In Vitro Analysis of Translational Rate and Accuracy with an Unmodified tRNA†

Kristi M. Harrington,‡ Irina A. Nazarenko,‡ Daniel B. Dix,§ Robert C. Thompson,§ and Olke C. Uhlenbeck*,‡

Departments of Chemistry and Biochemistry and Molecular, Cellular, and Development Biology, University of Colorado, Campus Box 215, Boulder, Colorado 80309-0215

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ABSTRACT: *Escherichia coli* $tRNA^{Phe}$ transcript lacking all the modified nucleosides was investigated in an *in vitro* translation system. To estimate the affinity of tRNA toward EF-Tu, K_d and k_{-1} were measured by the nuclease protection assay, and it was shown that the absence of modifications decreases ternary complex stability less than 2-fold. The activity of unmodified Phe-$tRNA^{Phe}$ on *E. coli* ribosomes was compared to modified Phe-$tRNA^{Phe}$ using the framework of the kinetic proofreading mechanism (Thompson & Dix, 1982) with both cognate and noncognate codons. Values of the individual rate constants in the elongation process showed that the modifications increased the accuracy of translation by (1) decreasing the rate of dipeptide synthesis and (2) increasing the rate of rejection with noncognate codons.

The function of the many posttranscriptional modifications on *Escherichia coli* tRNAs (Figure 1) is beginning to be understood. Mutants defective in individual modifying enzymes are generally viable but show decreased growth rates or are not able to compete in mixed cultures (Colby et al., 1976; Turnbough et al., 1979; Johnston et al., 1980; Kersten et al., 1981). As recently reviewed by Bjork (1992), a large number of genetic and biochemical experiments with tRNAs missing individual modifications indicate that a major role of tRNA modifications is to subtly affect the rate and accuracy of translation. An important goal for biochemists is to obtain an explanation for such "tuning" in terms of the translation mechanism. It is clear that this goal will be a difficult one. Not only are there many different modifications but each one could contribute a different amount to one or more steps in the translational mechanism. Furthermore, since modifications are rarely essential, the effect on an individual rate constant is often likely to be small so very sensitive assays will be needed.

Experiments with totally unmodified tRNAs prepared by *in vitro* transcription have been useful in evaluating the aggregate effect of all modifications in various biochemical processes. Unmodified tRNAs are usually, but not always, good substrates for aminoacyl-tRNA synthetases (Pallanck & Schulman, 1992; Giegé et al., 1993). Unmodified tRNA precursor are substrates for tRNA processing enzymes (McClain et al., 1987; Mattocia et al., 1988; Reyes & Abelson, 1988). Unmodified tRNAs have been shown to be active in unfractionated translation extracts (Samuelsson et al., 1988; Noren et al., 1989). In this work, we examine the activity of unmodified *E. coli* $tRNA^{Phe}$ for binding EF-Tu[1] and use a well-defined kinetic system (Thompson & Dix, 1982) to quantitatively evaluate the activity of an unmodified tRNA in the initial steps of peptide elongation. In this system, dipeptide synthesis is described by (1) an initial recognition step where reversible binding of the ternary complex to programmed ribosomes is followed by irreversible GTP hydrolysis and (2) a proofreading step where either dipeptide is made or the tRNA is rejected from the ribosome. The mechanism is therefore described as shown Scheme I. By determination of the individual rate constants for both the modified and unmodified $tRNA^{Phe}$'s, an explanation for how the modifications enhance translational accuracy can be proposed.

