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UNIVERSITY OF CALIFORNIA
SANTA CRUZ

**THE ROLE OF THE GTPASE FUNCTION OF ELONGATION FACTOR G
IN RIBOSOMAL TRANSLOCATION**

A dissertation submitted in partial satisfaction
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

CHEMISTRY

by

Gillian Kathleen Rexroad

March 2025

The Dissertation of Gillian Rexroad is
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ABSTRACT

The Role of the GTPase Function of Elongation Factor G in Ribosomal Translocation

Gillian Rexroad

During protein synthesis, the ribosome must move the tRNAs and mRNA together in single codon steps after the addition of each amino acid to the polypeptide. This process of translocation is catalyzed by the GTPase elongation factor G in prokaryotes. EF-G hydrolyzes GTP during each round of translocation, yet the purpose of this energy expenditure is unclear. Here, we first ask how inhibiting GTP hydrolysis using GTP analogs and a mutant form of EF-G impacts the structural rearrangements of the ribosome that take place during translocation, monitored with Förster resonance energy transfer (FRET). We find that hydrolysis is required only for reverse rotation of the 30S head domain, an event that occurs late in translocation after the tRNAs and mRNA have completed the bulk of their movement. We then investigate the specific role and timing of phosphate (Pi) release from EF-G, which is delayed relative to hydrolysis and is the step responsible for the bulk of the energy derived from hydrolyzing GTP. We determine the timing of Pi release relative to the structural rearrangements of the ribosome by monitoring structural dynamics with FRET and in parallel observing the kinetics of Pi release with a fluorescence-based reporter. We find that Pi release occurs after forward head rotation and, surprisingly, is coupled to reverse intersubunit rotation. Further, we show that both Pi release and EF-G dissociation are required for reverse head rotation. To account for these findings, we propose that delayed Pi release prevents premature dissociation of EF-G; this ensures that the codon-anticodon duplex is stabilized by EF-G throughout its movement to prevent a frameshift. We conclude that the GTPase function of EF-G, rather than driving tRNA movement, is crucial for enforcing accuracy during translocation. This function may well extend to other translational GTPases such as IF2 and EF-Tu, which also exhibit delayed Pi release and have critical roles in enforcing accuracy during different steps of protein synthesis.

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Candidate contributions: G.R. and H.F.N. designed the study, G.R. performed the experiments with the exception of the TLC assay (L.L.), G.R., J.P.D., and H.F.N. analyzed the data, and G.R., L.L., and H.F.N wrote the paper.

Chapter 1. Introduction

Overview of protein synthesis by the ribosome

In every organism, ribosomes synthesize diverse proteins based on nucleic acid sequences encoded in messenger RNAs (mRNAs). Ribosomes are large ribonucleoprotein complexes made up of two subunits, the small 30S subunit and large 50S subunit (in prokaryotes); each is composed of ribosomal RNA (rRNA) and proteins (Fig. 1A).

Protein synthesis has three main phases: initiation, elongation, and termination. During initiation, the ribosome and an initiator transfer RNA (tRNA) assemble at the start codon of the mRNA (reviewed in (Aitken and Lorsch, 2012; Milón and Rodnina, 2012; Caban and Gonzalez, 2015; Gualerzi and Pon, 2015; Hashem and Frank, 2018; Brito Querido et al., 2024)). In each round of elongation, an amino acid is added to the polypeptide. Elongation factor Tu (EF-Tu) (eEF-1A in eukaryotes) delivers a tRNA carrying an amino acid to the ribosome; selection of the correct tRNA based on the corresponding mRNA codon is monitored by the ribosome and aided by EF-Tu/eEF-1A (reviewed in Voorhees and Ramakrishnan, 2013; Maracci and Rodnina, 2016). Once the tRNA is accommodated, the ribosome catalyzes transfer of the amino acid to the growing polypeptide chain (Maden et al., 1968; Noller et al., 1992; reviewed in Rodnina et al., 2007). The ribosome must then move the mRNA and tRNAs (Fig. 1B) by precisely one codon in a process called translocation, which is catalyzed by elongation factor G (EF-G) in prokaryotes (eEF2 in eukaryotes) (Gupta et al., 1971). Finally, during termination, the synthesized protein is released with the assistance of release factors when the ribosome reaches a stop codon (reviewed in Korostelev, 2011; Hellen, 2018).

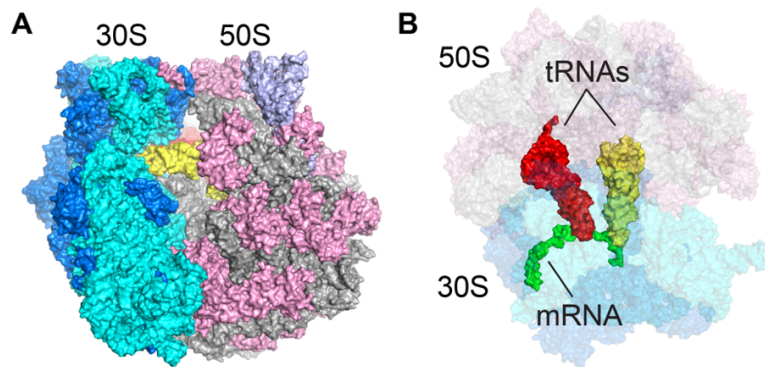


Figure 1. Structure of the ribosome bound with mRNA and tRNAs. (A) The bacterial ribosome has a small 30S subunit and large 50S subunit. (B) The ribosome bound with tRNAs and mRNA. 16S rRNA (cyan); 30S proteins (blue); 23S rRNA (grey); 5S rRNA (light purple); 50S proteins (pink); A-site tRNA (yellow); P-site tRNA (red); mRNA (green). (PDB 7ST7) (Carbone et al., 2021)

The mechanism of ribosomal translocation

During elongation, incoming tRNAs delivering amino acids bind to the A (aminoacyl) site of the ribosome. During peptidyl transfer, the polypeptide, which is initially connected to the tRNA in the P (peptidyl) site, is transferred to the A-site tRNA, leaving a deacylated tRNA in the P site (Fig. 2). The ribosome must then move the tRNAs and mRNA together by exactly one codon in a process termed translocation, which will be the focus of this thesis. During translocation, the tRNA in the A site carrying the polypeptide moves to the P site, leaving the A site empty for the next incoming aminoacyl-tRNA to pair with a newly exposed mRNA codon. The deacylated tRNA in the P site moves to the E (exit) site, from which it can dissociate.

The acceptor and anticodon ends of the tRNAs are moved in different steps, which are coupled to structural rearrangements of the ribosome. The sequence of events during translocation, including the identification of distinct translocation intermediates, has been enabled by a wealth of structural, biochemical, and kinetic data (reviewed in Noller et al., 2017a, 2017b; Korostelev, 2022; Noller, 2024). First, the acceptor ends of the tRNAs move on the large 50S subunit (Moazed and Noller, 1989; Frank and Agrawal, 2000) (Fig. 2); this is

coupled to forward intersubunit rotation (rotation of the entire 30S subunit relative to the 50S subunit) (Ermolenko et al., 2007; Carbone et al., 2021; Petrychenko et al., 2021; Rundlet et al., 2021) (Fig. 3A), and can happen spontaneously (Cornish et al., 2008). The resulting intermediate is termed the 'hybrid' state because the ends of each tRNA bind to different sites on the two subunits; for example, the A-site tRNA's anticodon end remains in the A site of the 30S subunit while its acceptor end has moved to the P site of the 50S subunit, hence its designation of A/P. The P-site tRNA similarly assumes a P/E hybrid state. Next, the anticodon ends of the tRNAs, paired with their associated mRNA codons, move relative to the 30S subunit body domain; this is facilitated by rotation of the 30S subunit head domain relative to the body domain (Ratje et al., 2010; Ramrath et al., 2013; Zhou et al., 2013, 2014) (Fig. 2; Fig. 3B). During this time, reverse intersubunit rotation begins (Ermolenko and Noller, 2011). By the end of this step, the A-site tRNA's anticodon end has moved to the P site relative to the 30S body domain but has not made contact with P-site elements of the head domain, leaving it in a 'chimeric hybrid' state (ap/P); the P-site tRNA assumes a comparable pe/E chimeric hybrid state. Reversal of the 30S head domain establishes final contacts between the tRNAs and the head domain (Gao et al., 2009; Zhou et al., 2014), completing the translocation sequence and leaving the tRNAs in their final states (P/P and E/E); both ends of the tRNA are in their final binding sites on both subunits (Fig. 2).

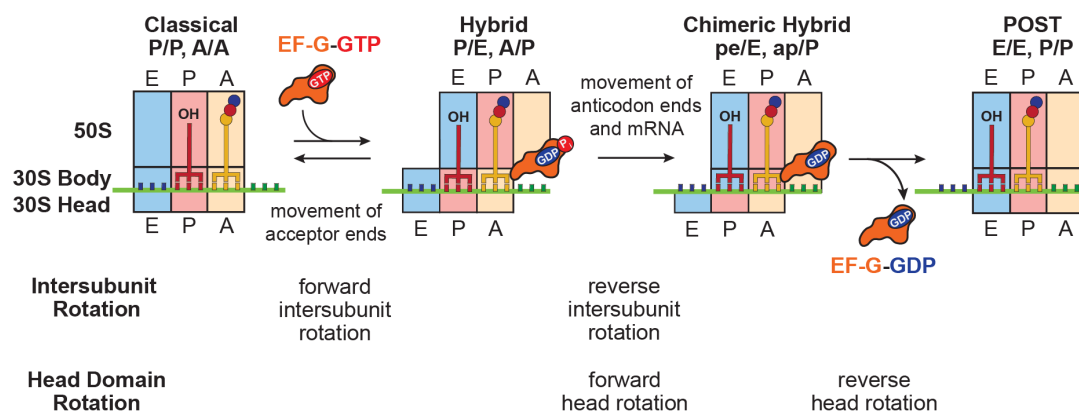


Figure 2. The mechanism of ribosomal translocation The acceptor and anticodon ends of the A- and P-site tRNAs (yellow and red, respectively) move on the ribosome in different steps. Movement is facilitated by structural rearrangements in the ribosome (intersubunit rotation and rotation of the 30S head domain). The ribosome is depicted using boxes to represent the 50S and 30S subunits. The 30S subunit is composed of two domains: the head and body. The A, P, and E sites are shown with yellow, red, and blue shading. EF-G (orange) catalyzes the process of translocation. mRNA is shown in green.

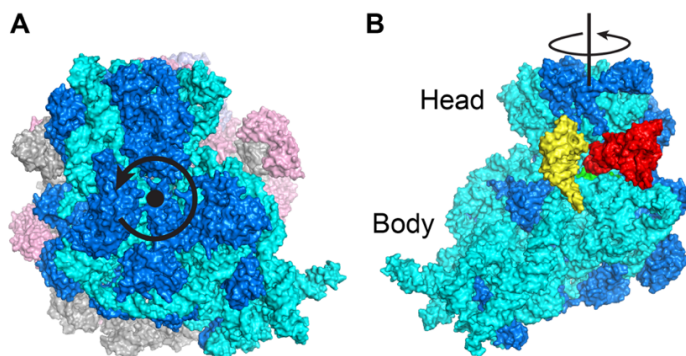


Figure 3. Structural rearrangements in the ribosome during translocation (A) Rotation of the small 30S subunit (blue, cyan) relative to the large 50S subunit (pink, gray). (B) Rotation of the 30S head domain relative to the body domain; 30S subunit is viewed from the subunit interface. Arrows depict the direction of forward rotation in both panels. Coloring scheme as in Fig. 1. (PDB 7ST7) (Carbone et al., 2021)

The debated role of GTP hydrolysis by EF-G

Although translocation can occur spontaneously *in vitro* under specific conditions (Gavrilova and Spirin, 1971; Cukras et al., 2003; Fredrick and Noller, 2003), elongation factor EF-G increases this rate by at least 4 orders of magnitude (Rodnina et al., 1997; Fredrick and Noller, 2003). EF-G is a 78 kDa protein with five domains, including a GTPase domain (Fig.

4A). EF-G binds the ribosome in complex with GTP (Fig. 2), and the ribosome acts similarly to a GTPase activating protein (GAP), activating EF-G and allowing it to rapidly hydrolyze GTP. The tip of domain IV of EF-G then contacts and stabilizes the A-site codon-anticodon duplex (Gao et al., 2009; Brilot et al., 2013; Ramrath et al., 2013; Zhou et al., 2014, 2019) (Fig. 4B) while the tRNAs and mRNA are moving to guard against a frameshift (Peng et al., 2019; Niblett et al., 2021). EF-G finally dissociates from the ribosome in its low-affinity GDP-bound form (Katunin et al., 2002; Savelsbergh et al., 2005) near the end of translocation (Fig. 2). Translocation still proceeds when GTP is replaced by a non-hydrolyzable GTP analog (Inoue-Yokosawa et al., 1974), albeit at rates ~50-fold slower (Rodnina et al., 1997; Katunin et al., 2002; Munro et al., 2010; Cunha et al., 2013); thus, hydrolysis is not required for translocation. Why, then, is GTP hydrolyzed?

The role of GTP hydrolysis in translocation is debated. It has been proposed that the energy derived from GTP hydrolysis drives tRNA movement through a conformational change in EF-G, akin to a “power stroke” in myosin, that actively pushes the tRNAs (Rodnina et al., 1997; Chen et al., 2013, 2016; Yao et al., 2013; Yin et al., 2019; Petrychenko et al., 2021). Other proposals describe how GTP hydrolysis is coupled to tRNA movement instead by inducing a structural rearrangement in the ribosome (Agrawal et al., 1999; Frank and Agrawal, 2000; Savelsbergh et al., 2003; Peske et al., 2004; Cunha et al., 2013; Holtkamp et al., 2014; Rodnina et al., 2020). An alternative model proposes that the tRNAs move spontaneously due to thermal energy in the environment; EF-G acts as a “pawl” in a Brownian ratchet mechanism of translocation, biasing movement of the freely diffusing tRNAs in the forward direction (Cornish et al., 2008; Spirin, 2009; Frank and Gonzalez, 2010; Ratje et al., 2010; Liu et al., 2014; Salsi et al., 2016; Wasserman et al., 2016; Carbone et al., 2021; Djumagulov et al., 2021; Ou and Petrov, 2023; Milicevic et al., 2024). In this model, GTP hydrolysis is used as a switch, enabling dissociation of EF-G. This model is consistent with findings that hydrolysis is essential only for events that occur near the *end* of translocation

(Flis et al., 2018), including dissociation of EF-G (Inoue-Yokosawa et al., 1974; Savelsbergh et al., 2009; Munro et al., 2010; Cunha et al., 2013).

In Chapter 2, we investigate the role of GTP hydrolysis by asking how blocking hydrolysis affects the structural rearrangements of the ribosome during translocation. We find that GTP hydrolysis is essential only for reverse rotation of the 30S head domain, an event that occurs very late in translocation. This finding contradicts proposals that hydrolysis drives tRNA movement and instead supports a Brownian ratchet model of translocation.

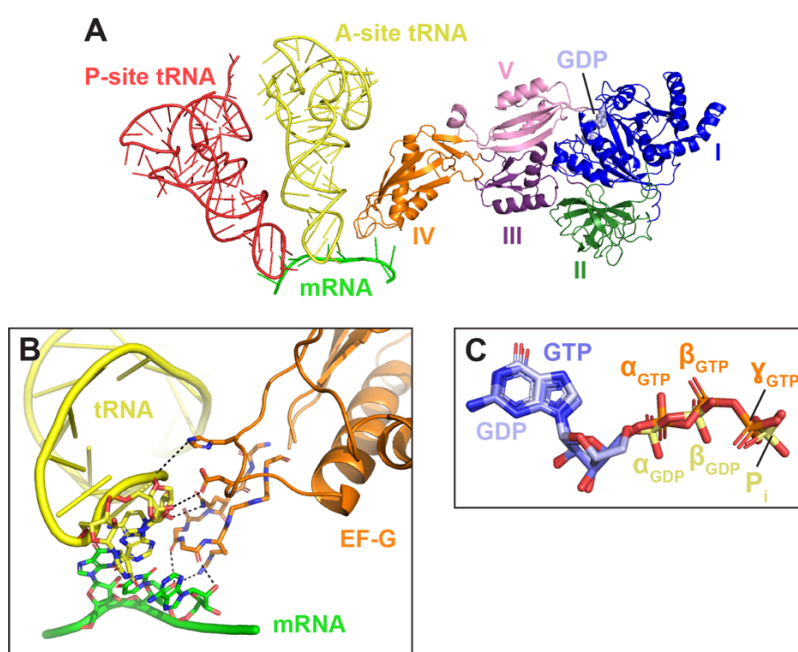


Figure 4. The GTPase EF-G stabilizes the codon-anticodon duplex during translocation (A) EF-G has five domains (I-V). The tip of domain IV of EF-G contacts the paired A-site tRNA anticodon and mRNA codon (PDBs 7ST7, 7SSD) (Carbone et al., 2021). (B) Interactions made between domain IV of EF-G and the A-site codon-anticodon duplex (PDB 4W29) (Zhou et al., 2014). (C) Overlay of GTP and GDP+Pi. The cleaved phosphate (Pi) is in almost exactly the same position as the uncleaved γ-phosphate. Phosphates in GTP and GDP+Pi are colored orange and yellow, respectively (PDBs 7ST7 (Carbone et al., 2021), 7N2V (Rundlet et al., 2021)).

The timing of phosphate release from EF-G

In our study of GTP hydrolysis, we made the puzzling observation that although GTP is hydrolyzed very rapidly upon EF-G·GTP binding, its effects are not felt until much later in

translocation. We note that although hydrolysis is rapid, the cleaved phosphate (Pi) remains bound to EF-G in almost exactly the same position as in unhydrolyzed GTP (Carbone et al., 2021; Petrychenko et al., 2021; Rundlet et al., 2021) (Fig. 4C). Pi is only released following a delay and allows EF-G to dissociate in its low-affinity GDP form (Katunin et al., 2002; Savelsbergh et al., 2005). The bulk of the energy derived from hydrolyzing GTP results from the charge separation due to release of Pi, rather than the hydrolytic event itself (Ross, 2006; Fontecilla-Camps, 2022). Thus, nearly all of the energy of GTP hydrolysis is preserved until late in translocation when Pi is released. Notably, delayed Pi release has also been observed for other translational GTPases, including elongation factor EF-Tu (Kothe and Rodnina, 2006) and initiation factors IF2 (Tomšić et al., 2000) and eIF2 (Algire et al., 2005) and appears to extend to many other classes of NTPases, including myosin (Brune et al., 1994; Takagi et al., 2004; Houdusse and Sweeney, 2016), F1 ATP synthase (Masaike et al., 2002), kinesin (Gilbert et al., 1995; Milic et al., 2014), and Ras when in complex with the GTPase activating protein neurofibromin (Allin et al., 2001; Kraemer et al., 2002; Phillips et al., 2003).

These observations raise several questions: (i) Why is Pi release delayed relative to hydrolysis? (ii) What event triggers Pi release? and (iii) What is the energy of Pi release used for? To investigate the role of Pi release, we ask when Pi is released with respect to the structural rearrangements of the ribosome during translocation. Its timing has been debated, with some proposing that it induces forward head rotation and tRNA movement (Petrychenko et al., 2021) and others concluding that it occurs later following reverse intersubunit rotation (Carbone et al., 2021). We also ask whether the structural dynamic events are dependent on or control phosphate release. The culmination of these two studies on GTP hydrolysis (Rexroad et al., 2022) and Pi release (Rexroad et al., in preparation) reveals a new understanding of the GTPase function of EF-G in protein synthesis.

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Chapter 2. The role of GTP hydrolysis by EF-G in ribosomal translocation

ABSTRACT

Translocation of tRNA and mRNA through the ribosome is catalyzed by the GTPase elongation factor G (EF-G) in bacteria. Although GTP hydrolysis accelerates translocation and is required for dissociation of EF-G, its fundamental role remains unclear. Here, we use ensemble FRET to monitor how inhibition of GTP hydrolysis impacts the structural dynamics of the ribosome. We use FRET pairs S12-S19 and S11-S13, which unambiguously report on rotation of the 30S head domain, and the S6-L9 pair, which measures intersubunit rotation. Our results show that, in addition to slowing reverse intersubunit rotation, as shown previously, blocking GTP hydrolysis slows forward head rotation. Surprisingly, blocking GTP hydrolysis completely abolishes reverse head rotation. We find that the S13-L33 FRET pair, which has been used in previous studies to monitor head rotation, appears to report almost exclusively on intersubunit rotation. To account for our finding that blocking GTP hydrolysis abolishes a rotational event that occurs after the movements of mRNA and tRNAs are essentially complete, we propose that the primary role of GTP hydrolysis is to create an irreversible step in a mechanism that prevents release of EF-G until both the tRNAs and mRNA have moved by one full codon, ensuring productive translocation and maintenance of the translational reading frame.

INTRODUCTION

Following formation of each peptide bond, the mRNA and tRNAs are translocated through the ribosome to advance the message by one codon. This process is catalyzed by a GTPase, the elongation factor EF-G (Gupta et al., 1971). Translocation is coupled to several large-scale conformational changes in the ribosome, including overall rotation of the 30S subunit relative to the 50S subunit (Frank and Agrawal, 2000; Horan and Noller, 2007) and rotation of the head domain of the 30S subunit (Spahn et al., 2004; Ratje et al., 2010).

Intersubunit rotation, which can occur spontaneously (Cornish et al., 2008), is coupled to translocation of the acceptor ends of the tRNAs on the 50S subunit (Ermolenko et al., 2007). Rotation of the 30S head domain, which requires participation of EF-G (Guo and Noller, 2012), is coupled to translocation of the anticodon ends of the tRNAs and their associated mRNA codons on the 30S subunit (Ratje et al., 2010; Ramrath et al., 2013; Zhou et al., 2013, 2014).

While GTP hydrolysis by EF-G is not required for a single round of translocation, as shown originally by Kaziro and co-workers using the non-hydrolyzable GTP analog GDPNP (Inoue-Yokosawa et al., 1974), it is required for the multiple turnover reaction as it is essential for dissociation of EF-G. Additionally, kinetic experiments show that GTP hydrolysis both precedes and accelerates translocation, suggesting that it plays a role in driving tRNA movement (Rodnina et al., 1997). The physical basis of this acceleration, however, has been debated. It has been proposed that GTP hydrolysis induces a conformational change in EF-G that either actively moves the tRNAs in a “power stroke” (Rodnina et al., 1997; Chen et al., 2013, 2016; Yao et al., 2013; Yin et al., 2019; Petrychenko et al., 2021) or that it induces a structural rearrangement in the ribosome that facilitates tRNA movement (Agrawal et al., 1999; Frank and Agrawal, 2000; Savelsbergh et al., 2003; Peske et al., 2004; Chen et al., 2013; Cunha et al., 2013; Holtkamp et al., 2014a; Rodnina et al., 2020). Others have proposed that GTP hydrolysis is coupled to tRNA-mRNA movement in a Brownian ratchet mechanism of translocation, in which movement is driven by thermal energy and EF-G acts as a pawl to prevent backwards movement (Cornish et al., 2008; Spirin, 2009; Frank and Gonzalez, 2010; Ratje et al., 2010; Liu et al., 2014; Salsi et al., 2016; Wasserman et al., 2016; Carbone et al., 2021; Rundlet et al., 2021). Although EF-G accelerates translocation by at least 10,000-fold (Rodnina et al., 1997; Fredrick and Noller, 2003), GTP hydrolysis contributes only about a 50-fold rate enhancement (Rodnina et al., 1997; Katunin et al., 2002; Munro et al., 2010; Cunha et al., 2013). Thus, it is evident that driving tRNA movement is not the primary role of GTP hydrolysis.

