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The Interface between *Escherichia coli* Elongation Factor Tu and Aminoacyl-tRNA

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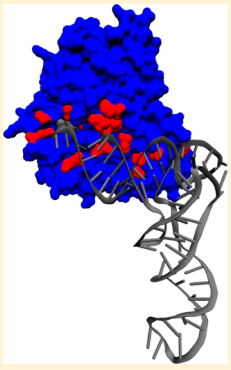
Supporting Information

ABSTRACT: Nineteen of the highly conserved residues of *Escherichia coli* (*E. coli*) Elongation factor Tu (EF-Tu) that form the binding interface with aa-tRNA were mutated to alanine to better understand how modifying the thermodynamic properties of EF-Tu−tRNA interaction can affect the decoding properties of the ribosome. Comparison of $\Delta\Delta G^{o}$ values for binding EF-Tu to aa-tRNA show that the majority of the interface residues stabilize the ternary complex and their thermodynamic contribution can depend on the tRNA species that is used. Experiments with a very tight binding mutation of $tRNA^{Tyr}$ indicate that interface amino acids distant from the tRNA mutation can contribute to the specificity. For nearly all of the mutations, the values of $\Delta\Delta G^{o}$ were identical to those previously determined at the orthologous positions of *Thermus thermophilus* (*T. thermophilus*) EF-Tu indicating that the thermodynamic properties of the interface were conserved between distantly related bacteria. Measurement of the rate of GTP hydrolysis on programmed ribosomes revealed that nearly all of the interface mutations were able to function in ribosomal decoding. The only interface mutation with greatly impaired GTPase activity was R223A which is the only one that also forms a direct contact with the ribosome. Finally, the ability of the EF-Tu interface mutants to destabilize the EF-Tu−aa-tRNA interaction on the ribosome after GTP hydrolysis were evaluated by their ability to suppress the hyperstable T1 $tRNA^{Tyr}$ variant where EF-Tu release is sufficiently slow to limit the rate of peptide bond formation (k_{pep}) . In general, interface mutations that destabilize EF-Tu binding are also able to stimulate k_{pep} of T1 $tRNA^{Tyr}$, suggesting that the thermodynamic properties of the EF-Tu−aa-tRNA interaction on the ribosome are quite similar to those found in the free ternary complex.



Bacterial elongation factor Tu (EF-Tu) is an important component of the multistep ribosomal decoding pathway that ensures rapid and accurate translation. In its GTP bound form, EF-Tu preferentially binds the aminoacylated form of each elongator tRNA (aa-tRNA), and the resulting ternary complex binds ribosomes with the correct codon−anticodon match at a rate substantially faster than free aa-tRNA. A cocrystal structure between *Escherichia coli* EF-Tu and aminoacyl-tRNA (aa-tRNA) shows an extensive interface that can be considered to have three parts.[1] First, 16 residues in Domains 1 and 3 of the protein form hydrogen bonds and ion pairs primarily with the helical backbone of the acceptor and T-stems of aa-tRNA. Second, 6 residues form a network of hydrogen bonds with the single-stranded NCCA terminus of aa-tRNA. Finally, 5 residues form a pocket large enough to accommodate the 20 different side chains of the esterified amino acid. Numerous experiments with EF-Tu mutants and modified aa-tRNAs have established that, while all three regions contribute to the affinity of aa-tRNA to EF-Tu, the relative amount that each region contributes depends strongly upon the identity of both the tRNA body and its esterified amino acid.[2] For each bacterial tRNA species, the sequence of three base pairs in the T-stem modulates the protein binding affinity in a manner that offsets the variable affinity of the esterified amino acid such that the overall affinity of all aa-tRNAs are nearly uniform.[3,4] This "thermodynamic compensation" mechanism appears to be conserved in bacteria[5] and has evolved to ensure that all cellular aa-tRNAs can compete equally for the common elongation factor.[6]

When the EF-Tu−aa-tRNA-GTP ternary complex binds encoded *E. coli* 70S ribosomes, a conformation change occurs resulting in structural changes in both the tRNA and the protein portions of the complex.[7−11] Nevertheless, the structural details of much of the interface are unchanged compared to the free complex. This includes nearly all the interactions between EF-Tu and the acceptor and T-stems as well as the general structure of the amino acid binding pocket. Only the positions of single-stranded residues C74 and C75 and the associated amino acids of EF-Tu are significantly changed upon ribosome binding. In addition to the similar structure of the interface, there is some data indicating that the thermodynamic properties of the interface between aa-tRNA and EF-Tu are retained after ribosome binding. Complexes made using mutations in the T-stem of *E. coli* $tRNA^{Val}$ that hyperstabilize binding to EF-Tu by differing amounts were found to bind *E. coli* ribosomes and hydrolyze GTP normally,

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but release from EF-Tu-GDP more slowly than normal and thereby have correspondingly slower rates of dipeptide bond formation.[4] Thus, the mechanism of thermodynamic compensation that evolved to ensure uniform aa-tRNA binding to EF-Tu may also be used to maintain a uniform rate of release from EF-Tu during ribosomal decoding. The affinity of EF-Tu to aa-tRNA has therefore been optimized by evolution in response to two opposing selective pressures. It must be tight enough to initially bind aa-tRNAs but not so tight that it cannot be disrupted during the ribosomal decoding process.

This contribution presents an initial characterization of the biochemical properties of 19 point mutants of *E. coli* EF-Tu amino acids that make up much of the interface with aa-tRNA. By measuring their affinities to two different aa-tRNAs, an overview of how this protein achieves its affinity and specificity for its substrate is obtained. In addition, by comparing these data with a similar set of experiments previously performed with EF-Tu from the distantly related thermophile, *Thermus thermophilus*,[12] a detailed thermodynamic comparison of how two orthologous, but structurally somewhat different proteins bind the same aa-tRNA. Finally, the activity of these mutant proteins on *E. coli* ribosomes, shows how the stability of this protein−aa-tRNA interface has been optimized for translation.

MATERIALS AND METHODS

EF-Tu Mutagenesis, Expression, and Purification. The expression vector pROEX HTb (Invitrogen) contained a TEV protease cleavage site between the 6x His tag, and *E. coli* EF-Tu gene was provided by Rachel Green.[13] After TEV cleavage, the N-terminal amino acid sequence of the recombinant protein becomes GGASK, which differs from the native MSK sequence by three residues. All 19 EF-Tu mutants were generated using QuikChange XL site-directed mutagenesis kit (Agilent Technologies). The sequence of each mutant was confirmed by DNA sequencing, and they were expressed in BL21-Gold (DE3) cells (Agilent Technologies).[12] Cells were grown in LB media supplemented with 100 μg/mL ampicillin at 37 °C with continuous shaking. They were induced after the optical density at 600 nm reached 0.5 using 1 mM IPTG and harvested after 3 h of induction. For all of the mutations, analysis of lysates on SDS gels showed an amount of the slightly longer recombinant protein that exceeded the amount of the abundant native EF-Tu. For R223A mutant, protein expression levels were at least 3-fold less than the other mutations.

