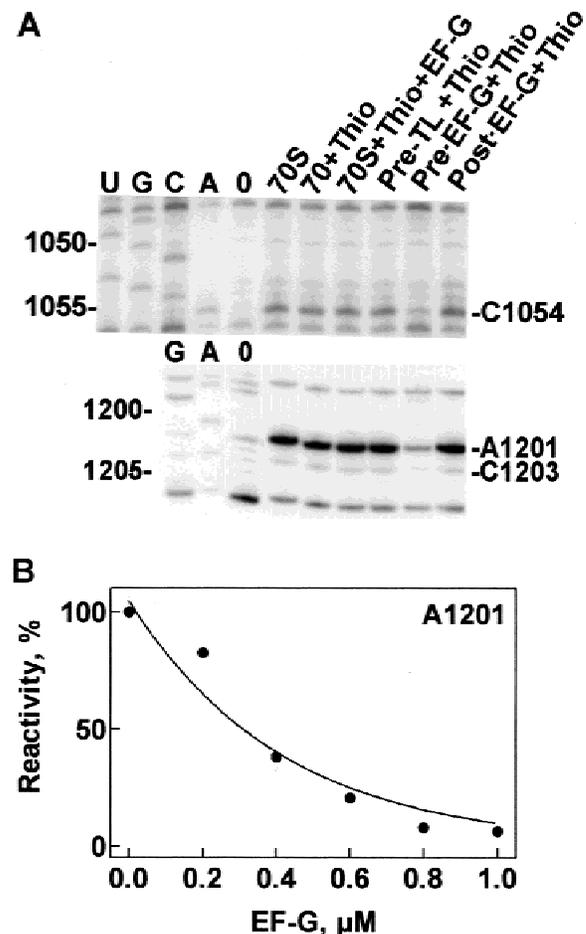


FIGURE 1. DMS protections by EF-G in helix 34 of 16S rRNA. **A:** Protection of positions 1054, 1201, and 1203. U, G, C, and A: sequencing lanes; 0: control without DMS. **B:** Dependence of A1201 protection on EF-G concentration.

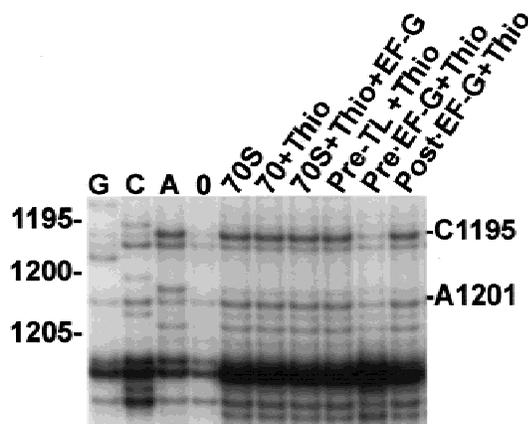


FIGURE 2. Hydroxyl radical protections by EF-G in helix 34 of 16S rRNA. 70S: vacant ribosomes; Thio: thiostrepton added; G, C, and A: sequencing lanes; 0: control without reagent.

finity of about 0.5 μM determined kinetically (Rodnina et al., 1997), and protection was almost complete at 1 μM EF-G, indicating full occupancy of the pretranslocation complex with EF-G (Fig. 1B). Thus, the protections observed in the pre complex were caused (directly or indirectly) by EF-G binding. DMS protections in other parts of 16S and 23S rRNA were not observed. No rRNA protections due to EF-G binding were observed when probing was performed with kethoxal (data not shown).

Complementary results were obtained by hydroxyl radical probing. Again the observed alterations in the chemical modification pattern were restricted to the pretranslocation complex containing EF-G and to helix 34 (Fig. 2). The sugar-phosphate backbone between residues 1195/1196 and 1201/1202 was protected from cleavage in the pre complex with EF-G; changes in other regions of rRNA were not observed (data not shown). No changes were observed in other ribosome complexes studied, including the thiostrepton-stabilized posttranslocation complex of EF-G (Fig. 2).

