The Affinity of Elongation Factor Tu for an Aminoacyl-tRNA Is Modulated by the Esterified Amino Acid†

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ABSTRACT: When different mutations were introduced into the anticodon loop and at position 73 of YFA2, a derivative of yeast $tRNA^{Phe}$, a single tRNA body was misacylated with 13 different amino acids. The affinities of these misacylated tRNAs for *Thermus thermophilus* elongation factor Tu (EF-Tu)•GTP were determined using a ribonuclease protection assay. A range of 2.5 kcal/mol in the binding energies was observed, clearly demonstrating that EF-Tu specifically recognizes the side chain of the esterified amino acid. Furthermore, this specificity can be altered by introducing a mutation in the amino acid binding pocket on the surface of EF-Tu. Also, when discussed in conjunction with the previously determined specificity of EF-Tu for the tRNA body, these experiments further demonstrate that EF-Tu uses thermodynamic compensation to bind cognate aminoacyl-tRNAs similarly.

Elongation factor Tu (EF-Tu)[1] is the bacterial G protein responsible for delivering all elongator aminoacyl-tRNAs (aa-tRNAs) to the ribosomal A site during translation. When EF-Tu is complexed with GTP, EF-Tu binds cognate elongator aa-tRNAs with similar, high affinities, despite differences in the tRNA sequences and properties of the amino acid side chains (*1*, *2*). Thus, while EF-Tu requires the presence of the esterified amino acid for tight tRNA binding, it has been considered nonspecific with respect to the identity of the aa-tRNA. However, recent experiments have demonstrated that EF-Tu shows high specificity for both the esterified amino acid and the tRNA body. LaRiviere et al. (*3*) found that tRNAs acylated with noncognate amino acids bind EF-Tu over a broad range of affinities, varying from 60-fold weaker to 120-fold tighter than tRNAs acylated with their cognate amino acid. Quantitative analysis of the binding of 4 cognate aa-tRNAs and 12 misacylated tRNAs to EF-Tu revealed that the thermodynamic contributions of the esterified amino acid and the tRNA body are independent of one another and that the affinities are arranged in such a way that cognate aa-tRNAs have a uniform affinity for EF-Tu. Thus, an amino acid that has a strong thermodynamic contribution to the overall affinity is esterified to a cognate tRNA that has a weak thermodynamic contribution, and vice versa. This phenomenon has been termed thermodynamic compensation (*3*).

One implication of thermodynamic compensation is that there is a range of thermodynamic contributions made by the esterified amino acid to the overall affinity of an aa-tRNA for EF-Tu. The available X-ray cocrystal structures of the EF-Tu•GDPNP•Phe-$tRNA^{Phe}$ and EF-Tu•GDPNP•Cys-$tRNA^{Cys}$ complexes show that the side chains of both phenylalanine and cysteine bind in the same pocket on the surface of EF-Tu (*4*, *5*). This suggests that each esterified amino acid will interact with EF-Tu at this site by making slightly different contacts with the pocket, thus resulting in differences in binding affinities of the amino acid for EF-Tu. The range of amino acid affinities could be as large as the 700-fold difference in tRNA affinities that was deduced using 19 different *E. coli* elongator tRNAs esterified with the same amino acid (*6*). The goal of the present paper was to directly test the specificity of *Thermus thermophilus* EF-Tu for the esterified amino acid. This aim was accomplished by misacylating a $tRNA^{Phe}$ variant, YFA2, with a number of different of amino acids and determining the affinities of these aa-tRNAs for EF-Tu. When a constant tRNA body was maintained, any changes in the aa-tRNA binding affinity could be attributed to changes in the amino acid identity. The ability of EF-Tu to interact differentially with the various esterified amino acids was further examined by making a mutation in the amino acid binding pocket of EF-Tu and determining the effect on the specificity of amino acid binding.

EXPERIMENTAL PROCEDURES

tRNA Mutagenesis and Preparation. Variants of the YFA2 gene (*7*) were created using site-directed mutagenesis (QuikChange Site-Directed Mutagenesis Kit, Stratagene). Transcription templates were prepared by PCR amplification of plasmid DNA. In vitro transcription (*8*) was performed in reactions containing 40 mM Tris-HCl (pH 8.0), 10 mM dithiothreitol (DTT), 20 mM $Mg(OAc)_2$, 25 mM NaCl, 2 mM spermidine, 2.5 mM of each NTP, 20 mM GMP, 50 μg/mL bovine serum albumin, and 30 μg/mL T7 RNA polymerase. tRNAs were purified on 10% denaturing polyacrylamide gels.

Aminoacylation Reactions. Aminoacylation reactions were performed with 30 mM Hepes (pH 7.0), 30 mM KCl, 15 mM $MgCl_2$, 5 mM DTT, 4 mM ATP, 2−3 μM tRNA,

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[1] Abbreviations: EF-Tu, elongation factor Tu; aa-tRNA, aminoacyl-tRNA; aaRS, aminoacyl-tRNA synthetase.

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10−25 μM [^{3}H]amino acid (10−100 Ci/mmol), 10 U/mL of inorganic pyrophosphatase (Sigma), 250 nM−2 μM of purified aminoacyl-tRNA synthetase (aaRS), and (in some cases) 20% DMSO. After a 15 min incubation at 37 °C, reactions were quenched with phenol/choloroform (pH 5.5) and subjected to phenol/chloroform and chloroform extractions. Reactions were precipitated with ethanol, the pellet was resuspended in 5 mM NaOAc (pH 5), and the samples were divided into aliquots and stored at −80 °C.