The method for affinity purification of the mutant proteins was somewhat altered from the one previously used[12] in order to minimize aggregation of EF-Tu and thereby improve TEV cleavage efficiency and avoid contamination by native EF-Tu. After cell lysis, 6xHis-tagged EF-Tu was bound to Ni-NTA column in 50 mM HEPES at pH 7.5, 1 M KCl, 10 mM $MgCl_2$, 20% glycerol, 10 mM imidazole, 5 mM β-mercaptoethanol (BME), 100 μM GDP, and 0.5% Tween-20. The proteins were eluted from the column with 50 mM HEPES pH 7.5, 500 mM KCl, 10 mM $MgCl_2$, 500 mM imidazole, 20% glycerol, 5 mM β-mercaptoethanol, and 100 μM GDP. After elution of the EF-Tu from the Ni-NTA resin, the His-tag was proteolytically removed from EF-Tu in the elution buffer by reacting His-tagged TEV with His-tagged EF-Tu at a 1:50 molar ratio for about 20 h at 4 °C. The glycerol in the buffers minimized aggregation,[14] and the high concentrations of imidazole and KCl were crucial to improve the accessibility of TEV protease to the TEV cleavage site and thereby increase the yield of the cleavage reaction. Following proteolysis, proteins were dialyzed into elution buffer that lacked imidazole and contained 200 mM KCl. TEV protease cleaved EF-Tu was separated from uncleaved protein by passing the protease cleavage reaction again over a Ni-NTA column and collecting the flow through fraction. The amount of aggregation of purified EF-Tu mutants was determined using an analytical Superose 6 column (GE Healthcare), and over 80% of the protein was monomeric. The final purification used a Superdex 75 column equilibrated with 50 mM HEPES pH 7.5, 200 mM KCl, 10 mM $MgCl_2$, 10% glycerol, 5 mM BME, and 100 μM GDP. The proteins were stored in 50 mM HEPES pH 7.5, 200 mM KCl, 10 mM $MgCl_2$, 50% glycerol, 5 mM DL-Dithiothreitol (DTT), and 100 μM GDP. The concentration of each mutant protein was determined using the Pierce 660 nm Protein Assay.[15]

Preparation of RNA. Yeast $tRNA^{Phe}$ was purchased from Sigma-Aldrich and resuspended and stored in 1× TE buffer. *E. coli* $tRNA^{Tyr}$ and the hyperstabilized T1 $tRNA^{Tyr}$ were prepared by *in vitro* transcription from DNA templates prepared by primer extension of two annealed synthetic oligonucleotides as previously described except that 1 U/μL RNasin was included in the transcription reaction.[3,12,16] The transcription reaction products were purified on a 10% denaturating polyacrylamide gel. After ethanol precipitation, each transcript was resuspended and stored in 1× TE buffer. tRNAs were aminoacylated with [^{3}H]-phenylalanine (MP Biomedicals) or [^{3}H]-tyrosine (PerkinElmer) using Ni-NTA purified yeast phenylalanyl synthetase or *E. coli* tyrosyl synthetase.[17] Aminoacylated tRNAs were resuspended and stored in 5 mM NaOAc buffer at pH 5 after phenol/chloroform extraction and ethanol precipitation steps.

^{32}P-labeled T1 $tRNA^{Tyr}$ was prepared to measure rates of dipeptide bond formation of EF-Tu variants.[3] T1 $tRNA^{Tyr}$ was labeled with [α-^{32}P]-ATP at the 3′ -CCA end and aminoacylated with tyrosine using published methods.[17,18]

Ribosomes from *E. coli* MRE600 cells were purified as described by Powers and Noller[19] to be used in GTP hydrolysis and dipeptide bond formation assays. In order to program ribosomes, tightly coupled 70S ribosomes were heat activated prior to mRNA and fMet-$tRNA^{fMet}$ addition.[18,20]

A chemically synthesized RNA oligomer with the sequence GGC AAG GAG GUA AAA AUG UAC GCA CGU (IDT) was resuspended in TE buffer and used as the mRNA for the GTPase and k_{pep} experiments.

Dissociation Rate Constant (k_{off}) Measurements. One μM of EF-Tu, 0.1−0.4 μM of [^{3}H]-Phe-$tRNA^{Phe}$ or [^{3}H]-Tyr-$tRNA^{Tyr}$, and 10 μM GTP were mixed to form ternary complex in 50 mM HEPES pH 7, 30 mM KCl, 70 mM NH_4Cl, 10 mM $MgCl_2$, 10 μM GTP, 3 mM phosphoenolpyruvate, 50 μg/mL of pyruvate kinase, and 1 mM DTT. Dissociation rate constants were measured using ribonuclease protection assay[21] in a 96-well microtiter plate format on ice.[3,12,17,22] For a more accurate error analysis, k_{off} measurements were repeated at least three times and the standard deviation is reported for each mutant.

GTP Hydrolysis Rate Measurement. Ternary complex was formed by mixing 0.4 μM EF-Tu with 1.2 μM Tyr-$tRNA^{Tyr}$ or 1.2 μM Tyr-T1 Tyr-$tRNA^{Tyr}$, and 50 μM γ-^{32}P-GTP in 50 mM HEPES pH 7, 30 mM KCl, 70 mM NH_4Cl, 10 mM $MgCl_2$, 10 μM GTP, 3 mM phosphoenolpyruvate, 50 μg/mL of pyruvate kinase, and 1 mM DTT according to published protocols.[4,13,23,24] Heat activated ternary complex solution was passed through two Bio-Spin 6 columns (BIO-RAD) to remove excess [γ-^{32}P]-GTP. At each specific time point, equal volumes of 0.4 μM ternary complex and 2 μM programmed ribosomes

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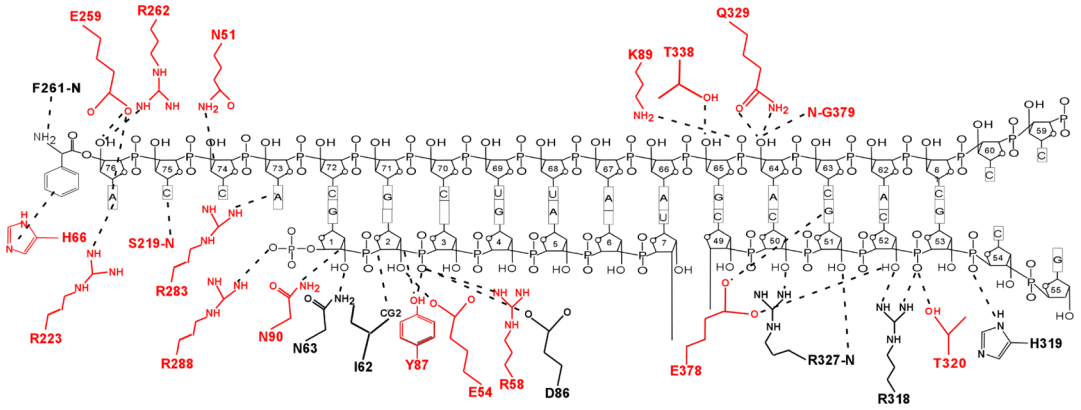

Figure 1. Diagram of the interface between *E. coli* EF-Tu and yeast Phe-tRNA^{Phe}. All main chain and side chain residues of EF-Tu within 5 Å of the aa-tRNA in the X-ray crystal structure are shown (pdb 1OB2). Dotted lines indicate the closest functional groups that could potentially form stabilizing interactions. The residues marked in red were mutated in this study.

