Perturbation of the tRNA Tertiary Core Differentially Affects Specific Steps of the Elongation Cycle

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The tRNA tertiary core region is important for both tRNA stability and activity in the translation elongation cycle. Here we report the effects of mutating each of two highly conserved base pairs in the tertiary core of Phe-tRNA\(^{\text{Phe}}\), 18–55 and 19–56, on rate and equilibrium constants for specific steps of this cycle, beginning with formation of aminoacyl-tRNA-EF-Tu-GTP ternary complexes and culminating with translocation of A-site-bound peptidyl-tRNA into the P-site. We find that codon-dependent binding of aminoacyl-tRNA to the A/T-site and proofreading of near-cognate tRNA are sensitive to perturbation of either base pair; formation of the ternary complex and accommodation from the A/T to the A-site are sensitive to 18–55 perturbation only, and translocation of peptidyl-tRNA from the A- to P-site is insensitive to perturbation of either. These results underline the importance of the core region in promoting the efficiency and accuracy of translation, and they likely reflect different requirements for structural integrity of the core during specific steps of the elongation cycle.

The L-shaped conformation of tRNA is maintained by interactions between the D- and T-loops within the “tertiary core” region of the molecule. These interactions include two universally conserved base pairs, G18-U55 (Ψ is a modification of U) and G19-C56, and a stacking interaction of three purines at the 18, 57, and 58 positions (Fig. 1A). The L-shaped conformation allows ribosome-bound tRNA to contact simultaneously the 30 S subunit decoding center, via its anticodon loop, and the 50 S subunit peptidyltransferase center, via its CCA end. Ribosome-bound tRNA, however, assumes conformations distinct from the classical L-shaped structure. Indeed, tRNA has been likened to a flexible spring that reversibly changes conformation during the process leading to recognition of cognate aminoacyl-tRNA at the A-site and peptide bond formation (1). Departures from the classical tRNA structure have also been seen for tRNA bound at the P- and E-sites (2–4).

Recent molecular dynamics studies have suggested that maintaining a balance between tRNA flexibility and rigidity is essential for optimizing the rate and accuracy of cognate tRNA recognition (5, 6), and it is reasonable to suppose that this balance might be important for other steps of the elongation cycle as well, such as translocation. Mutations in the tertiary core and D-arm that could affect this balance are known to affect tRNA function during elongation. Thus, maintenance of the geometries of one or both of the 18–55 and 19–56 bp is essential for optimal aminoacyl-tRNA-EF-Tu-GTP ternary complex (TC)\(^{4}\) formation (7, 8) and for rapid translocation of P-site tRNA (9). Related studies on bacterial cells have also demonstrated the importance of tertiary core interactions for the overall rate of translation (8, 10). Similarly, elevated error rates have been observed for D-arm variants (11, 12, including the Hirsh mutation (G24A) (13), which, although little affecting TC binding to a cognate codon, allows decoding of a near-cognate codon with relatively high efficiency (14). Despite these earlier examples, relatively little is known (15) about the role of the tertiary core in tRNA function at the A-site.

Here, using single and multiple turnover kinetics and binding experiments, we examine the effects of mutations in the G18-U55 and G19-C56 bp on each of four major steps comprising the elongation cycle (Fig. 1B) as follows: (i) TC formation; (ii) pre-accommodation (PRE-AC) complex formation which proceeds in three resolvable steps, an initial 2nd order binding step of the TC in which there is no codon-anticodon recognition, followed by two first-order steps in which cognate codon-anticodon recognition is rapidly followed by GTP hydrolysis (16); (iii) pretranslocation complex (PRE-TR) formation, in which peptidyl-tRNA and discharged tRNA are located in the A- and P-sites, respectively. PRE-TR formation from the PRE-AC complex proceeds via P release from EF-Tu followed by a rapid conformational change in EF-Tu that results in dissociation of aminoacyl-tRNA from EF-Tu (17) and either accommodation of aminoacyl-tRNA into the 50 S A-site followed by rapid peptide bond formation (18) or dissociation of aminoacyl-tRNA from the ribosome prior to peptide bond formation, the latter constituting a form of proofreading; and (iv) EF-G-GTP-dependent translocation, in which discharged tRNA and peptidyl-tRNA move from the P- to E-site and A- to P-site, respectively, completing the elongation cycle. Translocation proceeds via a hybrid state intermediate, with the 3′ ends of tRNAs moving toward the P- and E-sites on the 50 S subunit, whereas the anti-

4 The abbreviations used are: TC, ternary complex (aminoacyl-tRNA-EF-Tu-GTP); 70SIC, 70 S initiation complex; PRE-AC, pre-accommodation; PRE-TR, pre-translocation; fMet-tRNA\(^{\text{fMet}}\) (prf), fMet-tRNA\(^{\text{fMet}}\) labeled with proflavin; Phe-tRNA\(^{\text{Phe}}\) (prf), Phe-tRNA\(^{\text{Phe}}\) labeled with proflavin; WT, wild type.

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Codons of tRNAs remain in A- and P-sites on the 30 S subunit (19).

