Understanding the Sequence Specificity of tRNA Binding to EF-Tu using tRNA Mutagenesis[†]

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Abstract

Measuring the binding affinities of 42 single base pair mutants in the acceptor and T-stems of *S. cerevisiae* tRNA[Phe] to *T. thermophilus* EF-Tu revealed that most of the specificity for tRNA occurs at the 49-65, 50-64, and 51-63 base pairs. Introducing the same mutations at the three positions into *E. coli* tRNA[Leu]$_{CAG}$ resulted in similar changes in the binding affinity. Swapping the three pairs from several *E. coli* tRNAs into yeast tRNA[Phe] resulted in chimeras with EF-Tu binding affinities similar to the donor tRNA. Finally, analysis of double and triple base pair mutants of tRNA[Phe] shows that the thermodynamic contributions at the three sites are additive, permitting reasonably accurate prediction of the EF-Tu binding affinity for all *E. coli* tRNAs. Thus, it appears that the thermodynamic contributions of three base pairs in the T-stem primarily account for tRNA binding specificity to EF-Tu.

Keywords

EF-Tu; tRNA Mutagenesis; tRNA Protein Interaction; T-Stem; Thermodynamic compensation

Introduction

The bacterial translation factor EF-Tu is a GTPase that ensures rapid binding and accurate decoding of aminoacyl-tRNAs (aa-tRNAs) on the ribosome.[1,2] The GTP-bound form of the protein binds to its structurally diverse set of aa-tRNA substrates with nearly uniform affinity by combining quite variable thermodynamic contributions of the esterified amino acid and tRNA body.[3,4] Experiments with misacylated tRNAs established that the different esterified amino acids contribute up to 2.5 kcal/mol,[5] and the corresponding cognate tRNA bodies contribute up to 3.6 kcal/mol to the overall binding affinity.[6] Therefore, the sequence of each tRNA has evolved to possess a binding affinity that compensates for its cognate amino acid such that a uniform affinity for each aa-tRNA is achieved.

How EF-Tu achieves sequence specificity is not completely understood. Crystal structures of *T. aquaticus* EF-Tu complexed with either yeast (*S. cerevisiae*) Phe-tRNA[Phe] or *E. coli* Cys-tRNA[Cys] indicate that the protein interacts with the acceptor (A) and TΨC (T) helices of both tRNAs, but with a slightly different pattern of hydrogen bonds for the two tRNA bodies.[7; 8]

This suggests that each tRNA may bind the protein in a thermodynamically distinct manner, although this possibility has not been tested. Alanine scanning mutagenesis has identified 14 amino acids in the interface between EF-Tu and tRNA that contribute towards the affinity to yeast Phe-tRNA[Phe].[9] Furthermore, at least three of the EF-Tu residues interact with other tRNAs in a sequence-dependent manner.[10] Limited tRNA mutagenesis experiments have shown that the 51-63 and 49-65 base pairs in the T helix strongly contribute to the specific recognition of several tRNAs,[11,12] but it is not known whether additional sites are used by other tRNAs. In addition, it remains unclear how the individual recognition elements combine to achieve the overall tRNA specificity.

In this paper, individual base pairs in the A and T-stems of yeast tRNA[Phe] and *E. coli* tRNA[Leu]$_{CAG}$ are systematically mutated, and the effect on EF-Tu affinity is determined with the goal of obtaining a comprehensive view of the sequence-specific binding of EF-Tu to all bacterial tRNAs. The thermodynamic additivity of potential recognition contacts is assessed, and a recognition model is tested by introducing the proposed recognition elements from different tRNA backgrounds into yeast tRNA[Phe].

Results

Because of the available high resolution X-ray co-crystal structure and extensive biochemical data, mutations were initially made in yeast tRNA[Phe] (Figure 1). With the exception of the universally conserved G53-C61 base pair that is essential for proper folding of the T loop,[13] all twelve base pairs in the A and T helices were mutated even though the existing co-crystal structures only show contacts with nine of them. This was done because it is not known whether all tRNAs interact with EF-Tu in an identical manner. Other tRNA sequences could potentially form stabilizing hydrogen bonds or destabilizing steric clashes not seen in the two available structures. In addition, non-contacting residues could influence binding affinity indirectly by affecting the structure or the dynamics of the free tRNA.

Analysis of more than 5000 non-redundant tRNA sequences representing about 200 fully sequenced bacterial genomes from tRNA datamart (http://trnamart.uoregon.edu/) revealed that while the A and T helices predominantly contain Watson-Crick base pairs, a significant fraction of GU, AC, and other non Watson-Crick base pairs is present (Table 1). In order to limit the total number of mutants, base pair changes at a given position were only selected if they were present in greater than 5% of all tRNAs or greater than 25% of tRNAs in any individual amino acid acceptor family. This selection ensured that all commonly used sequence elements were tested, including those used in only a few tRNA types. For example, the G3-U70 pair that is present only in 5% of all tRNAs was included because it is present in all tRNA[Ala] species, and this could potentially affect the binding of this tRNA to EF-Tu. tRNA mutations at position 1-72 that initiated with either pyrimidine were not included to simplify synthesis but may be important for some tRNAs (see below).

Forty-two mutants of yeast tRNA[Phe] were prepared by *in vitro* transcription and were aminoacylated with [^{3}H]-Phe using yeast PheRS, which does not contain identity elements in the A and T helices. Unmodified tRNA transcripts could be used since unmodified tRNA[Phe] binds just as well as fully modified tRNA[Phe] [14]. Each [^{3}H]-Phe-tRNA was bound to *T. thermophilus* EF-Tu●GTP, and a ribonuclease protection assay[15,16] was used to determine a k_{off} for each mutant in Buffer A at 0°C (Figure 2A). For those mutations where k_{off} was either faster than 0.01 s^{-1} or slower than 0.0005 s^{-1}, k_{off} values were determined at several lower or higher NH$_4$Cl concentrations, and the value at 0.5 M was extrapolated from an ln k_{off} versus ln [NH$_4$Cl] plot (Figure 2B). To calculate K_d values from the k_{off} values, the value of k_{on} for EF-Tu binding to several tRNA[Phe] mutants was measured using a ribonuclease protection assay. Varying concentrations of EF-Tu●GTP were mixed with 2.5 nM [^{3}H]-Phe-tRNA in

Buffer A at 0°C. Reactions were terminated at varying times by addition of RNase A, incubated for an additional 20s, quenched, and filtered. The amount of [^{3}H]-Phe-tRNA bound as a function of time gave a k_{obs} at each EF-Tu concentration (Figure 2C). A plot of k_{obs} versus initial EF-Tu•GTP concentration allowed calculation of k_{on} (Figure 2D). The value of k_{on} was found to be similar for wild-type tRNA^{Phe} and all eight mutants tested. Thus, it was assumed that the average k_{on} value of 0.11 μM^{-1}sec^{-1} could be used to calculate K_d and $\Delta G°$ for all the tRNA mutants (Table I). Errors ranged between ± 0.1 and 0.4 kcal/mol for the calculated $\Delta G°$ values.

The free energies of binding of the yeast tRNA^{Phe} mutants to EF-Tu are compared graphically in Figure 3. Individual mutation of seven base pairs in the acceptor stem of Phe-tRNA^{Phe} resulted in modest effects on $\Delta G°$. In all seven cases, the range of $\Delta G°$ between the mutant and wild-type tested was less than 0.4 kcal/mol, which is only slightly greater than the error of measurement. While this does not eliminate the possibility that EF-Tu can derive some sequence specificity from this region by combining the small effects, to a first approximation the protein interacts with all tRNAs similarly in this part of the molecule. The only exception to this conclusion is the tRNAs which contain certain 1-72 base pairs that were not tested. Indeed, introducing the U1-A72 pair weakens the affinity to *E. coli* EF-Tu,[17] and U1-A72 in tRNA^{Gln} is an important determinant in defining the weak binding of tRNA^{Gln} to *T. thermophilus* EF-Tu (L. Behlen, unpublished data).

Mutations in four of the base pairs in the T-stem show substantial differences in their binding affinity to EF-Tu. The range of $\Delta G°$ values spans 0.8 kcal/mol for the 49-65 pair, 1.7 kcal/mol for the 50-64 pair, 1.0 kcal/mol for the 51-63 pair, and 0.4 kcal/mol for the 52-62 pair (Figure 3, Table 1). Comparable $\Delta G°$ effects were previously observed for a more limited number of mutations of the 49-65 and 51-63 pairs in tRNA^{Leu}, tRNA^{Asn}, tRNA^{Asp}, and tRNA^{Phe}.[11,12] However, since the U52-A62 pair present in tRNA^{Phe} is rarely found among bacterial tRNAs, it is unlikely that the 52-62 position contributes to EF-Tu binding specificity for most bacterial tRNAs since the AU and GC pairs commonly found at this position have virtually identical $\Delta G°$ values. Thus, this comprehensive mutagenesis experiment reveals that only the 49-65, 50-64 and 51-63 base pairs contribute substantially to the aa-tRNA binding specificity. The same three base pairs were previously identified as the primary sites that ensured weak binding of tRNA^{Sec} to *T. thermophilus* EF-Tu using minihelix substrates.[18]

To better understand how different base pairs combine to achieve the cognate $\Delta G°$, all eight combinations of the tightest and weakest of the three specificity base pairs were introduced into tRNA^{Phe}. Since the native tRNA^{Phe} sequence uses the weakest alternative (C-G) at position 49-65, and the tightest (G-C) at position 51-63, two of the eight $\Delta G°$ values had already been measured as single base-pair mutations. The $\Delta G°$ values for the remaining six tRNA^{Phe} derivatives were determined, and the data for all eight derivatives are shown in Figure 4. It is clear from this experiment that the three base pairs combine to achieve the overall affinity. As a result, it is possible that relatively similar $\Delta G°$ values can be obtained by different combinations of base pairs. For example, as shown in Figure 4, a $\Delta G°$ of about -9 kcal/mol can be achieved by using each combination of two weak and one strong base pair. It is likely that a $\Delta G°$ of -9 kcal/mol could also be achieved by combining three intermediate base pairs. Thus, many different tRNA sequences can be used to obtain an intermediate overall $\Delta G°$.

