Mutations at the accommodation gate of the ribosome impair RF2-dependent translation termination

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ABSTRACT

During protein synthesis, aminoacyl-tRNA (aa-tRNA) and release factors 1 and 2 (RF1 and RF2) have to bind at the catalytic center of the ribosome on the 50S subunit where they take part in peptide bond formation or peptidyl-tRNA hydrolysis, respectively. Computer simulations of aa-tRNA movement into the catalytic site (accommodation) suggested that three nucleotides of 23S rRNA, U2492, C2556, and C2573, form a "gate" at which aa-tRNA movement into the A site is retarded. Here we examined the role of nucleotides C2573 of 23S rRNA, a part of the putative accommodation gate, and of the neighboring A2572 for aa-tRNA binding followed by peptide bond formation and for the RF2-dependent peptide release. Mutations at the two positions did not affect aa-tRNA accommodation, peptide bond formation, or the fidelity of aa-tRNA selection, but impaired RF2-catalyzed peptide release. The data suggest that the ribosome is a robust machine that allows rapid aa-tRNA accommodation despite the defects at the accommodation gate. In comparison, peptide release by RF2 appears more sensitive to these mutations, due to slower accommodation of the factor or effects on RF2 positioning in the A site.

Keywords: translation; ribosome; decoding; accommodation; release factor

INTRODUCTION

The catalytic site of the bacterial ribosome—the peptidyl transferase center—is located in domain V of 23S rRNA in the large ribosomal subunit (50S). During peptide bond formation, the α-amino group of the A-site aa-tRNA attacks the carbonyl carbon of the P-site peptidyl-tRNA (pept-tRNA) to produce a new, 1-amino acid-longer pept-tRNA in the A site and a deacylated tRNA in the P site. The second enzymatic activity associated with the peptidyl transferase center is the hydrolytic cleavage of the ester bond in pept-tRNA during termination of protein synthe-

sis. Peptide bond formation (the aminolysis reaction) is an intrinsic activity of the ribosome that requires that the 3′-ends of the two tRNAs are correctly positioned in the A and P sites of the peptidyl transferase center, but does not involve an auxiliary factor. In contrast, peptide release from pept-tRNA is promoted by release factors, RF1 or RF2, which upon recognition of a termination codon in the 30S decoding site reach into the active site on the 50S subunit. In both aminolysis and hydrolysis reactions, one substrate is pept-tRNA positioned in the P site of the ribosome in the course of the preceding round of elongation. The respective reaction partner in the A site, aminoacyl-tRNA or RF1/2, has to bind, recognize the A-site codon, and accommodate in a way poised for the reaction. Because the peptidyl transferase center is located in a cleft at the interface side of the 50S subunit, this requires that the reactive domains, the 3′-end of aa-tRNA or the tip of domain 3 of RF1/2, move ~70 Å from the tRNA/factor entry site into the peptidyl transferase center of the ribosome in a process that has been termed accommodation of aa-tRNA.

The mechanism of the accommodation is not known in detail. Computer simulation of the aa-tRNA movement from

its A/T position, where the 3′-end of the aa-tRNA is attached to EF-Tu at the periphery of the ribosome, into the A/A position with the 3′-end positioned in the peptidyl transferase center suggested a step-wise movement through a corridor of conserved rRNA bases, which interact with the tRNA during the accommodation movement (Sanbonmatsu et al. 2005). The final accommodation of aa-tRNA in the A site of the peptidyl transferase center appears to be impeded by the interaction between the 3′-CCA end of aa-tRNA and the A-loop (helix 92) of 23S rRNA, in particular, the universally conserved bases U2492, C2556, and C2573, which act as a gate, causing the acceptor stem to pause before allowing entrance into the peptidyl transferase center (Sanbonmatsu et al. 2005). According to that simulation, the acceptor end of the aa-tRNA pauses upon interaction with U2492 and C2556 of 23S rRNA until the U2492–C2556 gate opens allowing the CCA end to proceed through. The CCA end then immediately interacts with C2573, causing it to pause for a second time (Fig. 1; Sanbonmatsu et al. 2005). Previous mutagenesis studies (O'Connor and Dahlberg 1995) indicated that substitutions of U2492 and U2555 affect the fidelity of translation, supporting the view that the accommodation gate may attenuate aa-tRNA binding. These results prompted us to further test the role of the 23S rRNA bases forming the gate by introducing mutations at critical positions that have not been previously studied by mutagenesis.

