Molecular mechanism of viomycin inhibition of peptide elongation in bacteria

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Viomycin is a tuberactinomycin antibiotic essential for treating multidrug-resistant tuberculosis. It inhibits bacterial protein synthesis by blocking elongation factor G (EF-G) catalyzed translocation of messenger RNA on the ribosome. Here we have clarified the molecular aspects of viomycin inhibition of the elongating ribosome using pre-steady-state kinetics. We found that the probability of ribosome inhibition by viomycin depends on competition between viomycin and EF-G for binding to the pretranslocation ribosome, and that stable viomycin binding requires an A-site bound tRNA. Once bound, viomycin stalls the ribosome in a pretranslocation state for a minimum of ~45 s. This stalling time increases linearly with viomycin concentration. Viomycin inhibition also promotes futile cycles of GTP hydrolysis by EF-G. Finally, we have constructed a kinetic model for viomycin inhibition of EF-G catalyzed translocation, allowing for testable predictions of tuberactinomycin action in vivo and facilitating in-depth understanding of resistance development against this important class of antibiotics.

Significance

Antibiotics are widely used to treat bacterial infections, but their mechanisms of action are often poorly understood. Viomycin is a tuberactinomycin antibiotic used for treating multidrug-resistant tuberculosis. Using an in vitro translation system that displays kinetic rates comparable to those in living cells we have characterized the mechanism of action of viomycin and constructed a kinetic model for how viomycin inhibits the translocation step of the peptide elongation cycle. Our results are vital for understanding the mechanism of the antimicrobial activity of viomycin and its sister drugs in living bacteria as well as resistance mechanisms against them, which can have strong implications for global health.

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time in the seconds range and was treated as a background reaction (SI Materials and Methods).

When viomycin was included in the factor mixture, so that all ribosomes were viomycin-free before mixing in the quench-flow instrument, we observed a large effect on the kinetics of tripeptide formation (orange through violet traces in Fig. 1D), whereas the rate and amplitude of dipeptide formation remained unaffected (Fig. S1). The relative amplitude of the fast phase gradually decreased from around 85% in the absence of viomycin to essentially 0 at 50 μM viomycin, whereas its mean time remained virtually unaltered, indicating that the fast phase represents tripeptide formation on viomycin-free ribosomes. A new slow phase appeared with a mean time of several tens of seconds, likely representing tripeptide formation by viomycin-free ribosomes, which escape inhibition. The decrease in the amplitude of this phase with increasing viomycin concentration reflects the increasing fraction of viomycin-bound ribosomes. The overall amplitude decrease with increasing viomycin concentration is due to read through of the stop codon present after the Met-Phe-Thr reading frame. Solid lines represent fits of Eq. 51 to the data. (E) The fraction of ribosomes inhibited by viomycin at different EF-G concentrations as estimated by subtraction of the amplitude of the fast phase with viomycin from that without it, plotted as a function of the viomycin concentration. Solid lines represent fits of Eq. 2 to the data. (Inset) The same data but in the concentration range from 0–10 μM viomycin. (F) Fraction of viomycin-inhibited ribosomes at 10 μM EF-G with and without preincubation of the 70S ribosome with the drug at two viomycin concentrations. All error bars represent SEM.
Fig. 3. (A) Time traces of multiple turnover GTP hydrolysis by EF-G on viomycin-stalled pretranslocation complexes at four different EF-G concentrations. A vertical offset of 20 has been added to separate the lines for clarity of presentation. (B) Rate of turnover GTP hydrolysis plotted as a function of EF-G concentration. (C) Fold change in average elongation cycle time and GTP molecules hydrolyzed per elongation cycle calculated using Eqs. 4 and 5 plotted as a function of viomycin concentration. The uninhibited elongation rate is assumed to be 20 amino acids per second and the free EF-G concentration is set to 10 μM; all other model parameters are those from the main text. All error bars represent SEM.

The drug was included only in the factor mixture. Notably, the fraction of viomycin-inhibited ribosomes was exactly the same (Fig. 1F), meaning that preincubation of viomycin with the initiation complex did not confer any advantage to the drug in its competition with EF-G for ribosome binding. It also implies that ribosomes with an empty A-site bind only a negligible amount of viomycin at the concentrations used, indicating that stable viomycin binding requires an A-site bound tRNA.