It is striking that the weakest and tightest tRNA^{Phe} derivatives in Figure 4 differ in $\Delta G°$ by -3.2 kcal/mol, which is comparable to the -3.6 kcal/mol range of $\Delta G°$ values seen among 19 valylated *E. coli* tRNAs assayed previously.[6] This suggests that the three base pairs identified by tRNA^{Phe} mutagenesis may explain the majority of tRNA sequence specificity observed among the bacterial tRNAs. To test this idea, we selected several tight, moderate, and weak *E. coli* tRNA bodies identified previously[6] and transplanted their base pairs at 49-65, 50-64,

and 51-63 into yeast tRNA[Phe]. The resulting tRNA[Phe] chimeras with 49-65, 50-64, and 51-63 sequences from tRNA[Asp], tRNA[Gly], tRNA[Leu], tRNA[Pro], tRNA[Tyr], tRNA[Trp], and tRNA[Gln] were phenylalanylated, and values for $\Delta G°$ with *T. thermophilus* EF-Tu were obtained. As shown in Figure 5, the $\Delta G°$ values of the tRNA[Phe] chimeras correlated closely to the $\Delta G°$ values obtained previously for the valylated *E. coli* tRNAs in nearly identical buffer conditions. [6] The difference in the absolute value of $\Delta G°$ of the two data sets is primarily accounted for by the fact that tRNAs esterified with valine bind EF-Tu more weakly than the same tRNAs esterified with phenylalanine.[19,20] However, the slope of the best fit line for the seven data points is 1.4, suggesting that some aspect of the bodies of several of the tRNAs outside of base pairs 49-65, 50-64, and 51-63 may also contribute to the binding affinity.

The availability of $\Delta G°$ values for the 11 double or triple base-pair mutations in tRNA[Phe] presented in Figures 4 and 5 makes it possible to evaluate whether the thermodynamic contributions of the three base pairs are additive. To do this, the positive or negative $\Delta\Delta G°$ values between tRNA[Phe] and each of the single base-pair mutations were calculated. The $\Delta G°$ of a given double or triple mutant could then be predicted by adding the appropriate $\Delta\Delta G°$ values to the $\Delta G°$ of tRNA[Phe]. As shown in Figure 6, the agreement between predicted and measured values is excellent over a wide range of $\Delta G°$. This shows that the thermodynamic contributions of the three base pairs are nearly independent of one another.

While the above experiments support the idea that the three base pairs are the primary specificity determinants in the binding of most aa-tRNAs to EF-Tu, all experiments thus far were performed in the background of yeast tRNA[Phe]. While this tRNA has an overall "D_4V_5" architecture that is typical of the majority of bacterial tRNAs,[21] it was important to test whether the three specificity base pairs made a similar relative contribution in a different aa-tRNA background. *E. coli* Leu-tRNA[Leu]$_{CAG}$ was chosen because it has a very different type II architecture with an extended variable loop and lacks aminoacyl tRNA synthetase recognition elements in the T-stem (Figure 7). The presence of different tertiary interactions in the core of tRNA[Leu] [22] can potentially affect either the structure or dynamics of the free tRNA or affect the strength and position of the hydrogen bonds in the bound complex, thereby altering the contributions of the specificity base pairs to the overall binding free energy. The measured affinities of the different Leu-tRNA[Leu]$_{CAG}$ mutants are reported in Table II and are compared to the corresponding Phe-tRNA[Phe] mutants in Figure 7. In general, the effect of a given mutation at each of the three positions is similar in the two tRNAs; however, it is notable that several mutants do not agree perfectly. For example, both A51-U63 and C50-G64 bind tighter in tRNA[Phe] than in tRNA[Leu]$_{CAG}$. Nevertheless, the fact that the effects of mutations in the two tRNA backgrounds are quite similar suggests that any structural and/or dynamic differences between the two tRNAs only minimally affects their interaction with EF-Tu.

Because the $\Delta\Delta G°$ values at the three specificity sites were additive, and were quite similar in another tRNA, it is of interest to use the tRNA[Phe] data to calculate the $\Delta G°$ values for all *E. coli* tRNAs and compare them to those measured previously.[6] To do this, the $\Delta\Delta G°$ values for the 49-65, 50-64, and 51-63 base pairs for each *E. coli* tRNA were added to the $\Delta G°$ of tRNA[Phe] to calculate their overall affinity. In general, since the *E. coli* tRNAs that bind EF-Tu tightly contain tight binding pairs and the weak binding tRNAs contain weak binding pairs, a reasonably good fit was expected. Indeed, most of the calculated values are close to the affinity of the valylated tRNAs, considering the error of the calculation (Figure 8). This is rather remarkable considering the overall structural differences between the *E. coli* tRNA bodies. However, tRNA[Glu]$_{UUC}$, tRNA[Met]$_M$, tRNA[Tyr]$_{GUA}$, and tRNA[Ile]$_{GAU}$ were rather far (>0.8 kcal/mol) outside of their calculated values. Such a lack of fit could be due to many factors. First, as shown above for tRNA[Leu]$_{CAG}$, the $\Delta\Delta G°$ of a given pair determined in tRNA[Phe] may not be the same in other tRNAs. This may be due to either slightly different complex structures or differences in structure or dynamics of the free tRNAs. Support for this

possibility includes the observation that Ala mutations in Arg 274 and Thr 332, which contact the tRNA in invariant positions, contribute different amounts in different tRNAs.[10] Secondly, the small $\Delta\Delta G°$ values observed at several sites in the acceptor stem could combine to slightly alter the overall affinity of certain tRNAs. Finally, it is possible that the extrapolated data for certain tRNA^X or tRNA^{Phe} mutants may be incorrect. For example, extrapolation of data obtained at much higher or lower NH_4Cl concentrations may not accurately give the correct value of $\Delta G°$ at 0.5 M NH_4Cl. Considering the many potential sources of error, the fit of calculated and experimental data in Figure 8 is quite good.

Discussion

Based on 53 mutations in yeast tRNA^{Phe} and 12 in *E. coli* tRNA^{Leu}, we have identified three base pairs in the T-stem of tRNA that modulate the affinity of tRNA to *T. thermophilus* EF-Tu in a sequence-specific manner. The data support and extend previous more limited tRNA and minihelix mutagenesis experiments[11,12,18,23] and molecular dynamics/bioinformatics analysis[24] that identified the same region. Other base pairs that contact EF-Tu do not contribute significantly to the sequence specificity, although there is evidence that certain mutations in the 1-72 base pair that were not tested here also have an effect.[23; 25] Nevertheless, the three base pairs in the T-stem quantitatively account for the characteristic affinity of most bacterial tRNAs to EF-Tu. Transplanting the three specificity base pairs from several *E. coli* tRNAs into yeast tRNA^{Phe} successfully converts tRNA^{Phe} into a molecule that has a binding affinity similar to the donor tRNA. Analysis of the free energy of binding for several double and triple base-pair substitutions suggests that the contribution of each base pair is thermodynamically independent. As a result, it was possible to use the single base-pair substitution data to predict the affinities of many bacterial tRNAs with reasonable accuracy.

The tRNA mutagenesis data presented here agree very well with experiments examining the effects of EF-Tu mutations on the specificity of tRNA binding.[10] Single Ala substitutions for amino acids in the tRNA binding cleft of *T. thermophilus* EF-Tu were tested for their ability to bind four tRNA bodies with different intrinsic affinities. Five "specificity" amino acids were identified that differentially affected $\Delta G°$ of binding to the four tRNAs. Two of the amino acids (Thr 332 and Arg 274) contact tRNA backbone functional groups (phosphate oxygens at C75 and G53) at the edges of the EF-Tu binding site that are conserved in all tRNAs and therefore must confer specificity indirectly. However, it is striking that three of the five "specificity" amino acids - Glu 390, Gln 341 and Thr 350 - interact with functional groups of the three "specificity" base pairs identified in this work. Glu 390 interacts with an amino group of G when present in the minor groove of the 51-63 base pair, while Gln 341 and Thr 350 interact with 2′ hydroxyl groups of the ribose at 64 and 65, respectively.[7] This agreement between the EF-Tu and tRNA mutagenesis data strongly supports the conclusion that the major part of the EF-Tu●tRNA interface responsible for sequence specificity has been identified.

