Exploring the Specificity of Bacterial Elongation Factor Tu for Different tRNAs†

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ABSTRACT: In order to identify amino acids in *Thermus thermophilus* elongation factor Tu which contribute to its specificity for different tRNAs, the binding affinities of 20 point mutations were compared to that of wild type protein using four tRNAs of differing affinities. The observed specificity for tRNA is the result of the varying contributions of five amino acids which make contacts with the T-stem and the 3′ terminus of tRNA. For three of the amino acids the test tRNAs differ in sequence at the site of contact, presumably explaining the specificity. However, the remaining two amino acids contact tRNA at conserved positions, suggesting that more global structural or dynamic properties of the free tRNA contribute to specificity.

Bacterial elongation factor Tu (EF-Tu) shows substantial specificity for different tRNAs. This is best illustrated by the observation that when 19 different *Escherichia coli* tRNAs were aminoacylated with valine, the resulting valylated tRNAs bound EF-Tu with binding free energies ranging from −8.1 to −11.7 kcal/mol (*1*). It has been proposed that this specificity for the different tRNA bodies evolved in order to compensate for the variable contribution of the different esterified amino acids to the binding affinity (*2*, *3*). As a result, tRNAs acylated with their cognate amino acid bind EF-Tu with similar binding affinities (*4*).

It is not known how the tRNA specificity of EF-Tu is achieved. The cocrystal structures of *T. aquaticus* EF-Tu bound to yeast Phe-tRNA^{Phe} or Cys-tRNA^{Cys} show a slightly different pattern of hydrogen bonds between the nucleic acid and the protein (*5*, *6*). While the tRNA^{Cys} and tRNA^{Phe} bodies happen to bind EF-Tu with similar affinities (*1*), the structures do suggest that specificity may arise from the protein making different contacts with different tRNAs. However, analysis of the effects of mutations in EF-Tu (*7*) or deoxynucleotide substitutions in tRNA^{Phe} (*8*) suggests that many, if not all, of the hydrogen bonds that are different between the two structures show little contribution to the binding affinity. Thus, it remains possible that all tRNAs interact with EF-Tu with the same set of thermodynamically important contacts and specificity arises either from certain contacts having variable free energies or from differences in the structure or dynamics of the free tRNAs.

In this paper we make use of 20 different site directed mutants of *T. thermophilus* EF-Tu to remove amino acid side chains capable of forming electrostatic or hydrogen bonding interactions with the tRNA body to better understand how the protein achieves specificity. Previous experiments examining the affinity of these same mutant proteins with yeast Phe-tRNA^{Phe} have identified 10 amino acids which contribute to the binding free energy (*7*). While several of the thermodynamically relevant contacts occur between the protein and the acceptor stem of tRNA^{Phe}, the majority involve the T-helix. By assessing how well these mutant proteins interact with other tRNA bodies with different overall affinities, sites which potentially contribute to specificity can be identified.

MATERIALS AND METHODS

EF-Tu Mutagensis and Purification. Mutagenesis of *T. thermophilus* EF-Tu and preparation of mutant proteins were described previously (*7*).

Preparation of tRNAs. Purified *E. coli* tRNA$^{Glu}_2$, tRNA^{Phe}, and tRNA$^{Tyr}_1$ were purchased from Sigma-Aldrich. The aminoacylation of the purified *E. coli* tRNAs with their cognate [^{3}H] amino acid was performed as in Dale et al. (*3*), except the final tRNA concentration was 5 μM rather than 2 to 3 μM. *E. coli* aminoacyl-tRNA synthetases were purchased as a synthetase mixture from Sigma-Aldrich.

EF-Tu Assays. The dissociation rate constant of each of the three *E. coli* aa-tRNAs was determined as described in Sanderson and Uhlenbeck (*7*).

RESULTS

Three modified, cognate *E. coli* aa-tRNAs were chosen for this work, Tyr-tRNA^{Tyr}, Phe-tRNA^{Phe}, and Glu-tRNA^{Glu} (Figure 1). Additionally, the affinity of yeast Phe-tRNA^{Phe} determined previously is also presented for comparison (*7*). Among the 19 *E. coli* tRNAs bodies previously analyzed, tRNA^{Tyr} was the weakest, tRNA^{Glu} was the tightest, and tRNA^{Phe} bound with an intermediate affinity (*1*). The use of the two extreme examples presumably maximizes the potential to identify residues important for specificity, and the choice of *E. coli* tRNA^{Phe} allows for a comparison with the previous data on yeast tRNA^{Phe}. The sequences of the four test tRNAs vary significantly in the region of the molecule that contacts EF-Tu. Only two of the 12 base pairs in the EF-Tu binding site were the same among the four tRNAs tested (Figure 1). One of these conserved base pairs, G53-C61, is present in all tRNAs and thus is unlikely to