Table 1. Dissociation Rate Constants (k_{off})[a] and Corresponding Differences in Free Energies ($\Delta\Delta G^{o}$)[b] with Respect to WT EF-Tu for Complexes Containing Mutant Proteins and Different aa-tRNAs

	yeast Phe-tRNA[c]		*E. coli* Tyr-tRNA[c]		T1 Tyr-tRNA[c]	
	$k_{off} \times 10^4$ (s^{-1})	$\Delta\Delta G^{o}$ (kcal/mol)	$k_{off} \times 10^4$ (s^{-1})	$\Delta\Delta G^{o}$ (kcal/mol)	$k_{off} \times 10^4$ (s^{-1})	$\Delta\Delta G^{o}$ (kcal/mol)
WT	8.7 ± 2.5	0.0 ± 0.2	19 ± 4	0.0 ± 0.2	0.16[d]	0.0
E54A	5.7 ± 0.9	−0.2 ± 0.2	21 ± 2	0.1 ± 0.1	a[e]	–
E55A	34 ± 18	0.7 ± 0.3	41 ± 4	0.4 ± 0.1	a	–
E259A	16 ± 1	0.3 ± 0.2	36 ± 6	0.4 ± 0.1	11 ± 2	2.3 ± 0.1
E378A	32 ± 11	0.7 ± 0.2	14 ± 2	−0.2 ± 0.1	4.2 ± 1.6	1.8 ± 0.2
H66A	115 ± 7	1.4 ± 0.2	342 ± 100	1.6 ± 0.2	10 ± 1	2.3 ± 0.2
K89A	12 ± 4	0.2 ± 0.2	30 ± 8	0.3 ± 0.2	a	–
N51A	10 ± 1	0.1 ± 0.2	16 ± 2	−0.1 ± 0.1	a	–
N90A	20 ± 9	0.5 ± 0.3	18 ± 2	−0.0 ± 0.1	a	–
Q329A	79 ± 19	1.2 ± 0.2	105 ± 27	0.9 ± 0.2	17 ± 7	2.6 ± 0.2
R58A	24 ± 12	0.6 ± 0.3	39 ± 4	0.4 ± 0.1	a	–
R223A	4.3 ± 2.1	−0.4 ± 0.3	7.5 ± 1.0	−0.5 ± 0.1	a	–
R262A	38 ± 3	0.8 ± 0.2	121 ± 45	1.0 ± 0.2	3.5 ± 0.8	1.7 ± 0.1
R377A	215 ± 35	1.8 ± 0.2	437 ± 140	1.7 ± 0.2	41 ± 5	3.0 ± 0.1
R283A	23 ± 3	0.5 ± 0.2	58 ± 5	0.6 ± 0.1	a	–
R288A	39 ± 7	0.8 ± 0.2	223 ± 85	1.3 ± 0.2	a	–
S219A	11 ± 3	0.1 ± 0.2	25 ± 7	0.2 ± 0.2	a	–
T320A	47 ± 1	0.9 ± 0.2	74 ± 23	0.7 ± 0.2	5.3 ± 0.8	1.9 ± 0.1
T338A	20 ± 1	0.5 ± 0.2	21 ± 5	0.1 ± 0.2	a	–
Y87F	11 ± 3	0.1 ± 0.2	25 ± 1	0.2 ± 0.1	a	–

[a] k_{off} is determined in 50 mM HEPES pH 7, 30 mM KCl, 70 mM NH_4Cl, 10 mM $MgCl_2$, 10 μM GTP, 3 mM phosphoenolpyruvate, 50 μg/mL of pyruvate kinase, and 1 mM DTT at 0 °C. [b] Standard deviations are calculated from at least three independent determinations. [c] $\Delta\Delta G^{o} = -RT \ln(K_{Dwt}/K_{Dmut})$, $K_D = k_{off}/(1.1 \times 10^5\ M^{-1}\ s^{-1})$ [d] Estimated value for k_{off}.[4] [e] a = value too slow to measure ($k_{off} < 2.4 \times 10^4$/s).

were mixed and quenched with 40% formic acid using KinTek quench flow instrument at 20 °C. The hydrolyzed GTP fraction was calculated by analyzing the samples on TLC plates coated with PEI cellulose. The reaction rates were calculated by plotting the fraction of GTP hydrolyzed vs time and fitting the data to a single exponential curve.[13]

Peptide Bond Formation Rate (k_{pep}) Measurement. After heat activation, 2 μM ribosomes were combined with 6 μM mRNA and 4 μM fMet-tRNA^{fMet} in order to program ribosomes for k_{pep} measurements.[18] Ternary complex was formed by mixing 1 μM of GTP bound EF-Tu with 40 nM of [3′ ^{32}P]-Tyr-T1 tRNA^{Tyr}. The slow kinetics of Tyr-T1 tRNA^{Tyr} allowed us to perform the experiments on benchtop in a 96-well microtiter plate at room temperature. For each time point, 4 μL of ternary complex was reacted with 4 μL of programmed ribosomes complex in 50 mM HEPES pH 7, 30 mM KCl, 70 mM NH_4Cl, 10 mM $MgCl_2$, 10 μM GTP, 3 mM phosphoenolpyruvate, 50 μg/mL of pyruvate kinase, and 1 mM DTT, and the reaction was quenched using 24 μL of 5 mM NaOAc at pH 5 and 100 μM EDTA buffer. The products were digested using Nuclease P-1 from *Penicillium citrinum* (Sigma-Aldrich) and separated on Flexible TLC Plates coated with PEI-cellulose (Sigma-Aldrich) in 70% isopropanol and 1.5 M HCl. The k_{pep} was determined from the slope of the fraction of dipeptide produced vs time plot that was fitted to a single exponential equation.[4,18,24] The k_{pep} values for each mutant were repeated at least three times, and the standard deviation was calculated.

RESULTS

Purification and aa-tRNA Binding Properties of Mutant *E. coli* EF-Tu Proteins. The crystal structure of *E.*

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coli EF-Tu GTP bound to yeast Phe-tRNA^{Phe} (pdb 1OB2) was used to choose 19 residues along the protein−RNA interface that were close enough to potentially alter the binding affinity to aa-tRNA. The mutated residues and the closest tRNA functional groups are highlighted in a diagram of the interface in Figure 1. All of the mutated residues are highly conserved among bacterial EF-Tus.[25] In every case, the native amino acid was mutated to a smaller side chain (usually alanine), thereby removing the potential stabilizing contact without introducing steric clash. A few of these *E. coli* EF-Tu mutant proteins had been biochemically characterized previously[26−30] and the aa-tRNA binding properties of many of the orthologous mutations in *T. thermophilus* EF-Tu have been determined.[12]

All 19 mutations were inserted into an expression vector containing the EF-Tu gene with an upstream 6x His followed by the TEV protease cleavage site.[13] As discussed in the Materials and Methods, the buffers used in the purification protocol were altered from those previously used[12] to minimize protein aggregation. The known ability of *E. coli* EF-Tu to aggregate[14,31,32] is a particular problem for the isolation of EF-Tu mutants expressed in *E. coli* because of the potential for the abundant untagged endogenous cellular protein to associate with the tagged mutant protein and thereby be retained on the Ni-NTA resin and contaminate the final preparation of mutant protein. To evaluate the efficacy of our revised protocol, we used it to purify the H84A mutant protein. Since H84 coordinates the scissile phosphate in the GTPase reaction,[9,10,33] its mutation reduces its GTPase activity by many orders of magnitude.[14,34−36] As others have found,[34,36] we observed that our H84A protein preparation binds GTP and aa-tRNA very similarly to the wild-type protein, but the complex produces undetectable amounts of dipeptide product on ribosomes (figure in Supporting Information). On the basis of the detection limits of the dipeptide assay, the H84A protein contains less than 1% contaminating wild-type protein, thereby validating our revised purification protocol.