Protein Purification. Histidine-tagged *E. coli* GluRS, ArgRS, and GlyRS were purified from plasmids provided by T. Yokogawa and K. Nishikawa (Gifu University, Japan). A total of 100 mL of BL21 pLysS cells was grown to A_{600} = 0.7, followed by an incubation for 4 h in the presence of 0.5 mM IPTG. Harvested cells were resuspended in 10 mL of 20 mM Tris-HCl (pH 7.6), 1 mM $MgCl_2$, 200 mM NaCl, 10 mM β-mercaptoethanol, 20 mM imidazole, 0.01% Triton X-100, and 5% glycerol (Buffer A), containing 50 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride (ABSF), and lysed by sonication. The lysate was cleared by centrifugation at 10000*g* for 30 min. A total of 1 mL of Ni-NTA Agarose (Qiagen) was added to the supernatant, and the slurry was mixed gently for 1 h at 4 °C. The slurry was loaded onto an empty column, washed twice with 4 mL of Buffer A, and eluted with 2 mL of Buffer A containing 300 mM imidazole. Purified proteins were dialyzed into Buffer A with 50% (v/v) glycerol. Yeast PheRS and *E. coli* AlaRS were purified as described (*7*). Purified *E. coli* GlnRS and ValRS were provided by J. Perona (University of California, Santa Barbara, CA). Purified *Deinococcus radiodurans* AspRS and *Chlamydia trachomatis* LysRS were a gift from D. Söll and G. Raczniak (Yale University, CT). P. Schimmel and L. Nangle (Scripps Institute) provided purified *E.coli* IleRS, MetRS, TrpRS, and the misacylating T222P mutant ValRS (*9*). Purified *Methanococcus jannaschi* ProRS was received from K. Musier-Forsyth (University of Minnesota, MN), as well as from Y. M. Hou and V. Scully (Thomas Jefferson University, PA).

EF-Tu from *T. thermophilus* was overexpressed and purified as described previously (*10*) with the following exception: Ammonium sulfate was slowly added to 30%, and the sample was centrifuged at 13000*g* for 15 min to remove the contaminating protein. Ammonium sulfate was then added to the supernatant to 70% to precipitate the EF-Tu as before.

EF-Tu Assays. EF-Tu stored in its GDP-bound form was converted to its GTP-bound form immediately prior to use. The GTP-bound form of EF-Tu was obtained by incubating 0.5 μM EF-Tu in 50 mM Hepes (pH 7.0), 20 mM $MgCl_2$, 0.5 M NH_4Cl, 5 mM DTT, 20 μM GTP, 0.6−3 mM phosphoenolpyruvate, and 50 μg/mL pyruvate kinase (Buffer B) at 37 °C for 3 h.

Dissociation rate constants of the EF-Tu·GTP·aa-tRNA complex were determined using a modified version of a ribonuclease protection assay (*1*). Ternary complex was formed by incubation of 0.5 μM EF-Tu·GTP with <0.1 μM [^{3}H]aa-tRNA in 100 μL reactions of Buffer B for 20 min on ice. After addition of 10 μL of 0.5−1 mg/mL RNAse A (Sigma), 10 μL aliquots were removed at various times, quenched into 110 μL of ice-cold 10% tricholoracetic acid containing 0.1 mg/mL unfractionated tRNA, and filtered through a nitrocellulose membrane. Samples were washed and counted as described (*10*). Dissociation rate constants were determined as the negative of the slope of a ln(counts per minute) versus time plot (*3*). Dissociation rate constants were measured at least three times, and the mean value and standard deviation for each k_{off} was calculated. K_D values were calculated using the experimental k_{off} and the previously determined value of k_{on}, 1×10^5 M^{-1} s^{-1} (*3*, *11*).

Relative K_D measurements of the misacylated tRNAs Phe-YFA2^{Phe}, Gly-YFA2^{Gly}, Lys-YFA2^{Lys}, and Trp-YFA2^{Trp} were determined using the method described in Pleiss and Uhlenbeck (*10*). Twelve different EF-Tu·GTP concentrations (30 nM−10 μM) in Buffer B at 0 °C were each mixed with ~10 nM [^{3}H]aa-tRNA in 50 μL reactions. After equilibrating the samples for 20 min, 5 μL of 0.1 mg/mL RNAse A (Sigma) was added and quenched 20 s later with 60 μL of 10% trichloroacetic acid containing 0.1 mg/mL unfractionated bulk tRNA. The protein-protected [^{3}H]aa-tRNA was collected on filters and quantified as described previously (*10*).

EF-Tu Mutagenesis. The Glu226Gln mutation of the *T. thermophilus* EF-Tu *tuf1* gene was prepared using site-directed mutagenesis (QuikChange Site-Directed Mutatgenesis Kit, Stratagene) and was confirmed by DNA sequence analysis. Overexpression and purification of the mutant EF-Tu, as well as the determination of its binding affinities to aa-tRNAs, were conducted in the same manner as the wild-type protein.