The location of the positions in 16S rRNA that are affected by EF-G binding to the pretranslocation complex is shown in Figure 3. Helix 34 contains four regions that are not involved in Watson-Crick base pairing, U1049, C1054-A1055, A1196, and A1201-U1202, and the accessibility for chemical modification of the latter three residues is strongly decreased by EF-G binding. These residues are located on two sides of helix 34; positions 1054, 1195, and 1196 point towards the shoulder of the 30S subunit in the vicinity of the ribosome decoding center and the 530 region of 16S rRNA, whereas positions 1201 and 1203 are located on the opposite face of the helix.

Role of GTP hydrolysis

The activity of EF-G in translocation is strongly increased by ribosome-stimulated GTP hydrolysis (Rod-

nina et al., 1997). To test whether the EF-G-induced structural change of helix 34 depends on GTP hydrolysis, EF-G was bound to pretranslocation complexes with a nonhydrolyzable analog of GTP, GDPNP, with or without thiostrepton, and the accessibility for DMS modification of the characteristic residue A1201 was monitored (Fig. 4). In the pretranslocation state stabilized by thiostrepton, A1201 was almost completely protected by binding of EF-G·GDPNP. After translocation, induced by prolonged incubation (45 min) in the presence of thiostrepton, or short incubation (5 min) in the absence of the antibiotic, the protections in helix 34 were no longer observed, in agreement with the results of Figure 1. These data suggest that the observed protections in helix 34 are brought about by the binding of EF-G to the ribosome, and that GTP hydrolysis in the presence of thiostrepton has no further effect, at least not in the relatively long time window defined by the modification reaction.

Influence of mutations in EF-G that inhibit translocation

Domain 4 of EF-G is important for the function of the factor in translocation (Kimata & Kohno, 1994; Rodnina et al., 1997; Savelsbergh et al., 2000). Replacement of histidine 583 at the tip of domain 4 with lysine or arginine decreases the translocation rate more than 100-fold, whereas other EF-G functions, including binding to the ribosome, GTP hydrolysis, and turnover, remain unchanged (Savelsbergh et al., 2000). In translocation experiments with EF-G (H583K/R), the addition of thiostrepton decreased the rate of translocation further, such that the extent of translocation remained below 10% after 5 min incubation at 37 °C, and did not exceed 20–30% after 45 min at 37 °C, compared to 90% observed with wild-type EF-G (data not shown). In pretranslocation complexes with EF-G (H583K/R), prepared in the presence of thiostrepton, the DMS protections in helix 34 were no longer observed (Fig. 5). This suggests that the interaction of the tip of domain 4 with the small ribosomal subunit, as observed by cryo-EM (Stark et al., 2000), are important for inducing the protections in helix 34. Because the extent of translocation remained low with the EF-G mutants, this establishes a correlation between the EF-G-induced protections in helix 34 and the efficiency of translocation.

The role of conformational changes of EF-G for inducing the DMS footprint in helix 34 was studied by using an EF-G mutant with a disulfide crosslink between domains 1 and 5, EF-G(XL) (Peske et al., 2000). EF-G(XL) binds to the ribosome, hydrolyzes GTP, and releases P_i with practically the same rates as wild-type EF-G, but is unable to promote translocation and remains firmly bound to the ribosome after one round of GTP hydrolysis, as demonstrated by DMS protections in the 1067 region of 23S rRNA (Peske et al., 2000).