To evaluate how well the mutant EF-Tu proteins bound aa-tRNA, 1 μM of each protein was activated with GTP, mixed with 0.1−0.2 μM yeast Phe-tRNA^{Phe}, and the dissociation rate measured using a ribonuclease protection assay at 0 °C.[21] The high concentration and excess of protein over aa-tRNA favors complex formation even if the protein is only partially active or has a weak K_D. For all mutant proteins, full RNase protection of Phe-tRNA^{Phe} was observed and a k_{off} value determined (Table 1). The $\Delta\Delta G^o$ of each mutation compared to the wild-type protein was also calculated using the value of k_{on} = 0.11 μM^{-1} s^{-1} determined previously.[3,12] Of the 19 interface mutations tested, one bound EF-Tu somewhat more tightly than the wild-type protein ($\Delta\Delta G^o$ = −0.4 kcal/mol). Six showed little or no effect ($\Delta\Delta G^o$ = −0.3 to +0.3 kcal/mol), nine showed a clear reduction in binding affinity ($\Delta\Delta G^o$ = +0.3 to +1.0 kcal/mol), and three had a large effect on Phe-tRNA^{Phe} affinity ($\Delta\Delta G^o$ = >1.0 kcal/mol).

The fact that the majority of the interface mutations caused a change in the binding affinity supports the notion that the X-ray crystal structure depicts the interface formed in solution. However, not all of the mutations lead to a significant $\Delta\Delta G^o$, and there is no clear correlation between the "quality" of the crystallographic contact and the value of $\Delta\Delta G^o$. For example, even though the amino nitrogen of K89 is within 2.9 Å of the OP1 of G65 of tRNA and thus clearly forms a charged hydrogen bond, the $\Delta\Delta G^o$ of the K89A mutation is very small. In contrast, a very large $\Delta\Delta G^o$ is seen for the E55A mutation although the carboxylate is more than 6 Å from the nearest tRNA residue. Such inconsistencies may simply reflect differences between the crystal and solution structures due to a rearrangement caused by the crystal lattice contacts. Additionally, the much higher ionic strength used in crystallization compared to the solution binding experiments could alter the occupancy of ions essential for protein folding or tRNA binding. However, several other explanations are possible. Mutation of a contacting amino acid to alanine not only removes a putative stabilizing contact, but also introduces a space in the interface that is occupied by one or more solvent molecules. Thus, the net effect of an alanine mutation on ΔG^o will depend on the identity of the original amino acid as well as the energetics of sequestering the solvent molecules. Another potentially important contributor to ΔG^o is any change in the structure of free EF-Tu that occurs upon aa-tRNA binding. For example, R58 makes an ion pair with E54 in the free form of EF-Tu (pdb 1EXM) that is disrupted when it binds the OP1 of G3 of the aa-tRNA.[37] Thus, the $\Delta\Delta G^o$ of the R58A mutation reflects the net free energy change of disrupting both these interactions. Finally, since a crystal structure of a complex emphasizes the static features of the interaction characterized by ΔH and not the changes in the dynamics of the two components characterized by ΔS, it is not always a good predictor of ΔG^o. Indeed, a wide range of $\Delta\Delta G^o$ values and apparent inconsistencies with the crystal structure of the complex have been observed when other protein−protein[38−40] or protein−nucleic acid[41] interfaces are "scanned" with alanine mutations.

In order to evaluate how *E. coli* EF-Tu achieves specificity for other aa-tRNAs, k_{off} values for each of the 19 mutant EF-Tu proteins bound to *E. coli* Tyr-tRNA^{Tyr} were determined (Table 1). On the basis of experiments with misacylated tRNAs, it is known that the tRNA^{Tyr} body binds EF-Tu about 1.1 kcal/mol weaker than the tRNA^{Phe} body,[42] so the two tRNAs are expected to respond differently to the panel of EF-Tu mutations. The data in Table 1 show that, within the accuracy of the measurements, 15 of the interface mutations have the same values of $\Delta\Delta G^o$ for the two tRNAs. However, there are four mutations at two different interface locations where the $\Delta\Delta G^o$ values differ. First, E378A and T338A both have a substantial (0.5 kcal/mol) effect on Phe-tRNA^{Phe} affinity and almost no (0 and 0.1 kcal/mol) effect on Tyr-tRNA^{Tyr} affinity. Since E378 and T338 both make contacts with the T-stem where most of the RNA sequence specificity occurs, these data can be understood by the different T-stem sequences of the two tRNAs. E378 stabilizes binding to tRNAs by forming a hydrogen bond to the amino group of guanine at either position of the 51−63 base pair.[43] Since tRNA^{Phe} has a G51-C63 pair while tRNA^{Tyr} contains an A51-U63 pair and therefore lacks an amino group, only tRNA^{Phe} can be destabilized by the E378A mutation. The hydroxyl group of T338 forms a hydrogen bond with the 2′ hydroxyl of ribose 65, and this interaction contributes to the sequence specificity at the 49−65 base pair. Since the C49-G65 pair present in tRNA^{Phe} contributes about 0.5 kcal/mol more to binding than the G49-C65 pair present in tRNA^{Tyr},[3] the T338A mutation has a much larger effect on tRNA^{Phe}.

The second region of the interface where the panel of EF-Tu mutants appears to respond differently with the two tRNAs involves the adjacent contacts of R288 and N90 with the 5′ terminal G residue present in in both tRNAs. The guanidinium group R288 forms an ion pair with the 5′ terminal phosphate of

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tRNA, while the amide group of N90 interacts with the 2′ O of ribose 1 (Figure 1). The R288A mutation destabilizes $tRNA^{Tyr}$ by 1.3 kcal/mol and $tRNA^{Phe}$ by 0.8 kcal/mol. In contrast, the N90A mutation destabilizes $tRNA^{Phe}$ by 0.5 kcal/mol and has no effect on $tRNA^{Tyr}$ binding. While no ternary complex structure of EF-Tu with $tRNA^{Tyr}$ is available, a simple explanation of the data is that the positions of the 5′ ends of the two tRNAs differ slightly such that $tRNA^{Tyr}$ only contacts R288, while $tRNA^{Phe}$ contacts both amino acids. This could be the result of a small change in the helical pitch resulting from the two different acceptor T-helix sequences. Alternatively, the stronger T-stem contacts that form with $tRNA^{Phe}$ could slightly change the position of the 5′ end of the molecule.

Introducing the T-stem base pairs of $tRNA^{Gly}$, one of the tightest binding tRNA bodies, into $tRNA^{Tyr}$, one of the weakest tRNA bodies, creates the T1 $tRNA^{Tyr}$ mutation. When aminoacylated with the tight-binding amino acid Tyr, the resulting Tyr-T1 $tRNA^{Tyr}$ binds exceptionally tightly to *E. coli* EF-Tu.[3,4] Under normal conditions, the dissociation rate is so slow that it is difficult to obtain accurately. However, estimates based upon sequence[4] or by extrapolation from k_{off} values obtained at different conditions suggest that that the k_{off} for the Tyr-T1 $tRNA^{Tyr}$ is from 60- to 120-fold slower than wild-type Tyr-$tRNA^{Tyr}$, corresponding to a $\Delta\Delta G^{o}$ from 2.2 to 2.6 kcal/mol.[3,4] Since T1 $tRNA^{Tyr}$ is such an extreme example of a substrate for EF-Tu, it was of interest to determine how it bound to the panel of EF-Tu mutants. In addition, these data help to interpret experiments that assay the function of T1 $tRNA^{Tyr}$ on the ribosome discussed in the next section.

