Think again

Synchronous tRNA movements during translocation on the ribosome are orchestrated by elongation factor G and GTP hydrolysis

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The translocation of tRNAs through the ribosome proceeds through numerous small steps in which tRNAs gradually shift their positions on the small and large ribosomal subunits. The most urgent questions are: (i) whether these intermediates are important; (ii) how the ribosomal translocase, the GTPase elongation factor G (EF-G), promotes directed movement; and (iii) how the energy of GTP hydrolysis is coupled to movement. In the light of recent advances in biophysical and structural studies, we argue that intermediate states of translocation are snapshots of dynamic fluctuations that guide the movement. In contrast to current models of stepwise translocation, kinetic evidence shows that the tRNAs move synchronously on the two ribosomal subunits in a rapid reaction orchestrated by EF-G and GTP hydrolysis. EF-G combines the energy regimes of a GTPase and a motor protein and facilitates tRNA movement by a combination of directed Brownian ratchet and power stroke mechanisms.

Keywords:
GTP hydrolysis; hybrid tRNA states; ribosome; ribosome motions; thermal fluctuations; translation

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Abbreviations:
A site, aminoacyl-tRNA binding site; **CHI,** chimeric state; **cryo-EM,** cryo-electron microscopy; **EF-G,** elongation factor G; **EF-Tu,** elongation factor Tu; **E site,** tRNA exit site; **GDPCP,** guanylyl 5′-(β,γ-methylene-diphosphonate), a non-hydrolyzable GTP analog; **GDPNP,** guanosine 5′-(β,γ-imido) triphosphate, another non-hydrolyzable analog of GTP; **INT,** intermediate state; **Pmn,** puromycin; **PRE,** pre-translocation state; **P site,** peptidyl-tRNA binding site; **POST,** post-translocation state; **SRL,** sarcin-ricin loop of 23S rRNA.

Introduction

The ribosome is a complex nanoparticle that consists of RNA and proteins arranged in two subunits: the small subunit (30S in bacteria) and the large subunit (50S in bacteria). During the elongation phase of protein synthesis, the ribosome translates the codon sequence of the mRNA into the amino acid sequence of a protein, using aminoacyl-tRNAs as adaptors. Each time an mRNA codon has been read and the respective amino acid incorporated into the nascent peptide chain, the mRNA, together with two tRNAs, moves through the ribosome by one codon in a process called translocation. The movement entails the rapid and precisely coordinated movement from the pre-translocation (PRE) to the post-translocation (POST) state, i.e. peptidyl-tRNA moves from the A site to the P site and deacylated tRNA from the P site to the E site, from where it dissociates (Fig. 1A). The propensity for translocation is an inherent property of the ribosome as a molecular machine, which can perform spontaneous – albeit extremely slow – translocation in both forward and backward directions [1–4]. In the cell, translocation is promoted by the GTPase elongation factor G (EF-G) (Fig. 1B), accelerating the reaction by several orders of magnitude and favoring the POST state.

Prior to and during translocation, the tRNAs on the ribosome assume intermediate configurations, including hybrid A/P and P/E states [5], and the

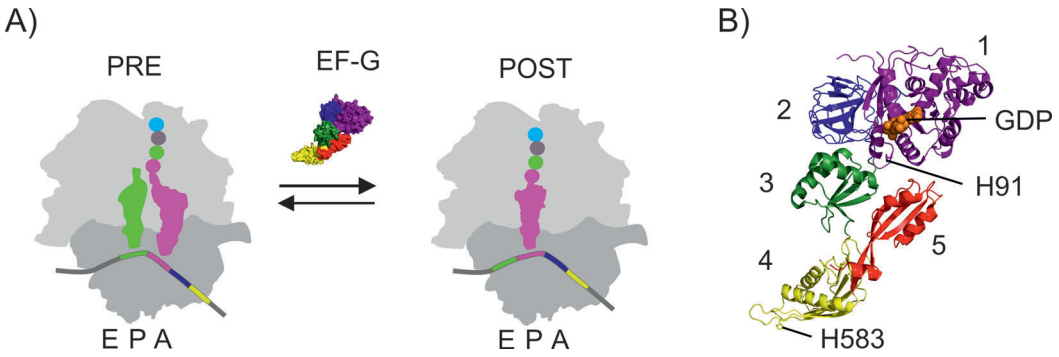

Figure 1. A: Overall scheme of translocation. In the pre-translocation state (PRE), deacylated tRNA (green) is bound in the P site (P) and peptidyl-tRNA (magenta, nascent chain indicated by colored circles) in the A site (A), both bound to their cognate codons (green or purple, respectively) in the mRNA. Following the binding of EF-G-GTP to the pre-translocation complex, translocation takes place. In the post-translocation state (POST), peptidyl-tRNA has moved to the P site, whereas deacylated tRNA has dissociated, as have inorganic phosphate (Pi) and EF-G-GDP. The 30S subunit is shaded in grey, the 50S subunit in light grey. GTP, GDP, and Pi are omitted. **B**: Structure of EF-G-GDP [90]. Domains 1–5 of EF-G are color-coded, GDP is shown in orange. Amino acids that are important for translocation (H583) or GTPase activity (H91) are indicated.

ribosome undergoes dynamic structural changes [6–9]. Despite the large body of information that has been gathered over four decades of research, key features of the molecular mechanism of translocation remain unclear. Among those are how the tRNAs and mRNA move through the ribosome, how EF-G promotes translocation, what the functional role of intermediate tRNA states is, and how GTP hydrolysis is coupled to forward movement.