RESULTS

Variants of Yeast tRNA^{Phe} Can Be Misacylated. The initial task of this paper was to prepare a series of aa-tRNAs that had a similar tRNA body but varied in the identity of the esterified amino acid. The tRNA background chosen for these experiments was YFA2, a yeast tRNA^{Phe} variant containing three nucleotide changes in the acceptor stem that allows it to be recognized by both phenylalanyl-tRNA synthetase (PheRS) and alanyl-tRNA synthetase (AlaRS) (*7*). To confer recognition of various tRNA sythetases onto YFA2, 11 different YFA2^X variants containing mutations in the anticodon loop and at position 73 were prepared (Figure 1 and Table 1). The mutated residues were chosen because they are important identity determinants for most tRNA synthetases (*12*, *13*), and changes at these positions do not affect aa-tRNA binding to EF-Tu (*3*). While changes in the anticodon of YFA2 generally abolished recognition by PheRS, the identity nucleotides for AlaRS recognition were retained. Therefore, each YFA2^X variant was designed to be a dual-specific tRNA that could be aminoacylated by a "cognate" aaRS to form X-YFA2^X and by AlaRS to form Ala-YFA2^X. The availability of the alanylated form of each YFA2^X served as a control to confirm that the mutations did not alter the affinity of the tRNA to EF-Tu.

Using a series of different synthetase concentrations, each YFA2^X was tested for its ability to be aminoacylated by its "cognate" aaRS and by AlaRS. The maximum percentage of aminoacylations obtained in these trial experiments are summarized in Table 1. With the exception of YFA2^{Glu}, all of the tRNAs could be acylated by a purified "cognate" synthetase to at least 10%, which was sufficient to perform EF-Tu binding experiments. It should be noted that many of these YFA2^X variants were relatively poor substrates for

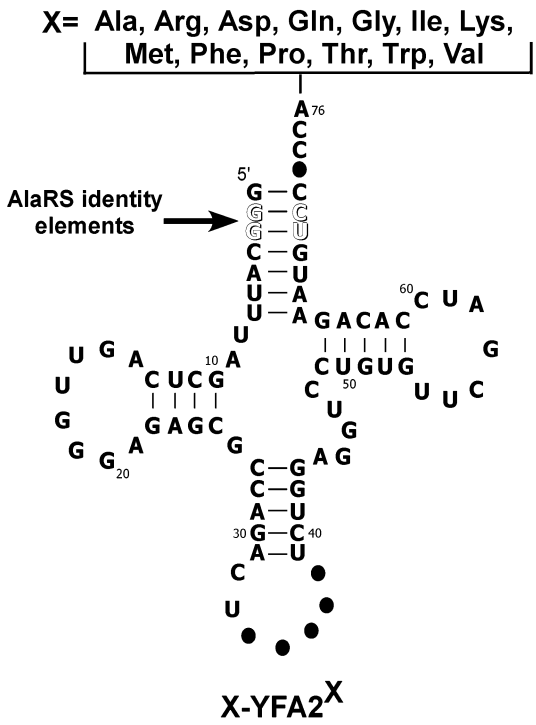

FIGURE 1: General framework of YFA2 tRNA derivatives. Black dots indicate nucleotides that were changed to facilitate recognition of YFA2 by noncognate aa-tRNA synthetases (see Table 1). Each YFA2 variant was named YFA2^X, where "X" describes the newly esterified amino acid. Outlined nucleotides (G2, G3, U70, and C71) are recognized by AlaRs, allowing each YFA2^X tRNA to also be esterified with alanine.

the aaRS when compared to the cognate tRNAs. However, introducing additional mutations into these YFA2^X tRNAs to improve aminoacylation could have affected EF-Tu affinity. The inability to acylate YFA2^{Glu} with GluRS was probably due to the absence of the U13·G22·A46 base triple that has been proposed to be an important element for *E. coli* GluRS recognition (*14*). Most of the variants could be aminoacylated with AlaRS to greater than 10%. The poor activity of YFA2^{Glu} and YFA2^{Gln} was probably due to the presence of G73, because AlaRS prefers an A at position 73 (*15*). Interestingly, however, YFA2^{Asp} contains G73 and was an adequate (though poor) substrate for AlaRS.

Misacylated tRNAs Show a Range of Binding Affinities for EF-Tu. Each aminoacyl-YFA2^X that could be prepared was bound to *T. thermophilus* EF-Tu·GTP in a 0.5 M NH_4Cl buffer at 0 °C, and the dissociation rate constant (k_{off}) was measured using a ribonuclease protection assay. The buffer conditions were chosen to permit comparison with similar studies (*3*, *6*). The measured k_{off} values and a previously reported k_{on} of 1×10^5 M^{-1} s^{-1} (*3*, *11*) were used to calculate K_D and $\Delta G°$ values (Table 2). To test whether the amino acid identity could affect k_{on}, the K_D values of four of the X-YFA2^X variants were also determined directly using a series of EF-Tu·GTP concentrations as described previously (*10*). Because this assay was complicated by requiring an esterified amino acid with a high specific activity and an independent determination of the fraction of active EF-Tu·GTP (*10*), it was not as accurate as the k_{off} determination. Nevertheless, the relative K_D values ($n = 4$) are comparable to the relative k_{off} values, indicating that $k_{on} = k_{off}/K_D$ is unaffected by the amino acid identity (Table 3).