The observation that the thermodynamic contributions of the three specificity base pairs were nearly independent of each other was surprising considering that they are adjacent to one another. Since the precise position of the base and backbone functional groups of a base pair in an RNA helix depends upon the identity of its neighboring pairs,[26] one would expect that the contribution of a protein contact to the binding free energy would be sensitive to the identity of neighboring pairs, and hence not be the same in all sequence contexts. Furthermore, examination of the structural details of the EF-Tu●tRNA^{Phe} complex reveals that while Glu 390 interacts with the 51-63 pair independently of other amino acids, Gln 341 and Thr 350 form a hydrogen bond with one another as well as with the 2′ hydroxyls 49-65 and 50-64 base pairs. Therefore, it is rather unexpected that sequence changes can modulate the strength of the 49-65 contact independently of the 50-64 contact. It would be valuable to obtain the

structures of EF-Tu complexes with different T-stem sequences to further understand this surprising absence of neighbor effects.

It is now possible to understand the total free energy of binding of *T. thermophilus* EF-Tu with a given aa-tRNA, $\Delta G°$(total), in terms of five thermodynamic contributions that are reasonably independent. First, there is a basal binding free energy, $\Delta G°$(tRNA) of about -8.0 kcal/mol associated with the aa-tRNA body that reflects the majority of the stabilizing contacts with the protein but is approximately the same for all aa-tRNAs. The contacts occur throughout the interface between the tRNA and EF-Tu, including the 5′ phosphate, the CCA terminus, part of the acceptor helix, and part of the T hairpin. $\Delta G°$(tRNA) also includes several stabilizing contacts with the amino group of the esterified amino acid. The second contribution, $\Delta G°$(a.a.), is due to the side chain of the esterified amino acid that was deduced directly by comparing misacylated tRNA^{Phe} species[5] or indirectly by comparing tRNAs acylated with their cognate amino acid and with Val[19]. The values of $\Delta G°$(a.a.) are the result of idiosyncratic interactions made with the large amino acid pocket in EF-Tu and vary by 2.5 kcal/mol[5]. The final three contributions to $\Delta G°$(total) are from the three T-stem base pairs studied in this work: $\Delta G°$(49-65), $\Delta G°$(50-64), and $\Delta G°$(51-63). Each of the terms depends strongly on the sequence of the pair, but the three can contribute up to a total of -3.6 kcal/mol, which is approximately the same as the range of free energies observed among *E. coli* tRNA bodies[6]. This range of free energies also approximates the range observed for $\Delta G°$(a.a.), and as discussed previously, evolution has adjusted the sequences of natural tRNAs such that the differences in $\Delta G°$(total) for natural, correctly esterified aa-tRNAs are quite small.[3,19] It is likely that the above dissection of $\Delta G°$(total) into five independent components is an oversimplification that ignores the cooperative structural and dynamic effects of both the free and bound components. However, as shown in Figure 8 and in [reference 19], the model is a useful tool to approximately calculate $\Delta G°$(total) for different tRNA sequences esterified with various amino acids.

T-stem sequences present in *E. coli* tRNAs vary considerably. As would be expected, the tight binding tRNA bodies such as tRNA^{Asp} or tRNA^{Gly} use the most strongly stabilizing base pairs at all three positions, while weak binding tRNAs such as tRNA^{Gln} and tRNA^{Trp} use the corresponding weakest alternatives. More interesting are the tRNA bodies with intermediate affinity which often use quite different combinations of the three base pairs to achieve the same binding affinity. Indeed, there are many cases where different isoacceptor tRNAs, which would be expected to have similar values for $\Delta G°$(total), have different sequences in the three critical base pairs. For example, tRNA$^{Ser5}_{GGA}$ uses G49-C65 G50-C64 G51-C63 in its T-stem and has a calculated $\Delta G°$(total)= -10.8 kcal/mol, while tRNA$^{Ser1}_{UGA}$ uses C49-G65 A50-U64 G51-C63 to give a similar calculated $\Delta G°$(total)= -10.2 kcal/mol. The fact that a given value of $\Delta G°$(total) can be achieved using so many combinations of base pairs is a distinguishing feature of EF-Tu recognition of tRNA, which presumably reflects its need to interact with all tRNAs with a characteristic affinity.

It is interesting to contrast the tRNA recognition properties of EF-Tu with those of aminoacyl tRNA synthetases which use a limited number of identity nucleotides to ensure that the small number of cognate tRNAs bind tightly and all other non-cognate tRNAs bind with a 10^3 to 10^4-fold weaker affinity.[27] Thus, both proteins can be considered to be sequence- specific tRNA binding proteins that use a similar number of tRNA residues to elicit a similar range in binding affinities. The major difference is that synthetases must distinguish a limited number of tRNAs from all others while EF-Tu must recognize every tRNA with a certain affinity. In general, tRNA synthetases do not use the three T-stem base pairs for specific recognition, so the two systems could have evolved independently. An interesting exception to this conclusion appears to be tRNA^{Gln}, which contains the rare U1-A72 pair that is needed for tight binding by their synthetases[28,29] and weak binding by EF-Tu[17](L. Behlen, unpublished data).

The rules for *T. thermophilus* EF-Tu binding to different tRNAs outlined above are likely to be the same for all bacteria. The main specificity residues Glu 390, Gln 341, Thr 350, and Thr 332 are universally conserved, and Arg 274 is highly conserved in bacterial EF-Tu sequences. The T-stem sequences of tRNAs that bind EF-Tu very tightly or very weakly are also highly conserved. For example, the tight binding tRNA^{Asp}s contain 95% G51-C63 91% C50-G64 and 90% G49-U65 which are all tight base pairs. Similarly, the weak binding tRNA^{Gln}s [6] contain 92% A51-U63 or U51-A63, 28% G50-U64 or U50-G64, and 91% G49-C65 or C49-G65, which are all weak base pairs. In contrast, tRNAs with intermediate binding affinity often show a wider variety of sequences in the T-stem. For example, bacterial tRNA^{Lys}s, which show intermediate affinity to EF-Tu [6], have variable T-stems that contain varying combinations of tight, weak, and intermediate base pairs with no pair present greater than 48% of the time at any of the three sites. There are a few interesting examples of tRNAs whose T-stem sequences are predicted to be very different from other members of their acceptor families. For example, a tRNA^{Glu} from *H. pylori* has G49-C65 G50-U64 A51-U63, which is much weaker than most bacterial tRNA^{Glu}. Also, tRNA^{Asn} from *B. Japonicum* has G49-C65 C50-G64 G51-C63, which is predicted to bind EF-Tu much more tightly than all other tRNA^{Asn}s. It is possible that these two tRNAs are related to the transamidation pathway present in those organisms that is used to produce Gln-tRNA^{Gln} and Asn-tRNA^{Asn} [30].

The conserved mechanism of tRNA specificity for bacterial EF-Tus does not extend to eukaryal and archaeal EF-Tus. Since Glu 390 in bacteria is an Asp in eukaryal and archaeal EF-Tus, it is possible that a similar recognition contact may occur. However, the remaining recognition residues Gln 341, Thr 350, Arg 274, and Thr 332 are quite variable in eukaryotes and archae. Furthermore, eukaryotic tRNA^{Asp}s, tRNA^{Glu}s, and tRNA^{Gly}s sequences do not have the tight binding G49-U65 present in bacteria, but usually contain the weaker C49-G65. Conversely, in eukaryotes, many tRNA^{Trp}s, which bind weakly in *E. coli*, contain the tight binding G49-U65 C50-G64 G51-C63. This suggests that the eukaryal and archaeal EF-Tus either recognize tRNAs in a slightly different manner or a specific interaction does not occur.

It is not known whether the selective pressure for the evolution of uniform binding of aa-tRNAs is solely to form the initial complex with EF-Tu. It is possible that the interactions of EF-Tu with aa-tRNA on the ribosome also played an important role in the evolution of the interface. On the ribosome, EF-Tu●GTP●aa-tRNA is rapidly bound to the A/T site, and upon correct codon-anticodon interaction, a signal is sent from the decoding center to EF-Tu to hydrolyze GTP and release the aa-tRNA into the A site.[2] It could be that a precise affinity of aa-tRNA for EF-Tu is essential for the correct rates of GTP hydrolysis or release from EF-Tu●GDP. For example, if the EF-Tu●tRNA interaction were too tight, dissociation of the complex could limit the rate of peptide bond formation. It will thus be interesting to evaluate the properties of tRNA mutations that either weaken or strengthen EF-Tu binding on the rates of GTP hydrolysis and peptide bond formation.