The Time a Ribosome Remains Inhibited After Viomycin Binding Depends Only on the Viomycin Concentration. To estimate the mean time for translocation after viomycin binding to the pretranslocation ribosome we designed a two-step tripeptide formation assay. First, viomycin-stalled pretranslocation ribosomes were formed by incubating initiated 70S ribosomes programmed with a truncated MFT mRNA and P-site bound [3H]Met-tRNA\textsuperscript{Met} for 5–10 s at 37 °C with Phe-tRNA\textsuperscript{Phe}–containing ternary complex and varying concentrations of viomycin. The resulting viomycin-stalled pretranslocation complexes were then mixed with EF-G, Thr-tRNA\textsuperscript{Thr}–containing ternary complex, and viomycin, leading to slow translocation of [3H]Met-Phe-tRNA\textsuperscript{Phe} followed by rapid formation of [3H]Met-Phe-Thr tripeptide. An mRNA truncated directly after the Thr codon was used to avoid viomycin-mediated read-through of the stop codon (Fig. S2). The reactions were stopped at different incubation times by formic acid and the relative amount of each peptide was determined as described above.

In these experiments we observed a complete absence of the fast phase of tripeptide formation (Fig. 2A), implying that virtually all ribosomes were viomycin-bound even at the lowest viomycin concentration (1 μM). In contrast, when 70S initiation complex with empty A-site and P-site bound [3H]Met-tRNA\textsuperscript{Met} was preequilibrated with viomycin in the previous section, the fraction of inhibited ribosomes did not change (Fig. 1F), indicating negligible drug binding. Taken together these results demonstrate that the affinity of viomycin to the ribosome greatly increases upon A-site binding of a tRNA. The mean time of tripeptide formation on viomycin-stalled ribosomes was estimated by fitting a single exponential function to each of the curves in Fig. 2A. The mean time increased linearly with viomycin concentration from around 45 s at 1 μM to around 120 s at 100 μM (Fig. 2B), indicating that after dissociation the drug could reassociate to the pretranslocation ribosome and prolong the stall. In contrast to the fraction of viomycin-inhibited ribosomes, which displayed strong negative correlation with EF-G concentration (Fig. 1E), the mean times displayed no EF-G concentration dependence in the 2.5–10 μM range (Fig. 2B). This suggests that viomycin dissociation and reassociation occur on an EF-G bound pretranslocation ribosome.

The Average Time of an Elongation Cycle Increases Nonlinearly with Viomycin Concentration. A major determinant of the growth inhibitory effect of ribosome targeting antibiotic drugs is their ability to slow down peptide elongation. Viomycin is a “slow” inhibitor of mRNA translation that stalls the ribosome for a time, \( \tau_s \), substantially longer than that required to carry out an uninhibited elongation cycle, \( \tau_0 \) (Fig. 2B). Therefore, its effect on the average time of one elongation cycle, \( \tau_{\text{avg}} \), can be conveniently written as (27)

\[
\tau_{\text{avg}} = \tau_0 + P_1 \cdot \tau_s.
\]

Here, \( P_1 \) is the probability that the ribosome is stalled by viomycin during one elongation cycle (Fig. 1E). The average time increased nonlinearly with increasing viomycin concentration (Fig. 2C) in a manner also observed in the case of fusidic acid inhibition of elongating ribosomes (27). The average time (\( \tau_{\text{avg}} \)) increased rapidly at low viomycin concentration as a consequence of the rapidly increasing probability that the ribosome becomes stalled (Fig. 1E). In contrast, at high viomycin concentration where the ribosome is virtually guaranteed to stall \( \tau_{\text{avg}} \) increased slowly due to viomycin rebinding events (Fig. 2B). This nonlinear response of the average elongation cycle time to viomycin concentration confirms that viomycin can bind to two ribosomal states during the elongation cycle. First, the drug binds with high efficiency to a transient state, likely the EF-G–free pretranslocation ribosome (Fig. 4). This first binding event is rapidly followed by transition to a downstream stalled state, likely the EF-G–bound pretranslocation ribosome (Fig. 4). In this state, the stalling time increased linearly with viomycin concentration due to repeated slow dissociation from and fast reassociation of viomycin to the stalled ribosome.