Efforts to dissect the impact of GTP hydrolysis on the kinetics of the large-scale structural dynamics of the ribosome have used FRET-based approaches to follow intersubunit rotation and rotation of the head domain of the 30S subunit (Ermolenko and Noller, 2011; Belardinelli et al., 2016; Wasserman et al., 2016; Belardinelli and Rodnina, 2017). While the effects of GTP hydrolysis on intersubunit rotation have been measured directly using the S6-L9 FRET pair (Ermolenko et al., 2007; Ermolenko and Noller, 2011; Belardinelli et al., 2016; Belardinelli and Rodnina, 2017; Levi et al., 2017), 30S head rotation has been inferred indirectly from changes in FRET efficiencies from fluorescent labels attached to sites that undergo more than one mode of movement (Belardinelli et al., 2016; Wasserman et al., 2016; Belardinelli and Rodnina, 2017). Here, we clarify how inhibition of GTP hydrolysis affects the kinetics of head rotation using the previously described FRET pairs S12(Alx488)-S19(Alx568) and S11(Alx488)-S13(Alx568) (Guo and Noller, 2012). These constructs each report directly and unambiguously on rotation of the 30S head domain, measuring energy transfer between one fluor in the head domain and another in the body domain. In parallel, we monitor intersubunit rotation using the previously reported S6-L9 FRET pair (Ermolenko et al., 2007).

We observe that blocking GTP hydrolysis modestly inhibits forward head rotation and slows reverse intersubunit rotation, as shown previously (Ermolenko and Noller, 2011). Surprisingly, we find that blocking GTP hydrolysis completely abolishes reverse rotation of the 30S head domain. We also show that the S13-L33 FRET pair previously used for monitoring 30S head rotation (Belardinelli et al., 2016) appears to report mainly on intersubunit rotation and has thus masked the effects of GTP hydrolysis on reverse head rotation. Based on our finding that blockage of GTP hydrolysis abolishes a rotational event that occurs after the movements of mRNA and tRNAs are essentially complete, we propose that the primary role of GTP hydrolysis is to enforce the accuracy and completion of each round of translocation.

RESULTS

Monitoring conformational dynamics in the ribosome with FRET

To monitor how GTP hydrolysis by EF-G affects the structural dynamics of the ribosome during translocation, we used ensemble FRET stopped-flow kinetics. Forward and reverse 30S head rotation were followed using the S12-S19 and S11-S13 FRET pairs (Guo and Noller, 2012) (Fig. 1A,B). The S6(Cy5)-L9(Cy3) FRET pair (Ermolenko et al., 2007) was used to follow intersubunit rotation (Fig. 1C). We also constructed the S13(Atto540Q)-L33(AIx488) FRET pair (Fig. S1) to directly compare our results to previous reports that used this pair to monitor 30S head rotation (Belardinelli et al., 2016).

A pre-translocation hybrid-state complex was formed with labeled ribosomes, a 39-nucleotide mRNA, deacylated elongator tRNA^{Met} bound in the P/E state and NAc-Met-Val-tRNA^{Val} in the A/P state (Methods). EF-G in the presence of GTP or various GTP analogs was rapidly delivered to the pre-translocation complex, and the fluorescence signal was recorded over a single round of translocation.

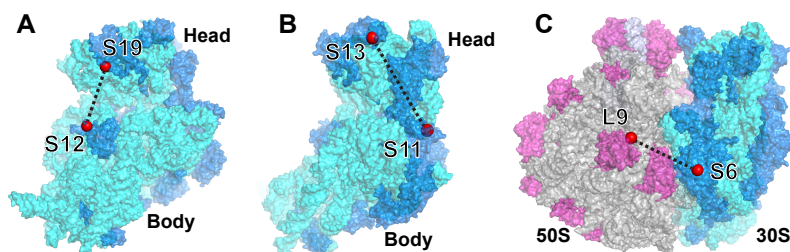


Figure 1. Positions of FRET pairs used to monitor ribosome dynamics. (A) S12-S19 and (B) S11-S13 FRET pairs report on rotation of the 30S head domain (Guo and Noller, 2012). (C) S6-L9 FRET pair reports on intersubunit rotation (Ermolenko et al., 2007). Ribosomal components are 16S rRNA (cyan), 30S proteins (blue), 23S rRNA (gray), 5S rRNA (light blue) and 50S proteins (magenta). Positions of fluorophores are indicated by red spheres. (PDB 4V9D) (Dunkle et al., 2011)

Intersubunit rotation proceeds in the absence of GTP hydrolysis

We first investigate the effects of inhibition of GTP hydrolysis on intersubunit rotation using the S6-L9 FRET pair (Ermolenko et al., 2007) (Fig. 1C) in order to directly compare

these rates with those from our head rotation reporters, described below. Since our pre-translocation complex has already undergone forward intersubunit rotation, we measure the rate constant of reverse intersubunit rotation by following the increase in acceptor fluorescence (Fig. 2A). With wild-type EF-G and GTP, the apparent rate constant for reverse intersubunit rotation is $\sim 4.3 \text{ s}^{-1}$ at 22°C (Table 1), in close agreement with that reported previously (Ermolenko and Noller, 2011). Although this rate is slower than that reported by Belardinelli et al. (Belardinelli et al., 2021) for the same pair ($\sim 47 \text{ s}^{-1}$), this difference can be explained by the lower temperature and concentration of EF-G (10-fold molar excess over ribosomes) used in our study compared to the conditions used by Belardinelli et al. (37°C and 80-fold excess).

To inhibit hydrolysis, we began by substituting GTP with the non-hydrolyzable analogs GDPNP and GDPCP and the slowly-hydrolyzed GTP analog GTP γ S. We also blocked GTP hydrolysis using EF-G carrying the GTPase-defective mutation H91L, identified previously in a screen for dominant-lethal mutations in EF-G (Niblett et al., 2021). We tested the ribosome-dependent GTP hydrolysis activity of EF-G (H91L) and found that it was undetectable over background, even after several minutes at 37°C (Fig. S2). In parallel, we tested the EF-G mutant H91A and also found it to be completely defective in GTP hydrolysis, in agreement with previous findings (Cunha et al., 2013; Salsi et al., 2014).

Inhibition of GTP hydrolysis using the analogs GDPNP, GDPCP and GTP γ S, or mutant EF-G(H91L) generally caused only modest (~ 2 - 4 -fold) decreases in the rate of reverse intersubunit rotation, except for the case of GDPCP, which reduced the rate by 39-fold (Fig. 2; Table 1), in general agreement with previous reports (Ermolenko and Noller, 2011; Belardinelli et al., 2016). These results show that although GTP hydrolysis modestly accelerates reverse intersubunit rotation, it is not required.

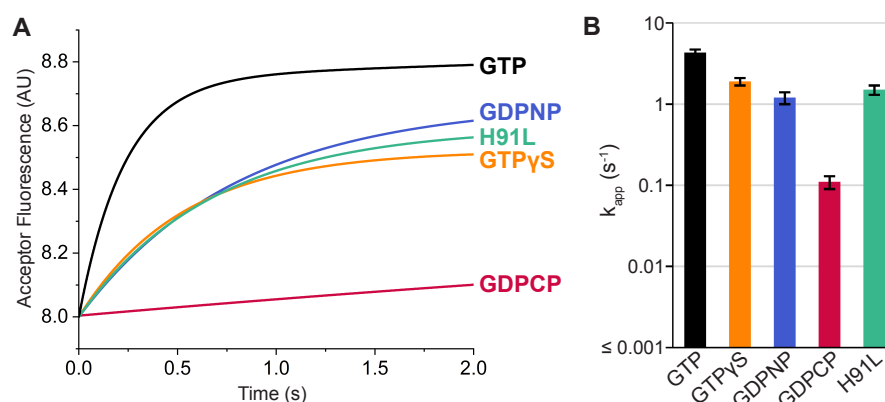


Figure 2. Effects of inhibition of GTP hydrolysis on reverse intersubunit rotation. (A) Reverse intersubunit rotation was monitored using the S6-L9 FRET pair (Ermolenko et al., 2007). At $t = 0$, a pre-translocation complex was rapidly mixed with either EF-G·GTP, EF-G·GDPNP, EF-G·GDPCP, EF-G·GTPγS, or EF-G(H91L)·GTP and changes in ensemble FRET efficiency were measured using a stopped-flow fluorometer (Methods). Curves are single-exponential functions fitted to the experimental traces (Fig. S3). With the exception of GDPCP, inhibiting GTP hydrolysis had only modest effects on the rate of reverse intersubunit rotation. (B) Pseudo-first order rate constants for the traces from panel (A) (Fig. S3; Table 1). Error bars represent standard deviation about the mean.

Table 1. Effects of Inhibition of GTP Hydrolysis on the Rates of Intersubunit and 30S Head Rotation¹

	Reverse Intersubunit Rotation	Forward 30S Head Rotation		Reverse 30S Head Rotation	
	S6·L9	S11·S13	S12·S19	S11·S13	S12·S19
GTP	4.3 ± 0.4	25 ± 10	23 ± 4	4.3 ± 0.6	4.9 ± 0.5
GTPγS	1.9 ± 0.2	2.3 ± 0.2	2.07 ± 0.07	0.24 ± 0.01	0.16 ± 0.03
GDPNP	1.2 ± 0.2	0.05 ± 0.01	0.059 ± 0.004	< 10 ⁻³	< 10 ⁻³
GDPCP	0.11 ± 0.02	0.25 ± 0.03	0.30 ± 0.08	< 10 ⁻³	< 10 ⁻³
H91L	1.5 ± 0.2	2.5 ± 0.4	2.2 ± 0.3	< 10 ⁻³	< 10 ⁻³

¹ Rotational rates represent apparent rate constants, given in s⁻¹, derived from single- or double-exponential functions fitted to the experimental traces (Figs. S3-5 and Figs. 2-4). Errors represent standard deviation about the mean.

Forward rotation of the 30S head domain proceeds in the absence of GTP hydrolysis

We next ask how inhibition of GTP hydrolysis affects the rates of rotation of the 30S subunit head domain. Upon delivery of EF-G·GTP to a pre-translocation complex containing the S12-S19 FRET pair, the FRET efficiency decreased with an apparent rate constant of 23

s^{-1} , indicating rapid forward rotation of the 30S subunit head domain (Fig. 3A, Table 1). This was followed by a slower (4.9 s^{-1}) increase in FRET efficiency, corresponding to reverse head rotation (Guo and Noller, 2012). Using ribosomes reconstituted with the S11-S13 FRET pair, similar forward and reverse rotational rate constants were observed (Fig. 3B, Table 1), but changes in FRET were anti-correlated with the S12-S19 pair, as expected (Guo and Noller, 2012).

Substituting GTP with the slowly-hydrolyzed GTP analog GTP γ S slowed the rate of forward head rotation by 11-fold, and the non-hydrolyzable GTP analogs GDPNP and GDCPC more strongly reduced the rate, by 450-fold and 90-fold, respectively (Figs. 3, 4A; Table 1). Surprisingly, the GTPase-deficient EF-G mutant H91L slowed forward head rotation substantially less (10-fold) than did the non-hydrolyzable analogs (Figs. 3, 4A). Our findings are consistent with previous biochemical evidence showing slow single-round translocation in the absence of GTP hydrolysis (Inoue-Yokosawa et al., 1974; Rodnina et al., 1997; Cunha et al., 2013) and structural studies showing 30S head rotation in the presence of GDPNP or GDCPC (Zhou et al., 2013; Demo et al., 2021).

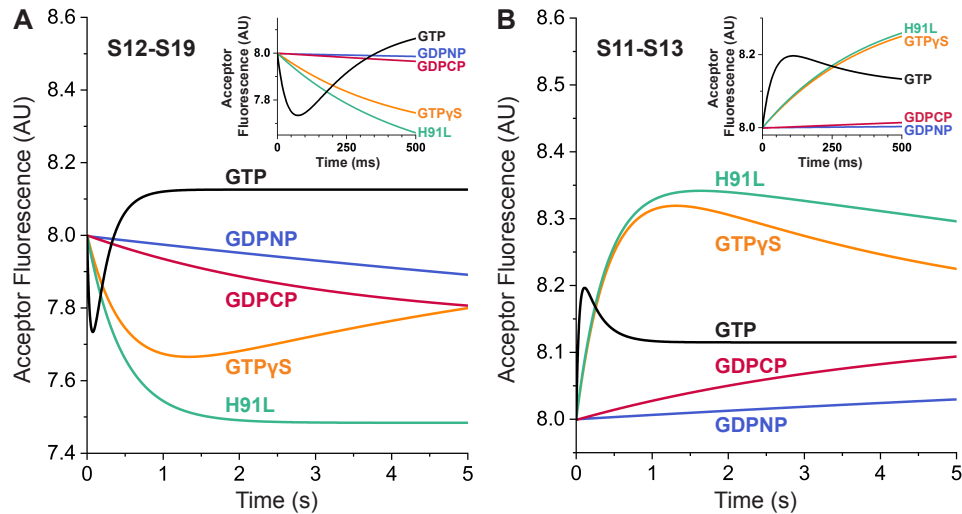


Figure 3. Effects of inhibition of GTP hydrolysis on 30S head rotation. Forward and reverse 30S head rotation were monitored with two anti-correlated FRET pairs (Guo and Noller, 2012). (A) For the S12-S19 FRET pair, decreasing and increasing acceptor fluorescence indicate forward and reverse rotation, respectively. (B) For the S11-S13 FRET pair, increasing and decreasing acceptor fluorescence indicate forward and reverse rotation,

respectively. Experimental traces were fitted to double-exponential functions (Fig. S4-5 and Table 1). Insets show expanded views of the first 500 ms.

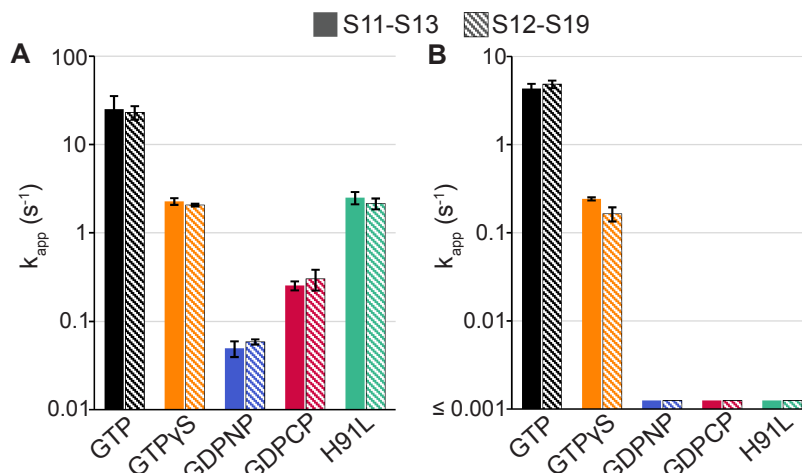


Figure 4. Reverse head rotation is abolished in the absence of GTP hydrolysis. Apparent rate constants for (A) forward and (B) reverse head rotation were obtained from fitting the traces from Fig. 3 to double-exponential functions (Fig. S4-5; Table 1). Error bars represent standard deviations about the mean.

Blocking GTP hydrolysis abolishes reverse rotation of the 30S head domain

Rates of reverse head rotation were obtained from the second phase of the traces in Figure 3, where changes in FRET efficiency reversed direction upon reversal of 30S head rotation (Figs. 3A,B). When GTP was replaced with the slowly hydrolyzed GTPγS, the rate of reverse head rotation was slowed by 23-fold, in general agreement with a previous report (Belardinelli et al., 2016). This demonstrates that slowing the rate of GTP hydrolysis impacts reverse head rotation to a greater degree than it affects forward head rotation (11-fold), as suggested previously (Belardinelli et al., 2016). But when GTP hydrolysis was blocked using the non-hydrolyzable GTP analogs GDPNP and GDPCP or the H91L mutant form of EF-G, reverse head rotation was undetectable (Figs. 3, 4). These results show that, in contrast to forward head rotation or intersubunit rotation, blockage of GTP hydrolysis abolishes reverse head rotation.

The S13-L33 FRET pair primarily reports on intersubunit rotation

Several of our observations involving 30S head rotation differ from those reported previously. For example, it was previously reported that forward head rotation was not observable in the presence of the non-hydrolyzable analogs GDPNP and GDCP and that reverse head rotation was slowed, but not abolished (Belardinelli et al., 2016). This contrasts with our findings of slowed forward head rotation but complete blockage of reverse head rotation with these analogs. In their study, 30S head rotation was inferred indirectly using a FRET pair with labels on proteins S13 in the 30S head domain and L33 on the 50S subunit (Belardinelli et al., 2016). Since this pair contains labels on both subunits, changes in FRET efficiency could be the result of distance changes due to intersubunit rotation, head rotation, or some combination of both. To test this possibility, we directly compared the results from our S12-S19 and S11-S13 pairs with the S13-L33 pair.

Upon delivery of EF-G·GTP to a labeled pre-translocation complex containing the S13-L33 FRET pair, we observed a biphasic increase in the fluorescence of Alexa488 (Fig. 5A), similar to the observations of Belardinelli et al. (Belardinelli et al., 2016). When GTP was replaced with GDPNP or the mutant EF-G(H91L) was used, we continued to observe fluorescence changes, although with decreased amplitudes and modestly (~2-3-fold) decreased rates (Fig. 5A; Table S1). These results contrast with those obtained with the S11-S13 and S12-S19 pairs, which show complete blockage of reverse head rotation (Figs. 3, 4). The persistence of large changes in FRET efficiency from the S13-L33 pair under two different conditions, which in our hands abolish reverse head rotation, appears to indicate that the S13-L33 pair is an unreliable reporter for 30S head rotation.

As can be seen in Fig. 5B, the apparent rate constants for the S13-L33 FRET pair for the GTP, GDPNP, and H91L conditions correlate most closely with those for reverse intersubunit rotation monitored using the S6-L9 FRET pair and differ from those measured directly for either forward or reverse head rotation. We conclude that the signal from the S13-L33 pair observed with GDPNP and H91L is mainly attributable to reverse intersubunit

rotation, which could account for the apparent discrepancies between the observations made using the different FRET reporters.

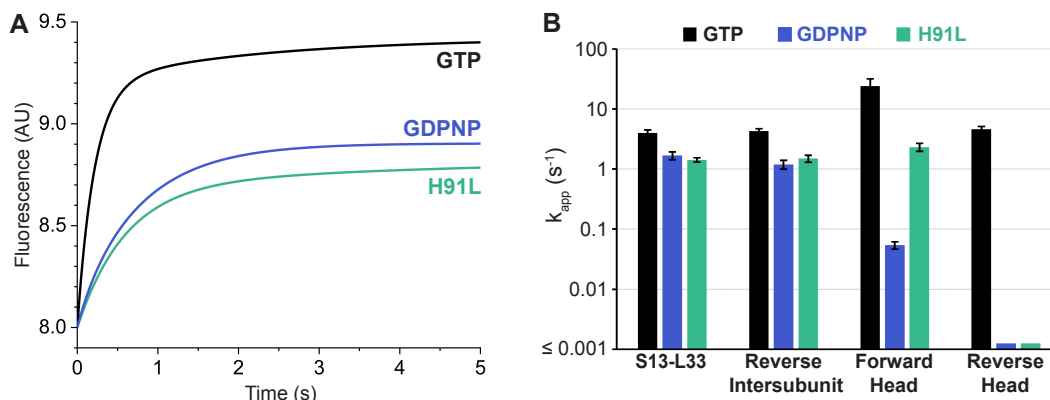


Figure 5. The S13-L33 FRET pair likely reports primarily on reverse intersubunit rotation. (A) A pre-translocation complex containing ribosomes with the S13(Atto540Q)-L33(Alexa488) FRET pair was combined with either EF-G-GTP (GTP), EF-G-GDPNP (GDPNP) or EF-G(H91L)-GTP (H91L) in a stopped-flow fluorometer. The fluorescence signal from Alexa488 was monitored over a single round of translocation. (B) The weighted average apparent rate constants (k_{app}) for the biphasic FRET changes from the S13-L33 FRET pair for GTP, GDPNP, and H91L conditions (Table S1, Methods) correlate most closely with the respective rates of reverse intersubunit rotation monitored using the S6-L9 FRET pair and differ from those measured directly for either forward or reverse head rotation (Table 1).

DISCUSSION

GTP hydrolysis by EF-G has previously been proposed to drive tRNA movement (Abel and Jurnak, 1996; Rodnina et al., 1997). Some investigators have proposed that hydrolysis is coupled to a conformational change in EF-G that either actively moves the tRNAs and mRNA in a “power stroke” (Chen et al., 2013; Yao et al., 2013; Yin et al., 2019) or induces a conformational change in the ribosome that provides the energy for tRNA and/or mRNA movement (Agrawal et al., 1999; Savelsbergh et al., 2003; Peske et al., 2004; Chen et al., 2013; Cunha et al., 2013; Holtkamp et al., 2014a; Rodnina et al., 2020). Others have suggested a Brownian ratchet mechanism driven by thermal energy in which EF-G acts as a pawl to prevent backwards movement of the tRNAs (Cornish et al., 2008; Spirin, 2009; Frank and Gonzalez, 2010; Ratje et al., 2010; Liu et al., 2014; Salsi et al., 2016; Wasserman et al.,

2016; Carbone et al., 2021; Rundlet et al., 2021). Still others have suggested mechanisms that combine elements of both (Holtkamp et al., 2014a, 2014b; Chen et al., 2016; Rodnina et al., 2020; Petrychenko et al., 2021). However, EF-G accelerates translocation by over ~10,000-fold (Rodnina et al., 1997; Fredrick and Noller, 2003) while GTP hydrolysis contributes only a ~30-50-fold increase in rate (Rodnina et al., 1997; Katunin et al., 2002; Munro et al., 2010; Cunha et al., 2013). Although the latter rate increase may well be explained by such models, we conclude that the principal role of GTP hydrolysis is not to drive tRNA or mRNA movement. Indeed, it has been argued that EF-G not only does not *push* the tRNAs through the ribosome, but actually *restrains* their movement (Zhou et al., 2019).

In their pioneering studies showing a complete round of translocation in the presence of GDPNP, Kaziro and co-workers suggested that the primary role of GTP hydrolysis is to enable cycling of EF-G on and off the ribosome (Inoue-Yokosawa et al., 1974; Kaziro, 1978). The strict requirement of GTP hydrolysis for dissociation of EF-G (Inoue-Yokosawa et al., 1974; Savelsbergh et al., 2009; Munro et al., 2010; Cunha et al., 2013) strongly suggests that the role of GTP hydrolysis must indeed be linked to EF-G occupancy; yet, merely facilitating release of EF-G would not seem to justify such a costly expenditure of energy. Finally, EF-G remains bound to the ribosome long after GTP hydrolysis, which occurs rapidly upon EF-G binding (Savelsbergh et al., 2003), pointing to the importance of the persistence of EF-G on the ribosome. GTP hydrolysis must, therefore, serve some other crucial function.

Here, we sought to clarify how blocking GTP hydrolysis affects structural rearrangements that occur in the ribosome during translocation using FRET pairs that directly report on 30S head rotation. Surprisingly, we find that the single step that requires GTP hydrolysis is reverse rotation of the 30S subunit head domain. That both dissociation of EF-G and reverse head rotation require GTP hydrolysis implies that they are linked. Thus, EF-G remains on the ribosome throughout forward head rotation and completion of movement of

tRNA and mRNA (Fig. 6), consistent with recent time-resolved cryo-EM structures of translocation intermediates (Carbone et al., 2021; Petrychenko et al., 2021).

But why must EF-G remain on the ribosome during this time? The most serious error that can occur during protein synthesis is shifting of the translational reading frame, which causes incorrect reading of all subsequent codons, including out-of-frame stop codons, thus creating potentially toxic truncated polypeptide products. The reading frame is most vulnerable to frameshifting during movement, when neither of the two codon-anticodon duplexes is stabilized by their ribosomal binding sites. During movement of the duplexes, EF-G acts to maintain the reading frame by contacting the minor groove of the A-site codon-anticodon duplex and stabilizing it until movement is complete (Gao et al., 2009; Brilot et al., 2013; Ramrath et al., 2013; Zhou et al., 2014, 2019; Peng et al., 2019; Niblett et al., 2021). The vulnerability of the reading frame during movement has been captured in the structures of two translocation intermediates undergoing -1 and +1 frameshifting events, respectively (Zhou et al., 2019; Demo et al., 2021).

