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An Elongation Factor G-Induced Ribosome Rearrangement Precedes tRNA-mRNA Translocation

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Summary

The elongation cycle of protein synthesis is completed by translocation, a rearrangement during which two tRNAs bound to the mRNA move on the ribosome. The reaction is promoted by elongation factor G (EF-G) and accelerated by GTP hydrolysis. Here we report a pre-steady-state kinetic analysis of translocation. The kinetic model suggests that GTP hydrolysis drives a conformational rearrangement of the ribosome that precedes and limits the rates of tRNA-mRNA translocation and Pi release from EF-G·GDP·Pi. The latter two steps are intrinsically rapid and take place at random. These results indicate that the energy of GTP hydrolysis is utilized to promote the ribosome rearrangement and to bias spontaneous fluctuations within the ribosome-EF-G complex toward unidirectional movement of mRNA and tRNA.

Introduction

The translocation step of the elongation cycle is promoted by elongation factor G (EF-G), a GTPase that hydrolyzes GTP during the reaction. During translocation, an extensive structural rearrangement takes place, as two tRNAs bound to mRNA move together from their pre- to their posttranslocation positions on the ribosome. The reaction is slow without GTP hydrolysis (Katunin et al., 2002; Rodnina et al., 1997) and extremely slow, but significant, without EF-G under certain conditions in vitro (Gavrilova et al., 1976; Southworth et al., 2002). This indicates that the basic reaction is intrinsic to the ribosome and requires a particular conformation that is unstable and induced by EF-G binding and further promoted by GTP hydrolysis (Katunin et al., 2002).

How structural changes of the ribosome, induced by EF-G, and tRNA-mRNA movement may be related is not clear. Apart from the scarcity of direct structural information, this is due to the fact that the sequence of partial reactions of translocation is not known. In previous kinetic work, the partial reactions of EF-G binding to the pretranslocational ribosome, GTP hydrolysis, Pi release, tRNA movement, and dissociation of EF-G from the posttranslocational ribosome were measured individually, yielding apparent rate constants for the steps following rapid GTP hydrolysis (Katunin et al., 2002; Rodnina et al., 1997; Savelsbergh et al., 2000a). Because Pi release and tRNA movement exhibited the same rates, we assumed that the former step was rate limiting for the latter (Wintermeyer et al., 2001). However, since true rate constants, and therefore the sequence, of those steps were not determined, that model remained preliminary.

Here we report the kinetic analysis of translocation that provides rate constants for the steps up to and including tRNA-mRNA movement. Several rapid kinetic assays were used to monitor different partial reactions of translocation. GTP hydrolysis in EF-G was analyzed by the quench-flow technique using [γ-^{32}P]GTP (Rodnina et al., 1997). Release of Pi following the cleavage was monitored by stopped-flow using a fluorescence change of MDCC-labeled phosphate binding protein (PBP) from *E. coli* (Brune et al., 1994). tRNA translocation was followed by the fluorescence change of proflavin attached to the D loop at the elbow region of the tRNA (tRNA^{Phe}(Prf16/17)) which reports the movement of the tRNA from the A to the P site (Robertson et al., 1986). Finally, the movement of the mRNA during translocation was followed directly by a newly developed assay whereby the fluorescence change of fluorescein attached to the 3′ end of a short mRNA fragment was monitored. For each observable, the concentration dependence of the observed rate was measured and yielded rate constants for EF-G binding (k_1, k_{-1}), GTP hydrolysis (k_2), and a rearrangement step (k_3). According to the kinetic results, the rearrangement step, which depends on GTP hydrolysis, limits the rates of translocation and Pi release both of which are rapid and independent of each other.

Results

Kinetics of GTP Hydrolysis, Pi Release, and Translocation

When ribosomes were rapidly mixed with EF-G and GTP, one round of GTP cleavage proceeded at a rate of about 80 s^{-1} at nonsaturating EF-G concentration (Figure 1A). Rapid, single-round Pi release ("burst") took place after a prolonged delay and at a rate of 20 s^{-1}, i.e., was much slower than GTP hydrolysis (Figures 1A and 1B). The following turnover reaction of GTP hydrolysis was manifested in a slower phase of Pi release that proceeded linearly for a few seconds, because liberated Pi was taken up by PBP present in excess, and eventually leveled off due to saturation of PBP with Pi (cf. Figure 3A, inset). The rate of the first round of translocation, as monitored by fluorescence labels in either tRNA or mRNA (Experimental Procedures), was identical to that of rapid Pi release, about 20 s^{-1} (Figure 1B). The rates of Pi release coincided with the rates of tRNA-mRNA