**EXPERIMENTAL PROCEDURES**

**Ribosomes and Proteins**—Tight-coupled ribosomes were prepared from *Escherichia coli* MRE600 cells as described (9). Clones of *E. coli* His-tagged proteins EF-G, EF-Tu, IF1, IF2, and IF3 were obtained as described (9), and the proteins were purified on nickel-nitrilotriacetic acid (Qiagen) columns, except that EF-G went through an additional fast protein liquid chromatography Mono Q column with a gradient of 50–350 mM KCl in buffer containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 0.5 mM EDTA, and 6 mM 2-mercaptoethanol. EF-Ts was a gift from Dr. Yale E. Goldman (University of Pennsylvania).

mRNAs—mRNA022UUU was prepared from JM109 cells transformed with pT7Z18 plasmid containing the sequence under a T7 promoter, which was provided by Dr. C. Gualerzi (University of Camerino). The plasmid was extracted, restricted, and then transcribed using the EPICENTRE AmpliscrIBE T7 flash transcription kit. mRNA was purified by precipitation with 2.5 M LiCl on ice (30 min). mRNA022CUC was made by mutation of UUU to CUC, using the QuickChange protocol (Qiagen) followed by a preparation procedure essentially identical to that described for making mRNA022UUU.

**tRNAs**—Native *E. coli* tRNA⁰Met and tRNA⁰Phe were acquired from Chemical Block (Moscow, Russia). Mutations were introduced into plasmid pTFMa (a derivative of pUC18) containing *E. coli* tRNA⁰Phe sequence and its variants were purified, restricted with BstNI, and transcribed by T7 RNA polymerase.

**Measurement of Kₐ for TC Formation**—Kinetic analysis of TC binding to 70SIC was measured by using the formula

\[
K_a = \frac{[\text{Phe-tRNA}] + [\text{EF-Tu}] + K_a - \sqrt{([\text{Phe-tRNA}] + [\text{EF-Tu}] + K_a)^2 - 4[\text{Phe-tRNA}][\text{EF-Tu}]}{2}
\]

Unlabeled and prf-labeled tRNA⁰Met were charged and formylated with partially purified *E. coli* tRNA synthetase containing MetRS and formyltransferase by incubating 20 μM tRNA⁰Met, 80 μM [³⁵S]methionine (2000 dpm/pmol), 720 μM folic acid (as a formyl donor), and 1/10 volume of crude *E. coli* aminocyl-tRNA synthetase in Buffer C’ (Buffer C plus 10 mM KCl) at 37 °C for 30 min. Crude synthetase was prepared from the supernatant resulting from ultracentrifugation of *E. coli* MRE600 cell lysate (40,000 rpm, 18 h, Beckman rotor Ti70.1) through 6 ml of 1 M sucrose in 20 mM Tris-HCl (pH 7.5), 500 mM NH₄Cl, 10 mM magnesium acetate, 0.5 mM EDTA, and 3 mM 2-mercaptoethanol. The supernatant was passed through a DEAE-cellulose column, and fractions with A₂₈₀/A₂₆₀ ≥ 1.5 were pooled.

**Complex Formation**—All complexes were made up in Buffer A (50 mM Tris-HCl (pH 7.5), 70 mM NH₄Cl, 30 mM KCl, 7 mM MgCl₂, 1 mM dithiothreitol) at 37 °C. 70SIC was formed by incubating 2 μM 70 S ribosome, 8 μM mRNA, 3 μM each of IF1, IF2, IF3, and Met-tRNA⁰Met, and 1 mM GTP for 25 min. TC was formed by incubating 5 μM EF-Tu, 1 μM [³⁵S]tRNA⁰Phe, 1 μM GTP, 1.5 mM phosphoenolpyruvate, and 0.5 μl pyruvate kinase for 5 min, unless otherwise specified. PRE-TR complex was formed by incubating 0.8 μM 70SIC with 0.4 μM TC. Prior to use in stoichiometric or kinetic assays, 70SIC and PRE-TR complex were purified by centrifugation through a 1.1 M sucrose cushion (450,000 × g, 40 min, 4 °C) in Buffer A and Buffer B (same as Buffer A but with 20 mM MgCl₂), respectively.

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\]
accommodation of tRNAPhe transcripts required a double 
U55A, 75–90% of the total fluorescence change occurs in the 
with [3H]Phe-labeled TC (0.12 M) incubating 4 l.

70SIC, 0.4 M; EF-Tu, 1 M; [3H]Phe-tRNAPhe 0.2 M; GTP, 100 
M; volume, 20 l. Samples were incubated for 1 min at 
37 °C before filtering. Dipeptide formation was measured using 
the chromatographic method described below.

Kinetics—All kinetics experiments were carried out in Buffer 
A at 25 °C. All concentrations given below are final, after mix-
ning. Rapid kinetics experiments were performed either with 
a Kintek quenched flow apparatus (fMet-Phe formation, GTP 
hydrolysis, fMet-Phe-puromycin formation) or with an 
Applied Photophysics SX.18MV stopped-flow spectrophotometer 
(accommodation and translocation). The slow dissociation 
of fMet-[3H]Phe-tRNAPhe from the A-site was monitored by 
nitrocellulose filtration.

fMet-Phe Formation—[3H]Phe-labeled TC complex (0.2 l) 
was rapidly mixed with 70SIC (0.4 M). Reactions 
were quenched with 5 mM NH4OH, lyophilized, taken up in 500 l of 
water, applied to a cation exchange column (Bio-Rad 50W-X8, 
400 l) that had been prewashed with 0.01M HCl and water, 
and eluted with water. [3H]-Labeled fMet-Phe eluted in the flow-
through and ~5 column volumes. Values shown are corrected 
for a background measured in the absence of 70SIC.

GTP Hydrolysis and Accommodation—TC was formed by 
incubating 4 l EF-Tu, 4 l [3H]Phe-tRNAPhe, and 0.8 l 
[γ-32P]GTP, 0.2 M TC (calculated from the concentration of 
[γ-32P]GTP) was rapidly mixed with 0.4 M 70SIC. After 
quenching with 0.6 M HCl, aliquots (50 l) were mixed with 
20 mM sodium molybdate (150 l), the resulting dodecamolyb-
date complex was extracted with an equal volume of ethyl ace-
etate, and radioactivity was determined in a scintillation counter. 
Accommodation was monitored by changes in the fluorescence 
on rapid mixing TC (0.2 l) with 70SIC (0.4 M) containing 
fMet-tRNAPhe (prf).