We propose that the principal role of GTP hydrolysis is to create a checkpoint to ensure that the mRNA and tRNAs have translocated by one full codon before EF-G is released. Since GTP hydrolysis occurs much earlier in the sequence of events, the actual signal for release of EF-G is likely release of inorganic phosphate (P_i). Consistent with this is the finding that inhibiting P_i release blocks multiple turnover translocation (Savelsbergh et al., 2009). If dissociation of EF-G is required for reverse rotation of the 30S head domain, it would explain why GTP hydrolysis is required for reverse head rotation. By preventing release of EF-G until the duplex has moved by precisely one codon, GTP hydrolysis enforces maintenance of the reading frame and a round of productive translocation, justifying the expenditure of this chemical energy.

Our model is consistent with a recent time-resolved cryo-EM structural study of translocation dynamics by Korostelev and co-workers (Carbone et al., 2021). The authors infer that GTP hydrolysis is used as a switch controlling the ability of EF-G to bind and leave

the ribosome rather than for driving tRNA movement. They similarly conclude that EF-G remains bound to the ribosome until reaching a final stage of 30S head rotation to support the tRNA-mRNA helix and prevent frameshifting. Our results now provide direct kinetic evidence that reveals a fundamental connection between GTP hydrolysis and reverse head rotation. Our results are also in agreement with the finding that late steps in translocation on mammalian ribosomes are stalled in the absence of GTP hydrolysis (Flis et al., 2018). However, that same study concluded that late steps of translocation on bacterial ribosomes are merely slowed when GTP hydrolysis is abolished, in contrast to our findings. We suspect that use of a FRET pair containing labels on A-site tRNA and protein uS13, which would be sensitive to both tRNA movement and 30S head rotation, may have obscured the effect of blocking GTP hydrolysis on reverse head rotation. Our results demonstrate that GTP hydrolysis is required for resolving late steps in bacterial translocation, just as in the mammalian mechanism.

Given the seriousness of losing the translational reading frame, it should not be surprising to learn that other and perhaps even all translational GTPases are in some way involved in guarding against frameshifting. Initiation factor IF2 helps to ensure selection of the initiator tRNA at the start codon, establishing the reading frame at the outset of translation (Caban and Gonzalez, 2015). Elongation factor EF-Tu helps to maintain the reading frame (Hughes et al., 1987; Vijgenboom and Bosch, 1989) by preventing binding of non-cognate tRNAs to the A site (Tapio and Kurland, 1986; Voorhees and Ramakrishnan, 2013), which can result in frameshifting (Weiss and Gallant, 1983). And release factor RF3 has been shown to elicit termination following miscoding and frameshifting events (Zaher and Green, 2009, 2011), protecting the cell against miscoded proteins. It thus seems possible that the seemingly costly expenditure of GTP throughout all steps of protein synthesis may have a common basis in avoiding the dangers of translational frameshifting.

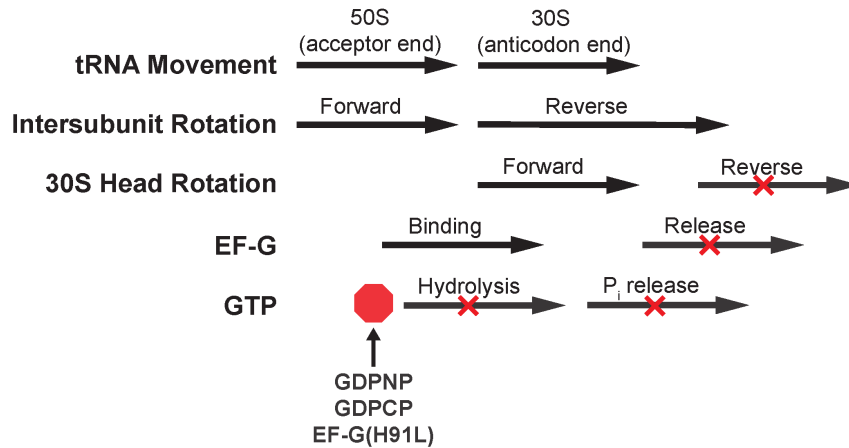


Figure 6. Effects of blocking GTP hydrolysis on events during translocation. Blocking GTP hydrolysis specifically abolishes reverse rotation of the 30S subunit head domain (Figs. 3,4), likely due to inhibition of release of EF-G, which requires release of P_i (Inoue-Yokosawa et al., 1974; Savelsbergh et al., 2009; Munro et al., 2010; Cunha et al., 2013). Arrows indicate proposed order of events during a single round of translocation. Red octagon symbol indicates blockage of GTP hydrolysis by GDPNP, GDPCP, or EF-G(H91L). Red X marks indicate steps abolished by blockage of GTP hydrolysis.

MATERIALS & METHODS

Tight-couple MRE600 70S ribosomes and 50S and 30S subunits were purified as described (Moazed and Noller, 1989; Lancaster et al., 2002; Hickerson et al., 2005).

Elongator N-Acetyl-Met-tRNA^{Met} (transcribed) and Val-tRNA^{Val1} (Sigma) were prepared as described (Niblett et al., 2021). mRNA mv39

(5'GGCAAGGAGGUAAAAAUGGUAUACAAAAAAUCAAUAUC) was transcribed from DNA oligonucleotides (5')-TTCTAATACGACTCACTATAGGCAAG

GAGGTAAAAATGGTATACAAAAAATCAAAATC and (5')-GATTTTGATTTTTTGT

ATACCATTTTTACCTCCTTGCCTATAGTGAGTCGTATTAGAA (IDT).

QuikChange site-directed mutagenesis (Stratagene) was used to create mutations in S13 (C85S, P112C) and L33 (P31C) from plasmids pET24b::S13 (Culver and Noller, 2000) and pET21b::L33 (Cornish et al., 2009), respectively. The mutant proteins were over-expressed and purified essentially as described (Culver and Noller, 2000; Hickerson et al., 2005). 6His-wild-type EF-G (Wilson and Noller, 1998), 6His-EF-G(H91A), and 6His-EF-

G(H91L) (Niblett et al., 2021) were purified as described (Guo and Noller, 2012). Note that we use residue numbering including the amino-terminal Met residue for ribosomal proteins, but for EF-G our numbering excludes its N-terminal Met to maintain conformity with previous studies (Peng et al., 2019).

Fluorescent labeling of ribosomal proteins was done as described (Niblett et al., 2021) at cysteine residues introduced at the following positions: S12(K108C) and S11(R106C) with Alexa488 (Invitrogen), S13(D11C) and S19(Q56C) with Alexa568 (Invitrogen), and S6(D41C) and L9(N11C) with Cy5 and Cy3 (Amersham GE), respectively. S13(P112C) and L33(P31C) were labeled with Atto540Q (Sigma) and Alexa488, respectively (Belardinelli et al., 2016).

Doubly-labeled S12-S19 and S11-S13 70S ribosomes were constructed by *in vitro* reconstitution of 30S subunits from 16S rRNA, and 20 purified ribosomal proteins, including fluorescently-labeled S12(AIx488) and S19(AIx568) or S11(AIx488) and S13(AIx568), followed by association with natural 50S subunits, as described (Niblett et al., 2021). Doubly-labeled S6-L9 ribosomes were purified by reconstituting 30S (Δ S6) and 50S (Δ L9) subunits isolated from S6 and L9 deletion strains, respectively, with fluorescently-labeled S6(Cy5) and L9(Cy3), as described (Niblett et al., 2021). Doubly-labeled 70S-S13-L33 ribosomes were purified by reconstituting 30S (Δ S13) and 50S (Δ L33) isolated from S13 and L33 deletion strains, respectively, with fluorescently-labeled S13(Atto540Q) and L33(AIx488). 50S subunits lacking L33 were purified from the L33 deletion strain CSH142 Δ *rpmG* (Cornish et al., 2009), and 30S subunits lacking S13 were purified from the S13 deletion strain MG1655 Δ *rpsM::kan* kindly provided by Rachel Green (Cukras and Green, 2005) essentially as described (Moazed and Noller, 1989; Hickerson et al., 2005). 30S (Δ S13) and 50S (Δ L33) subunits were reconstituted with S13(Atto540Q) and L33(AIx488), respectively, by incubating in buffer containing 330 mM NH₄Cl, 10 mM MgCl₂, 1 mM DTT, 0.01% (w/v) Nikkol and 25 mM Tris·Cl (pH 7.5) at 42°C for 1 hour. The subunits were then concentrated using 0.5 mL 100K MWCO concentrators (Amicon). Reconstituted 30S subunits were brought to 100 mM NH₄Cl,

20 mM MgCl₂, 1 mM DTT, 0.01% Nikkol (w/v), 25 mM Tris·HCl (pH 7.5) and heat-activated by incubating at 42°C for 15 min. Subunits were associated and 70S ribosomes purified as described previously (Hickerson et al., 2005; Niblett et al., 2021).

GTP Hydrolysis Assay

Hydrolysis of α -[³²P]-GTP (Perkin Elmer) was monitored by thin-layer chromatography. Ribosomes (6 pmol) were combined with 6 pmol EF-G in a total volume of 10 μ L in 25 mM Tris·HCl (pH 7.5), 75 mM NH₄Cl, 8 mM MgCl₂ and 1 mM DTT and incubated at 37°C. 5 μ L of 15 μ M GTP in the same buffer were added and 0.75 μ L was withdrawn and spotted on a polyethyleneimine-cellulose TLC plate (Sigma) at 10 seconds and at each subsequent time point. The TLC plate was developed in 0.5 M potassium phosphate, pH 3.5 (Rodnina et al., 1999) and visualized by autoradiography.

Stopped-Flow Kinetics Measurements

Rotation of the 30S head or body domains was followed by changes in FRET efficiency using an Applied Photophysics SX-20 stopped-flow fluorometer. Pre-translocation complexes were formed by incubating 0.25 μ M 70S ribosomes with 0.5 μ M mv39 mRNA (or 0.25 μ M mv24-pyrene mRNA) and 0.5 μ M N-Ac-Met-tRNA^{Met} for 30 min at 37°C in 100 mM NH₄Cl, 20 mM MgCl₂, 1 mM DTT, 0.01% Nikkol, and 25 mM Tris·HCl pH 7.5. Val-tRNA^{Val} was added to a final concentration of 0.5 μ M and the complex was further incubated for 30 min at 37°C before being placed on ice. EF-G was incubated at a concentration of 0.75 μ M with each nucleotide for 5 min at 37°C before being placed on ice; the same buffer was used as for pre-translocation formation, with the exception that 50 mM Tris·HCl (pH 7.5) was used.

Prior to each stopped-flow experiment, the pre-translocation complex was diluted to give a final concentration of 75 nM 70S ribosomes in 100 mM NH₄Cl, 10 mM MgCl₂, 1 mM DTT, 0.01% Nikkol, and 50 mM Tris·HCl pH 7.5. Both the pre-translocation complex and EF-G-nucleotide mix were allowed to equilibrate to room temperature for 5 min prior to each

experiment. Rapid mixing in the stopped-flow fluorometer produced final concentrations of 37.5 nM 70S and 375 nM EF-G. GTP, GDPNP, GDPCP, and GTP γ S (Sigma) were used at final concentrations of 0.5 or 1 mM, which are both saturating concentrations (Katunin et al., 2002; Cunha et al., 2013).

For S12-S19 and S11-S13 experiments, Alexa488 was excited at 496 nm and emission was collected using a 590 nm long-pass filter. For S6-L9 experiments, Cy3 was excited at 550 nm and emission as collected using a 645 nm long-pass filter. For S13(Atto540Q)–L33(Alexa488), Alexa488 was excited at 488 nm and emission was collected using a 515 nm long-pass filter. Between 2 and 13 independent experiments were performed with wild-type EF-G and GTP. For all other conditions, 2-3 experiments were performed with the exceptions of S11-S13 and S12-S19, for which 1 or 2 experiments were performed per condition per pair. In each experiment, 2 to 9 traces were recorded.

Individual traces were fit to either single- or double-exponential functions to obtain apparent rate constants, essentially as described (Niblett et al., 2021). A linear term reporting the slow change in fluorescence due to bleaching and fluorescence drift was additionally included if the value associated with this term converged during the fitting process. For the GTP condition of the S11-S13 experiments, traces were smoothed by binning prior to being fitted. Rate constants were averaged for each experiment, and the mean of these means was reported. A standard deviation was calculated for each independent experiment, and the pooled standard deviation was reported, calculated with the equation $\sigma_{\text{pooled}} = \sqrt{(\sigma_1^2 + \sigma_2^2 + \sigma_3^2 + \dots / n)}$, where n = total number of independent experiments. For all S13-L33 traces and traces longer than 5 seconds, data between 0 and 0.1 sec were not included in the fit. End cutoff times were chosen such that the total fitting range was not more than five times the half-life of the change in fluorescence.

The fold inhibitions in rates of forward and reverse head rotation were calculated after averaging equivalent rates obtained using the S11-S13 and S12-S19 pairs. The weighted average apparent rate constants (k_{ave}) for S13-L33 were calculated according to the equation

$k_{ave} = (a_1k_1 + a_2k_2)/(a_1 + a_2)$, where a_1 and a_2 are the amplitudes of the phases with rates k_1 and k_2 , respectively.

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SUPPLEMENTAL INFORMATION

Table S1. Effects of Inhibitors of Reverse Head Rotation on the S13-L33 Signal

	k_1	a_1	k_2	a_2	k_{ave}
GTP	4.7 ± 0.3	1.2 ± 0.1	0.48 ± 0.04	0.23 ± 0.02	4.0 ± 0.5
GDPNP	6 ± 1	0.08 ± 0.01	1.29 ± 0.08	0.83 ± 0.08	1.7 ± 0.3
H91L	1.68 ± 0.06	0.69 ± 0.04	0.19 ± 0.05	0.14 ± 0.01	1.4 ± 0.1

Pseudo-first-order rate constants are in s^{-1} . Experimental traces (Fig. 5A, S6) were fitted to double-exponential functions, yielding apparent rate constants k_1 and k_2 . a_1 and a_2 are the amplitudes of change associated with rates k_1 and k_2 , respectively. Errors represent standard deviations about the mean. (k_{ave}) weighted average apparent rate constant (Methods).

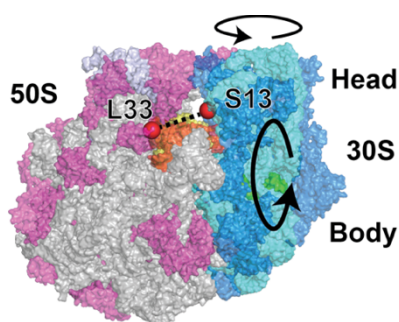


Figure S1. Locations of dyes in S13-Atto540Q-L33-Alexa488 FRET pair. The S13-L33 signal is influenced by both intersubunit rotation and head domain rotation due to placement of the labels on S13 in the 30S head domain and on L33 in the 50S subunit. Coloring is as in Fig. 1. A- and P-site tRNAs are shown in yellow and red, respectively; mRNA is shown in green.

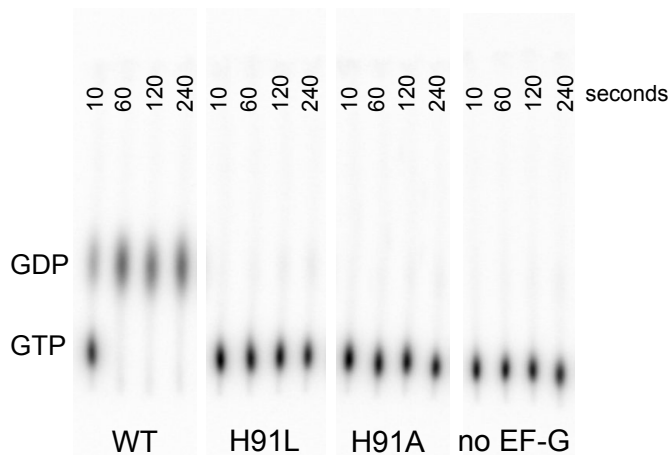


Figure S2. The mutant EF-G(H91L) is GTPase-deficient. Ribosome-dependent hydrolysis of α -[^{32}P]-GTP by EF-G was monitored by TLC (Methods). Both H91L and H91A mutants do not hydrolyze GTP above background over 4 min at 37°C. (WT) wild-type EF-G; (H91L) EF-G(H91L); (H91A) EF-G(H91A); (no EF-G) ribosomes only.

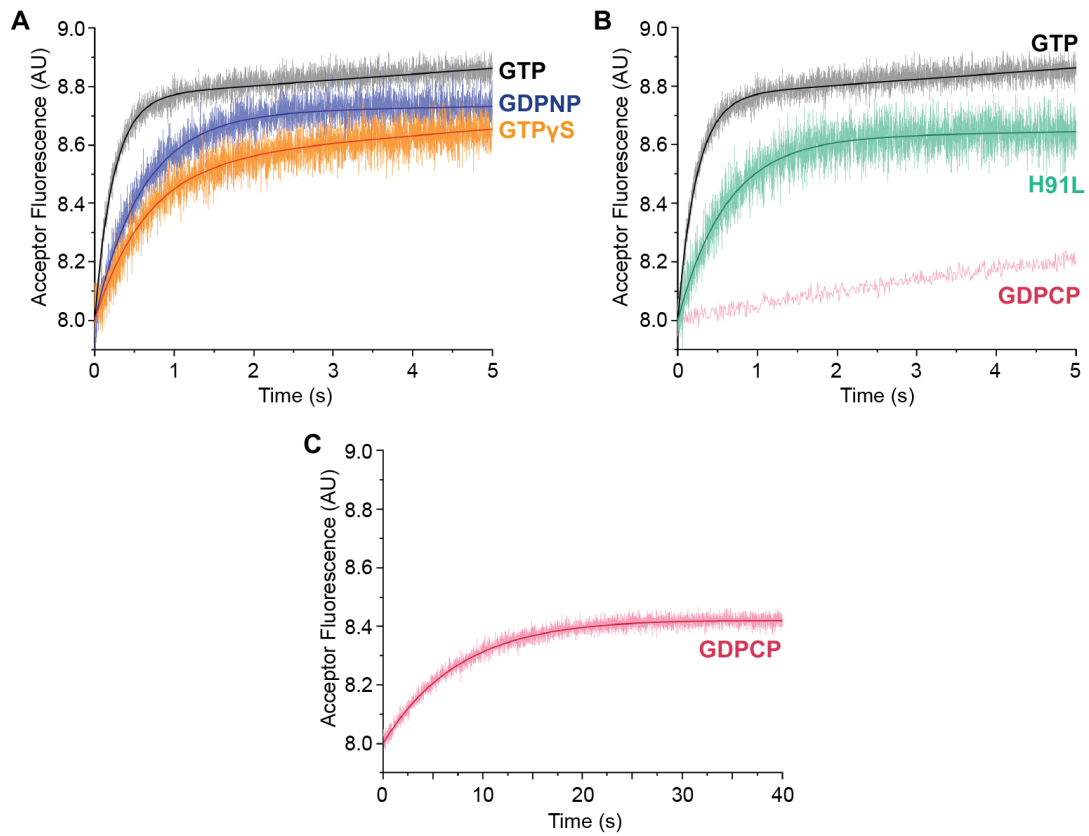


Figure S3. Fluorescence traces and fitted curves from S6-L9 FRET pair reporting on intersubunit rotation. Shown are averaged traces for each condition and single exponential fitted curves overlaid. (A,B) Traces over 5 sec are separated between panels for clarity. (C) Traces for the GDPCP condition were fitted over 40 sec.

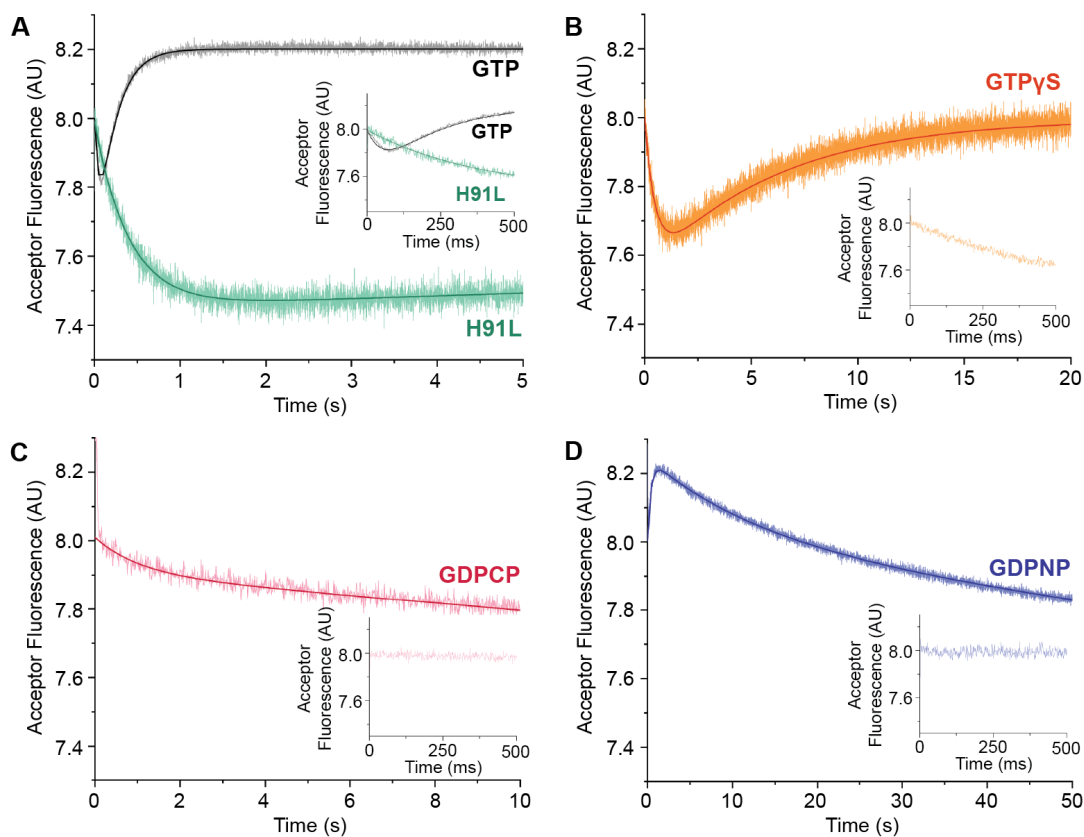


Figure S4. Fluorescence traces and fitted curves from S12-S19 FRET pair reporting on rotation of the 30S head domain. Shown are averaged traces for each condition and single- or double- exponential fitted curves overlaid. Insets show traces over 500 msec. (A) Traces for GTP and H91L conditions were fit over 5 sec or less. Traces for (B) GTPyS, (C) GDPCP, and (D) GDPNP conditions were fit over longer timespans. For these conditions, traces were also collected over 5 sec while the pressure was held by the stopped-flow apparatus to obtain higher resolution data near the beginning. The first 500 msec of these traces are shown in the insets.

