Fusidic Acid Targets Elongation Factor G in Several Stages of Translocation on the Bacterial Ribosome*

Anneli Borg1,5, Mikael Holm2, Ikue Shiroyama3, Vasili Hauryliuk1, Michael Pavlov1, Suparna Sanyal1, and Måns Ehrenberg4,2

From the 1Department of Cell and Molecular Biology, Biomedical Center, Uppsala University, Box 596, 751 24 Uppsala, Sweden and 2H Biomedical AB, Dag Hammarskjölds Väg 34A, Uppsala Science Park, 751 83 Uppsala, Sweden

Background: FA inhibits ribosomal elongation and recycling.

Results: The mechanism of FA inhibition of peptide elongation was determined.

Conclusion: FA acts as a strong inhibitor by targeting several EF-G-containing states of the peptide elongation cycle.

Significance: This study places FA-stalled ribosomal structures in a functional context and enables in vivo modeling of FA action. Techniques for studying slow inhibitors were developed.

The antibiotic fusidic acid (FA) targets elongation factor G (EF-G) and inhibits ribosomal peptide elongation and ribosome recycling, but deeper mechanistic aspects of FA action have remained unknown. Using quench flow and stopped flow experiments in a biochemical system for protein synthesis and taking advantage of separate time scales for inhibited (10 s) and uninhibited (100 ms) elongation cycles, a detailed kinetic model of FA action was obtained. FA targets EF-G at an early stage in the translocation process (I), which proceeds unhindered by the presence of the drug to a later stage (II), where the ribosome stalls. Stalling may also occur at a third stage of translocation (III), just before release of EF-G from the post-translocation ribosome. We show that FA is a strong elongation inhibitor ($K_{50\%} \approx 1$ μM), discuss the identity of the FA targeted states, and place existing cryo-EM and crystal structures in their functional context.

The bacterial ribosome is targeted by a large set of antibiotics (1) and is attractive as a potential target for novel drugs in the race against the ever emerging antibiotic resistance among bacterial pathogens (2). Some translation-targeting drugs lock the ribosome in long lived states via binding to translation factors. Among these is the steroid-like antibiotic fusidic acid (FA),3 which stalls the ribosome in complex with EF-G and GDP (3). FA has low affinity to EF-G off the ribosome but forms a strong complex with ribosome-bound EF-G (3, 4). EF-G plays double roles in bacterial protein synthesis by both catalyzing mRNA translocation during the peptide elongation cycle and, aided by ribosome recycling factor, splitting the post-termination ribosome into subunits (5) for a new round of initiation of mRNA translation (6, 7). In line with this, FA inhibits both ribosomal peptide elongation and ribosome recycling (8). Here, we focus on FA inhibition of the mRNA translocation process.

Fundamental discoveries of the action of EF-G in translocation of mRNA have benefitted from studies of the inhibitory action of FA (9–11). In experiments with naked ribosomes, it was shown that FA allows for a first round of GTP hydrolysis on ribosome-bound EF-G (12) and then traps the ribosome in a complex with GDP-bound EF-G from which FA dissociates slowly (3). The $K_{50\%}$ value for FA inhibition of multiple rounds of GTP hydrolysis as catalyzed by the naked ribosome was estimated as 1 μM (4, 13). In line with the early observation that FA does not inhibit the first round of GTP hydrolysis (12), it was demonstrated that FA-bound EF-G promotes movement of A site-bound peptidyl-tRNA into the P site (10). Savelbergh et al. (8) used a multicycle assay in which EF-G at rate-limiting concentration promoted ribosomal translocation. Here, the $K_{50\%}$ value for FA inhibition of the EF-G cycle was estimated as 200 μM, a value much higher than the $K_{50\%}$ value for FA action on ribosomal recycling (0.1 μM (8)), leading to the suggestion that the dominant cause of bacterial growth inhibition by FA is impairment of ribosomal recycling rather than inhibition of peptide elongation (8).

Early cryo-EM visualizations show FA-stalled ribosomes in both classical conformation with unrotated subunits and in ratcheted conformation with rotated subunits (14). All ribosomes were assembled with EF-G, GDP, and FA. Those with peptidyl-tRNA in the P site displayed classical conformation, whereas those with deacylated tRNA in the P site displayed ratcheted conformation. A later crystal structure of the ribosome in complex with EF-G, GDP, FA, and deacylated tRNA$^{1\text{Met}}$ displays the ribosome in classical conformation with unrotated subunits and tRNA$^{1\text{Met}}$ in both the P/P and the E/E site (15). Ramrath et al. (16) reported a cryo-EM structure of the bacterial ribosome in a partially rotated translocation state formed by EF-G-driven translocation in the presence of FA.

In the present work, rapid kinetics techniques (quench flow and stopped flow) were applied to an optimized system for in vivo modeling of FA action. Techniques for studying slow inhibitors were developed.

*This work was supported by the Swedish Research Council (to M. E., A. B., S. S., and V. H.); the Knut and Alice Wallenberg Foundation, RiboCORE (to M. E. and S. S.); the European Regional Development Fund through Centre of Excellence in Chemical Biology, Estonian Science Foundation, Grants ETF9012 and PUT37 (to V. H.); and Umeå University (to V. H.).

1 Present address: Dept. of Molecular Biology, Umeå University, 901 87 Umeå, Sweden.
2 To whom correspondence should be addressed: Dept. of Cell and Molecular Biology, Biomedical Center, Uppsala University, Box 596, 751 24 Uppsala, Sweden. Tel.: 46-18-471-4213; E-mail ehrenberg@xray.bmc.uu.se.
3 The abbreviations used are: FA, fusidic acid; EF, elongation factor; PEP, phosphoenolpyruvate; PK, pyruvate kinase; MK, myokinase; IF, initiation factor; LeuRS and PheRS, leucyl- and phenylalanyl-tRNA synthetase, respectively; GDPNP, guanosine 5’-[(β,γ)-imido]triphosphate.
vitro protein synthesis with *Escherichia coli* components of high purity (17) and *in vivo*-like function (18, 19). We studied the inhibitory action of FA in the authentic elongation cycle of the bacterial ribosome. FA is a “slow” inhibitor (20) of protein synthesis, which allowed for the design of a detailed model for FA action involving, in particular, several distinct modes of ribosome targeting by the drug and precise estimates of the major kinetic parameters relevant to FA inhibition of bacterial growth. We interpret previous biochemical data and structural information from cryo-EM and x-ray crystallographic techniques in the novel conceptual framework provided by the rich mechanistic insights of the present work.

**MATERIALS AND METHODS**

**Experimental Design**

**Reagents and Buffer Conditions—**Ribosomes (*E. coli* MRE600) were prepared according to Ref. 19. fMet-tRNA<sup>fmot</sup> was prepared according to Ref. 21, with minor modifications. Initiation factors, elongation factors, and aminoacyl-tRNA synthetases were overexpressed in His-tagged form and purified by nickel affinity chromatography. All concentrations for translation factors in the reaction mixtures were based on the Bradford assay. Purified tRNA<sup>Phe</sup> was from Chemical Block (Moscow, Russia). Bulk tRNA was prepared as described previously (22). [3H]Met and [3H]GDP were from Biotrend (Germany). ATP and GTP were from GE Healthcare. FA sodium salt, phosphoenolpyruvate (PEP), pyruvate kinase (PK), myokinase (MK), GDP, and unlabeled amino acids were from Sigma-Aldrich. All other chemicals were from Merck or Sigma-Aldrich. All experiments were performed at 37 °C in polyvinyl buffer containing 95 mM KCl, 5 mM NH<sub>4</sub>Cl, 0.5 mM CaCl<sub>2</sub>, 8 mM putrescine, 1 mM spermidine, 5 mM potassium phosphate, 1 mM dithioerythritol, and 5 mM Mg(OAc)<sub>2</sub>.

mRNA templates, encoding fMet-Leu-Phe (MLF), were prepared by transcription from double-stranded DNA primers with overlapping sequences by PCR essentially as described (21). Preparation of the transcription reaction mixture and purification of the mRNA on a poly(d1) column were performed as described previously (18) with minor modifications. The forward primer sequence was GGTACCAGAAATTAATACGCTCAGTATA-GGAAATTGGGCTTTGTAAACATTAAGGAG (5’ to 3’), and the reverse primer sequence was TTTTTTTTTTTTTTCTGAATTTAAACAGCATTAACTCTTTAATTTTGTTAAACAGGCCC (5’ to 3’, overlap underlined). The pyrene-labeled mRNA was from IBA GmbH and had the sequence AACAAUUAAGGGAUAAAUUGCUUGUUUUA (5’ to 3’).

**Assembly of 70S Initiation Complexes—**To prepare ribosomal (70S) initiation complex with MLF mRNA, a reaction mixture containing GTP (1 mM), ATP (1 mM), PEP (10 mM), PK (50 μg/ml), MK (2 μg/ml), IF1 (8 μM), IF2 (4 μM), IF3 (8 μM), 70S ribosomes (4 μM), [3H]Met-tRNA<sup>fmot</sup> (5 μM), and MLF mRNA (16 μM) was prepared. The mixture was incubated for 15 min at 37 °C and then chilled on ice. 500 μl of the mixture was applied to 400 μl of 1.1 M sucrose cushion in polyvinyl buffer, and the initiation complexes were collected by centrifugation at 259,000 × g for 2 h at 4 °C in a 55S rotor (Sorvall, RC M150 GX). The supernatant was removed, and the pellet was washed with and dissolved in polyvinyl buffer. The initiation complexes were aliquoted, shock-frozen in liquid nitrogen, and stored at −80 °C.