Translocation—PRE-TR complex (0.12 l) containing 
tRNAPhe was rapidly mixed with 1 l EF-G, 1 mM GTP, and 5 
mM puromycin. To prevent dissociation of fMet-Phe-tRNAPhe 
before translocation, the synringe containing pretranslocation 
complex was cooled in an ice bath. fMet-[3H]Phe-puromycin 
was extracted into ethyl acetate, and radioactivity was determined.

fMet-Phe-tRNAPhe Dissociation—70SIC (0.2 l) was mixed with 
[3H]Phe-labeled TC (0.12 l) and incubated at 25 °C. Al-
liquots were analyzed by nitrocellulose filtration at different time 
points (1–180 min). [3H]Phe in the filtrate measures dissociation 
of fMet-[3H]Phe-tRNAPhe (24).

Determination of Rate Constants and Functional 
Heterogeneity—With one clear exception, the U55A variant, 
each Phe-tRNAPhe preparation (WT or variant) gave rates and 
stoichiometries of ribosome-dependent reactions consistent 
with complete or near-complete functional homogeneity. 
Thus, rates of fMet-Phe-tRNA dissociation, GTP hydrolysis, 
fMet-Phe formation, and translocation were well fit to single 
exponential equations, giving apparent first-order rate con-
stants, with stoichiometries per TC formed similar to those 
observed with native or WT Phe-tRNAPhe. Fitting the rates of 
accommodation of tRNAPhe transcripts required a double 
exponential equation. However, for all of the transcripts except 
U55A, 75–90% of the total fluorescence change occurs in the 
rapid first phase of reaction, so even with this assay the extent of 
any putative functional heterogeneity would be quite limited. In 
contrast, the U55A variant preparation could have considerable 
functional heterogeneity, because its ribosome-dependent 
reactions proceed with low stoichiometry, and the fluorescent 
change accompanying accommodation does not show a domi-
nant first phase. Fits to exponential equations were carried out 
with Igor-Pro (Wavemetrics). kcat and Kcat values were obtained 
by fitting the curves of kcat′ as a function of 70SIC concentration 
to the Michaelis-Menten equation.

RESULTS

tRNA Variant Preparation—tRNAPhe was transcribed and 
contained the wild-type sequence (WT-tRNAPhe) or variations in 
the 18, 19, 55, and/or 56 positions (Fig. 1A). WT transcript 
differs from the native tRNAPhe, isolated from E. coli cells, in 
having no post-transcriptional modification, and it is used for 
direct comparison with transcribed variants.

Initial Screening of Variants—Four assays (Table 1) were 
used to test each of the 12 tRNAPhe variants for functional Phe-
tRNA interaction with the A-site, following addition of the 
 corresponding [3H]Phe-tRNAPhe-EF-Tu-GTP to a 70 S initia-
 tion complex (70SIC) containing fMet-tRNAPhe in the 
P-site and an empty A-site programmed with an UUU codon as 
follows: (i) the initial stoichiometry of Phe-tRNA binding to 
the A-site via the TC complex; (ii and iii) the stoichiometry and 
rate (kcat′) of dipeptide formation; and (iv) the rate of peptidyl-
tRNA dissociation (kdp) from the A-site following dipeptide 
formation. Here an increased value indicates destabilization of 
peptidyl-tRNA binding to the A-site.

Measured at an EF-Tu concentration of 1 l, mutation 
effects in reducing initial Phe-tRNA binding are, for the 
most part, modest (Table 1). The strongest effects on TC bind-
ing are found for U55A (4-fold decrease) and C56G and G18U/
U55A, suggesting that this mutation directly perturbs Phe-
tRNA interaction with the A-site. Additional evidence for 
such a perturbation is provided by the larger negative effect that 
U55A mutation has on the stoichiometry of dipeptide forma-
tion than on the stoichiometry of initial Phe-tRNA binding. 
This contrasts with the largely similar effects that almost all of 
the other mutants (G19C excepted) have on both stoichiome-
tric assays.

More pronounced effects are seen on apparent rate constants 
(Table 1). For kdp, the largest decreases are for U55A, G18U/
U55A, and G19C (8–10-fold) and G18A, C56A, and C56G (3–4-fold), whereas for kcat′ the largest increases (5–9-fold) are 
found for U55G, G18U/U55A, G19U, and C56G. The dissociation 
rate for U55A was not determined accurately because of its 
low binding stoichiometry but is likely to be relatively rapid, 
given the evidence for perturbed Phe-tRNA binding to the 
A-site. In both assays, WT function is fully or partly restored in
19–56 double variants that preserve Watson-Crick base pairing.

Selection of Variants for Further Study—Five variants (U55A, G18U/U55A, G19U, C56A, and G19U/C56A) were selected for particularly close scrutiny, to determine which step or steps in the overall process of PRE-TR complex formation were responsible for the effects observed. The first four variants were selected because, with the exception of U55A, they have relatively large effects on the rate and smaller effects on the stoichiometry of dipeptide formation (U55A strongly affects both), allowing us to concentrate our analysis on the rate constants. The G19U/C56A variant was selected to more fully examine the rescuing effect of a second mutation that restores a 19–56 Watson-Crick base pair.

Binding of Phe-tRNA\textsuperscript{Phe} to the ribosomal A-site, leading to PRE-TR complex formation, includes three readily distinguishable steps (Fig. 1B) as follows: (a) formation of the TC, (b) formation of the PRE-AC complex, and (c) formation of the PRE-TR complex. Below we determine the effects of the selected mutations on each of these three steps.