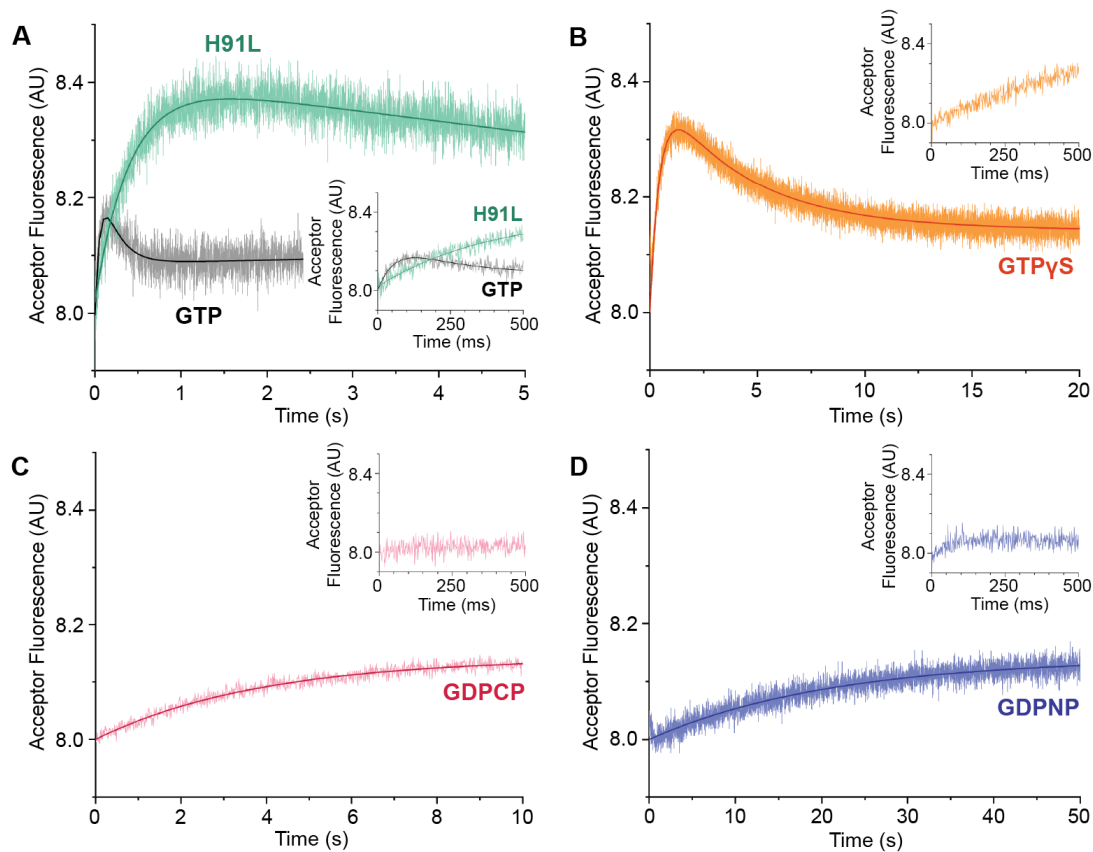


Figure S5. Fluorescence traces and fitted curves from S11-S13 FRET pair reporting on rotation of the 30S head domain. Shown are averaged traces for each condition and single- or double- exponential fitted curves overlaid. Insets show traces over 500 msec. (A) Traces for GTP and H91L conditions were fit over 5 sec or less. Traces for (B) GTP γ S, (C) GDPCP, and (D) GDPNP conditions were fit over longer timespans. For these conditions, traces were also collected over 5 sec while the pressure was held by the stopped-flow apparatus to obtain higher resolution data near the beginning. The first 500 msec of these traces are shown in the insets.

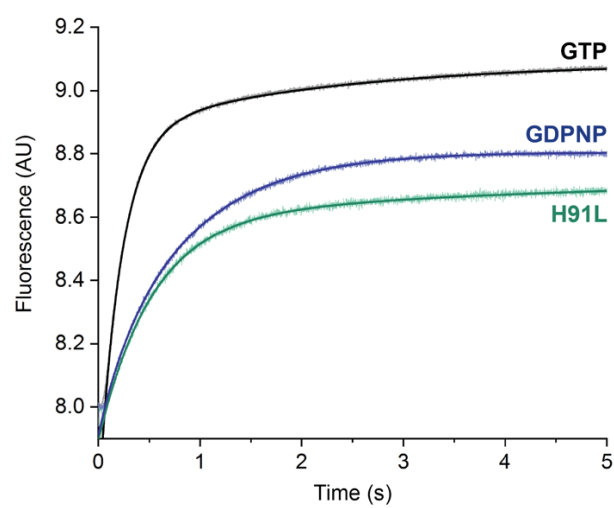


Figure S6. Fluorescence traces from S13-L33 FRET pair. Shown are averaged traces for each condition and double exponential fitted curves overlaid.

Chapter 3. Characterization of a widely-used fluorescence-based translocation assay

ABSTRACT

The kinetics of translocation can be monitored using a fluorescence-based assay in which the signal from a pyrene label at the 3'-terminus of the mRNA is quenched upon movement by one codon. However, it remains unclear what specific event causes the fluorescence quenching in this assay. Here, we ask which structural dynamic event during translocation correlates with mRNA quenching and find that the quenching signal correlates most closely with reverse intersubunit rotation.

INTRODUCTION

To unravel the mechanism of translocation, it is integral to have assays that allow observation of the kinetics of mRNA and tRNA movement. One such assay uses puromycin, an analog of the A-site tRNA aminoacyl moiety and terminal nucleoside. Puromycin reacts with a peptidyl group in the P site but not in the A site (Traut and Monroe, 1964), thus distinguishing whether the peptidyl tRNA has translocated from the A to P site. Other assays use fluorescently-labeled tRNAs to monitor tRNA movement. For example, tRNA^{Phe} (Robertson et al., 1986) and tRNA^{fMet} (Pan et al., 2007) labeled with proflavin have been used to report on movement of the A-site tRNA and P-site tRNA, respectively. Similarly, fluorescein-labeled tRNA^{fMet} has been used in combination with a fluorescence quencher attached to 30S subunit protein S13 to observe movement of the P-site tRNA to the E site (Belardinelli et al., 2016). Fluorescence reporters are also used to monitor mRNA movement by labeling the 3'-terminus of the mRNA with a fluorophore (Savelsbergh et al., 2003; Studer et al., 2003). These assays have two main advantages for tracking translocation: (i) fluorescent-labeled mRNA is easily synthesized and (ii) the assay does not restrict the type of tRNAs that can be used.

One of these widely-used assays monitors translocation through quenching of the signal from a pyrene-labeled mRNA (Studer et al., 2003). In this assay, the signal from a pyrene at position +9 (relative to the first nucleotide of the P-site codon at position +1) at the 3' end of a 24-nucleotide mRNA is quenched upon translocation by one codon to the +6 position. The decay in fluorescence can be fit to a double exponential function. Because movement of the mRNA and tRNAs is coupled to forward rotation of the 30S subunit head domain (Ratje et al., 2010; Ramrath et al., 2013; Zhou et al., 2013), it initially seemed plausible that the quenching signal corresponded to forward head rotation. However, forward head rotation is significantly faster than mRNA quenching and therefore cannot account for the signal (Guo and Noller, 2012). Instead, the rate constant for the fast phase of quenching corresponds most closely to that for reverse rotation of the 30S subunit head domain (Guo and Noller, 2012). To rationalize this observation, it was suggested that the environment surrounding the pyrene fluorophore remains constant throughout forward head rotation and only changes upon reverse head rotation, at which point the fluorescence is quenched. However, this model has not been tested, leaving the physical basis for the mRNA quenching signal uncertain.

RESULTS

We first asked whether reverse head rotation could account for the quenching signal upon mRNA movement, as previously hypothesized (Guo and Noller, 2012). To test this, we asked how the mRNA quenching signal was affected by slowing or abolishing reverse head rotation. Reverse head rotation was inhibited by replacing GTP with GTP analogs (GDPNP, GDPCP, GTP γ S) or using the catalytic H91L mutant form of EF-G, as described in Chapter 2. Surprisingly, under conditions that abolish reverse head rotation, rate constants for mRNA quenching are decreased by only ~2-3-fold, except in the case of GDPCP, which reduced the rate constant more substantially (11-fold; Fig. 1; Table 1), in general agreement with the findings of Salsi et al. (Salsi et al., 2016). The persistence of mRNA quenching under

conditions where reverse head rotation is abolished shows that mRNA quenching cannot be caused by reverse head rotation.

We next asked which of the different ribosomal rotational events correlates most closely with mRNA quenching. We observe that the rate constants for mRNA quenching and reverse intersubunit rotation are impacted to similar extents under each condition (Fig. 1B), suggesting that these events are connected.

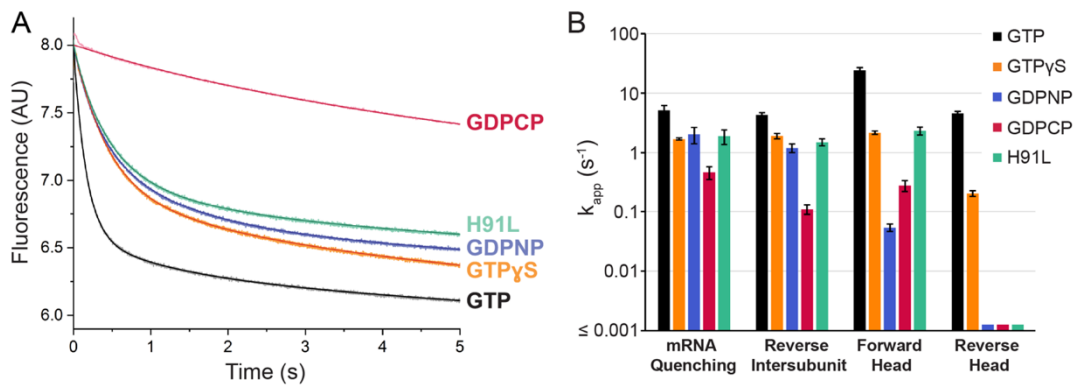


Figure 1. mRNA quenching correlates most closely with reverse intersubunit rotation. (A) Translocation was monitored by quenching of a fluorophore attached to the 3' end of a 24-nt mRNA. The biphasic experimental traces are shown with double-exponential fitted curves overlaid. (B) Weighted average apparent rate constants (k_{ave}) for mRNA quenching (Methods) correspond most closely to those for reverse intersubunit rotation (Table 1; see also Table 1 in Chapter 2).

Table 1. Effects of Inhibition of GTP Hydrolysis on mRNA Quenching¹

	k_1	k_2	k_{ave}	a_1+a_2	% k_1	% k_2
GTP	6.2 ± 0.3	0.82 ± 0.09	5 ± 1	1.7	81	19
GDPNP	3.5 ± 0.8	1.0 ± 0.1	2.0 ± 0.6	1.4	42	58
GDPCP	3.3 ± 0.7	0.21 ± 0.05	0.5 ± 0.1	0.93	8	92
GTPyS	2.38 ± 0.06	0.34 ± 0.02	1.70 ± 0.07	1.6	67	33
H91L	3.3 ± 0.8	1.0 ± 0.1	1.9 ± 0.5	1.3	38	62

¹mRNA quenching traces (Fig. 1) were fitted to double-exponential functions. Errors represent standard deviation about the mean. a_1 and a_2 are the amplitudes of change associated with rates k_1 and k_2 , respectively. % k_1 and % k_2 represent the relative contributions of these phases to the overall quenching signal. (k_{ave}) weighted average apparent rate constant.

DISCUSSION

Based on kinetic evidence, it has been speculated previously that the signal in the widely-used mRNA quenching translocation assay may be attributed to reverse rotation of the 30S subunit head domain (Guo and Noller, 2012). Here, use of different conditions in which GTP hydrolysis is slowed or abolished allowed us to determine which structural dynamics could be responsible for quenching. We show that the quenching signal from this assay cannot be the result of reverse head rotation; instead, the kinetics of fluorescence quenching correlate most closely with those of reverse intersubunit rotation, consistent with previous findings (Ermolenko and Noller, 2011). The discrepancy between our conclusions and those of Guo et al. is likely attributed to differences in the kinetic values compared. In the study from Guo et al., the kinetics of the fast phase (k_1) of quenching was compared to kinetics for the structural dynamics during translocation. By contrast, here we use a weighted average apparent rate constant (k_{ave}), which incorporates the contributions of both fast and slow phases.

Our results provide important context for previous work conducted using this mRNA quenching assay and for its use going forward. However, there remain questions about the mRNA quenching assay. First, the difference between the two phases of the biphasic quenching signal remains unknown. Additionally, the physical mechanism by which reverse intersubunit rotation is connected to quenching is unclear; understanding the connection between reverse intersubunit rotation, mRNA movement, and fluorescence quenching may reveal further insights into the mechanism of translocation.

MATERIALS & METHODS

Materials

Doubly-labeled S12-S19 and S11-S13 70S ribosomes were constructed by *in vitro* reconstitution of 30S subunits from 16S rRNA, and 20 purified ribosomal proteins, including fluorescently-labeled S12(Alx488) and S19(Alx568) or S11(Alx488) and S13(Alx568),

followed by association with natural 50S subunits. Doubly-labeled S6-L9 ribosomes were purified by reconstituting 30S (Δ S6) and 50S (Δ L9) subunits isolated from S6 and L9 deletion strains, respectively, with fluorescently-labeled S6(Cy5) and L9(Cy3). The 3'-amino-modified mRNA mv24 (Dharmacon) was labeled with pyrene essentially as described (Guo and Noller, 2012). Details for all other materials are described in the methods of Chapter 2.

Methods

For FRET assays, pre-translocation complexes were formed from fluorescent-labeled ribosomes, a 39-nt transcribed mRNA, tRNA^{Met} in the P/E state, and N-Ac-Met-Val-tRNA^{Val} in the A/P state. S6-L9 ribosomes were used to measure intersubunit rotation, and S12-S19 and S11-S13 ribosomes were used to measure 30S head rotation. For mRNA quenching assays, pre-translocation complexes were formed with natural tight-couple 70S, a 24-nt mRNA containing a 3'-terminal pyrene, and the same tRNAs as above. Fluorescence changes over a single round of translocation following rapid mixing of the pre-translocation complex with EF-G and GTP (or GTP analog) were monitored using an Applied Photophysics SX20 stopped-flow fluorometer at 22°C. Pyrene was excited at 343 nm and emission was collected using a 375 nm long-pass filter. GTP hydrolysis was blocked using the non-hydrolyzable analogs GDPNP and GDPCP or the H91L mutant of EF-G, or slowed using the GTP analog GTP γ S. Fluorescence traces were fit to single- or double-exponential functions to obtain apparent rate constants. The weighted average apparent rate constants (k_{ave}) for mRNA quenching were calculated according to the equation $k_{ave} = (a_1k_1 + a_2k_2)/(a_1 + a_2)$, where a_1 and a_2 are the amplitudes of the phases with rates k_1 and k_2 , respectively. % k_1 and % k_2 were calculated according to the equations % $k_1 = a_1 / (a_1 + a_2) \times 100$ and % $k_2 = a_2 / (a_1 + a_2) \times 100$. Additional information can be found in the methods of Chapter 2.

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Chapter 4. The timing of phosphate release from EF-G during protein synthesis

ABSTRACT

During catalysis of ribosomal translocation by elongation factor G (EF-G), GTP hydrolysis occurs rapidly upon binding of EF-G-GTP; however, the cleaved γ -phosphate (Pi) remains bound to EF-G in almost exactly the same position as in unhydrolyzed GTP, thus preserving most of the energy of GTP hydrolysis. Release of Pi is required for dissociation of EF-G-GDP, but its timing remains unclear. Here, we determine the timing of Pi release with respect to the structural rearrangements of the ribosome during translocation and the interdependence of these events. We find that Pi remains bound until the final steps of translocation, that Pi release occurs after forward head rotation, that it is coupled to reverse intersubunit rotation, and that both Pi release and EF-G dissociation are necessary for reverse head rotation. We propose that the delay of Pi release prevents premature dissociation of EF-G, ensuring that each round of translocation is productive and accurate. Finally, this strategy may extend to other translational GTPases such as IF2 and EF-Tu, which also exhibit delayed Pi release and whose roles are also centered primarily on the accuracy of key events in protein synthesis.

INTRODUCTION

Protein synthesis on the ribosome is mediated by numerous translation factors, including the GTPases initiation factor IF2 and elongation factors EF-Tu and EF-G. It remains a mystery why these translational GTPases (trGTPases), which carry out seemingly diverse functions, all require GTP hydrolysis. Thus, IF2 promotes joining of the 50S subunit to the 30S initiation complex (reviewed in Caban and Gonzalez, 2015) and EF-Tu enforces accurate selection of aminoacyl-tRNAs (Tapio and Kurland, 1986; Hughes et al., 1987; Vijgenboom and Bosch, 1989; Gromadski and Rodnina, 2004), while EF-G catalyzes translocation of

mRNA and tRNAs (Gupta et al., 1971). Here, we focus on the role of the GTPase function of EF-G. Our findings have implications for the common requirement for GTP hydrolysis by multiple translation factors.

Following the formation of each peptide bond during protein synthesis, the ribosome must move the mRNA and tRNAs by precisely one codon in a process catalyzed by EF-G (eEF2 in eukaryotes) (Gupta et al., 1971). This moves the A-site tRNA carrying the polypeptide to the P site, leaving the A site vacant for the next incoming aminoacyl-tRNA, and moves the deacylated tRNA from the P site to the E site, from which it can dissociate. Translocation is coupled to large-scale structural rearrangements in the ribosome. First, rotation of the entire 30S subunit facilitates movement of the acceptor ends of the A- and P-site tRNAs into the P and E sites of the 50S subunit (Moazed and Noller, 1989; Frank and Agrawal, 2000; Ermolenko et al., 2007; Carbone et al., 2021; Petrychenko et al., 2021; Rundlet et al., 2021). Next, rotation of the 30S subunit head domain moves the anticodon ends of the tRNAs along with the mRNA relative to the 30S body domain (Ratje et al., 2010; Ramrath et al., 2013; Zhou et al., 2013, 2014). During this step, the 30S subunit also begins to reverse its rotation (Ermolenko and Noller, 2011). Finally, reverse head rotation restores the contacts of the anticodon ends of the tRNAs with the 30S head domain (Gao et al., 2009; Zhou et al., 2014).

Although GTP is rapidly hydrolyzed upon binding of EF-G·GTP to the ribosome, (Rodnina et al., 1997), the purpose of its hydrolysis is confounded by two puzzling facts. First, hydrolysis is essential only for events that occur near the *end* of translocation (Flis et al., 2018), including dissociation of EF-G (Inoue-Yokosawa et al., 1974; Katunin et al., 2002; Cunha et al., 2013) and reverse rotation of the 30S head domain (Rexroad et al., 2022). Second, following GTP hydrolysis, the cleaved phosphate (Pi) remains bound in almost exactly the same position as in unhydrolyzed GTP (Carbone et al., 2021; Petrychenko et al., 2021; Rundlet et al., 2021), thus preserving nearly all of the energy of GTP hydrolysis until late in the translocation process. Following a delay, Pi is then released, which allows EF-G to

dissociate in its low-affinity GDP form (Katunin et al., 2002; Savelsbergh et al., 2005). These observations raise several questions: (i) Why is Pi release delayed relative to hydrolysis? (ii) What event triggers Pi release? and (iii) What is the energy of Pi release used for?

Here, we use a fluorescence-based method (Brune et al., 1994) to follow the kinetics of Pi release and in parallel monitor intersubunit rotation and 30S head rotation using ensemble FRET. Additionally, we test the interdependence of Pi release and ribosome structural dynamics using antibiotics, EF-G mutants, and the Pi analog BeF_3^- . We find that Pi release occurs after forward 30S head rotation but precedes reverse head rotation. Unexpectedly, we find that Pi release appears to be coupled to reverse intersubunit rotation. Finally, we show that Pi release is required for reverse 30S head rotation. We conclude that Pi release is delayed so that EF-G remains on the ribosome sufficiently long to ensure accurate completion of translocation. A similar strategy may extend to the GTPase functions of IF2 and EF-Tu, both of which are involved in translational fidelity (Tapio and Kurland, 1986; Hughes et al., 1987; Vijgenboom and Bosch, 1989; Caban and Gonzalez, 2015) and exhibit delayed Pi release (Tomšić et al., 2000; Kothe and Rodnina, 2006).

RESULTS

Phosphate release occurs well beyond completion of forward head rotation

To elucidate the role of Pi release from EF-G during translocation, we first asked when Pi is released with respect to the structural dynamic events of the ribosome. Translocation was initiated by stopped-flow delivery of EF-G and GTP to pre-translocation ribosome complexes, as described in Methods. To monitor the kinetics of Pi release, we employed a previously developed method, which uses the increase in fluorescence of a coumarin-labeled phosphate binding protein (PBP) upon transition from its apo to Pi-bound state (Brune et al., 1994). An exponential increase due to rapid release of Pi during the single round of productive translocation is followed by a linear turnover phase in which EF-G unproductively binds the ribosome and hydrolyzes GTP (Fig. 1A).

To monitor the large-scale rotational movements within the ribosome during translocation we used ensemble FRET. Reverse intersubunit rotation was monitored with the S6-Cy5-L9-Cy3 FRET pair, which contains fluorophores on ribosomal proteins S6 and L9 in the 30S and 50S subunits, respectively (Ermolenko et al., 2007). An increase in acceptor fluorescence reflects the increase in FRET efficiency upon reverse intersubunit rotation (Fig. 1A). Rotation of the 30S head domain with respect to the body domain was monitored with S11-Alexa488-S13-Alexa568 and S12-Alexa488-S19-Alexa568 pairs, which each contain one label in the head domain and one label in the body domain of the 30S subunit (Guo and Noller, 2012). A rapid increase in fluorescence followed by a relatively slower decrease is seen with the S11-S13 pair due to forward and reverse head rotation, respectively (Fig. 1A). The opposite behavior is seen for the anti-correlated S12-S19 pair (Fig. 1A). To account for possible variations in activity between ribosomes with different labeling schemes, we monitored Pi release from each doubly-labeled ribosome construct (Fig. 1C; S1; Table 1).

As seen previously, forward head rotation is the fastest measurable rotational event (Guo and Noller, 2012) (Fig. 1; Table 1). Forward head rotation occurs much faster than Pi release, showing that forward head rotation occurs before Pi release. Interestingly, the rate constant for Pi release is most similar to that of reverse intersubunit rotation, suggesting that these two events may be coupled (Fig. 1C, Table 1); we explore this connection further below. Additionally, the rate constants for Pi release and reverse head rotation indicate that Pi release likely occurs concomitantly with or immediately prior to reverse head rotation (Fig. 1C, Table 1).

Having determined the approximate timing of Pi release from EF-G with respect to the structural dynamics during rapid translocation, we wondered whether this order would persist when translocation was slowed. To slow translocation, we used the aminoglycoside antibiotic paromomycin (Cabañas et al., 1978; Peske et al., 2004). Paromomycin binds h44 in the 30S subunit, inducing the 'flipped-out' conformations of 16S rRNA bases A1492 and A1493, which contact the A-site tRNA (Fourmy et al., 1996; Carter et al., 2000; Pape et al.,

2000; Selmer et al., 2006). Paromomycin thus slows translocation by stabilizing binding of tRNA in the A-site (Peske et al., 2004).

We observe that paromomycin causes Pi release from EF-G to be slowed, producing an initial burst phase less pronounced compared to the linear turnover phase (Fig. 1B, S2A). To more accurately measure Pi release in the presence of paromomycin, we introduced fusidic acid, which prevents dissociation of EF-G following Pi release (Bodley et al., 1969; Gao et al., 2009; Zhou et al., 2013) (Fig. S2A), thereby reducing the contribution of the turnover phase. Control experiments show that fusidic acid does not significantly affect the rates of reverse intersubunit rotation or single-round Pi release (Fig. S3; Table S1). Fusidic acid was therefore used in all experiments monitoring Pi release or reverse intersubunit rotation but was omitted from experiments monitoring head rotation because of its inhibitory effect on reverse head rotation, as described below (Fig. 5).

We find that in the presence of paromomycin, the rates of all rotational events (forward and reverse head rotation and reverse intersubunit rotation) as well as the rate of Pi release from EF-G are slowed by ~3-4-fold (Fig. 1C; Fig. S4; Table S1). Notably, forward head rotation, although slowed, remains the fastest rotational event observed and is still significantly faster than Pi release. Additionally, the similarity between the rates of Pi release, reverse intersubunit rotation, and reverse head rotation that we observe in unperturbed translocation persists in the presence of paromomycin. Importantly, paromomycin is not expected to directly affect the rate of GTP hydrolysis by EF-G. Therefore, the decreased rate of Pi release must result from the inhibitory effects of paromomycin on translocation itself. This strongly suggests a functional link between the structural dynamics of the ribosome and release of Pi from EF-G.