Materials and Methods

Expression and purification of *T. thermophilus* EF-Tu was performed as described previously. [9] Formation of the EF-Tu●GTP complex and determination of the fraction of the complex capable of binding aa-tRNA were determined as previously described.[9] Double-stranded DNA templates for T7 transcription of tRNAs were prepared by annealing two oligonucleotides (Integrated DNA Technologies) with at least 20 base pairs of overlap and extending with Taq DNA polymerase. *In vitro* transcription was performed as described previously,[12] except in the synthesis of the A start transcript where 12.5 mM AMP was used to prime the transcription instead of GMP. The transcripts were purified on a 10% denaturing polyacrylamide gel. The tRNAs were aminoacylated with purified yeast PheRS or *E. coli* LeuRS and [^{3}H]phenylalanine (123 Ci/mmol) or [^{3}H]leucine (160 Ci/mmol) (Amersham) as described previously.[5]

The dissociation rate (k_{off}) constants of [3H]-aa-tRNAs from EF-Tu•GTP were determined as previously described [9] at 0°C in buffer A (50 mM Hepes pH 7.0, 20 mM MgCl2, 0.5 M NH$_4$Cl, 5 mM DTT, 20 µM GTP, 3 mM Phosphoenolpyruvate, and 50 µg/mL pyruvate kinase). For those tRNAs where k_{off} was either faster than 0.01 s^{-1} or slower than 0.0005s^{-1}, k_{off} values were determined at several lower or higher NH$_4$Cl concentrations, and the value at 0.5 M was extrapolated from an ln k_{off} versus ln [NH$_4$Cl] plot.[4]

The association rate constants (k_{on}) for aa-tRNAs to EF-Tu•GTP were determined using a ribonuclease protection assay in buffer A at 0°C. After activation to form EF-Tu•GTP, the protein was serially diluted in buffer A to concentrations ranging from 100 nM to 800 nM. To initiate the binding reaction, a 25 µL aliquot of protein was mixed with 25 µL of 2.5nM [^{3}H]-aa-tRNA and subsequently quenched at multiple time points by the addition of 10 µL of 1mg/mL RNase A. This concentration of RNase digests free aa-tRNA within 20 seconds, but the ternary complex that formed during the initial incubation was stable and resistant to digestion. After the incubation with RNase, 150 µL of 10% (w/v) trichloroacetic acid (TCA) with 0.1 mg/mL unfractionated yeast tRNA (Sigma Aldrich) was added, filtered over a nitrocellulose membrane presoaked in 5% TCA and washed three times with 100 µL ice cold TCA. The dried filters were quantified using a Molecular Dynamics Storm Phosphoimager and ImageQuant software (Amersham Biosciences). The amount of [^{3}H]-aa-tRNA bound to EF-Tu as a function of time was fitted with a single exponential yielding a k_{obs} for each EF-Tu concentration (Figure 2). The association rate constants were determined using the slopes of k_{obs} versus concentration of EF-Tu (Figure 2d). All measurements were done in triplicate. Control experiments in the absence of EF-Tu showed that RNase A digested >95% of aa-tRNA within 15 seconds.

References

1. Krab IM, Parmeggiani A. EF-Tu, a GTPase odyssey. Biochim Biophys Acta 1998;1443:1–22. [PubMed: 9838020]

2. Pape T, Wintermeyer W, Rodnina MV. Complete kinetic mechanism of elongation factor Tu-dependent binding of aminoacyl-tRNA to the A site of the *E. coli* ribosome. EMBO J 1998;17:7490–7. [PubMed: 9857203]

3. Louie A, Ribeiro NS, Reid BR, Jurnak F. Relative affinities of all *Escherichia coli* aminoacyl-tRNAs for elongation factor Tu-GTP. J Biol Chem 1984;259:5010–6. [PubMed: 6370998]

4. LaRiviere FJ, Wolfson AD, Uhlenbeck OC. Uniform binding of aminoacyl-tRNAs to elongation factor Tu by thermodynamic compensation. Science 2001;294:165–8. [PubMed: 11588263]

5. Dale T, Sanderson LE, Uhlenbeck OC. The affinity of elongation factor Tu for an aminoacyl-tRNA is modulated by the esterified amino acid. Biochemistry 2004;43:6159–66. [PubMed: 15147200]

6. Asahara H, Uhlenbeck OC. The tRNA specificity of *Thermus thermophilus* EF-Tu. Proc Natl Acad Sci USA 2002;99:3499–504. [PubMed: 11891293]

7. Nissen P, Kjeldgaard M, Thirup S, Polekhina G, Reshetnikova L, Clark BF, Nyborg J. Crystal structure of the ternary complex of Phe-tRNAPhe, EF-Tu, and a GTP analog. Science 1995;270:1464–72. [PubMed: 7491491]

8. Nissen P, Thirup S, Kjeldgaard M, Nyborg J. The crystal structure of Cys-tRNACys-EF-Tu-GDPNP reveals general and specific features in the ternary complex and in tRNA. Structure 1999;7:143–56. [PubMed: 10368282]

9. Sanderson LE, Uhlenbeck OC. Directed Mutagenesis Identifies Amino Acid Residues Involved in Elongation Factor Tu Binding to yeast Phe-tRNA(Phe). J Mol Biol 2007;368:119–30. [PubMed: 17328911]

10. Sanderson LE, Uhlenbeck OC. Exploring the specificity of bacterial elongation factor Tu for different tRNAs. Biochemistry 2007;46:6194–200. [PubMed: 17489561]

11. Roy H, Becker HD, Mazauric MH, Kern D. Structural elements defining elongation factor Tu mediated suppression of codon ambiguity. Nucl Acids Res 2007;35:3420–30. [PubMed: 17478519]

12. Sanderson LE, Uhlenbeck OC. The 51-63 base pair of tRNA confers specificity for binding by EF-Tu. RNA 2007;13:835–40. [PubMed: 17449728]

13. Romby P, Carbon P, Westhof E, Ehresmann C, Ebel JP, Ehresmann B, Giege R. Importance of conserved residues for the conformation of the T-loop in tRNAs. J Biomol Struct Dyn 1987;5:669–87. [PubMed: 3078237]

14. Harrington KM, Nazarenko IA, Dix DB, Thompson RC, Uhlenbeck OC. *In vitro* analysis of translational rate and accuracy with an unmodified tRNA. Biochemistry 1993;32:7617–22. [PubMed: 7688564]

15. Pleiss JA, Uhlenbeck OC. Identification of thermodynamically relevant interactions between EF-Tu and backbone elements of tRNA. J Mol Biol 2001;308:895–905. [PubMed: 11352580]

16. Vorstenbosch EL, Potapov AP, de Graaf JM, Kraal B. The effect of mutations in EF-Tu on its affinity for tRNA as measured by two novel and independent methods of general applicability. J Biochem Biophys Methods 2000;42:1–14. [PubMed: 10647810]

17. Seong BL, RajBhandary UL. Mutants of *Escherichia coli* formylmethionine tRNA: a single base change enables initiator tRNA to act as an elongator *in vitro*. Proc Natl Acad Sci USA 1987;84:8859–63. [PubMed: 3321059]

18. Rudinger J, Hillenbrandt R, Sprinzl M, Giege R. Antideterminants present in minihelix(Sec) hinder its recognition by prokaryotic elongation factor Tu. EMBO J 1996;15:650–7. [PubMed: 8599948]

19. Asahara H, Uhlenbeck OC. Predicting the binding affinities of misacylated tRNAs for *Thermus thermophilus* EF-Tu. GTP Biochemistry 2005;44:11254–61.

20. Dale T, Uhlenbeck OC. Binding of misacylated tRNAs to the ribosomal A site. RNA 2005;11:1610–5. [PubMed: 16244128]

21. Kim SH, Sussman JL, Suddath FL, Quigley GJ, McPherson A, Wang AH, Seeman NC, Rich A. The general structure of transfer RNA molecules. Proc Natl Acad Sci USA 1974;71:4970–4. [PubMed: 4612535]

22. Tukalo M, Yaremchuk A, Fukunaga R, Yokoyama S, Cusack S. The crystal structure of leucyl-tRNA synthetase complexed with tRNALeu in the post-transfer-editing conformation. Nat Struct Mol Biol 2005;12:923–30. [PubMed: 16155583]

23. Stortchevoi A, Varshney U, RajBhandary UL. Common location of determinants in initiator transfer RNAs for initiator-elongator discrimination in bacteria and in eukaryotes. J Biol Chem 2003;278:17672–9. [PubMed: 12639964]

24. Eargle J, Black AA, Sethi A, Trabuco LG, Luthey-Schulten Z. Dynamics of Recognition between tRNA and elongation factor Tu. J Mol Biol 2008;377:1382–405. [PubMed: 18336835]

25. Fischer W, Doi T, Ikehara M, Ohtsuka E, Sprinzl M. Interaction of methionine-specific tRNAs from *Escherichia coli* with immobilized elongation factor Tu. FEBS Lett 1985;192:151–4. [PubMed: 3902504]

26. Xin Y, Olson WK. BPS: a database of RNA base-pair structures. Nucl Acids Res. 200810.1093/nar/gkn676Advance Access published October 9, 2008

27. Schulman LH. Recognition of tRNAs by aminoacyl-tRNA synthetases. Prog Nucleic Acid Res Mol Bio l 1991;41:23–87.

28. Jahn M, Rogers MJ, Soll D. Anticodon and acceptor stem nucleotides in tRNA(Gln) are major recognition elements for E. coli glutaminyl-tRNA synthetase. Nature 1991;352:258–60. [PubMed: 1857423]

29. Rould MA, Perona JJ, Soll D, Steitz TA. Structure of *E. coli* glutaminyl-tRNA synthetase complexed with tRNA(Gln) and ATP at 2.8 A resolution. Science 1989;246:1135–42. [PubMed: 2479982]