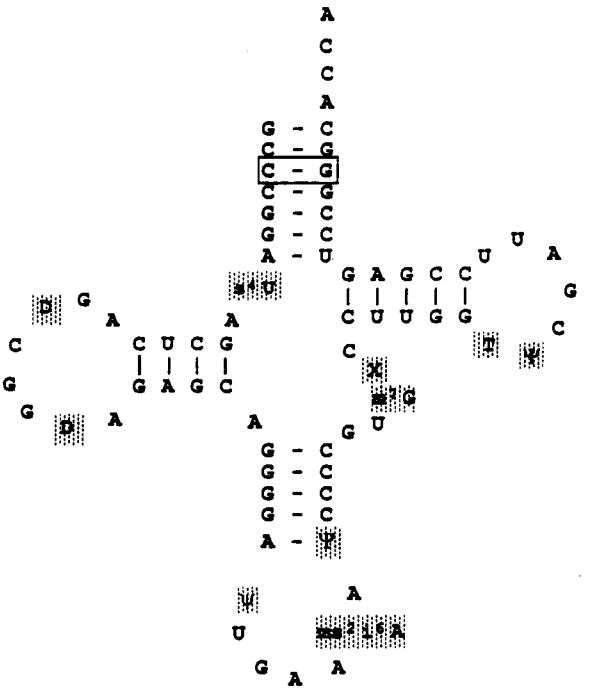

FIGURE 1: Sequence of *E. coli* $tRNA^{Phe}$ with the 10 modified nucleotides shaded. The C_3-G_{70} pair was changed to G_3-C_{70}.

MATERIALS AND METHODS

Unmodified *E. coli* $tRNA^{Phe}$ was prepared by transcription of plasmid DNA by T7 RNA polymerase (Sampson & Uhlenbeck, 1988). In order to prepare tRNAs with 5′-monophosphate termini, transcription was primed with 5′-GMP (Sampson & Uhlenbeck, 1988). tRNA transcripts were purified on denaturing polyacrylamide gels and stored in deionized water. Prior to aminoacylation, the tRNA was heated at 85 °C for 2 min and allowed to renature, and aminoacylation was carried out in reactions containing 1–5 μM tRNA and [^{3}H]phenylalanine (45–72 Ci/mmol, Amersham) as previously described (Peterson & Uhlenbeck, 1992).

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‡ Department of Chemistry and Biochemistry.

§ Department of Molecular, Cellular, and Developmental Biology.

[1] Abbreviations: EF-Tu, elongation factor Tu from *Escherichia coli*; Phe-$tRNA^{Phe}$, phenylalanyl-tRNA; fMet-tRNA, formylmethionyl-tRNA; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate).

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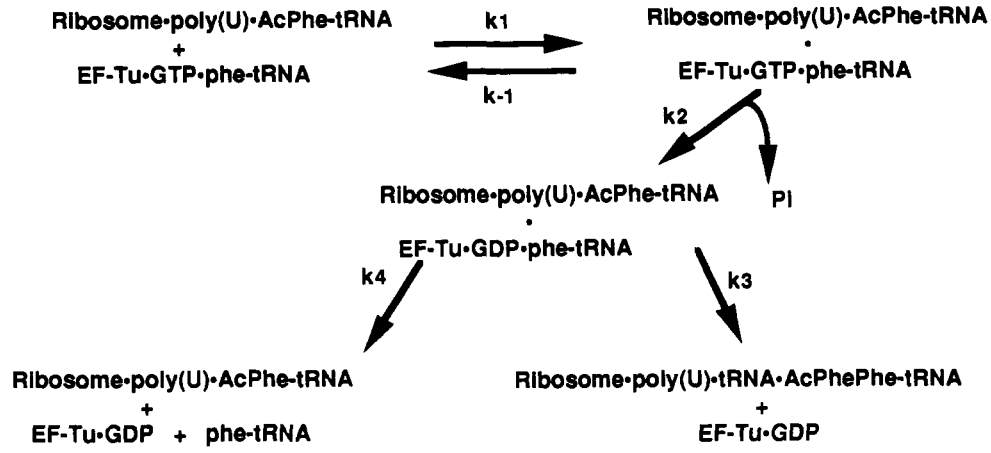
Scheme I



Phe-tRNA^{Phe} was purified by applying the reaction mixture to a 50-μL TSK Fractagel DEAE column equilibrated in 50 mM NaCl and 50 mM sodium acetate, pH 7.0, washing with 500 μL of 150 mM NaCl in the same buffer, and eluting with a 120-μL wash of 800 mM NaCl in the same buffer. The modified *E. coli* tRNA^{Phe} (Sigma) was purified and aminoacylated by the same protocols.