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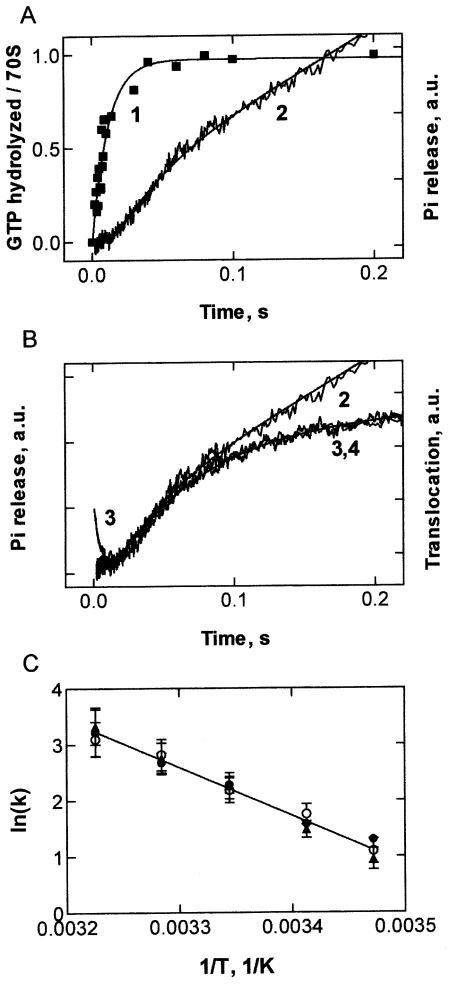

Figure 1. Kinetics of Elemental Steps of Translocation

Reactions were performed under single-round conditions in excess of EF-G (1 μM).

(A) GTP hydrolysis and Pi release. Hydrolysis of [γ-^{32}P]GTP (1, ■) was measured by quench-flow. Pi release (2) following GTP hydrolysis was measured by stopped-flow monitoring the fluorescence change of MDCC-PBP due to binding Pi (Experimental Procedures).

(B) tRNA and mRNA movement. In stopped-flow experiments, fluorescence changes of proflavin in fMetPhe-tRNA^{Phe}(Prf16/17) (3) or fluorescein attached to the 3′ end of the MF-mRNA (MF-mRNA-3′Flu) (4) were monitored; a trace for Pi release (2) is shown for comparison.

(C) Temperature dependence of apparent rate constants of Pi release (▲) and tRNA/mRNA movement as measured with fMetPhe-tRNA^{Phe}(Prf16/17) (○) or MF-mRNA-3′Flu (●).

movement at all temperatures in the range from 15°C to 37°C at saturating concentrations of EF-G (Figure 1C). This result strongly suggests that either one reaction, Pi release or tRNA movement, was limiting the rate of the other, or that there was a common rate-limiting step that was not observed directly and preceded Pi release and tRNA-mRNA movement.

Different kinetic models that would be consistent with these results are depicted in Figure 2. Model A assumes that, following the binding of EF-G·GTP (step 1) and rapid GTP hydrolysis (2), tRNA-mRNA movement (3) precedes and limits the rate of Pi release (4), which is rapid

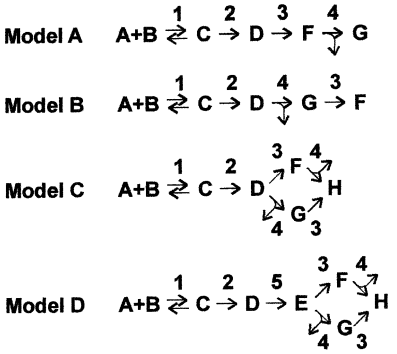

Figure 2. Alternative Kinetic Schemes of Translocation

For the identification of steps 1–5, see text.

intrinsically. In model B, the two steps are reversed and Pi release (4) limits the rate of movement (3). The branched model C assumes that rapid GTP hydrolysis (2) forms an intermediate, D, from which Pi release (4) and tRNA-mRNA movement (3) take place randomly. Finally, in model D, movement (3) and Pi release (4) are assumed to be rapid and independent of each other, but both are limited by the rate by which a common intermediate, E, is formed (5).