30. Cathopoulis TJ, Chuawong P, Hendrickson TL. Conserved discrimination against misacylated tRNAs by two mesophilic elongation factor Tu orthologs. Biochemistry 2008;47:7610–6. [PubMed: 18627126]
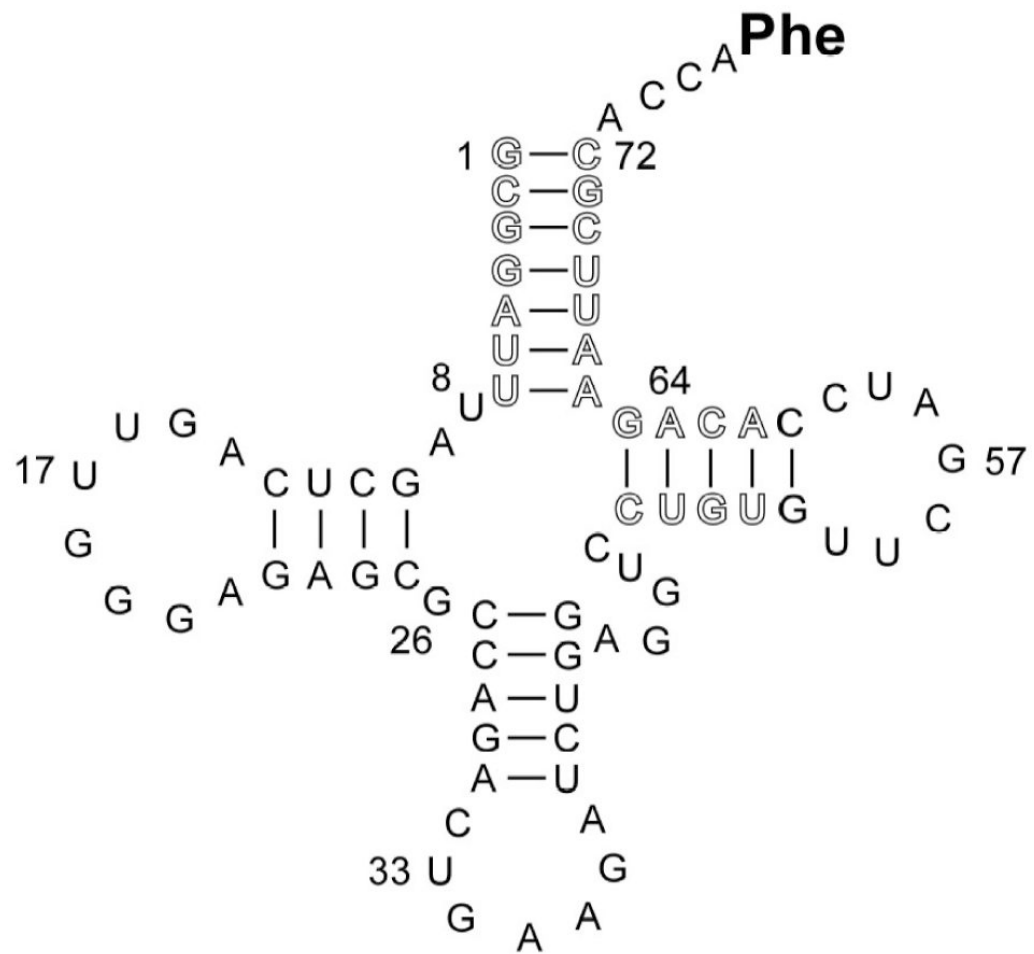
Figure 1.
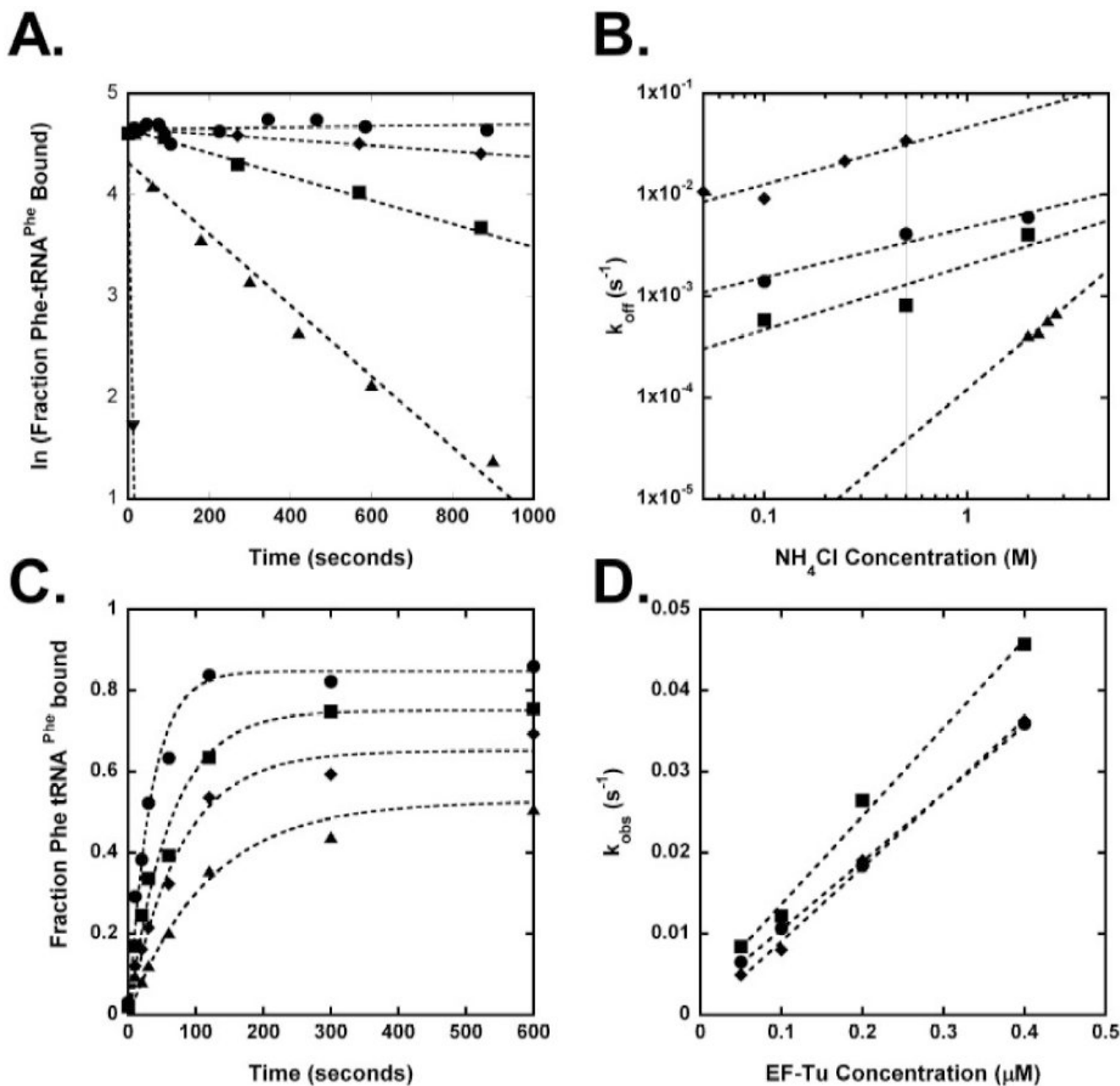Sequence of *S. cerevisiae* tRNA^{Phe} transcript used in this study with the sites of mutation outlined.