The base of C2573 of 23S rRNA is oriented toward the accommodation corridor through which aa-tRNA passes on its way into the A site on the 50S subunit. Substitution of C2573 for a larger purine base should create an obstacle for the movement and thus decrease the accommodation rate. In contrast, deletion of C2573 is expected to promote accommodation by facilitating the access of the tRNA into the A site. Also nucleotide A2572, although it is not a part of the accommodation gate, is of interest as a target for mutagenesis. A2572 is adjacent to C2573, but is oriented away from the accommodation gate due to the sharp kink

of the backbone between the two nucleotides. There is evidence suggesting that A2572 is involved in conformational changes in the vicinity of the peptidyl transferase center. A2572 becomes more accessible to chemical modification by dimethyl sulfate (DMS) when the pH is lowered (which impairs peptide bond formation) (Katunin et al. 2002) or upon inactivation of the 50S subunits in a low-salt buffer (Zamir et al. 1974). The reactivity of A2572 toward DMS also increases as a consequence of various unrelated base exchanges, such as A960C (Sergiev et al. 2000), A2451U (*Mycobacterium smegmatis*) (Beringer et al. 2005), A2531C and A2531U (Chan et al. 2006), or the K63E mutation of protein L4 (Gregory and Dahlberg 1999). In the two crystal forms of the *Escherichia coli* 70S ribosome (Borovinskaya et al. 2007), A2572 occupies two positions that differ in their orientation toward ribosomal protein L3. In the structure where A2572 is involved in the stacking interaction with Gln150 of protein L3, the latter residue is methylated. Residue mGln150 of protein L3 in *E. coli* corresponds to Trp255 of protein L3 in yeast ribosomes, for which various A-site-related mutant phenotypes have been observed (Meskauskas and Dinman 2007). These observations may suggest that A2572 is involved in long-range communications within the large ribosomal subunit, which might affect the size of the gate and hence the accommodation efficiency, or even aa-tRNA selection directly. Accommodation of RF1/2 in the A site may also require movement through the accommodation corridor; however, this has not been tested so far. Here, we have studied the effects of mutations of 23S rRNA residues 2573 and 2572 on ribosome function, in particular, on aa-tRNA accommodation during peptide elongation and RF2-dependent termination.

RESULTS AND DISCUSSION

Site-directed mutagenesis and effects of mutations in vivo

Using site-directed mutagenesis we changed the base of A2572 to a pyrimidine (U or C) or deleted the nucleotide. Nucleotide C2573 was replaced by three other nucleotides or deleted. The plasmid carrying the mutated rRNA operon was introduced by plasmid exchange into an *E. coli* strain that lacked all seven chromosomal rRNA operons (Asai et al. 1999). The strains were viable and did not show significant impairment in cell growth, except for the two deletion mutations, Δ2572 and Δ2573, both of which were lethal.