Viomycin Inhibition Leads to Multiple Rounds of Futile GTP Hydrolysis by EF-G Before Translocation. EF-G can bind to the viomycin-stalled pretranslocation ribosome (12, 14, 17). We wished to know whether such binding leads to multiple cycles of GTP hydrolysis before successful translocation. Therefore, we designed an experiment to follow multiple turnover GTP hydrolysis by EF-G in the presence of viomycin-stalled pretranslocation ribosomes. For that, initiated 70S ribosomes programmed with MFT mRNA and P-site bound [3H]Met-tRNA\textsuperscript{Met} were rapidly mixed in a quench-flow instrument with a mixture of Phe-tRNA\textsuperscript{Phe}–containing ternary complex, viomycin, [3H]GTP, and varying concentrations of EF-G. Viomycin-stalled pretranslocation complex was formed within 20 ms (Fig. S1). The reactions were stopped at different incubation times by formic acid and the relative amounts of [3H]GTP and [3H]GDP were determined by anion exchange chromatography with on-line radiation detection.

At all EF-G concentrations [3H]GDP accumulated linearly with time (Fig. 3A), showing that steady-state cycling of EF-G on the ribosome was established rapidly. The turnover rate of GTP hydrolysis, zero in the absence of EF-G, saturated already at the lowest EF-G concentration used (0.625 μM), as expected from the lack of an EF-G concentration dependence of the stalling time observed above (Fig. 2B). We observed a small linear increase in the GTP hydrolysis rate at higher EF-G concentrations from 3.9 s\(^{-1}\) at
0.625 μM to 6.6 s⁻¹ at 7.5 μM EF-G (Fig. 3B), likely due to EF-G reacting with free 50S subunits. Accounting for this side reaction the maximal rate of GTP hydrolysis by EF-G on viomycin-stalled pretranslocation ribosomes, \( k_{\text{GTP}} \), was estimated as 3.75 ± 0.20 s⁻¹ from the y axis intercept of the straight line in Fig. 3B. This estimate corresponds to a dwell time of 270 ± 15 ms for EF-G on the viomycin-stalled ribosome, in agreement with previously published dwell time estimates based on single-molecule FRET experiments (12, 28).

A Kinetic Model for Viomycin Action on Translating Ribosomes. The simplest model for viomycin inhibition of protein synthesis that accounts for all of the in vitro results described in the present work is shown in Fig. 4. The total probability that a ribosome becomes inhibited by viomycin during an elongation cycle is a combination of the probabilities that viomycin binds either to the first or to the second sensitive state and is given by

\[
P_i = 1 - \frac{K_{I1} \cdot [\text{EF-G}]}{K_{I1} \cdot [\text{EF-G}] + [\text{Vio}]} - \frac{K_{I2}}{K_{I2} + [\text{Vio}]}.
\]

The second term on the right side is the product of the probabilities that the ribosome escapes inhibition in the first and the second state. The inhibition constant, \( K_{I1} \), is the ratio between the binding rate constants of EF-G and viomycin to the pretranslocation ribosome (\( k_G \) and \( k_{Vio} \) in Fig. 4). The inhibition constant, \( K_{I2} \), is the ratio between the rate constants for the forward step required for the ribosome to escape from the second viomycin-sensitive state and for viomycin binding to that state (\( k_{\text{trans}} \) and \( k_{Vio} \) in Fig. 4).

The time that a ribosome remains inhibited by viomycin is given by

\[
\tau_i = \frac{1}{q_{Vio} \cdot \left( 1 + \frac{[\text{Vio}]}{K_{I2}} \right)}.
\]

Here \( q_{Vio} \) is the rate constant for viomycin dissociation from the second viomycin-sensitive state, the EF-G-bound ribosome. The average elongation cycle time can now be written in terms of the elementary rate constants of the translocation process in the presence of viomycin as

\[
\tau_{\text{avg}} = \tau_i + \tau_1 = \tau_0 + \frac{1}{q_{Vio} \cdot \left( 1 + \frac{[\text{Vio}]}{K_{I1} \cdot [\text{EF-G}] + [\text{Vio}]} \right)} \cdot \frac{[\text{Vio}]}{[\text{Vio}]} + \frac{[\text{Vio}]}{K_{I1} \cdot [\text{EF-G}] + [\text{Vio}]},
\]

(4)

The parameters \( K_{I1} \) and \( K_{I2} \) for viomycin dissociation from the EF-G-bound ribosome, \( q_{Vio} \), is 0.022 ± 0.0005 s⁻¹. The inverse of this rate constant estimates the residence time for viomycin on this complex as 44 ± 1 s. From Eq. 4 we determined IC₅₀ values for translocation inhibition by viomycin, as defined as the viomycin concentration required to double the duration of an average elongation cycle. IC₅₀ values at 2.5, 5, and 10 μM EF-G are 5, 6, and 9 nM viomycin, respectively.

