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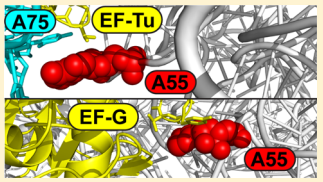
Highly Conserved Base A55 of 16S Ribosomal RNA Is Important for the Elongation Cycle of Protein Synthesis

Bhubanananda Sahu, Prashant K. Khade, and Simpson Joseph*

Department of Chemistry and Biochemistry, University of California at San Diego, 9500 Gilman Drive, La Jolla, California 92093-0314, United States

ABSTRACT: Accurate decoding of mRNA requires the precise interaction of protein factors and tRNAs with the ribosome. X-ray crystallography and cryo-electron microscopy have provided detailed structural information about the 70S ribosome with protein factors and tRNAs trapped during translation. Crystal structures showed that one of the universally conserved 16S rRNA bases, A55, in the shoulder domain of the 30S subunit interacts with elongation factors Tu and G (EF-Tu and EF-G, respectively). The exact functional role of A55 in protein synthesis is not clear. We changed A55 to U and analyzed the effect of the mutation on the elongation cycle of protein synthesis using functional assays. Expression of 16S rRNA with the A55U mutation in cells confers a dominant lethal phenotype. Additionally, ribosomes with the A55U mutation in 16S rRNA show substantially reduced *in vitro* protein synthesis activity. Equilibrium binding studies showed that the A55U mutation considerably inhibited the binding of the EF-Tu·GTP·tRNA ternary complex to the ribosome. Furthermore, the A55U mutation slightly inhibited the peptidyl transferase reaction, the binding of EF-G·GTP to the ribosome, and mRNA−tRNA translocation. These results indicate that A55 is important for fine-tuning the activity of the ribosome during the elongation cycle of protein synthesis.



The ribosome is a macromolecular enzyme composed of three rRNAs (16S, 23S, and 5S rRNAs in bacteria) and 54 ribosomal proteins. The ribosome binds a mRNA and interacts with aminoacyl tRNAs and several translation factors to synthesize the protein encoded by the mRNA. During the elongation cycle of protein synthesis, the ribosome sequentially interacts with the EF-Tu·GTP·aminoacyl tRNA ternary complex (EF-Tu ternary complex) and EF-G·GTP. The EF-Tu ternary complex delivers the aminoacyl tRNAs to the ribosomal A site for incorporation into the growing peptide chain. During this process termed decoding, the EF-Tu ternary complex binds initially to the ribosome in the A/T state, in which the anticodon of the tRNA forms base pairs with the mRNA codon in the A site while the acceptor end of the tRNA remains bound to EF-Tu.[1] Proper codon−anticodon interaction by the cognate EF-Tu ternary complex triggers GTP hydrolysis on EF-Tu, and EF-Tu·GDP dissociates from the ribosome. This allows the aminoacyl tRNA to bind to the A site in the 50S subunit and participate in peptide bond formation. In contrast, the anticodon of noncognate tRNAs does not form base pairs with the A site codon and rapidly dissociates from the ribosome without triggering GTP hydrolysis on EF-Tu.

Recent X-ray crystal structures of the EF-Tu ternary complex bound to the ribosome in the A/T state show how the EF-Tu ternary compex is stabilized on the ribosome.[2,3] One of the important interactions identified in these crystal structures is between the 3′ end of the A/T tRNA and the shoulder domain of the 16S rRNA. Residues 72−75 at the 3′ end of the A/T tRNA are distorted, which allows base C75 of the tRNA to pack between EF-Tu residue Thr219 and the flipped 16S rRNA base A55 in the shoulder domain of the 30S subunit (Figure 1A). Base A55 in helix 5 of 16S rRNA is highly conserved (>99%).[4] In addition, two loops in domain II of EF-Tu undergo a conformational change to contact the shoulder domain of the 30S subunit. One of these loops of EF-Tu (residues 219−226) interacts with bases A55, U56, G357, and U368 in the 16S rRNA. Specifically, A55 and U56 form hydrophobic interactions with the highly conserved residue Thr221 in domain II of EF-Tu. These interactions of the EF-Tu ternary complex with the shoulder domain of the 30S subunit have been proposed to play a crucial role during decoding.[2]