Order of Reactions

In order to determine the order of reactions and distinguish between models A–D, one of the steps, either tRNA movement (step 3, Figure 2) or Pi release (step 4), was selectively inhibited, and the effect on the other step was studied. tRNA movement was inhibited by the antibiotic viomycin which binds to the decoding region of 16S rRNA (Powers and Noller, 1994) and blocks tRNA movement by stabilizing the tRNA in the A site (Modolell and Vazquez, 1977; Y.P. Semenkov, personal communication), whereas it does not affect EF-G binding to the ribosome and GTP hydrolysis (Modolell and Vazquez, 1977; Rodnina et al., 1997). The rate of Pi release from EF-G was not changed by viomycin, both in the first round and the turnover reaction, although translocation was completely blocked (Figure 3A). Similar effects, i.e., inhibition of translocation and no effect on Pi release, were found previously for EF-G mutants with either a H583K replacement at the tip of domain 4 (Savelsbergh et al., 2000a) or with a disulfide crosslink between domains 1 and 5 (Peske et al., 2000). Furthermore, identical time courses of Pi release were observed with pretranslocation and vacant ribosomes, i.e., with or without translocation (data not shown). Thus, Pi release does not depend on translocation, excluding the sequential model A whereby Pi release follows translocation (Figure 2).

Another EF-G mutant that was strongly impaired in translocation, while ribosome binding and GTP hydrolysis were not affected, was a domain deletion mutant that lacked domains 4 and 5 (Savelsbergh et al., 2000a). When translocation was performed with EF-GΔ4/5, Pi release proceeded with the same very slow rate as translocation, which was several thousand-fold lower, compared to native EF-G (Figure 3B). This result is compatible with models A, B, or D, but disfavors the random

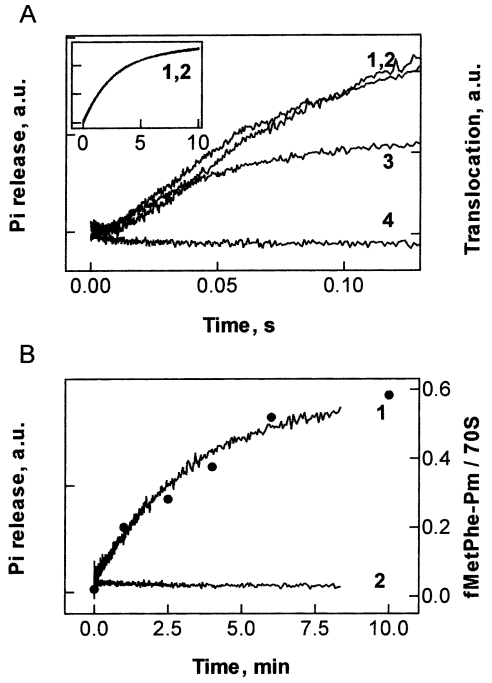

Figure 3. Effect of Translocation Inhibition on Pi Release

(A) Inhibition by viomycin. EF-G-dependent reactions were initiated by addition of EF-G·GTP (3 μM) to pretranslocation complex with fMetPhe-tRNA^{Phe}(Prf16/17) (0.1 μM) in the absence (1, 3) or presence (2, 4) of viomycin (100 μM). Pi release was measured by the fluorescence increase of MDCC-PBP (1, 2); translocation by the increase of proflavin fluorescence (3, 4). According to the puromycin assay, translocation was >80% and <10% in the absence and presence of viomycin, respectively. Inset: Multiple rounds of Pi release measured over 10 s.

(B) Inhibition by domain 4/5 deletion. Pi release with EF-G(Δ4/5) (1 μM) (1); the control was performed without factor (2). Translocation of fMetPhe-tRNA^{Phe} (●) induced by EF-G(Δ4/5) was measured by reaction with puromycin.

model C, which would imply that the mutation in EF-G has the same effect on two chemically different, kinetically independent reactions, which seems unlikely. Rather, the data are explained best by assuming that the truncation of EF-G affected a single reaction that was rate limiting for both translocation and Pi release.

Complementary results were obtained with ribosomes on which ribosomal protein L7/12 was replaced with mutant L7/12(K70A). Surprisingly, this mutation had a strong inhibitory effect on Pi release. The replacement of L7/12 was accomplished with about 85% efficiency (Mohr et al., 2002), and ribosomes carrying L7/12(K70A) were fully active in promoting single-round GTP hydrolysis by EF-G (data not shown). In contrast, the amplitude of the fluorescence change of MDCC-PBP during the burst phase, that is, the amount of Pi released in one round of reaction, was strongly decreased and was not much different from that observed with core ribosomes lacking L7/12 (Figure 4A). This was not due to impaired EF-G binding, as the amplitudes of rapid Pi release reached their (different) final levels at about the same EF-G concentration (Figure 4B). About 25% of the ribosomes reconstituted with L7/12(K70A) promoted rapid Pi release (Figure 4B), not much above the level attributed to the presence of about 15% ribosomes that still contained wt L7/12. In contrast, the extent of rapid translocation was the same on ribosomes with wt or mutant L7/12 (Figure 4C), and the rate was decreased only 2-fold by the mutation (Figures 4C and 4D). (Ribosomes reconstituted with wt L7/12 were slightly [2-fold] slower in translocation than native ribosomes for unknown reasons.) Thus, rapid translocation takes place on ribosomes that do not exhibit rapid Pi release, implying that Pi release cannot limit the rate of translocation. This result excludes the sequential model B (Figure 2).