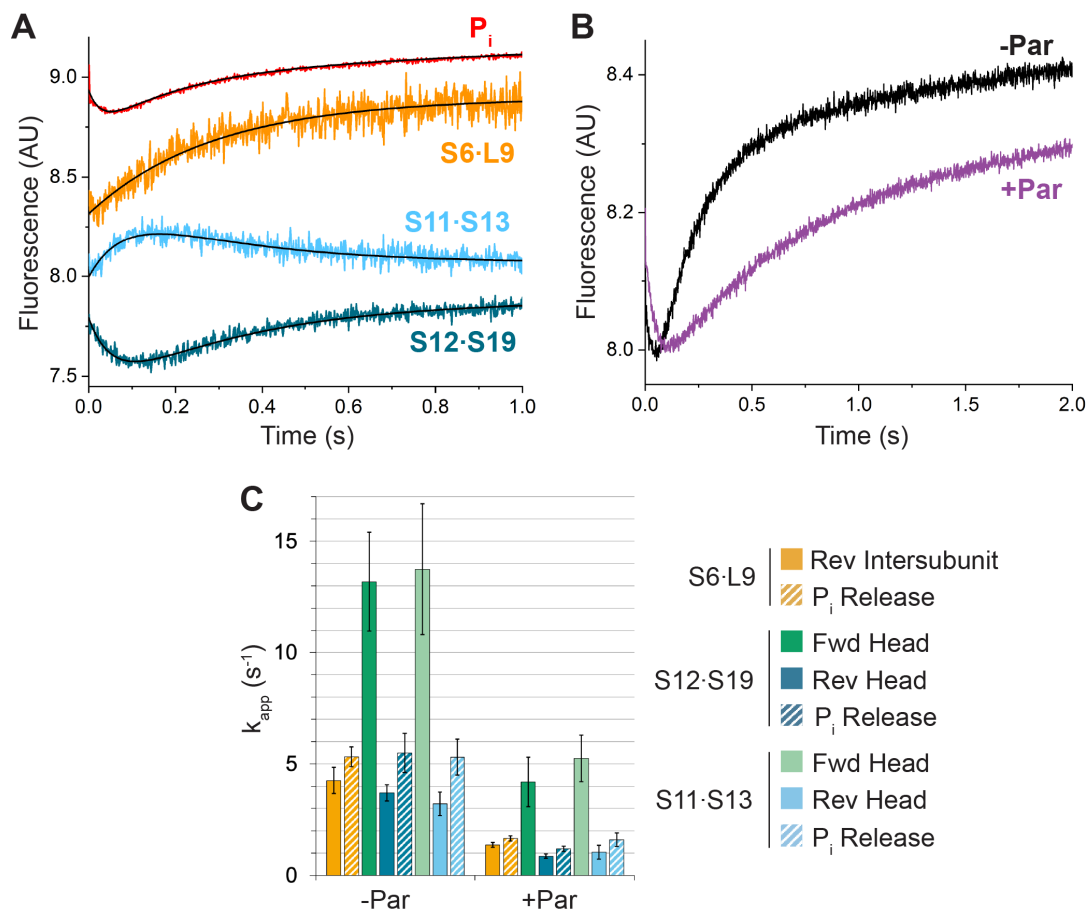


Figure 1. Kinetics of phosphate release and rotational events during ribosomal translocation (A) Representative averaged fluorescence traces with fitted exponential functions overlaid. For clarity, only the P_i release trace in the presence of S6-L9-labeled ribosomes is shown; traces for all constructs are shown in Fig. S1. (B) P_i release in the absence or presence of paromomycin (Par). For clarity, only traces obtained in the presence of the S6-L9 complex are shown. (C) Pseudo-first order rate constants in the absence or presence of paromomycin (Par). Fluorescence traces were fit to exponential functions to obtain rate constants, as detailed in Methods.

Table 1. Rate constants for rotational events and release of phosphate during translocation

FRET Pair	Rotational Event	k_1 (s ⁻¹)	Rotational Event	k_2 (s ⁻¹)	P _i Release (s ⁻¹)
S6-L9	forward intersubunit	fast	reverse intersubunit	4.3 ± 0.6	5.3 ± 0.4
S12-S19	forward head	13 ± 2	reverse head	3.7 ± 0.4	5.5 ± 0.9
S11-S13	forward head	14 ± 3	reverse head	3.2 ± 0.5	5.3 ± 0.8

Rotational events (reverse intersubunit rotation, forward head rotation, and reverse head rotation) were monitored using FRET during unperturbed translocation. Rate constants of P_i release from each type of labeled ribosome were measured using MDCC-PBP. Pseudo first order rate constants were obtained by fitting fluorescence traces to single or double exponential functions, as detailed in Methods.

Phosphate release is coupled to reverse intersubunit rotation

The observation that reverse intersubunit rotation, P_i release from EF-G, and reverse head rotation consistently occur at very similar rates (Fig. 1C; Table 1, S1) prompted us to investigate a potential functional link between P_i release and each rotational event. We began by asking how reverse intersubunit rotation and P_i release are affected when translocation is slowed using a novel strategy. Rather than slow translocation using an antibiotic, we created a mutant form of EF-G with impaired, but not abolished, GTPase activity. We chose to mutate Asp20 of EF-G, which is a conserved residue in the P loop of translational GTPases with an indirect role in catalyzing GTP hydrolysis (Maracci et al., 2014). Although this mutation has not previously been introduced to EF-G, the equivalent Asp residue in EF-Tu has been mutated previously to Asn and reduced the rate of GTP hydrolysis by ~6-fold (Maracci et al., 2014). We therefore expected this mutation to similarly reduce the rate of GTP hydrolysis by EF-G, and as a result, reduce the rate of P_i release. As predicted, we observe a reduction in the rate of P_i release from EF-G(D20N) (Fig. 2; Table S1). As in the paromomycin experiments, fusidic acid was used to minimize turnover of EF-G while monitoring P_i release and also introduced to corresponding experiments monitoring reverse intersubunit rotation (Fig. S2B).

Using EF-G(D20N) we find that reverse intersubunit rotation and Pi release are both slowed by ~3-fold; thus, the similarity in their rates persists (Fig. 2B, S4; Table S1). This observation provides further evidence that Pi release is coupled to reverse intersubunit rotation.

Reverse head rotation requires release of phosphate from EF-G

We next explored the possibility of a functional link between Pi release from EF-G and reverse head rotation. We again used the D20N mutant form of EF-G, which slows Pi release (Fig. 2), and asked how reverse head rotation was affected by slowed Pi release. Fusidic acid was again introduced to Pi release experiments but was omitted while observing reverse head rotation due to its inhibition of this step (Fig. 5). We observe that reverse head rotation and Pi release from EF-G(D20N) are similarly slowed (Figs. 3A, S4; Table S1), which suggests that Pi release has become rate-limiting for reverse head rotation.

We also asked how completely blocking the release of Pi from EF-G – without affecting the rate of GTP hydrolysis – affects reverse head rotation. BeF_3^- , a Pi analog, has been used previously for structural and mechanistic studies of NTPases, including EF-G (Bigay et al., 1987; Fisher et al., 1995; Kagawa et al., 2004; Gavriljuk et al., 2012; Salsi et al., 2016). Following normal hydrolysis of GTP and release of Pi, BeF_3^- rapidly replaces Pi on EF-G, maintaining stable binding of EF-G to the ribosome (Salsi et al., 2016) and mimicking a state of EF-G prior to release of Pi. In the presence of BeF_3^- , forward head rotation proceeds at a normal rate (Fig. 3B,C). This was expected since it precedes Pi release (Fig. 1). Likewise, the rate of reverse intersubunit rotation is unchanged (Fig. S5C). This observation is consistent with findings that reverse intersubunit rotation does not require GTP hydrolysis (Ermolenko and Noller, 2011; Rexroad et al., 2022).

Unlike the other rotational events, reverse head rotation is increasingly inhibited by increasing concentrations of BeF_3^- (Fig. S5A-B) and is completely abolished at 2 mM BeF_3^- (Figs. 3B-C, S5A-B). Taken together, these results show that stabilizing EF-G in a state

mimicking that prior to Pi release abolishes reverse head rotation. This result combined with the observation that Pi release is rate-limiting for reverse head rotation supports the conclusion that reverse head rotation requires Pi release. This is consistent with findings that reverse head rotation requires GTP hydrolysis by EF-G (Rexroad et al., 2022).

Because we showed that reverse intersubunit rotation and reverse head rotation are both linked to Pi release from EF-G and occur at similar rates in unperturbed and slowed translocation, we wondered if these events could themselves be coupled. This question was informed by observations made when Pi release was slowed with the slowly hydrolyzed GTP analog GTP γ S or with the H92Q mutant form of EF-G (Koripella et al., 2015). Unfortunately, rate constants for single-round Pi release cannot be obtained for either condition because the rate of single-round Pi release from EF-G(H92Q) is too slow to be distinguished from the turnover rate and the cleaved thiophosphate group from GTP γ S does not induce an increase in fluorescence from MDCC-PBP. However, in both conditions we observe that reverse intersubunit rotation occurs significantly faster than reverse head rotation (Fig. 3D). These results demonstrate that reverse intersubunit rotation and reverse head rotation can be uncoupled. This is consistent with our findings that reverse head rotation requires Pi release, while reverse intersubunit rotation does not.

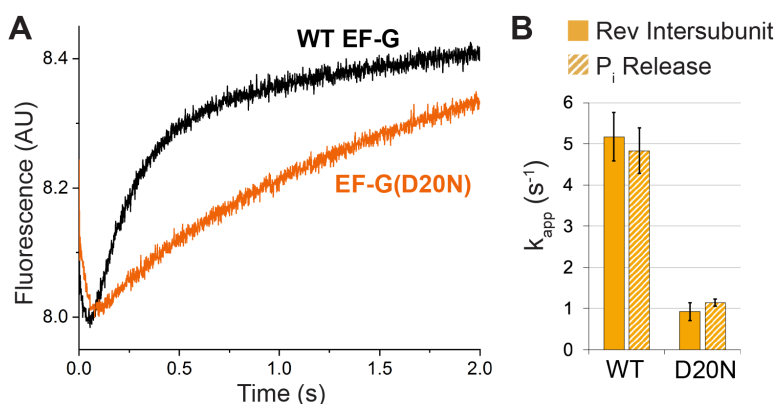


Figure 2. Reverse intersubunit rotation and Pi release from EF-G(D20N) (A) Pi release upon rapid mixing of pre-translocation complex with GTP and either WT EF-G or EF-G(D20N). For clarity, only traces obtained in the presence of the S6-L9 complex are shown.

(B) Pseudo-first order rate constants for reverse intersubunit rotation and Pi release were obtained from fluorescence traces (Fig. S4; Table S1). All experiments were conducted in the presence of 0.2 mM fusidic acid to minimize turnover of EF-G. We observed no effect of fusidic acid on the rates of reverse intersubunit rotation in the presence of WT EF-G or EF-G(D20N) (Fig. S3).

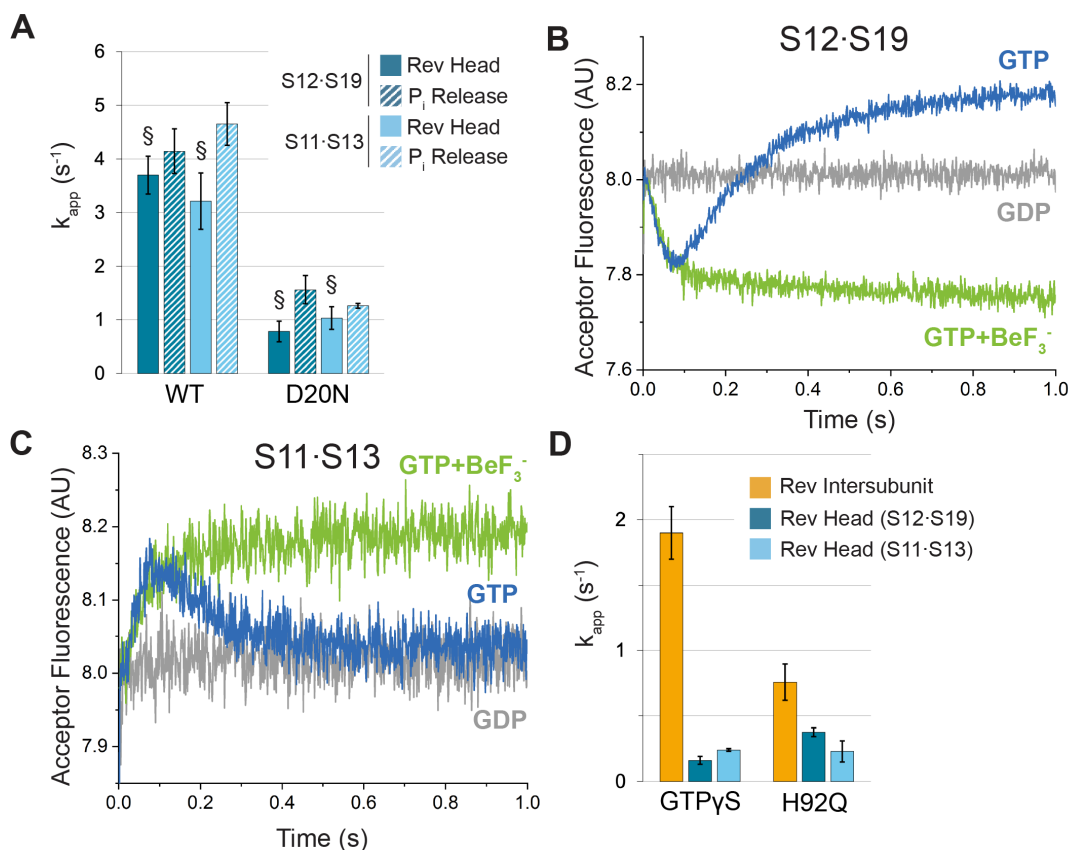


Figure 3. Pi release from EF-G is required for reverse head rotation. (A) Pseudo-first order rate constants for reverse head rotation and Pi release were obtained from fluorescence traces (Fig. S4; Table S1). Fusidic acid was introduced to Pi release experiments, but omitted from head rotation experiments (marked with §). (B,C) Rotation of the small subunit head domain was monitored with FRET pairs (B) S12-S19 and (C) S11-S13. Pre-translocation complexes were rapidly combined in a stopped-flow fluorometer with EF-G and either GTP, GTP plus 2 mM BeF₃⁻, or GDP. (D) Pseudo-first order rate constants for reverse intersubunit rotation and reverse head rotation in the presence of GTPγS or EF-G(H92Q). Data for GTPγS from Rexroad et al. (Rexroad et al., 2022)

EF-G dissociation is required for reverse head rotation

Since Pi release is required for EF-G dissociation (Katunin et al., 2002; Savelsbergh et al., 2005) and our results here suggest it is also required for reverse head rotation, we investigated the possible connection between EF-G dissociation and reverse head rotation. We first asked when EF-G dissociates from the ribosome with respect to reverse head rotation. To monitor the kinetics of EF-G dissociation, we constructed a novel FRET pair containing labels on ribosomal protein S12(K108C) in the 30S body domain and Q254C in domain 1 of EF-G (Fig. 4A). The two fluorophores are predicted to be ~54Å apart immediately prior to EF-G dissociation but farther apart upon initial EF-G binding. We therefore attribute the rapid increase ($19 \pm 2 \text{ s}^{-1}$) in acceptor fluorescence (Fig. 4B-C; Table S2) to a combination of binding and movement of EF-G on the ribosome. This is followed by a slower decrease ($6 \pm 2 \text{ s}^{-1}$) in fluorescence due to dissociation of EF-G (Fig. 4B-C; Table S2). This signal persists when the labeling scheme is reversed (Fig. S6B), and no change in fluorescence is observed from S12-Cy5 when combined with unlabeled EF-G(Q254C) (Fig. S6C). These observations indicate that the change in acceptor fluorescence can be attributed to a change in FRET efficiency. Additionally, when the experiment is performed in the presence of 0.2 mM fusidic acid, an antibiotic that prevents dissociation of EF-G, the decrease in fluorescence associated with dissociation is abolished (Fig. 4B), as expected. We compared the kinetics of reverse head rotation and EF-G dissociation using labeled EF-G-Cy5 in both experiments (Fig. 4C). The close similarity between the rates of reverse head rotation and dissociation of EF-G provides evidence that these events are coupled (Fig. 4C).

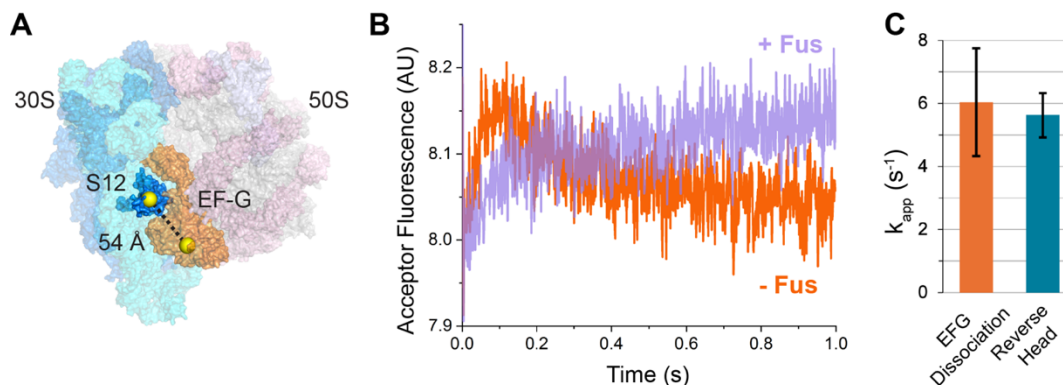


Figure 4. Kinetics of EF-G dissociation during ribosomal translocation. (A) The EF-G(Cy5)·S12(Cy3) FRET pair enables observation of EF-G dissociation. Positions of fluorophores are shown with yellow spheres (PDB 7SSD) (B) Changes in acceptor fluorescence of EF-G-Cy5 following rapid mixing of pre-translocation complex containing S12-Cy3 ribosomes with EF-G-Cy5·GTP in the presence or absence of fusidic acid (Fus). Traces over 5 sec shown in Fig. S6A. (C) Pseudo-first order rate constants for EF-G dissociation and reverse head rotation, monitored with EF-G·S12 and S12·S19 FRET pairs, respectively (Table S2).

Finally, we asked whether blocking EF-G dissociation inhibits reverse head rotation. EF-G contains a conserved element among GTPases termed switch I, which contacts GTP. Switch I also stabilizes contacts between domains I and III of EF-G, which prevents EF-G from dissociating from the ribosome (Laurberg et al., 2000; Zhou et al., 2013). Release of Pi and disordering of switch I allow EF-G to dissociate (Ticu et al., 2009; Carbone et al., 2021; Petrychenko et al., 2021). The antibiotic fusidic acid can bind in the space previously occupied by switch I and assume a similar function, which stabilizes EF-G on the ribosome (Zhou et al., 2013). Importantly, fusidic acid binds after Pi release and does not affect it (Fig. S3A). We therefore used it to ask whether reverse head rotation requires EF-G dissociation following normal Pi release. We observed that fusidic acid fully (S11·S13) or mostly (S12·S19) abolishes reverse head rotation (Fig. 5), strongly supporting a requirement for EF-G dissociation prior to reverse head rotation.

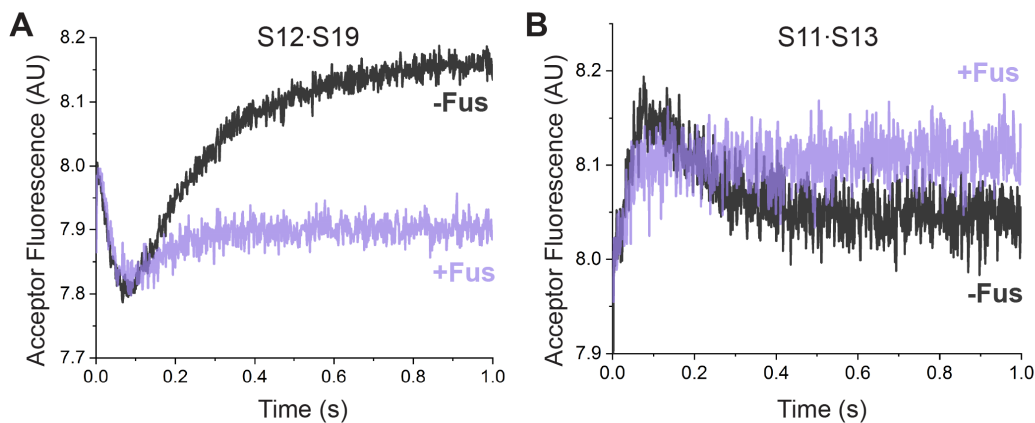


Figure 5. Fusidic acid inhibits reverse rotation of the 30S head domain. Head rotation was monitored using the (A) S12-S19 and (B) S11-S13 FRET pairs. Pre-translocation complex was mixed with EF-G and GTP in the absence or presence of fusidic acid (Fus). Traces over 5 sec are shown in Fig. S7.

DISCUSSION

During every round of EF-G-catalyzed translocation, EF-G hydrolyzes GTP (Nishizuka and Lipmann, 1966a, 1966b; Gupta et al., 1971). However, the purpose for consumption of such energy during each round of translocation has remained unclear. It has been proposed that the energy of hydrolysis is coupled to tRNA movement through a conformational change in EF-G – often termed a “power stroke” – that pushes the tRNAs (Rodnina et al., 1997; Chen et al., 2013, 2016; Yao et al., 2013; Yin et al., 2019). An alternative proposal is that translocation proceeds by a Brownian ratchet mechanism, in which the tRNAs move spontaneously, driven by thermal energy, and that EF-G acts as a “pawl” to bias the direction of their movement (Spirin, 2009; Frank and Gonzalez, 2010; Liu et al., 2014; Salsi et al., 2016; Wasserman et al., 2016; Djumagulov et al., 2021; Ou and Petrov, 2023). In the latter mechanism, GTP hydrolysis could provide a switch to prevent premature release of EF-G from the ribosome to ensure that translocation is productive and that the correct reading frame is maintained (Carbone et al., 2021; Rexroad et al., 2022; Milicevic et al., 2024).

Structural studies show that the cleaved γ -phosphate remains bound to EF-G in almost exactly the same position as in unhydrolyzed GTP (Carbone et al., 2021; Petrychenko et al.,

2021; Rundlet et al., 2021). Because the bulk of the energy derived from hydrolyzing GTP comes from the charge separation resulting from release of Pi, rather than the hydrolytic event itself (Ross, 2006; Fontecilla-Camps, 2022), we sought to investigate the timing of Pi release with respect to the series of structural rearrangements of the ribosome that occur during translocation.

To follow the structural dynamics of the ribosome, we used previously developed FRET pairs that directly report on intersubunit (Ermolenko et al., 2007) and 30S head (Guo and Noller, 2012) rotation. To monitor association and dissociation of EF-G from the ribosome, we developed a novel FRET pair (Fig. 4); and to measure the kinetics of Pi release we used a previously described fluorescent phosphate sensor (Brune et al., 1994). We then used a range of strategies to inhibit translocation, including antibiotics, mutant forms of EF-G, and the Pi analog BeF_3^- , to dissect the interdependence of the above events. Our findings clarify the order of events during the late steps of translocation and reveal how Pi release is functionally connected to the structural rearrangements of the ribosome. These results shed light on the roles of GTP hydrolysis and Pi release during translocation.

We proposed previously that Pi release could be an important signaling event that allows EF-G to dissociate only after completion of movement of the tRNAs and mRNA relative to the 30S body (Rexroad et al., 2022). During movement of the tRNAs and mRNA, pairing of the codon-anticodon duplex is stabilized by contact with domain IV of EF-G (Gao et al., 2009; Brilot et al., 2013; Ramrath et al., 2013; Zhou et al., 2014, 2019) to prevent frameshifting (Peng et al., 2019; Niblett et al., 2021). Thus, premature dissociation of EF-G could result in unproductive translocation or risk a potentially lethal frameshifting event. Our results here provide further evidence supporting this model.