Following decoding and peptide bond formation, the mRNA−tRNA complex is translocated by the ribosome. Translocation is catalyzed by EF-G·GTP, which hydrolyzes GTP to accelerate the reaction.[5] Translocation moves the deacylated tRNA and the peptidyl tRNA from the P and A sites to the E and P sites, respectively. This leaves an empty A site and completes one round of the elongation cycle of protein synthesis. Cryo-electron microscopy (cryo-EM),[6−9] X-ray crystallography,[10,11] and small molecule Förster resonance energy transfer (smFRET)[12−18] studies have indicated that the interaction of EF-G with the ribosome induces structural changes in the ribosome. Binding of EF-G to the ribosome induces a ratchetlike counterclockwise rotation of the 30S subunit relative to the 50S subunit.[6,18] Additionally, the head domain of the 30S subunit undergoes a swiveling motion,[10,11] and the L1 stalk of the 50S subunit moves toward the E-site.[12,15,17,19] Recently, many new intermediate steps in translocation have been identified by cryo-EM analysis, suggesting that this process consists of a complex metastable, energy landscape.[7,20,21] However, it is still not clear how conformational changes in the ribosome trigger GTP hydrolysis

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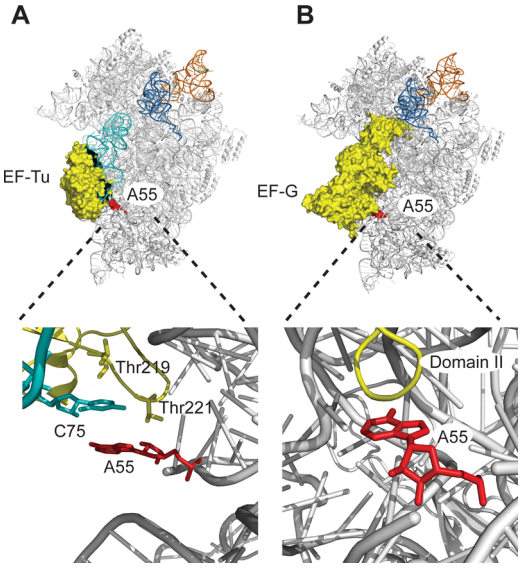

Figure 1. Interaction of 16S rRNA base A55 with tRNA, EF-Tu, and EF-G. (A) Structure of the 30S subunit showing the location of A55 of 16S RNA with respect to EF-Tu (PDB entry 2WRN). The enlarged figure below shows the details of the interaction of A55 with EF-Tu (residues Thr219 and Thr221) and tRNA (base C75). (B) Structure of the 30S subunit showing the location A55 of 16S RNA with respect to EF-G (PDB entry 2WRI). The enlarged figure below shows the details of the interaction of A55 with domain II of EF-G (residues 319–323). Shown are the 30S subunit (gray), A site tRNA (cyan), P site tRNA (pale blue), E site tRNA (brown), base A55 (red), EF-Tu (yellow), and EF-G (yellow).

on EF-G and direct the movement of the mRNA–tRNA complex.

Currently, the crystal structure of the ribosome·EF-G·GDP·fusidic acid complex determined by Ramakrishnan and co-workers provides a high-resolution view of the post-translocation state.[22] The antibiotic fusidic acid traps EF-G in a conformation that is intermediate between the GTP and GDP forms. In this structure, the ribosome is in a nonratcheted state and EF-G is stabilized by contacts with the 50S and 30S subunits. In particular, flipped out base A55 in helix 5 of 16S rRNA contacts domain II of EF-G (residues 319–323) (Figure 1B). Additionally, domain III of EF-G bridges the shoulder domain of the 30S subunit (h5/h15 and ribosomal protein S12) and the sarcin-ricin loop (SRL) in the 50S subunit. Because deletion of domain III reduces the GTPase activity of EF-G,[23] it was proposed that the interaction of domain III with both ribosomal subunits allows EF-G to distinguish between the ratcheted and nonratcheted states, and these contacts may play a role in GTP hydrolysis.[22] Because domain II of the EF-Tu ternary complex also interacts with the shoulder domain of the 30S subunit (discussed above), a common mechanism may be used to activate GTP hydrolysis or couple GTP hydrolysis to conformational changes in the ribosome.[2,22]