Two types of tRNA transcripts were made: with wild type and with C3-G70 to G3-C70 transversion in the acceptor stem, which permitted more efficient transcription by T7 polymerase (Milligan et al., 1987). Though all data presented in this paper were obtained with the G3-C70 transcript, controls showed no difference between this mutant and the wild-type transcript as far as ternary complex formation and cognate elongation reactions are concerned.

E. coli EF-Tu was prepared free of nucleotides as previously described (Thompson et al., 1981). The ternary complex was prepared with 0.5 μM EF-Tu, 0.5 μM [γ-^{32}P]GTP, and 0.5 μM [^{3}H]Phe-tRNA^{Phe} in either buffer A (50 mM Tris-HCl, pH 7.4, 150 mM NH_4Cl, 10 mM $MgCl_2$, 5 mM dithiothreitol) or buffer (50 mM TrishHCl, pH 7.2, 50 mM KCl, 25 mM NH_4Cl, 5 mM $MgCl_2$, 1 mM dithiothreitol). Dissociation rate constants and equilibrium dissociation constants of the ternary complex were measured by a pancreatic ribonuclease protection assay as described (Louie & Jurnak, 1985).

Single-turnover dipeptide synthesis experiments were performed in a rapid mixing apparatus at 5 °C (Eccleston et al., 1980). For each time point, 15 μL (30 pmol) of poly(U)-programmed 70S ribosome containing *N*-acetyl[^{14}C]Phe-tRNA^{Phe}, prepared as described (Thompson et al., 1981), was mixed with 5 μL (2.5 pmol) of the ternary complex containing modified or unmodified [^{3}H]Phe-tRNA^{Phe} (45–60 Ci/mmol) and [γ-^{32}P]GTP (40–60 Ci/mmol) in buffer B at 5 °C. The reaction was terminated by the addition of 10 μL of 0.5 M EDTA, and 12-μL aliquots were analyzed for GTP hydrolysis and *N*-acetyl[^{14}C]phenylalanyl[^{3}H]phenylalanine by previously described procedures (Eccleston et al., 1985; Thompson et al., 1986). Rate constants for GTP hydrolysis (k_{GTP}) and dipeptide formation (k_{PEP}) were determined by computer simulation of the reaction progress curves (Thompson et al., 1980), taking into account the concentrations of active ribosomes and the ternary complex.

The ternary complex with [^{35}S]GTPγS was prepared by incubating 90 pmol of GTPγS (30 Ci/mmol) with 90 pmol of EF-Tu and 90 pmol of [^{3}H]Phe-tRNA^{Phe} (45–60 Ci/mmol) in 90 μL of buffer B for 10 min at 5 °C (Thompson & Karim, 1982). To remove a background of ^{35}S counts (approximately 7%), the ternary complex was centrifuged through a 0.5-mL Bio-Gel P-6 column equilibrated with the same buffer. The rate of GTPγS hydrolysis was determined by mixing 30-μL aliquots of programmed ribosomes (36 pmol) and the radioactive ternary complex (30 pmol) at 5 °C and removing 5-μL aliquots at times between 0.17 and 35 min. The 5-μL aliquots were quenched with 20 μL of 0.5 M EDTA, and the amount of [^{35}S]thiophosphate was determined by a charcoal binding assay (Karim & Thompson, 1986). The rate of radioactive ternary complex dissociation from the ribosomes was determined by mixing 15 μL of the radioactive ternary complex (15 pmol) with 15 μL of programmed ribosomes (18 pmol) for 15 s at 5 °C and then adding 300 μL of the nonradioactive ternary complex (150 pmol). At times between 0.17 and 35 min, 27.5-μL aliquots were withdrawn, quenched with 20 μL of 0.5 M EDTA, and analyzed for [^{35}S]thiophosphate production.

