Supporting Information

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SI Materials and Methods

Materials and Sample Preparation. WT 70S ribosomes and subunits from *Escherichia coli* strain MRE600 were prepared as described (1, 2). WT *E. coli* ribosomal proteins were overexpressed and purified as described (3, 4). Site-directed mutagenesis (5) of plasmids encoding 30S proteins (4) was used to replace naturally occurring cysteine residues with phylogenetically tolerated nonreactive residues in S11 (C69A, C119V) and S13 (C85S), and to introduce single cysteine mutations at positions S11-R106C, S13-D11C, and S19-Q56C; S12-K108C was constructed as described (3). These labeling positions were chosen to be (*i*) surface-accessible, (*ii*) nonconserved, (*iii*) uninvolved in any known functional contacts, and (*iv*) located with interdye distances near the R_0 value (62 Å) for the Alexa 488-568 pair. Mutant 30S proteins were purified by FPLC ion-exchange chromatography and labeled by reaction of the introduced cysteine residues with maleimide derivatives of the respective donor and acceptor dyes, Alexa 488 and Alexa 568, as described (3). Excess dye was removed by repurification of the labeled protein by ion-exchange chromatography (3). Doubly fluorescent-labeled 30S ribosomal subunits containing the S11-D/S13-A and S12-D/S19-A FRET pairs were then constructed by in vitro reconstitution of the labeled proteins with 16S rRNA and the remaining 18 purified unmodified 30S ribosomal proteins, as described (3, 4), with the exception of the order of protein adding, for which all of the labeled and unmodified proteins were added at the same time instead of sequentially. Doubly labeled 70S ribosomes were then formed by association of the reconstituted labeled 30S subunits with natural unlabeled 50S subunits, followed by purification of the 70S peak by sucrose gradient sedimentation as described (3, 4). All of the reconstitutions were done with two fully labeled fluorescent proteins plus 18 nonlabeled proteins. Thus, the measured translocation rates from doubly labeled ribosomes cannot be influenced by contamination with WT or nonlabeled ribosomes. $tRNA^{Val}$ and GTP were purchased from Sigma, and $tRNA^{Met}$ from MP Biomedicals. Unmodified mv27 mRNA (5′-GGC AAG GAG GUA AAA AUG GUA AAA AAA-3′) and 3′-amino-modified mv24 mRNA (5′-GGC AAG GAG GUZ AAA AUG GUA AAA-NH_2-3′) were synthesized by Integrated DNA Technologies. The 3′-amino-modified mRNA was labeled with pyrene by reaction with pyrene succinimidyl ester (Molecular Probes), gel purified as described (6), and dissolved in water at 30 μM. *N*-acetyl-Val-$tRNA^{Val}$ was prepared as described (7). C-terminal-His_6-tagged EF-G (8) was prepared as follows. Cells were lysed in 25 mM Tris·HCl (pH 7.5), 50 mM KCl, 15 mM imidazole, 15% (vol/vol) glycerol, and 5 mM β-mercaptoethanol. Soluble EF-G was bound to Ni-NTA agarose resin (Qiagen) in the same buffer. The resin was washed with 25 mM Tris·HCl (pH 7.5), 500 mM KCl, 15 mM imidazole, 15% glycerol, 5 mM β-mercaptoethanol, and 0.01% (vol/vol) Nikkol. EF-G was eluted from the resin with 50 mM Tris·HCl (pH 7.5), 50 mM KCl, and 250 mM imidazole. EF-G was dialyzed against buffer A (25 mM Tris·HCl pH7.5, 20 mM KCl, and 5 mM β-mercaptoethanol), and further purified by FPLC chromatography using a 6 mL Resource-Q anion-exchange column (GE Healthcare Biosciences). EFG was eluted with a 120-mL salt gradient from 20 to 300 mM KCl in buffer A. The purified protein was dialyzed against 25 mM Hepes-KOH (pH 7.5), 150 mM NH_4Cl, 6 mM $MgCl_2$, and 6 mM β-mercaptoethanol and stored at −80 °C.

Stopped-Flow Kinetics Experiments. All stopped-flow experiments were conducted in buffer containing 20 mM Hepes-KOH (pH 7.5), 6 mM $MgCl_2$, 150 mM NH_4Cl, 6 mM β-mercaptoethanol, 2 mM spermidine, and 0.1 mM spermine, as described (9). For FRET measurements, the pretranslocation complex was constructed by incubating 1 μM 70S ribosomes with 2 μM mRNA (unlabeled) and 2 μM elongator $tRNA^{Met}$ at 37 °C for 20 min. *N*-acetyl-Val-$tRNA^{Val}$ (1.8 μM) was then added and the complex further incubated at 37 °C for 30 min. The pretranslocation complex was diluted to 100 nM in the previously mentioned buffer before FRET measurements. The pretranslocation complex used for the mRNA translocation assay was constructed in a similar way except that 1 μM pyrene-labeled mRNA was used. For experiments without A-site tRNA, 1 μM 70S ribosomes were incubated with 1.2 μM elongator $tRNA^{Met}$ and 2 μM unlabeled mRNA (or 1 μM pyrene-labeled mRNA) at 37 °C for 20 min.