**TC Formation**—The binding affinities to EF-Tu-GTP of native Phe-tRNA\textsuperscript{Phe}, its WT transcript, and the five selected variants were determined by the ribonuclease protection assay (22), giving the dissociation constants displayed in Table 2. The largest effects are seen for U55A and G18U/U55A, which have 15–20-fold lower affinities than WT-Phe-tRNA\textsuperscript{Phe} for EF-Tu-GTP. G19U and C56A are less affected, with 6- and 1.7-fold lower affinities, respectively, although for G19U/C56A binding affinity is restored to a level close to that of the WT transcript. Structural studies of the ternary complex (26) show a contact between EF-Tu and the backbone of nucleotide 54. Thus, although EF-Tu does not directly interact with the 18–55 bp, the comparatively large effects of 18–55 mutations on TC formation may reflect a perturbation of the stacking interaction.
TABLE 1
Preliminary screening: relative values for EF-Tu-GTP-Phe-tRNA\(^{\text{Phe}}\) binding, dipeptide formation, and fMetPhe-tRNA\(^{\text{Phe}}\) dissociation (UUU codon)

| tRNA\(^{\text{Phe}}\) | Initial Phe-tRNA\(^{\text{Phe}}\) binding | Dipeptide stoichiometry | \(K_{\text{GTP}}\)^a | \(K_{\text{acc}}\)^b |
|-----------------|--------------------------------------|------------------------|----------------|----------------|
|                 | EF-Tu, 1 \(\mu\)M                    | EF-Tu, 5 \(\mu\)M      | \(s^{-1}\)      | \(s^{-1}\)      |
| Native          | 1.06 ± 0.05                          | 1.04 ± 0.05            | (1.0)          | (1.0)          |
| WT transcript   | (1.0)                                | (1.0)                  |                |                |
| G18A            | 0.79 ± 0.06                          | 0.89 ± 0.05            | 0.26 ± 0.02    | 0.26 ± 0.02    |
| U55G            | 0.82 ± 0.03                          | 0.90 ± 0.04            | 0.74 ± 0.09    | 8.8 ± 1.0      |
| U55A            | 0.26 ± 0.02                          | 0.16 ± 0.03            | 0.10 ± 0.02    | ND†            |
| G18A/U55G       | 0.81 ± 0.04                          | 0.98 ± 0.06            | 0.00 ± 0.05    | 2.8 ± 0.4      |
| G18U/U55A       | 0.56 ± 0.02                          | 0.57 ± 0.06            | 0.12 ± 0.01    | 6.0 ± 0.5      |
| G19U            | 0.76 ± 0.02                          | 0.70 ± 0.05            | 0.58 ± 0.06    | 5.9 ± 0.5      |
| G19C            | 0.66 ± 0.02                          | 0.42 ± 0.03            | 0.12 ± 0.02    | 2.3 ± 0.4      |
| C56A            | 0.91 ± 0.04                          | 0.80 ± 0.05            | 0.35 ± 0.02    | 2.8 ± 0.3      |
| C56G            | 0.57 ± 0.03                          | 0.58 ± 0.02            | 0.29 ± 0.02    | 4.9 ± 0.4      |
| G19U/C56A       | 0.90 ± 0.03                          | 0.93 ± 0.06            | 1.09 ± 0.12    | 2.4 ± 0.5      |
| G19A/C56U       | 1.00 ± 0.05                          | 0.95 ± 0.04            | 1.20 ± 0.11    | 2.0 ± 0.2      |
| G19C/C56G       | 1.04 ± 0.03                          | 1.04 ± 0.04            | 1.07 ± 0.10    | 1.6 ± 0.2      |

a Apparent net rate constant for peptide bond formation is shown.

b Apparent net rate constant for fMetPhe-tRNA\(^{\text{Phe}}\) dissociation from the ribosome is shown.

c Preliminary screening: relative values for EF-Tu-GTP-Phe-tRNA\(^{\text{Phe}}\) binding, dipeptide formation, and fMetPhe-tRNA\(^{\text{Phe}}\) dissociation (UUU codon).

TABLE 2
Measures of tRNA\(^{\text{Phe}}\) variant functionality with UUU codon

| tRNA\(^{\text{Phe}}\) | \(K_{\text{GTP}}\)^a TC formation | \(K_{\text{GTP}}\)^b | \(K_{\text{acc}}\)^b | \(K_{\text{acc}}\)^c |
|-----------------|---------------------------------|-----------------|-----------------|-----------------|
|                 | TC formation                    | \(s^{-1}\)      | \(s^{-1}\)      | \(s^{-1}\)      |
| Native          | 0.06 ± 0.01                     | 11 ± 1.5        | 3.72 ± 0.56     | 5.6 ± 0.76      |
| WT transcript   | 0.07 ± 0.01†                    | 12 ± 2.7        | 2.82 ± 0.2      | 6 ± 1.1         |
| U55A            | 1.1 ± 0.2                      | 0.6 ± 0.2       | ≤0.16†          | ≤0.22†          |
| G18U/U55A       | 1.3 ± 0.2                      | 0.51 ± 0.08     | 0.18 ± 0.02     | 0.28 ± 0.08     |
| G19U            | 0.41 ± 0.06                    | 1.4 ± 2.2       | 1.3 ± 0.1       | ≥4              |
| C56A            | 0.12 ± 0.01                    | 0.95 ± 0.8      | 0.74 ± 0.7      | 4 ± 2           |
| G19U/C56A       | 0.09 ± 0.01                    | 4.5 ± 4.0       | 2.6 ± 0.2       | 6 ± 2           |

a Apparent net rate constant for GTP hydrolysis. Gromadzki and Rodnina (25) report a value of ~10 s\(^{-1}\) for native tRNA\(^{\text{Phe}}\) at 20 °C, also measured at 0.4 \(\mu\)M 70SIC. The absolute value for WT-tRNA\(^{\text{Phe}}\) GTPase stoichiometry is 0.72 ± 0.05 GTP hydrolyzed/TC added. Values for native and all variants except U55A were similar, when corrected for the amount of TC formation, using the \(K_{\text{GTP}}\) values in column 2. The GTPase stoichiometry/TC added for the U55A variant was 0.13.