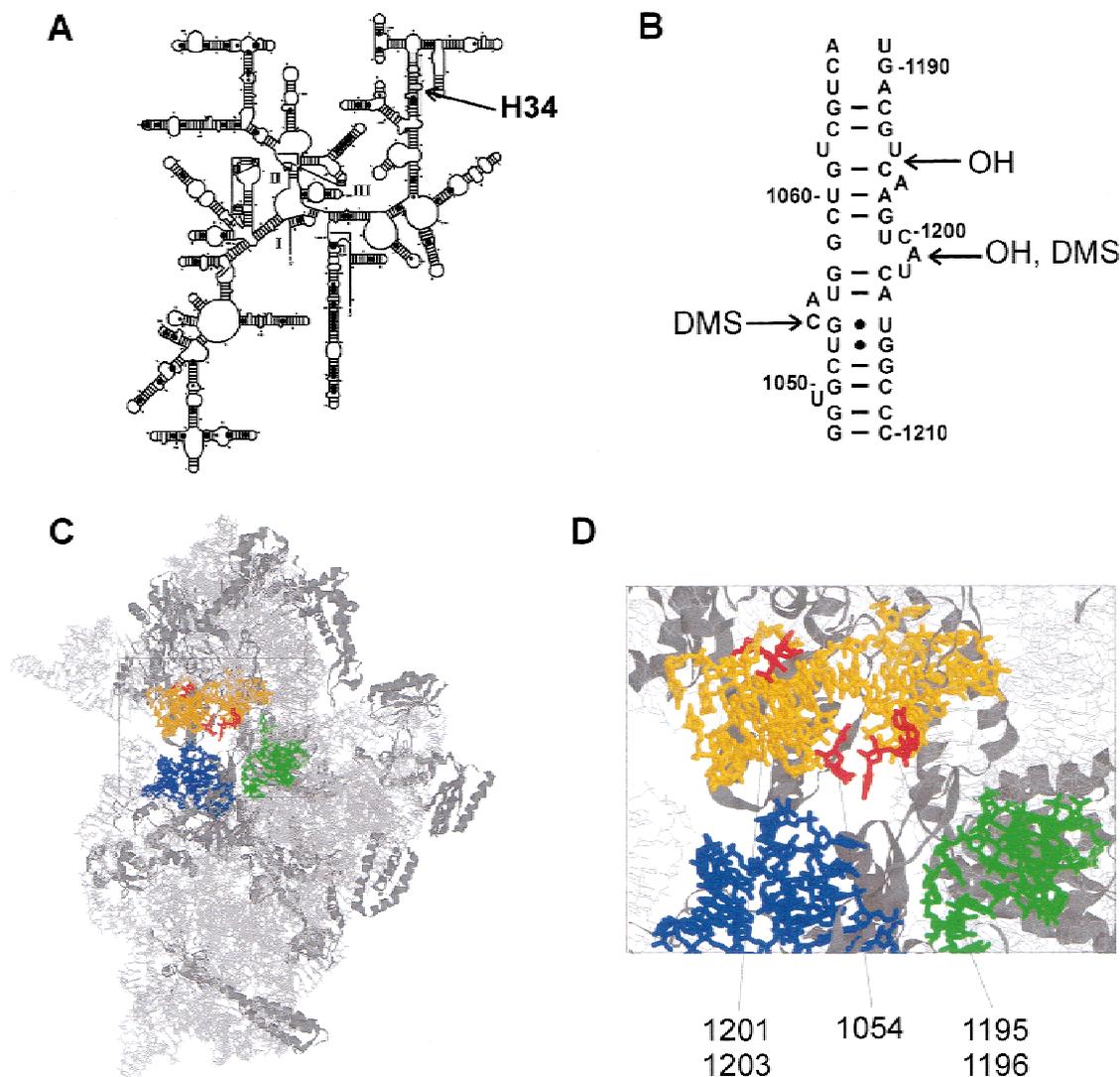


FIGURE 3. Location of protected positions in the 30S subunit. **A:** Secondary structure of 16S rRNA. The three domains of 16S rRNA are indicated by roman numerals; H34: helix 34. **B:** Summary of EF-G-dependent protections in helix 34. **C:** Location of helix 34 in the crystal structure of 30S subunit (Wimberly et al., 2000; PDB 1FJF). Light gray: 16S rRNA; dark gray: proteins; orange: helix 34; blue: 530 region; green: decoding region of helix 44; red: protected residues in helix 34. **D:** Positions of protected residues in the 30S crystal structure. Color code is as in **C**.