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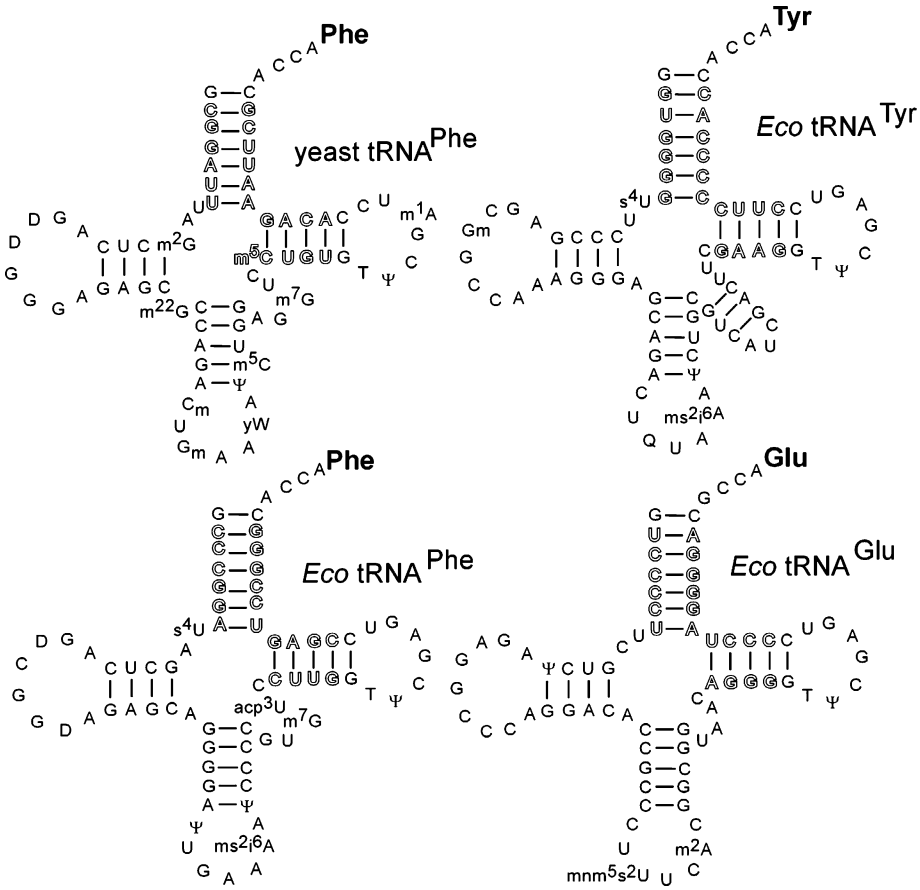

FIGURE 1: Cloverleaf representation of the modified *E. coli* tRNAs used in this study. Black outlined residues are positions in the acceptor and T-stems which vary in at least one of the four sequences. yW = wybutosine; Ψ = pseudouridine; ms^2i^6A = 2-methylthio-N6-isopentenyladenosine; D = dihydrouridine; s^4U = 4-thiouridine; T = thymine; mnm^5s^2U = 5-methylaminomethyl-2-thiouridine; Q = queuosine; Gm = 2'O-methylguanosine; m^5C = 5-methylcytosine; m^7G = 7-methylguanosine; m^2A = 2-methyladenosine; acp^3U = 3-(3-amino-3-carboxypropyl) uridine; m^2G = N2-methylguanosine; m^1A = 1-methyladenosine; $m^{22}G$ = N2,N2-dimethylguanosine; Cm = 2'O-methylcytosine.

contribute directly to specificity. The 1−72 base pair, which is conserved in the four test tRNAs, does differ in other tRNAs and thus could potentially contribute to their specificity. Indeed, there is evidence that the A1-U72 base pair contributes to the weaker binding of several *E. coli* tRNAs (*9*, *10*).

Previous experiments (*1*, *2*) establishing the tRNA specificity of EF-Tu made use of misacylated, unmodified tRNAs with altered anticodons; however, this work utilized fully modified tRNAs which where acylated with their cognate amino acids. Each aa-tRNA was combined with the GTP bound form of wild type *T. thermophilus* EF-Tu and 20 mutant proteins containing conservative point mutations in the tRNA binding cleft. The dissociation rate of each tRNA from each protein was determined by an RNase protection assay (*2*, *4*), and the resulting k_{off} values were converted to K_D values by assuming a constant k_{on} of 1.1×10^5 M^{-1} s^{-1} (*7*). These calculated K_D values are reported in Table 1. Previous experiments measuring the k_{off} and K_D values for a series of different tRNAs with wild type EF-Tu (*2*, *11*) or different mutants of EF-Tu with yeast Phe-$tRNA^{Phe}$ (*7*) have all obtained a similar value for $K_D/k_{off} = k_{on}$. Thus it is reasonable to assume that k_{on} is constant for all of the experiments presented in this work.