Mutations that change the efficiency of aa-tRNA accommodation in the A site were expected to have an effect on translation fidelity. We checked the effect of the mutations on the frequency of translation errors in vivo with the help of reporter plasmids containing mutant variants of the *lacZ* gene (Cupples and Miller 1989; O'Connor and Dahlberg 1993). In this system, active β-galactosidase could only be synthesized as a result of various translational errors, such
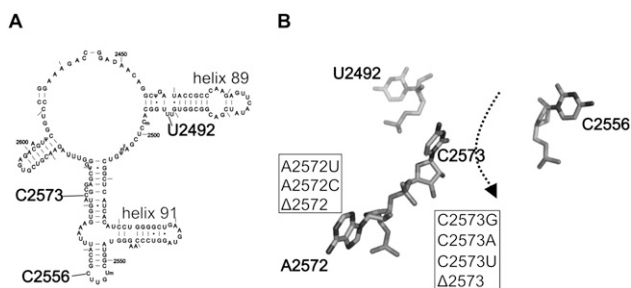

FIGURE 1. Positions of 23S rRNA bases U2492, C2556, and C2573 suggested to form the aa-tRNA accommodation gate (Sanbonmatsu et al. 2005) in the secondary (*A*) and tertiary (*B*) structures of 23S rRNA. The arrow indicates the direction of movement of the 3′-end of aa-tRNA. Substitutions of nucleotides studied in this work are listed in boxes in *B*.

as stop-codon readthrough, ±1 frameshifting, or codon misreading. Whereas the mutation C2573A slightly decreased the misreading frequency (Fig. 2), the effects of other mutations on misreading were insignificant. The efficiency of frameshifting or stop-codon readthrough was not affected significantly by any of the mutations (data not shown). The lack of misreading and frameshifting effects suggested that the mutations (except C2573A and the two deletion mutations) had no significant influence on the rates of aa-tRNA selection.

Effects of mutations on 23S rRNA structure

To test the effects of mutations on the structure of 23S rRNA and on elemental steps of translation in vitro, mutant as well as wild-type ribosomes from the same genetic background were prepared. The mutants were selected according to their expected effect on the aa-tRNA accommodation rate: C2573A, A2572U, Δ2572, and Δ2573; wild-type ribosomes were used for comparison. Because strains expressing ribosomes with the A2572U and C2573A mutations were viable in the absence of wild-type ribosomes, we prepared these mutant ribosomes directly from cells. The mutant ribosomes with lethal mutations Δ2572 and Δ2573 could only be expressed in the presence of the wild-type ribosomal operons, and the mutant ribosomes were prepared by affinity chromatography using a streptavidin-binding RNA aptamer. The presence of mutations in 23S rRNA was confirmed by primer extension analysis (Leonov et al. 2003).

To test whether the mutations affected the structure of 23S rRNA, we performed chemical probing with the base-specific reagents DMS, kethoxal, and 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluene sulfonate (CMCT). Virtually all nucleotides whose reactivity was changed by the mutations were located in the vicinity of the
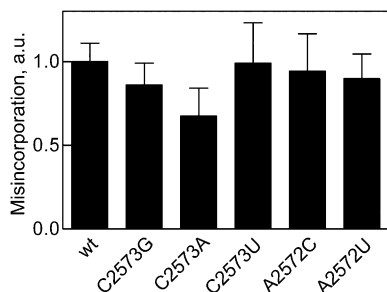

FIGURE 2. Effect of mutations in 23S rRNA on codon reading. Measured is the activity of β-galactosidase that appears as a result of Gln misincorporation caused by the misreading of a Glu codon. The activities measured with mutant strains are normalized to the activity measured with a strain containing wild-type ribosomes expressed in the same genetic background; "1.0" corresponds to 1.3 ± 0.1 Miller units. The activity of the wild-type (wt) ribosomes with the wt *lacZ* construct (no-error control) was 3980 ± 700 Miller units.

mutation site (Fig. 3), while the conformation of 23S rRNA regions distant from the mutated position was not affected (data not shown). In most cases, only a few bases changed their reactivities (Fig. 3). These results indicate that the mutations did not cause global distortions of the ribosome structure, but had limited local effects at the accommodation gate.