Of the remaining models C and D, model C predicts that the observed rates of either Pi release or translocation should decrease if either reaction is inhibited, because, at saturation, $k_{app} = k_4 + k_5$ (Cleland, 1975). Thus, the rate of Pi release should decrease when translocation is inhibited. This was not observed, as the rate of Pi release remained unaffected when translocation was blocked by viomycin in the presence of EF-G at saturating concentration (Figure 3), or when translocation was inhibited by mutations in EF-G (Peske et al., 2000; Savelsbergh et al., 2000a). Based on these results and those obtained with EF-GΔ4/5, discussed above, model C is considered unlikely. All results can be consistently explained by model D which features a rate-limiting step that follows GTP hydrolysis but precedes Pi release and tRNA-mRNA movement both of which are intrinsically rapid and take place independently of each other.

Determination of Rate Constants

Rate constants of translocation, k_1, k_{-1}, k_2, k_3, as defined by model D (Figure 6), were derived from the rates of EF-G binding, GTP hydrolysis, Pi release, and translocation, each measured over a large range of EF-G concentrations (up to 4 μM). Rate constants were determined analytically by solving the respective rate equations; this included the determination of k_3 from fluorescence measurements of tRNA or mRNA movement. The same value of k_3 was determined from Pi release by global fitting using numerical integration. Details of the kinetic analysis and the equations used for evaluation are given in the Supplemental Data at http://www.molecule.org/cgi/content/full/11/6/1517/DC1.

Both EF-G binding to pretranslocation ribosomes and tRNA movement were monitored by the fluorescence change of A site-bound fMetPhe-tRNA^{Phe}(Prf16/17), which was biphasic (Figure 1B, trace 3). The rate of the initial fluorescence decrease, which is due to binding (Rodnina et al., 1997), exhibited a linear concentration dependence (data not shown). Since subsequent rapid GTP hydrolysis precluded the accurate determination of rate constants of the binding step, values of $k_1 = 150 \pm 30\ \mu M^{-1}s^{-1}$ and $k_{-1} = 140 \pm 20\ s^{-1}$ were taken from a previous measurement with a nonhydrolyzable GTP analog, caged-GTP (Katunin et al., 2002). The slower fluorescence increase (Figure 1B, trace 3), which reports tRNA movement, is considered below.

For determining k_2, GTP hydrolysis was measured by quench-flow. The rate increased with EF-G concentration and approached saturation at concentrations of >3 μM (Figure 5A). Nonlinear fitting yielded $k_2 = 250 \pm 100\ s^{-1}$ and $K_{M2} = 3 \pm 1$ μM, where K_{M2} is the K_M for EF-G of step 2 of the model. The fitted value is consistent