**Inhibition of Dipeptide Formation by FA and EF-G—**To study FA binding to the 70S:EF-G complex in the presence of GDP, purified initiation complexes were used because the ribosomes could not be initiated properly in the absence of GTP. The initiation complex mixture contained GDP (100 μM), ATP (1.9 mM), PEP (10 mM), MLF initiation complexes (1 μM), EF-G (20 μM), and varying concentrations of FA (0–2 mM, as indicated). The elongation mixture contained GTP (1 mM), ATP (1 mM), PEP (10 mM), PK (50 μg/ml), MK (2 μg/ml), EF-Tu (20 μM, whereof ~40% was active in dipeptide formation), EF-Ts (2 μM), tBulk (110 μM) total tRNA, whereof 4 μM was tRNA<sub>Leu</sub><sup>LACG</sup> or tRNA<sub>Leu</sub><sup>LAGG</sup>, leucine (200 μM), and LeuRS (1.1 μM). The elongation mixture was incubated for 15 min at 37 °C and was then kept on ice. Equal amounts of the initiation complex mixture and the elongation mixture were rapidly mixed in a quench flow apparatus (RQF-3, KinTeK Corp.) and the reaction was quenched after different times by rapid mixing with 50% formic acid. All samples were centrifuged for 15 min at 20,800 × g to pellet the precipitates, and the supernatants were discarded. Each pellet was dissolved in 165 μl of 0.5 M KOH by vortexing and incubation at room temperature for 10 min. 13 μl of 100% formic acid was added, and precipitates were pelleted by centrifugation for 15 min at 20,800 × g. The supernatants were transferred into new tubes and the centrifugation was repeated. The peptides formed were detected by reversed phase HPLC separation with online scintillation counting (β-RAM model 3, IN/US Systems) on a C18 column (Merck) with isocratic elution in 42% MeOH, 58% H<sub>2</sub>O, 0.1% TFA. Not only was the fMet-Leu-dipeptide formed, but fMet-Leu-Leu tripeptide was also formed due to misreading of the Phe codon by tRNA<sub>CAG</sub> and tRNA<sub>LACG</sub>. In order to follow the time evolution of the dipeptide formation, the dipeptide and the tripeptide peak fractions were added.

To study FA binding to the 70S:EF-G complex in the presence of GTP, the 70S initiation complexes were prepared *in situ*. The initiation complex mixture contained GTP (100 μM), ATP (1.9 mM), PEP (10 mM), PK (50 μg/ml), MK (2 μg/ml), IF1 (2 μM), IF2 (1 μM), IF3 (2 μM), ribosomes (1 μM), [3H]Met-tRNA<sup>fmot</sup>(1.5 μM), MLF mRNA (4 μM), EF-G (20 μM), and FA at varying concentration (0–50 μM). Before it was loaded to the quench flow instrument, the initiation complex mixture was incubated for 15 min at 37 °C and then kept on ice.

To study competitive inhibition of peptide bond formation by EF-G and FA, these factors were present in the elongation mixture, EF-G at 40 μM and FA at 0 or 4 mM concentration. The tBulk concentration was in one case 110 μM, giving 4 μM tRNA<sub>CAG</sub> reading CUG, and in another case 55 μM.

**FA Inhibition of Translocation by EF-G in Single Round Mode—**To estimate the rate of translocation from tripeptide formation, initiation complexes were prepared *in situ*. The initiation complex mixture contained GTP (1 mM), ATP (1 mM), PEP (10 mM), PK (50 μg/ml), MK (2 μg/ml), IF1 (2 μM), IF2 (1 μM), IF3 (2 μM), ribosomes (1 μM), [3H]Met-tRNA<sup>fmot</sup>(1.5 μM), and MLF mRNA (4 μM). The elongation mixture contained GTP (1 mM), ATP (1 mM), PEP (10 mM), PK (50 μg/ml), MK (2 μg/ml), EF-Tu (30 μM), EF-Ts (2 μM), EF-G (40 μM), tBulk (110 μM) total tRNA, whereof 4 μM was tRNA<sub>Leu</sub><sup>LACG</sup> or
Fusidic Acid Inhibits Multiple Ribosomal States

FIGURE 1. General scheme for peptide elongation cycle "i" during ribosomal synthesis of a protein and its inhibition by FA. In the special case that i = 1, Fig. 1 describes the first elongation cycle in the synthesis of a protein, as in Fig. 4. The scheme contains parameters obtainable from the present experiments, also relevant for FA inhibition of protein synthesis in the living cell. Subscheme A shows the inhibition of peptide bond formation by EF-G and FA.

In the special case that i = 1, Fig. 1 describes the first elongation cycle in the synthesis of a protein, as in Fig. 4. The scheme contains parameters obtainable from the present experiments, also relevant for FA inhibition of protein synthesis in the living cell. Subscheme A shows the inhibition of peptide bond formation by EF-G and FA.

tRNA_{Leu} and 1.6 μM was tRNA_{Phe}, purified tRNA_{Phe} (2.4 μM), leucine (200 μM), phenylalanine (200 μM), LeuRS (1.1 μM), PheRS (0.5 μM), and FA at varying concentration (0–4 mM). The initiation mixture and the elongation mixture were incubated for 15 min at 37 °C and then kept on ice until loaded onto the quench flow instrument. The samples from the quench flow were treated as described for the dipeptide formation experiments, and the peptides were analyzed by reversed phase HPLC. The same buffer conditions as for the dipeptide samples were used (42% MeOH, 58% H2O, 0.1% TFA).

Binding of EF-G and FA to the Post-translocation Ribosome with Dipeptidyl-tRNA in the P Site—A post-translocation complex with dipeptidyl-tRNA in the P site was prepared in a mixture containing GTP (1 mM), ATP (1 mM), PEP (10 mM), PK (50 μg/ml), IF1 (2 μM), IF2 (4 μM), IF3 (8 μM), ribosomes (1 μM), 3H[M]Met-tRNA_{Met} (1.5 μM), MLF mRNA (4 μM), tBulk (27.5 μM), whereof 1 μM was tRNA_{Leu} or tRNA_{Phe}, purified tRNA_{Phe} (2.4 μM), LeuRS (1.1 μM), PheRS (0.5 μM), EF-Tu (37 μM), EF-G (20 μM), and leucine (100 μM). This mixture was incubated for 10 min at 37 °C before the addition of FA (0–2 mM) and for another 10 min after the addition. An elongation mixture was prepared containing GTP (1 mM), ATP (1 mM), PEP (10 mM), PK (50 μg/ml), if 3H[M]Met-tRNA_{Met} (1.5 μM), MLF mRNA (4 μM), tBulk (27.5 μM), whereof 1 μM was tRNA_{Leu} or tRNA_{Phe}, purified tRNA_{Phe} (3 μM), leucine (200 μM), phenylalanine (200 μM), LeuRS (1.1 μM), PheRS (0.5 μM), and FA (0 or 40 μM). The mixtures were incubated for 15 min at 37 °C and then kept on ice until loaded onto the quench flow instrument. The samples were treated and analyzed as described above for the tripeptide samples.

Mean Time Calculations

Inhibition of Dipeptide Formation by EF-G and FA—As described in the legend to Fig. 1, the 70S post-translocation complex R’ with peptidyl-tRNA in the P site and an empty A site binds ternary complex T3 with Michaelis-Menten rate constant k_{T3} (k_{cat}/K_m) and incorporates an amino acid in complex R_{pep}, with Michaelis-Menten rate constant k_{pep} (k_{cat}/K_m), leading to formation of the preribosomal complex R_{pep} with peptidyl-tRNA in the ribosomal A site. Alternatively, complex R’ binds EF-G with rate constant k_{G}^{-1}, forming the EF-G-inhibited complex R_{G}^{-1}, from which EF-G dissociates with rate constant q_{G}^{-1}. Association of FA to R_{pep} with rate constant k_{FA}^{-1} leads to the formation of R_{pep} from which FA dissociates with rate constant q_{FA}^{-1}.

We will first describe this inhibitory effect of EF-G and FA on the peptide bond formation (the transition from R’ to R_{pep}), which can be assayed separately from the FA inhibitory effects on the subsequent translocation process. To do this, we only need to consider part A of the scheme in Fig. 1. To describe the kinetics of R_{pep} accumulation, we set up the following linear differential equation system for the probabilities of being in the different ribosomal states (or, equivalently, for the fractional concentrations of the corresponding complexes).

\[ \frac{dp_{G}^{-1}}{dt} = -q_{G}^{-1}p_{G}^{-1} + k_{FA}^{-1}[FA]p_{G}^{-1} \]  (Eq. 1)
Fusidic Acid Inhibits Multiple Ribosomal States

Two different scenarios for peptide bond formation are described in the present work. In the first, the post-translocation ribosomal complex $R'$ is pre-equilibrated with FA and EF-G together with either GDP or GTP. Then ternary complex is added, and the time-dependent peptide bond formation is monitored by quench flow techniques. At low ternary complex concentrations, the last term in the last set of parentheses of Equation 13 can be neglected. Furthermore, we can assume that the occupancy of the $R'_{13}$ complex is very low, so that $p_{13}^{-1}(0) \ll p_{13}^{-1}(0)$. Taking also the inequality $q_{13}^{-1} \gg q_{13}^{-1}$ into account leads to the following approximations.

\[
\tau_{\text{pep}} = \frac{1}{k_{\text{pep}}} \left( \frac{k_{\text{pep}}}{k_{\text{pep}}} \frac{1}{(q_{13}^{-1} + [FA])} \right) \quad \text{Eq. (14)}
\]

\[
\tau_{\text{pep}} = \frac{1}{k_{\text{pep}}} \left( \frac{k_{\text{pep}}}{k_{\text{pep}}} \frac{1}{(q_{13}^{-1} + [FA])} \right) \quad \text{Eq. (15)}
\]

\[
\tau_{\text{pep}} = \frac{1}{k_{\text{pep}}} \left( \frac{k_{\text{pep}}}{k_{\text{pep}}} \frac{1}{(q_{13}^{-1} + [FA])} \right) \quad \text{Eq. (16)}
\]

\[
p_{\text{pep}} \approx p_{13}^{-1}(0) \approx [FA][G]/([FA][G] + k_{G}^{-1}) \quad \text{Eq. (17)}
\]

Here, the parameter $k_{G}^{-1} = K_{G}^{-1}K_{F}^{-1}$ describes the joint inhibition of the $R'$ complex by EF-G and FA. In the second scenario, when EF-G and FA are added together with ternary complexes, the initial probabilities $p_{13}^{-1}(0)$ and $q_{13}^{-1}(0)$ are zero, and Equation 13 simplifies to Equation 43 under “Results.”