In the present review, we will discuss some of these questions, taking into account the recent progress in ensemble kinetics, single-molecule, and structural studies of translocation. In particular, we will examine the functional role of intermediate states of translocation, as stabilized by antibiotics or the use of EF-G mutants. We will focus on the role of spontaneous and EF-G-induced conformational fluctuations of ribosomal complexes and how translocation on 50S and 30S ribosomal subunits is coupled to GTP hydrolysis. Based on recent kinetic results, we will argue against the frequently held assumption that the spontaneous movement of the tRNAs on the 50S ribosomal subunit into hybrid binding states already constitutes full translocation on the 50S subunit. We will show that, by contrast, 50S translocation is coupled to 30S translocation that is promoted by EF-G and GTP hydrolysis. We will also discuss conformational changes of EF-G taking place during translocation, and the unusual energy regimes of EF-G as a switch GTPase and/or motor protein.

Spontaneous motions of ribosome-tRNA complexes

The ribosome undergoes a variety of spontaneous thermal motions, which take place in the time range of milliseconds to seconds. Some movements are local, such as movements of the ribosome stalk comprising protein L1 (L1 stalk) [7, 10–13], or of the L11-binding region with the L12 stalk [14, 15]. Larger movements involve rotations of the 30S relative to the 50S subunits (previously termed "ratchet" [9]) and movements of the 30S head domain, called "swiveling" [16]. These motions are orchestrated by the movements of the tRNAs, and there are many different discrete positions that tRNAs can assume. In the ground state prior to translocation (PRE), the tRNAs – deacylated tRNA and peptidyl-tRNA – are bound in the P and A sites, respectively. Driven by thermal energy, the tRNAs can spontaneously move to so-called hybrid states [5]. In the hybrid states, the anticodon stem-loops of the tRNAs reside in the A and P sites of the 30S subunit, while the respective acceptor ends are oriented toward the P and E sites of the 50S subunit. Hybrid state formation correlates with the rotational movement of the ribosomal subunits relative to each other [17, 18].

In addition to the hybrid/rotated state, a number of other non-classical states have been identified. For example, hybrid states H1 and H2 [19] differ in the position of the two tRNAs (P/E and A/P vs. P/E and A/A, respectively). In states MSI and MSII [20] the position of protein L1 changes from an open arrangement characteristic for the classical state into a closed one where L1 interacts with the P/E tRNA. Further states R_0, R_1, R_2, and R_f differ in the orientation of the 30S subunit head and represent intermediate states of intersubunit rotation [21]. Structures determined by cryo-electron microscopy (cryo-EM) identified a number of states of the PRE complexes, e.g. classes 2, 4A, 4B, 5, and 6 [22], or PRE 1 to PRE 5 states [8] (Fig. 2), which differ in the orientation of the tRNAs, the degree of subunit rotation, the conformation of the 30S subunit, and the position of the L1 stalk of the 50S subunit. Also the POST state comprises a mixed population of complexes that differ in the position and orientation of the E-site tRNA and L1 [8, 13]. Computer simulations indicate that fluctuations of ribosomal elements are intrinsically rapid and proceed within microseconds, whereas translocation is limited by tRNA movements in the millisecond time range [13].

Hybrid states reflect essential dynamics of the ribosome

The transition of the ribosome from the classical into the hybrid/rotated PRE

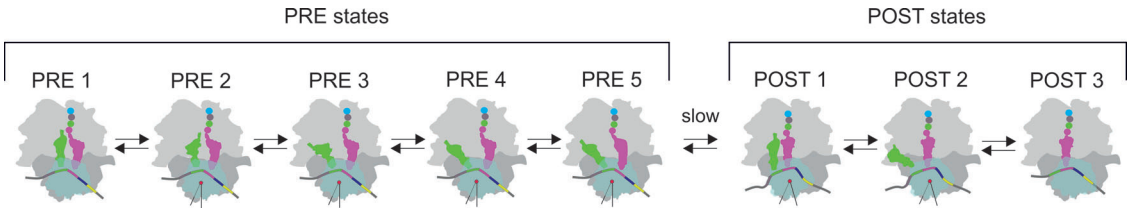

Figure 2. Spontaneous interconversion of PRE and POST states of the ribosome. States PRE 1–5 and POST 1–3 differ in tRNA positions, degree of subunit rotation, and extent of 30S head (30S area shaded blue) swiveling (indicated by the angle). The reversible transition between PRE 5 and POST 1 constitutes slow forward or reverse translocation. Structures of PRE and POST states were determined by cryo-EM [8].

state is essential for translocation, as blocking subunit rotation abolishes the reaction [23]. The importance of intermediate hybrid-state formation for translocation has been demonstrated for both the P/E state of the P-site tRNA [24–27] and the A/P state of the A-site peptidyl-tRNA [26, 28, 29]. While there is little doubt that formation of hybrid/rotated states is a prerequisite for translocation, their exact functional significance remains controversial, and there are several models, discussed below, that try to link hybrid state formation to translocation.

Model 1: Hybrid-state formation constitutes tRNA translocation on the 50S subunit

It is often assumed that the spontaneous formation of hybrid A/P and P/E states is a first, EF-G-independent, step of translocation that brings the 3' ends of peptidyl-tRNA and deacylated tRNA into their post-translocation positions on the 50S subunit. Consequently, the function of EF-G and GTP hydrolysis in such models is restricted to the acceleration of the coupled movement of the two tRNAs and the mRNA on the 30S subunit. However, as has been pointed out repeatedly, the peptidyl-tRNA in the A/P hybrid state is not ready to take part in a rapid peptidyl transferase reaction with puromycin [30–33], and thus cannot be positioned in the P site proper.

To monitor the dynamics of tRNA movements on both 30S and 50S subunits, we have introduced and validated a new dual-color labeling approach [32]. We used a Bodipy fluorophore attached to the N-terminal methionine of the dipeptidyl-tRNA to monitor 50S translocation in real time, and have related these kinetics to that of 30S translocation monitored with fluorescence-labeled mRNA [32] (Fig. 3A and B). These experiments showed that translocation promoted by EF-G-GTP proceeds at the same rates on 30S and 50S subunits. Furthermore, the rate of tRNA movement into the P site monitored by the change in Bodipy fluorescence coincided with the increase of puromycin reactivity, indicating that the label reported the movement into the authentic POST state. Thus, models assuming that the 50S POST state is reached by spontaneous hybrid state formation, prior to 30S translocation, are not supported by these data.