Table 1: Summary of the tRNAs Used in This Paper

tRNA	nucleotides changed from YFA2	aaRS recognition[a]	percent misacylation	percent alanylation
YFA2[b]		PheRS	80	60
YFA2^{Arg}	G34A, A35C, A36G, G37A	ArgRS	25	50
YFA2^{Asp}	A35U, A36C, A38C, A73G	AspRS	25	10
YFA2^{Gln} [c]	G34C, A35U, A36G, G37A, A38U, A73G	GlnRS	15	0
YFA2^{Glu}	G34U, A35U, A36U, G37A, A73G	GluRS	0	0
YFA2^{Gly}	A35C, A36C, A73U	GlyRS	20	60
YFA2^{Ile}	A36U	IleRS	15	70
YFA2^{Lys}	G34C, A35U, A36U	LysRS	35	50
YFA2^{Met}	G34C, A36U	MetRS	40	60
YFA2^{Pro}	G34U, A35G, A36G	ProRS	25	35
YFA2^{Thr} [d]	A36C	ValRS	25	60
YFA2^{Trp}	G34C, A35C	TrpRS	30	40
YFA2^{Val} [e]	A36C	ValRS	30	60

[a] All aminoacyl-tRNA synthetases are from *E. coli* except the following: PheRS, yeast; AspRS, *D. radiodurans*; LysRS, *C. trachomatis*; ProRS, *M. jannaschi*. [b] See ref *7*. [c] Superscripted names of YFA2 tRNAs indicate the amino acid esterified to that tRNA (see Figure 1). All tRNAs were also recognized by *E. coli* AlaRS, except YFA2^{Gln} and YFA2^{Glu}. [d] Contains the anticodon nucleotides recognized by ValRS and was misacylated with threonine using a ValRS containing a mutation in its editing site (*9*). [e] See ref *3*.

Table 2: Dissociation Constants and $\Delta G°$ Values for Misacylated and Alanylated YFA2^X tRNAs at 0.5 M NH_4Cl

	X-YFA2^X		Ala-YFA2^X	
YFA2^X	K_D (nM)	$\Delta G°$ (kcal/mol)[a]	K_D (nM)	$\Delta G°$ (kcal/mol)[a]
YFA2^{Arg}	17	−9.8	120	−8.8
YFA2^{Asp}	>150[b]	<−8.6	50	−9.3
YFA2^{Phe}	11	−10.1	100	−8.9
YFA2^{Gln}	1.9	−11.1	n.d.[c]	n.d.
YFA2^{Gly}	62	−9.1	70	−9.1
YFA2^{Ile}	8.1	−10.3	100	−8.9
YFA2^{Lys}	35	−9.4	70	−9.1
YFA2^{Met}	17	−9.8	120	−8.8
YFA2^{Pro}	15	−9.9	110	−8.8
YFA2^{Thr}	15	−9.9	120	−8.8
YFA2^{Trp}	3.6	−10.7	90	−8.9
YFA2^{Val}	17	−9.8	120	−8.8

[a] $\Delta G°$ values were calculated using the equation $\Delta G° = -RT\ln(1/K_D)$. [b] 150 nM is the upper limit of the assay (see text). [c] n.d. = not determined because of insufficient aminoacylation (see Table 1).

Thus, the differences in the free energy values in Table 2 for the different X-YFA2^X variants are due to the amino acid identity.

Table 2 also shows the calculated K_D values and the free energy values for each of the Ala-YFA2^X tRNAs that serve as controls. Because the goal of this paper was to keep the tRNA constant and vary only the amino acid, it was crucial that tRNA recognition by EF-Tu be unaffected by the mutations made in YFA2 at the anticodon and position 73. All of the alanylated YFA2^X tRNAs bound EF-Tu with similar affinities, indicating that the mutations made in YFA2

Table 3: Relative k_{on} Values Calculated for Four X-YFA2^{X} tRNAs

X-tRNA^{X}	relative k_{off}	relative K_D	relative k_{on}[a]
Phe-YFA2^{Phe}	1	1	1
Gly-YFA2^{Gly}	7.9	5.6	0.71
Lys-YFA2^{Lys}	5.3	3.2	0.60
Trp-YFA2^{Trp}	0.46	0.33	0.72

[a] Relative k_{on} = (relative k_{off})/(relative K_D).

to facilitate misacylation did not affect its interaction with EF-Tu.

The binding of YFA2 to EF-Tu shows at least an 80-fold range in the K_D values depending on the identity of the amino acid. This range may be even greater because Asp-YFA2^{Asp} dissociates too rapidly from EF-Tu·GTP to be accurately measured at 0.5 M NH_4Cl. Although the affinities of weakly binding aa-tRNAs can often be estimated by extrapolation of the data at lower ionic strengths (*3*, *6*), this was not possible for Asp-YFA2^{Asp} because it showed virtually no dependence of k_{off} on NH_4Cl concentration (data not shown). Therefore, the k_{off} for Asp-YFA2^{Asp} can only be estimated to be less than the assay's limit of $k_{off} = 1$ min^{-1}, which corresponds to a K_D of greater than 150 nM. The next two weakest amino acids are the two smallest, alanine and glycine, which have nearly identical K_D values of 80 ± 20 nM. All other amino acids show tighter binding, which is consistent with their larger size. The tightest amino acid tested is glutamine at 1.9 nM, followed by tryptophan, isoleucine, and phenylalanine.