Rate constants for GTP hydrolysis and proofreading ratios for the reaction with the near-cognate codon were determined as described previously (Thomas et al., 1988). The 52-nucleotide mRNA was designed to have a Shine–Delgarno sequence, an AUG initiation codon in the ribosomal P site, and a CUC codon in the A site. The mRNA was prepared by *in vitro* transcription with T7 RNA polymerase and purified by phenol and ether extraction, ethanol precipitation, and elution through a 0.5-mL Bio-Gel (Bio-Rad) P-6 column equilibrated in 20 mM KOAc, pH 4.5, containing 50 mM KCl. Ribosomes (180 pmol) were enzymatically initiated with initiation factors, GTP, and fMet-tRNA for 10 min at 37 °C in 120 μL of buffer B. To determine the rate of GTP hydrolysis, 157.5 pmol of programmed ribosomes was mixed with 35 pmol of the ternary complex in 150 μL of buffer B at 5 °C. At times between 0.17 and 110 min, 10-μL aliquots were withdrawn and quenched with 20 μL of 0.5 M EDTA, and the amount of GTP hydrolyzed was determined. To obtain proofreading ratios, 65 pmol of the ternary complex was incubated with 22.5 pmol of programmed ribosomes in 80 μL of buffer B for 2 h at 5 °C. Reactions were quenched with 50 μL of 0.5 M EDTA and analyzed for the amount of GTP hydrolyzed and dipeptide formed.

Each rate constant reported here is a result of at least three determinations, with the variation in absolute values up to 20%, depending on the preparations used. The comparative experiments with the modified and unmodified tRNAs were always performed side by side with the same preparation of EF-Tu and ribosomes. In this case, even a 20–30% difference between rate or equilibrium constants appeared to be highly reproducible.

Table I: Binding of Modified and Unmodified *E. coli* Phe-tRNA^{Phe} to *E. coli* EF-Tu·GTP

	buffer A			buffer B		
	K_d (M × 10^9)	k_{-1} (s^{-1})	k_1 (calcd) (M^{-1} s^{-1} × 10^{-5})	K_d (M × 10^9)	k_{-1} (s^{-1})	k_1 (calcd) (M^{-1} s^{-1} × 10^{-5})
modified	6	0.0016	2.6	55	0.008	1.5
unmodified	9	0.0028	3.1	80	0.014	1.7

RESULTS

Stability of the Ternary Complex. Initial attempts to form a complex between EF-Tu·GTP and unmofieid *E. coli* [^{3}H]-Phe-tRNA^{Phe} were unsuccessful due to the tendency of the unmodified (but not the modified) *E. coli* tRNA^{Phe} to adopt a denatured conformation (Peterson & Uhlenbeck, 1992). While the ternary complex could be efficiently formed when Tu·GTP was added directly to an aminoacylation reaction, the denaturants present in standard protocols for purifying aminoacyl-tRNA were sufficient to inactivate the unmodified Phe-tRNA^{Phe}. Since the high temperatures needed to renature Phe-tRNA^{Phe} would cause deacylation, an alternative protocol involving DEAE column chromatography had to be developed to purify the aminoacylated tRNA^{Phe} transcripts.

The ribonucleas protection assay (Louie & Jurnak, 1985) was used to determine the dissociation rate constant (k_{-1}) and the equilibrium dissociation constant (K_d) of both the modified and unmodified Phe-tRNA^{Phe} binding to *E. coli* EF-Tu·GTP. Since conditions optimal for ternary complex formation often differ from those used for the elongation reaction on the ribosomes, two different buffers were used. Buffer A was identical to that used previously (Louie & Jurnak, 1985) to characterize the stability of the ternary complex while buffer B was the one used (Thompson et al., 1981; Dix et al., 1986) to determine elemental rate constants of aa-tRNA binding. The values of k_{-1} and K_d determined for the modified *E. coli* Phe-tRNA^{Phe} in buffer A (Table I) are virtually identical to the values of 1.7×10^{-3} s^{-1} and 1.1 nM reported by Louie and Jurnak (1985) using the same method. Although the ribonuclease protection assay is indirect, very similar values of $k_{-1} = 0.7 \times 10^{-3}$ s^{-1} and $K_d = 0.4$ nM were recently determined directly using a fluorescent quenching assay (Ott et al., 1989). The lower ionic strength and lower Mg^{2+} concentration in buffer B result in an approximately 9-fold higher K_d for the ternary complex formed with modified Phe-tRNA^{Phe}. This is consistent with several previous studies which have shown that a high concentration of ammonium salts stabilizes the ternary complex (Antonsson & Leberman, 1982; Delaria et al., 1991).