Due to time scale separation between the rate of FA dissociation $q_{13}^{-1}$ and other elemental rate constants in subsequence A of Eq. 1, the kinetics of peptide bond formation is biphasic with the fractional amplitude of the slow phase given by $p_{13}^{-1}$ (see “Parameter Estimation from Experimental Data”).

FA Inhibition of Translocation by EF-G in Single Round Mode—Here we describe FA inhibition of translocation by EF-G in single round mode (see Fig. 1). EF-G binds to complex $R'_{13}$ with Michaelis-Menten parameter $K_{G}^{-1}/K_{G}$, which leads to formation of the post-translocation complex $R'_{13}$ via complexes $R'_{13}$, $R'_{13}$, $R'_{13}$, and $R'_{13}$ with Michaelis-Menten constant $k_{G}$, where $1/k_{G} = 1/k_{G} + 1/k_{G} + 1/k_{G} + 1/k_{G}$. The latter multistep reaction can be inhibited by FA binding to $R'_{13}$ with rate constant $k_{G}$, leading to complex $R'_{13}$, which rapidly transforms to complex $R'_{13}$. Alternatively, FA may directly form $R'_{13}$ by first binding to complex $R'_{13}$; FA dissociates slowly from $R'_{13}$ with rate $q_{13}^{-1}$; leading to complex $R'_{13}$. FA may then bind to $R'_{13}$, before $R'_{13}$ proceeds to the downstream complex $R'_{13}$. Therefore, FA may then bind again to form complex $R'_{13}$. When, eventually, EF-G dissociates from $R'_{13}$ with rate constant $q_{13}$, the inhibited reaction reaches the post-translocation complex $R'_{13}$. After this, peptide bond formation occurs as described for cycle “ii” in Fig. 1. When $i = 1$, the scheme illustrates the first cycle of protein synthesis, in which $R'$ has initiator tRNA (Met-tRNA^{Met}) in the P site (see Fig. 4).

In analogy with the dipeptide case described above, we can evaluate the kinetics of translocation by setting up a system of differential equations for the time-dependent probabilities, $p_{\text{pep}}$ $p_{\text{pep}}$, $p_{\text{pep}}$, $p_{\text{pep}}$ $p_{\text{pep}}$ $p_{\text{pep}}$ $p_{\text{pep}}$ $p_{\text{pep}}$, and $p_{\text{pep}}$, that the translocation process is in ribosomal states $R'_{13}$ $R'_{13}$, $R'_{13}$, $R'_{13}$, $R'_{13}$, $R'_{13}$, and $R'_{13}$, respectively. Integration of these equations from time 0 to infinity yields results in an algebraic equation.
system for the average times \( t'_{\text{pep}} \), \( t'_{\text{G}} \), \( t'_{\text{G1}} \), \( t'_{\text{11}} \), \( t'_{\text{G2}} \), \( t'_{\text{22}} \), \( t'_{\text{G3}} \) and \( t'_{\text{33}} \) that the system is in the corresponding states in a single round experiment. Solving this algebraic system with the initial condition \( p'_{\text{pep}}(0) = 1 \) gives the following for the average time of translocation.

\[
\tau'_{G} = \tau'_{\text{pep}} + \tau'_{G0} + \tau'_{G1} + \tau'_{11} + \tau'_{G2} + \tau'_{22} + \tau'_{G3} + \tau'_{33} = \tau'_{AG} + \tau'_{IG}(\text{Eq. 18})
\]

\[
\tau'_{AG} = \frac{K_G}{k_G[G]} + \frac{1}{k_G G} + \frac{1}{k_G G} + \frac{1}{q_{G3} G} + \frac{k_{G0}^{i+1} K_G[G]}{k_{G0}^{i} [T_3 + 1] q_{G3} G} (\text{Eq. 19})
\]

The terms for both the uninhibited translocation, \( \tau'_{AG0} \) and the time increment due to FA inhibition, \( p'_{IG}\tau'_{IG} \), that depend on the concentration of ternary complexes can be neglected due to the high efficiency of \( T_3 \) binding in comparison with EF-G rebinding to the post-translocation state “\( i + 1 \)”. Neglecting these terms and also \( d_{\text{f}3} \) in comparison with \( k'_{G3} \) in Equations 18–20 gives the following,

\[
\tau'_{AG} = \frac{1}{k_G G} (K_G + 1) (\text{Eq. 21})
\]

\[
p'_{IG}\tau'_{IG} = \frac{1}{q_{G2} G} \frac{[FA] + K_{G1} [FA]}{K_{G1}} + \frac{1}{q_{G3} G} K_{G1} [FA] (\text{Eq. 22})
\]

where

\[
K_{G1} = k_{G1} / k_{G3} (\text{Eq. 23})
\]

\[
K_{G2} = k_{G2} / k_{G3} (\text{Eq. 24})
\]

\[
K_{G3} = q_{G3} / k_{G3} (\text{Eq. 25})
\]

The probability of one FA binding event during translocation is given by Equation 45 under “Results.” The expression for \( p'_{IG} \) used for fitting of experimental data can hence be obtained from Equations 22 and 45. In our model of FA inhibition of the translocation process, the time increment due to FA inhibition is non-linear in the FA concentration, as can be seen from Equation 22. For a different model where \( k'_{G3} \) cannot be reached directly from \( k_{G1} \), this increment \( (p'_{IG}\tau'_{IG}) \) would have a strictly linear dependence on the FA concentration.

**FA Inhibition of Translocation by EF-G in Multicycle Mode—**

We consider the experimental situation when preformed pre-translocation complex, \( R'_{pep} \), is transformed to the post-translocation complex, \( R' \), by multiple cycling of EF-G at low concentration in the absence or presence of FA. At the start of incubation, the concentration, \( [G_{A}]_0 \), of uninhibited EF-G is equal to the total EF-G concentration, \( [G_{A}] \). In the initial phase of the incubation, the concentration \( [G_{A}] \) relaxes to its steady state value as governed by the following, approximate, differential equation,

\[
\frac{d[G_{A}]}{dt} = - \frac{[G_{A}] p_{IG}}{\tau_{AG}} + \frac{[G_{A}]}{\tau_{AG}} (\text{Eq. 26})
\]

where \( [G_{A}] \) is the concentration of the FA stalled EF-G, so that the following is true.

\[
[G_{A}] = [G_{A}]_0 - [G_{A}] (\text{Eq. 27})
\]

The time \( \tau_{AG} \) is principally similar to \( \tau'_{AG} \) in Equations 21, but with the ribosomal concentration \( R'_{pep} \) replacing the EF-G concentration \( G \) and the Michaelis-Menten constants \( k_{G} \), \( K_{G} \) and \( K_{G'} \), which take the rate of GDP to GTP exchange on EF-G into account, replacing \( k_{G} ' \) and \( K_{G} ' \).

\[
\tau_{AG} = G_{A} \frac{1}{k_{G} [R'_{pep}] + 1} (\text{Eq. 28})
\]

In a parameter and time range where \( \tau_{AG} \) is approximately constant, the solution to Equation 26 is approximated by the following,

\[
[G_{A}] = [G_{A}]_0 e^{-k_{rel} t} + \frac{[G_{A}]}{\tau_{AG} + p_{IG} \tau_{IG}} (1 - e^{-k_{rel} t}) (\text{Eq. 29})
\]

where \( k_{rel} \) is the rate constant by which the system relaxes to its quasi-steady state.

\[
k_{rel} = \frac{p_{IG} \tau_{AG} + 1}{\tau_{AG}} (\text{Eq. 30})
\]

Here \( \tau_{G} = \tau_{AG} + p_{IG} \tau_{IG} \) is the average EF-G cycle time in the presence of FA and the product \( p_{IG} \tau_{IG} \) is given by Equation 22. The translocation flow rates in the absence (\( j_{AG} \)) and presence (\( j_{IG} \)) of FA are given by the following.

\[
\frac{j_{AG}}{\tau_{AG}} = \frac{[G_{A}]}{[G_{A}]_0 t} (1 + (p_{IG} \tau_{IG} / \tau_{AG}) e^{-k_{rel} t}) (\text{Eq. 31})
\]

The equations for fitting of the accumulation of tripeptides with time in Fig. 7 are then obtained by integration.