Using Eq. 4 it is possible to account for the futile cycles of GTP hydrolysis by EF-G on viomycin-stalled ribosomes. The average number of GTP molecules hydrolyzed per elongation cycle as a function of the viomycin concentration is given by

\[
GTP_{\text{avg}} = GTP_0 + k_{\text{GTP}} \cdot \frac{P_i \cdot \tau_i}{q_{Vio} \cdot \left( 1 + \frac{[\text{Vio}]}{K_{I1} \cdot [\text{EF-G}] + [\text{Vio}]} \right)}.
\]

(5)

Here \( GTP_0 \) is the number of GTP molecules hydrolyzed during an uninhibited elongation cycle, that is, 2, \( k_{\text{GTP}} \) is the (saturated) turnover rate of GTP hydrolysis by EF-G on the viomycin-stalled ribosome and \( P_i \cdot \tau_i \) is the average time per elongation cycle the ribosome is viomycin-bound and stimulating futile GTP hydrolysis by EF-G. Considerably more viomycin is required to double the GTP cost of an elongation cycle than to double its duration (Fig. 3C): 15, 30, and 60 nM at 2.5, 5, and 10 μM EF-G, respectively.
Discussion
From the in vitro results presented here we constructed a simple yet powerful model for viomycin inhibition of translocation and estimated its three kinetic parameters, KI1, KI2, and qV/2 (Fig. 4 and Eq. 4). According to this model there are two viomycin-sensitive states in the elongation cycle. Elongating ribosomes first become viomycin-sensitive upon aminoacyl tRNA delivery to the ribosomal A site by EF-Tu. This EF-G–free pretranslocation state is the most viomycin-sensitive state. Viomycin and EF-G compete for first binding to this state such that the translocation state is the most viomycin-sensitive state. Viomycin first becomes viomycin-sensitive upon aminoacyl tRNA delivery and EF-G binding brings the ribosome to the second viomycin-sensitive pretranslocation state, where viomycin-bound ribosomes stall for a minimum time of ~45 s. In this state, rebinding of viomycin prolongs the stalling time, which increases linearly with viomycin concentration and is doubled when the viomycin concentration reaches KI2, here estimated as 66 μM (Eq. 3). We note that the minimal stalling time of ~45 s after a successful viomycin attack is equivalent to the time required to translate roughly 900 codons in rapidly growing Escherichia coli cells with an average codon translation time of 50 ms (29). This greatly exceeds the average distance of 14–26 codons between ribosomes on mRNA (30), and therefore viomycin binding to a ribosome is expected to lead to queuing of trailing ribosomes behind it.

The existence of two viomycin-sensitive states during the elongation cycle, one before and one after binding of EF-G to the ribosome, is supported by two recent structures of the viomycin-bound pretranslocation ribosome. One is a 3.3-Å crystal structure (18) containing three tRNAs and no EF-G, and the other is a 7-Å cryo-EM structure (17) containing two tRNAs and EF-G. In the former structure the ribosome is observed in the nonrotated state and the bound tRNAs are in the classical A, P, and E sites, whereas in the latter structure the ribosome is in the rotated state and the tRNAs are in the hybrid A/P and P/E states. Because FRET experiments suggested that viomycin binding induces rotation of the ribosomal subunits (11), the functional significance of the nonrotated viomycin-bound structure is unclear. Further structural work is required to understand the significance of different viomycin-bound states of the ribosome and also to clarify how the drug affects large-scale ribosome dynamics during translocation.

Our conclusion that viomycin binds tightly to the ribosome only after a tRNA has been delivered to the A site by EF-Tu also finds support in the crystal structure of the viomycin-bound ribosome (18). Here viomycin occupies the space between H69 and h44 that is vacated by the monitoring bases A1492 and A1493 when they engage with the codon–anticodon minihelix during mRNA decoding (21, 22, 31). This suggests that A-site binding of a tRNA liberates the viomycin binding site, which would otherwise be occupied by the monitoring bases. Further evidence that A-site binding of tRNA increases viomycin affinity to the ribosome comes from data presented in a recent single-molecule study (13). From their data we estimate a dissociation constant for viomycin binding to 70S ribosomes with an empty A site as large as 20 μM. In contrast, we observe that when viomycin is equilibrated with ribosomes with peptidyl tRNA in the A site, drug binding is fully saturated already at 1 μM, implying a dissociation constant much smaller than 1 μM.