A convenient way to compare how the four different aa-tRNAs interact with the set of mutant proteins is to subtract the binding free energy of each mutant protein to a given tRNA from the binding free energy of the wild type protein to the same tRNA. The resulting $\Delta\Delta G°$ value ($\Delta\Delta G° = -RT \ln(K_{Dwt}/K_{Dmut})$) indicates how much the mutated amino acid affects the affinity to the given aa-tRNA. These $\Delta\Delta G°$ values for the four tRNAs are also given in Table 1, but the data is most conveniently viewed graphically in Figure 2, where the position on the graph is related to the four regions on the yeast Phe-$tRNA^{Phe}$ binding interface as defined previously and summarized in the diagram in Figure 3.

Of the 20 mutants tested, three (Arg389Ala, Arg330Ala, and Glu271Ala) impaired binding to all four tRNAs by so much that it was not possible to obtain an accurate value for $\Delta\Delta G°$ at 0.5 M NH_4Cl. Thus, the dissociation rates of these three EF-Tu mutants, two controls (Gln341Ala and Thr350Val), and wild type EF-Tu were measured with each of the three tRNAs in a 50 mM NH_4Cl buffer where the binding is significantly tighter. The $\Delta\Delta G°$ values for these five mutants in the lower ionic strength buffer are shown in Figure 2C. Under these tight binding conditions, reliable $\Delta\Delta G°$ values for Glu271Ala and Arg389Ala and the two controls were obtained, but the binding of the Arg330Ala protein was still too weak to be measured and thus no conclusions could be made about this mutation. Since the relative $\Delta\Delta G°$ values for the three tRNAs in the low ionic strength buffer using the two control mutant proteins were similar to that obtained in the standard buffer, it is likely

Table 1: Binding Values of Mutant EF-Tu to *E. coli* tRNAs[a]

	K_D (nM)			ΔΔG° (kcal/mol)			
	Glu-tRNA^{Glu}	Phe-tRNA^{Phe}	Tyr-tRNA^{Tyr}	*Eco* Glu-tRNA^{Glu}	*Eco* Phe-tRNA^{Phe}	*Eco* Tyr-tRNA^{Tyr}	yeast Phe-tRNA^{Phe} [b]
wild type	16 ± 7.5	13 ± 6.8	13 ± 6.8				
Lys52Ala	30 ± 2.9	15 ± 4.1	26 ± 12	0.3	0	0.3	0.2
Glu55Ala	11 ± 1.7	11 ± 2.8	19 ± 8.5	−0.2	−0.1	0.1	−0.3
Arg59Ala	18 ± 3.4	12 ± 2.9	26 ± 6.7	0.1	−0.1	0.3	−0.1
Asn64Ala	6.3 ± 1.3	10 ± 4.0	8.6 ± 2.4	−0.5	−0.2	−0.4	−0.2
Tyr 88Phe	19 ± 6.4	12 ± 2.3	29 ± 7.2	0.1	−0.1	0.3	−0.2
Lys90Ala	45 ± 12	42 ± 4.0	66 ± 29	0.6	0.6	0.8	0.5
Asn91Ala	121 ± 17	60 ± 18	116 ± 52	1.1	0.8	1.1	0.9
Thr232Val	9.5 ± 1.8	13 ± 1.8	18 ± 0.9	−0.3	−0.1	0.1	−0.1
Glu271Ala	>300	>300	>300	>1.8	>1.8	>1.8	>1.8
Arg274Ala	152 ± 35	153 ± 48	206 ± 66	1.2	1.3	1.4	0.9
Arg300Ala	39 ± 17	27 ± 7.1	28 ± 15	0.5	0.4	0.3	0.2
Arg330Ala	>300	>300	>300	>1.8	>1.8	>1.8	>1.8
His331Val	180 ±24	103 ± 37	134 ± 42	1.3	1.1	1.1	0.8
Thr332Val	>300	104 ± 28	287 ± 35	>1.8	1.1	1.6	1.0
Arg339Ala	11 ± 3.8	11 ± 2.5	32 ± 2.3	−0.2	−0.1	0.4	0.1
Gln341Ala	>300	236 ± 44	261 ± 53	>1.8	1.5	1.5	1.0
Thr350Val	>300	241 ± 56	156 ± 14	>1.8	1.6	1.2	1.1
Lys376Ala	21 ± 5.1	16 ± 28	23 ± 4.8	0.1	0.1	0.2	0.1
Arg389Ala	>300	220 ± 53	>300	>1.8	1.5	>1.8	>1.8
Glu390Ala	>300	88 ± 28	2.4 ± 0.7	>1.8	1.0	−0.8	1.0