Initiation and aminoacyl-tRNA accommodation

The activity of mutant ribosomes in translation initiation was tested by measuring the binding of initiator f[^{3}H]Met-tRNA^{fMet} to the P site using nitrocellulose filtration. None of the mutations affected the formation of the 70S initiation complex (Fig. 4A), indicating that the activity of the ribosomes in initiation was not impaired by the mutations. The ability to synthesize the dipeptide fMet-Phe upon addition of the ternary complex EF-Tu·GTP·[^{14}C]Phe-tRNA^{Phe} to the initiation complex was also not affected by the mutations (Fig. 4B), implying that the final level of Phe-tRNA^{Phe} binding, and hence the proportion of ribosomes with an active A site, was not affected. These observations indicated that the overall activity of the ribosomes was not compromised by the mutations and allowed us to measure the specific effect on the rate of peptide bond formation, which at the present conditions is limited by the rate of the preceding accommodation step (Bieling et al. 2006). The rate of fMet-Phe formation was measured by quench–flow by mixing the initiation complex with the ternary complex and stopping the reaction after the desired times by addition of the quencher. The observed rate of dipeptide formation was approximately the same for wild-type and mutant ribosomes; $k_{app} = 2.3 \pm 0.2$ sec^{-1} (wt), $k_{app} = 2.0 \pm 0.2$ sec^{-1} (Δ2573), $k_{app} = 2.1 \pm 0.2$ sec^{-1} (C2573A), $k_{app} = 2.8 \pm 0.3$ sec^{-1} (Δ2572), and $k_{app} = 1.9 \pm 0.2$ sec^{-1} (A2572U) (Fig. 4C). Thus, none of the mutations of nucleotides 2573 and 2572 in 23S rRNA affected the rate of aa-tRNA accommodation to any appreciable extent. Together with the results of the in vivo assays, these results indicate that the presumed accommodation gate at C2573 is not rate limiting for aa-tRNA accommodation, at least not for Phe-tRNA^{Phe}. It is not excluded, however, that a step different from the one suggested by molecular dynamics simulations limits the rate of accommodation (Sanbonmatsu et al. 2005), or that mutations at the gate cause accommodation to take an alternative pathway. Furthermore, it is possible that the accommodation of aa-tRNAs other than Phe-tRNA^{Phe} is indeed affected, for instance by the C2573A mutation, which would explain its effect on misreading in vivo.

Peptide release by RF2

During the termination of protein synthesis, RF1 or RF2 recognizes a stop codon in the A site and promotes the
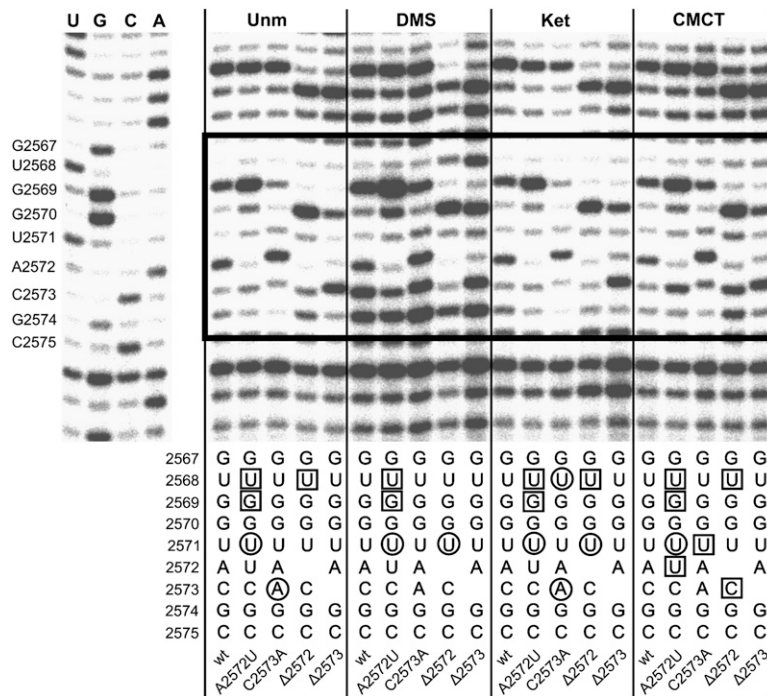
FIGURE 3. Chemical probing of the 23S rRNA structure. Fragment of the sequencing gel showing the changes in chemical reactivity in the vicinity of the mutation. Source of the 50S subunits is indicated at the *bottom*. U, G, C, and A denote the respective sequencing lanes. Unm, unmodified rRNA; DMS, after modification with DMS; Ket, after modification with kethoxal; and CMCT, after modification with CMCT. The region in the sequence where changes in chemical reactivity are found is indicated by frame and the effects are summarized in the primary sequence diagram below the gel. Increased chemical reactivity is indicated by squares, decreased reactivity by circles. Shown is sequencing using primers complementary to the 2730–2749 and 2591–2611 regions of 23S rRNA, which cover the region of 23S rRNA in which nucleotides forming the accommodation gate are located.