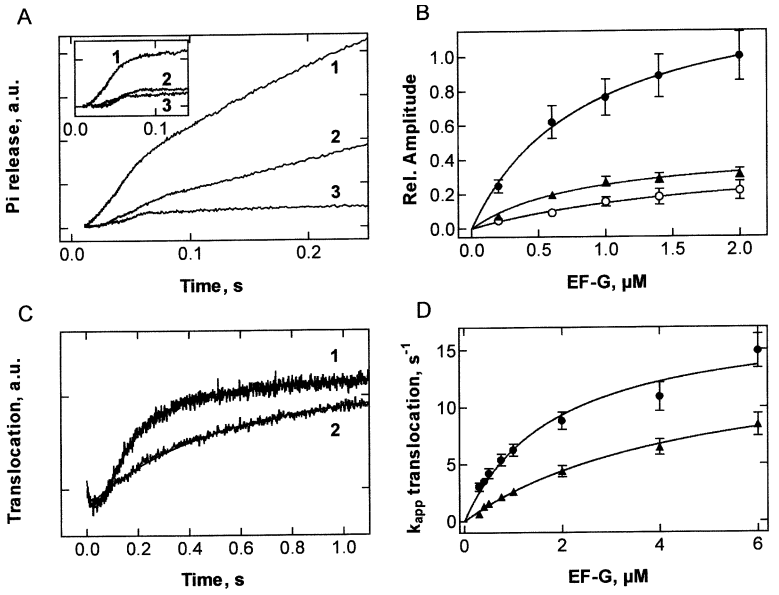

Figure 4. Effect of Inhibiting Pi Release on Translocation

(A) Time courses of Pi release with EF-G·GTP (2 μM) and ribosomes (0.2 μM) reconstituted with either wt L7/12 (1) or with L7/12(K70A) (2) or core ribosomes lacking L7/12 (3). Inset: Fluorescence change due to rapid Pi release. Traces 1 and 2 were corrected for turnover by subtracting the linear phase of the turnover reaction, as described below (B).

(B) Relative amplitudes of rapid Pi release on ribosomes reconstituted with wt L7/12 (●) or L7/12(K70A) (▲), and on 70S cores lacking L7/12 (○). Fluorescence amplitudes due to rapid Pi release were determined from the transients ([A], initial 0.1 s) after subtraction of the linear function accounting for the turnover reaction between 0.2 and 0.5 s (see examples in the inset of [A]), and were normalized by setting the maximum amplitude to 1.

(C) Time courses of translocation (EF-G·GTP, 2 μM) measured by the fluorescence increase of fMetPhe-tRNA^{Phe}(Prf16/17) on ribosomes (0.2 μM) reconstituted with wt L7/12 (1) or L7/12(K70A) (2).

(D) Concentration dependence of translocation rates (k_{app} translocation) on ribosomes reconstituted with wt L7/12 (●) or L7/12(K70A) (▲). Rates were obtained from time courses as in Figure 3C by exponential fitting.

with the value calculated using two rate constants that were determined directly, k_1 and k_{-1}: $K_{M2} = (k_{-1} + k_2)/k_1 = 2.6 \pm 0.8$ μM.

According to model D, Pi release (step 4, Figure 2) and tRNA-mRNA movement (step 3) are intrinsically rapid, and their rates are determined by a preceding step (step 5). Thus, the value for the rate constant of this step (k_3 in the following evaluations) can be obtained from the kinetics of either Pi release or tRNA/mRNA movement. As described below, the same value was obtained in the two cases, supporting the validity of the approaches used.

First, the value of k_3 was determined from the concentration dependence of the translocation rate, monitored by fluorescent labels in either the A site peptidyl-tRNA or the mRNA (cf. Figure 1B). Time courses were measured at increasing EF-G concentration, and rates of tRNA or mRNA movement were evaluated by exponential fitting. The two labels gave identical results. Rates increased with increasing EF-G concentration and, according to the nonlinear fit, saturated at $k_{sat} = 30\ s^{-1}$ with $K_{M3} = 0.6 \pm 0.1$ μM (Figure 5B), where K_{M3} is the K_M for EF-G of step 3. For model D, $k_{sat} = k_2 \cdot k_3/(k_2 + k_3)$ (Cleland, 1975); taking $k_2 = 250 \pm 100\ s^{-1}$ from the GTPase measurements, the calculation yielded $k_3 = 34 \pm 4\ s^{-1}$, the same value as obtained from Pi release. A similar value of $K_{M3} = K_{M2} \cdot k_3/(k_2 + k_3) = 0.40 \pm 0.25$ μM was calculated from the values of the rate constants k_1, k_{-1}, k_2, and k_3, determined from GTP hydrolysis and Pi release.

The same value of k_3 was determined from Pi release. Time courses of Pi release, measured at various concentrations of EF-G, showed a delay phase followed by the rapid first round of Pi release and a slower, quasilinear phase due to multiple turnover (Figure 5C). The rate of the first round of Pi release at EF-G saturation was estimated to 30–40 s^{-1} by exponential fitting, which was possible because at high concentration the delay was negligible. At lower concentrations of EF-G, however, the delay was significant and exponential fitting was not feasible. Therefore, a global fit of all time courses of Pi release measured at different concentrations of EF-G was performed by numerical integration (see the Supplemental Data at http://www.molecule.org/cgi/content/full/11/6/1517/DC1). The calculations yielded the following values for the rate constants: $k_1 = 120 \pm 30\ s^{-1}$, $k_2 = 250 \pm 50\ s^{-1}$, and $k_3 = 35 \pm 5\ s^{-1}$. As shown by the calculated curves in Figure 5C, the whole set of time courses, measured at various EF-G concentrations, is well represented by these values. The same values were obtained with vacant ribosomes (data not shown).