Figure 2.
(A.) Measurement of dissociation rates at 0°C in buffer A for: no RNase A (●), Phe-tRNAPhe with (■) and without (▼) EF-Tu●GTP, Phe-tRNA^{Phe}(G49-U65) (◆), and Phe-tRNA^{Phe}(A51-U63) (▲). Average k_{off} values are given in Table I. (B.) NH$_4$Cl concentration dependence of dissociation rates at 0°C for Phe-tRNA^{Phe} (●), Phe-tRNA^{Phe} (G50-U64) (■), Phe-tRNA^{Phe} (G50-U64 U51-A63) (◆), Phe-tRNA^{Phe} (G49-U65 C50-G64) (▲). Interpolated values at 0.5 M NH$_4$Cl (vertical line) are shown in Table I. (C.) Observed rate of association for Phe-tRNA^{Phe} at 0°C in buffer A at: 0.4 μM (●), 0.2 μM (■), 0.1 μM (◆), 0.05 μM (▲) EF-Tu*GTP. (D.) Association rate dependence on EF-Tu concentration at 0°C in buffer A for: Leu-tRNA$^{Leu}_{CAG}$ (●), Phe-tRNA^{Phe} A49-U65 (■), Phe-tRNA^{Phe} (◆). Slopes give k_{on}= 0.08, 0.11 and 0.09 μM^{-1}s^{-1} respectively.
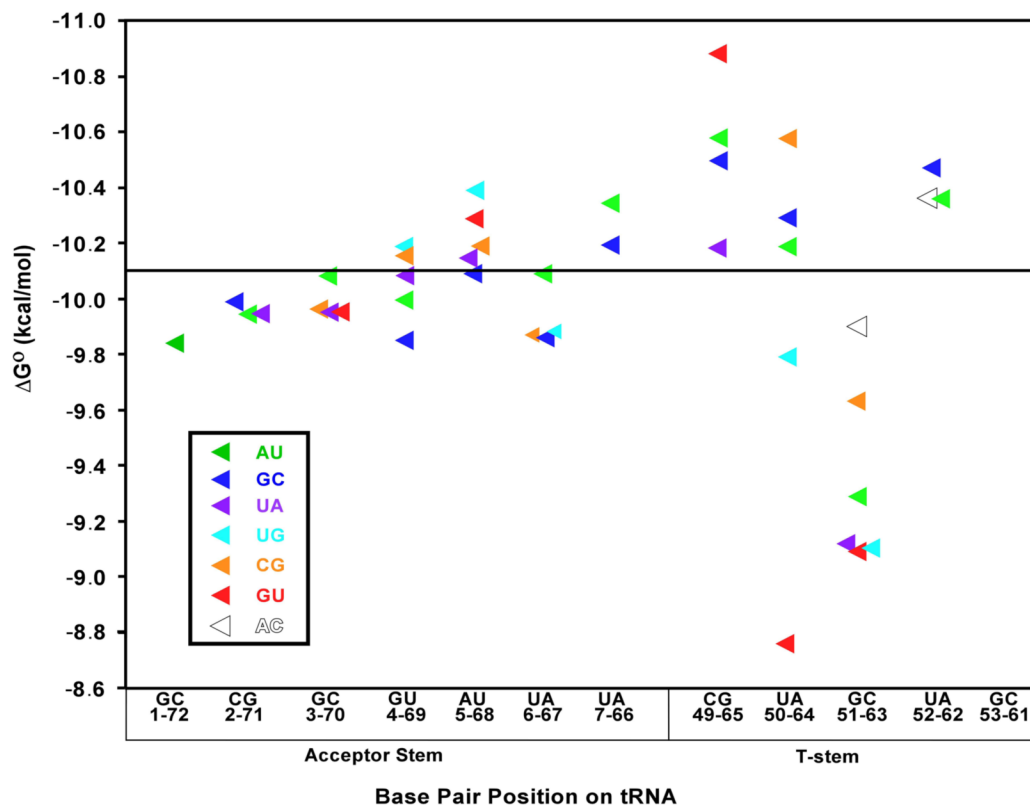
Figure 3.
Effects of single base pair substitutions in yeast tRNA[Phe] on $\Delta G°$ of binding to *T. thermophilus* EF-Tu in buffer A at 0°C. The solid horizontal line marks the $\Delta G°$ for wild-type tRNA[Phe] with the sequence given on the x axis, while colored triangles indicate the $\Delta G°$ of each mutant at the indicated base pair. Numerical data are shown in Table I.
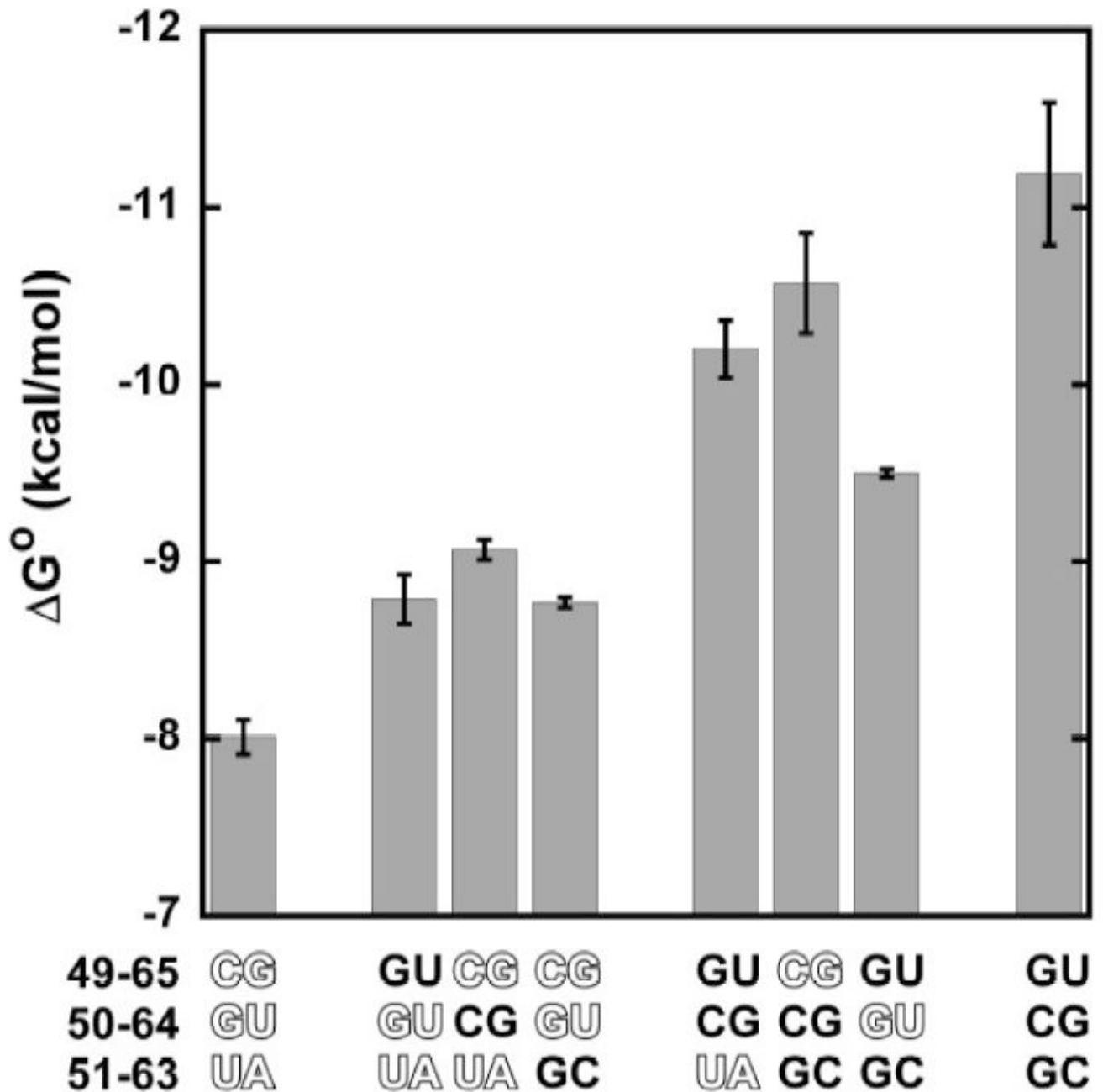
Figure 4.
Free energy of binding to *T. thermophilus* EF-Tu in buffer A at 0°C of different combinations of strong and weak (outlined) base pairs at the three specificity positions inserted into tRNA[Phe]. Numerical data in Table I.
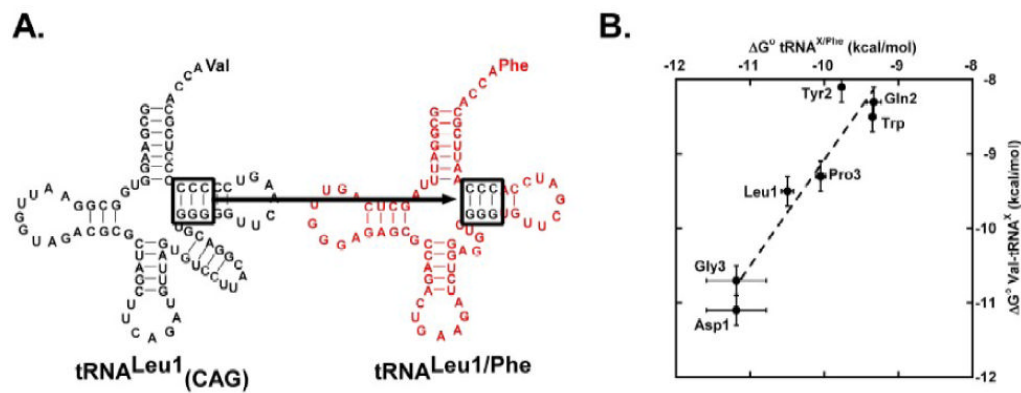
Figure 5.
Specificity swap experiment. (A.) Three specificity pairs in tRNA$^{Leu}_{CAG}$ were transplanted into tRNA^{Phe} to give tRNA$^{Leu/Phe}$. (B.) Comparison of $\Delta G°$ for binding seven Phe-tRNA$^{X/Phe}$ chimeras to *T. thermophilus* EF-Tu with the $\Delta G°$ for Val-tRNA^{X} determined previously [6]. The slope of the best fit line is 1.4 with a correlation coefficient of 0.95. Numerical data in Table I.
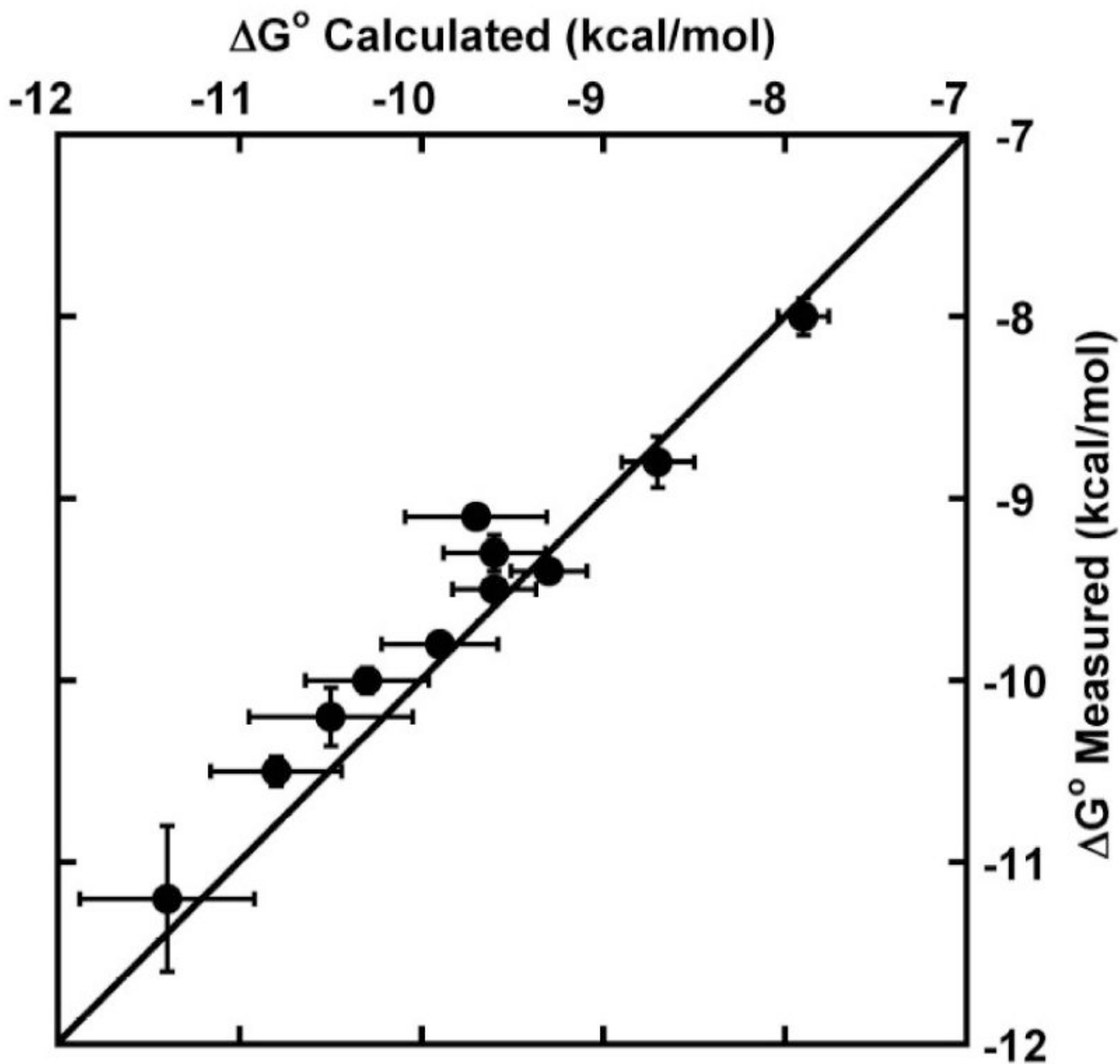
Figure 6.
Additivity test. Comparison of measured ΔG° for double and triple base pair mutants of
tRNA[Phe] with the value calculated assuming a linear combination of effects found with single
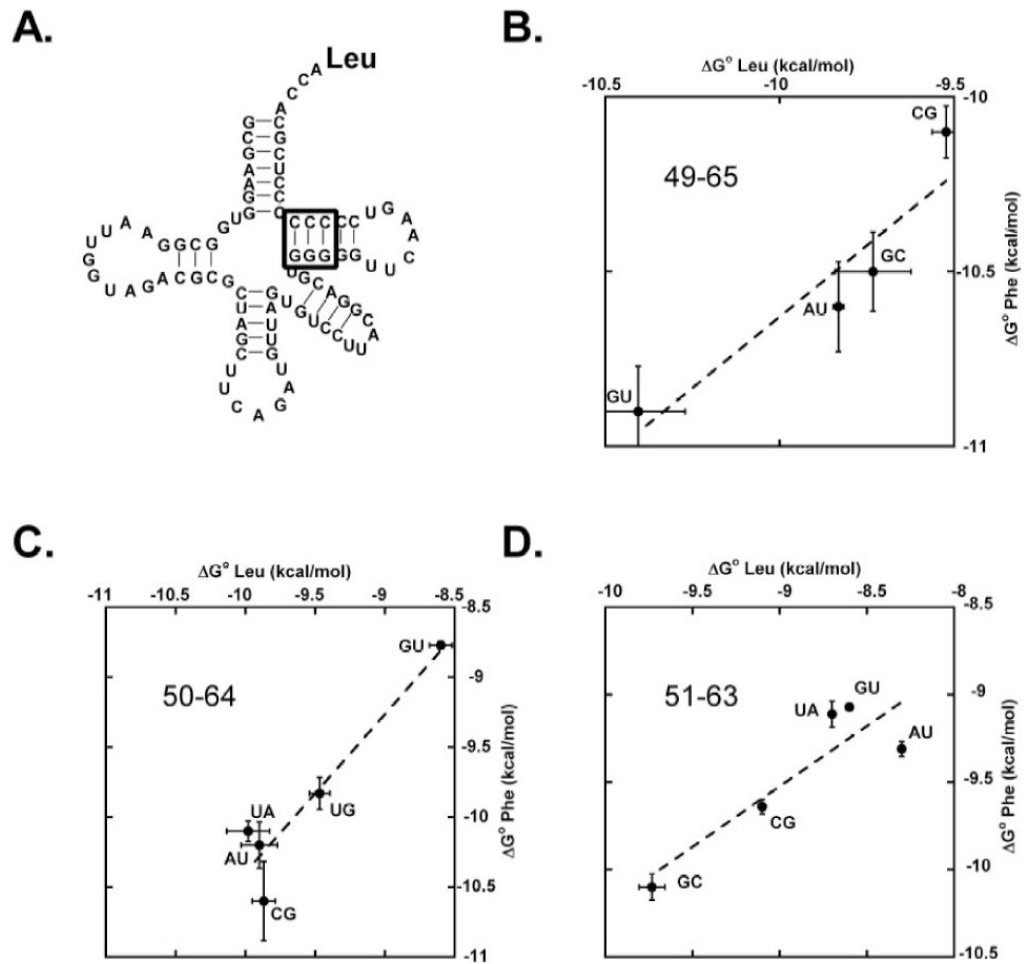base pair mutations. Line indicates perfect correlation between measured and calculated ΔG°.