The unmodified Phe-tRNA^{Phe} associates with EF-Tu·GTP only slightly less well than the modified Phe-tRNA^{Phe}: in both buffers, K_d is approximately 1.5-fold higher and the dissociation rate constant is correspondingly faster. Thus, the modifications stabilize the ternary complex, but the effect is very small.

Kinetic Properties of the Cognate Reaction. Ternary complexes contaiing [γ-^{32}P]GTP and either modified or unmodified [^{3}H]Phe-tRNA^{Phe} were prepared to assay their activity on *E. coli* ribosomes. Using a rapid mixing device (Eccleston et al., 1980), each ternary complex was injected into a vessel containing an excess of poly(U)-programmed *E. coli* ribosomes with *N*-acetyl-Phe-tRNA^{Phe} in the P site. At various times, the reaction was quenched, and the amounts of GTP hydrolyzed and peptide formed were determined. The reaction progress curves in Figure 2 show that the rates of GTP hydrolysis are very similar for the two ternary complexes,

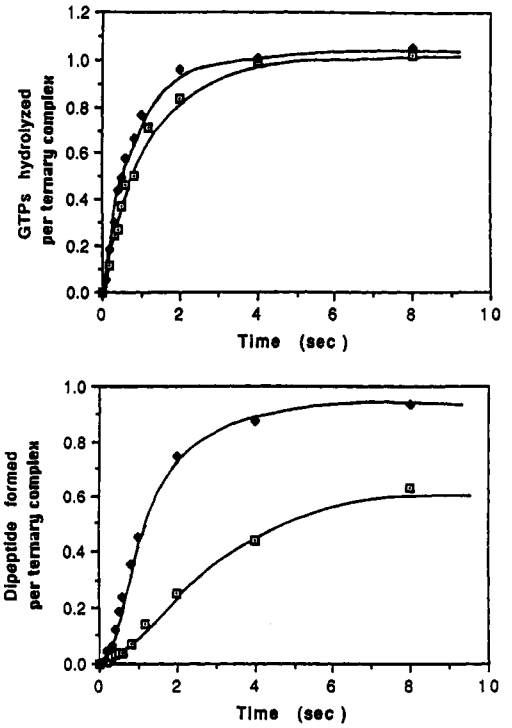


FIGURE 2: GTP hydrolysis (panel A, top) and dipeptide formation (panel B, bottom) of ternary complexes formed with modified (□) or unmodified (◆) *E. coli* Phe-tRNA^{Phe} on poly(U)-programmed *E. coli* ribosomes.

Table II: Rate Constants for the Reaction of Poly(U)-Programmed Ribosomes with the Ternary Complex, Containing GTP and Modified or Unmodified Phe-tRNA^{Phe}

	k_{GTP} (M^{-1} s^{-1})	k_{PEP} (s^{-1})
modified	5×10^6	0.65
unmodified	3.1×10^6	1.7

while there is a larger difference in the rates of dipeptide formation. Computer simulation of the reaction progress curves, taking into account the concentration of active ribosomes and ternary complexes, was performed in order to determine k_{GTP}, the apparent second-order rate constant for GTP hydrolysis, and k_{PEP}, the apparent first-order rate constant for peptide bond formation. The values of k_{GTP} determined in the simulation were 5.0×10^6 and 3.1×10^6 M^{-1} s^{-1} for the modified and unmodified Phe-tRNA^{Phe}'s, respectively. k_{PEP} values were 0.65 and 1.7 s^{-1} for the modified and unmodified Phe-tRNA^{Phe}'s, respectively (Table II).