Discussion

Kinetic Model of Translocation

Based on the present kinetic data and results reported previously (Katunin et al., 2002; Rodnina et al., 1997; Savelsbergh et al., 2000a), we propose the following kinetic model of translocation (Figure 6). First, EF-G·GTP binds to the ribosome rapidly and reversibly. Subsequently, GTP is hydrolyzed. The release of Pi from EF-G·GDP·Pi is delayed and much slower than GTP hydrolysis. Pi release and tRNA-mRNA movement take place at the same rates, and, according to the kinetic analysis, both reactions are rapid intrinsically and rate limited by a preceding rearrangement of the ribosome-EF-G·GDP·Pi complex. Despite equal apparent rates, rearrangement and actual movement (or Pi release) are distinct events with different rate constants, i.e., 35 s^{-1} and much faster than that, respectively. The rate-limiting step is referred to as unlocking rearrangement, taking up a term introduced by Spirin (1968), because most likely it entails changes at the interface between the subunits, although the actual changes will almost certainly be different from those envisioned at the time. According to the model, Pi release and tRNA-mRNA movement take place at

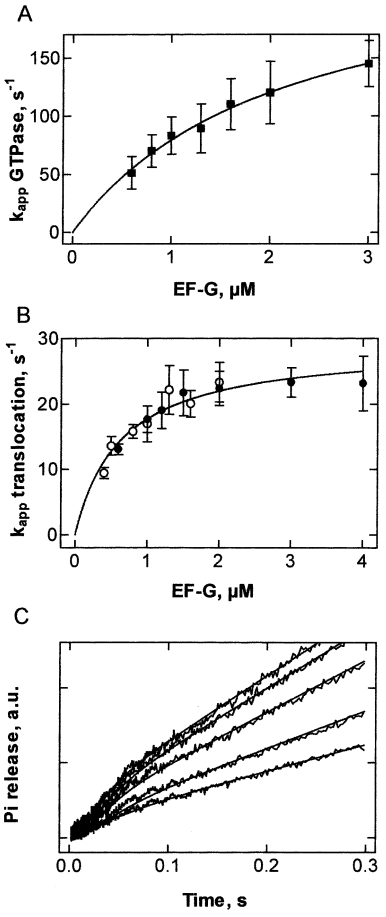

Figure 5. Concentration Dependence of Partial Reactions of Translocation

(A) Hydrolysis of [γ-^{32}P]GTP.

(B) Concentration dependence of k_{app} of translocation. Values for k_{app} were obtained by exponential fitting of the traces measured by the fluorescence of fMetPhe-tRNA^{Phe}(Prf16/17) (○) or mRNA(3′-Flu) (●).

(C) Pi release. Pi release was measured by stopped-flow at increasing concentrations of EF-G (bottom to top: 0.4, 0.6, 1.0, 1.2, and 1.5 μM). Smooth lines show fits obtained by global analysis using numerical integration of these and additional data (0.8, 2.0, 3.0, and 4.0 μM EF-G), as described in the Supplemental Data at http://www.molecule.org/cgi/content/full/11/6/1517/DC1.

random, and both reactions have to occur in order to allow subsequent steps, including a presumed "relocking" step, that ultimately lead to the dissociation of EF-G·GDP from the ribosome to take place rapidly.

The major feature of the proposed kinetic model is that actual movement is rate limited by a preceding rearrangement of the ribosome-EF-G complex. Assuming that the same structural changes are required for translocation to take place with or without GTP hydrolysis (Katunin et al., 2002), the model suggests that the acceleration of translocation by GTP hydrolysis (Rodnina et al., 1997) is due to the acceleration of the unlocking rearrangement. Since the rearrangement also precedes Pi release, the form of EF-G that brings about the rearrangement is the GDP·Pi form. This indicates that the formation of EF-G·GDP·Pi on the ribosome induces a conformational change in EF-G that drives the rearrangement of the ribosome-EF-G complex. Unlocking and movement are kinetically distinct events, indicating that movement is not directly coupled to the rearrangement promoted by GTP hydrolysis and may be spontaneous.

The kinetics of the steps following Pi release and tRNA-mRNA movement (k_6–k_9) have not been fully characterized yet. It is known that the dissociation of EF-G, as measured by turnover, is much faster with GTP than with nonhydrolyzable GTP analogs or with GDP (Katunin et al., 2002), indicating that GTP hydrolysis promotes one of the steps preceding the dissociation of EF-G·GDP. The rate-limiting step seems to be another rearrangement (relocking, Figure 6) which according to fluorescence data obtained with mant-GTP involves a conformational change of EF-G (B. Wilden, A.S., M.V.R., and W.W., unpublished data). The mechanism of coupling GTP hydrolysis to this step is not clear. Part of the coupling seems to be accomplished by retaining Pi in the binding pocket in domain 1 of EF-G, as Pi release is rate limiting for the relocking rearrangement (our unpublished data).