Structural data suggest that the interaction of the EF-Tu ternary complex and EF-G with the shoulder domain of the 30S subunit (and particularly with A55 of 16S rRNA) is important for protein synthesis. Consistent with the structural data, the A55G mutation was isolated in a genetic screen for mutations that cause a defect in translation.[24] Another study also showed that changing A55 to G, C, or U dramatically inhibited the synthesis of a reporter protein *in vivo*.[25] However, it is not clear why mutation of A55 results in the inhibition of protein synthesis. Here, we analyzed the defects of the A55U mutation using several functional assays. Our studies show that the A55U mutation substantially inhibited the binding of the EF-Tu ternary complex to the ribosome. Additionally, the A55U mutation caused modest defects in peptide bond formation, in binding of EF-G·GTP to the ribosome, and in mRNA–tRNA translocation. These results show that A55 is important for improving the overall performance of the ribosome during the elongation cycle of protein synthesis.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis. Mutation A55U in 16S rRNA was introduced by site-directed mutagenesis with the QuickChange polymerase chain reaction mutagenesis kit (Stratagene). Plasmid pLK35-16S-MS2 was used as the template for introducing the mutation.[26,27] Clones with the A55U mutation were identified by sequencing, and the sequence of the entire 16S rRNA operon of the correct construct was also verified by DNA sequencing.

Purification of MS2-Tagged 30S Subunits. Because the A55U mutation in 16S rRNA is lethal, the mutant 30S subunits were purified from POP2136 cells using the MS2 affinity tag as described previously.[26,27] The purity of the MS2-tagged 30S subunit was assayed by primer extension analysis using the primer 5′-CCCGTCCGCCACTCGTCAGC-3′, as described previously.[26,27]

***In Vitro* Translation of Reporter Protein.** *In vitro* translation of reporter gene *Renilla* luciferase was performed to test the activity of the purified ribosome as described previously.[28] The *in vitro* translation reaction is based on the *Escherichia coli* S-100 extract to which activated wild-type or A55U mutant ribosomes had been added to initiate protein synthesis. The synthesis of the luciferase reporter enzyme was monitored by measuring the luminescence every 2 min with a 96-well plate reader (Genios, Tecan). All the reactions were performed in duplicate, and the experiments were repeated more than three times.

To validate our results, we performed the *in vitro* translation assay with [^{35}S]methionine. The reaction was conducted as described above but with 1 μL of L-[^{35}S]methionine (1175 Ci/mmol, Perkin-Elmer EasyTide, catalog no. NEG709A) added instead of nonradioactive L-methionine. The reaction mixture was incubated at 30 °C for 90 min. Samples were denatured by adding an equal volume of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer and heated at 90 °C for 5 min. Two microliters of sample was separated on 15% SDS–polyacrylamide gel. To determine the molecular weight of the product, a molecular weight ladder was also loaded on the gel. The gel was stained with Coomassie blue, washed with water, and dried. The dried gel was exposed to a phosphorimager screen for 4–5 days and quantitated using a phosphorimager (Bio-Rad).

Determination of the K_D for Binding of the EF-Tu Ternary Complex to the Ribosome. For the filter binding experiments, a modified 96-well dot blot apparatus (Shleicher and Schuell) with an upper nitrocellulose membrane (BA-85, Whatman) and a lower nylon membrane (Hybond-N+, Amersham) was used, as described previously.[29] *E. coli* tRNA^{Phe} was labeled with [α-^{32}P]ATP as described previously.[30,31] The EF-Tu ternary complex was prepared with a 10-fold excess of EF-Tu (H84A) over Phe-[^{32}P]tRNA^{Phe} in buffer B [50 mM Tris-HCl (pH 7.5), 15 mM $MgCl_2$, 70 mM NH_4Cl, 30 mM KCl, 8 mM putrescine, 2 mM DTT, 1 mM GTP, and 0.5 mM

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spermidine]. The ternary complex (0.2 nM) was incubated for 1 min at room temperature with P site-blocked ribosomes (0–150 nM) prepared in the same buffer without GTP (final concentration of 0.3 mM after mixing). Using a multichannel pipet, 25 μL of the reaction mixture was applied to the 96-well dot blot apparatus and washed three time with 100 μL of buffer B. The membranes were removed from the 96-well dot blot apparatus, dried, and then exposed to a phosphorimager screen (Bio-Rad) to determine the amount of EF-Tu ternary complex bound to the ribosome. The K_D was calculated by fitting the data to the one-site binding hyperbolic equation $Y = B_{max} \times X/(K_d + X)$ using GraphPad Prism.