Model 2: EF-G binding is restricted to the hybrid/rotated state of the ribosome

This model was initially suggested on the basis of biochemical experiments [34] and a cryo-EM structure that captured EF-G bound to the hybrid/rotated ribosome, whereas the complex with the classical state of the ribosome could not be obtained [35]. More recently, similar conclusions were reached by two single-molecule studies, which suggested that (i) formation of the hybrid state (strictly speaking, closing of the L1 stalk) was correlated with the recruitment of EF-G [36] and (ii) translocation was initiated only on rotated ribosomes [37]. These conclusions were challenged by another single-molecule study, which observed translocation from either classical or hybrid state [38]. When EF-G was recruited to the classical state, the ribosome transiently visited the hybrid state before moving to the POST state. This report raised significant doubts concerning the preferential recruitment of EF-G to the hybrid state. Furthermore, pre-steady-state kinetic data suggest that EF-G can bind to the pre-translocation complex in either state, hybrid or classical, and that the state of the ribosome prior to EF-G binding has little effect on the rate of translocation [32]. This is in agreement with previous reports that revealed similar association rate constants and affinities of EF-G binding to ribosomes in different conformational states [27, 38–40]. Thus, on the balance of the arguments, exclusive recruitment of EF-G to the ribosome in the hybrid state is not supported by most of the available data.

Model 3: The transition from the classical to the hybrid/rotated state is rate-limiting for translocation

The most recent argument in favor of this model was put forward in a single-molecule study reporting that the rate at which the ribosome spontaneously achieves the hybrid state (based on the closure of the L1 stalk) is closely correlated with the rate at which EF-G engages in the A site [36]. In contrast, the ensemble kinetic analysis shows that the rate of 50S translocation is largely independent of whether the ribosome assumes the classical or hybrid/rotated state [32]. Similarly, mutations in 23S rRNA or tRNA that disfavor hybrid-state formation affected the rate of translocation only moderately [27, 41, 42]. The rate of GTP hydrolysis by EF-G was similar on ribosomes in hybrid and classical states, and about 10 times faster than the fluctuations between the hybrid and classical states [43, 44], suggesting that EF-G was recruited regardless of the state [27, 39, 40].