While all 19 EF-Tu mutations were able to fully protect Tyr-T1 $tRNA^{Tyr}$ from ribonuclease digestion, 12 of the complexes were so stable that no significant digestion occurred after 60 min of incubation, preventing a reliable determination of k_{off}. However, while dissociation rates for the remaining seven mutants were also quite slow, they were sufficiently fast to obtain reproducible k_{off} values (Table 1). $\Delta\Delta G^{o}$ values for these mutations are also reported, but since they had to be calculated using an estimated value for the wild type protein, they are less reliable than those obtained with Tyr-$tRNA^{Tyr}$. A comparison of the binding properties of the panel of EF-Tu mutants to Tyr-$tRNA^{Tyr}$ and Tyr-T1 $tRNA^{Tyr}$ reveals that the two tRNAs interact with EF-Tu in a very similar manner. Thus, with three exceptions, the EF-Tu mutations that greatly weaken $tRNA^{Tyr}$ binding ($\Delta\Delta G^{o}$ = +0.7 kcal/mol or more) were found to bind weakly enough to T1 $tRNA^{Tyr}$ that a k_{off} could be obtained, while the EF-Tu mutations that have a modest or no effect on $tRNA^{Tyr}$ binding ($\Delta\Delta G^{o}$ = +0.6 kcal/mol or less) were found to bind so tightly to T1 $tRNA^{Tyr}$ that no k_{off} could be obtained. Of the three exceptions, the fact that E378A substantially weakens T1 $tRNA^{Tyr}$ and not $tRNA^{Tyr}$ is expected because the T1 mutation introduces a G51-C63 pair that forms an additional stabilizing contact with E378. The second exception, R288A, has a large (+1.3 kcal/mol) effect in $tRNA^{Tyr}$ binding, but is sufficiently smaller to not be detectable with T1 $tRNA^{Tyr}$. As discussed above, this reduced contribution of R288 was also seen for $tRNA^{Phe}$ and may be the result of a slight change in the position of the 5′ phosphate that results from the tighter contact in the T-stem. The final exception is that E259A has a very large effect on binding to T1 $tRNA^{Tyr}$ but only a modest effect on binding to $tRNA^{Tyr}$ or $tRNA^{Phe}$. Taken together, the data indicate that, while this unusually stable $tRNA^{Tyr}$ mutation interacts with EF-Tu in a manner that is generally similar to that of $tRNA^{Tyr}$, the additional binding energy derives not only from a new contact made in the region of the mutation but also from changes in the strengths of contacts at distal sites.

Activity of EF-Tu Interface Mutations on *E. coli* Ribosomes. Although the structure of the interface between EF-Tu and aa-tRNA is largely unchanged when the ternary complex binds ribosomes and undergoes the conformational change that activates GTP hydrolysis,[8,10] it is possible that the energetics of this thermodynamically tuned interface may in some way participate in this first phase of the decoding reaction. Thus, it was important to evaluate whether the interface mutants could affect the rate of GTP hydrolysis on the ribosome. For an initial screen, ternary complexes containing Tyr-$tRNA^{Tyr}$ and [γ-^{32}P] GTP was prepared for 17 of the mutants and the rate of GTP hydrolysis measured using ribosomes with fMet-$tRNA^{fMet}$ in the P site. Several of the mutants were also assayed using Tyr-T1 $tRNA^{Tyr}$. Since the experiments used 0.4 μM EF-Tu and a small excess of aa-tRNA, most of the protein will be in ternary complex for wild type EF-Tu but significant amounts of free protein will be present for the weaker binding mutants. The ternary complexes were then mixed with 1 μM ribosomes, and time points taken to give a rate of GTP hydrolysis. These reaction conditions are subsaturating for ribosome binding in order to maximize the sensitivity of the assay for changes in activity due to the mutations.

GTPase progress curves for wild-type and four mutant proteins are shown in Figure 2, and the calculated values of k_{obs}

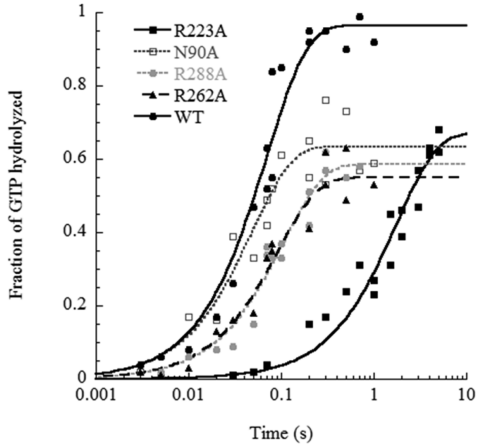

Figure 2. Time courses of ribosome-catalyzed GTP hydrolysis of ternary complexes containing Tyr-$tRNA^{Tyr}$ and the indicated EF-Tu mutations. Experiments were performed in 50 mM HEPES pH 7, 30 mM KCl, 70 mM NH_4Cl, 10 mM $MgCl_2$, 10 μM GTP, 3 mM phosphoenolpyruvate, 50 μg/mL of pyruvate kinase, and 1 mM DTT at 20 °C. Lines for each mutant indicate the best fit to a single binding equilibrium adjusted to an extent and a k_{obs}. Values for all mutants are given in Table 2

are summarized in Table 2. With one exception, all of the mutations show robust GTPase activities with reaction rates and extents within 2-fold of those of the wild-type protein. Since the GTPase assays in this screen were only performed under a single set of reaction conditions, it is not possible to interpret the differences in rate and extent. For example in Figure 2, the low reaction extents for the R262A and R288A mutations are likely to be the result of incomplete formation of complex due to their weak aa-tRNA binding, while the low extent observed for the N90A mutation, which binds aa-tRNA normally, may result from incomplete GTP binding[12] or a

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Table 2. Rates of GTP Hydrolysis (k_{obs}) of EF-Tu Mutants at Subsaturating Encoded Ribosomes[a]

	Tyr-tRNA^{Tyr}[b]	T1 Tyr-tRNA^{Tyr}[b]
	k_{obs} (s^{-1})	k_{obs} (s^{-1})
WT	14 ± 4	12 ± 4
E54A	11 ± 4	–
E55A	13 ± 2	–
E259A	14 ± 3	8.0 ± 4.4
E378A	8.7 ± 4.8	–
H66A	a[c]	–
K89A	16 ± 2	7.2 ± 3.4
N51A	11 ± 5	–
N90A	21 ± 4	22 ± 3
Q329A	17 ± 4	–
R223A	0.8 ± 0.2	–
R262A	13 ± 5	–
R283A	12 ± 3	13 ± 4
R288A	14 ± 2	17 ± 1
S219A	12 ± 3	–
T320A	15 ± 4	–
T338A	9.5 ± 1.6	–
Y87F	13 ± 2	–

[a]Experiments are performed using 1 μM encoded ribosomes in 50 mM HEPES pH 7, 30 mM KCl, 70 mM NH_4Cl, 10 mM $MgCl_2$, 10 μM GTP, 3 mM phosphoenolpyruvate, 50 μg/mL of pyruvate kinase, and 1 mM DTT at 20 °C. [b]Standard deviations are calculated from at least three independent determinations. [c]a = insufficient ternary complex formed.

partially denatured protein. Nevertheless, all but one of the mutations are effective GTPases and therefore do not significantly interfere with the conformational change and subsequent GTP hydrolysis associated with the decoding reaction. The five mutations assayed with Tyr-T1-tRNA^{Tyr} also showed rates of GTP hydrolysis comparable to those of the wild-type protein.

R223A is the single EF-Tu interface mutation that shows a dramatically reduced rate of GTP hydrolysis. As shown in Figure 2, while the reaction extent is substantial, its k_{obs} is at least 15-fold slower than that of the wild type despite the fact that it efficiently forms ternary complex and dissociates from Tyr-tRNA^{Tyr} similarly to wild-type protein. While additional experiments will be required to understand the underlying reason for this low activity, it is interesting that R223 is the only interface amino acid that also contacts the ribosome. In the ternary complex R223 interacts with E259 prior to ribosome binding, whereas after ribosome binding R223 contacts U368 of 23S rRNA.[9,10,44] Thus, the most straightforward explanations for the reduced GTPase of the R223A ternary complex are that it either binds the ribosome poorly or is unable to maintain the reoriented conformation needed for GTP hydrolysis.