Figure 7.
Comparison of ΔG° values for base pair substitutions in Leu-tRNA$^{Leu}_{CAG}$ (numerical data in Table II) and Phe-tRNA^{Phe} (numerical data in Table I). (A.) Sequence of *E. coli* tRNA$^{Leu}_{CAG}$ with sites of base pair substitutions indicated. (B.) The 49-65 base pair. Line has a slope of 0.82 and a correlation coefficient of 0.93. (C.) The 50-64 base pair. Line has a slope of 1.1 and a correlation coefficient of 0.94. (D.) The 51-63 base pair. Line has a slope of 0.69 and a correlation coefficient of 0.93.
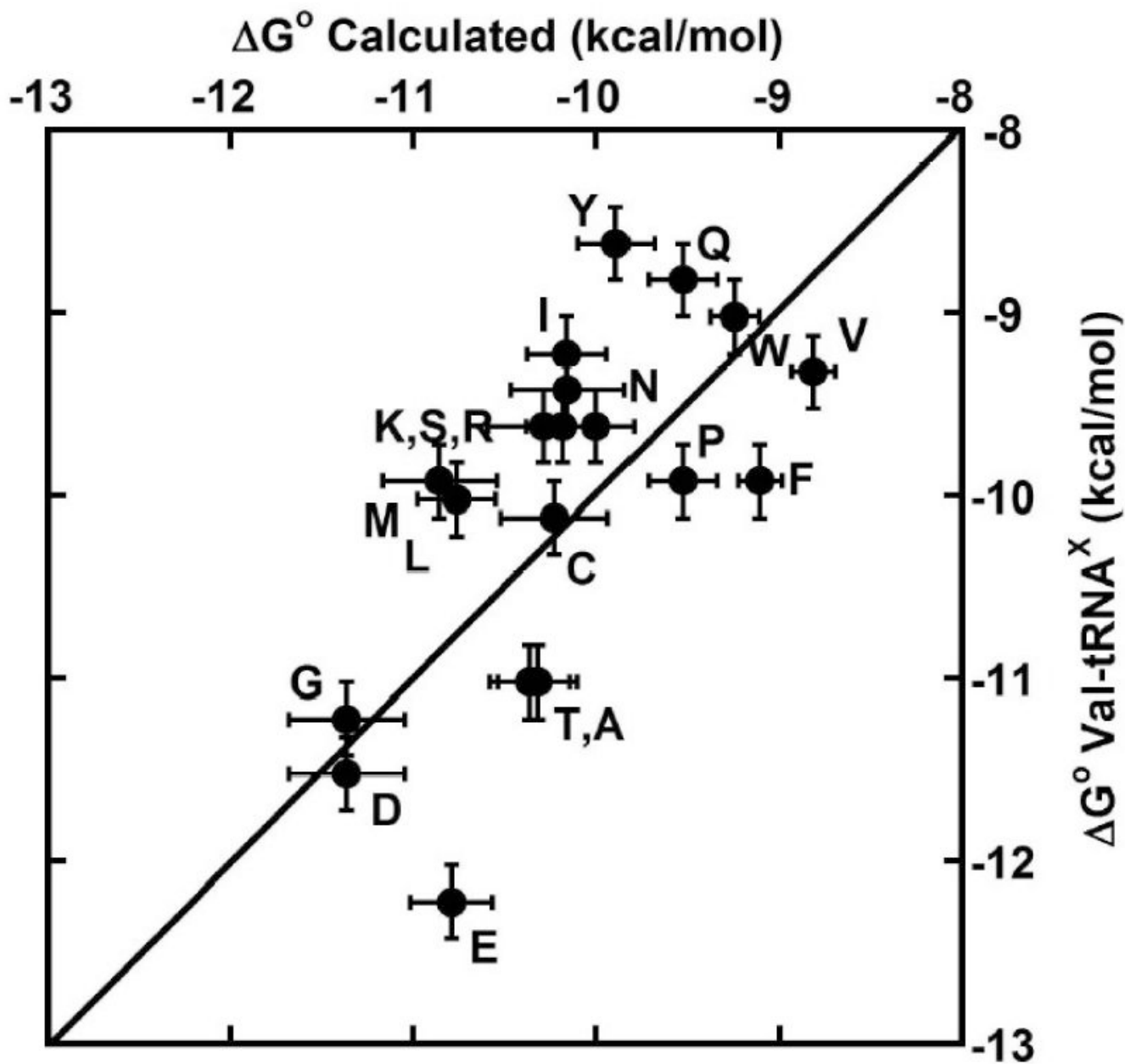
Figure 8.
Comparison of previously measured ΔG° of Val-tRNA^X 6 with the calculated ΔG° of the tRNA based on the $\Delta\Delta G^\circ$ values of the 49-65, 50-64, and 51-63 base pairs determined from single base pair mutations. tRNAs are labeled with the one letter code.