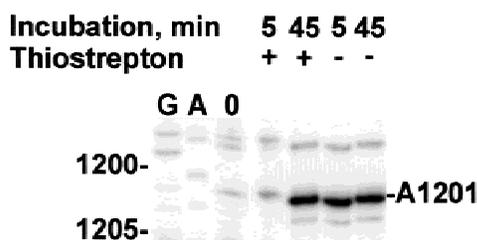


FIGURE 4. Protection against DMS of A1201 in helix 34 by EF-G-GDPNP. Pretranslocation ribosomes were incubated with EF-G and GDPNP in the absence or presence of thiostrepton. In the presence of thiostrepton, the extent of translocation was <10% after 5 min and >70% after 45 min incubation, as measured by puromycin, whereas in the absence of thiostrepton, translocation was complete within 5 min. In controls without EF-G, the extent of translocation was <5% after 45 min incubation. G and A: sequencing lanes; 0: control without DMS.

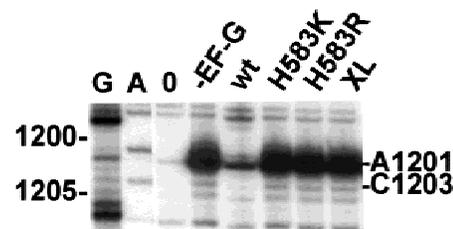


FIGURE 5. Effect of mutations in EF-G on DMS modifications in helix 34. Pretranslocation complexes were incubated with wild-type EF-G, EF-G(H583K/R), or EF-G(XL) in the presence of thiostrepton for 5 min at 37 °C and then subjected to reaction with DMS (Materials and Methods). According to the puromycin assay, the extent of translocation was <10% in all samples. G and A: sequencing lanes; 0: control without DMS.

Thus, EF-G(XL) forms a stable complex with the pretranslocation ribosome. As shown in Figure 5, the formation of this complex does not induce the protections in helix 34. This result once more establishes the correlation between helix 34 protections and translocation and indicates that conformational flexibility of EF-G, which is restricted by the crosslink in EF-G(XL), is required for the effect to be induced.

DISCUSSION

Helix 34 is known to play an important role in translation. Mutations at different positions in helix 34 severely impair cell growth by diverse effects on translation, including reduced incorporation of ribosomes into polyosomes, erroneous termination, decreased fidelity of protein synthesis by increased readthrough of stop codons, and increased frameshifting (Prescott & Kornau, 1992; Moine & Dahlberg, 1994). Helix 34 comprises the binding site of spectinomycin, an antibiotic that inhibits translocation by binding to helix 34 (Bollen et al., 1968; Sigmund et al., 1984; Carter et al., 2000). EF-G-induced protections against chemical modification of helix 34 residues are only observed in complexes capable of translocation, that is, with wild-type EF-G, both with GTP and GDPNP, whereas no protections are observed with EF-G mutants that are strongly impaired (583 mutations in domain 4) or completely inactive (crosslinked EF-G) in translocation. These findings suggest that helix 34 is involved in the promotion of translocation by EF-G.

Are the protections of helix 34 residues induced by EF-G binding to the pretranslocation complex direct or indirect? The 18-Å cryo-EM reconstruction of the same complex (Stark et al., 2000) reveals that the only contact EF-G makes with the 30S subunit is at the shoulder. The contact region is at least 20 Å away from the protected residues of helix 34, suggesting the protections are indirect. The loss of the protections caused by a single amino acid exchange at position 583 in domain 4 of EF-G indicates that, in keeping with the model derived from cryo-EM (Stark et al., 2000), it is probably domain 4 that binds to the 30S shoulder, thereby inducing a conformational change in helix 34 of 16S rRNA.