Peptidyl Transferase Assay. Peptidyl transferase experiments were performed at 20 °C in ribosome buffer {50 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) (pH 7.0), 70 mM NH_4Cl, 30 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, and 0.5 mM GTP} with a quench-flow instrument (μQFM-400, BioLogic) as described previously.[31,32] Briefly, $tRNA^{Phe}$ was labeled with [α-^{32}P]ATP by exchanging the 3′-terminal A76 of $tRNA^{Phe}$ with [α-^{32}P]AMP using the enzyme tRNA nucleotidyl transferase. The ^{32}P-labeled $tRNA^{Phe}$ was aminoacylated with phenylalanine and used for the formation of the EF-Tu ternary complex. Ribosomes were programmed with a mRNA having the codons for methionine and phenylalanine in the P and A sites, respectively. fMet-$tRNA^{fMet}$ was bound to the ribosomal P site to form the initiation complex. The initiation complex and EF-Tu ternary complex were rapidly mixed with the quench-flow instrument. The concentrations of the initiation complex and EF-Tu ternary complex after mixing were 0.5 and 0.05 μM, respectively. Two microliters of the quenched product was digested with 1 μL of S1 nuclease. S1 nucleases degrade the $tRNA^{Phe}$, leaving behind an adenine monophosphate linked to the fMet-Phe dipetide. The product was separated by thin layer chromatography using PEI-cellulose plate with a 2-propanol/HCl/water mixture (70:15:15) for 4 h. The solvent can resolve the reaction mixture into an upper band of fMet-Phe-[^{32}P]AMP (the expected dipeptide product) and a middle band of [^{32}P]AMP and [^{32}P]aminoacyl-AMP. The time course of peptide bond formation was fit to a single-exponential equation to determine the extent of dipeptide formation for the mutant ribosome.

Determination of the K_D for Binding of EF-G to the Ribosome. EF-G containing a single cysteine at position 591 was labeled with 5-iodoacetamidofluorescein as described previously.[33] The K_D for binding of EF-G to the ribosome was determined as described previously.[32]

Toeprinting Assay. Translocation was monitored by the toeprinting assay as described previously.[34]

Translocation Kinetics. The rapid kinetic translocation experiments were performed as described previously.[35,36] The experiments were conducted at 25 °C in transloction buffer [20 mM HEPES-KOH (pH 7.6), 150 mM NH_4Cl, 6 mM $MgCl_2$, 4 mM β-mercaptoethanol, 2 mM spermidine, and 0.05 mM spermine]. Briefly, 80 μL of the pretranslocation complex (0.25 μM, after mixing) was rapidly mixed with 80 μL of EF-G·GTP (1.25 μM EF-G and 0.5 mM GTP, after mixing) using a stopped-flow instrument (μSFM-20, BioLogic). The stopped-flow instrument was set up to excite the sample at 343 nm and to monitor the fluorescence intensity above 360 nm using a long-pass filter (361 AELP, Omega Optical). From each experiment, approximately five traces were averaged, and the experiments were independently repeated at least three times. The rate of translocation was determined by fitting the data to the double-exponential equation $Y = ax + b + A_1 \exp(-k_1x) + A_2 \exp(-k_2x)$ using Bio-Kine (BioLogic).

RESULTS

The A55U Mutation in 16S rRNA Is Dominant Lethal. To investigate the role of A55 in the elongation cycle of protein synthesis, we introduced the A55U transversion mutation by site-directed mutagenesis. A55 is in a loop region, and the A55U mutation does not change the structure of the loop. The mutation was made in plasmid pLK35-16S-MS2 to facilitate purification of the mutant ribosomes using the MS2 affinity tag method.[28] The mutation was confirmed by sequencing the entire 16S rRNA operon.