b Apparent net rate constant for accommodation as measured by fluorescence change. The magnitude of the fluorescence change/GTP hydrolyzed was similar for all tRNAs tested except U55A, which was about 1/5 of the WT transcript.

c Apparent rate constant is for accommodation (see Equation 2).

d Pleiss and Uhlenbeck (22) report a value of 0.025 \(\mu\)M for Thermus thermophilus EF-Tu.

That the 18–55 bp makes with the intraloop 54–58 bp (Fig. 1A). The smaller effects of mutations at 19–56 are consistent with their being even further from EF-Tu within the TC, and with earlier results showing that the G19C and C56G mutations have only minor effects on TC formation (15). The similarity in the values for native and WT-Phe-tRNA\(^{\text{Phe}}\) binding to EF-Tu is also in accord with earlier results (27).

PRE-AC Complex Formation—Rates of PRE-AC complex formation for the selected variants were determined by quenched flow rapid kinetic studies of EF-Tu-dependent GTP hydrolysis (Fig. 2A and Table 2). The stoichiometries of GTP hydrolysis per TC bound to the ribosome were similar for all of the transcribed tRNAs and for native tRNA, except for the markedly lower stoichiometry of the U55A variant. Measured at a single 70SIC concentration (Fig. 2A), the apparent net rate constant for GTP hydrolysis (\(k'_{\text{GTP}}\), see Table 2), relative to that for WT transcript, is 2-fold larger for native tRNA, 10-fold smaller for the U55A and G18U/U55A variants, and 5-fold smaller for the G19U and C56A variants, the latter effects being fully rescued by the G19U/C56A double mutation. Measuring \(k'_{\text{GTP}}\) as a function of increasing 70SIC concentration for WT transcript, G18U/U55A, and C56A allowed effects on the net rate constant \(k_{\text{cat,GTP}}\) to be distinguished from effects on \(K_{\text{GTP}}\) values (defined as the concentration of 70SIC giving half-maximal velocity) (Fig. 2B). For G18U/U55A \(k_{\text{cat,GTP}}\) is decreased 12-fold with little change in \(K_{\text{GTP}}\), but for C56A both \(k_{\text{cat}}\) (3-fold decrease) and \(K_{\text{GTP}}\) values (3-fold increase) are affected.

PRE-AC Complex Formation—Rates of PRE-AC complex formation were determined by measurement of the rate of accommodation. In accord with earlier results (16, 18), this rate is indistinguishable from that of dipeptide formation (data not shown). Accommodation was measured by the change in the fluorescence of P-site-bound fMet-tRNA\(^{\text{Phe}}\)(prf) for a constant amount of TC added (Fig. 3). Previously we showed that this change occurs at the same rate as the fluorescent change of Phe-tRNA\(^{\text{Phe}}\)(prf), a prolyl derivative of Phe-tRNA\(^{\text{Phe}}\) (see supplemental Fig. 1C in Ref. 19) that signals accommodation into the A-site (16). For the TC formed with the WT transcript, the change in fMet-tRNA\(^{\text{Phe}}\)(prf) fluorescence proceeds in two phases as follows: a large, fast decrease followed by a slow, small decrease, and it is the former that corresponds to accommodation. The two phases are well separated for all the variants but U55A, permitting reliable estimation of the stoichiometries of accommodated Phe-tRNA\(^{\text{Phe}}\) from the magnitude of the first phase decrease in fluorescence, as well as of the apparent net rate constants for accommodation (\(k'_{\text{acc}}\)) (Table 2). \(k'_{\text{acc}}\) is reduced 15-fold in G18U/U55A and 2–4-fold in the 19–56 single variants, which are once again rescued by the G19U/C56A double mutation. For U55A, the two phases of fluorescence decrease are not well separated, allowing only upper limit estimates of the stoichiometry of accommodation and \(k'_{\text{acc}}\), the latter being ≥18-fold less than for the WT transcript.

The differences in the apparent net rate constants for GTP hydrolysis (\(k'_{\text{GTP}}\)) and accommodation allow calculation of...
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FIGURE 2. Mutation effects on EF-Tu-GTP hydrolysis with cognate UUU codon. A, time course of GTP hydrolysis. Initiation complex, 0.4 μM. Ternary complex was formed by preincubating EF-Tu, Phe-tRNA\textsuperscript{Phe}, and GTP in the molar ratio 5:5:1. TC concentration was calculated using \(k_b\) values in Table 2. Curves are fit to single exponential equations. B, concentration dependence of the rate of GTP hydrolysis. The curves are fit to the Michaelis-Menten equation, yielding the following results: WT transcript, \(k_{cat} = 1.4 \pm 0.1 \text{ s}^{-1}, K_m = 0.6 \pm 0.1 \text{ μM}\); C56A, \(k_{cat} = 6 \pm 2 \text{ s}^{-1}, K_m = 2.4 \pm 0.9 \text{ μM}\).