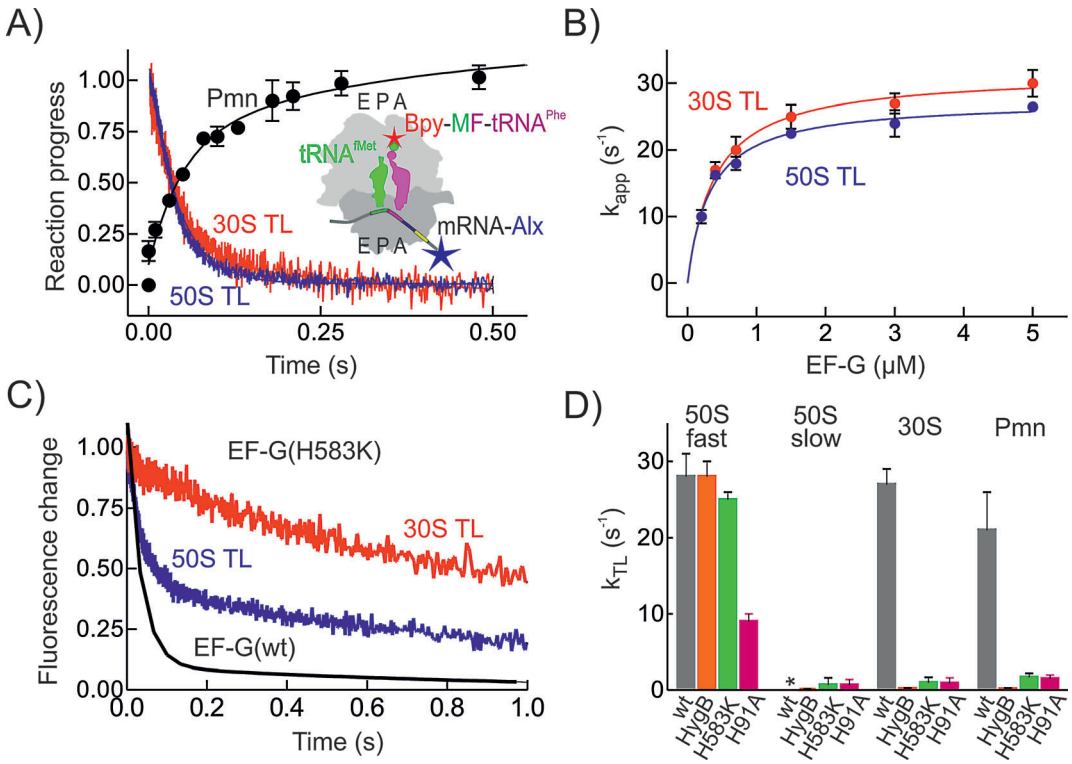

Figure 3. Rapid kinetics of EF-G-dependent translocation on 30S and 50S ribosomal subunits. **A**: Time courses of translocation on 30S (red) and 50S (blue) subunits measured by fluorescence stopped-flow, and time course of the Pmn reaction carried out by quench-flow. The rate of 50S translocation measured by Pmn was $20 \pm 6\,s^{-1}$. Fluorescent reporters were Alexa 405 (Alx) at the 3′ end of a short mRNA construct (30S translocation) or Bodipy FL (Bpy) at the N-terminal methionine of MetPhe(MF)-tRNA^{Phe} (inset). **B**: Dependence of 30S and 50S translocation on EF-G concentration. Apparent rate constants (k_{app}) saturated at the same rate of $28 \pm 3\,s^{-1}$ (37°C), indicating that 30S and 50S translocation were coupled. **C**: Inhibition of translocation by the H583 mutation in domain 4 of EF-G. 30S and 50S translocation was measured by fluorescence stopped-flow as in A. 50S translocation with wt EF.G is shown for comparison. **D**: Apparent rates of 50S and 30S translocation (k_{TL}) as determined by stopped-flow or rapid Pmn reaction. EF-G(wt), gray; EF-G(wt) + HygB, orange; EF-G(H583K), green; EF-G(H91A), magenta; *, slow 50S translocation not observed with EF-G(wt). Data taken from [32].

Furthermore, molecular dynamic simulations suggest that spontaneous subunit rotation and movements within the 30S subunit during the inter-conversion of classic and hybrid states are very rapid, taking place in the sub-microseconds time range [45]. Together, these results are inconsistent with the notion that the formation or stabilization of the hybrid/rotated state constituted the rate-limiting step of EF-G-dependent translocation.

We also note that the rates of EF-G binding to the ribosome observed by single-molecule methods are generally much lower than those measured in ensemble kinetics [39, 46–48]. This may indicate that the transitions observed in single-molecule studies [36, 37] represent later steps of EF-G interaction with the ribosome, which correlate with EF-G engagement and the rate-limiting unlocking of the 30S subunit [32, 48].

Model 4: Dynamic fluctuations as prerequisites for translocation

One possible explanation for the existence of the hybrid states is that loosely coupled dynamic fluctuations are manifestations of the ribosome as a thermally driven machine, and are prerequisites for any directional motion. However, these fluctuations alone are not sufficient to generate directed motion, and hence coupling of ribosome fluctuations to conformational changes of EF-G is required. One notable observation is that EF-G engagement halts the fluctuation between the classical and hybrid state [38], which may contribute to the forward commitment of translocation and favor the transition to the POST state. Thus, while the hybrid/rotated states are authentic intermediates of translocation reflecting the intrinsic dynamics of the ribosome, they probably indicate the range of dynamic conformations that are accessible by spontaneous fluctuations of the ribosome.

Intermediate states of EF-G–dependent translocation

Recruitment of EF-G to the ribosome and GTP hydrolysis remodel the energy landscape of the ribosome in such a way that the tRNAs can rapidly move into their POST states. With EF-G and GTP, the reaction is completed within milliseconds [39, 48, 49] (Fig. 3), which makes it difficult to isolate translocation intermediates. However, using a