The free concentration of viomycin required to significantly reduce the average peptide elongation rate is very low, in the nanomolar range under in vivo-like conditions (Fig. 3C). In addition to cell permeability factors, drug efflux pumps and drug degradation pathways the sensitivity of bacteria to viomycin will depend on the concentration of intracellular ribosomes and their sensitivity to the drug, as determined by the numerical values of the three parameters, KI1, KI2, and qV/2. Here it is relevant to consider the clinical drug target M. tuberculosis, which is known to be significantly more sensitive to viomycin and its sister drug capreomycin than E. coli (32). Slow-growing mycobacteria such as M. tuberculosis maintain a much lower intracellular ribosome concentration than the fast-growing E. coli (33). This by itself would increase the drug susceptibility of M. tuberculosis because at any intracellular drug concentration a larger fraction of the ribosomes would be disabled. However, the drug susceptibility of individual ribosomes does play a major role, as evidenced by multiple resistance mutations present either in ribosomal RNA (34) or in the tRNA methylase ThyA (32, 35). In our model such resistance mutations would lead to changes in the numerical values of the parameters KI1, KI2, and qV/2. Decreased drug binding rate or decreased lifetimes of the two drug sensitive states would be reflected by larger KI1 and KI2 constants. Other members of the tuberactinomycin family such as capreomycin and enniomycin share the same binding site on the bacterial ribosome and have effects on bacterial cells similar to those of viomycin. Thus, our model likely describes translocation inhibition by the entire tuberactinomycin class of antibiotics, and differences in drug efficacy are likely caused by different values of the three parameters rather than different mechanisms.

Our results show that viomycin inhibition confers an extra energy cost due to the futile cycling of EF-G on viomycin-stalled ribosomes. However, much higher concentrations of viomycin are required to increase the GTP cost of an elongation cycle than are required to reduce the elongation rate (Fig. 3C). In vivo implication of this extra energy loss by viomycin-bound ribosomes is hard to estimate, but it could be significant under energy-limited conditions.

In summary, we have characterized the mechanism of viomycin inhibition of the elongation step of bacterial protein synthesis. The kinetic model we present here along with estimates of its three key parameters provides a quantitative basis for understanding the antimicrobial activity of viomycin, also applicable in vivo. We are optimistic that our model is general enough to be instrumental also for characterization of other tuberactinomycin antibiotics and resistance mechanisms that have evolved also against these viomycin-related drugs. Finally, our model will aid characterization of more effective elongation inhibitors, which is one of the main goals of global antibiotics research.

Materials and Methods
Reagents and Buffers. All experiments were performed at 37 °C in Heps-polymixin buffer [95 mM KCl, 5 mM NH4Cl, 0.5 mM CaCl2, 8 mM putrescine, 1 mM spermidine, 5 mM potassium phosphate, 5 mM Mg(OAc)2, 1 mM dithiothreitol, and 5 mM Heps, pH 7.5]. All reaction mixes except those for the GTP hydrolysis experiments contained 1 mM ATP, 1 mM GTP, 10 mM phospho-
 enolpyruvate (PEP), 1 μM pyruvate kinase (PK), and 0.1 μM myokinase (MK). In the GTP hydrolysis experiments the reaction mixes contained 1.5 mM ATP, 0.5 mM [3H]GTP, and 10 mM PEP but no PK or MK. His-tagged initiation factors IF1, IF2, and IF3; elongation factors EF-G, EF-Tu, and EF-Ts; and acyl-
tRNA-synthetases (ThrRS and PheRS) were purified using nickel-affinity chromatography (HiTrap; GE Healthcare). All protein concentrations were determined using the Bradford assay. Ribosomes (E. coli MR6000) and [3H]Met-
tRNA[Met] were prepared according to ref. 36, and ribosome concentration was determined spectrophotometrically. XR7 mRNA with the coding sequence Met-
Phe-Thr-Stop (AUG-UUU-ACG-AUU) and the truncated mRNA coding MFT were used as templates for translation in a cell extract. The truncated mRNA coding MFT (AUG-UUU-ACC) were used as template for transcription of the ribosome was purchased from IBA. Bulk tRNA was prepared from E. coli MR6000 according to ref. 37. The [3H]Met and [3H]GTP were prepared from Perkin-Elmer, viomycin was from USP, and all other chemicals were from either Merck or Sigma-Aldrich.