\[
\int_{0}^{t} j_{AG} du = \frac{[G_{A}]_0 t}{\tau_{AG}} = j_{AG} t (\text{Eq. 33})
\]

\[
\int_{0}^{t} j_{IG} du = \frac{[G_{A}]_0 t}{1 + p_{IG} \tau_{IG} / \tau_{AG}} + \frac{j_{AG} p_{IG} \tau_{rel} / \tau_{AG} (1 - e^{-k_{rel} t})}{k_{rel} (1 + p_{IG} \tau_{IG} / \tau_{AG})} = \frac{j_{AG} t}{k_{rel} \tau_{IG}} (1 - e^{-k_{rel} t}) (\text{Eq. 34})
\]

At low FA concentrations, where the inhibition is dominated by the binding to the first FA-sensitive state, the approximation \( \tau_{IG} \approx 1 / q_{f2} \) is valid.
In the steady state, FA inhibition of the rate of EF-G cycling is given by the ratio \( \frac{j_{IC}}{j_{AG}} \).

\[
\frac{j_{IC}}{j_{AG}} = \frac{[GA]}{[GO]} = \frac{\tau_{AG} + p_{IG}}{\tau_{AG}} = \frac{\tau_{AG}}{\tau_{AG} + \frac{1}{q_{f3}} \left( \frac{[FA]}{[FA] + K_{f1}} + \frac{[FA]}{[FA] + K_{f2}} \right) + \frac{1}{q_{f3}} K_{f1}}
\] (Eq. 35)

At low FA concentration, where inhibition is dominated by the binding to the first FA-sensitive state we get the following,

\[
\frac{j_{IC}}{j_{AG}} = \frac{1}{1 + \frac{[FA]}{\tau_{AG} q_{f3} K_{f1}}} = \frac{1}{1 + \frac{[FA]}{\tau_{AG} q_{f3} K_{f1}}} (\text{Eq. 36})
\]

where

\[
K_{50%} = \tau_{AG} q_{f3} K_{f1}.
\] (Eq. 37)

From this, it follows that a \( K_{50%} \) value estimated from an EF-G-cycling experiment is proportional to the EF-G cycling time, \( \tau_{AG} \), in the absence of FA. Estimates of the in vivo \( K_{50%} \) value for FA inhibition based on in vitro systems must therefore be calibrated to compensate for any significant difference between the in vivo cycle time, which for \( E.\ coli \) is about 50 ms (24), and the in vitro cycle time (see “Discussion”).

Parameter Estimation from Experimental Data

Inhibition of Dipeptide Formation by EF-G and FA—In the absence of FA, the time dependence of dipeptide formation is biphasic; there is a fast phase with a large amplitude \( a_1 (1 - u_0) \) and a small average time \( \tau_{Apep}^1 \), and a slow phase with a small amplitude \( u_1 u_0 \) and a much larger average time \( \tau_s \) (Figs. 2A and 3). We describe dipeptide formation in the absence of FA as follows.

\[
dip_A(t) = a_1 ((1 - u_0) f(\tau_{Apep}^1)) + u_0 f(\tau_{Apep}^1 \tau_s) + bg_1
\] (Eq. 38)

In the presence of FA (with or without preincubation), a fraction \( \left(p_{Apep}^1 \right) \) of the ribosomes is bound to EF-G and FA. These ribosomes pass through another sequential slow step with time constant \( \tau_{Apep}^1 \) corresponding to the rate of release of FA. The unbound fraction \( (1 - p_{Apep}^1) \) has the same behavior as in the absence of FA. Hence, we describe dipeptide formation in the presence of FA as follows.

\[
dip_{A}(t) = a_2 (1 - p_{Apep}^1) ((1 - u_0) f(\tau_{Apep}^1) + u_0 f(\tau_{Apep}^1 \tau_s)) + p_{Apep}^1 ((1 - u_0) f(\tau_{Apep}^1 \tau_s) + u_0 f(\tau_{Apep}^1 \tau_s \tau_{pep}^1)) + bg_2
\] (Eq. 39)

The functions \( f(x), f(x,y), \) and \( f(x,y,z) \) in Equations 38 and 39, where \( x, y, \) and \( z \) represent time constants, describe the accumulation of the end product for a scheme of irreversible sequential reactions with the number of steps and time constants indicated. Simultaneous fitting of Equations 38 and 39 to dipeptide formation curves obtained in parallel in the absence and presence of FA estimated the parameters \( p_{Apep}^1, \tau_{Apep}^1 \) and \( \tau_{pep}^1 \). These estimates where used as input for the average time analysis described above. Because Equations 38 and 39 share the term describing the additional slow phase with time constant \( \tau_s \) and fractional amplitude \( u_0 \), it will appear as a common background term of the two curves.

FA Inhibition of Translocation by EF-G in Single Round Mode—Tripeptide formation in the absence of FA can be described as a three-step process with time constants \( \tau_{Apep}^1, \tau_{AG}^1 \), and \( \tau_{pep}^1 \). Because FA and EF-G compete poorly with ternary complex binding and do not affect the peptide bond formation rate under the conditions used, the increased tripeptide formation time in the presence of FA originates mainly from slower translocation. This slow translocation is seen in the tripeptide formation curves as a slow phase with time constant \( \tau_{AG}^1 \). Thus, in analogy with the description of the dipeptide formation, fitting functions for tripeptide formation in the absence or presence of FA can be set up.

\[
trip_A(t) = a_1 ((1 - u_0) f(\tau_{Apep}^1, \tau_{AG}^1, \tau_s)) + u_0 f(\tau_{Apep}^1, \tau_{AG}^1, \tau_s) + bg_1
\] (Eq. 40)

\[
trip_{A}(t) = a_2 (1 - p_{IG}^1) ((1 - u_0) f(\tau_{Apep}^1, \tau_{AG}^1 (1 - p_{IG}^1), \tau_{pep}^1)) + u_0 f(\tau_{Apep}^1, \tau_{AG}^1 (1 - p_{IG}^1), \tau_{pep}^1 \tau_s) + p_{IG}^1 ((1 - u_0) f(\tau_{IG}^1)) + u_0 f(\tau_{IG}^1) + bg_2
\] (Eq. 41)

Here, as before, the functions \( f(x), f(x,y), f(x,y,z) \), and \( f(x,y,z,u) \) are the solutions for the end product of a differential equation system describing a scheme of irreversible sequential reactions with the number of steps and time constants indicated. We noted that the translocation time constant was reduced in the presence of FA in a way that was related to the bound fraction of ribosomes, and the translocation time was scaled accordingly \((\tau_{AG}^1 (1 - p_{IG}^1)) \). The tripeptide formation curve alone does not contain enough information to resolve all of the substeps of tripeptide formation \( \tau_{Apep}^1, \tau_{AG}^1 \), and \( \tau_{pep}^1 \). However, the total time of uninhibited tripeptide formation \( \tau_A \) can be obtained by substituting the time constant of one of the substeps (e.g. \( \tau_{Apep}^1 \)) by \( \tau_{Apep}^1 = \tau_A - \tau_{AG}^1 - \tau_{pep}^1 \) in Equations 40 and 41. Parallel fitting of the expressions in Equations 40 and 41 to tripeptide formation curves obtained in the absence and presence of FA gave good estimates for \( \tau_A, p_{IG}^1 \), and \( \tau_{IG}^1 \) that were used for the total time analysis.

RESULTS

Inhibition of Dipeptide Formation by EF-G and FA—In the protein elongation cycle, EF-G catalyzes the movement of peptidyl-tRNA from the A to the P site of the ribosome. In the presence of FA, EF-G remains stably bound to the post-translocated ribosome, thereby blocking ternary complex (T,) binding to the A site and formation of the next peptide bond. To characterize this inhibitory effect on peptide bond formation, we first studied the joint binding of EF-G and FA to the ribosomal 70S initiation complex \( (R^I) \) as a mimic for the post-translocation ribosome (Figs. 1 (with \( t = 1 \) and 2E). In these experiments, EF-G (20 \( \mu M \)) and FA (0–2 \( mM \)) were pre-equilibrated.
with the $R^1$ complex, containing initiator tRNA ($[^3]$H]Met-tRNA$^{Met}$) in the P site and a Leu codon (CUG) in the empty A site, in the presence of GDP or GTP (100 μM). The pre-equilibrated mixtures (containing different fractions of FA-free ($R^3$) and EF-G/FA-inhibited ($R^*_I$) ribosomal complex as determined by the FA concentration) were rapidly mixed with a factor mixture, containing Leu-tRNA$^{Leu}$ (4 μM), GTP (1 mM), and EF-Tu (20 μM), in a quench flow instrument. The extent of $[^3]$H]Met-Leu formation at different incubation times was then quantified as described under “Materials and Methods.” The time courses of dipeptide formation were distinctly biphasic, as shown in Fig. 2A for the GDP case. The fractional amplitude of the slow phase ($p^I_{lep} \text{ (0)}$) increased hyperbolically from 0 in the absence of FA to near 1 as the FA concentration increased to higher values (Fig. 2B). The slow phase amplitude increase was very similar when these experiments were performed in the presence of GDP or GTP (Fig. 2B). A residual slow phase, observed also in the absence of FA (Fig. 2A), was treated as described under “Materials and Methods.” All time courses were analyzed in terms of average times for the slow ($t^I_{lep}$) and fast ($\tau_{lep}$) phases of peptide bond formation along with their respective amplitudes $p^I_{lep}$ and $1 - p^I_{lep}$ (see “Materials and Methods”). The slow phase time, $t^I_{lep}$, increased linearly with increasing FA concentration from about 6 s to about 20 s at 2 mM FA concentration (Fig. 2C), and the time of the fast phase, $\tau_{lep}$, reflecting ternary complex binding and peptide bond formation on EF-G free ribosomes, was 32 ms. The total average time for dipeptide formation, $\tau^I_{lep}$, increased sharply with increasing FA concentration in the low concentration range from its initial value, $\tau^I_{lep}(0)$, and gradually in the high FA concentration range (Fig. 2D). To interpret these findings in terms of the elemental rate constants in Fig. 2E, we approximated $\tau^I_{lep}$ as follows (see also Equations 14–17).