hydrolysis of P-site-bound pept-tRNA in the peptidyl transferase center. To enter the peptidyl transferase center, RFs, like aa-tRNAs, may also have to use the accommodation corridor. This prompted us to study the effect of mutations on the efficiency of RF2-catalyzed peptide release in vitro, using a model system with ribosomes containing fMet-tRNA^{fMet} bound to an AUG codon in the P site and a UAA stop codon in the A site (Fig. 4D). The mutation A2572U decreased the rate of peptide release fourfold (k_{app} = 0.10 ± 0.01 sec^{-1}) compared with the reaction on wild-type ribosomes (k_{app} = 0.46 ± 0.04 sec^{-1}), while the mutations C2573A, Δ2572 and Δ2573 only slightly decreased the rate of peptide release (k_{app} = 0.37 ± 0.03 sec^{-1}, k_{app} = 0.29 ± 0.03 sec^{-1}, and k_{app} = 0.33 ± 0.05 sec^{-1}, respectively). The effect is statistically significant and highly reproducible. It may reflect an impaired accommodation of RF2 in the A site or a slower chemistry step. The interpretation is complicated by the fact that it is not known which of the two steps is rate limiting for RF2-dependent peptide release. The reported strong dependence of the reaction rate on the nature of the nucleophile (Shaw and Green 2007) argues against RF2 binding or rearrange-

ments being rate limiting for the reaction. This would suggest that the observed rate decrease may reflect the effect on the chemical step of pept-tRNA hydrolysis. Because the activity of the peptidyl transferase center in peptide bond formation was not impaired, the inhibition of peptide release by mutations should involve group(s) specific for the hydrolysis reaction, e.g., nucleophile positioning in the active center. On the other hand, if the hydrolysis reaction itself is not affected by the mutations and is rate limiting on the wild-type ribosomes, the true inhibition of RF2 accommodation by the mutation of RF2 accommodation may be larger than that observed upon monitoring the peptide release, because the accommodation step may become rate limiting on mutant ribosomes. In this case, the fourfold decrease in the rate of peptide release can be considered as the lower limit for the effect on the accommodation of RF2. It should be noted that the strongest decrease in the release rate was observed for mutation of nucleotide 2572, which is not participating in the formation of the accommodation gate or of the core of the catalytic center (Korostelev et al. 2008; Weixlbaumer et al. 2008). Nucleotide 2572 may play an important role in the regulation of the gate size or effect the positioning of RF2 in some way. Impairment in RF-dependent termination may also explain why mutations of other residues at the accommodation gate, U2492 and U2555, result in increased stop-codon readthrough in vivo (O'Connor and Dahlberg 1993, 1995)

In summary, the present data indicate that the ribosome is quite robust in dealing with mutations at the accommodation gate, as we have not observed any effect on Phe-tRNA^{Phe} accommodation. In comparison, the RF2-dependent peptide release is affected by the mutations, although it is not clear which step of the reaction (the accommodation of the factor in the A site or the chemistry step) is affected. This identifies RFs as potential interesting ligands for molecular dynamics simulations of their accommodation during termination.