Fluorescence measurements were carried out at 22 °C using an Applied Photophysics SX-20 stopped-flow apparatus, as described (9). For both the FRET and quenching experiments, 50 μL of a solution containing 1 μM EF-G and 1 mM GTP was rapidly mixed with 50 μL of 100 nM pretranslocation complex and the change in fluorescence emission was recorded. For FRET experiments, the Alexa 488 donor was excited at 490 nm and the Alexa 568 acceptor fluorescence emission was collected using a 590-nm long-pass filter. mRNA translocation rates were obtained using the method of Studer et al. (6) by following quenching of fluorescence of the pyrene residue attached to position +9 of the mRNA. The pyrene dye was excited at 343 nm and fluorescence emission recorded using a 375-nm long-pass filter. The kinetics results for each experiment were obtained by averaging 10 different stopped-flow traces. Each FRET experiment was repeated at least twice, using independently reconstituted labeled ribosome constructs. Averaged kinetics traces were analyzed and fitted with Pro-Data-Viewer software (Applied Photophysics), as shown in Fig. S3.

1. Moazed D, Noller HF (1989) Interaction of tRNA with 23S rRNA in the ribosomal A, P, and E sites. *Cell* 57(4):585–597.
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8. Rodnina MV, et al. (1999) Thiostrepton inhibits the turnover but not the GTPase of elongation factor G on the ribosome. *Proc Natl Acad Sci USA* 96(17):9586–9590.
9. Ermolenko DN, Noller HF (2011) mRNA translocation occurs during the second step of ribosomal intersubunit rotation. *Nat Struct Mol Biol* 18(4):457–462.

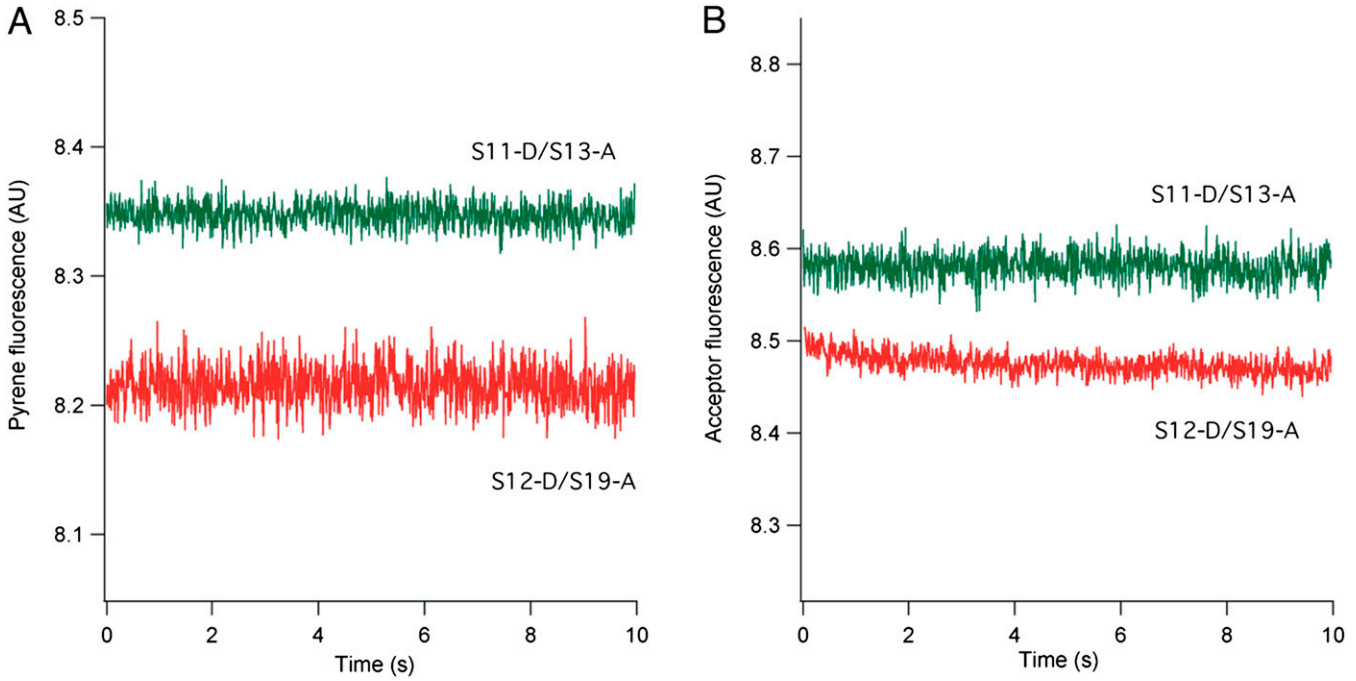

Fig. S1. Control reactions lacking A-site peptidyl-tRNA. Pretranslocation complexes were made as in Fig. 2 and mixed with EF-G GTP at time = 0, except that no *N*-acetyl-Val-tRNA was bound to the A site. Little change in (*A*) fluorescence of pyrene-labeled mRNA or (*B*) intrasubunit FRET was observed.