The Glu226Gln EF-Tu Mutant Shows an Altered Specificity. The specificity of EF-Tu for the identity of the esterified amino acid must be the result of interactions between the amino acid side chain and the amino acid binding pocket on

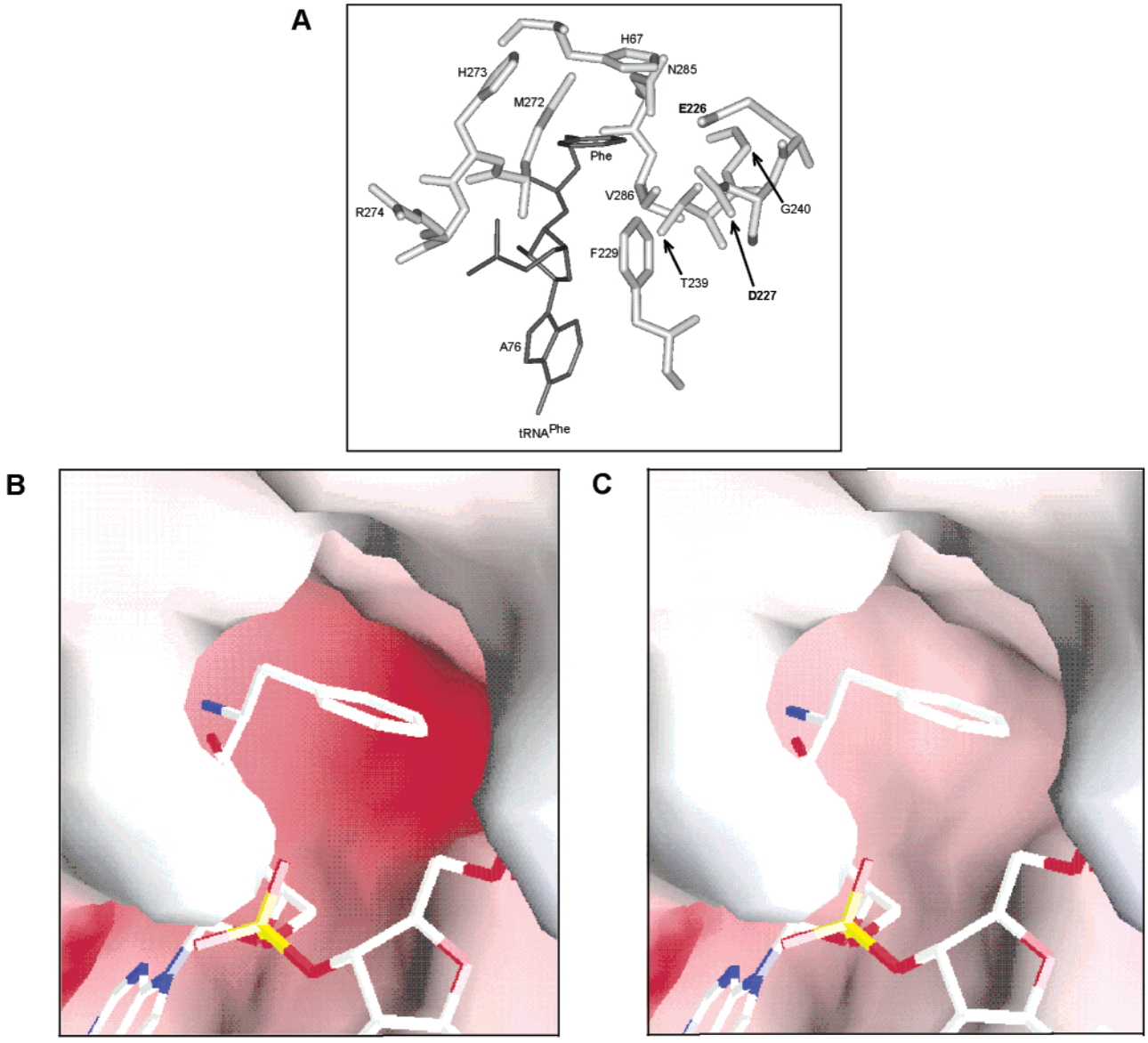

FIGURE 2: (A) EF-Tu binding pocket for Phe-tRNA^{Phe}, taken from Nissen et al. (*4*). Labels for amino acids are placed next to or point to their respective side chain. The back and sides of the pocket consist mostly of hydrophobic residues, while His67 forms the top. The pocket is closed by two basic residues, Lys52 (excluded for clarity) and Arg274. There are two acidic residues, Glu226 and Asp227 (labeled in bold), whose side chains face the pocket. One letter abbreviations: R = Arg, H = His, M = Met, N = Asn, E = Glu, G = Gly, D = Asp, V = Val, T = Thr, F = Phe, and A76 = adenosine 76. (B) A charge surface representation of the amino acid binding pocket of wild-type EF-Tu and (C) the Glu226Gln EF-Tu mutant using GRASP (*33*). Negative charges are represented in red and positive charges, in blue. Phe-tRNA^{Phe} is shown in stick form.

Table 4: Relative K_D Values for the Glu226Gln EF-Tu Mutant Compared to the Wild-Type EF-Tu at 0.5 M NH_4Cl

X-YFA2^X	K_D of wild-type EF-Tu (nM)	K_D mutant/K_D wild-type
Ala-YFA2^{Ala}	100	0.9
Arg-YFA2^{Arg}	17	1.2
Phe-YFA2^{Phe}	11	1.3
Gln-YFA2^{Gln}	1.9	3.4
Gly-YFA2^{Gly}	62	0.9
Ile-YFA2^{Ile}	8.8	2.8
Lys-YFA2^{Lys}	35	0.9
Met-YFA2^{Met}	17	0.7
Pro-YGA2^{Pro}	15	0.8
Thr-YFA2^{Thr}	15	0.9
Trp-YFA2^{Trp}	3.6	2.8
Val-YFA2^{Val}	17	1.0

the surface of EF-Tu. Figure 2A shows the portion of the X-ray structure of EF-Tu·GDPNP·Phe-tRNA^{Phe} containing the esterified phenylalanine and the surrounding amino acids (*4*). The back and sides of the pocket consist primarily of main-chain atoms and hydrophobic side-chain residues, while His67 forms the top of the pocket and stacks on the phenylalanine. The pocket is closed by Lys52 and Arg274, whose charged side chains point away from the pocket and make ion pairs with the tRNA body. The local charge in the pocket is predominantly negative (Figure 2B) as a result of the nearby Glu226 and Asp227, whose side chains face the pocket.