MATERIALS AND METHODS

Buffers

The following buffers were used: buffer A: 50 mM Tris-HCl (pH 7.5), 70 mM NH$_4$Cl, 30 mM KCl, 7 mM MgCl$_2$; and buffer B:
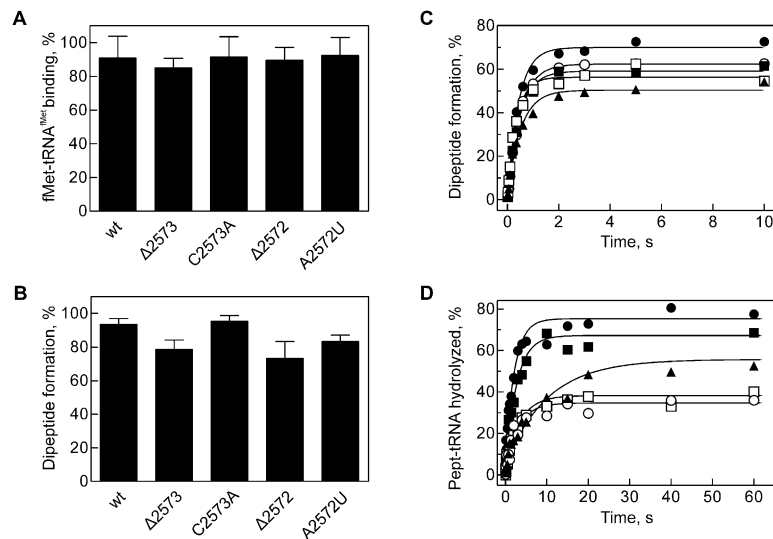
FIGURE 4. Effect of mutations on the elemental steps of translation. (A) fMet-tRNA[fMet] binding to the P site of 70S ribosomes in the presence of initiation factors. (B) Peptide bond formation upon addition of EF-Tu•GTP•[^{14}C]Phe-tRNA[Phe] to the 70S initiation complexes formed with wild-type (wt) ribosomes, or mutants as indicated. (C) Time courses of dipeptide formation. Symbols: wt ribosomes (●), Δ2573 (○), C2573A (■), Δ2572 (□), and A2572U (▲). (D) Kinetics of peptide release. Symbols: wt ribosomes (●), Δ2573 (○), C2573A (■), Δ2572 (□), and A2572U (▲).

20 mM Hepes-KOH (pH 7.5), 200 mM NH_4OAc, 1 mM magnesium acetate, 4 mM β-mercaptoethanol. All experiments were carried out at 37°C.

Mutagenesis and ribosome purification

Mutations were introduced by site-directed mutagenesis into a SphI-BamHI fragment of the *E. coli rrnB* operon cloned into M13 mp18 phage (Kunkel 1985). The mutated fragment was cloned into the pLK1192U plasmid containing a streptavidin-binding aptamer ("strep tag") inserted in helix 25 of 23S rRNA (Leonov et al. 2003). The plasmid contains the *rrnB* operon of rDNA under the control of the phage λ P_L promoter. Transformation of the AVS69009 strain (Asai et al. 1999), plasmid substitutions, and testing the purity of mutant rRNA in the cells were carried out as previously described (Sergiev et al. 2000).

Ribosomes and 50S ribosomal subunits were prepared as previously described (Rodnina and Wintermeyer 1995; Milon et al. 2007). To prepare ribosomes with lethal mutations (Δ2572, Δ2573, and UUU2491-3UC), all steps preceding the zonal centrifugation were carried out as previously described (Rodnina and Wintermeyer 1995), followed by the isolation of mutant 50S subunits by strep-tag affinity chromatography (Leonov et al. 2003).