$$
\tau^I_{lep} = \tau^I_{lep(0)} + p^I_{lep} \cdot \tau^I_{lep(0)} = \left(1 + \frac{1}{K^I_{lep(0)}} \right) \left( 1 + \frac{K^I_{lep}}{[T_{lep}^I]} \right) + \left( \frac{[G][FA]}{[G][FA] + K^I_{lep}} \right) \left( 1 + \frac{(k^I_{lep}/q^I_{lep(0)})[FA]}{q^I_{lep}} \right)$$

(Eq. 42)

The total probability that the reaction was inhibited by FA, $p^I_{lep}$, was here approximated as the probability $p^I_{lep}(0)$ that the ribosome was EF-G/FA-bound already in the preincubation phase (i.e. in complex $R^*_I$) (Figs. 1 and 2E). The parameters $q^I_{lep}$ and $K^I_{lep}$ are the first order rate constant for dissociation of FA from $R^*_I$ and the second order rate constant for association of FA to $R^*_I$, respectively. The parameter $q^I_{lep}$ is the first order rate constant for dissociation of EF-G from its complex with $R^I$ (Fig. 2E), and $K^I_{lep}$ in Equation 42 is the combination, $K^I_{lep} = q^I_{lep}/q^I_{lep}/K^I_{lep}$.

Fitting of the parameters of Equation 42 to the experiments in Fig. 2 estimated the average time of FA release from the $R^*_I$ complex, $1/q^I_{lep}$, as 6.1 s, the inhibition constant $K^I_{lep} = q^I_{lep}/K^I_{lep}$ as 1.2 mM, and the equilibrium constant for binding of EF-G and
FA to the initiation complex, $K_0^{0}$, as 55 $\mu M^2$ in the presence of GDP (all parameter estimates are summarized in Table 1). In the presence of GTP, $K_0^{0}$ was estimated as 74 $\mu M^2$ by fitting of a hyperbolic binding curve to the data in Fig. 2B (inset). We note that the EF-G/FA-bound ribosomal complex $R_3^{0}$ contained GDP, irrespective of the nucleotide used to form the complex, as confirmed experimentally by sample centrifugation through a sucrose cushion followed by TLC analysis (data not shown).

From the estimates of $q_{eff}, q_{G3}/k_{F3}$, and $K_0^{0}$, and based on an active EF-G fraction of 51%, the rate constant $k_{G3}^{0}$ was estimated as 3.7 $\mu M^{-1} s^{-1}$ (Table 1). The fraction of active EF-G was estimated from nitrocellulose filter binding experiments as the fraction of EF-G able to form 70S-EF-G(GDP)-FA complex (data not shown).

To characterize the roles of EF-G and FA as authentic competitive inhibitors of ternary complex binding, we performed dipeptide formation experiments also in the absence of a pre-equilibration step. Here, a solution containing the initiation complex, $R_1$, was rapidly mixed with a solution containing EF-Tu(GTP)-Leu-tRNA$^{Leu}$ ternary complex ($T_{1,2,3}$ or $4$, or $5$), EF-G (40 $\mu M$), GTP (1 mM), and FA (0 or 4 mM) in the quench flow instrument. Formation of the $[^3H]Met-Leu$ dipeptide was monitored as described above, and representative time traces obtained at 2 mM FA concentration are shown in Fig. 3. In the absence of FA, the fast phase time of peptide bond formation, $\tau_{pep}$, was estimated as 29 or 60 ms at 2 $\mu M$ or 1 $\mu M$ final concentration of $T_3$, respectively. The inverse proportionality between the peptide bond formation time and the ternary com-

**TABLE 1**

| Parameter | From dipeptide formation (Fig 2) | Interpretation | Parameter | From tripeptide formation (Fig 5) | Interpretation |
|-----------|----------------------------------|----------------|-----------|----------------------------------|----------------|
| $K_0^{0}$ (GDP) = $q_{G3}^{0}k_{F3}^{0}$ | $k_{G3}^{0}k_{F3}^{0}$ | 55 ± 3 $\mu M^2$ | Duration time of FA in the | Duration time of FA in the | Equilibrium constant for |
| $K_0^{0}$ (GTP) = $q_{G3}^{0}k_{F3}^{0}$ | $k_{G3}^{0}k_{F3}^{0}$ | 74 ± 26 $\mu M^2$ | $R_1^{0}$ | dipeptidyl post- | binding of EF-G and FA to |
| $q_{F2}^{0}$ | $q_{F3}^{0}$ | 6.1 ± 0.3 s | $R_1^{0}$ | translocation complex | the initiation complex ($R_1^{0}$). |
| $k_{G3}^{0}$ | $k_{F3}^{0}$ | 3.7 ± 0.5 $\mu M^{-1} s^{-1}$ | Duration time of FA in the | Duration time of FA in the | Rate constant for binding |
| $k_{G3}^{0}$ | $k_{F3}^{0}$ | 1.2 ± 0.1 mM | intermediate translocation | intermediate translocation | of EF-G to the initiation |
| $k_{G3}^{0}$ | $k_{F3}^{0}$ | 2.8 ± 0.9 mM | $R_2^{0}$ | complex ($R_2^{0}$). | complex ($R_0^{0}$). |
| $k_{G3}^{0}$ | $k_{F3}^{0}$ | 2.0 ± 0.1 mM | Inhibition constant for the | Inhibition constant for the | Equilibrium constant for |
| $k_{G3}^{0}$ | $k_{F3}^{0}$ | 0.12 ± 0.01 mM | binding of FA to the first | binding of FA to the second | binding of FA to the |
| $k_{G3}^{0}$ | $k_{F3}^{0}$ | 3.5 ± 1.7 mM | FA sensitive translocation | FA sensitive translocation | ternary complex ($R_3^{0}$). |
| $k_{G3}^{0}$ | $k_{F3}^{0}$ | 2.8 ± 0.9 mM | state ($R_1^{0}$) | state ($R_2^{0}$) | ($R_0^{0}$). |
| $k_{G3}^{0}$ | $k_{F3}^{0}$ | 2.0 ± 0.1 mM | Inhibition constant for the | Inhibition constant for the | ($R_3^{0}$). |
| $k_{G3}^{0}$ | $k_{F3}^{0}$ | 0.12 ± 0.01 mM | binding of FA to the EF-G | binding of FA to the EF-G | ($R_1^{0}$). |
| $k_{G3}^{0}$ | $k_{F3}^{0}$ | 3.5 ± 1.7 mM | bound dipeptidyl post- | bound dipeptidyl post- | ($R_2^{0}$). |
Fusidic Acid Inhibits Multiple Ribosomal States

FIGURE 3. Diapetide formation in the absence of preincubation of initiation complexes with EF-G and FA. Time traces were obtained after mixing of initiated ribosomes (0.5 μm) with EF-G (20 μm) and EF-Tu(GDP)-tRNA\textsuperscript{Leu} in ternary complexes (1 (circles) or 2 μm (squares)) in the presence (2 μm; red traces) or absence (black traces) of FA. (The concentrations are the effective concentrations after mixing.)

- FA, 2 μM T3
- FA, 1 μM T3
- FA, 1 μM T3
- FA, 2 μM T3
- FA, 1 μM T3
- FA, 1 μM T3