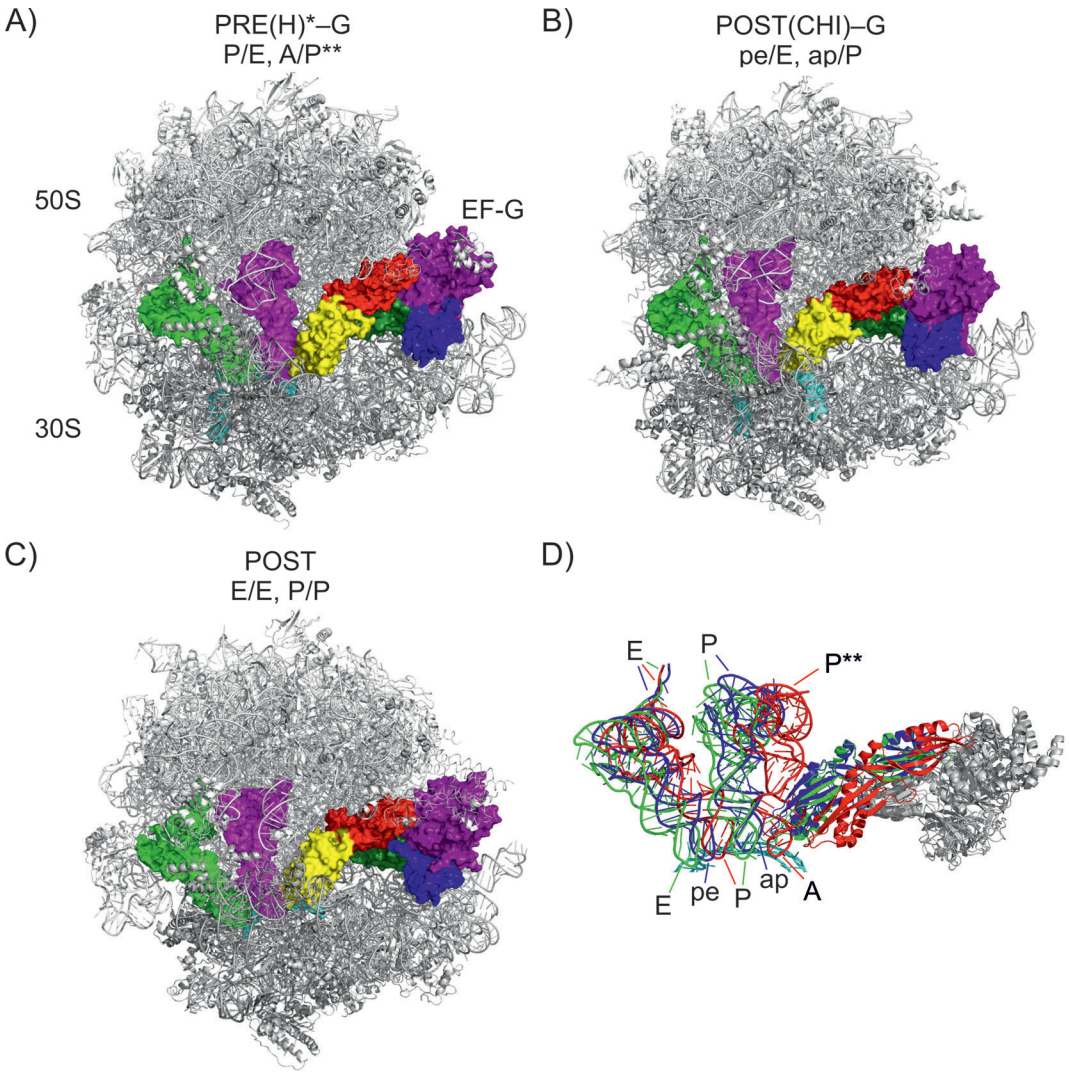

Figure 4. Arrangement of tRNAs and EF-G in PRE, early POST, and late POST states. **A**: Arrangement of EF-G and tRNAs in PRE. The complex was formed with GTP, stabilized by the addition of viomycin and fusidic acid, and analyzed by cryo-EM [56]. Peptidyl-tRNA is present in an intermediate A/P** state, deacylated tRNA in the P/E hybrid state. **B**: Arrangement of EF-G and tRNAs in chimeric ap/P and pe/E states in an early POST state stabilized by fusidic acid. The structure was determined by cryo-EM [51]. **C**: Crystal structure of a POST complex stabilized by fusidic acid [71]. The complexes are color-coded as in Fig. 1. **D**: Overlay of tRNAs and EF-G from the structures displayed in panels A–C, aligned on domain 1 of EF-G. The positions of tRNAs are indicated. Note the different positions of domain 4 of EF-G in PRE (red), early POST (blue) and in late POST (green).

toolbox of antibiotics, non-hydrolyzable GTP analogs and EF-G mutants allowed the dissection of the translocation pathway into a number of intermediates. Although the exact placement of each of these complexes on or off the main translocation pathway should be critically assessed in each case, the locations of tRNAs in those EF-G-induced states are notably distinct from their PRE (both classical and hybrid) and POST positions (Fig. 4). Several crystal and cryo-EM structures revealed translocation intermediates where a swiveling motion of the 30S head domain shifted the tRNA anticodon domain into a position between the P and E site on the 30S subunit, hence resulting in the pe/E state of the tRNA; similarly, the A-site tRNA attained a transient ap/P state and domain 4 of EF-G reached into the 30S A site close to the anticodon arm of the A-site tRNA [21, 50–53]. While the nomenclature for such states is complicated and likely to change with each further identified intermediate, we shall, in the following discourse, adopt the term "chimeric" (CHI) for those intermediates [21], but will distinguish them as PRE or POST depending on whether or not the peptidyl-tRNA can react rapidly with puromycin – a diagnostic tool for the authentic P-site position.

The first intermediate that forms upon EF-G binding is most probably not yet CHI. Binding of EF-G to the PRE complex favors the formation of hybrid/rotated states [36–38, 42–44, 54, 55], but may not per se lead to the formation of a CHI intermediate. Recently, a candidate for an early state was visualized by cryo-EM of the complex stalled by the antibiotic viomycin with two tRNAs bound in their hybrid position (as

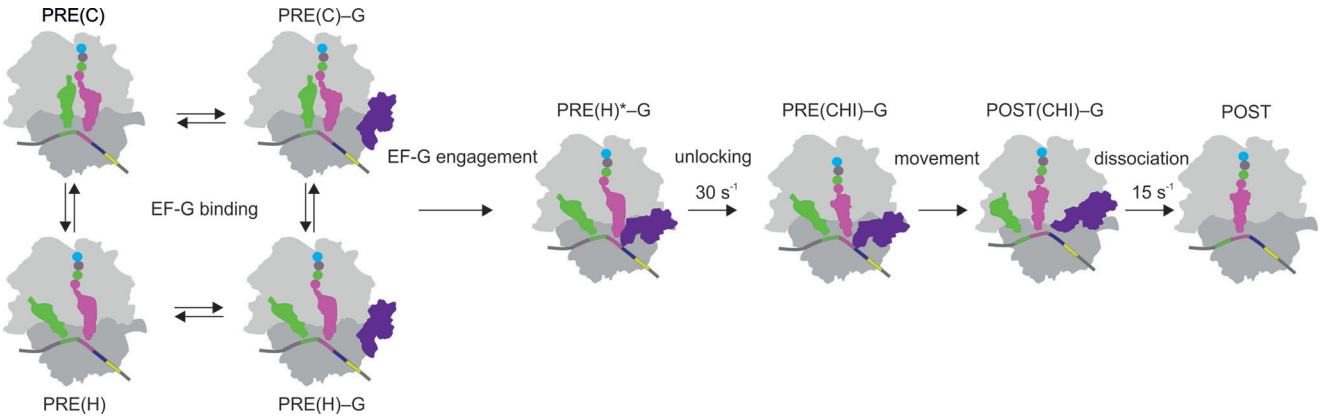

Figure 5. Intermediate states of EF-G-dependent translocation. Intermediates of 30S and 50S translocation were identified by cryo-EM, rapid kinetics, single-molecule experiments, and crystallography. For details see text.

in the PRE5 complex [8]) and EF-G occupying the intersubunit space between the L12 stalk and the A-site tRNA (Fig. 5A) [56]. It is not clear whether the viomycin-stalled complex represents an authentic intermediate of translocation, because viomycin alone has dramatic effects on the rotational status of the ribosome and on the mobility of tRNAs [57, 58].