For the reaction mechanism shown above, the complex rate constant $k_{GTP} = k_1k_2/(k_{-1} + k_2)$ and $k_{PEP} = k_3 + k_4$. Since the rate of chemical cleavage of GTP (k_2) on cognate codons is much faster than the rate of ternary complex dissociation from the ribosomes (k_{-1}) (Thompson et al., 1980; Thompson & Dix, 1982; Thompson & Karim, 1982), the expression of k_{GTP} simplifies to k_1. Thus, the 1.6-fold slower k_{GTP} for the unmodified tRNA is due to a decrease in the rate of ternary complex binding to the ribosomes. Furthermore, when the codon–anticodon match is correct, very little proofreading occurs (Thompson et al., 1980; Thompson & Dix, 1982), so k_4 is very small and k_{PEP} is entirely due to k_3, the rate of dipeptide formation. Thus, the nucleotide modifications *decrease* the rate of dipeptide bond formation by a factor of 2.6.

In order to determine the effect of nucleotide modifications on the initial binding of the ternary complex to ribosomes, it is necessary to use the ternary complex with GTPγS instead

Table III: Rate Constants for Ribosome Binding to Modified and Unmodified Phe-tRNA^{Phe} Ternary Complexes Prepared by GTPγS

	k_2 (s^{-1})	$k_2 + k_{-1}$ (s^{-1})	k_{-1} (s^{-1})
modified	0.0029	0.0089	0.0060
unmodified	0.0033	0.022	0.019

Table IV: Proofreading of Modified and Unmodified Phe-tRNA^{Phe} with a mRNA Containing a CUC Codon

	k_{GTP} (M^{-1} s^{-1})	R^a	k_3 (s^{-1})	k_4 (s^{-1})
modified	0.060×10^6	63	0.65	40
unmodified	0.025×10^6	7.3	1.7	11

[a] R = the proofreading ratio, the number of moles of GTP hydrolyzed per peptide bond formed.

of GTP. This results in a decrease in the rate of the chemical cleavage step on the ribosome from about 20 s^{-1} to 3×10^{-3} s^{-1} and thereby makes it rate-limiting in the reaction (Thompson et al., 1980; Thompson & Karim, 1982). As shown in Table III, the rates of [^{35}S]GTPγS cleavage measured for the modified and unmodified ternary complexes are virtually identical. Thus, as would be expected, the tRNA nucleotide modifications have no effect on the rate of the chemical cleavage of GTPγS (and presumably GTP) on the ribosome.

Substituting GTPγS for GTP in the ternary complexes allows the effect of nucleotide modifications on the rate of ternary complex dissociation from the ribosome (k_{-1}) to be determined in a chase experiment (Karim & Thompson, 1986). The [^{35}S]GTPγS ternary complex is allowed to bind to programmed ribosomes, followed by addition of a 10-fold excess of the unlabeled GTPγS ternary complex. Control experiments showed that the this excess of unlabeled ternary complex is enough to prevent the reassociation of ^{35}S ternary complex dissociated from the ribosomes. Under these conditions, the apparent rate of [^{35}S]thiophosphate production is equal to $k_2 + k_{-1}$, so k_{-1} can be calculated by subtracting k_2 determined in the previous experiment. The results of these experiments, shown in Table III, demonstrate that nucleotide modifications decrease the rate of ternary complex dissociation from the ribosome (k_{-1}) by a factor of 3.2.

Kinetic Properties of a Noncognate Reaction. Because individual nucleotide modifications are known to affect ribosomal proofreading (Bjork, 1992), it was important to examine the effect of modifications on k_4, the rate of rejection of a noncognate tRNA. This was done with the same modified and unmodified tRNA^{Phe} ternary complexes but using a synthetic mRNA with a Shine–Delgarno sequence, an AUG codon for the P site and a noncognate CUC codon for the A site (Thomas et al., 1988). Ribosomes with this mRNA were prepared with fMet-tRNA in the P site using initiation factors and then assayed in the normal fashion. As shown in Table IV, the modifications increase the rate of GTP hydrolyis on the noncognate codon by 2.4-fold. Since this is somewhat larger than the 1.6-fold increase in GTP hydrolysis caused by modifications with the cognate codons (Table II), we can conclude that the modifications cause a modest decrease in accuracy in the initial recognition step. Since k_{-1} is quite fast with respect to k_2 for noncognate codons, it is inappropriate to assume that $k_{GTP} = k_1$ as was done for the cognate reaction. Due to the relatively low amount of ternary complex bound to ribosomes with the noncognate codons, we have not attempted to further dissect k_{GTP}.