Table I

Rates of dissociation (k_{off}) of mutants of Phe-tRNA^{Phe} from *T. thermophilus* EF-Tu in buffer A at 0°C. Asterisk (*) refers to data extrapolated from NH_4Cl dependence (see Figure 2). Frequency refers to the appearance of that base pair in greater than 5000 non-redundant bacterial tRNA gene sequences (http://trnamart.uoregon.edu/). N is the number of independent determinations. $\Delta G°$ is calculated from k_{off} assuming $k_{on} = 0.11$ $\mu m^{-1}s^{-1}$ (see text). All errors are standard errors.

Mutation	Frequency	k_{off} ($s^{-1} \times 10^4$)	$\Delta G°$ (kcal/mol)	N
WT		9.6 ± 0.7	-10.1 ± 0.07	5
A1-U72	0.03	14.9 ± 1.4	-9.8 ± 0.09	3
G2-C71	0.50	11.8 ± 1.2	-10.0 ± 0.10	3
U2-A71	0.03	11.3 ± 0.4	-10.0 ± 0.04	3
A2-U71	0.02	12.5 ± 0.2	-9.9 ± 0.02	3
C3-G70	0.30	11.6 ± 0.3	-10.0 ± 0.03	3
A3-U70	0.14	9.3 ± 0.3	-10.1 ± 0.03	3
U3-A70	0.08	12.3 ± 0.2	-9.9 ± 0.02	3
G3-U70	0.05	12.2 ± 0.2	-9.9 ± 0.02	3
U4-A69	0.08	9.7 ± 0.3	-10.1 ± 0.03	3
G4-C69	0.49	14.6 ± 0.5	-9.8 ± 0.03	3
A4-U69	0.06	11.1 ± 1.0	-10.0 ± 0.09	3
U4-G69	0.03	7.2 ± 0.9	-10.2 ± 0.12	3
G5-C68	0.33	8.5 ± 0.5	-10.1 ± 0.06	3
C5-G68	0.30	7.1 ± 0.5	-10.2 ± 0.06	3
G5-68U	0.03	6.6 ± 1.2	-10.3 ± 0.17	3
U5-G68	0.03	4.9 ± 0.7	-10.4 ± 0.15	3
U5-A68	0.06	7.6 ± 1.1	-10.2 ± 0.14	3
A6-U67	0.16	8.7 ± 0.7	-10.1 ± 0.08	3
G6-C67	0.24	14.4 ± 0.9	-9.9 ± 0.06	3
C6-G67	0.28	14.6 ± 0.8	-9.8 ± 0.05	3
U6-G67	0.004	14.4 ± 1.5	-9.9 ± 0.11	3
A7-U66	0.23	5.4 ± 0.2	-10.4 ± 0.04	3
G7-C66	0.48	7.4 ± 0.5	-10.2 ± 0.07	3
G49-C65	0.46	4.0 ± 0.5	-10.5 ± 0.11	3
G49-U65	0.24	2.3 ± 0.3	-10.9 ± 0.13	3
A49-U65	0.20	3.8 ± 0.5	-10.6 ± 0.13	3
U49-A65	0.01	8.3 ± 0.7	-10.2 ± 0.08	3
A50-U64	0.13	8.1 ± 1.3	-10.2 ± 0.17	3
G50-C64	0.19	6.6 ± 1.1	-10.3 ± 0.17	3
C50-G64	0.34	4.1 ± 1.2	-10.6 ± 0.28	3
G50-U64	0.11	106 ± 3.1	-8.8 ± 0.03	4
U50-G64	0.04	15.3 ± 1.7	-9.8 ± 0.11	3
C51-G63	0.15	21.3 ± 0.9	-9.6 ± 0.04	3
A51-U63	0.22	39.2 ± 1.7	-9.3 ± 0.04	3
U51-A63	0.09	52.5 ± 2.0	-9.2 ± 0.04	3
G51-U63	0.03	60.9 ± 1.3	-9.1 ± 0.02	3
U51-G63	0.02	57.3 ± 4.2	-9.1 ± 0.07	3
A51-C63	0.01	13.7 ± 0.7	-9.9 ± 0.05	3
A52-U62	0.13	5.0 ± 0.4	-10.4 ± 0.08	3
G52-C62	0.84	4.3 ± 0.2	-10.5 ± 0.06	3
A52-C62	0.01	5.3 ± 1.3	-10.4 ± 0.25	3
Double Mutants		k_{off} ($s^{-1} \times 10^4$)	$\Delta G°$ (kcal/mol)	N
G50-U64 U51-A63*		428 ± 41.6	-8.0 ± 0.10	3
C50-G64 U51-A63		61.0 ± 3.4	-9.1 ± 0.06	4
G49-U65 G50-U64		28.6 ± 0.7	-9.5 ± 0.02	3
G49-U65 C50-G64*		1.2 ± 0.5	-11.2 ± 0.40	3
G49-C65 G50-C64		4.4 ± 0.4	-10.5 ± 0.08	5
G49-C65 G50-U64		36.2 ± 1.7	-9.4 ± 0.05	5
G50-C64 A51-U63		37.5 ± 3.7	-9.3 ± 0.10	6
Triple Mutants		k_{off} ($s^{-1} \times 10^4$)	$\Delta G°$ (kcal/mol)	N
G49-U65 G50-U64 U51-A63		102 ± 14.3	-8.8 ± 0.14	3
G49-U65 C50-G64 U51-A63		7.6 ± 1.2	-10.2 ± 0.16	3
G49-U65 G50-C64 A51-U63		10.0 ± 0.7	-10.0 ± 0.07	5
G49-C65 A50-U64 A51-U63		16.8 ± 0.7	-9.8 ± 0.04	5

Table II

Rates of dissociation (k_{off}) of mutants of Phe-tRNA^{Phe} from *T. thermophilus* EF-Tu in buffer A at 0°C. Asterisk (*) refers to data from reference [12]. N is the number of independent determinations. ΔG° is calculated from k_{off} assuming $k_{on} = 0.11$ μm^{-1}s^{-1} (see text). All errors are standard errors.

Mutation	k_{off} (s^{-1} × 10^4)	ΔG° (kcal/mol)	N
WT	17.9 ± 1.9	-9.7 ± 0.11	7
A49-U65	14.9 ± 0.2	-9.8 ± 0.02	3
C49-G65	26.4 ± 1.1	-9.5 ± 0.04	3
G49-U65	5.2 ± 07	-10.4 ± 0.14	3
C50-G64	14.0 ± 1.2	-9.9 ± 0.08	4
U50-A64	11.4 ± 1.8	-10.0 ± 0.15	4
U50-G64	29.2 ± 2.1	-9.5 ± 0.07	3
G50-U64	142 ± 11.6	-8.6 ± 0.08	3
A50-U64	12.8 ± 1.7	-9.9 ± 0.13	3
C51-G63*		-9.1	
G51-U63*		-8.6	
A51-U63*		-8.3	
U51-A63*		-8.7	