[a] k_{off} values were determined at 0.5 M NH_4Cl, 20 mM $MgCl_2$, 50 mM HEPES pH 7.0, 4 °C. K_D calculated as $k_{off}/(1.1 \times 10^5\ M^{-1}\ s^{-1})$ and $\Delta\Delta G^\circ = -RT \ln(K_{Dwt}/K_{Dmut})$. [b] Data taken from Sanderson and Uhlenbeck (2006).

that buffer does not affect specificity. Thus, the absence of any specificity for Glu271Ala and Arg389Ala mutations for the three tRNAs should also be observed in the standard buffer.

Of the 19 EF-Tu mutations where $\Delta\Delta G^\circ$ values could be obtained, 14 showed similar (within 0.5 kcal/mol) $\Delta\Delta G^\circ$ values for all four tRNAs tested. Thus we can conclude that the corresponding amino acids do not contribute differentially to the binding affinity of EF-Tu to this set of tRNAs. Nine of these 14 mutations had $\Delta\Delta G^\circ$ values close to zero and thus do not make thermodynamically significant contacts to any of the tested tRNAs. The remaining five of these 14 mutations, Lys90Ala, Asn91Ala, Glu271Ala, His331Val, and Arg389Ala, weakened the binding to EF-Tu by a similar amount for all four tRNAs and thus contribute to affinity, but not to specificity. It is likely that the corresponding amino acids make identical contacts with each of the tRNAs. It remains possible, however, that these five mutations may show different $\Delta\Delta G^\circ$ values when tested with other tRNAs.

Five mutations (Arg274Ala, Thr332Val, Gln341Ala, Thr350Val, and Glu390Ala) show $\Delta\Delta G^\circ$ values which differ by more than 0.5 kcal/mol for at least two of the four tRNAs tested. These differential effects suggest that the corresponding amino acids contribute to specificity of tRNA binding. While it is difficult to relate the values of $\Delta\Delta G^\circ$ for a given mutation with the amount that the corresponding amino acid contributes to specificity in a simple way, two features of the data stand out. First, it is striking that for four of the five "specificity" mutations, the value of $\Delta\Delta G^\circ$ is the greatest for tRNA^{Glu}, the tightest binding tRNA body. This suggests that the tight binding of tRNA^{Glu} is the result of an increased thermodynamic contribution at each of these four sites. However, the trend is not consistent since the Arg274Ala mutation has the greatest deleterious effect on the weak binding tRNA^{Tyr}. Thus it appears that specificity may be achieved by a complex sum of contributions of many sites along the EF-Tu/tRNA interface.

A second striking feature is the effect of the Glu390Ala mutation. Unlike the other four mutations where the differences in the $\Delta\Delta G^\circ$ are modest and only differ for one of the tRNAs, Glu390Ala has a very large $\Delta\Delta G^\circ$ effect that is quite different for each tRNA. In the case of Glu-tRNA^{Glu}, the mutation is strongly destabilizing, while with Phe-tRNA^{Phe} the destabilizing effect is much less. Interestingly, in the case of Tyr-tRNA^{Tyr}, the mutation actually stabilizes the complex. Among the four tRNAs tested, the range of $\Delta\Delta G^\circ$ was nearly 3 kcal/mol. While we cannot directly relate these values to the total tRNA binding energy, it is likely that Glu390 acts as a major specificity residue to distinguish among these four tRNAs.

It is reasonable to expect that differences in the structures and dynamics of the four tRNAs are responsible for their differential binding to *T. thermophilus* EF-Tu. While the structures of *E. coli* tyrosyl, phenylalanyl, and glutamyl tRNAs either free in solution or bound to EF-Tu are not known, they are likely to interact with the protein in a manner similar to yeast Phe-tRNA^{Phe} and *E. coli* Cys-tRNA^{Cys} binding to *T. aquaticus* EF-Tu where X-ray cocrystal structures are available. In these structures, the amino acid side chains of the protein interact with the acceptor and T-stems of the two tRNAs similarly, but not identically. The two structures share nine electrostatic or hydrogen bonding protein−nucleic acid contacts, but also make from four contacts that are unique to tRNA^{Cys} and seven contacts that are unique to tRNA^{Phe}. Similar differences may be sufficient to cause differences in the binding free energy and account for the specificity of the four test tRNAs.