FIGURE 3. Mutation effects on accommodation rate with cognate UUU codon. Accommodation was monitored by fMet-tRNA\textsuperscript{Met}(prf) fluorescence change. Initiation complex, 0.4 μM EF-Tu, 1 μM Phe-tRNA\textsuperscript{Phe}, 0.2 μM GTP, 200 μM. With one exception (U55A) curves are fit to a double exponential equation. For WT Phe-tRNA\textsuperscript{Phe} and most variants, the large decrease in the first reaction phase corresponds to accommodation, and it is followed by a small, slow decrease in the second phase of unclear origin, possibly because of a minor amount of misfolded tRNA. For U55A, which binds ternary complex slowly and with low stoichiometry, the first phase is not clearly distinguishable from the second, and results are fit with a single exponential. Relative amplitudes are observed changes for 0.2 μM ternary complex added and are thus corrected for incomplete TC formation, using the \(K_d\) values in Table 2. Monitoring of P-site tRNA\textsuperscript{Phe} does not permit observation of initial binding of Phe-tRNA\textsuperscript{Phe} to the A/T-site.

apparent rate constants for the accommodation step itself (\(k^*_{acc}\)) from Equation 2 (Table 2).

\[
k^*_{acc} = (k'_{GTPase} - k_{acc})/(k'_{GTPase}k_{acc})
\]  

(Eq 2)

The results emphasize the dramatic difference between the marked effects of the 18–55 variants (U55A and G18U/U55A) on \(k_{acc}\) (20-fold decreases) versus the total lack of effect of the 19–56 variants.

**Misreading**—To investigate the effect of mutations in the core region of tRNA\textsuperscript{Phe} on misreading, we formed 70 S initiation complexes containing a near-cognate CUC codon (coding for Leu) at the A-site in place of the original cognate UUU codon and determined, following individual addition of Phe-tRNA\textsuperscript{Phe}s, the following: (i) the rates of single turnover GTPase; (ii) the rates of single and multiple turnover dipeptide formation; and (iii) the proofreading ratios \(r\), formed/dipeptide formed. The results of these studies are presented in Fig. 4 and Table 3.

Replacing cognate UUU with CUC decreases \(k'_{GTPase}\) for each of the Phe-tRNA\textsuperscript{Phe}s tested (Fig. 4A). Although the reductions are larger for native and WT Phe-tRNA\textsuperscript{Phe} (6–8-fold), than for the variants G18U/U55A and C56A (2–4-fold), the order of reactivity for the CUC codon is unchanged from that for the UUU codon, native > WT > G18U/U55A, C56A.

More dramatic effects are seen on dipeptide formation (Fig. 4, B and C), which proceeds much more slowly and for which the order of reactivity for the CUC codon, G18U/U55A, C56A > WT > native is inverted with respect to that found for the UUU codon (Fig. 5), indicating that mutations in the tRNA tertiary core promote misreading.

The biphasic character of near-cognate dipeptide formation, evident in Fig. 4, B and C, is accounted for quantitatively by scheme 1 in Fig. 4D. Here \(r\), which is the ratio of the rate constant for accommodation and peptide bond formation to that for aminoacyl-tRNA release, provides a measure of proofreading. The very slow rate of second phase reaction in Fig. 4B is determined by the rate of EF-Tu-GDP recycling to EF-Tu-GTP and the value of \(r^{-1}\). For experiments performed in Buffer A, estimates of \(r\) were determined either by fitting the results in Fig. 4, A and B, to scheme 1 in Fig. 4D or by directly determining \(R\), the measured ratio of \(P_i\) formed/dipeptide formed, and setting \(r\) equal to \(R^{-1}\). Both methods yielded values that agree within experimental error (Table 3). Very similar values of \(r\) were obtained in a “high fidelity” polyamine-containing buffer (25).

It is the comparatively poor proofreading by the G18U/U55 and C56A variants (\(r = 3–4\)) that accounts for their higher rates of dipeptide synthesis as compared with WT-tRNA (\(r = 7\)) and native (\(r = 28\)) (Fig. 5). That proofreading is considerably more stringent for native versus WT-tRNA agrees with earlier results (27). Finally, using the overall rate of dipeptide formation with the CUC codon as a criterion, it is clear that proofreading is also decreased in the G18A and G19U variants, but it is maintained in the G19U/C56A double variant that restores the 19–56 Watson-Crick base pair (Fig. 5).

**Translocation**—Apparent rate constants of translocation (\(k'_{tr}\)) using cognate mRNA were determined by quenched flow...
kinetic measurements of fMet-Phe-puromycin formation following rapid mixing of the PRE-TR complex with EF-G/GTP in the presence of puromycin (Fig. 6). Although the WT transcript fMet-Phe-tRNAPhe is translocated 3-fold more slowly than native, none of the 12 mutations studied further decreased the rate by more than 2-fold, and some showed slightly increased rates over WT transcript. This result is in marked contrast to the large decreases in translocation rate that we previously observed with the corresponding tRNAfMet variants bound in the P-site (9) (Fig. 6), especially at the 18 and 55 positions.