Ensemble kinetics of unperturbed, EF-G-catalyzed translocation indicated that the stabilization of the hybrid intermediate by EF-G is manifested in subunit rotation [59, 60]. As suggested by ensemble kinetics, the rate of rotation is remarkably high: $>80\,s^{-1}$ (22°C) [60]. Compared to the reported rates of spontaneous fluctuations from the classical/non-rotated to hybrid/rotated states ($1–2\,s^{-1}$; [7, 19, 36, 43, 44]), this implies that EF-G binding greatly accelerates the classical-to-hybrid transition. This effect – to our knowledge – has not been noticed so far. The subsequent movement of the 30S subunit head ($80\,s^{-1}$; [60]) probably coincides with GTP hydrolysis (estimated between 30 and $100\,s^{-1}$; [47, 61]). Thus, the formation of an early EF-G-induced translocation intermediate PRE(H)–G is rapid compared to the spontaneous conformational fluctuations of the ribosome. One potential caveat that has to be considered here is the extent to which the rates obtained by the current single-molecule setups are comparable to those from ensemble kinetics. The advantage of the latter technique is the better time resolution and generally less stringent limitations concerning buffer conditions and concentrations of ligands accessible experimentally. One potential disadvantage is the averaging over a whole population of molecules, some of which may be inactive. This, however, would lead to an underestimation, rather than overestimation of rates – i.e. the apparent difference between ensemble and single-molecule kinetic data would be even larger. Alternatively, the rates obtained in single-molecule experiments represent lower-limit estimates due to limited time resolution. We also note that the addition of spermine, which is often used in single-molecule experiments, slows down translocation substantially (our unpublished data). These points should be considered in future experiments to obtain kinetic parameters that can be compared from the two techniques.

The events following the formation of the PRE(H)–G state are mostly known from pre-steady-state ensemble kinetics. GTP hydrolysis induces rapid (about $30\,s^{-1}$), concerted movements of the tRNAs on both subunits into a presumed early POST state [32, 48, 61] (Figs. 3 and 4). The latter complex undergoes further rearrangements, which entail further movement of deacylated tRNA through the E site, backward rotation of the subunit head, and dissociation of EF-G [60, 61], which occur at a rate of about $15\,s^{-1}$ [61] (for comparison of kinetic parameters obtained at different conditions, see [47, 60, 62]). The rates of tRNA and mRNA movement into the early POST state coincide with the rate of Pi release [48]. Although the intrinsic rates of the three reactions may be different, they are synchronized (to $30\,s^{-1}$) by the preceding rate-limiting step of 30S unlocking [48]. In comparison, the movement of tRNAs in the unlocked state of the ribosome appears intrinsically rapid [36, 48, 63]. In the final step, deacylated tRNA dissociates from the ribosome [37, 64–66] – probably coupled to the backward swiveling of the 30S head, counter-rotation of the 30S body, and dissociation of EF-G [37, 59–61]. This results in the final POST state.

Another translocation intermediate was obtained by crystallizing the ribosomes with only one tRNA bound to the P site and with EF-G stabilized by non-hydrolyzable GTP analogs [50, 53, 67]. These structures probably represent the pre-hydrolysis state. Nevertheless, the positions of the tRNAs on the 30S subunit are already changed toward the pe state, i.e. the complex can be classified as PRE(CHI). Alternatively, as translocation can proceed slowly in the absence of GTP hydrolysis [39] the state isolated with the non-hydrolyzable GTP analog may be a POST(CHI) state. One further PRE(CHI) intermediate was identified by kinetic studies, when translocation was slowed down either by adding the antibiotic hygromycin B or by introducing mutations in EF-G (EF-G(H91A) and EF-G(H583K)), and monitoring translocation on both 50S and 30S subunits [32, 61] (Fig. 3C and D). The CCA end of the A-site peptidyl-tRNA in those complexes moves in at least two steps (PRE(H) → PRE(CHI) → POST) [32] (Fig. 4). During the first step, which can take place independently of 30S translocation, the CCA end of peptidyl-tRNA moves to an ap state on the 50S subunit; in that state, the reaction with

puromycin remains very slow, suggesting that the state differs from the final POST state and from the CHI state stalled in the presence of fusidic acid (see below). EF-G–GTP binding – independent of GTP hydrolysis – is sufficient to promote the movement into the ap state on the 50S subunit, probably by affecting the landscape of spontaneous thermal motions within the ribosome. It is likely that the P-site tRNA concomitantly moves to a state that has been designated INT [32, 47], although this movement may be only partial in the absence of GTP hydrolysis [47]. The energy of EF-G–GTP binding alone is not sufficient to promote rapid movement on the 30S subunit. The second step, which entails both 30S translocation and tRNA movement on the 50S subunit from an intermediate into the POST state, is driven by GTP hydrolysis, which couples conformational rearrangements of EF-G to the engagement of domain 4 with the codon–anticodon complex in the 30S A site [32]. Although binding of hygromycin B to the ribosome or introducing the H91A and H583K mutations in EF-G inhibit tRNA movement [61, 68, 69], the state is likely to be on the main translocation pathway, because the completion of translocation is only slowed down (by 30-fold with EF-G(H91A) and (H583K) or 400-fold with hygromycin B), rather than blocked. The structure of this intermediate is currently not known.

Another CHI intermediate, with two tRNAs and EF-G bound, was visualized by cryo-EM of the fusidic acid-stabilized complex (Fig. 5B). Fusidic acid freezes a POST state of the complex [51, 70]. It is believed that fusidic acid binds to EF-G in a specific conformation that forms transiently late in translocation, after the tRNAs have moved [49, 70]. However, while the CCA ends of tRNAs in that complex were observed in their E and P positions on the 50S subunit, the tRNA anticodons were bound in states intermediate between the E, P, and A sites, referred to as pe/E, ap/P [51] (Fig. 4). That structure can be interpreted as a late POST(CHI) intermediate. Finally, the crystal structure of the fusidic acid-stabilized POST state shows the tRNAs in their post-translocation positions, E/E and P/P [71] (Fig. 5C). It remains puzzling that the structures determined by cryo-EM and crystallography captured different states of the complex, although both complexes were stabilized by fusidic acid. This may be explained by slight differences in the conditions of complex formation or by different sensitivity of *Escherichia coli* versus *Thermus thermophilus* ribosomes to EF-G stalling by fusidic acid.