The results above demonstrate strong inhibition of translocation by EF-G in single round mode—The results above demonstrate strong inhibition of translocation by EF-G in single round mode. In vivo, there is no such preincubation, and the rebinding of EF-G and FA to the post-translocation ribosome always occurs in competition with T\textsubscript{3} and is therefore weak. Here we describe the inhibitory effect of FA on tripeptide formation, which mainly reflects the impact of the drug on the translocation process itself. In tripeptide formation experiments, FA inhibition of EF-G-dependent translocation with average time $\tau_{G0}$ was estimated from the total average time, $\tau_{tot} = \tau_{pep} + \tau_{G0} + \tau_{pep}^2$ for the ribosome to move from the 70S initiation complex $R$\textsuperscript{1} to the tripeptide complex $R_{pep}$. This time includes the first peptide bond formation time $\tau_{pep}$, all translocation steps, including EF-G dissociation from the post-translocated ribosome, with overall average time $\tau_{G0}$; and the second peptide bond formation step with average time $\tau_{pep}^2$ (Figs. 1 (with $i = 1$) and 4). For this we prepared, as described under “Inhibition of Dipeptide Formation by FA and EF-G,” a ribosome mixture containing ribosomal initiation complex $R$\textsuperscript{1} with initiator tRNA (f\textsuperscript{3}H)Met-tRNA\textsuperscript{Met}(M\textsuperscript{SS}) in the P site programmed with MLF mRNA. We also prepared a factor mixture containing EF-Tu (30 μM), EF-G (40 or 10 μM), Leu-tRNA\textsuperscript{Leu} (4 μM), Phe-tRNA\textsuperscript{Phe} (4 μM), and FA (0–4 mM). Rapid mixing of the ribosome and the factor mixtures in the quench flow instrument led to formation of f\textsuperscript{3}HMet-Leu, followed by translocation and formation of f\textsuperscript{3}HMet-Leu-Phe (Fig. 4). The reaction was quenched after different incubation times, and the amounts of f\textsuperscript{3}HMet-Leu diapetide and f\textsuperscript{3}HMet-Leu-Phe tripeptide formed were determined by HPLC as described above. The time curves for f\textsuperscript{3}HMet-Leu-Phe formation obtained at different FA concentrations displayed biphasic kinetics (Fig. 5A). The curves were, as in the case of diapetide formation, analyzed in terms of the average times of their fast ($\tau_{G1}$) and slow phase ($\tau_{G2}$) and the fractional amplitudes of the two phases, (1 – $p_{G1}$) and $p_{G1}$, respectively. The slow phase amplitude $p_{G1}$, which approximated the per cycle probability that the ribosome was inhibited by FA, increased sharply with increasing FA concentration from 0 to near 1 in a nearly hyperbolic manner in the low FA concentration range (Fig. 5B). The fast phase time remained approximately constant, and the slow phase time, $\tau_{G2}$, increased linearly with increasing FA concentration from an initial value of about 9 s (Fig. 5C). The contribution of the slow phase to the total tripeptide formation time is given by $p_{G1}\tau_{G2}$. In the presence of FA, the total average time of tripeptide formation, $\tau_{tot} = \tau_{pep} + \tau_{G0} + \tau_{pep}^2$ (Figs. 1 (with $i = 1$) and 4) increased sharply with increasing FA concentration in the low concentration range (Fig. 5D), due to the sharply increasing amplitude, $p_{G1}$, of the slow phase (Fig. 5B). The following linear increase (Fig. 5D) in $\tau_{tot}$ was due to the linear increase in the slow phase time, $\tau_{G2}$ (Fig. 5C), in the high FA concentration range where $p_{G1} = 1$. The FA dependences of the total tripeptide formation time (Fig. 5D) and the slow phase time, $\tau_{G2}$ (Fig. 5C), were the same at EF-G concentrations of 5 and 20 μM. This implies that the FA- and EF-G-dependent increase of the peptide bond formation time described by Equation 13, was negligible in relation to the FA-dependent but EF-G-independent increase in $\tau_{G2}$ in the translocation cycle. Furthermore that the FA-dependent increase in $\tau_{G2}$ was dominated by the increase in $\tau_{G2}$. From this, we conclude that $p_{G1} = p_{G0}$ and $\tau_{G1} = \tau_{G0}$. Fig. 5 also reveals that FA was released before EF-G from the inhibited ribosomal complex because $\tau_{G2}$ would otherwise have depended on the EF-G concentration.
FIGURE 4. Schematic of FA inhibition of the first peptide elongation steps (see also Fig. 1). The FA inhibition modes I, II, and III (IIIA and IIIB) are enclosed in red boxes. The 70S initiation complex (R1) with fMet-tRNA and in the P site may bind ternary complex and form ribosomal complex R1G1 by peptide bond formation. Alternatively, EF-G may bind to R1 with the rate constant kG1 and form complex R1G1, from which EF-G dissociates with the rate constant qG1. FA may bind to R1G1 with the rate constant kF1 to form the complex R1G1F, which slowly dissociates with the rate constant qG1. These reactions define the third inhibition mode of FA (III). Ribosomal complex R1G1F may, via the complexes R1G1F, R1G2F, and R1G3, be rapidly translocated by EF-G to ribosomal complex R1F with Michaelis-Menten parameters kF1 and qF1. Alternatively, FA may bind to R1G1, with association rate constant kF1 and form R1G1F, which is rapidly translocated to R1G2 in which the ribosome is stalled until FA release with rate constant qG2. After this, FA may bind to R1G2, or the reaction may continue downstream to the post-translocation state R1G3 with the rate constant kG3. The slow phase time to theFA-bound ribosome bound to EF-G and FA to a preformed post-translocation ribosome. If this were true, it would be possible to assemble the inhibited complex R1G2 also by binding of EF-G and FA to a preformed post-translation ribosome, R2 (Fig. 4). To test this proposal, the post-translocation complex, R2, was formed by first incubating the 70S initiation complex, R1, with Leu ternary complex (1 μM) and EF-G (20 μM), allowing fMet-Leu formation and translocation. Subsequently, the R2 complex together with EF-G was incubated with FA at concentrations in the 0–2 mM range to form the FA-bound post-translocation complex. Then the slow phase time of Met-Leu-Phe formation following Phe ternary complex addition was determined. It was shown to vary linearly with the FA concentration with a y axis intercept and a slope distinct from that obtained in the tripeptide formation experiments (see Fig. 5C). This means that the state R1G2 in which the ribosome was stalled by FA attack during the translocation process was different from the ribosomal state reached by binding of EF-G and FA to the post-translocation ribosome, R2. From this, we conclude that there was a third translocation state, R1G3, competent in FA binding downstream of and distinct from state another ribosomal complex in the translocation process, R1G2F, downstream of R1G1. This conclusion follows from the consideration that if FA had dissociated from and reassociated with R1G1, the time t1 would have increased linearly with a constant slope at all FA concentrations (see “Materials and Methods”).

A model (Fig. 4) that quantitatively accounts for the complex translocation inhibition by FA (Fig. 5) is as follows. During tripeptide formation, FA bound to R1G1 with probability [FA]/(K1G1 + [FA]), where the inhibition constant K1G1 is the ratio between the forward rate constant, kG1, from R1G1 and the rate constant, kF1, for binding of FA to R1G1 (Fig. 4). Then the FA-bound ribosomal complex R1G1F rapidly moved downstream in the translocation process to the FA-bound complex R1G2F, in which the EF-G-bound ribosome was stalled by the action of FA. Dissociation of FA from R1G2F was, before dissociation of EF-G, followed by low efficiency binding of the drug to the downstream complex R1G2 with probability [FA]/(K1G2 + [FA]). Here, the inhibition constant K1G2 is the ratio of the forward rate constant, kG2, and the rate constant, kF2, for FA binding to R1G2F. An inhibition mechanism with an efficient first drug binding event to state R1G1 followed by much less efficient rebinding to state R1G2 would explain both the initial sharp increase and the following gradual, linear increase of the total tripeptide formation time, t1, in response to increased FA concentration (Fig. 5D). We conclude from this set of experiments that FA must have bound to at least two states in the translocation process and that FA binding to the first did not inhibit the transition to the second state.
translocation. From the data in Fig. 5, we estimated the following.

\[ \frac{1}{q_{f2}} \left( \frac{[FA]}{[FA] + K_{11}} + \frac{[FA]}{K_{12}} \right) + 1 \frac{[FA]}{K_{13}} \]  

(Eq. 44)

Here, \( r_{1} \) is the uninhhibited tripeptide formation time, and \( r_{1} \) is the average slow phase time of translation. The FA inhibition probability \( p_{1} \) of the translation process is given by the following.

\[ p_{1} = 1 - \frac{K_{11}}{K_{11} + [FA]} \cdot \frac{K_{12}}{K_{12} + [FA]} \cdot \frac{K_{13}}{K_{13} + [FA]} \]  

(Eq. 45)

The second term on the right side is the product of the probabilities of no inhibition by FA in states \( r_{1} \), \( r_{2} \), and \( r_{3} \) during translocation. From the data in Fig. 5, we estimated \( r_{1} \) as 120 ms; the average FA release time from the second FA-sensitive state \( r_{2} \), \( 1/q_{f2} \) as 8.9 s; the average FA release time from the third FA-sensitive state \( r_{3} \), \( 1/q_{f3} \) as 6.1 s; the inhibition constant for the first FA-sensitive state, \( K_{11} \), as 0.12 mM; the inhibition constant for the second FA-sensitive state, \( K_{12} \), as 3.5 mM; and the inhibition constant for the third FA-sensitive state, \( K_{13} \), as 2.8 mM (see Table 1). We note that \( 1/q_{f2} \), which dominated the inhibition, was about 100 times larger than \( r_{1} \), suggesting a 2-fold reduction in the average ribosome speed at a FA concentration of about 1.3 \( \mu \)M (see “Discussion”).

**FA Inhibition of Translocation by EF-G in Single Round Mode with Fluorescence-labeled mRNA**—In order to specifically monitor the effect of FA on the mRNA movement step in ribosomal translocation, we prepared a ribosome mixture as described under “Inhibition of Dipeptide Formation by EF-G and FA” but with the MLF mRNA replaced by a 3’-pyrene-labeled and truncated MLF-encoding mRNA (see “Materials and Methods”) (26). The ribosome mixture was rapidly mixed with a factor mixture containing EF-G (40 \( \mu \)M), FA (0–0.8 \( \mu \)M), and ternary complex formed from EF-Tu-GTP (10 \( \mu \)M) and Leu-tRNA-Leu (4 \( \mu \)M), in a stopped flow or, to monitor dipeptide formation, in a quench flow instrument. During the reaction, initial formation of the fMet-Leu dipeptide was followed by translocation of the mRNA in relation to the ribosomal frame, back-ratching of the ribosome to the post-translocation state, and rapid or slow release of EF-G from FA-free or FA-bound ribosomes, respectively. A single round of translocation was ensured by omitting Phe-tRNA-Phe, the cognate tRNA for the second codon, from the reaction mixture. The fluorescence time traces measured in the stopped flow instrument displayed two phases; an initial fast intensity increase was followed by a decrease associated with the mRNA movement in the ribosomal frame (Fig. 6A). Steps occurring after mRNA movement, such as back-ratching and EF-G release, are not visible in the fluorescence traces.