DISCUSSION

Effects of 18–55 and 19–56 Mutations on tRNA Function during Elongation—Here we demonstrate that mutations of the strictly conserved 18–55 and 19–56 bp in the tRNA tertiary core differentially affect specific steps of the elongation cycle. The present results, taken together with related earlier results describing mutational effects on translocation from the P-site (9), may be summarized as follows (Fig. 1B). (i) The overall rate of PRE-AC complex formation, as measured by kGTP, the stability of peptidyl-tRNA bound to the A-site, as measured by kpb, and the rate of dipeptide formation in response to a near-cog-

TABLE 3

| tRNA Tertiary Core Affects the Elongation Cycle |
|-----------------------------------------------|
| tRNA Tertiary Core Affects the Elongation Cycle |
|-----------------------------------------------|
| kGTPase | r′ | r″ | r‴ |
| Native | 1.3 ± 0.2 | 46 ± 24 | 28 ± 6 | 30 ± 2 |
| WT transcript | 0.9 ± 0.1 | 6.9 ± 0.8 | 7 ± 2 | 6.5 ± 0.2 |
| G18U/U55A | 0.23 ± 0.05 | 2.7 ± 0.3 | 4 ± 1 | 5 ± 2 |
| C56A | 0.24 ± 0.03 | 2.9 ± 0.4 | 3 ± 1 | 2.9 ± 0.6 |

a r′ is the ratio of the rate constant for accommodation and peptide bond formation to that for aa-tRNA release, is determined by fitting results in Fig. 4, A and B, to scheme 1 in Fig. 4D.

b r″ = R − 1, where R is equal to P, formed/dipeptide formed. It was determined on a reaction mixture incubated for 50 min that was identical to that used for misreading assays (Fig. 4) except that 20 μM GTP was used instead of 200 μM GTP to reduce background GTP hydrolysis contributing to P formation.

c r‴ = R = 1 as determined in high fidelity polyamine buffer (50 mM Tris-HCl (pH 7.5), 70 mM NH4Cl, 30 mM KCl, 3.5 mM MgCl2, 0.5 mM spermidine, 8 mM putrescine, 2 mM dithiothreitol). Here 0.5 μM EF-Ts was added, and determinations were made after a 6-min incubation.
The changes in function accompanying mutation of any of the 4 bases in the 18–55 or 19–56 bp appear to result mainly from perturbations in local tRNA structure, rather than from disruptions of specific interactions of these bases with either EF-Tu or the ribosome. Thus, the deleterious effects of single mutations in the G19-C56 Watson–Crick base pair at the periphery of the tertiary core structure (28, 29) (Fig. 1A) are largely or fully rescued by double mutations that restore Watson–Crick pairing. As well, all four Watson-Crick pairs at the 19–56 position yield similar values for the stoichiometry of TC binding and for the stoichiometry and apparent rate constant of dipeptide formation (Table 1), and the double variant G19U/C56A has very similar values to WT-tRNA for TC formation, \( k'_{\text{GTP}} \) and proofreading (Table 2 and Fig. 5). The situation is less straightforward for the G18-U55 base pair because these nucleotides form a bifurcated, three center interaction between the 2 carbonyl of U55 and N1 and the 2-amino group of G18 (30), rather than a Watson–Crick base pair. Moreover, G18 makes a stacking interaction with bases G57 and A58, which form a so-called “purine trap” (8). As a result, the G18U/U55A double variant does not restore the disruption in the tRNA structure that results from U55A substitution, accounting for its general failure to reverse the effects of U55A mutation on TC formation, \( k'_{\text{GTP}} \), \( k'_{\text{GTP}} \) and \( k'_{\text{acceptance}} \) (Tables 1 and 2). On the other hand, we find high relative activities for the G18A/U55G variant, in accord with modeling studies showing that the geometry of the A-G base pair is similar to that of the G-U base pair within the tRNA tertiary core (8) and with several functional studies demonstrating the near interchangeability of the A-G and G-U (i.e. WT) base at the 18–55 position pairs (7–9, 31). An analogous explanation may account for the higher activity of the U55A variant than the U55A variant with respect to both Phe-tRNA\textsuperscript{Phe} interaction with the A-site (Table 1) and translocation of P-site-bound tRNA\textsuperscript{tRNA}\textsuperscript{Met} (9) (Fig. 6), because in vivo selection of effective variant suppressor tRNAs yields some G-G pairs but no G-A pairs in positions equivalent to 18–55 (8, 32).

Comparing the Present Results with Those of Nazarenko et al. (15)—The importance of the 18–55 and 19–56 bp for tRNA function in elongation was less evident in an earlier study (15). This latter work, which carried out two measurements (the stoichiometries of GTP hydrolysis and dipeptide formation) on a limited set of variants (U55C, G19C, and G19C/C56G), concluded that the conserved nucleotides 18, 19, 55, and 56 of tRNA\textsuperscript{Phe} are not essential for functional Phe-tRNA\textsuperscript{Phe} interaction with the ribosome. Our more extensive study of these base pairs, including determination of the effects of mutation on reaction rates as well as stoichiometries, clearly show that some disruptions of 18–55 and 19–56 bp have major impacts on Phe-tRNA\textsuperscript{Phe} interaction with the ribosome. That U55A substitution, studied here, has a more deleterious effect on function than the U55C substitution studied by Nazarenko et al. (15) is consistent with modeling studies (31) showing that A-G, G-U, and G-C are the only three combinations of 18–55 bases that form hydrogen bonds within the constraints of the elbow structure of tRNA\textsuperscript{Phe}.

Despite the different conclusions reached in the two studies, there is only one apparent disagreement between the present results and those of Nazarenko et al. (15). This concerns the effect of G19C mutation on dipeptide stoichiometry, with
Nazarenko et al. (15) reporting no effect, although we find a 2.5-fold decrease (Table 1). This relatively minor difference is likely attributable to different experimental protocols; Nazarenko et al. (15) used poly(U)-programmed ribosomes, N-acetyl-Phe-tRNA
Phe
 as peptide donor, and yeast Phe-tRNA
Phe
 as peptide acceptor, whereas we employ 0.22mRNA-programmed ribosomes containing fMet-tRNA
Phe
 as peptide donor and E. coli Phe-tRNA
Phe
 as peptide acceptor. In addition, Nazarenko et al. (15) performed their assays at relatively high concentrations of ribosomes (up to 2 \( \mu \)M) and Mg\(^{2+}\) (10 mM), conditions that would be expected to minimize decreases observed in the stoichiometry of dipeptide formation arising from weaker TC affinity for the ribosome. By comparison, the experiments reported in Table 1 utilized 0.4 \( \mu \)M ribosomes and 7 mM Mg\(^{2+}\).