For three of the five "specificity" amino acids, the four tRNAs differ in sequence at or near the sites of contact potentially explaining the different $\Delta\Delta G^\circ$ values. The situation seems the clearest for Glu390, where the identity of the 51-63 base pair is G-C for tRNA^{Glu} and yeast tRNA^{Phe}, U-G in *E. coli* tRNA^{Phe}, and A-U in tRNA^{Tyr}. In both cocrystal structures, the side chain of Glu390 lies within the minor groove of the tRNA at base pair 51-63 and is close to

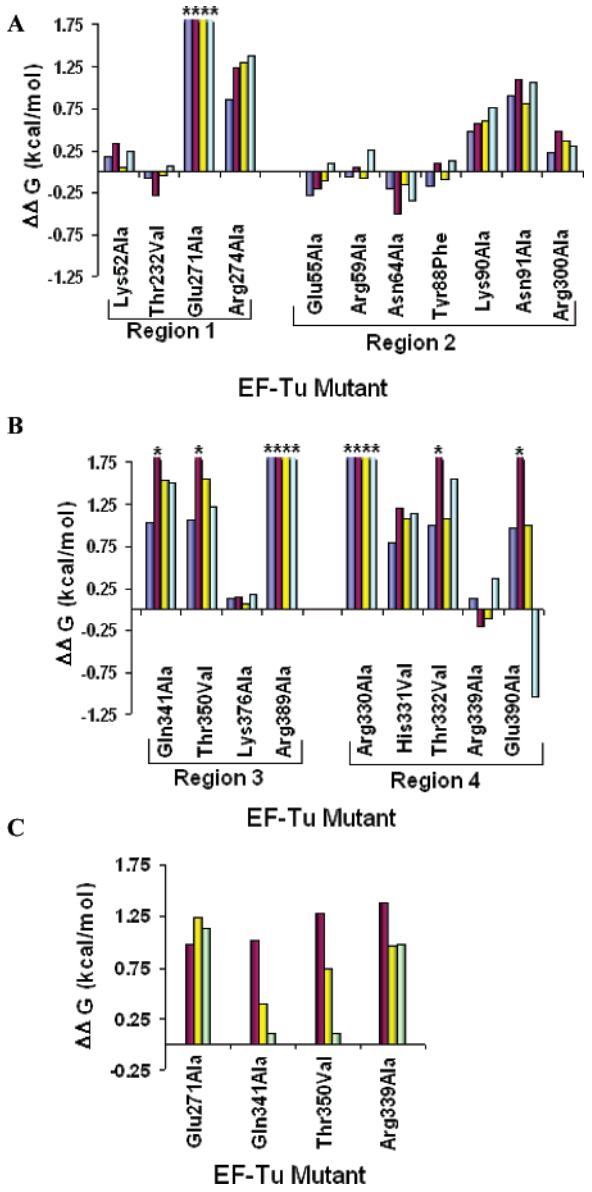

FIGURE 2: Graphical representation of tRNA binding data in Table 1. $\Delta\Delta G°$ graph of EF-Tu mutations in (**A**) regions 1 and 2 and (**B**) 3 and 4 binding to four different aa-tRNAs at 0.5 M NH_4Cl, 20 mM MgCl2, 50 mM HEPES pH 7.0 at 4 °C. (**C**) $\Delta\Delta G°$ graph of weak binding EF-Tu mutants binding three aa-tRNAs at 50 mM NH_4Cl. Asterisk represents $\Delta\Delta G°$ effects beyond the range of the assay (>1.8 kcal/mol). Yeast Phe-$tRNA^{Phe}$ (blue), *E. coli* Glu-$tRNA^{Glu}$ (red), *E. coli* Phe-$tRNA^{Phe}$ (yellow), *E. coli* Tyr-$tRNA^{Tyr}$ (green).

the N2 exocyclic amine group of the guanine in either the C51-G63 base pair of yeast $tRNA^{Phe}$ or the G51-C63 base pair of *E. coli* $tRNA^{Cys}$ (Figure 4A and Figure 4B). Thus the positive $\Delta\Delta G°$ observed for the Glu390Ala mutation with $tRNA^{Glu}$ and both $tRNA^{Phe}$ is consistent with the loss of this stabilizing hydrogen bond. However, it is less clear why the $\Delta\Delta G°$ value differs by nearly 2-fold between $tRNA^{Glu}$ and yeast $tRNA^{Phe}$, which have exactly the same G51-C63 base pair. Perhaps the position of this base pair is subtly influenced by the fact that the two flanking base pairs are U-A in yeast $tRNA^{Phe}$ and G-C in $tRNA^{Glu}$. Since $tRNA^{Tyr}$ lacks an exocyclic amino group in the minor groove of the 51-63 base pair, it does not exhibit a positive $\Delta\Delta G°$ for the Gln390Ala mutation and presumably derives little binding energy from Glu390. Indeed, based on the negative $\Delta\Delta G°$ observed for the Glu390Ala mutation, it appears that the presence of a glutamate at position 390 actually weakens the binding of $tRNA^{Tyr}$. Thus, not unexpectedly, it appears that this sole base-specific contact in the EF-Tu−tRNA interface is an important contributor to the tRNA sequence specificity.