To check the growth phenotype, the mutant plasmid was transformed into *E. coli* strain POP2136 that carries the temperature-sensitive λ repressor gene (*cI857*). In this strain, transcription of the plasmid-borne rRNA operon is under the control of the λ P_L promoter. The growth rate of POP2136 containing either the wild-type (WT) or A55U mutant plasmid was normal at 30 °C because the λ repressor is active at 30 °C and represses the expression of the plasmid-encoded rRNA operon. In contrast, at 42 °C the λ repressor is inactive and expression of the plasmid-encoded 16S rRNA with the A55U mutation conferred a dominant lethal phenotype (Figure 2A).

The A55U Mutation Inhibits Protein Synthesis. To evaluate whether the mutant ribosome is active in protein synthesis, we performed an *in vitro* translation assay. In this *in vitro* translation assay, we monitored the time course of *Renilla* luciferase synthesis by the WT and mutant ribosomes by bioluminescence[28] (Figure 2B). *Renilla* luciferase catalyzes the oxidation of coelenterazine to produce light, which was monitored in real time with a plate reader. Our results show that the A55U mutant ribosome is inactive in synthesizing the *Renilla* luciferase protein. We confirmed these results using L-[^{35}S]methionine to label the *Renilla* luciferase protein produced by the *in vitro* translation reaction and separating the products by SDS–PAGE (Figure 2C). The amount of *Renilla* luciferase produced by the A55U mutant ribosomes was reduced by ≈73% compared to the amount produced by WT ribosomes.

The A55U Mutation Inhibits the Binding of the EF-Tu Ternary Complex to the Ribosome. The crystal structure of the EF-Tu ternary complex bound to the ribosome in the A/T state showed that base C75 of tRNA is stacked between residue Thr219 of EF-Tu and base A55 of 16S rRNA[2] (Figure 1A). Base A55 flips out of helix 5 of 16S rRNA to make this stacking interaction. In addition, base A55 also makes a hydrophobic interaction with Thr221 of EF-Tu. To investigate whether these interactions that A55 makes are important for binding of the EF-Tu ternary complex to the ribosome, we determined the binding affinity using a nitrocellulose filter binding assay. We used the EF-Tu (His84Ala) mutant that is defective in GTP hydrolysis in the filter binding assay because the EF-Tu ternary complex binds reversibly to the ribosome in the A/T state.[37] Filter binding experiments showed that the equilibrium binding constants (K_D) for WT and A55U ribosomes were 3.5 ± 0.4 and 21 ± 4 nM, respectively (Figure 3A,B). Thus, the A55U ribosome is ∼6-fold defective in binding the EF-Tu ternary complex.

The A55U Mutation Affects the Peptidyl Transferase Reaction. In the fully accommodated state, base C75 of A site tRNA forms a base pair with G2553 of 23S rRNA, which is important for the peptidyl transferase reaction.[38,39] However, as

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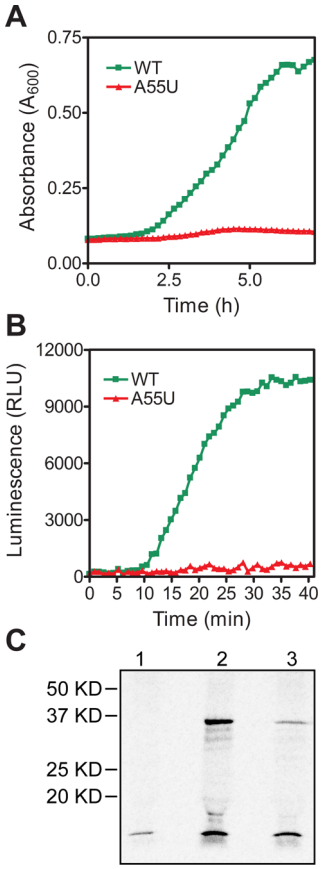

Figure 2. Effect of the A55U mutation on cell growth and *in vitro* translation. (A) Growth curve of the *E. coli* POP2136 strain containing the WT and A55U mutant plasmid at 42 °C. (B) *In vitro* translation by WT and A55U mutant ribosomes. Time course showing the synthesis of *Renilla* luciferase by the WT and mutant ribosomes. The *y*-axis shows the relative luminescence in arbitrary units (RLU). Data for the WT and A55U mutant are colored green and red, respectively. (C) SDS–PAGE showing the *in vitro* synthesis of ^{35}S-labeled *Renilla* luciferase: lane 1, control reaction mixture without ribosomes; lane 2, WT ribosomes; lane 3, A55U mutant ribosomes. The bottom band is free [^{35}S]methionine and the top band is *Renilla* luciferase, which has a molecular mass of 36 kDa.