How could helix 34 be affected by EF-G interacting with the shoulder region? The shoulder region contains helix 16 of 16S rRNA. Thus, EF-G binding could induce an allosteric alteration of the structure of 16S rRNA that could be transmitted to helix 34. Proteins may be involved as well, in particular protein S4, which is located in the shoulder region. S4 is bound to the junction of five helical elements of 16S rRNA (S4 junction; Heilek et al., 1995; Powers & Noller, 1995; Wimberly et al., 2000) of which one comprises helix 18 of 16S rRNA, a region known to contain a pseudoknot (530 region pseudoknot; Powers & Noller, 1991) and to be crucially important for ribosome function (Powers & Noller, 1991;

O'Connor et al., 1995). It is conceivable that induced motion at the S4 binding region, by altering the pseudoknot structure, may lead to an overall conformational change of the subunit, including the head and the neck region, as suggested by cryo-EM (Stark et al., 2000). Conformational coupling could also involve protein S5, which, by its C-terminal domain, contacts protein S4 and, by its N-terminal domain, approaches helix 34 (Wimberly et al., 2000). Protein S5 has been implicated in EF-G function and translocation (Marsh & Parmegiani, 1973), in keeping with the observation that mutations in protein S5 confer resistance to spectinomycin (Bollen et al., 1968; Brink et al., 1994), suggesting that disturbing the interaction between protein S5 and helix 34 interferes with translocation.

A rearrangement in helix 34 may also be caused by the interaction of EF-G with the 50S subunit. In such a case, the conformational signal could be transmitted via intersubunit bridges of which several were identified in the structure of the 70S ribosome. Among those, helix 27 of 16S rRNA (intersubunit bridge B2a; Cate et al., 1999) may be particularly important, because it can assume alternative base-pairing arrangements (Lodmell & Dahlberg, 1997). The conformational transition of helix 27 is accompanied by structural rearrangements in 16S rRNA near the decoding region (Lodmell & Dahlberg, 1997), including the cooperative opening and closing of double helices, such as helices 44, 18, and 34, which are connected to proteins S4 and S5. Thus, there may be signal transmission to helix 34 through another network of interactions, originating on the 50S subunit and involving helix 27, and it seems possible that dynamic changes of helix 27 contribute to translocation catalysis, in addition to the effect on the accuracy of tRNA selection (Lodmell & Dahlberg, 1997).

Helix 34 is an essential component of the decoding region, and by direct interactions with the codon-anticodon complex on the ribosome (Carter et al., 2000), it may contribute to the ~10-fold stabilization of the tRNA·mRNA complex in the A-site (Pape et al., 1998, 1999). Indeed, crosslinking data indicate that nucleotides A1196 and C1052 of helix 34 are in the close vicinity of the mRNA, and C1052 is close to the A-site mRNA codon (Dontsova et al., 1992), and the high-resolution crystal structure of the 30S subunit reveals that C1054 interacts with G34 in the 5' position of the anticodon of an anticodon stem loop bound to the A-site (Ogle et al., 2001); the same interaction is suggested by the crystal structure of 70S ribosome-tRNA complexes (Yusupov et al., 2001). The present results suggest that the binding of EF-G induces a structural change in helix 34 that releases these interactions and destabilizes the tRNA in the A-site, thereby facilitating tRNA movement in translocation. There are most likely other parts of the ribosome involved in structural changes related to translocation that are not observed in the present experiments because they are either inhibited by the presence of thiostrepton or do not

lead to accessibility changes for the chemical reagents used.