Structural Changes Related to Translocation

Following tRNA-mRNA movement, EF-G undergoes a reorientation and moves from a pretranslocation position outside the 30S A site (Stark et al., 2000; see also the different arrangement for EF-G·GDPCP proposed by Agrawal et al.,1999) to its posttranslocation position where domain 4 reaches into the 30S A site, occupying the site of the anticodon arm of A site tRNA (Agrawal et al., 1998; 1999; Stark et al., 2000; Wilson and Noller, 1998). As suggested earlier (Rodnina et al., 1997), the movement of domain 4 of EF-G into the 30S A site may have the function of preventing backward tRNA movement which may otherwise be significant in the unlocked state of the ribosome. This way, EF-G might bias spontaneous tRNA movement on the ribosome, driving forward movement by a Brownian ratchet mechanism. This raises the interesting possibility that EF-G has a dual motor function: a mechanochemical one in coupling GTP hydrolysis to ribosome unlocking and a ratchet function in biasing tRNA-mRNA movement.

There are numerous interactions between tRNAs, mRNA, and the ribosome that have to rearrange during translocation. In the decoding site, ribosomal residues interact with the mRNA and the tRNA anticodon regions (Ogle et al., 2001; Stark et al., 2002; Valle et al., 2002; Yusupov et al., 2001). In the peptidyl transferase center, residues of 23S rRNA form base-specific interactions with the 3′-terminal CCA sequences of the tRNAs (Kim and Green, 1999; Samaha et al., 1995; Yusupov et al., 2001). At the subunit interface, a number of bridges, most of them made up of rRNA, connect the two subunits and also contact the tRNAs (Stark et al., 2002; Yusupov et al., 2001). Cryo-EM data indicate that EF-G induces large-scale structural rearrangements of the ribosome (Frank and Agrawal, 2000; Stark et al., 2000). Extensive alterations are seen in the 30S subunit where the head moves relative to the body (Agrawal et al., 1999; Stark et al., 2000), and a rotational rearrangement of the subunits relative to one another upon binding of EF-G·GDPCP has been observed (Frank and Agrawal,

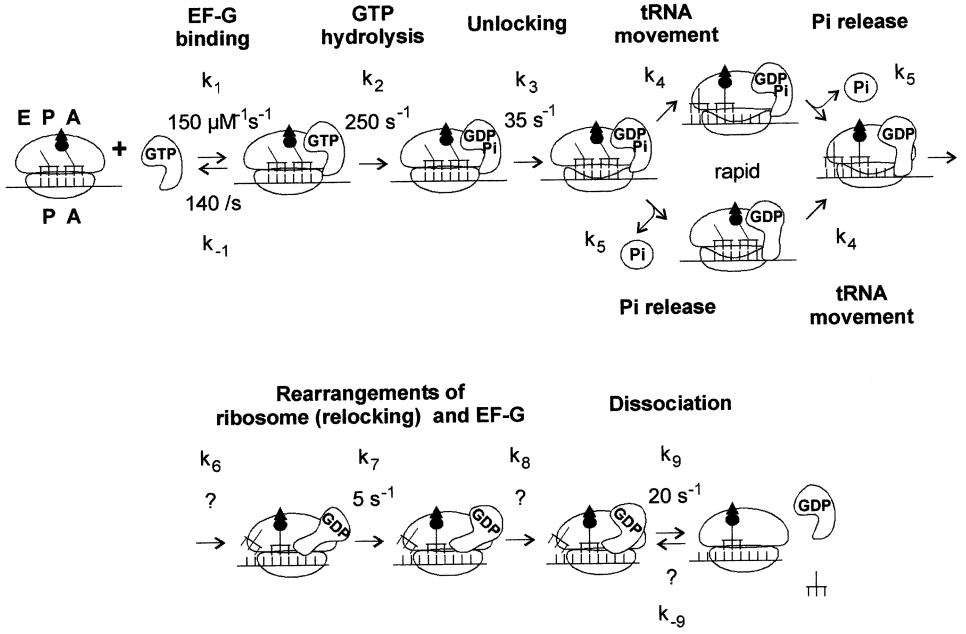

Figure 6. Kinetic Model of Translocation

Ribosomes are depicted in two conformations, closed and open, to indicate unlocking. EF-G is depicted in different conformations and orientations on the ribosome, based on cryo-EM models (Stark et al., 2000). Values of k_7 and k_9 are preliminary (B. Wilden, A.S., W.W., and M.V.R., unpublished data).