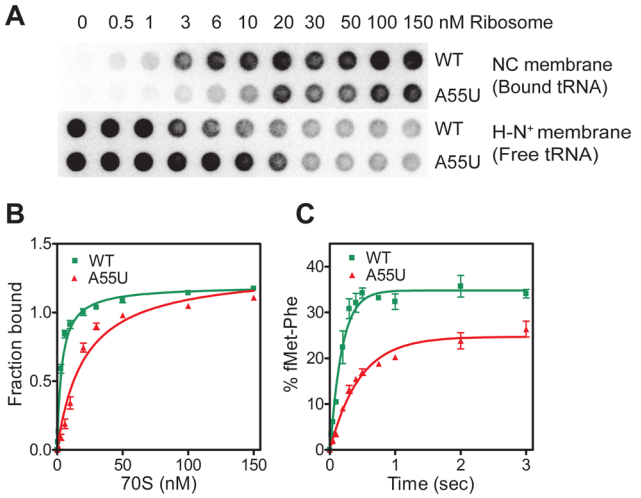

Figure 3. Effect of the A55U mutation on the binding of the EF-Tu ternary complex to the ribosome and peptide bond formation. (A) Representative dot blot showing the nitrocellulose membrane with the EF-Tu ternary complex bound to the ribosome (top) and the HN$^+$ membrane with the free tRNAs bound (bottom). The dots for the WT and A55U mutant are indicated. (B) Graph showing the equilibrium binding of the EF-Tu·GTP·Phe-tRNA ^{Phe} ternary complex to WT and mutant ribosomes. The standard deviations from four experiments are shown. (C) Time course of peptide bond formation by the WT and mutant ribosomes. The lines were best fit to a single-exponential equation. Data for the WT and A55U mutant are colored green and red, respectively. The standard deviations from two experiments are shown.

described above, in the A/T state base C75 of tRNA interacts with A55 of 16S rRNA. We analyzed the effect of the A55U mutation on the peptidyl transferase reaction using a pre-steady state kinetic assay. The EF-Tu·GTP·Phe-tRNA^{Phe} ternary complex was rapidly mixed with a saturating concentration of ribosome with fMet-tRNA^{fMet} in the P site in a quench-flow machine. The Phe-tRNA^{phe} was labeled with α-^{32}P at the 3′ end (described in Experimental Procedures).[30] The time course of the fMet-Phe-[α-^{32}P]-AMP dipeptide formed was fit to a single-exponential equation to determine the rate of dipeptide formation (Figure 3C). The rate of peptide bond formation was 5.5 ± 0.9 s^{-1} for WT ribosomes and 2.2 ± 0.6 s^{-1} for A55U ribosomes. Thus, the A55U mutation decreased the rate of peptide bond formation by 2.5-fold, showing that base A55 is moderately important for peptide bond formation.

The A55U Mutation Inhibits the Binding of EF-G to the Ribosome and Translocation. The crystal structure of the ribosome·EF-G complex showed that A55 of 16S rRNA flips out of helix 5 and interacts with domain II of EF-G.[22] To determine whether the A55U mutation affects the binding of EF-G·GTP to the ribosome, we used a fluorescence-based equilibrium binding assay.[32,40] Equilibrium binding experiments were performed with a fixed concentration of EF-G and varying concentrations of the ribosome (Figure 4A). The K_D values for binding of EF-G to the WT and A55U mutant ribosomes were 2.4 ± 0.8 and 7.9 ± 1 nM, respectively. These results show that the A55U mutation has an only ≈3-fold defect in binding EF-G.

To investigate the role of A55 in translocation, we initially used a toeprinting assay to monitor EF-G-dependent translocation of the mRNA–tRNA complex[34] (Figure 4B). Pretranslocation complexes were programmed with a fragment of the T4 gene 32 mRNA containing tRNA^{fMet} in the P site and tRNA^{Phe} in the A site. The extent of translocation was found to be similar for WT and A55U mutant ribosomes (>95% translocation). The toeprinting result shows that the mutant ribosome forms an authentic pretranslocation complex and is able to translocate the mRNA–tRNA complex upon addition of EF-G.