Box 1

GTP/GDP binding and conformation of EF-G

The nucleotide binding properties of EF-G are somewhat unusual compared to e.g. small Ras-like GTPases or another translational GTPase, EF-Tu. EF-G binds GTP and GDP with similar affinities [88, 89]. The rate of GDP dissociation from free EF-G is extremely high, $300\,s^{-1}$ [89]. Given the similar affinities to GTP and GDP, the high excess of GTP over GDP in the cell, and rapid GDP dissociation, EF-G is likely to be predominantly present in the GTP-bound form in vivo. Rapid GDP dissociation explains why – in contrast to many other GTPases – EF-G does not need a nucleotide exchange factor. The structures of free EF-G in the apo-, GDP-, and GDPNP-bound forms appear to be quite similar [90–93]. However, this apparent similarity may be misleading, and result either from using a mutant of EF-G for crystallization or from using GDPNP as a GTP analog [90]. With GDPNP, the interaction between the P-loop of the factor and the β–γ bridging oxygen of GTP, as found in other GTPases, cannot form. As a consequence, conformational rearrangements that accompany GTP binding may be impaired (similarly to what was recently suggested for another translational GTPase, eIF5B [94]). In fact, isothermal titration calorimetry suggested that GTP binding to EF-G induces a significant conformational change of the factor, and this rearrangement is different from that in the presence of GDP [88].

Conformational changes of EF-G on the ribosome

While structural differences between the GTP- and GDP-bound forms of free EF-G are uncertain (Box 1), on the ribosome EF-G appears to undergo substantial conformational changes-driven by GTP hydrolysis and Pi release – that are important for driving the functional cycle of the factor. The initial recruitment of EF-G – as of several translation factors – is likely to involve the L12 stalk [14, 72], and, in fact, in several structures the C-terminal domain of L12 is found attached to the GTP-binding domain of EF-G [53, 56, 71]. However, the affinity of EF-G for isolated L12 is rather low [72], and the major stabilization of the factor on the ribosome is provided by interactions with the sarcin-ricin loop (SRL) on the 50S subunit. Crystal structures show how interactions with the SRL may lead to the GTPase activation of EF-G [50, 53]. Key residues in EF-G and EF-Tu form a nearly identical catalytic site, suggesting the conservation of a basic GTPase activation mechanism [50, 53, 73]. A histidine residue in the switch II region, His91 in *E. coli* EF-G, plays an essential role in the reaction [61], similarly to the homologous residue His84 in EF-Tu [74]. In the absence of the ribosome, the catalytic His is oriented away from the crucial water molecule, which attacks the γ-phosphate during hydrolysis. The SRL may stabilize the activated conformation of the factor with His91 rotated toward and positioning the hydrolytic water [73]. However, beyond this point the analogy vanishes: the GTPase activity of EF-Tu is activated when there is correct codon–anticodon interaction and is stringently controlled [75], whereas EF-G hydrolyzes GTP rapidly upon binding to the ribosome, independent of its functional state [39, 40, 47, 76]. Also the chemical mechanism of catalysis is under debate [77] and has not been thoroughly studied with EF-G.

The most urgent question concerning the conformational rearrangements of EF-G is how EF-G binding, GTP hydrolysis, and Pi release alter the structure of EF-G and promote tRNA

movement through the ribosome. In all GTPases, GTP binding and hydrolysis is sensed by the switch I and II regions [78]. In free EF-G, these crucial sensors are disordered and become ordered in the complex with a non-hydrolyzable GTP analog, GDPCP, on the ribosome [50, 53]. This causes a reorientation of EF-G such that the tip of domain IV moves and the CHI state of the ribosome is stabilized. Thus, these structures provide the first insight as to how EF-G and the ribosome might interact [50, 53]. Further rearrangements of EF-G occur upon movement into the POST state, during which the tip of domain IV moves by another ~6.6 Å into the A site [53, 67, 71] (Fig. 5D). This further movement is a result of the rotation of the superdomain 1–2 relative to domains 3–5 that presumably occurs upon GTP hydrolysis [50]. After GTP hydrolysis and translocation, switch I becomes disordered [71], which accelerates the release of EF-G/GDP from the post-translocation ribosome. Thus, the GTPase cycle controls the position and orientation of domain 4 of EF-G through the rearrangements of the switch regions that are communicated through the domains 2/3 and 1/5 interfaces. The conformational changes of EF-G are essential for function, because blocking the relative domain rearrangement, e.g. by cross-linking domains 1 and 5, abrogates translocation [33].

The role of EF-G and GTP hydrolysis

EF-G is an unconventional GTPase, because it combines the characteristics of switch GTPases and motor proteins. As is the case with most GTP-binding proteins, EF-G is expected to change its conformation in response to GTP binding, GTP cleavage, and Pi release, which in turn regulates its progression through translocation. In fact, one of the most compelling manifestations of the GTPase-like function of EF-G is the importance of GTP hydrolysis for EF-G dissociation, which is strongly inhibited when GTP hydrolysis is abolished [61, 79]; this was, for a long time, thought to be the only function of GTP hydrolysis. On the other hand, EF-G acts as a motor protein by rectifying energy into directed movement. At some point, it is difficult to distinguish between the two energy regimes, because GTP hydrolysis is coupled to the movement not only energetically, but also conformationally, which may be an action assigned to either a conformationally driven GTPase or an entropic motor.