The total average time for the initial intensity increase and the following intensity decrease, estimated as 75 ms from the sum of the relaxation times for these two phases (27), was unaf-
Fusidic Acid Inhibits Multiple Ribosomal States

From quench flow experiments, the average time of dipeptide formation ($\tau^p_{\text{pep}}$) was estimated as 32 ms. Subtraction of the dipeptide formation time (32 ms) from the time from incubation start to completion of mRNA movement (75 ms) estimated the average time from peptide bond formation to mRNA movement as 43 ms. Given the total tripeptide formation time for uninhibited ribosomes ($\tau^p_{\text{pep}}$) of 120 ms and a time of the second peptide bond formation ($\tau^p_{\text{pep}}$) of 20 ms (data not shown), the remaining time was 25 ms ($120 - 75 - 20 = 25$ ms). This 25-ms period includes all steps after movement of the mRNA, required for the ribosome to be ready to bind the next ternary complex.

Although the kinetics of fluorescence change remained unaltered, the final fluorescence intensity after mRNA movement, $F_{\text{FA}}(\infty)$, increased with increasing FA concentration. The difference in final fluorescence in the presence and absence of FA was normalized to the total fluorescence amplitude in the absence of FA to form the intensity ratio $\Delta_{\text{FA}}(\infty) = (F_{\text{FA}}(\infty) - F_0(\infty))/(F_0(0) - F_0(\infty))$. This ratio displayed an FA concentration dependence similar to that displayed by the fraction of FA-bound ribosomes observed in the tripeptide formation experiments (Fig. 6B). However, at very high FA concentration, where all ribosomes were FA-bound, roughly half of the fluorescence decrease due to mRNA movement remained (see Fig. 6A). This, together with the unaltered kinetics of mRNA movement, implies that the reduction in fluorescence amplitude was due to higher specific fluorescence from the FA-bound than from the FA-free ribosome rather than slower mRNA movement on FA-bound ribosomes.

**FA Inhibition of Translocation by EF-G in Multicycle Mode**

In all experiments described above, we studied the effect of FA on a single cycle of peptide elongation with EF-G present in large molar excess over the ribosome. A great advantage of this approach is that, due to separation of time scales for inhibited and uninhibited translocation, we could determine the probability of FA inhibition per elongation cycle from the slow phase fraction of tripeptide formation. Here, in contrast, we studied the effect of FA on multiple cycling of a small amount of EF-G acting on pretranslocation ribosomes present in large molar excess over EF-G. For this, we prepared a mixture with 70S initiation complex (4 µM) programmed with MLF-encoding mRNA and a factor mixture with Leu-tRNA$^{\text{Leu}}$ (5 µM), Phe-tRNA$^{\text{Phe}}$ (5 µM), EF-Tu (37 µM), EF-G (0.1 µM), and FA (0 or 40 µM). After rapid mixing of equal volumes of these two solutions in the quench flow instrument, the ribosomes rapidly reached the pretranslocation state ($R^1_{\text{pre}}$) with fMet-Leu-tRNA$^{\text{Leu}}$ in the A site and decacylated tRNA$^{\text{Phe}}$ in the P site. Translocation was then stimulated by multiple cycles of EF-G action, rapidly followed by tripeptide formation as monitored by HPLC and online radiometry.

In the absence of FA, the number of tripeptides formed per active EF-G molecule increased linearly in time from 0 to about 15 during the first 3 s of incubation (Fig. 7, black curve). Then the cycling rate decreased due to the combined effects of reduced concentration of pretranslocation ribosomal complex by translocation and by dipeptidyl-tRNA drop-off from the A site. In the presence of FA (gray curve), tripeptide formation displayed a burstlike phase, during which the initial rate was equal to that in the uninhibited case. Then tripeptide formation was linear in time until 8 s of incubation at a rate 8-fold slower than that in the absence of FA. The absence of a rate decrease in the long time range in the inhibited case was due to a slower reduction of the concentration of pretranslocation ribosomes and a smaller sensitivity to this reduction in the presence than in the absence of FA (see “Discussion”). The burst phase reflected the comparatively long time, $\tau_{\text{rel}}$, to establish the steady state fractions of free EF-G and EF-G inhibited in ribosome-EF-G-GDP-FA complexes, a phenomenon associated with “slow” or tight binding (20) enzyme inhibitors, like FA. It can be shown (see Equation 30) that $\tau_{\text{rel}}$ in the presence of FA was determined by the rate constant $q_{\text{eff}}^{\text{AG}}$ of FA dissociation from the inhibited complex and by the times of the uninhibited ($\tau_{\text{AG}}$) and inhibited ($\tau_{\text{AG}}$) G cycle in the steady state as follows.

$$\tau_{\text{rel}} \approx \frac{1}{(q_{\text{eff}}^{\text{AG}} \tau_{\text{AG}})}$$

(Eq. 46)

From the curves in Fig. 7, we estimated the uninhibited EF-G cycle time ($\tau_{\text{AG}}$) as 193 ms, the duration time of FA on inhibited ribosomes ($1/q_{\text{eff}}^{\text{AG}}$) as 5.3 s, and the relaxation time to the steady state fractions of free EF-G and EF-G inhibited in ribosome-EF-G-GDP-FA complexes, a phenomenon associated with “slow” or tight binding (20) enzyme inhibitors, like FA.
Fusidic Acid Inhibits Multiple Ribosomal States

state ($\tau_{rel} = 1/k_{rel}$) as 0.67 s (see Equations 33 and 34), which corresponds well to the relaxation time predicted by Equation 46. In the uninhibited and inhibited cases, incubation times up to 3 and 8 s, respectively, were used for the curve fitting. Considering FA inhibition of EF-G as a simple, uncompetitive mechanism (25), the ratio of the slopes of the linear part of the curves in Fig. 7 estimated the FA concentration at which the cycling rate was reduced by a factor of 2, $K_{50FA}$, as about 3 $\mu$M. The reason why this $K_{50FA}$ value was more than 2 times larger than the $K_{50FA}$ value obtained for the single turnover, tripeptide formation experiments described under “FA Inhibition of Translocation by EF-G in Single Round Mode” was the slow EF-G binding to the ribosome in the cycling experiment (Fig. 7), which greatly increased the cycling time of the factor (see “Discussion”).

DISCUSSION

We have characterized the inhibitory action of the antibiotic drug fusidic acid on the elongation cycle of the E. coli ribosome. We used quench flow and stopped flow techniques for quantitative analysis of FA-dependent inhibition of peptide elongation in single turnover as well as in EF-G-cycling experiments. The results reveal previously unknown mechanistic aspects of FA action in translocation and pave the way for quantitative modeling of FA action in the living cell.

High Resolution Kinetics by Separation of Time Scales in Single Turnover Experiments—Here we used single turnover experiments for the whole elongation cycle of the ribosome and for the partial reactions leading from ternary complex binding to peptidyl transfer. All experiments were strictly interpreted in terms of average times, which makes the approach compatible with steady state situations in vivo and in vitro. There was a clear separation of time scales for FA-inhibited and uninhibited ribosomes; the elongation cycle lasted about 100 ms for an uninhibited ribosome and about 6 s or longer for an inhibited ribosome, as also observed with single molecule in vitro experiments (28). This made it possible to determine the probability, $p_{rel}^1$, of the ribosome inhibition by FA per elongation cycle as well as the FA-dependent inhibition time, $\tau_{rel}^1$, with high precision. A major finding by this experimental approach is that FA targeted ribosome-bound EF-G in multiple states of the peptide elongation cycle. The methodology developed here can be used for studying other translocation-targeting antibiotics that act as tight binding or “slow” inhibitors.

FA Targets Ribosome-bound EF-G in Multiple States of the Elongation Cycle—We have found that FA bound with high efficiency to EF-G already in an early stage of the translocation process (Fig. 4, target mode I). This state, $R_{G1}^3$, we tentatively identify as a ribosome structure with rotated subunits, containing EF-G in complex with GDP and ready to move the mRNA in relation to the 30 S subunit frame. The rationale for this proposal is that according to our fluorescence data (Fig. 6), this state was comparatively long lived. This would neatly account for the high efficiency by which state $R_{G1}^3$ was targeted by FA, as determined by the inhibition constant $K_{50FA}$ in Equation 44, equal to the ratio between the mRNA translocation rate constant ($k_{G1}^3$) and the FA association rate constant ($k_{FA}^3$) (Fig. 4). Subsequently, the FA-bound complex $R_{G1}^3$ rapidly continued to a downstream state, $R_{G2}^3$, in which FA remained bound on the average 9 s before it dissociated from the still EF-G-bound ribosome. After dissociation, FA could rebind to the EF-G-containing ribosome (target mode II, state $R_{G2}^3$, Fig. 4), albeit at reduced efficiency compared with that of the first target state $R_{G1}^3$, thereby prolonging the inhibition time $\tau_{rel}^3$ in an EF-G concentration-independent manner (Fig. 5C). The rapid downstream movement of the FA-bound ribosome from state $R_{G1}^3$ to $R_{G2}^3$, the subsequent dissociation of FA from $R_{G2}^3$, and the inefficient rebinding of FA to the $R_{G2}^3$ or $R_{G3}$ state downstream of $R_{G1}^3$ are reflected in the very sharp initial increase of the average elongation time at low FA concentrations followed by its gradual, linear increase in the high FA concentration range. We tentatively identify $R_{G2}^3$ with the intermediate translocation complex presented by Ramrath et al. (16), different from the EF-G-GDP-bound post-translocation complex here identified as $R_{G3}^3$, visualized in a crystal structure by Gao et al. (15) (Fig. 4). This suggestion is based on our experiments revealing complexes $R_{G2}^3$ and $R_{G1}^3$ to be distinct. In other words, the minimal inhibition time of complex $R_{G3}^3$ was longer (9 s) than that of $R_{G2}^3$ (6 s), although they had very similar inhibition constants. Notably, the minimal inhibition time of the dipeptidyl post-translocation complex, $R_{G4}^3$, was very similar to the one obtained with the initiation complex, with fMet-tRNA$^{fMet}$ in the P site, $R_{G4}^1$. This could be a consequence of their similar configuration. The proposition that a ribosome targeted by FA during translocation was stalled in a different state ($R_{G3}^3$) than a ribosomal state ($R_{G2}^3$) obtained by back binding of EF-G and FA to the post-translocation ribosome is further discussed under “FA Action and Ribosome Structures”.