Quench-Flow Tripeptide Formation Experiments. Two mixtures were prepared. The initiation mixture contained 70S ribosomes (0.3 μM), IF1, IF2, and IF3 (1 μM each), [3H]Met-tRNA[Met] (1.5 μM), and MFT mRNA (0.7 μM). The elongation mixture contained EF-Tu (2 μM), EF-Ts (2 μM), EF-G (10, 20, or
40 μM), threonine (200 μM), phenylalanine (200 μM), ThrRS (0.5 μM), PheRS (0.5 μM), bulk tRNA (150 μM), of which tRNA^Phe and tRNA^Thr made up 4 μM and tRNA^Thr, 1.7 μM, and 2.3 μM of overexpressed tRNA^Thr. Viomycin (1–100 μM) was added either to the elongation mixture or to both mixes as indicated. After 15-min incubation at 37 °C equal volumes of the two mixes were rapidly mixed and the reaction quenched at different time points with formaldehyde (17% final concentration) using a quench-flow instrument (RQF-3; KinTek Corp.). After quenching the samples were centrifuged at 20,800 × g for 10 min. The supernatant was re-suspended in 165 μL 0.5 M KOH to cleave the peptides from the tRNA. After 10 min 13C3H6O/MeOH/trifluoroacetic acid (62/38/0.1 by volume) mobile phase and a C-18 column (Merck) with on-line scintillation counting (β-RAM model 3; INUS Systems) to quantify the remaining amounts of f[3H]Met, f[3H]Met-Phe, and f[3H]Met-Phe-Thr peptides.

Sequential Tripeptide Formation Experiments. Three mixes were prepared. The initiation mixture contained ribosomes (0.9 μM), IF1, IF2, and IF3 (1 μM each), f[3H]Met-tRNA^Met (0.8 μM), and truncated MFT mRNA (1 μM). Two elongation mixes were prepared. The first contained phenylalanine (200 μM), PheRS (0.5 μM), tRNA^Phe (4 μM), EF-Tu (5 μM), EF-Ts (2 μM), and viomycin (2–200 μM). The second contained threonine (200 μM), ThrRS (0.5 μM), EF-Tu (8 μM), EF-Ts (2 μM), bulk tRNA (225 μM), of which tRNA^Thr and tRNA^Phe made up 6 μM, viomycin (1–100 μM), and EF-G (15–60 μM). All three mixes were incubated for 15 min at 37 °C. During the experiment, one volume of the initiation mixture was mixed with one volume of the first elongation mixture, and the resulting mixture was incubated for 5–10 s and then one volume of the second elongation mixture was added. The reaction was quenched at different time points after the addition of the second elongation mixture using formaldehyde (17% final). The samples were treated identically to the quench-flow samples above.

GTP Hydrolysis Experiments. Two mixes were prepared. The initiation mixture contained 70S ribosomes (0.5 μM), fMet-tRNA^Met (0.8 μM), and MFT mRNA (0.8 μM). The elongation mixture contained phenylalanine (200 μM), PheRS (0.5 μM), overexpressed tRNA^Phe (4 μM), EF-Tu (8 μM), EF-Ts (1 μM), EF-G (1–40 μM), and viomycin (600 μM). Both reaction mixes contained 0.5 mM [γ-32P]GTP, 1.5 mM ATP, and 10 mM PEP. After 15-min incubation at 37 °C equal volumes of the two mixes were rapidly mixed and the reaction was quenched at different time points with formaldehyde (17% final concentration) using the quench-flow instrument. The acid-quenched samples were centrifuged at 20,800 × g. The supernatant containing the [γ-32P]GTP and [γ-32P]GDP was analyzed by anion exchange chromatography with on-line scintillation counting (β-RAM model 3; INUS Systems). A Mono-Q GL column (GE Healthcare) was used and the mobile phase was a multistep gradient of 0–2 M NaCl in 20 mM Tris (pH 7.5).

Data Analysis and Curve Fitting. All curve fitting was done in MATLAB R2014b (MathWorks) using the Leonardt–Marquardt algorithm as implemented in the curve-fitting toolbox. Detailed descriptions of curve-fitting procedures and derivations of the equations in the main text can be found in SI Materials and Methods.