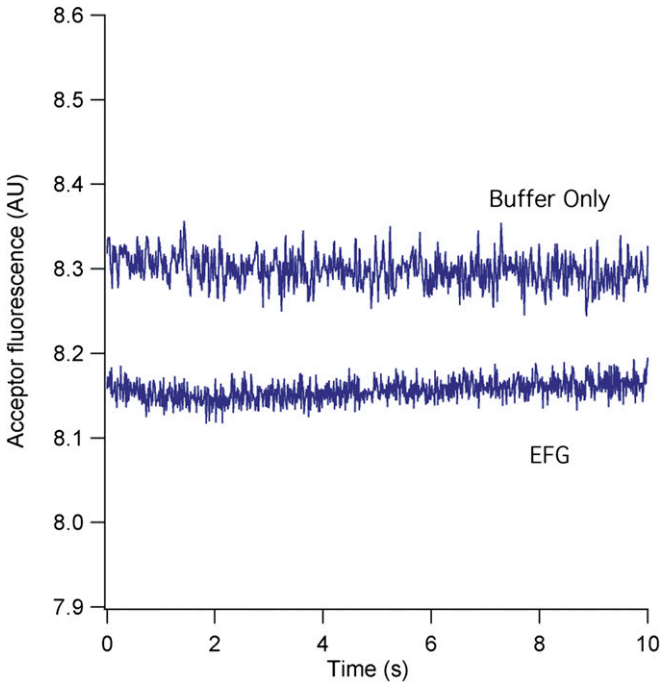

Fig. S2. Control FRET reactions lacking EF-G and GTP. Pretranslocation complexes were made as in Fig. 2, except that the pretranslocation complex was rapidly mixed with buffer only (upper trace) or with EF-G in the absence of GTP (lower trace).

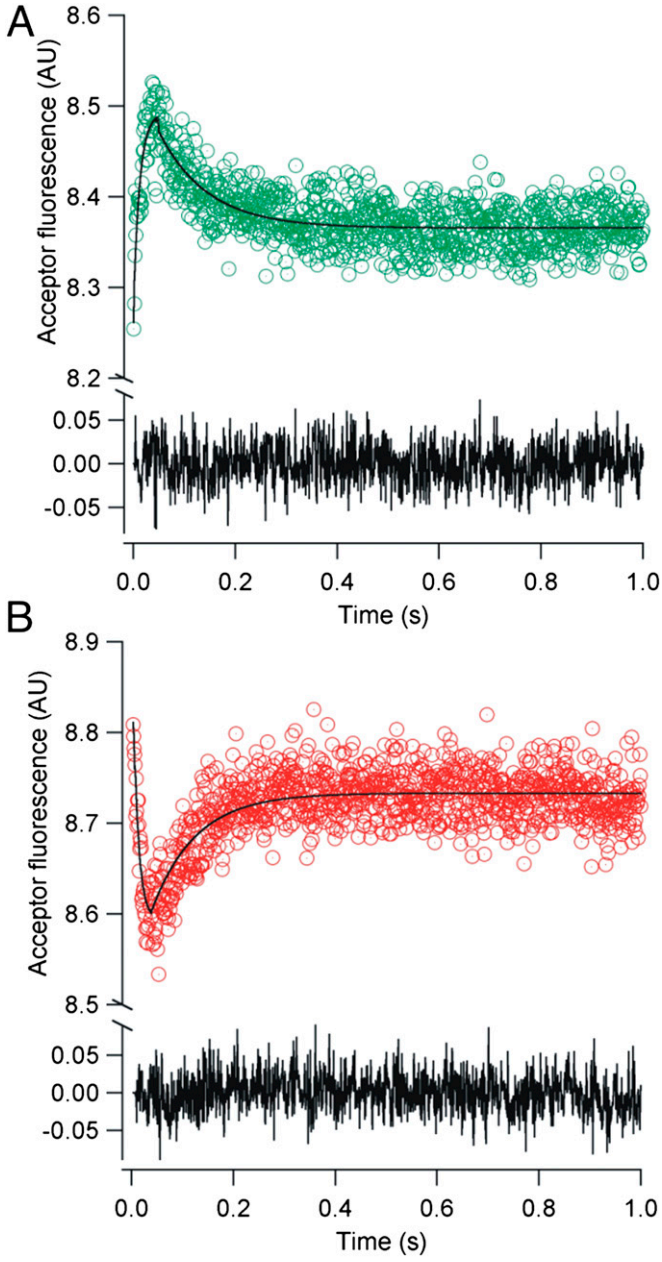

Fig. S3. Fitting of curves to kinetic data. Stopped-flow kinetic data were fitted to two single-exponential curves as described in *Materials and Methods*. (*A*) S11-D/S13-A construct. (*B*) S12-D/S19-A construct. The residual noise (black trace), shown at the bottom for each fit, is randomly distributed around 0.