The differential $\Delta\Delta G°$ values that are observed for the Glu341Ala and Thr350Val mutations among the four tRNAs indicate that Gln341 and Thr350 both contribute to the tRNA specificity of EF-Tu. This is a clear case of "indirect readout" since Gln341 and Thr350 appear to only contact the phosphodiester backbone near residues 64 and 65. Since all four of the test tRNAs have a different combination of base pairs at positions 49-65 and 50-64, small difference in the positioning of the backbone due to the effect of neighboring nucleotides may account for the variable $\Delta\Delta G°$ values and resulting differences in overall tRNA binding affinity. Based on a comparison of the X-ray cocrystal structures of *T. aquaticus* EF-Tu complexed with either yeast Phe-$tRNA^{Phe}$ or *E. coli* Cys-$tRNA^{Cys}$ which have different base pairs at 49-65 and 50-64, the actual network of hydrogen bonds may differ as well. As shown in Figure 4C and Figure 4D, the two amino acids only interact with ribose 65 in $tRNA^{Cys}$, but interact with riboses 64 and 65 in $tRNA^{Phe}$. Thus it seems possible that different tRNAs may make a different set of interactions with these two amino acids.

Surprisingly, the remaining two "specificity" amino acids interact with the tRNA at positions where the sequences are identical among the four test tRNAs. The first is Thr332, which appears to interact with the phosphate group that lies between the invariant G53 and the highly conserved G52 in all three of the EF-Tu·aa-tRNA cocrystal structures. The range of $\Delta\Delta G°$ values for the Thr332Val mutation varies by at least 0.7 kcal/mol, but the pattern of effects does not relate to the local sequence in a clear way. The different value of $\Delta\Delta G°$ for yeast Phe-$tRNA^{Phe}$ may possibly be understood by the fact that it contains a U52-A62 base pair. However, the other three tRNAs all contain the same G52-C62 base pair but show varying values of $\Delta\Delta G°$. A similar situation is observed for the final "specificity" residue, Arg274, which forms an ion pair with the 3′ terminal phosphate-76 that is located within the universally conserved CCA terminus of the tRNA. Nevertheless, the Arg274Ala mutation shows a relatively small, but experimentally significant, difference among the four tRNAs tested.

How could amino acids such as Thr332 and Arg274, which interact with regions of the tRNA where the local sequence does not change, contribute to the overall specificity of the interaction? One possible explanation is that the effect is a manifestation of variations in the structure and/or dynamics of the different tRNAs in their unbound form. In this scenario, each of the free tRNAs would have a different conformation that would have to distort in order to bind the protein. If this were the case, the amount that residues would contribute to the specificity would depend on the degree to which they are needed to maintain the distorted state. For example, the four test tRNAs considered here have quite different sets of base pairs at 7-66 and 49-65, the helical junction of the acceptor and T-stems. This could, in turn result in a different angle between the stems in the free