To test whether residues within the amino acid binding pocket could affect the specificity of EF-Tu for the esterified amino acid, the Glu226Gln mutation was created. This mutation was chosen because it was conservative enough to preserve the structure of the pocket while dramatically reducing the local negative charge. The difference between the wild-type and the mutant was confirmed by charge surface representations of the proteins that were calculated assuming the mutation imparted no structural changes on the protein (Figure 2C). The prediction was that the less-negative pocket of Glu226Gln EF-Tu would increase the affinity of negatively charged amino acids and weaken the affinity of amino acids with positively charged side chains.

Using the tight-binding aa-tRNA, Gln-YFA2^{Gln}, the Glu226Gln mutant showed similar aa-tRNA binding properties as wild-type EF-Tu, including a similar active-site titration curve (data not shown). These controls suggest that the Glu226Gln mutation did not dramatically alter the protein structure. Table 4 compares the binding affinities of the Glu226Gln mutant to the wild-type EF-Tu. While most of the aa-tRNAs showed very similar affinities to the two proteins, the Glu226Gln mutant bound Gln-YFA2^{Gln}, Trp-YFA2^{Trp}, and Ile-YFA2^{Ile} approximately 3-fold weaker than wild-type EF-Tu. As with the wild-type protein, the Glu226Gln mutant bound several Ala-YFA2^X tRNAs similarly (data not shown). Thus, the mutant protein shows a narrower range of free energies and a subtly altered amino acid hierarchy (Figure 3).

Because the Glu226Gln mutation was expected to affect the local charge, the binding of charged esterified amino acids was of particular interest. Surprisingly, arginine and lysine both bound the wild-type and mutant proteins similarly, despite the reduction of the negative charge in the amino acid binding pocket. The k_{off} of Asp-YFA2^{Asp} was still too

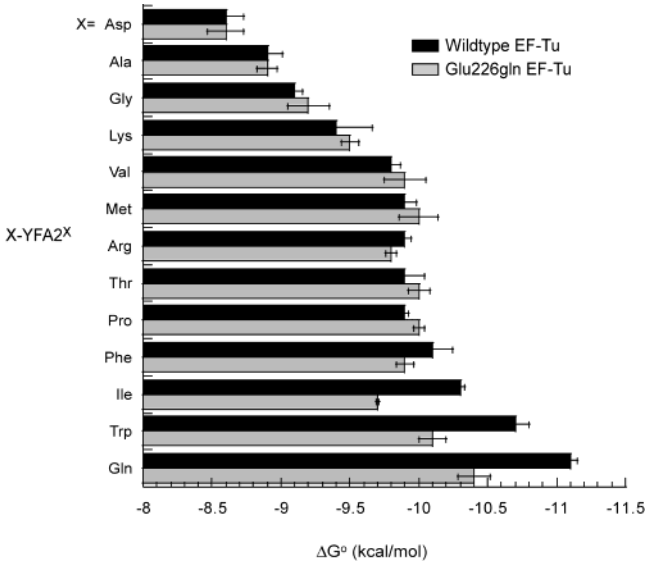

FIGURE 3: Binding energies of X-YFA2^X tRNAs for wild-type EF-Tu (black) and the Glu226Gln mutant EF-Tu (gray). Mutating residue 226 changes the hierarchy of thermodynamic contributions from the esterified amino acid and reduces the total range of binding energies.

fast to measure; therefore, it was not possible to determine whether aspartate binds more tightly to the Glu226Gln EF-Tu compared to the wild-type protein.

DISCUSSION

To determine the thermodynamic contributions of each amino acid to the overall affinity of an aa-tRNA for EF-Tu, it was necessary to esterify a series of different amino acids to the same tRNA body. Although well-developed chemical methods are available to do this (*16*, *17*), they are difficult to adapt to the preparation of [^{3}H]aa-tRNA substrates with the high specific activity needed for the binding assay. Aminoacyl-tRNA synthetases can efficiently produce [^{3}H]aa-tRNAs, but they only recognize their cognate amino acid and tRNA substrates. This complicated their use for the current paper, which required misacylation of a single tRNA body with many different amino acids. The use of mutant synthetases that have decreased specificity for their cognate amino acid side chains (*18*−*20*) is one solution to the problem. However, there are only a limited number of such synthetases, and none are able to utilize a large number of different amino acids. We did, however, make use of the misacylating T222P mutant ValRS (*9*) to introduce threonine onto YFA2^{Val}.