Since the second phase of decoding involves disruption of the interface between EF-Tu and aa-tRNA, accommodation of the aa-tRNA into the A site, and the subsequent formation of the dipeptide bond, it seemed possible that EF-Tu interface mutants could alter k_{pep}, the rate of dipeptide bond formation. However, many experiments[45−47] suggest that the observed value of k_{pep} reflects the rate of accommodation, the conformational change that accompanies the movement of aa-tRNA into the A site, and not the rates of earlier steps such as release of Pi[48] or release of aa-tRNA from EF-Tu[45−47] which are significantly faster. In support of this, mutations in the T-stem of tRNA^{Val} that dissociate faster from EF-Tu than wild-type tRNA^{Val} had the same values of k_{pep} as that of wild-type tRNA^{Val},[4] consistent with the idea that k_{pep} is limited by the subsequent slower accommodation step in both cases. Since all but one of the EF-Tu interface mutants either weaken EF-Tu binding or have no effect, they would also not be expected to affect k_{pep}. Indeed, k_{pep} values reported for H66A and E378A EF-Tu were found to be identical to cthose of the wild-type protein[4,24] and N90A, E259A, and H66A have activities comparable to those of wild-type protein using a polyU directed translation assay.[27,29]

In order to compare the thermodynamic properties of the interface between EF-Tu and aa-tRNA on the ribosome with those of the free complex, we made use of the T1 tRNA^{Tyr} mutant, which binds free EF-Tu at least 60-fold tighter than tRNA^{Tyr}. Since this tRNA showed a 57-fold slower k_{pep}, it was proposed that in this case k_{pep} reflected release from EF-Tu and not accommodation.[4] Subsequent experiments measuring the release of a fluorescent derivative of EF-Tu from a hyperstabilized tRNA^{Val} support this proposal.[46] Thus, an EF-Tu interface mutation that destabilized its interaction with Tyr-T1 tRNA^{Tyr} during decoding would be expected to increase its rate of release and thereby show a faster k_{pep}. Similarly, interface mutations that had little or no effect on binding Tyr-T1 tRNA^{Tyr} would continue to show the very slow k_{pep} observed for wild type EF-Tu. Therefore, by measuring the k_{pep} values of Tyr-T1 tRNA^{Tyr} for the panel of interface mutations, a view of the thermodynamic behavior of the ternary complex during the aa-tRNA release step of decoding can be obtained.

Ternary complexes formed between 1 μM of each of the 19 EF-Tu interface mutations and 40 nM [3′-^{32}P]Tyr-T1 tRNA^{Tyr} were mixed with 2 μM ribosomes containing fMet-tRNA^{fMet} in the P site and the rates of formation of dipeptide determined using a TLC assay.[18] Since k_{pep} is so slow for wild-type EF-Tu, manual pipetting could be used for time points between 5 and 200 s. As shown for the wild-type EF-Tu in Figure 3, a nearly complete progress curve could be obtained and a k_{pep} calculated of 0.087/s. This value is much slower than the k_{pep} of about 1/s that we typically obtain for native Tyr-tRNA^{Tyr},[4] therefore indicating that release from EF-Tu is limiting k_{pep} with the

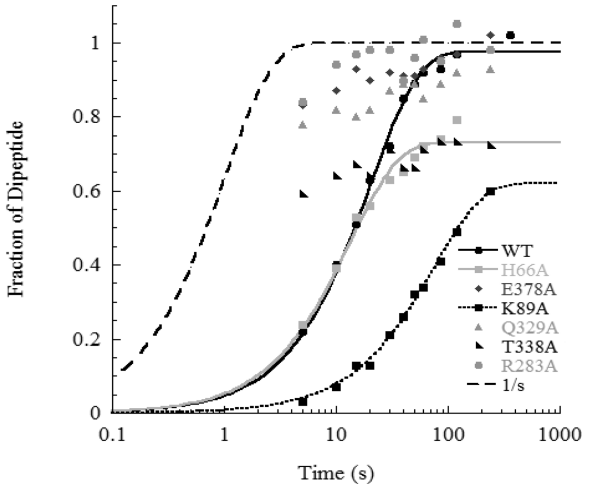

Figure 3. Time courses of fMet-Tyr formation using ternary complexes containing Tyr-T1 tRNA^{Tyr} and the indicated EF-Tu mutations. Experimentally determined extents are normalized relative to WT values. Rates are given in Table 3

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hyperstable T1 tRNA^{Tyr}. Figure 3 shows several examples of EF-Tu interface mutants which also show k_{pep} > 0.25/s and thus are also rate limited by product release. Also shown are several other EF-Tu mutants that were substantially complete by the 5 s time point and thus are able to partially or fully suppress the effect of the hyperstable T1 tRNA^{Tyr}. Data for 18 of the 19 mutants are summarized in Table 3. The R223A mutation did not give appreciable amounts of dipeptide, consistent with the observation that it showed very poor GTPase activity.

Table 3. Rates of fMet-Tyr Formation with EF-Tu Mutants Complexed with Tyr-T1 tRNA^{Tyr}[a]

	peptide bond formation[b]
	k_{pep} (s^{-1})
WT	0.055 ± 0.026
K89A	0.015 ± 0.003
E54A	0.096 ± 0.011
E55A	0.052 ± 0.016
E259A	>0.25
E378A	>0.25
H66A	0.073 ± 0.008
N51A	0.045 ± 0.004
N90A	0.17 ± 0.05
Q329A	>0.25
R58A	0.099 ± 0.006
R223A	0.002 ± 0.001
R262A	>0.25
R283A	>0.25
R288A	>0.25
R377A	>0.25
S219A	0.084 ± 0.046
T320A	>0.25
T338A	>0.25
Y87F	0.056 ± 0.034

[a]Experiments performed in 50 mM HEPES pH 7, 30 mM KCl, 70 mM NH_4Cl, 10 mM $MgCl_2$, 10 μM GTP, 3 mM phosphoenolpyruvate, 50 μg/mL of pyruvate kinase, and 1 mM DTT at 20 °C. [b]Standard deviations are calculated for at least 3 independent determinations.

For the majority of the interface mutants, the k_{pep} data with Tyr-T1 tRNA^{Tyr} are very consistent with the corresponding k_{off} data summarized in Table 1. Eight of the mutations which show k_{pep} values slower than 0.25/s and thus appear to be rate limiting for release from EF-Tu are all mutations that have little or no effect ($\Delta\Delta G^{o}$ < 0.5 kcal/mol) on aa-tRNA binding and therefore behave similarly to the wild-type protein on the ribosome. In addition, eight mutations that show very fast k_{pep} values and thus suppress the effect of the hyperstable Tyr-T1 tRNA^{Tyr} are all proteins that substantially weaken binding to aa-tRNA ($\Delta\Delta G^{o}$ > 0.6 kcal/mol). This striking correlation between the k_{pep} and k_{off} data for 16 of the EF-Tu mutations suggests that the thermodynamic "profile" of the interface mutants on the ribosome after GTP hydrolysis is very similar to the one observed for the formation of the free ternary complex. A similar correlation between k_{pep} and k_{off} was observed for a series of tRNA^{Val} mutations that hyperstabilize binding to EF-Tu to differing extents.[4]

However, there are two clear exceptions to this correlation between the k_{pep} and k_{off} data. First, the H66A mutation, which destabilizes binding to Tyr-T1 tRNA^{Tyr} by more than 2 kcal/mol, is unlike the other eight strongly destabilizing mutations in that it shows a slow k_{pep} value of 0.073/s similar to that of wild-type EF-Tu. Since H66 stacks on the esterified amino acid, the H66A mutation is known to strongly destabilize aa-tRNAs esterified with aromatic amino acids,[24] so the effect on the k_{off} of Tyr-T1 tRNA^{Tyr} was expected. However, the position of H66 is unchanged in the ribosome-bound complex after GTP hydrolysis[10,44] so the stacking interaction would be expected to still be stabilizing. Furthermore, the H66A mutation showed a normal k_{pep} value when assayed with Ala-tRNA^{Ala},[24] indicating that the mutation does not hinder the ribosome from performing peptide bond formation in some unknown manner.