MATERIALS AND METHODS

Materials

70S ribosomes from *Escherichia coli* MRE 600, MF-mRNA (codons used are AUGUUU, coding for fMetPhe), Phe-tRNA^{Phe}, initiation factors (IF) 1–3, EF-G (Rodnina et al., 1999; Rodnina & Wintermeyer, 1995), and fMet-tRNA^{fMet} (Rodnina et al., 1994) were prepared as described. EF-Tu (Boon et al., 1992), EF-G mutants H583K, H583R (Savelsbergh et al., 2000), and a double mutant with a disulfide bridge engineered between domains 1 and 5 (Peske et al., 2000), all containing a C-terminal (His)₆-tag, were expressed and purified as described.

Preparation of pre- and posttranslocation complexes

To prepare 70S initiation complexes, ribosomes (0.25 μ M) were programmed with 0.75 μ M MF-mRNA (Calogero et al., 1988) in the presence of 0.4 μ M of each IF1, IF2, IF3, and 0.38 μ M f[³H]Met-tRNA^{fMet}, and 1 mM GTP in buffer A (20 mM K-HEPES, pH 7.5, 30 mM KCl, 70 mM NH₄Cl, 7 mM MgCl₂) for 30 min at 37 °C. Initiation complex was isolated by centrifugation through 400 μ L 1 M sucrose in buffer A for 1 h at 150,000 \times *g* in a Sorvall M120GX centrifuge. Supernatant was removed and the pellets dissolved in buffer A to a concentration of 0.6 μ M initiation complex. Ternary complex, EF-Tu·GTP·[¹⁴C]Phe-tRNA^{Phe}, was prepared by incubating 1.1 μ M EF-Tu, 1 mM GTP, 3 mM phosphoenol pyruvate, 0.1 mg/L pyruvate kinase and 0.66 μ M [¹⁴C]Phe-tRNA^{Phe} for 10 min at 37 °C. Ternary complex was added to the initiation complex, and incubated for 1 min at 37 °C to form 0.3 μ M pretranslocation complex. The amounts of [¹⁴C]Phe-tRNA^{Phe} or f[³H]Met-tRNA^{fMet} bound to the ribosomes were determined by nitrocellulose filtration by directly applying aliquots of the reaction mixture to the filters (Sartorius) and subsequent washing with buffer A. Filters were dissolved and radioactivity measured in QS361 scintillation cocktail (Zinsser Analytic). The occupancy of the pretranslocation complexes was close to 100% for both ³H and ¹⁴C labels. According to HPLC analysis after liberating peptides by alkaline hydrolysis, fMetPhe-tRNA^{Phe} formation was quantitative. Puromycin analysis (1 mM puromycin, 10 s at 37 °C) without or with prior addition of EF-G·GTP revealed that >90% of fMetPhe-tRNA^{Phe} was located in the A-site and could be translocated to the P-site.

EF-G was stabilized on pre- or posttranslocation ribosome complexes by thiostrepton (Rodnina et al., 1999). To prepare the pretranslocation complexes with bound EF-G, 0.5 μ M EF-G, preincubated with 1 mM GTP (or GDPNP) for 15 min at 37 °C, were added to 0.25 μ M pretranslocation complex in the presence of 50 μ M thiostrepton and 2% DMSO and incubated for 5 min at 37 °C; >80% of fMetPhe-tRNA^{Phe} remained in the A-site, as measured by the formation of fMetPhe-puromycin. The posttranslocation complex was obtained by incubating pretranslocation complex containing thiostrepton with 2 μ M EF-G for 45 min at 37 °C; the extent of translocation was >70%.

Chemical probing

Modification of ribosome complexes (0.25 μ M) with DMS was carried out for 10 min at 37 °C (Stern et al., 1988). Ribosomal RNA was purified by repeated phenol extractions, and methylated sites were determined by primer extension sequencing with AMV reverse transcriptase (Stern et al., 1988), using a set of oligodeoxyribonucleotide primers spaced such as to cover the entire length of both 16S and 23S rRNA. RNA cleavage by hydroxyl radicals generated by the Fe(II)-EDTA reaction with hydrogen peroxide was performed as described (Powers & Noller, 1995), and cleavage sites were determined by primer extension sequencing as above.

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