A more practical approach was to mutate a given tRNA in various ways to make it a substrate for different synthetases. However, this approach had the restriction that the mutations must not affect the binding to EF-Tu. Because we knew that EF-Tu has significant specificity for different tRNA bodies (*6*) but did not understand how that specificity is achieved, it was important to restrict the mutagenesis to the anticodon and position 73, which are regions of the tRNA currently known not to affect EF-Tu affinity when mutated (*3*). While this restriction clearly limits the ability of certain synthetases to efficiently misacylate a tRNA, the use of high concentrations of purified synthetases made it possible to obtain sufficient quantities of the YFA2 body esterified with 13 different amino acids. Only one of the attempted amino

acids, glutamate, could not be successfully esterified onto YFA2. Of the remaining six amino acids, three required tRNA modifications that were expected to affect EF-Tu binding: SerRS recognizes a long variable arm in the tRNA (*21*), HisRS requires an extra guanosine at the −1 position (*22*), and a major determinant for yeast TyrRS is a C1-G72 base pair (*23*). In addition, experiments on cysteine, asparagine, and leucine were not conducted because either the tritiated amino acid or the synthetase was unavailable. Similar experiments with one or more additional tRNA bodies would be required to analyze the contributions of the untested seven amino acids.

The binding free energy of *T. thermophilus* EF-Tu·GTP to the YFA2^{X} tRNAs esterified with 13 different amino acids varied from less than −8.6 to −11.1 kcal/mol. This range of 2.5 kcal/mol clearly shows that EF-Tu gains substantial binding energy from the amino acid side chain. Indeed, if one uses the free energy value of −7.1 kcal/mol estimated for deacylated tRNA^{Phe} binding to EF-Tu (*24*) and the observed value of −9.1 kcal/mol for Gly-YFA2^{Gly}, then the tight binding Gln-YFA2^{Gln} ($\Delta G° = -11.1$ kcal/mol) can be estimated to derive about 2 kcal/mol from the glutamine side chain and an additional 2 kcal/mol from the remainder of the esterified amino acid. Thus, the contribution of 4 kcal/mol for an esterified glutamine would be about 40% of the total binding energy of the cognate Gln-tRNA^{Gln} ($\Delta G° = -10.1$ kcal/mol).

The data obtained here agree well with a number of similar experiments that compared the affinity of EF-Tu to a tRNA body acylated with its cognate and a single noncognate amino acid. Studies by Pingound and Urbanke (*25*) and Wagner and Sprinzl (*26*) indicate that Phe-tRNA^{Lys} binds *E. coli* EF-Tu 5-fold more tightly than Lys-tRNA^{Lys}, which agrees well with our observation that Phe-YFA2^{Phe} binds 3.5-fold more tightly than Lys-YFA2^{Lys}. Also, Knowlton and Yarus (*27*) found that Gln-*su*$^{+}$*7* tRNA bound EF-Tu 1.5-fold tighter than Trp-*su*$^{+}$*7* tRNA, which agrees with the 2-fold difference observed between Gln-YFA2^{Gln} and Trp-YFA2^{Trp}. Finally, Becker and Kern (*28*) and Stanzel et al. (*29*) observed that misacylated Asp-tRNA^{Asn} and Glu-tRNA^{Gln} did not form stable complexes with EF-Tu, while the cognate Asn-tRNA^{Asn} and Gln-tRNA^{Gln} did. This suggests that aspartate and glutamate make much weaker contributions to EF-Tu binding than asparagine and glutamine. We confirm here that glutamine is indeed a very "tight" amino acid and aspartate a very "weak" one, but we were unable to obtain quantitative information on glutamate and asparagine.

In general, our demonstration of the variable thermodynamic contributions of the different amino acids esterified to a common tRNA body nicely complements the studies completed by Asahara and Uhlenbeck (*6*), which measured the range of thermodynamic contributions of the different tRNA bodies esterified with a common amino acid, valine. As shown in Figure 4, the 3.6 kcal/mol range of free energy values observed among the 19 elongator tRNA bodies is slightly greater than the 2.5 kcal/mol range of free energy values measured here. However, the contributions of one potentially strong amino acid, tyrosine, and one potentially weak amino acid, glutamate, were not measured, and the weakest aa-tRNA, Asp-YFA2^{Asp}, bound too weakly to measure accurately. Thus, the overall range of free energy values for the amino acids may be greater than 2.5 kcal/mol.

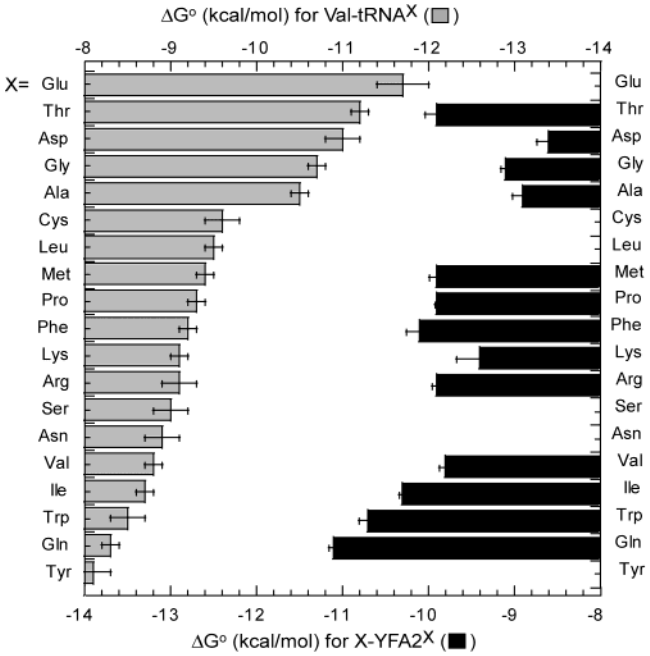

FIGURE 4: Comparison of the tRNA body and amino acid binding energies for *T. thermophilus* EF-Tu. Gray bars depict binding energies of different *E. coli* tRNA bodies acylated with valine, taken from Asahara and Uhlenbeck (*6*). Black bars show the binding energies of the misacylated YFA2^{X} tRNAs from this paper. In general, a "weak" binding tRNA body is matched to a "strong" binding amino acid, and vice versa.