A second mutation in which k_{pep} and k_{off} do not correlate is T338A, which has little effect on Tyr-tRNA^{Tyr} binding and binds Tyr-T1 tRNA^{Tyr} tighter than our limit of measurement. Nevertheless, T338A shows a fast (<0.25/s) k_{pep} value with Tyr-T1 tRNA^{Tyr}, suggesting that the complex on the ribosome is destabilized sufficiently so that release from EF-Tu is not rate limiting. Interestingly, in the ternary complex T338 contacts the T-stem directly and also helps position adjacent amino acids which form thermodynamically important contacts. However, the position of T338 is unchanged in the ribosome-bound complex, so it is unclear why the complex is less stable.

DISCUSSION

The X-ray crystal structure of the *E. coli* EF-Tu−GTP-Phe-tRNA^{Phe} complex allowed the identification of alanine mutations at 13 different positions within the protein−nucleic acid interface that significantly altered the stability of the complex and 6 that had little or no effect. Although the many possible ways that a mutation can affect the observed binding affinity prohibited a straightforward interpretation the $\Delta\Delta G^{o}$ values in terms of the crystal structure of the complex, it is clear that the stability of the complex is the result of interactions that span the entire interface from the esterified amino acid to the T-loop 53 Å away. Since the formation and disruption of this complex is essential to the mechanism of ribosomal decoding, this panel of mutations is a useful tool for understanding several features of this mechanism.

Thirteen of the aa-tRNA interface mutants in *E. coli* EF-Tu studied here were previously made at the orthologous positions of *T. thermophilus* EF-Tu,[49] permitting comparison of the aa-tRNA binding properties of the same mutations in EF-Tus from two distantly related bacteria. Although the *T. thermophilus* protein is 13 residues longer and the two proteins differ in sequence at 105 additional positions,[25] 23 of the 26 interface residues are identical, including all 13 of the sites of common mutations. Since crystal structures of yeast Phe-tRNA^{Phe} bound to both proteins are available (pdb 1OB2 and 1TTT),[33] this aa-tRNA was chosen to make the comparison. The calculated $\Delta\Delta G^{o}$ value between wild type and mutant protein at each of the 13 positions in the two systems are compared in Figure 4. It is striking that for 10 of the mutations the $\Delta\Delta G^{o}$ values in the two systems were the same within the error of measurement despite the many sequence differences between the two proteins and the fact that one is from a mesophile and the other from a thermophile. Only three mutations showed significantly different $\Delta\Delta G^{o}$ values in the two systems. The E259A mutation has a much larger deleterious effect on Phe-tRNA^{Phe} binding when made in *T. thermophilus* EF-Tu than when made in *E. coli* EF-Tu. In contrast, the R58A and R288A mutations affect Phe-tRNA^{Phe} binding to the *E. coli* EF-Tu much more than when they are

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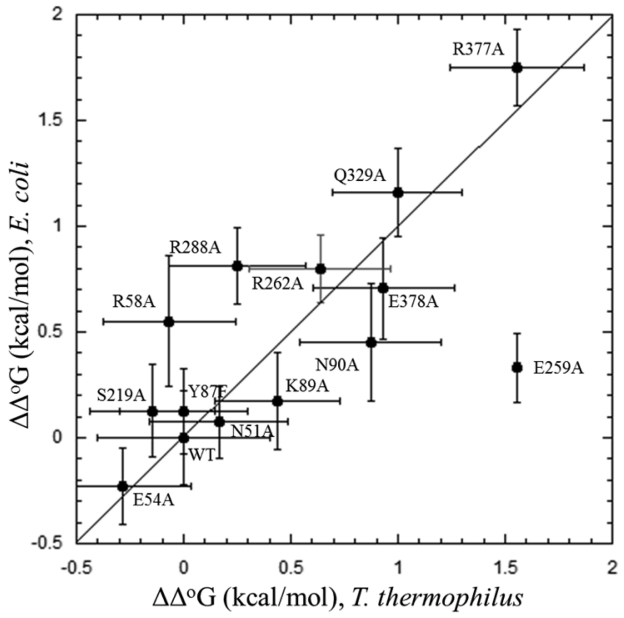

Figure 4. Comparison of yeast Phe-tRNA^{Phe} binding to mutations made in *E. coli* EF-Tu (Table 1) with identical mutations made at orthologous sites in *T. thermophilus* EF-Tu.[49] *E. coli* numbering is used. Experiments performed in 50 mM HEPES pH 7, 30 mM KCl, 70 mM NH_4Cl, 10 mM $MgCl_2$, 10 μM GTP, 3 mM phosphoenolpyruvate, 50 μg/mL of pyruvate kinase, and 1 mM DTT at 0 °C for *E. coli* EF-Tu. $\Delta\Delta G^o$ values for each mutation were calculated relative to those of corresponding wild-type proteins (ΔG^o = 10.1 ± 0.3 for *E. coli* and 9.8 ± 0.2 for *T. thermophilus*). Since the complex was not stable enough, $\Delta\Delta G^o$ value displays the lower limit for *T. thermophilus* E259A, and the actual value is larger than 1.8.[49]

introduced into the *T. thermophilus* protein. Although the crystal structures of the two complexes reveal several small differences in the positions of the interface amino acids, it is unlikely that they explain the differing response to the E259, R58, and R288 mutations as the positions of these three side chains are very similar in the two structures. It is interesting that the *E. coli* E259A and R288A mutations, which showed different $\Delta\Delta G^o$ values than in *T. thermophilus*, also showed different $\Delta\Delta G^o$ values when Phe-tRNA^{Phe} and Tyr-tRNA^{Tyr} were compared. As is further discussed below, this may reflect a slightly different mode of aa-tRNA binding that is shared by all EF-Tu proteins. If this is the case, the thermodynamic properties of binding aa-tRNAs to the distantly related *E. coli* and *T. thermophilus* EF-Tu proteins are nearly identical, suggesting that they are identical for all bacterial species. This conclusion agrees with our recent analysis of the T-stem sequences of bacterial tRNAs which concludes that there is a "code" for the sequence-specific recognition of tRNA that is the same in all bacteria.[5]

Since the free energy of binding of different tRNA bodies to EF-Tu varies considerably, it could be anticipated that tRNAs would respond differently to the panel of 19 interface mutations. This expectation was born out when the $\Delta\Delta G^o$ values comparing each mutant to wild type were measured using Phe-tRNA^{Phe}, a tighter binding tRNA, and Tyr-tRNA^{Tyr}, a weaker binding tRNA. While 15 of the mutants showed the same $\Delta\Delta G^o$ values for the two aa-tRNAs, there were four "specificity" amino acids where the $\Delta\Delta G^o$ values differed between the two tRNAs. Two of these residues are involved in contacts with the T-stem where the sequences of the two tRNAs were different. Since tRNA mutagenesis studies have established that the T-stem sequence defines EF-Tu binding specificity,[3] it is not surprising that mutations in the contacting amino acids will differentially effect binding to the two tRNAs. More interestingly, the other two "specificity" amino acids participated in contacts at the 5′ end of tRNA where the sequences of the two tRNAs were identical. Although the underlying reason for this source of specificity is not yet understood, it is clear that the two aa-tRNAs tested interact with EF-Tu in a thermodynamically distinct manner using contacts throughout the interface and are not limited to the regions where the two tRNA sequences differ. It seems likely that when the binding of other aa-tRNAs to the panel of interface mutants are tested, other unique specificity amino acids will be identified. Indeed, when the panel of interface mutants of *T. thermophilus* EF-Tu were tested with four different aa-tRNAs, five "specificity" amino acids were identified, including the expected residues that contact the T-stem as well as the orthologs of T320 and R262 which were not identified in this work.[49] Interestingly, the orthologs of R288A and N90A identified here were not found to be "specificity" amino acids in the *T. thermophilus* experiments.