EF-G binding, without GTP hydrolysis, promotes slow and possibly incomplete translocation. When GTP hydrolysis is suppressed, e.g. in the GTPase-defective EF-G mutant H91A, the tRNAs are transiently trapped in a PRE(CHI) position with the CCA end of the A-site peptidyl-tRNA in the ap state on the 50S subunit [32]. The dissociation of the E-tRNA is slow and the fluorescence change of the mRNA reporter is reduced, suggesting partial mRNA movement [61]. Although the intermediate formed with EF-G(H91A) is transient, it may be similar to the state PRE(CHI) (pe/E) stalled by GDPCP or GDPNP [50, 53, 67], although there remains an ambiguity related to the lack of the A-tRNA in the structures and to the different lifetime of the stalled complexes. In some conditions, the E-site tRNA remains stably bound to the ribosome in the presence of EF-G-GDPNP [54, 80]. Regardless of the exact nature of the intermediate, it forms because EF-G can rectify the intrinsic Brownian motions of the ribosome into directed movement.

GTP hydrolysis accelerates translocation 20–50-fold [36, 39, 47, 61] and synchronizes tRNA and mRNA movements on the two ribosomal subunits [32]. This suggests that GTP hydrolysis accelerates the transition from PRE(CHI) to POST. The ribosome together with EF-G and GTP can generate a significant mechanical force during translocation [81]. At almost 90 pN, this force is large in magnitude but small in working distance, which is estimated at 0.5 nm – much less than the full travelling length of one codon, which is 2 nm [81]. This may be rationalized by assuming that a significant distance from PRE(H) to PRE(CHI) state is already covered by the preceding Brownian motion, whereas generation of the GTP-powered directional force – i.e. a power stroke – is required to move the tRNA anticodons and the mRNA on the 30S subunit.

As described above, binding of EF-G to the ribosome, and GTP hydrolysis, promote conformational changes of EF-G, placing its domain 4 in the proximity of the codon–anticodon complex in the A site. The rate of 30S translocation closely correlates with the ability of domain 4 of EF-G to engage with the A site [36], whereas the rate of EF-G engagement is controlled by conformational changes of EF-G [33, 82, 83]. There are several models as to what exactly EF-G does to promote the motion on the 30S subunit. It can (i) actively push the A-site tRNA; (ii) change the conformation of the ribosome, or (iii) act as a "door-stop" by following the spontaneous movement of the tRNA out of the A site with its domain 4 and restricting the backward movement of the tRNA. Model (i) is attractive, as it would be consistent with force generation during translocation [81] and with cryo-EM studies suggesting that EF-G may disrupt the interaction of the mRNA–tRNA duplex with a universally conserved base (A1492) in the ribosomal decoding center [51, 83]. It is also corroborated by the results of mutational analyses suggesting that point mutations at key positions in the tip of domain 4 impair the translocation step PRE(CHI) to POST [32]. Moreover, the translocation rate was increased by cleavage of 16S rRNA between nucleotides A1493 and G1494 [84] or by disrupting the interactions between the A-site codon and ribosomal residues [85]. On the other hand, there is also strong evidence in favor of model (ii): EF-G domain 4 engagement may promote 30S translocation by altering the conformation of the subunit, e.g. by opening the mRNA-binding cleft, by stabilizing the open conformation of the E-site gate, or displacing ribosome elements that act as hurdles for 30S translocation [16, 21, 50, 52, 53, 68]. EF-G may also facilitate 30S head domain movements, consistent with recent findings suggesting that the movement of the 30S head coincides with mRNA movement [52, 60], or by altering the inter-subunit bridges B1a, B4, B7a, and B8 [86]. On the other hand, the position of the domain 4 during and after translocation and the conformational restrictions on the backward movement of domain 4 argue in favor of the "door-stop" model (iii) [9, 50, 53, 67, 71]. In principle, all three models may be non-exclusive. An additional possibility is that GTP hydrolysis –

coupled through delayed Pi release [48] – is required for the backward rotation of the 30S subunit head, which would complete the transition [60, 87]. Thus, the translocating ribosome–EF-G complex combines features of a Brownian machine and a power-stroke motor with that of the switch GTPase. EF-G-GTP orchestrates the rapid synchronous progression of tRNAs and mRNA through the ribosome by combining these different energy regimes.

Conclusions and outlook

Recent work on translocation provides two important lessons. First, the ribosome is a dynamic machine that spontaneously fluctuates between different loosely coupled conformations. EF-G – even when GTP hydrolysis is prevented – rectifies reversible Brownian fluctuations into directed motion through intermediate states of translocation (CHI). Second, EF-G-dependent movements of the mRNA–tRNA module on both ribosomal subunits take place in a synchronous manner, 50S translocation being coupled to 30S translocation. Domain movements of EF-G, which are driven by GTP hydrolysis, play a crucial role in accelerating translocation, possibly by disrupting interactions of the tRNAs with the ribosome, changing the conformation and rotational orientation of the 30S subunit, or restricting backward movement of the tRNAs. Conformational changes of EF-G upon binding to the ribosome, GTP hydrolysis, and Pi release may be considered as typical for a switch GTPase. However, the energy provided by GTP hydrolysis is used to promote forward movement of the tRNAs by a combination of Brownian ratchet and power stroke mechanism. Thus, EF-G combines different energy regimes to change the energy landscape for translocation and promote forward movement.

A number of challenging questions remains. The trajectory of tRNAs through the ribosome during EF-G-promoted translocation should be visualized, and the details of coupled conformational changes revealed. The functional role of structural elements of the ribosome, particularly the L1 and L12 stalks, should be unraveled. We have to understand how the ribosome, with the help of EF-G, and presumably powered by GTP hydrolysis, moves through secondary structure elements of the mRNA, including hairpins and tightly packed structures as pseudoknots. Finally, one of the most challenging questions is how the ribosome maintains the reading frame during translocation. The ribosome is a case study for nanomachines in general, and solving these questions will deepen our understanding of the fundamentals of molecular motion in biological systems.

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