The fastest event we observe is forward head rotation, followed by slower reverse intersubunit rotation and release of Pi from EF-G. This timing is consistent with our proposed model. Since release of Pi allows dissociation of EF-G, delaying release of Pi until completion of forward head rotation, which is coupled to movement of the codon-anticodon duplex, would

maintain binding of EF-G to the ribosome throughout tRNA movement. This finding also shows that Pi release cannot induce forward head rotation, as was suggested in a recent structural study (Petrychenko et al., 2021). However, if Pi release only needs to be delayed until after tRNA movement, we might expect it to be triggered by completion of forward head rotation. Surprisingly, we find that Pi release is instead coupled to reverse intersubunit rotation. This result is consistent with the results of a recent time-resolved cryo-EM study (Carbone et al., 2021). In the structure of a pre-translocation *E. coli* ribosome bound with EF-G·GDP·Pi, which has a high degree ($\sim 10^\circ$) of intersubunit rotation, switch I of EF-G bridges the sarcin-ricin loop (SRL) of the 50S subunit and h14 of the 30S subunit. During the transition to the following intermediate, the ribosome undergoes reverse intersubunit rotation and release of Pi from EF-G. The authors propose that reverse intersubunit rotation moves h14 in the 30S subunit out of contact with switch I, consequently disrupting this stabilizing interaction; the structure of a similar translocation intermediate observed in *Giardia* (Majumdar et al., 2023) is consistent with this finding. The loss of interaction between switch I and the 30S subunit causes switch I to become disordered and for Pi, whose binding is stabilized by an ordered switch I, to be released from EF-G. Our finding that Pi release is coupled to reverse intersubunit rotation supports such a mechanism.

It is noteworthy that Pi release and reverse intersubunit rotation are ~ 2 -3-fold slower than forward head rotation, indicating a delay between completion of forward head rotation and release of Pi. The functional significance of this timing is unclear. It is possible that some unidentified event takes place before completion of reverse intersubunit rotation. It is also possible that this timing evolved simply to ensure that translocation on the small subunit body has been completed before Pi is released. This delay may also provide time for thermodynamically favorable interactions between the tRNA and mRNA to be re-established in case any slippage in the reading frame has occurred during movement.

Finally, our kinetic observations suggest that Pi release, EF-G dissociation, and reverse head rotation occur in succession at similar rates. Release of Pi allows EF-G to

progressively dissociate from the ribosome (Savelsbergh et al., 2005), evidenced by a stepwise decrease in the observed buried surface area by EF-G over the final sequential translocation intermediates (Carbone et al., 2021). We find that Pi release is required for reverse head rotation, which is explained by our finding that reverse head rotation also requires dissociation of EF-G (Fig. 5). Although it has been reported previously that the antibiotic fusidic acid, which prevents EF-G dissociation, slows reverse head rotation (Belardinelli and Rodnina, 2017), here we show that it blocks completion of reverse head rotation (Fig. 5). This discrepancy may be attributed to a difference in the FRET pairs used in the two studies. In the previous report, the FRET pair used to monitor head rotation has since been shown to report primarily on reverse intersubunit rotation (Rexroad et al., 2022). By contrast, both FRET pairs used here report directly and exclusively on head rotation. The requirement for dissociation of EF-G prior to reverse head rotation is consistent with the observation that EF-G stabilizes the head in its rotated state (Ratje et al., 2010; Ramrath et al., 2013; Zhou et al., 2013). Since reverse head rotation is the final step in the translocation process and establishes the final interactions between the tRNAs and the ribosome, preventing its reversal until the tRNAs are in their final positions would be important for accomplishing productive translocation. However, an intriguing remaining question is: what prevents the tRNAs from reversing during reversal of the head domain?

Our results are consistent with a role for Pi release in regulating the timing of release of EF-G from the ribosome, in agreement with the findings of Carbone et al. (Carbone et al., 2021). Perhaps surprisingly, the role of EF-G must be to *restrain* the tRNA, ensuring that the motions of the tRNAs are coordinated precisely with those of the mRNA. In fact, during spontaneous movement of peptidyl-tRNA in the absence of EF-G (Zhou et al., 2019), the tRNA moves *further* than in EF-G-catalyzed translocation. Like catalysis of peptide bond formation (Sievers et al., 2004), the challenge for the ribosome in translocation must be largely entropic in nature.

Finally, our findings have implications for a possible underlying functional commonality among the translational GTPases, which share extensive structural homology (Leipe et al., 2002). Notably, delayed Pi release has also been observed for elongation factor EF-Tu (Kothe and Rodnina, 2006) and initiation factors IF2 (Tomšic et al., 2000) and eIF2 (Algire et al., 2005), whose roles are also centered primarily on the accuracy of key events in protein synthesis (Tapio and Kurland, 1986; Hughes et al., 1987; Vijgenboom and Bosch, 1989; Caban and Gonzalez, 2015).

MATERIALS & METHODS

Materials

Doubly-labeled S11(Alx488)-S13(Alx568) and S12(Alx488)-S19(Alx568) and singly-labeled S12(Cy3) or S12(Cy5) ribosomes were formed by in vitro reconstitution, and doubly-labeled S6-L9 ribosomes were formed by combining 30S(Δ S6) and 50S(Δ L9) with S6-Cy5 and L9-Cy3, respectively, as previously described (Niblett et al., 2021).

Transcribed mRNA mv39 and NAcMet-tRNA^{Met}, as well as Val-tRNA^{Val1} (Sigma) were prepared as described (Niblett et al., 2021; Rexroad et al., 2022). GTP and GDP were purified by anion-exchange chromatography on a Resource Q column (Pharmacia Biotech).

Mutations Q254C and H92Q were introduced to EF-G through QuikChange Mutagenesis (Stratagene). The mutation D20N in EF-G was created using the Kunkel method (Kunkel, 1985). The Q254C mutation was made in a -Cys background (Wilson and Noller, 1998). EF-G(Q254C) was then expressed in BLR(DE3) and purified essentially as described (Guo and Noller, 2012), with the exception that β ME was omitted from the storage buffer. The H92Q and D20N mutant forms of EF-G were expressed in DH10B and purified essentially as described (Niblett et al., 2021).

Phosphate was removed from all EF-G used in Pi release experiments by purification on a S75 size-exclusion column (Pharmacia Biotech). EF-G(Q254C) was labeled with Cy3 or Cy5, and excess dye was removed by size-exclusion chromatography on a S75 column

(Pharmacia Biotech). Fluorescent-labeled EF-G was purified from unlabeled EF-G on a phenyl column by binding EF-G in buffer containing 25 mM Tris (pH 7.5), 1M (NH₄)₂SO₄, 400 mM NH₄Cl, and 5 mM β ME and applying a step-wise gradient of decreasing concentrations of (NH₄)₂SO₄. S12(K108C) was labeled with Cy3 or Cy5 essentially as described (Rexroad et al., 2022).

Stopped-Flow Kinetics

Pre-translocation complexes containing mRNA, tRNA^{Met} in the P site, and NAcMet-Val-tRNA^{Val} in the A site were formed as described previously (Rexroad et al., 2022) and rapidly mixed with EF-G and GTP in a SX20 stopped-flow fluorometer (Applied Photophysics) at 22°C. Paromomycin (Sigma) and fusidic acid were used at concentrations of 5 μ M and 0.2 mM, respectively.

For S12-S19 and S11-S13 experiments, Alexa488 was excited at 496 nm and emission was collected with a 590 nm long-pass filter. For S6-L9 and EF-G-S12 experiments, Cy3 was excited at 550 nm and emission was collected with a 645 nm long-pass filter. For Pi release experiments, coumarin was excited at 430 nm and emission was collected with a 455 nm long-pass filter. For experiments containing only Cy5, Cy5 was excited at 600 nm and emission was collected with a 645 nm long-pass filter.

All stopped-flow experiments were conducted as described previously (Rexroad et al., 2022), with the following changes. In experiments monitoring Pi release, 250 nM MDCC-PBP (phosphate binding protein labeled with N-[2-(1-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide) (Invitrogen) was present in both syringes prior to mixing. The final concentrations after mixing in stopped-flow were 50 nM ribosomes, 250 nM EF-G, 250 nM MDCC-PBP, and 50 μ M GTP. For FRET experiments directly compared with Pi release experiments, identical conditions were used, but MDCC-PBP was omitted.

For assays containing BeF₃⁻, BeF₂ and NaF were combined and allowed to sit for several min at 22°C before being added to EF-G and GTP (or GDP). The final concentrations

after mixing in stopped-flow were 2 mM BeF₂ and 5 mM NaF, unless noted otherwise. GDP was purified from GTP on an anion-exchange column. GDP, BeF₂, and NaF were all used at final concentrations of 0.5 mM in experiments containing both GDP and BeF₃⁻.

For assays monitoring EF-G dissociation with the EF-G-S12 FRET pair, 70S-(S12-Cy3) and EF-G-Cy5 were combined in stopped-flow. Experiments monitoring head rotation for comparison with EF-G dissociation kinetics used 70S-(S12-Alx488)-(S19-Alx568) and EF-G-Cy5.

Kinetic Analysis

Fluorescence traces for Pi release were fit to double exponential functions of the form $y = c + a_1e^{-k_1t} + a_2e^{-k_2t} + c_3t$. An initial decrease in fluorescence was seen in many traces, and was included in the fit to account for its effect on the rate constant associated with the burst phase (k_2). Each curve was first fit over a range of endpoints, and the endpoint chosen was the earliest time at which all kinetic parameters appeared to have relatively stable values. For each condition, the corresponding FRET traces were fit over similar timescales. Head rotation and reverse intersubunit rotation data were fit to double and single exponential functions, respectively. An added linear term (c_3*t) accounting for drift in the signal was included in the fit when needed. For S11-S13 FRET experiments with either EF-G(D20N) or paromomycin and EF-G-S12 FRET experiments, individual traces had high levels of noise; to more accurately fit these traces, traces from each experiment were averaged and then fit.

Rate constants and errors were obtained from a minimum of 3 independent experiments, each with at least 3 separate traces. Rate constants were averaged for each experiment, and the means were averaged to obtain reported values. A pooled standard deviation was calculated with the equation $\sigma_{\text{pooled}} = \sqrt{(\sigma_1^2 + \sigma_2^2 + \sigma_3^2 + \dots) / n}$, based on the standard deviations for n independent experiments. Additionally, a standard deviation of the means for independent experiments was calculated. The larger of these two values was

reported as the error. Figures containing fluorescence traces and fit curves were created using OriginLab.

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SUPPLEMENTAL INFORMATION

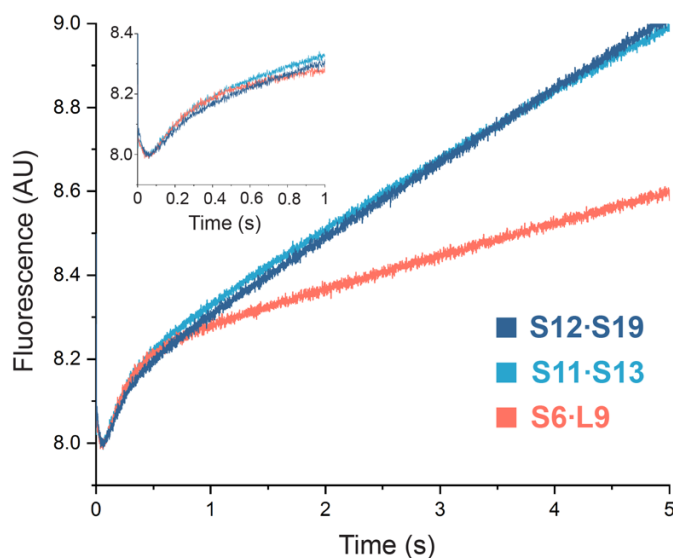


Figure S1. Kinetics of rapid Pi release from EF-G does not depend on FRET labeling scheme. Pi release was monitored using MDCC-PBP in the presence of ribosomes with each of three FRET labeling schemes (S12-S19, S11-S13, and S6-L9). Inset shows first 1 sec.

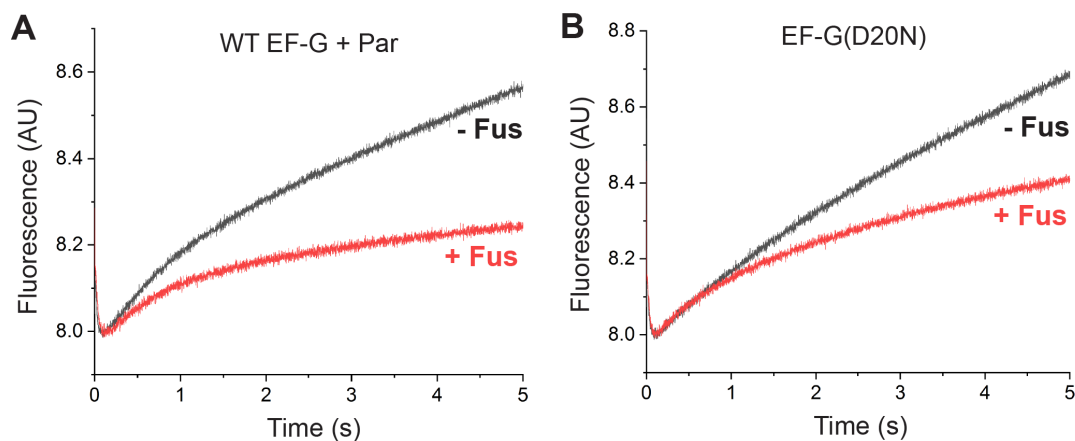


Figure S2. Fusidic acid lessens the turnover phase during phosphate release. To minimize the contribution of the linear turnover phase to the overall signal in Pi release experiments, fusidic acid was added to (A) paromomycin (Par) and (B) EF-G(D20N). Pi release in the absence (-Fus) and presence (+Fus) of fusidic acid are shown.

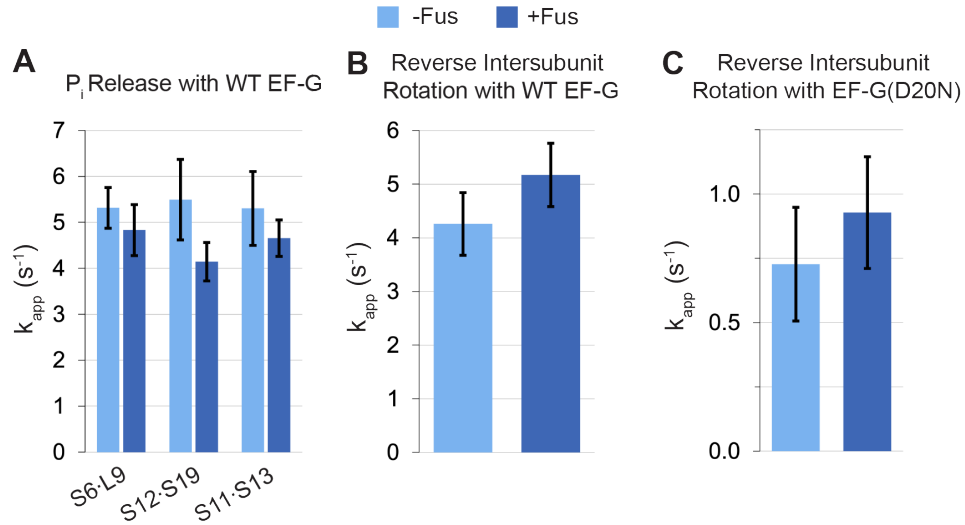


Figure S3. Fusidic acid has no significant effect on rate constants for phosphate release or reverse intersubunit rotation. (A) Rate constants for P_i release obtained from each fluorescently-labeled ribosome construct. (B,C) Rate constants for reverse intersubunit rotation, monitored with S6-L9 FRET pair in the presence of (B) WT EF-G and (C) EF-G(D20N). Experiments were conducted in the absence (light blue) or presence (dark blue) of fusidic acid.

Table S1. Kinetics of rotational events and release of phosphate

Event	Reverse intersubunit rotation (s ⁻¹)	Forward head rotation (s ⁻¹)		Reverse head rotation (s ⁻¹)		Pi Release (s ⁻¹)		
FRET Pair	S6-L9	S12-S19	S11-S13	S12-S19	S11-S13	S6-L9	S12-S19	S11-S13
WT EF-G	4.3 ± 0.6 [5.2 ± 0.6]	13 ± 2	14 ± 3	3.7 ± 0.4	3.2 ± 0.5	5.3 ± 0.4 [4.8 ± 0.6]	5.5 ± 0.9 [4.1 ± 0.4]	5.3 ± 0.8 [4.7 ± 0.4]
EF-G (D20N)	0.7 ± 0.2 [0.9 ± 0.2]	9 ± 2	5.5 ± 0.8	0.8 ± 0.2	1.0 ± 0.2	[1.15 ± 0.08]	[1.6 ± 0.3]	[1.26 ± 0.05]
Par	[1.4 ± 0.1]	4 ± 1	5 ± 1	0.9 ± 0.1	1.0 ± 0.3	[1.7 ± 0.1]	[1.2 ± 0.1]	[1.6 ± 0.3]
GTPyS	1.9 ± 0.2	2.07 ± 0.07	2.3 ± 0.2	0.16 ± 0.03	0.24 ± 0.01	ND	ND	ND
EF-G (H92Q)	0.8 ± 0.1 [0.9 ± 0.2]	1.0 ± 0.2	1.2 ± 0.2	0.37 ± 0.03	0.23 ± 0.08	ND	ND	ND

Pseudo-first order rates constants for reverse intersubunit rotation, forward and reverse head rotation, and P_i release. Rotational movements were monitored by FRET and P_i release was monitored by MDCC-PBP using ribosomes with each FRET labeling scheme. Brackets denote experiments that were conducted in the presence of 0.2 mM fusidic acid. (Par) paromomycin. (ND) indicates rates that could not be determined, as explained in the main text. Data for GTPyS from Rexroad et al. 2022.

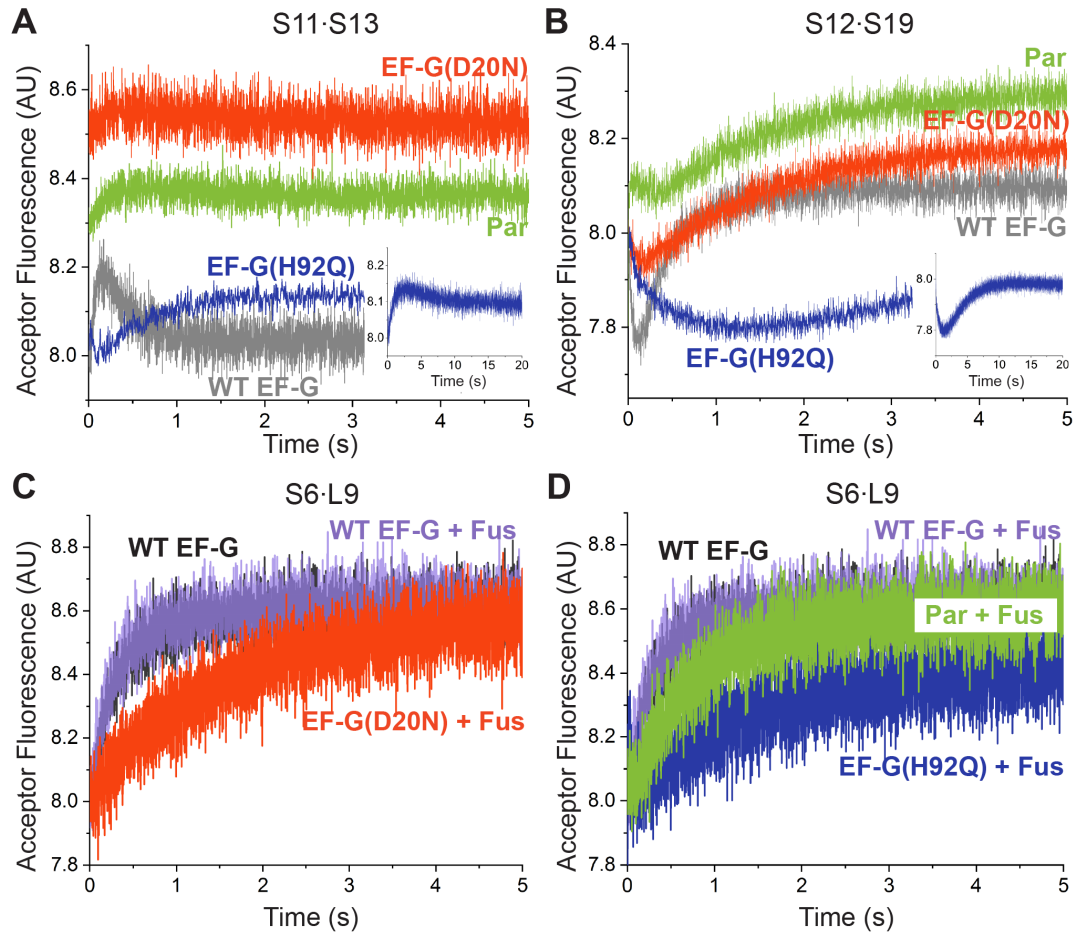


Figure S4. FRET experiments with EF-G mutants and paromomycin. Head rotation was monitored with (A) S11·S13 and (B) S12·S19 FRET pairs. (C,D) Reverse intersubunit rotation was monitored with the S6·L9 FRET pair. Wild-type or mutant EF-G and GTP were rapidly combined with pre-translocation complex. Experiments containing an antibiotic (Fus and/or Par) also contained WT EF-G and GTP. Fusidic acid was only used where noted. Insets show traces for EF-G(H92Q) over 20 seconds. Fusidic acid (Fus); paromomycin (Par).

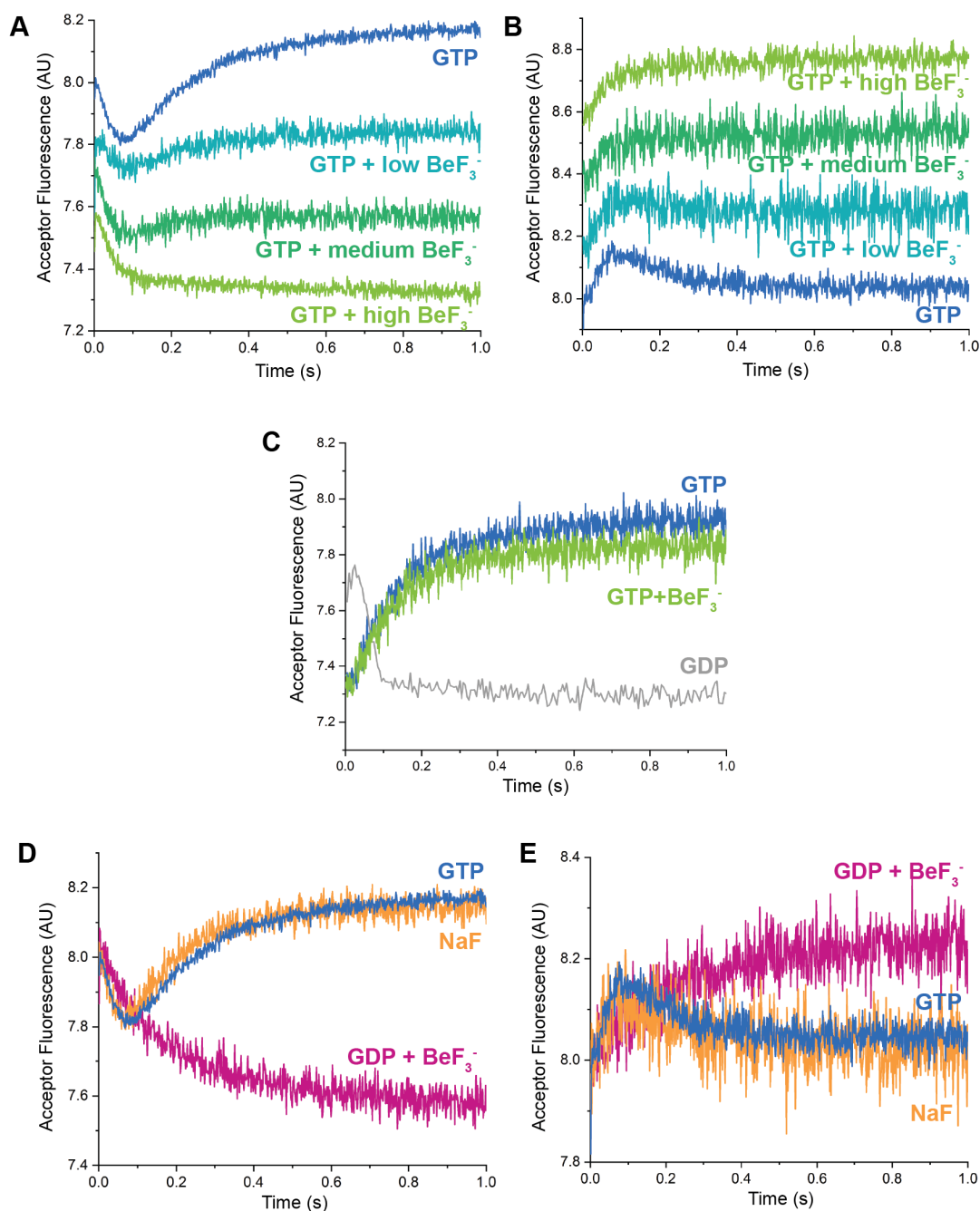


Figure S5. Additional control experiments conducted with BeF₃⁻ (A,B) Head rotation was monitored with the S12·S19 (A) and S11·S13 (B) FRET pairs in the presence of increasing amounts of BeF₂ and NaF. With increasing concentrations, reverse head rotation diminishes until it is completely abolished. 0.5 mM BeF₂ + 0.5 mM NaF (low BeF₃⁻); 1 mM BeF₂ + 4 mM NaF (medium BeF₃⁻); 2 mM BeF₂ + 5 mM NaF (high BeF₃⁻). (C) Reverse intersubunit rotation was monitored in the presence of GTP, GTP and BeF₃⁻, or GDP. BeF₃⁻ did not affect the kinetics of reverse intersubunit rotation. The drop in fluorescence in the GDP trace during the first 100 ms is an artifact of not engaging the pressure hold on the stopped-flow fluorometer

during long observation times. (D,E) Head rotation was monitored with S12·S19 (D) and S11·S13 (E) FRET pairs. Head rotation was unchanged in the presence of NaF alone. EF-G pre-incubated with GDP and BeF_3^- was able to support slow forward head rotation, but not reverse head rotation.