The two sets of data defining the amino acid and tRNA contributions also strongly support the idea of thermodynamic compensation outlined in LaRiviere et al. (*3*). As depicted in Figure 4, the relative thermodynamic contributions made by the amino acids determined here are, in general, inversely related to the contributions of their respective cognate tRNA bodies. Thus, the weakly binding amino acids, such as aspartate, alanine, and glycine, have correspondingly tight binding cognate tRNA bodies, while the strongly binding amino acids, such as glutamine and tryptophan, have weakly binding tRNA bodies. As a result, tRNAs acylated with their cognate amino acid all bind within a small range of K_D values; however, tRNAs acylated with noncognate amino acids can either bind very strongly or very weakly. A more thorough analysis of the implications of thermodynamic compensation for EF-Tu and all 400 possible aa-tRNAs will be published elsewhere.

It appears from Figure 4 that there is an exception to thermodynamic compensation. On the basis of the experiments presented here, threonine is of intermediate affinity; therefore, one would expect tRNA^{Thr} to display a weaker affinity than that determined by Asahara and Uhlenbeck (*6*). However, it is likely that the relatively high affinity of tRNA^{Thr} is a result of using *E. coli* tRNA^{Thr} with *T. thermophilus* EF-Tu. Indeed, *T. thermophilus* tRNA^{Thr} binds *T. thermophilus* EF-Tu much less well than its *E. coli* counterpart, indicating that the concept of thermodynamic compensation holds in homologous systems (Wolfson and Asahara, unpublished results).

It is of interest to understand why the amino acids make such different energetic contributions to the overall binding energy of aa-tRNAs to EF-Tu. Alanine and glycine may bind weakly because of their small side chains, while valine and

threonine are larger and bind more tightly. Large, nonpolar amino acids, such as tryptophan and phenylalanine, probably bind tightly because of the hydrophobic nature of the pocket and the availability of His67 for stacking. The weak binding of acidic aspartate can be understood given the overall negative charge in the pocket. Last, glutamine may gain additional binding energy from some specific interaction with the binding pocket.

There are some amino acids that make unexpected contributions to EF-Tu binding. For example, positively charged lysine and arginine have intermediate binding energies, irrespective of a negatively charged pocket. Proline also has an intermediate binding energy, despite the loss of the putative hydrogen bond between the α-amino group of the amino acid and a main-chain amide in the pocket (*4*, *5*). In such cases, it may be pertinent to consider that there are likely to be structural and energetic differences between the EF-Tu-bound form of a given aa-tRNA and the same aa-tRNA free in solution. When an aa-tRNA is bound to EF-Tu, the esterified amino acid and the 3′ terminal adenosine each have their own binding pocket on the surface of EF-Tu, resulting in the physical separation of the amino acid from the terminal adenosine and the rest of the tRNA (*4*, *5*). However, in the absence of EF-Tu, the esterified amino acid may interact with the tRNA. Experiments using tRNAs containing a fluorescent 3′-terminal formycin residue suggest that the amount of stacking of the terminal adenosine onto the rest of the tRNA is influenced by the presence of the esterified amino acid (*30*). Further, it is possible that charged amino acids, such as lysine, interact with the tRNA by forming a salt bridge with the phosphate backbone. For the esterified lysine to bind EF-Tu, this salt bridge would have to be broken, and an energetic penalty would be paid. Binding energy would then be gained upon interaction of the esterified lysine with the amino acid binding pocket on EF-Tu. Thus, the thermodynamic values presented in Table 2 include both the differences in free energies of the free and bound conformations of the aa-tRNA, as well as the free energy change associated with the binding of the amino acid to EF-Tu.

The amino acids in EF-Tu that form the binding pocket for the esterified amino acid are highly phylogenetically conserved, suggesting that there may be strong selective pressure to maintain differential binding of amino acids. If this is true, certain mutations in the binding pocket could affect the amino acid specificity of EF-Tu. The results in Table 4 and Figure 3 show that Glu226Gln is an example of such a mutation. Compared to the wild-type protein, the binding affinities of esterified glutamine, tryptophan, and isoleucine are weaker for the Glu226Gln mutant. While it is difficult to explain why these particular amino acids are affected, these results show that even a conservative mutation in the binding pocket can affect the amino acid hierarchy and the total range of binding affinities.

Interestingly, the affinities of wild-type EF-Tu for lysine and arginine were similar compared to the Glu226Gln mutant, despite an anticipated reduction in the negative charge of the amino acid binding pocket. Either the basic residues do not interact with Glu226, or the pocket is flexible enough to compensate for any lost binding energy. Small movements of this pocket appear likely based on the structural differences between free EF-Tu·GDPNP, EF-Tu·GDPNP·Phe-tRNA^{Phe}, and EF-Tu·GDPNP·Cys-tRNA^{Cys} (*4*, *5*, *31*). Modeling studies conducted by Nissen et al. (*32*) also suggest that minor rearrangements in the binding pocket may occur to accommodate each of the esterified amino acids. A search for additional examples of hierarchy mutations in the amino acid binding pocket of EF-Tu and an examination of their physiological properties are interesting areas for future research.

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