Because there was no difference in the extent of translocation between the WT and A55U mutant ribosomes, we performed a pre-steady state kinetic experiment to determine if the rate of translocation was affected. The rate of translocation was investigated by using a fluorescence-based, stopped-flow method.[35] The pretranslocation complex was made by programming the ribosome with a short mRNA having a pyrene dye attached at the 3′ end, tRNA^{fMet} in the P site, and fMet-Phe-tRNA^{Phe} in the A site. The pretranslocation complex was rapidly mixed with a 10-fold excess of EF-G in a stopped-flow instrument, and the decrease in the fluorescence intensity of the pyrene dye was monitored. The change in fluorescence intensity showed two phases with apparent rate constants of k_{obs1} and k_{obs2} for the fast and slow phases, respectively. The rate of the rapid phase is consistent with the rate of

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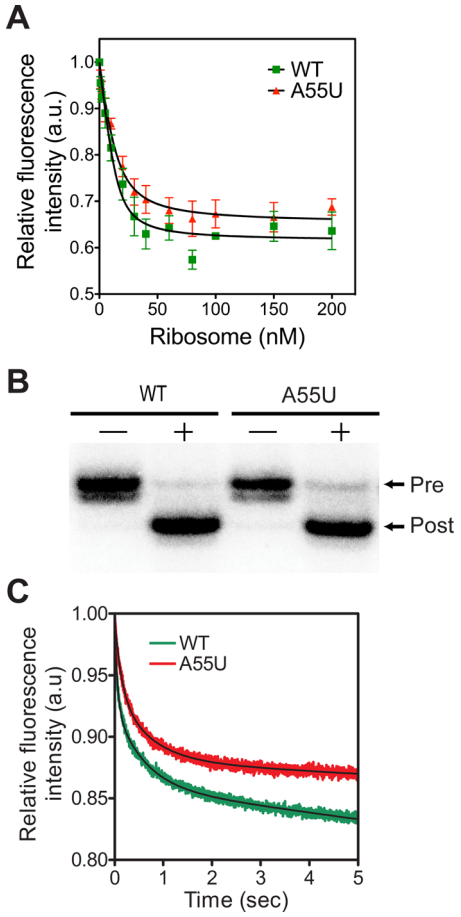

Figure 4. Effect of the A55 mutation on the binding of EF-G to the ribosome and mRNA−tRNA translocation. (A) Equilibrium binding of EF-G to the WT and A55U mutant ribosomes determined using a fluorescence-based assay. The binding reactions were performed with GTP and fusidic acid. The starting fluorescence intensity was normalized to 1, and the change in fluorescence intensity is shown in arbitrary units (a.u.). The standard deviations are from three independent experiments. Data for the WT and A55U mutant are colored green and red, respectively. (B) Toeprint assay showing the extent of translocation by WT and A55U mutant ribosomes. Bands corresponding to the pretranslocation (Pre) and the post-translocation (Post) complex are indicated by arrows. Minus signs and plus signs indicate the absence and presence of EF-G, respectively. (C) Representative time course of EF-G-dependent translocation. The pre-steady state rate of translocation was monitored by the decrease in fluorescence intensity. Data for the WT and A55U mutant are colored green and red, respectively.

translocation measured using fluorescently labeled tRNAs.[41] The data were analyzed by fitting the decrease in fluorescence intensity to a double-exponential equation to calculate k_{obs1} and k_{obs2} for the WT and mutant ribosomes. The WT ribosome translocated with a rate of 19.4 ± 1.6 s^{-1} (Figure 4C). In contrast, the A55U mutant ribosome translocated at a rate that is 2-fold slower than that of the WT (8.0 ± 0.6 s^{-1}). This suggests that A55 is moderately important for EF-G-dependent translocation.