Table S2. Rate constants for reverse head rotation, EF-G movement, and EF-G dissociation

FRET Pair	Event	k_1 (s^{-1})	Event	k_2 (s^{-1})
S12·S19	Forward head	22 ± 4	Reverse head	5.6 ± 0.7
EFG·S12	EF-G Movement	19 ± 2	EF-G Dissociation	6 ± 2

Pseudo first order rate constants obtained from experiments in Fig. 4. EF-G(Q254C)-Cy5 was used in both experiments. Errors represent pooled standard deviation (S12·S19) or standard deviation of fitted average traces (EFG·S12), as detailed in Methods.

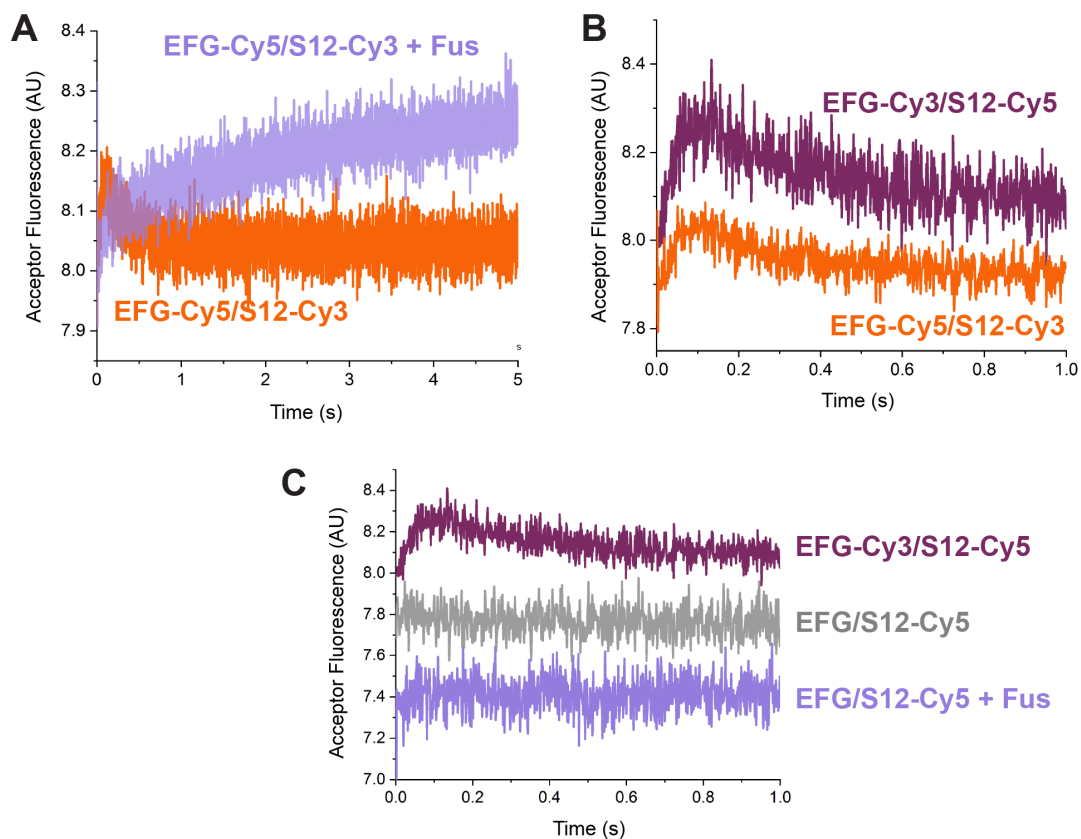


Figure S6. Validation of EF-G-S12 FRET pair. (A) Ribosomes containing Cy3-labeled S12 were rapidly combined with EF-G-Cy5 and GTP in the presence or absence of fusidic acid (Fus). (B) Similar fluorescence signatures were observed when the labels (Cy3 and Cy5) were reversed on S12 and EF-G. (C) Ribosomes containing S12-Cy5 were rapidly combined with unlabeled EF-G(Q254C) and GTP, in the presence or absence of fusidic acid (Fus). The fluorescence of directly excited Cy5 was unchanged in both experiments.

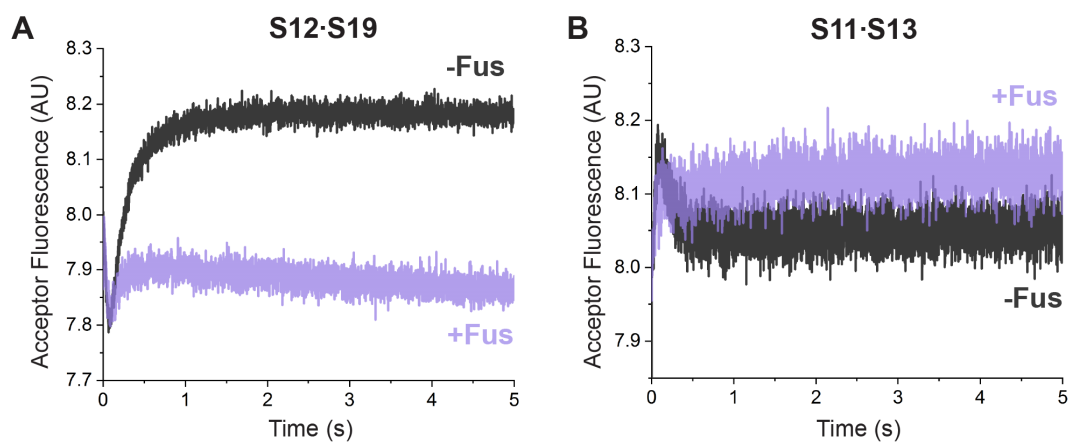


Figure S7. Fusidic acid inhibits reverse head rotation. Head rotation was monitored with S12-S19 (A) and S11-S13 (B) FRET pairs by combining pre-translocation complex with EF-G and GTP in the presence or absence of fusidic acid (Fus). The first 1s of the above traces can be seen in Fig. 5.

Chapter 5. A method for construction of active ribosomes containing site-specific fluorescent labels in 16S ribosomal RNA

ABSTRACT

Site-specific internal fluorescent labeling of ribosomal RNA (rRNA) would greatly aid studies of the ribosome by expanding the set of potential probe positions in fluorescence-based assays. However, introducing fluorescent labels to rRNA while maintaining its structural and functional integrity is a major challenge. Here, we adapt trans-acting site-specific nucleoside transferase ribozymes to label 16S rRNA. We have developed conditions for efficient labeling and for removing the annealed ribozymes from 16S rRNA following the reaction that preserve the activity of the labeled rRNA. We further demonstrate efficient incorporation of the fluorescently-labeled 16S rRNA into functional ribosomes. This method may also prove useful for labeling of other large, structured, functional RNAs.

INTRODUCTION

Following structural changes within the ribosome is critical for studying translation. Förster resonance energy transfer (FRET) is an integral technique for observing changes to the structure of the ribosome and for monitoring the binding of factors. FRET pairs containing at least one dye attached to the ribosome have been used to study these events during initiation (Hickerson et al., 2005; Marshall et al., 2008; Ling and Ermolenko, 2015; MacDougall and Gonzalez, 2015), tRNA selection (Chen et al., 2011), and translocation (Ermolenko et al., 2007; Cornish et al., 2008, 2009; Fei et al., 2009; Guo and Noller, 2012; Belardinelli et al., 2016; Wasserman et al., 2016). Fluorescent labels are typically introduced to the ribosome by attaching maleimide-derivatized fluorophores to non-native cysteine residues in ribosomal proteins. For example, to monitor intersubunit rotation, fluorescent dyes are attached to ribosomal proteins S6 in the small 30S subunit and L9 in the large 50S

subunit (Ermolenko et al., 2007). These ribosomes are easily produced by isolating subunits from deletion strains ($\Delta S6$ or $\Delta L9$) and reconstituting with fluorescent-labeled S6 and L9. However, this strategy is not available for all reporters, as many ribosomal proteins are essential.

When deleting a target protein is not possible, the ribosome must be reconstituted completely from its individual components. The 30S subunit can be formed by combining purified 16S rRNA and twenty independently expressed and purified ribosomal proteins (Culver and Noller, 2000). This strategy was used to assemble ribosomes for monitoring rotation of the 30S subunit head domain; fluorophores were attached to S11 and S13 or S12 and S19 in the body and head domains of the 30S subunit, respectively (Guo and Noller, 2012).

The ability to label rRNA would expand the set of possible positions for labels as well as obviate the need for labor-intensive reconstitutions that require separate purification of twenty 30S proteins. One such method introduces labels by hybridizing fluorescent-labeled oligonucleotides to helical extensions of the rRNA; this strategy has been used to observe intersubunit rotation by labeling h44 in the 30S subunit and H101 in the 50S subunit. However, the requirement for a helical extension greatly limits the available labeling positions, and hybridization of oligonucleotides to rRNA disrupts its structure. We therefore sought to explore strategies for *directly* labeling rRNA with a probe through a covalent linkage.

Labeling rRNA poses several challenges. First, fluorescently-labeled nucleotides cannot be incorporated into rRNA using solid-phase synthesis due to limitations on length (16S rRNA is 1542 nucleotides). Second, rRNA is highly structured, so mild conditions and minimal manipulation are essential to preserve its structure. Not surprisingly, there are few approaches for post-transcriptionally labeling long RNAs with site-specificity at internal positions. Methods involving chemical modification (Zhao et al., 2018), a methyltransferase (Tomkuvienė et al., 2012), or an alkyltransferase ribozyme (Okuda et al., 2023) have been used to introduce an alkyne group to a targeted adenine, to which an azide-derivatized

fluorophore can be conjugated; use of hybridization or guide strand(s) can then provide site-specificity to such approaches. In another method, a deoxyribozyme (Büttner et al., 2014) adds a fluorescent-labeled nucleoside to the 2' hydroxyl of a targeted adenosine. However, the deoxyribozyme-catalyzed addition is accelerated by a terbium (Tb^{3+}) cofactor, which is less ideal for use with sensitive RNAs.

More recently, a ribozyme-catalyzed reaction, also adding a fluorescent-labeled nucleoside to the 2'-hydroxyl of a targeted adenosine under mild conditions, has been developed (Ghaem Maghami et al., 2019). This method also uses commercially available reagents, has been tested on 5S rRNA, and at the time this study began was the most easily adaptable strategy for labeling rRNA; we therefore elected to pursue it. This trans-acting nucleoside transferase ribozyme, developed through *in vitro* evolution, is composed of a conserved catalytic region flanked by two arms that can be designed to hybridize to any targeted RNA sequence, conferring site-specificity (Fig. 1A). This ribozyme can use as a substrate an ATP analog containing a fluorescent moiety attached to its N^6 position (Fig. S1). The ribozyme forms a 2'-5' phosphodiester bond between a targeted internal adenosine and the modified adenosine substrate (Fig. 1B), releasing pyrophosphate in the process.

Here, we adapt the ribozymes developed by Ghaem Maghami et al. (2019) for efficient labeling of 16S rRNA, which can then be incorporated into functional ribosomes. We optimized the efficiency of the ribozyme in adding a Cy(3/5)-derivatized adenosine to rRNA, developed a procedure for quantitative removal of the ribozymes from rRNA under mild conditions, and achieved efficient reconstitution of the labeled 16S rRNA into active ribosomes. This method greatly expands the set of potential positions for fluorescent labeling of ribosomes for biophysical studies. Moreover, our approach should be applicable to site-specific fluorescent labeling of other large, structured, functional RNAs.

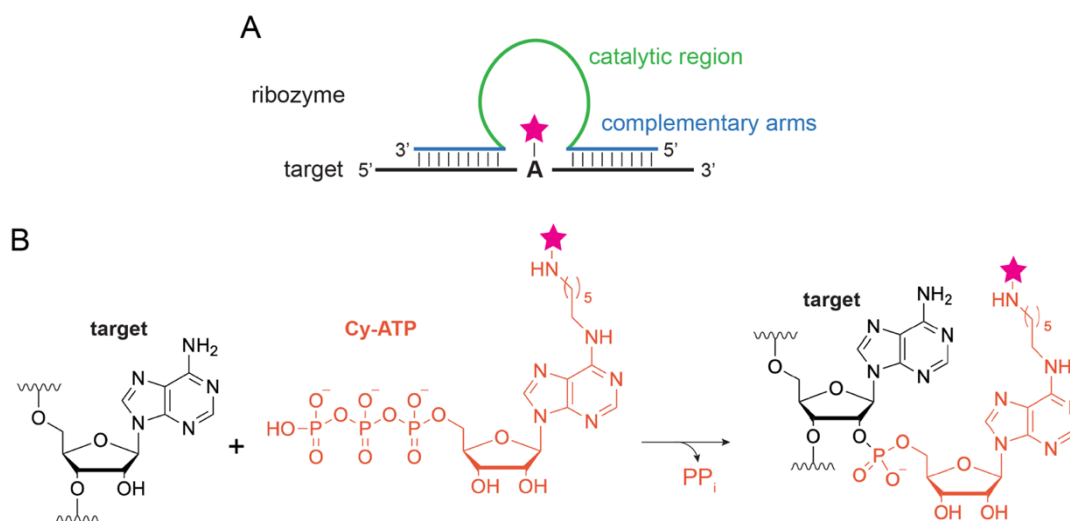


Figure 1. Site-specific fluorescent labeling of RNA by trans-acting ribozymes. (A) Nucleoside transferase ribozymes developed by Ghaem Maghami et al. (2019) contain a conserved catalytic region (green) flanked by arms (blue) designed to hybridize to the targeted RNA (black), enabling site-specific labeling at an internal adenosine. (B) Ribozymes use N⁶-Cy(3/5)-modified ATP (“Cy-ATP”) as a substrate to create a 2'-5' phosphodiester bond at the targeted adenosine, releasing pyrophosphate. Pink stars represent fluorophores. Full structures of N⁶-modified ATP analogs are shown in Fig. S1.

RESULTS

Ribozymes modify 16S rRNA with site-specific fluorescent labels

We chose two sites, A415 and A1019, in 16S rRNA with low conservation and occurring in different types of secondary structure as targets for labeling (Fig. 2A). We initially tested the ribozymes on short 35-45 nucleotide RNA targets designed based on the corresponding regions of 16S rRNA (Fig. S2). The ribozymes (hereafter termed Rz415 and Rz1019) efficiently modified the small target RNAs using Cy3-ATP as a substrate (Fig. 2B). Having established their activity on short RNAs, we next asked whether the ribozymes could modify full-length 16S rRNA.

After reacting 16S rRNA with the ribozymes, we assessed the ribozymes' site-specificity using a primer extension assay, in which extension by reverse transcriptase is blocked by bases modified with Cy(3/5). The position of labeling of 16S rRNA was followed by di-deoxynucleotide sequencing in parallel (detailed in Methods). Primer extension products

showed strong bands corresponding to the bases immediately prior to the targeted adenosines (G416 and G1020 for targets A415 and A1019, respectively) (Fig. 2C). This indicates that the reverse transcriptase cannot proceed past the modified nucleoside, as expected, showing that both ribozymes modified precisely their targeted adenosines.

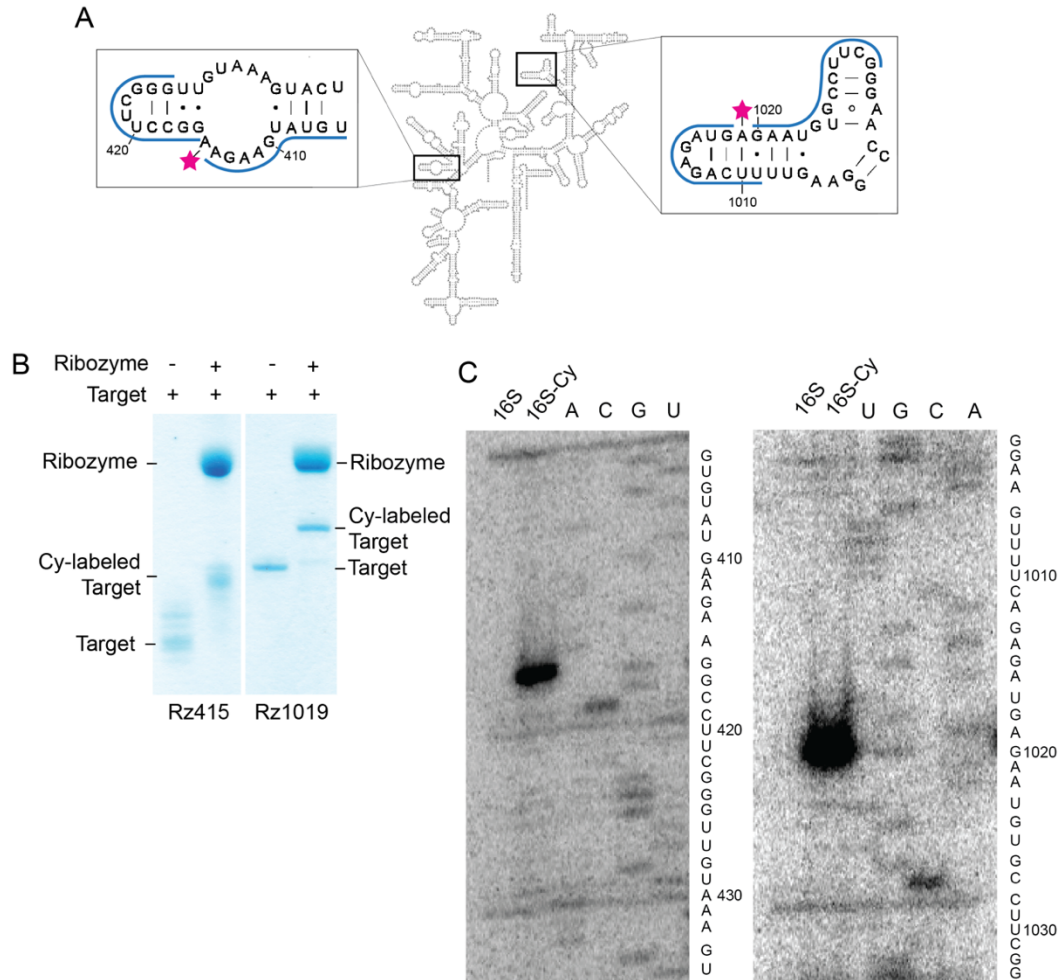


Figure 2. Ribozymes targeting two positions in 16S rRNA are site-specific. (A) Targeted adenosines in 16S rRNA (A415 and A1019), marked with pink stars. Blue lines indicate where the ribozymes' complementary arms were designed to anneal. (B) Modification of short RNAs by ribozymes visualized on a denaturing acrylamide gel stained with methylene blue. (C) Primer extension on 16S rRNA prior to (16S) and after (16S-Cy) modification by each ribozyme (left: Rz415; right: Rz1019). Sequencing lanes are labeled with A, C, G, and U. Sequence of relevant region of 16S rRNA is shown on right.

Optimization of the modification efficiency on 16S rRNA

Having established that the ribozymes modify 16S rRNA with site-specificity, we next sought to optimize the reaction conditions and labeling efficiency. Our first consideration was that the relatively high concentration (40 mM) of MgCl_2 used in our initial assays might cause degradation of the rRNA after prolonged incubation at 37°C. We tested the stability of 16S rRNA following incubation for 4h at 37°C in various concentrations of MgCl_2 . We observed that 16S is increasingly degraded with higher concentrations of MgCl_2 (Fig. 3A), so reducing the concentration of MgCl_2 would be beneficial. However, the ribozymes require Mg^{2+} for the reaction and thus their efficiency is highly sensitive to its concentration (Ghaem Maghami et al., 2019).

To find out if the ribozymes retained activity at a lower $[\text{Mg}^{2+}]$, we tested their activity modifying the short RNAs described above at various concentrations of MgCl_2 (Fig. 3B). Additionally, we compared their activity using two different ATP analog substrates: 6AH-ATP (N^6 -(6-aminoethyl)-ATP) and Cy5-ATP (N^6 -(6-aminoethyl)-Cy5-ATP) (Fig. S1; Fig. 3B). We observed that although the ribozymes have efficiency near 100% at 10 mM MgCl_2 when using 6AH-ATP as a substrate, this same efficiency is only achieved with 20-40 mM MgCl_2 when Cy5-ATP is the substrate.

We asked whether changing other conditions in the reaction could reduce the ribozymes' Mg^{2+} dependence when Cy5-ATP is the substrate. We tested ribozyme activity at 10 mM MgCl_2 in the presence of different solvents (20% or 40% ethanol, 20% methanol, 50% dioxane, or 10% DMSO) or with the addition of the detergent Nikkol to 0.1%. We observe that ribozyme modification efficiency is near 100% in the presence of 20% ethanol, DMSO, and methanol (Fig. 3C). We then tested the ribozyme efficiency modifying 16S rRNA in 10 mM MgCl_2 with 20% ethanol. The use of 20% ethanol significantly increases the labeling efficiency on 16S rRNA (Fig. 3D). Our results also show that after collecting modified 16S rRNA from a sucrose gradient, concentration on a spin column, and ethanol precipitation, excess dye is effectively removed (Fig. 3D).