The effect of modifications on the proofreading step could be evaluated by determining the amount of GTP hydrolyzed and the relatively small amount of fMet-Phe formed with the noncognate codon at longer incubation times. These data allowed calculation of the proofreading ratio (R), the number of moles of GTP hydrolyzed per peptide bond formed (Table IV). The modifications increase R 8.6-fold and therefore substantially increase accuracy on a noncognate codon. The proofreading ratio can be related to elemental rate constants by the expression $R = (k_3 + k_4)/k_3$. Since k_3 does not depend on the nature of the codon–anticodon interaction (Thompson et al., 1986), we can use the values of k_3 for each tRNA in the cognate reaction (Table II) to calculate k_4. As shown in Table IV, the rate of aminoacyl-tRNA rejection from the ribosomes, k_4, is about 4-fold faster when the modifications are present.

DISCUSSION

The aggregate effect of the 10 nucleotide modifications in *E. coli* tRNA^{Phe} is to alter no less than four of the five elemental rate constants of the initial steps of protein synthesis. The modifications increase k_1, the rate of binding of the ternary complex to ribosomes, and k_4, the rate of rejection of noncognate tRNAs. The modifications decrease k_{-1} (the rate of dissociation of the ternary complex) and k_3 (the rate of dipeptide bond formation). Only k_2 (the rate of GTP hydrolysis) is unchanged. Although the magnitudes of these effects are small (less than 4-fold), the data are reproducible and a clear view of how the modifications increase accuracy by modulating rate constants emerges.

Interestingly, the modifications affect accuracy quite differently in the two steps of translation we have examined. The accuracy of the initial recognition step can be judged by comparing k_{GTP} on cognate and noncognate codons. Although modifications increase k_{GTP} in both cases, the effect is slightly greater with the noncognate codon. Thus, tRNA modifications actually cause a modest (1.5-fold) decrease in accuracy in the initial recognition step. On the basis of our data with the cognate codon, it appears that modifications cause tighter binding by both increasing the association rate (k_1) and decreasing the dissociation rate (k_{-1}). Although we could not dissect k_{GTP} for noncognate codons, it is resonable to expect that the same is true. Thus, by causing relatively tighter binding to noncognate codons, the modifications slightly reduce the accuracy of the initial recognition step.

In contrast, the effect of modifications on the proofreading step is to substantially increase accuracy. This is seen by examining the ratio of GTP hydrolyzed per peptide bond formed (R) for the cognate and noncognate reactions. For the cognate reaction, the ratio is close to 1 for both tRNAs, while for the noncognate reaction, R is 8.6-fold higher for the modified tRNA. When the individual rate constants are estimated, it is clear that the modifications increase the accuracy of proofreading in two independent ways. First, they decrease the rate of peptide bond formation (k_3) 2.6-fold, thereby allowing a longer time to reject incorrect codon–anticodon pairs. Second, they increase the rate of rejection of incorrect tRNAs (k_4) 3.6-fold. Since the increase in accuracy of the proofreading step is substantially greater than the decrease in accuracy of the initial binding step, the overall effect of tRNA modifications is, as expected, to substantially increase the accuracy of peptide synthesis.

Although we have not yet evaluated the role of tRNA modifications on other steps of translation, the effects seen here are large enough to conclude that tRNA modifications are crucial for translational accuracy. An 8.6-fold increase in misincorporation at each step of elongation will lead to a large increase in the number of incorrect proteins. For

example, if the normal misincorporation rate is assumed to be 10^{-3} (Kurland & Gallant, 1986), an 8.6-fold increase would result in one error per 116 amino acids. For a protein of 300 amino acids, less than 8% would then have the correct sequence. Since it has been estimated that about half of the misincorporation events lead to loss of activity (Miller, 1979), it is clear that the lack of all nucleotide modifications would have disastrous consequences for the cell.