2000), suggesting changes of contacts at the subunit interface.

The present kinetic results argue in favor of a model whereby a major rearrangement of the ribosome, i.e., the one that is driven by GTP hydrolysis, takes place prior to tRNA-mRNA movement. We consider unlikely, therefore, that the majority of tRNA-ribosome contacts are maintained throughout the movement, as has been proposed on the basis of similar chemical modification patterns of tRNA transcripts before and after translocation (Dabrowski et al., 1995, 1998). This does not exclude that the movement takes place in steps. Rather, given the large number of interactions that involve the 3′ end, the elbow region, and the anticodon arm of the tRNAs, it is likely that interactions are resolved and reformed in a sequential fashion (Noller et al., 2002). The sequence at present is unknown. The attainment of hybrid state positions of the 3′ ends of the tRNAs prior to translocation may indicate that movement on the 50S subunit takes place first (Noller et al., 2002), although other sequences are not excluded. According to the present model, the unlocking rearrangement of the ribosome has to take place before movement of either tRNA or mRNA can occur.

Experimental Procedures

Materials

Experiments were carried out in buffer A (50 mM Tris·HCl [pH 7.5], 70 mM NH_4Cl, 30 mM KCl, 7 mM $MgCl_2$, 1 mM DTT) at 37°C. f[^{3}H]Met-tRNA^{fMet}, [^{14}C]Phe-tRNA^{Phe}, and tRNA^{Phe}(Prf16/17) were prepared as described (Rodnina et al., 1994; Rodnina and Wintermeyer, 1995), as were ribosomes from *E. coli* MRE600, MF-mRNA (120 nt, codons used were AUG [fMet] and UUU [Phe]), initiation factors, mRNA, EF-Tu, and EF-G (Rodnina et al., 1999). Ribosomes were depleted of protein L7/12 and reconstituted as described (Mohr et al., 2002). The preparation of mutant L7/12(K70A) will be described elsewhere (Mohr et al., submitted). Wt and mutant protein L7/12 were expressed and isolated as described (Savelsbergh et al., 2000b); the purity was >90%.

To prepare fluorescently labeled mRNA, a short variant of MF-mRNA with a coding sequence of 14 nucleotides at the 3′ end was prepared by in vitro transcription using T7 RNA polymerase. Labeling of the periodate-oxidized mRNA with fluorescein-5-thiosemicarbazide (Molecular Probes) was carried out essentially as described (Lentzen et al., 1994).

Biochemical Assays

The preparation of pretranslocation complexes programmed with MF-mRNA (or fluorescein-labeled MF-mRNA) and carrying deacylated tRNA^{fMet} in the P site and fMetPhe-tRNA^{Phe} or fluorescent fMetPhe-tRNA^{Phe}(Prf16/17) in the A site was described previously (Katunin et al., 2002). Greater than ninety-five percent of the ribosomes carried the tRNAs as indicated. To induce translocation, EF-G, preincubated with 1 mM of GTP for 15 min at 37°C, was mixed with the pretranslocation complex at the indicated concentrations. The extent of translocation (>85%) was determined by reaction with puromycin (1 mM puromycin, 10 s, 37°C; Rodnina et al., 1997). Rates of Pi release were measured in the presence of 25 μM GTP and 2.5 μM MDCC-labeled PBP.

Rapid Kinetics

Fluorescence stopped-flow measurements were performed and the data evaluated as described previously (Rodnina et al., 1997). Fluorescence of proflavin or fluorescein was excited at 470 nm and measured after passing a KV500 filter (Schott); MDCC fluorescence was excited at 425 nm and measured after passing a KV450 filter. Experiments were performed in buffer A at 37°C by rapidly mixing equal volumes (60 μl) each of the pretranslocation complex and EF-G·GTP. The data were evaluated by exponential fitting using TableCurve software (Jandel Scientific) or by numerical integration using Scientist software (Micromath Scientific Software). Analytical solutions of rate equations were used to determine kinetic parameters from the concentration dependence of rates measured at

pseudo-first-order conditions (see the Supplemental Data at http://www.molecule.org/cgi/content/full/11/6/1517/DC1).

Rate constants of Pi release were estimated by global analysis of a combined data set comprising all time courses obtained at different EF-G concentrations, using numerical integration according to model D (Figure 6). Details are described in the Supplemental Data.

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