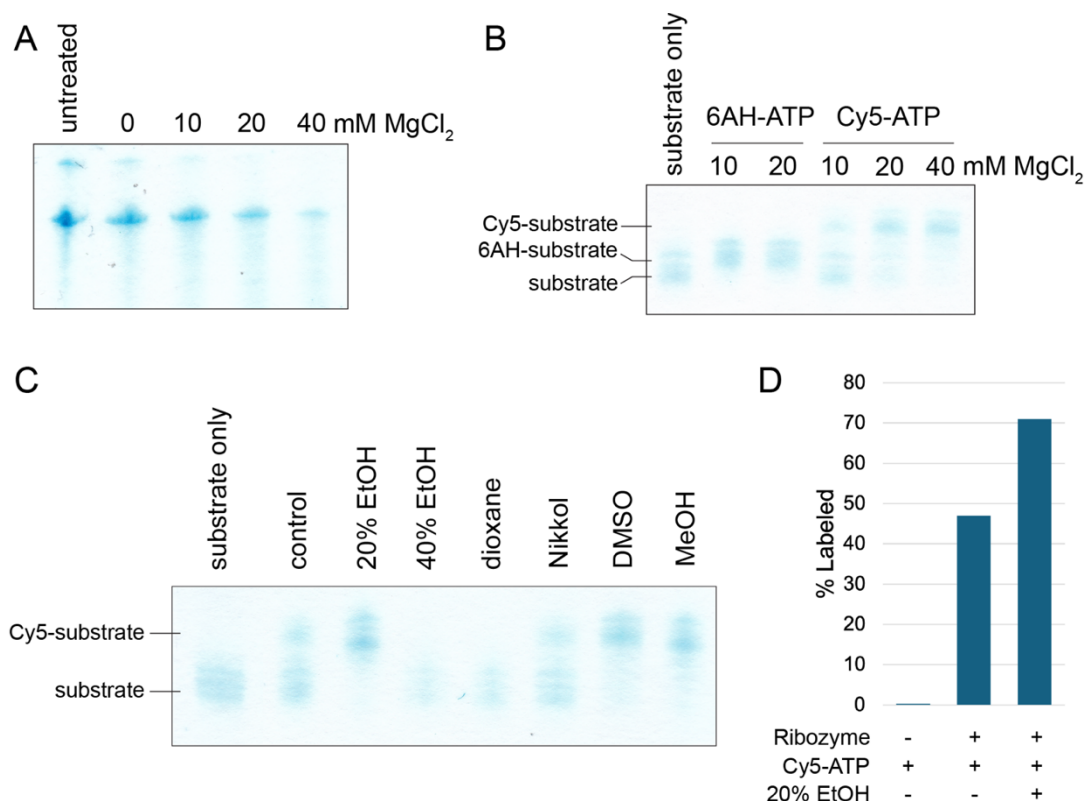


Figure 3. Optimizing efficiency of Cy dye labeling by ribozymes. (A) 16S rRNA visualized on acrylamide gel following incubation for 4h at 37°C in indicated concentrations of MgCl₂. Untreated sample was not incubated. (B) Modification of a short target RNA by Rz415 with 6AH-ATP (N⁶-(6-aminoethyl)-ATP) or Cy5-ATP (N⁶-(6-aminoethyl)-ATP-Cy5), visualized on an acrylamide gel. (C) Modification of a short RNA by Rz415 with Cy5-ATP in the presence of different additives and solvents. (D) Labeling efficiency of 16S rRNA by Rz1019 with Cy5-ATP in the presence or absence of 20% ethanol. Cy5-ATP was effectively removed on a sucrose gradient.

Anti-sense oligonucleotides efficiently remove ribozymes from target RNA

After the modification reaction is complete, the ribozymes remain annealed to the target RNA. This could pose a problem for two reasons: (i) annealed ribozymes disrupt the structure of the target rRNA and could therefore disrupt ribosomal function, and (ii) if a second fluorophore is to be added with a second ribozyme, the first ribozyme could also use this fluorophore at any unmodified original sites. To remove the ribozymes from the target RNA, we employed antisense DNA oligonucleotides (ASOs). Since the ribozymes are only annealed to the target RNA at their two complementary arms but additionally contain a

catalytic region, an ASO complementary to the entire ribozyme should be able to outcompete the target RNA (Fig. 4A). The ribozyme-ASO duplex can then be removed by collecting 16S rRNA from a sucrose gradient.

To test the efficacy of this strategy, ^{32}P -labeled ribozymes were annealed to 16S rRNA, to which ASOs were introduced. We observe efficient annealing of the ribozymes to 16S rRNA followed by efficient removal upon incubation with the ASOs (Fig. 4B). Additionally, ^{32}P -labeled ASOs did not show non-specific binding to 16S rRNA (Fig. 4C), allowing them to be used in excess of the ribozyme.

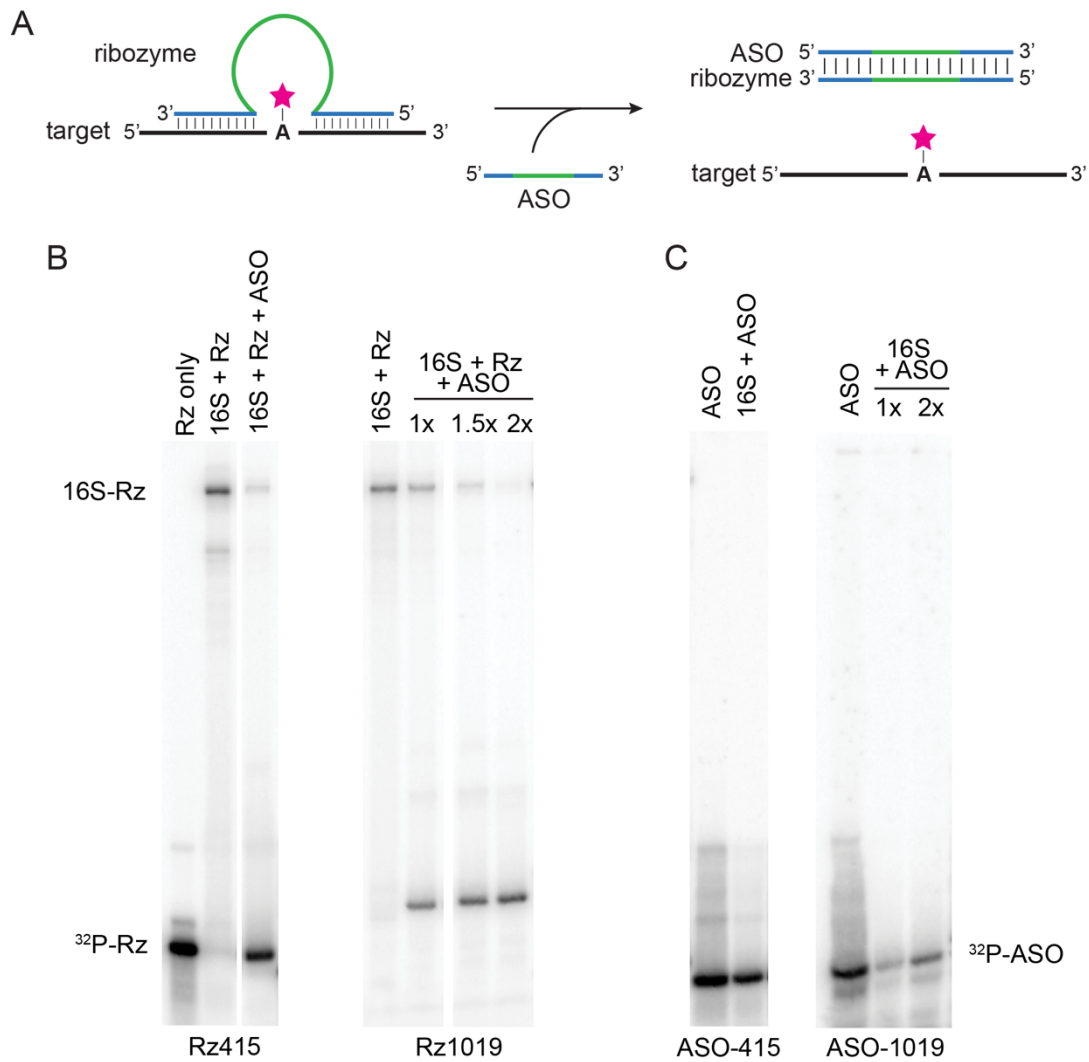


Figure 4. Removal of ribozymes from 16S rRNA with anti-sense oligonucleotides. (A) Schematic depicting removal of annealed ribozymes from 16S rRNA using ASOs. (B) Removal of ^{32}P -labeled ribozymes from 16S rRNA visualized on a non-denaturing acrylamide gel. Values indicate fold molar concentration of ASO over ribozyme. (C) ASOs do not bind to 16S rRNA, as visualized with ^{32}P -ASOs on a non-denaturing acrylamide gel. Values indicate fold molar concentration of ASO over 16S rRNA.

Reconstitution of ribosomes containing fluorescently-labeled 16S rRNA

After modifying 16S rRNA with Cy3 and Cy5 at the two targeted positions, 30S subunits were reconstituted using the labeled 16S and TP30 (total 30S ribosomal proteins extracted from natural 30S subunits). Reconstituted 30S subunits combine efficiently with natural 50S subunits to form 70S ribosomes that migrate similarly to natural tight-couple 70S ribosomes from *E. coli* (Fig. 5A). The reconstituted ribosomes are also able to synthesize a full-length 27 kD protein in a previously developed (Niblett et al., 2021) *in vitro* translation assay (Fig. 5B). Overall, these findings demonstrate that fluorescent-labeled 16S rRNA can be incorporated into highly active ribosomes for use in *in vitro* assays.

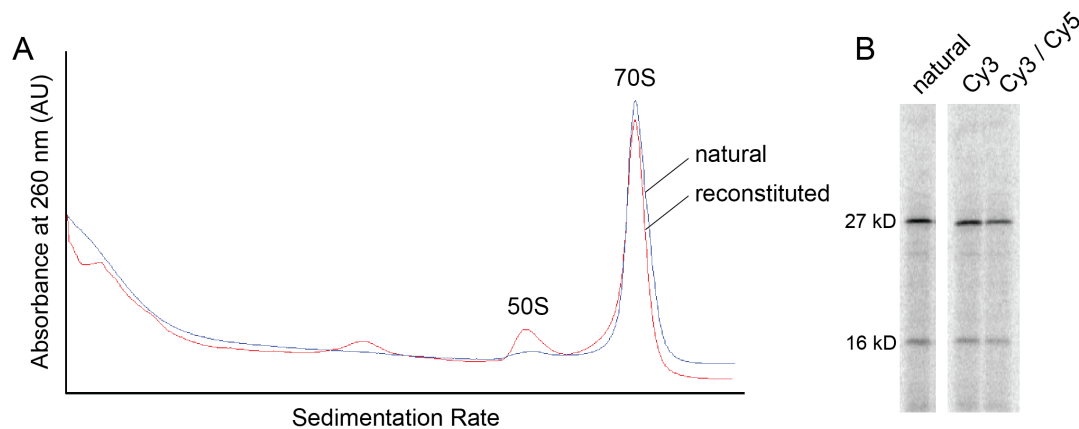


Figure 5. Fluorescent-labeled 16S rRNA can be reconstituted into active ribosomes. (A) 70S containing 16S labeled with Cy dyes at both A415 and A1019 sediments at a similar rate to natural tight-couple 70S. (B) Reconstituted ribosomes containing 16S-(A1019-Cy3) ("Cy3") or 16S-(A415-Cy3)-(A1019-Cy5) ("Cy3/Cy5") can synthesize a full length protein (27kD) in an *in vitro* translation assay. Natural tight-couple ribosomes were used for comparison. The 16kD product results from a frameshift at a slippery sequence introduced to the sequence (described in Methods); this aspect of the assay is not relevant for establishing activity of the ribosomes. (Assay performed by Dr. Alexandria Smart)

CONCLUSION

Here, we have adapted the ribozymes developed by Ghaem Maghami et al. (2019) for fluorescent labeling of 16S rRNA. We show that the ribozymes can label 16S rRNA with Cy dyes site-specifically and efficiently. We present a protocol for efficiently labeling 16S rRNA with Cy3/Cy5 and incorporating the labeled 16S rRNA into functional ribosomes. This protocol contains the following improvements and additional steps compared to the initial presentation of these ribozymes by Ghaem Maghami et al. (2019): (i) the reaction conditions have been altered to allow for a lower $[Mg^{2+}]$ to be used, minimizing degradation of the rRNA during incubation for extended periods of time; (ii) the efficiency of modification has been optimized at this lower Mg^{2+} concentration, resulting in average efficiencies of 60% and 75% for ribozymes targeting A415 and A1019, respectively; (iii) we developed a method for removal of the annealed ribozymes using ASOs, so that the risk of altering rRNA structure or function by annealed ribozymes is eliminated; (iv) efficient reconstitution of fluorescent-labeled 16S rRNA into 30S subunits has been demonstrated; and finally (v) ribosomes containing 16S rRNA with up to two fluorescent labels are shown to be active in translation.

This method unlocks many new potential sites for labeling the ribosome with fluorophores, which will be useful when designing reporters for future biophysical studies. While this method was being developed, a site-specific tenofovir transferase ribozyme was reported (Ghaem Maghami et al., 2020). Rather than using ATP analogs, this ribozyme uses analogs of the antiviral drug tenofovir, a non-natural nucleotide analog. The tenofovir transferase and nucleotide transferase ribozymes are orthogonal, presenting the possibility for future one-pot double labeling of rRNA.

Finally, the method presented here may be adaptable to labeling other long, functional RNAs with tags such as fluorophores, spin labels, and biotin. Specifically, optimization of the labeling efficiency with Cy dyes at lower $[Mg^{2+}]$ and the use of ASOs to remove the annealed ribozymes should aid future labeling efforts. While some optimization of

conditions may be necessary for each RNA of interest, the advancements made here represent a framework for labeling large, structured RNAs for use in functional assays.

MATERIALS AND METHODS

Materials

Ribozymes and short RNAs were transcribed *in vitro* with T7 RNA polymerase using oligonucleotide templates (see sequences below) followed by gel purification. Tight-couple MRE600 70S, 30S and 50S subunits, and 16S rRNA were purified as described (Moazed and Noller, 1989; Culver and Noller, 2000; Lancaster et al., 2002; Hickerson et al., 2005). TP30 was purified essentially as described (Nierhaus, 1990). Oligonucleotides were obtained from IDT. ATP analogs (N⁶-(6-aminohexyl)-ATP, N⁶-(6-aminohexyl)-ATP-Cy3, and N⁶-(6-aminohexyl)-ATP-Cy5) were obtained from Jena Biosciences.

Analytical Reactions on Short RNAs

Reactions in which the ribozymes modified short RNAs contained ~0.5 µg substrate RNA, 2-5x stoichiometric excess of ribozyme, 50 mM K⁺-Hepes pH 7.5, 0-5 mM NaCl, 50-120 mM KCl, 40 mM MgCl₂, and 200 µM 6AH-ATP or Cy-ATP, unless noted otherwise. 7 µL reactions were incubated 4h at 37°C, at which point they were combined with an equal volume of 'stop' solution composed of TBE and formamide, such that the final concentration of formamide was 40%. Reactions were run on a 15% 19:1 (acrylamide:bis-acrylamide) urea gel and stained with methylene blue. To test removal of the ribozymes with ASOs, ribozymes were labeled with ³²P and incubated with 16S in the above conditions (Cy-ATP was omitted) in a volume of 5 µL. 2 µL ASO was added to the indicated concentration and the reaction was incubated for 2h at 37°C. Reactions were run on a 4% 29:1 acrylamide non-denaturing gel and imaged on a Phosphorimager. Non-specific binding of ASOs to 16S rRNA was assayed similarly by incubating ³²P-labeled ASO with 16S rRNA for 2h at 37°C in the modification

reaction buffer described above. Labeling with ^{32}P at the 5'-terminus was carried out by incubation with T4 PNK (New England Biolabs) and [γ - ^{32}P]-ATP (Perkin-Elmer).

Primer Extension

Primer extension of modified 16S rRNA was carried out based on the method of (Hamlyn et al., 1978). Primers PE_496 and PE_1083 (sequences below) were 5'-labeled with ^{32}P as described above and extended with reverse transcriptase to visualize the regions of 16S rRNA modified by Rz415 and Rz1019, respectively. All reactions contained dNTPs, and each sequencing reaction was supplemented with a single ddNTP. The conditions of (Triman et al., 1989) were used with the following changes. Each dNTP was used at a final concentration of 32 μM , and each ddNTP was used at a final concentration of 3.2 μM . Reactions were run on a 6% (19:1 acrylamide:bis-acrylamide) urea gel.

Modification of 16S rRNA and ASO-directed Removal of Ribozymes

For modification of 16S rRNA, 100 pmol of 16S rRNA in a volume of 16 μL was incubated for 4h at 37°C in a PCR machine, with a 2- to 3-fold stoichiometric excess of ribozyme and 200 μM Cy-ATP in buffer containing 50 mM K^+Hepes pH 7.5, 50 mM KCl or 200 mM NH_4Cl , 10 mM MgCl_2 , and 20% ethanol. Although different concentrations of salt (KCl, NH_4Cl , and NaCl) were used, this was not observed to have an appreciable effect on labeling efficiency (data not shown). To remove ribozymes using ASOs, a 2- to 2.5-fold stoichiometric excess of ASO over ribozyme was added, the buffer was adjusted to 50 mM K^+Hepes , 100 mM NH_4Cl , 50 mM KCl, 5 mM MgCl_2 , and 10% ethanol, and the reaction was incubated for 2h at 37°C.

If the 16S rRNA was to be used for reconstitution of 30S subunits immediately following modification at a single site, then excess Cy-ATP was removed by applying ~100 μL sample to a ~0.8 mL S-200 size exclusion spin column equilibrated with buffer containing 20 mM Tris pH 7.5 and 10 mM MgCl_2 , and the ASO-directed ribozyme removal step was

skipped. If the 16S rRNA was to be modified at a second site, then the ribozyme was removed with an ASO, and the sample was then instead applied to a 10-30% (w/v) sucrose gradient containing 20 mM Tris pH 7.5 and 5 mM MgCl₂, which was spun on an SW60 rotor at 55,000 rpm for 2h at 4°C. The 16S rRNA peak was collected, concentrated on a 100kD MWCO concentrator (Amicon), ethanol precipitated, and resuspended in buffer containing 20 mM Tris pH 7.5 and 10 mM MgCl₂. The labeling efficiency was determined by comparing the absorbance at 260 nm to that at 550 or 650 nm for Cy3 and Cy5, respectively.

Second modifications of the 16S rRNA were carried out as described above, but excess Cy-ATP was removed on an S-200 column, as described, rather than on a sucrose gradient. Double-modified 16S rRNA was then used for reconstitution of 30S without removal of the ribozyme-ASO duplex.

Reconstitution of Ribosomes

Labeled 16S rRNA was heat-activated for 15 min at 42°C in 20 mM Tris pH 7.5, 100 mM KCl, and 10 mM MgCl₂. Reconstitution of 30S subunits from 16S rRNA and TP30 was carried out as described (Nierhaus, 1990). 30S and 50S subunits were associated and 70S ribosomes purified essentially as described (Hickerson et al., 2005; Niblett et al., 2021).

***In vitro* Translation Assay**

The *in vitro* translation assay was conducted as in Niblett et al., 2021 using [³⁵S]Met-tRNA^{Met} to visualize protein products. An mRNA encoding ribosomal protein S2 with an introduced slippery sequence was used, as described in (Niblett et al., 2021).

Table 1. Sequences of DNA oligonucleotides

Oligo Name	Use	Sequence
Rz1019 coding	<i>In vitro</i> transcription	5' CTAATACGACTCACTATAGGCGAAGGCACATTCC ACGCTGAGTAGACATACTTGCAAACGCTGCAAAC ATAGACATCTCTGAA
Rz1019 template	<i>In vitro</i> transcription	5' TTCAGAGATGTCTATGTTTGCAGCGTTTGCAAGTA TGTCTACTCAGCGTGGAATGTGCCTTCGCCTATA GTGAGTCGTATTAG
Rz415 coding	<i>In vitro</i> transcription	5' CTAATACGACTCACTATAGGCCCGAAGGCCACG CTGAGTAGACATACTTGCAAACGCTGCAAACATA GATCTTCATACA
Rz415 template	<i>In vitro</i> transcription	5' TGTATGAAGATCTATGTTTGCAGCGTTTGCAAGTA TGTCTACTCAGCGTGGGCCTTCGGGCCTATAGTG AGTCGTATTAG
Sub415 template	<i>In vitro</i> transcription	5' TTTACAACCCGAAGGCCTTCTTCATACACGCGGC CTATAGTGAGTCGTATTAG
Sub1019 template	<i>In vitro</i> transcription	5' CGAAGGCACATTCTCATCTCTGAAAACCTCCGTG GATGTCAACCTATAGTGAGTCGTATTAG
T7 promoter	<i>In vitro</i> transcription	5' CTAATACGACTCACTATAGG
PE_496	Primer extension on 16S rRNA	5' CGGAGTTAGCCGGTGCTTC
PE_1083	Primer extension on 16S rRNA	5' GCGGGACTTAACCCAAC
ASO_anti415	ASO for Rz415 removal	5' TGTATGAAGATCTATGTTTGCAGCGTTTGCAAGTA TGTCTACTCAGCGTGGGCCTTCGGGCC

ASO_anti1019	ASO for Rz1019 removal	5' TTCAGAGATGTCTATGTTTGCAGCGTTTGCAAGTA TGTCTACTCAGCGTGGAATGTGCCTTCGCC
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ACKNOWLEDGMENTS

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SUPPLEMENTAL FIGURES

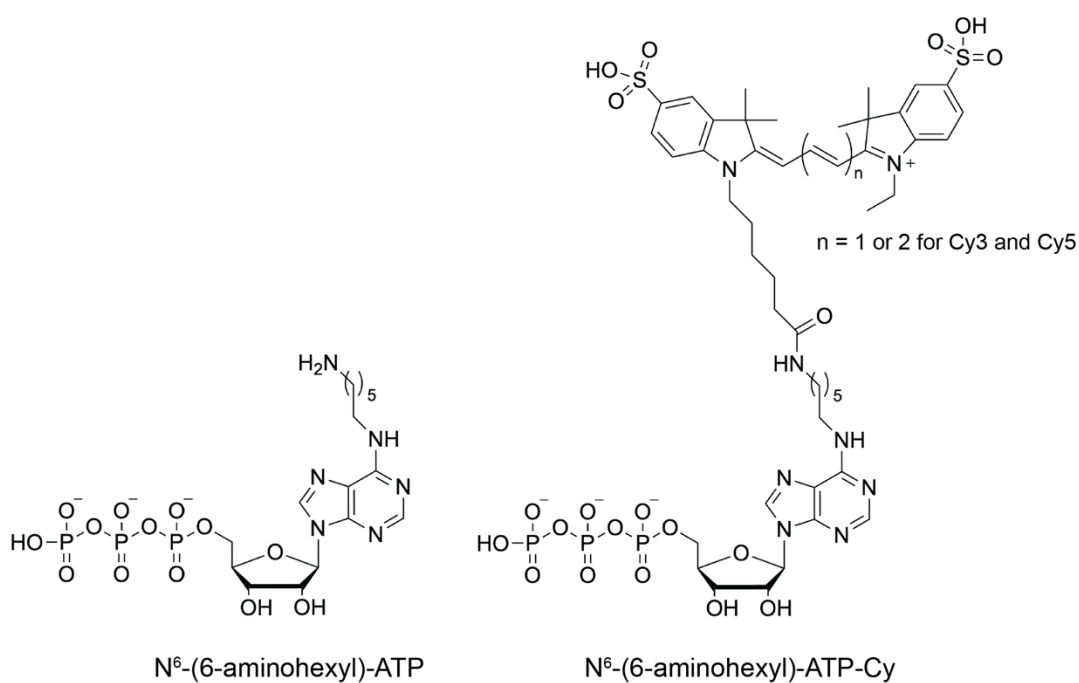


Figure S1. Modified ATP analogs used by ribozymes. ATP is modified at position N^6 with a linker containing a terminal amine (left), which can react with various NHS-(N-hydroxy-succinimide)-derivatized probes, including fluorophores, biotin, and spin labels, yielding analogs such as that shown on the right.

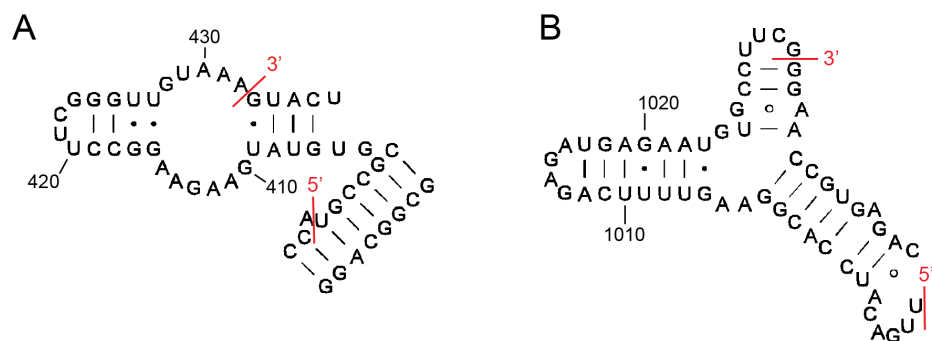


Figure S2. Short RNA targets based on the sequence of 16S rRNA used to test ribozyme activity. RNAs of 35-45 nt containing the targeted sites with sequences demarcated by red marks were transcribed *in vitro*. The short RNAs were used as simple targets to test the activity of ribozymes targeting A415 (A) and A1019 (B).