The biochemical steps of peptide bond formation we have studied can be related to different structural states on the ribosome defined by chemical modification (Moazed & Noller, 1989). The initial binding of this ternay complex to the ribosome results in the formation of the T/A state where the 16S rRNA has a typical A-site protection pattern, but the tRNA site on the 50S subunit is not occupied. Since EF-Tu does not interact with the anticodon hairpin (Joshi et al., 1984) and the 30S subunit gives A-site protection with the anticodon hairpin alone (Rose et al., 1983), the aminoacyl-tRNA in the T/A state has its anticodon hairpin interacting with the 30S subunit and the remainder interacting with EF-Tu. As expected from this structural model, the rate constants of the initial recognition steps k_1 and k_{-1}, are sensitive to changes in the codon–anticodon interaction such as having a mismatched codon (Thompson & Dix, 1982) or even a wobble codon (Thomas et al., 1988). It is therefore likely that one or more of the modifications in the anticodon hairpin are responsible for the increase in k_1 and decrease in k_{-1} that we observe. A number of studies using partially unmodified tRNAs support this conclusion (Gefter & Russell, 1969; Wilson & Roe, 1989).

For a cognate codon, the hydrolysis of GTP is followed by the dissociation of EF-Tu·GDP from ribosomes. This permits the upper part of the aminoacyl-tRNA to enter the 50S subunit and give the 23S rRNA protection pattern typical of the A/A state (Moazed & Noller, 1989). Dipeptide bond formation then occurs rapidly. Thus, the measured rate constant of dipeptide formation, k_3, describes several steps. Experiments with binary complexes (Thompson et al., 1986) suggest that the release of EF-Tu·GDP from the ribosomes is the rate-limiting step in the process and thus determines k_3. Since the release of EF-Tu·GDP from ribosomes presumably also involves the dissociation of EF-Tu·GDP from tRNA and we have shown the modifications stabilize the ternary complex, it is not surprising that the $tRNA^{Phe}$ modifications decrease k_3 2.6-fold. The correlation between ternary complex stability and k_3 has been noted previously for two mutants of EF-Tu which form less stable ternary complexes (Ott et al., 1990) and increased k_3 values (data not shown). Since EF-Tu is known to interact with the upper portion of the tRNA tertiary structure (Boutorin et al., 1981; Joshi et al., 1984; Wikman et al., 1987), all seven of the modified nucleotides in that region (Figure 1) may contribute to the slower k_3 values. Alternatively, all the modifications may act together to prevent a global "loosening" of the unmodified tRNA structure (Sampson & Uhlenbeck, 1988; Hall et al., 1989).

For noncognate codons, the codon–anticodon interaction is sufficiently weak that the aminoacyl-tRNA falls off the ribosome after GTP hydrolysis at a rate k_4 that greatly exceeds k_3. In several respects, k_4 is believed to resemble k_{-1}: both rate constants are greatly increased when the codon–anticodon match is incorrect (Thompson & Dix, 1982). In this regard, it is quite interesting that tRNA modifications increase k_4 while decreasing k_{-1}. It is possible, of course that k_4 and/or k_{-1} reflect multiple-step reactions that have different rate-limiting steps. It is interesting, however, that the modifications have a very different effect on binding the Tu–tRNA complex to ribosomes before and after GTP hydrolysis.

The data presented here provide a starting point for the biochemical analysis of individual tRNA modifications. This can be done either by analyzing the biochemical properties of tRNAs missing single modifications (Gefter & Russell, 1969; Lo et al., 1982; Diaz et al., 1986; Wilson & Roe, 1989; Diaz & Ehrenberg, 1991) or by introducing a single modification into the unmodified tRNA using purified modifying enzymes (Sakamoto & Okada, 1985). Whichever method is chosen, it appears that the effects will be quite small, so accurate assays are needed. It will also be interesting to determine the effects of tRNA modification on the rate constants of individual steps of translocation, E-site binding, and tRNA release.

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