The protein mutagenesis experiments therefore give a more nuanced view of how EF-Tu achieves its broad range of binding affinities for different tRNA bodies than was previously obtained from tRNA mutagenesis experiments. An analysis of nearly 100 base pair mutations in three different tRNA backgrounds led to the view that nearly all the variable affinity was due to the sequence of three or sometimes four base pairs in the T-stem, while binding to the remainder of the tRNA was essentially the same for all tRNAs.[3] Although the protein mutagenesis experiments confirm that the amino acids that contact the T-stem contribute strongly to the specificity, they also indicate that other interface amino acids that contribute to the binding affinity may also contribute to the specificity. Thus, rather than the specificity being solely due to the interactions in the T-stem, it can also result from variable thermodynamic contributions of contacts made throughout the entire interface that occur when the protein contacts the slightly different geometries of the acceptor and T-helices of the different tRNAs. In most cases these thermodynamic differences will be the result of a small change in the relative positions of the atoms in the same contact. However, it is possible that some tRNAs will actually form different contacts with the protein. Indeed, although they have not been thermodynamically analyzed, several of the proposed contacts present in the cocrystal structure of *E. coli* Cys-tRNA^{Cys} with *Thermus aquaticus* (*T. aquaticus*) EF-Tu (PDB 1B23)[50] are different from those seen with yeast Phe-tRNA^{Phe} with the same protein (PDB 1OB2). Thus, in order to bind the many different tRNA bodies, the interface residues of EF-Tu appear to have evolved to be thermodynamically and possibly structurally "malleable" so that the appropriate free energy of binding can be achieved.

Although the data were somewhat limited, $\Delta\Delta G^o$ values for the panel of interface mutations that compared the stabilizing T1 mutation of *E. coli* tRNA^{Tyr} and wild-type tRNA^{Tyr} revealed several "specificity" amino acids that contacted the acceptor stem, well away from the T1 mutation. This indicates that a sequence change made in one part of tRNA can modify the contribution of contacts at quite distal sites, suggesting that the thermodynamics of the entire interface was subtly altered. A similar phenomenon was observed when the affinities of certain mutations of MS2 coat protein were determined using a panel

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of phosphorothioate derivatives of the RNA hairpin binding site.[41] For several of the mutant proteins, the effect of substituting a phosphate with a phosphorothioate on the binding affinity was quite different from what had been measured with the wild-type protein. Thus, the protein mutation had subtly altered the strength of contacts elsewhere on protein−nucleic acid interface.

Two assays were chosen to provide a preliminary view of how the interface mutations affect the multistep ribosomal decoding pathway. The first measured the rate of GTP hydrolysis under conditions where the ternary complex was at least partially formed, but at subsaturating concentrations of encoded ribosomes. While the extents of reaction varied, all but one of the mutations showed a rate of GTP hydrolysis quite similar to that of wild-type protein. The sole exception (R223A) can be attributed to weaker binding of the ternary complex to the ribosome, resulting from disruption of a contact that forms between them. Thus, the energetics of the interface do not contribute to the rate-limiting GTPase activation step that precedes GTP hydrolysis.[45] This is consistent with observation that the structure of the interface just prior to GTP hydrolysis (pdb 2XQD)[10] is nearly the same as its structure in the ternary complex (pdb 1OB2 and 1TTT).[33] The conformational changes associated with GTPase activation are known to involve the nearby switch I loop and switch II region of EF-Tu, and mutation of these residues can substantially reduce the GTPase activity.[10,34−37,51−54]

The second assay to evaluate the interface mutations on the ribosome employed a variant of $tRNA^{Tyr}$ that when bound to wild-type EF-Tu is so stable that its release from EF-Tu GDP is sufficiently slow that, unlike normal aa-tRNAs, it limits the rate of peptide bond formation.[4] Thus, this synthetic system could evaluate the contribution of each interface residue to the interaction between aa-tRNA and EF-Tu-GDP on the ribosome by measuring how well they are able to destabilize the hyperstabilized complex and thereby restore the rate of peptide bond formation to its normal value. It was found that the effects on k_{pep} for all but two of the mutations were completely consistent with their stabilities in the free ternary complex. Thus, the thermodynamic properties of the interface between aa-tRNA and EF-Tu in the free ternary complex largely resemble those on the ribosome when aa-tRNA is released from EF-Tu after GTP hydrolysis. While this result is consistent with the observation that the positions of virtually all the interface residues in the ternary complex remain unchanged after GTP hydrolysis on the ribosome (pdb 2XQD, 2WRQ, and 2Y14), it is nonetheless striking considering that both the aa-tRNA and EF-Tu are making multiple additional contacts with the ribosome that apparently do not change the energetics of their interface.

The fact that both the structure and the thermodynamic properties of the EF-Tu-aa-tRNA interaction are largely unchanged as EF-Tu goes through its catalytic cycle may be a consequence of the complex evolutionary pressure that the interface is subjected to. For the formation of the ternary complex, the interface residues evolved so that they could interact with each tRNA body in a slightly different manner to give a characteristic binding affinity that complements the binding affinity of the cognate esterified amino acid and thereby ensures that the affinities of all aa-tRNAs are roughly uniform. After the ternary complex binds the ribosome and GTP is hydrolyzed, tRNA dissociates from the protein and accommodates into the A site at a rate that exceeds 7/s at 20 °C,[45] a rate that is substantially faster than the 3/s dissociation rate of EF-Tu from the ribosome under similar conditions.[45] Although the interface is substantially weaker, it appears likely that the balance between the affinities of the tRNA body and the esterified amino acid need to be maintained to ensure all aa-tRNAs release from the protein with a rate fast enough so as not to limit the rate of peptide bond formation. If the relative thermodynamic properties of the release step were to differ substantially from those of the binding step, certain aa-tRNAs could potentially show anomalously low incorporation rates. Thus, the similarity of binding affinities might be a universal mechanism to adjust incorporation rates of individual amino acid into the proteins. Indeed, genome-wide analysis of ribosome pausing sites in *E. coli* revealed little evidence of rate-limiting elongation kinetics dependent upon aa-tRNA identity.[55]

In the context of the above argument, it is intriguing that our screen revealed two mutations, H66A and T338A that affect the binding and release steps quite differently. H66, which strongly contributes to the specificity of the esterified amino acid in the binding step, appears not to contribute to stabilizing the complex prior to release. In contrast, T338 contributes little to aa-tRNA binding, but appears to be important in maintaining the stability of the complex after GTP hydrolysis. These two exceptions may be an indication of an additional step in either the binding or release pathways. Alternatively they may be some anomaly of either the type of mutant made or the particular assays employed in our screen. A more thorough analysis of the enzymatic properties of these mutant proteins is clearly a priority.

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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