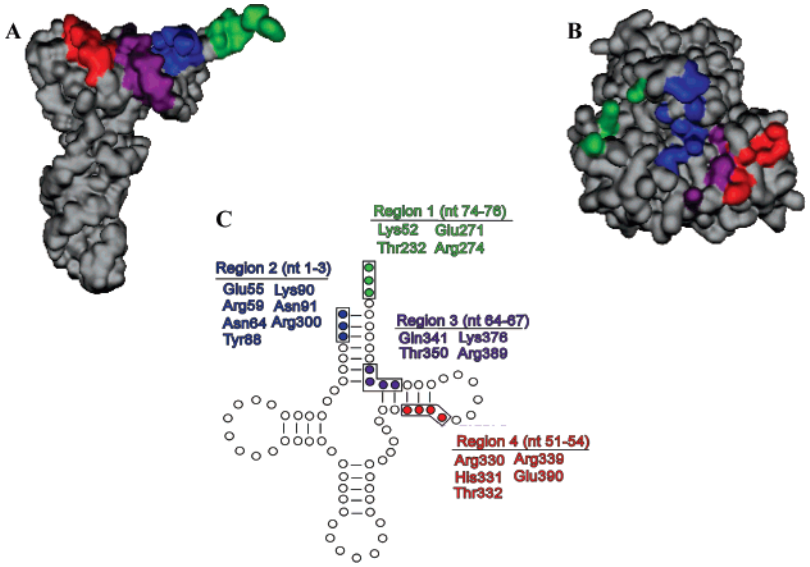

FIGURE 3: Surface representation of EF-Tu·aa-tRNA interaction regions. Region 1 (green), region 2 (blue), region 3 (purple), and region 4 (red) on (**A**) yeast Phe-tRNA^{Phe} and (**B**) *T. aquaticus* EF-Tu. (**C**) A cloverleaf representation of yeast tRNA^{Phe} showing the nucleotides and amino acids of each region.

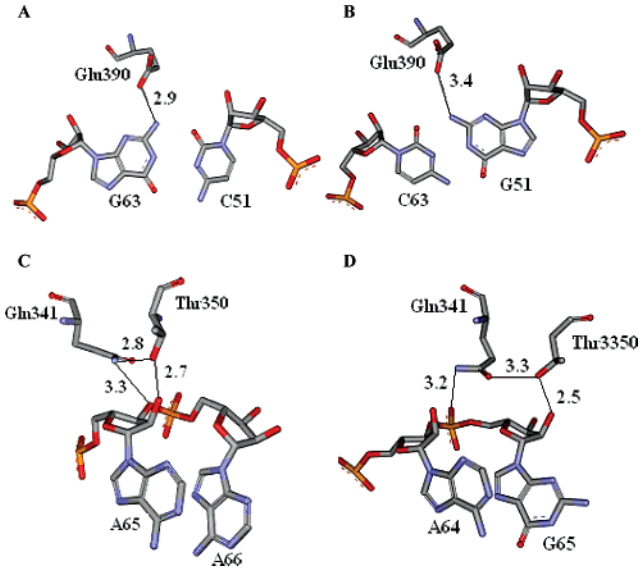

FIGURE 4: Molecular details of "specificity" contacts from co-crystal structures. Interaction of Glu390 with the 51-63 base pair of the tRNA T-stem from (**A**) EF-Tu·GDPNP·Cys-tRNA^{Cys} (PDB: 1B23) and (**B**) EF-Tu·GDPNP·Phe-tRNA^{Phe} (PDB: 1TTT) structures. Interactions of Gln341 and Thr350 with nucleotide 64 and 65 the helical junction of the T-stem from (**C**) the EF-Tu·GDPNP·Cys-tRNA^{Cys} and (**D**) the EF-Tu·GDPNP·Phe-tRNA^{Phe} structures. Black lines indicate distances between functional groups possibly involved in an interaction.

tRNAs that would have to be distorted to different degrees to bind the protein. Thus, it is conceivable that the thermodynamically relevant contacts formed at the ends of the acceptor and T-stems, such as those formed with Arg274 and Thr332, are critical to maintain the distorted conformation. This would lead to a variable $\Delta\Delta G°$ when these residues are mutated even though they interact at conserved positions.

DISCUSSION

By measuring the affinities of a series of EF-Tu mutants to several aa-tRNAs, we have obtained an initial view of how this protein achieves specificity for different tRNA bodies. As is the case for aminoacyl-tRNA synthetases, it appears that specificity is the result of contributions from several sites on the protein that are integrated to give a total binding free energy (*12*, *13*). Five EF-Tu mutations gave a differential response when tested with four different tRNA bodies so the corresponding amino acids are categorized as "specificity" residues. Since only a limited number of tRNAs were tested, it remains possible that additional amino acids will be used to help discriminate among other tRNAs. While one cannot easily relate the $\Delta\Delta G°$ value obtained from a protein mutation to the amount that the corresponding amino acid contributes to the total binding energy, the data suggest that the majority of the specificity amino acids act by stabilizing the protein−tRNA complex by different amounts. However, in one case, a specificity amino acid (Glu390) appears to destabilize the binding of an aa-tRNA (Tyr-tRNA^{Tyr}) to the protein. Thus a tight binding tRNA body appears to have tighter stabilizing contacts and/or fewer destabilizing contacts.