In vivo assays

Doubling times were measured by monitoring A_{600} in Luria–Bertani medium at 37°C, using three independent clones to monitor the growth rate of each strain. Doubling times were calculated by a least-squares linear approximation of the logarithms of the optical density of the cultures in their log phase. For translation fidelity assays, the cells were harvested at the same stage of growth, namely, the mid-log phase. Translational fidelity was measured

using a set of *LacZ* reporter strains (pSG or f′CSH) (O'Connor and Dahlberg 1993; Sergiev et al. 2005). *E. coli* strain MC140 carrying one of the reporter plasmids was transformed by one of the plasmids carrying a wild-type or mutant 23S rRNA gene. The activity of β-galactosidase was determined from at least three independent experiments as previously described (O'Connor and Dahlberg 1993).

Chemical probing

DMS, kethoxal, and CMCT modifications were carried out following Stern et al. (1988), with the exception that modifications were done for 10 min at 37°C. Ribosomal RNA was purified by repeated phenol extractions, and modified sites were determined by primer extension with AMV reverse transcriptase.

Biochemical methods

EF-Tu, initiation factors, f[^{3}H]Met-tRNA[fMet], and [^{14}C]Phe-tRNA[Phe] from *E. coli* were prepared as previously described (Rodnina et al. 1994; Rodnina and Wintermeyer 1995; Rodnina et al. 1999). f[^{3}H]Met-tRNA[fMet] and [^{14}C]Phe-tRNA[Phe] were purified to homogeneity by HPLC (Milon et al. 2007). mRNA-1 coding for fMet-Phe (5′-GGCAAGGAGGUAAAUA<u>AU GUUC</u>ACGAUU-3′ [coding sequence underlined]) and mRNA-2 coding for fMet-Stop (5′-GGCAAGGAGGUAAAUA<u>AUGUAA</u> ACGAUU-3′) were purchased from Dharmacon or Curevac. Initiation or pretermination complexes were formed by incubation of wild-type 30S subunits (1 μM) and mutant 50S subunits (1 μM) with mRNA-1 or mRNA-2 (1.5 μM) as appropriate, f[^{3}H]Met-tRNA[fMet] (1.5 μM), IF1, IF2, and IF3 (1.5 μM each), DTT (1 mM), and GTP (1 mM) in buffer A at 37°C for 45 min. The amount of f[^{3}H]Met-tRNA[fMet] bound to the P site was determined by nitrocellulose filtration. The ternary complex, EF-Tu•GTP• [^{14}C]Phe-tRNA[Phe], was prepared by incubating EF-Tu (3 μM) with GTP (1 mM), phosphoenol pyruvate (3 mM), and pyruvate kinase (0.1 mg/mL) in buffer A at 37°C for 15 min, followed by the addition of [^{14}C]Phe-tRNA[Phe] (2 μM). To measure dipeptide formation, the initiation complex (0.4 μM) was mixed with a ternary complex (0.6 μM) and incubated for 2 min at 37°C. The reaction was stopped with 0.5 M KOH, incubated for 30 min at 37°C, neutralized, and the dipeptide formed was analyzed by RP-HPLC (Katunin et al. 2002).

Rapid kinetics experiments

Quench–flow assays were performed at 37°C in a KinTek RQF-5 apparatus. To measure the time courses of peptide bond formation, the initiation complex (0.25 μM) was rapidly mixed with a ternary complex (0.375 μM). After different incubation times, reactions were stopped with KOH (0.5 M) and analyzed as described above. To measure the rate of fMet release, pretermination complexes (0.2 μM) were mixed with RF2 (0.4 μM). Reactions were stopped with 6% TCA, f[^{3}H]Met-tRNA[fMet] and

deacylated tRNA^{fMet} were precipitated by addition of three volumes of ethanol, and the amount of liberated f[^{3}H]Met was determined by measuring the radioactivity in the supernatant.

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