DISCUSSION

Protein synthesis is a complex process catalyzed by the ribosome with the help of several translational factors. The EF-Tu ternary complex and EF-G are the two translational factors that iteratively interact with the ribosome during the elongation cycle of protein synthesis. Both the EF-Tu ternary complex and EF-G interact with the shoulder domain of the 30S subunit. Helix 5 of 16S rRNA in the shoulder domain of the 30S subunit contacts domain II of the EF-Tu ternary complex and EF-G.[2,22] Interestingly, the highly conserved 16S rRNA base A55 in helix 5 is flipped out to interact with C75 of the tRNA in A/T state and with Thr221 of EF-Tu. These interactions have been proposed to be important for decoding by the ribosome.[2] Furthermore, A55 of 16S rRNA interacts with a conserved loop in domain II of EF-G (residues 319−323), suggesting that A55 may be important for mRNA−tRNA translocation.[22]

In agreement with the structural data, previous studies showed that mutations of A55 are deleterious to cells.[24,25] To further understand the role of A55 in protein synthesis, we introduced the A55U mutation and analyzed the effect of the mutation on distinct steps in the elongation cycle. Consistent with previous studies, our results show that the A55U mutation confers a dominant lethal phenotype. Previous studies also showed that mutation of A55 drastically reduced the extent of translation of a reporter protein *in vivo*.[24,25] In agreement with these results, we show that purified A55U mutant ribosomes poorly synthesize *Renilla* luciferase *in vitro*. Together, these results show that A55 is highly conserved because it is functionally important for translation.

Equilibrium binding studies showed that the A55U mutation increased the K_D for binding of the EF-Tu ternary complex to the ribosome by 6-fold. This suggests that the stacking interaction of A55 with C75 of A/T tRNA and the hydrophobic contact of A55 with Thr221 of EF-Tu are critical for stabilizing the EF-Tu ternary complex on the ribosome. The binding of the EF-Tu ternary complex in the A/T state to the ribosome is a key intermediate in decoding. In the A/T state, the EF-Tu ternary complex is largely stabilized by the interaction of the anticodon arm of the tRNA with the 30S subunit A site and of the acceptor arm of the tRNA bound to EF-Tu interacting with the 30S shoulder domain and the SRL region of the 50S subunit. Even though the binding affinity of the EF-Tu ternary complex for the A55U mutant ribosome is reduced by 6-fold, the rate of peptide bond formation was reduced by only 2.5-fold. This is because tRNA selection by the ribosome is kinetically driven and the thermodynamic differences in the stability of the EF-Tu ternary complex on the ribosome are not fully exploited.[42] Finally, our studies show that the A55U mutation inhibits the binding of EF-G to the ribosome by 3-fold and reduces the rate of translocation by 2-fold, suggesting that A55 is not critical for this process. Thus, the A55U mutation has only modest effects on peptide bond formation and translocation.

Then, why are mutations at A55 deleterious to cells, and why do they lead to strong inhibition of *in vivo* and *in vitro* protein synthesis? One possibility is that the A55U mutation inhibits the initiation step of protein synthesis by interfering with the function of initiation factor 2. This may explain the observed ≈73% reduction in the amount of full-length *Renilla* luciferase produced by the A55U mutant ribosomes compared to the amount produced by WT ribosomes (Figure 2C). However, we focused on the elongation cycle of protein synthesis and did not analyze the effect of the A55U mutation on translation initiation. Another possibility is that the A55U mutation inhibits the processivity of the ribosome in the elongation cycle. The *in vivo* and *in vitro* assays for measuring protein synthesis

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require the synthesis of a full-length enzyme (β-galactosidase or luciferase), which is much more demanding than catalyzing a single step of protein synthesis. The probability of synthesizing a complete protein may be reduced by the A55U mutation because the modest defects in decoding and translocation may be additive. Previous studies have observed a similar effect with mutations in the 23S rRNA that are lethal when expressed in cells but have only modest effects in functional assays.[43−45] Taken together, these results suggest that even subtle defects in individual steps of protein synthesis may become amplified because multiple rounds of peptide bond formation and translocation are required for the synthesis of a protein. Thus, A55 plays a small but important role in improving the performance of the ribosome during the elongation cycle of protein synthesis.

AUTHOR INFORMATION

Corresponding Author

*Address: 4102 Urey Hall, Department of Chemistry and Biochemistry, University of California at San Diego, 9500 Gilman Dr., La Jolla, CA 92093-0314. E-mail: sjoseph@ucsd.edu. Phone: (858) 822-2957.

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ABBREVIATIONS

rRNA, ribosomal RNA; mRNA, messenger RNA; tRNA, transfer RNA; EF, elongation factor; PDB, Protein Data Bank.

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