An evaluation of the molecular details of the interactions based on the tRNA sequences and the available X-ray data suggest that there may be two different strategies for how the protein achieves specificity for the tRNA. In the first, the "specificity" amino acids act by interacting differentially to sites on the tRNA where the sequence varies among the tRNAs. This includes one straightforward case where an amino acid side chain (Glu390) interacts with an amino group of a guanine in the minor groove of the 51-63 base pair. Since the sequences of the base pair and hence the presence and/or position of the amino group vary among the tRNAs, the contribution of this contact to the total binding energy is expected to vary. The second example of a "local" specificity contact involves the residues Gln341 and Thr350, which sense sequence differences in the 49-65 and 50-64 base pairs by contacting the phosphodiester backbone of residues 64 and 65. An understanding of how the sequence specificity is achieved by such an "indirect readout" mechanism will probably require additional X-ray crystal structures since the two available structures differ in this region. However, in this case and the previous one, the mechanism of tRNA

sequence specificity by EF-Tu resembles that of tRNA synthetases. A limited number of amino acids contact "recognition" nucleotides that vary among tRNAs. In these cases, the precise rules for recognition can presumably be deduced by measuring the binding affinity of wild type EF-Tu to a series of tRNA mutations.

Although less clearly established, a second strategy for how EF-Tu shows specificity for different tRNAs may depend upon the degree to which the structure and/or dynamics of the free tRNA needs to change in order to bind the protein and form the ternary complex. This need for a common ternary complex structure was first suggested by Janiak et al. based on the different amounts of quenching of a fluorophore attached to the 4-thiouridine at position 8 of different tRNAs (*14*). As discussed in the Results section, variability in the structure of the free tRNAs can explain why amino acid residues in EF-Tu can contribute to specificity even though they contact regions of the tRNA where the sequence does not vary. An additional example of how the structure of the free tRNA can modulate EF-Tu binding affinity may be the 1-72 base pair. Since EF-Tu makes numerous contacts with a closed 1-72 pair, it is not surprising that $tRNA^{fMet}$, which contains a mismatched C1-A72 base pair, binds poorly to EF-Tu (*4*, *15*−*17*). Interestingly, the U1-A72 mutation of $tRNA^{fMet}$ bound EF-Tu less well than the C1-G72 pair (*17*), perhaps because the terminal U-A base pair is partially "frayed" in the free form and part of the protein binding free energy is used to close the base pair. This mechanism may be used for several *E. coli* tRNAs containing A1-U72 or U1-A72 base pairs that are known to bind weakly to EF-Tu (*1*).

In common with most of the amino acids that make up the tRNA binding interface of EF-Tu, the five "specificity" residues identified in this work are highly conserved among bacteria, ranging from >80% for Thr332 and Arg274 to 100% for Gln341, Thr350, and Glu390. This suggests that the majority of bacterial EF-Tu's use the same mechanism to achieve tRNA specificity. Indeed, the specificity of *E. coli* EF-Tu for different *E. coli* tRNAs was found to be similar to that of the *T. thermophilus* protein for the same tRNAs (Alexey Wolfson, unpublished data). However, in contrast to the high sequence conservation of EF-Tu, different bacteria show remarkable diversity in the sequences of the acceptor and T-stems in each individual tRNA species. For example, among the $tRNA^{Tyr}$, sequences from 129 different bacteria, the identity of 10 of the 12 base pairs that compose the EF-Tu binding site varies considerably in sequence. Indeed, the three base pairs (49-65, 50-64, and 51-63) which contact the specificity amino acid residues identified here vary substantially among bacteria for nearly every tRNA species. Thus, while other bacterial EF-Tu's probably use the same amino acids to achieve specificity, it appears that the corresponding tRNAs do not use the same combination of sequences to reach the desired binding free energy. For example, in *E. coli*, the $tRNA^{Val}_{UAC}$ isoacceptor has a G51-C63 base pair while the $tRNA^{Val}_{GAC}$ isoacceptor has a U51-A63 base pair. Based on the data presented here, $tRNA^{Val}_{UAC}$ would be expected to derive more binding free energy from Glu390 than $tRNA^{Val}_{GAC}$. Previous work has shown that the two isoacceptors of *E. coli* $tRNA^{Val}$ bind EF-Tu with similar affinities (*16*), indicating that the thermodynamic contribution of the G51-C63 base pair of $tRNA^{Val}_{UAC}$ must be offset by weaker binding to one or more of its other specificity sites. It is interesting that $tRNA^{Val}_{UAC}$ has a G49-C65 base pair which has been proposed to weaken EF-Tu binding (*1*) while $tRNA^{Val}_{GAC}$ contains G49-U65 which may contribute more to binding. Thus, different sequences of the 49-65 and 51-63 base pairs, both of which contact specificity amino acids, may act together to modulate the binding of tRNA such that both value isoacceptors bind similarly. If this analysis is correct, the large sequence variability for a given tRNA species reflects the fact that the specificity of EF-Tu for tRNA is the result of a summation of contributions from multiple interactions. Since only the total binding free energy of a given tRNA species must have a characteristic value, different organisms use different combinations of sequences to achieve that value.

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