Target mode III is the binding of FA to the post-translocation ribosome in complex with EF-G (Fig. 4, III). This may occur during the translocation process but also following rebinding of EF-G to the post-translocation ribosome. In the latter case, FA and EF-G jointly acted as competitive inhibitors of the EF-Tu-GTP-facilitated delivery of aminoacyl-tRNA to the ribosomal A site. At a fixed FA concentration, EF-G therefore served as a competitive inhibitor of the ternary complex with an inhibition constant inversely proportional to the concentration
of FA. The competitive nature of this inhibition mode was validated by the observation that the inhibition sensitivity increased at reduced ternary complex concentration (Fig. 3). This mode of FA inhibition was comparatively weak and therefore unlikely to play a major role in the living cell except in situations of low ternary complex levels. It is, however, possible that the hypersensitivity to FA caused by overexpression of EF-G from E. coli or Mycobacterium tuberculosis in E. coli cells (29) was due to competitive inhibition of ternary complex binding to the post-translocation ribosome by EF-G and FA, but further experiments will be required to resolve this issue.

How Sensitive Is the Translocating Ribosome to Fusidic Acid?—In our tripeptide formation experiments, where the time of one elongation cycle was around 100 ms, the $K_{50\%}$ value, *i.e.* the drug concentration that doubled the elongation cycle time, was 1.3 $\mu$M (Equation 44 with parameter values from Table 1). This predicts strong inhibition of protein elongation in the living cell in the low FA concentration range, although the per elongation cycle probability of an inhibition event would be low. Assuming an *in vivo* elongation cycle time of about 50 ms, the $K_{50\%}$ value is only 0.6 $\mu$M. At a FA concentration of 1.2 $\mu$M, the per cycle probability, $p_{I}^{1}$, of inhibition is 0.01 (Equation 45), leading to a 3-fold increase of the average elongation cycle time (Equation 44). Even at this low inhibition probability, there will, on the average, be three inhibitory events per translation of an average sized mRNA encoding a 300-amino acid protein. The probability, $P_M$, of an uninhibited translation of such an mRNA is Poisson distributed and given by the following.

$$P_M = e^{-M \cdot p_I}$$  
(Eq. 47)

With $M = 300$ and $p_I^1 = 0.01$, the chance of an uninhibited translation would be only 5%. One may also keep in mind that an FA-stalled ribosome will cause queuing of trailing ribosomes, an effect that will become more severe with increasing frequency of initiation of translation of the mRNA. At an FA concentration of 6 $\mu$M, the per elongation cycle inhibition probability is 0.05, the average elongation time is 10-fold longer than in the uninhibited case, and almost every ribosome is stalled and induces queuing at least once during each mRNA translation round. This means that even low concentrations of FA inside a bacterial cell would effectively block protein synthesis and inhibit bacterial proliferation.

In the present work, inhibition of protein synthesis was quantified as the increase of the average elongation cycle time with increasing FA concentration (Fig. 5D and Equation 44). The inhibition mechanism is complex and cannot be characterized by a single $K_I$ value, but the FA concentration at which the peptide elongation time was increased by a factor of 2, the $K_{50\%}$ value, is well defined. It is here shown to be around 1 $\mu$M, an estimate 200-fold smaller than a previous estimate of the $K_{50\%}$ value (IC$_{50\%}$ value) as 200 $\mu$M by Savelsbergh et al. (8). The latter estimate was based on a multicycle assay in which EF-G at rate-limiting concentration promoted translocation of pretranslocation ribosomes in initial excess over EF-G during a single, fixed incubation time at varying FA concentration. The fraction of translocated ribosomes was probed by peptidyl transfer to the small aminoacyl-tRNA analog puromycin and not to an authentic aminoacyl-tRNA, as in the experiment of Fig. 7. The $K_{50\%}$ value estimated for FA inhibition of translocation as 200 $\mu$M was 3 orders of magnitude higher than that for FA inhibition of ribosomal recycling (8). It was therefore argued that the primary growth-inhibitory action of FA in bacterial cells is on ribosomal recycling by EF-G and ribosome recycling factor and not on translocation (8). However, the much higher $K_{50\%}$ value in their experiments in comparison with $K_{50\%}$ of about 3 $\mu$M obtained in our EF-G-cycling experiments conducted under *in vivo* like conditions is easily accounted for by three major differences between their and our experimental setup (see “Materials and Methods” for details). (i) The much lower ribosome concentration in their assay resulted in slow EF-G association with the ribosome and artificially slow factor cycling in the absence of FA, which by itself greatly increased the $K_{50\%}$ value. If, for example, the elongation cycle time was prolonged from 50 ms, as *in vivo* (30), to 1 s by slow EF-G association, this would increase the $K_{50\%}$ value 20-fold (see Equations 36 and 37). (ii) During the single, fixed incubation time in their assay, the concentration of pretranslocation complex was greatly reduced by the progress of the translocation reaction, prohibiting the establishment of an authentic steady state and further reducing the FA sensitivity of the assay. (iii) Slow relaxation toward the steady state (see Fig. 7 and Equation 46), typical of slow inhibitors at low concentration (20), further reduced the FA sensitivity of their assay.

**FA Action and Ribosome Structures**—It has been suggested that FA locks EF-G in the GTP conformation after GTP hydrolysis and thereby prevents the factor from dissociating from the post-translocation ribosome (15). However, early cryo-EM studies demonstrated that whereas EF-G-GDPFA could form a stable complex with both the post-translocation ribosome in classical, unratcheted conformation with peptidyl-tRNA in the P site and the ratcheted ribosome with decacylated tRNA in the P/E site, EF-G, when bound to the non-cleavable GTP analog GDPNP, could only form a stable complex with the ratcheted ribosome containing a decacylated tRNA in the P/E site (14). Furthermore, mRNA translocation proceeded poorly when catalyzed by EF-G in complex with the non-hydrolyzable GTP analog GDPNP (31) but was virtually unhindered by FA (Fig. 6). This suggests that the EF-G-GDP-FA and EF-G-GDPNP complexes are fundamentally different and accordingly that the translocation inhibitory action of FA is more complex and interesting than mere locking of EF-G in the GTP conformation.

There exist in the literature two ribosomal structures of particular interest in the present context. One was obtained by x-ray crystallography (15), and the other was obtained by cryo-EM (16). Ramakrishnan and collaborators (15) prepared a post-translocation-like complex with decacylated tRNA$^{fMet}$ in the P and E sites to which they added EF-G, GDP, and FA. The crystal structure displayed the ribosome in classical conformation with unrotated subunits and EF-G, bound to GDP and FA, in a conformation distinct from the crystal structure of free EF-G-GDP. In contrast, Spahn and collaborators (16) prepared a ribosomal complex, containing decacylated tRNA$^{fMet}$ in the P site and Val-tRNA$^{Val}$ in the A site. To this they added EF-G, GTP, and FA, incubated for 20 min, and placed the resulting
Fusidic Acid Inhibits Multiple Ribosomal States

ribosomal complex on the grid for multicomponent cryo-EM. The structure of this complex, containing EF-G-GDP, FA and two tRNAs, was in a novel intermediate translocation conformation with Val-tRNA Val in the ap/P state and tRNA D Met in the pe/E state. Here, ap/P represents an intermediate state between the A and the P sites in the small and a full P site in the large subunit, and pe/E represents an intermediate state between the P and the E site with respect to the small and a full E site state in the large subunit. This would suggest that the cryo-EM structure (16) may correspond to the $R_{12}$ complex and the crystal structure (15) to the $R_{13}$ complex of the present work (Fig. 4). However, this conclusion is uncertain because the cryo-EM structure was achieved by translocation of an aminoacyl-tRNA rather than a peptidyl-tRNA. It is known that translocation of an aminoacyl-tRNA is much slower than translocation of a peptidyl-tRNA (32), and the long incubation time (20 min) could have allowed for dissociation of FA and movement of the ribosome from the $R_{12}$ state to a downstream complex, followed by reassociation of FA. Furthermore, the ribosomal complex in the crystal structure (15) with its two molecules of decacylated tRNA D Met was not an authentic post-translocation complex, so that the interpretation that the crystal structure corresponds to $R_{13}$ and the cryo-EM structure to $R_{12}$ must be taken with caution.

Acknowledgments—We thank Venki Ramakrishnan, Jody Puglisi, Maria Selmer, and Tanel Tenson for valuable comments on the manuscript.

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