Effects of 18–55 and 19–56 Mutations on Misreading—Fidelity in decoding mRNA is accomplished via two processes, an initial selection of cognate tRNA via an induced-fit, leading to EF-Tu-dependent GTP hydrolysis and PRE-AC complex formation, and a kinetic proofreading step that involves partitioning of ribosome-bound aminoacyl-tRNA that has been released from EF-Tu between accommodation followed by dipeptide formation and dissociation from the ribosome (14, 33) (scheme 1 in Fig. 4D). The rate of EF-Tu-dependent GTPase provides one measure of the induced-fit process, whereas the overall rate of dipeptide formation provides a measure of proofreading. Our results show that when a near-cognate codon is used, mutations disrupting tertiary base pairing, while little affecting relative rates of GTP hydrolysis (variant versus WT-Phe-tRNA
Phe
) (Fig. 2A and Fig. 4A), result in enhanced relative rates of dipeptide synthesis (Fig. 5). Thus, the rate of dipeptide formation for the G18U/U55A variant relative to WT Phe-tRNA
Phe
 increases some 40-fold, from 0.06 to 2.5, when the cognate UUU codon is replaced by near-cognate CUC, whereas the corresponding increase for the C56A variant is 10-fold. These results provide the first indication of the importance of tRNA tertiary core structure in maintaining the fidelity of protein synthesis.

The induced-fit process is thought to proceed via large scale conformational changes of the 30 S subunit in response to cognate codon:anticodon recognition that trigger specific interactions between the TC and the 50 S subunit, resulting in GTP hydrolysis (1, 33, 34). The antibiotic kirromycin, while allowing formation of the PRE-AC complex and P release, prevents both the conformational change in EF-Tu-GDP that normally follows P release as well as EF-Tu-GDP dissociation from the ribosome (17). Cryo-EM studies of the kirromycin-stalled cognate complex reveals a distortion in the tRNA bound as part of the TC, relative to the crystal structure of tRNA alone, stemming from a kink near bases 26 and 44/45 (35). This has led to the hypothesis that the bending of aminoacyl-tRNA, which allows it to acquire the orientation required for codon recognition, is also a part of the communication between the decoding site and the GTPase activation center (1, 35). The elevated error rates that have been observed for D-arm variants (11–14), noted above, lend support to this hypothesis, if it is assumed that such variants adopt a kinked conformation even when binding to a near-cognate codon.

Portions of the GTPase-activation region of the 50 S subunit are in contact with the tertiary core of tRNA within the kirromycin-stalled complex (1, 35). Such contacts have led to the suggestion (6) that mutations in the tertiary core region could lead to reduced GTPase activity by distorting the three-way interaction between the switch region in EF-Tu, the acceptor arm of tRNA, and the sarcin-ricin loop. Our results demonstrating the importance of maintaining both the 19–56 and 18–55 bp in the transition state for GTP hydrolysis (Table 2) support this suggestion. Indeed, as judged by effects on the value of \( k^r_{\text{GTPase}} \) perturbing these base pairs by mutation has as strong an effect on the induced-fit process as does replacement of the cognate UUU codon by the near-cognate CUC codon (Fig. 3E).

A recent molecular dynamics simulation emphasizes the sensitivity of the rate of the accommodation process to the precise alignment of the acceptor arm relative to the 50 S subunit (5, 6). This is because of the narrow accommodation corridor through which the acceptor arm must navigate as it moves from the A/T-site to the A-site (34). As a result, although the tertiary structure of tRNA is required to flex during accommodation, it also must be sufficiently stiff to avoid significant steric clashes. Our results, showing the accommodation rate constant \( k^r_{\text{acc}} \) for a cognate codon-tRNA interaction to be sensitive to mutation in the 18–55 bp but not in the 19–56 bp (Table 2), suggest that the proper balance is achieved by maintaining the 18–55 bp, which is embedded within the tRNA tertiary core (Fig. 1A), and allowing the 19–56 bp at the periphery of the tRNA elbow to come apart, at least at the beginning of the accommodation process. This suggestion is consistent with results indicating that the 19–56 Watson-Crick base pair is not maintained in the kirromycin-stalled complex (36). On the other hand, the balance between accommodation and tRNA dissociation for a near-cognate codon-tRNA interaction is clearly altered by perturbation of both the 18–55 and 19–56 bp, with mutation leading to increased misreading. This suggests that optimal sensing of a noncognate codon-anticodon interaction, leading to tRNA dissociation, requires strict maintenance of the tertiary core base pair interactions.

EF-G-catalyzed translocation of tRNAs proceeds with GTP hydrolysis and involves large scale movements of each tRNA, which, given the distorted tRNA structures that have been found at the P- and E-sites (2–4), are likely to require some flexing of tRNA. This would be consistent with our results showing that, as is the case for accommodation, maintenance of the 19–56 bp is not required for the efficient translocation of either tRNA. On the other hand, translocation of A-site tRNA differs from translocation of P-site tRNA in also being insensitive to maintenance of the 18–55 bp (Fig. 6). This could be interpreted as indicating that translocation of A-site tRNA to the P-site requires more flexibility within the tertiary core than translocation of P-site tRNA to the E-site. It would be of interest to determine whether molecular dynamics simulations of translocation (37, 38) support this suggestion.

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