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37. For *BSR4*, the GenBank accession numbers are: AF394603 for RH, AF394604 for PRU, and AF394605 for CEP. For *SAG3*, the GenBank accession numbers are: AF340227 for RH, AF340228 for PRU, and AF340229 for CEP.
38. PCR amplification of all 15 single-copy, polymorphic loci was performed as described (*8*). Briefly, 35 reaction cycles consisting of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min was carried out using 1 μl of parasite DNA as template (~10^4 parasite equivalents). For the BSR4 primers, the annealing temperature was 52°C instead of 60°C. The majority of primers were selected to amplify the coding regions of the respective genes used in this study. The primers utilized for amplification are as follows (all sequences in 5′-3′ orientation): *SAG1*, Forward (F): CAATGTGCACCTGTAGGAAGC, Reverse (R): GTGGAATTCCTTTGTCGATTTGAG; *SAG2*, F: GAAATGTTTCAGGTTGCTGC, R: AACGTTTCACGAAGGCACA; *SAG3*, F: GACGAATTCACGAGGGAGCTTGCT, R: GCGCGTTGTTAGACAAGACA; *SAG4A*, F: TACGATTTCAAGAAGGCGCT, R: GTCTCGAGCTTCGCAGCATGATACA; *SAG4B*, F: CGTCCTGGTACTCAACGACG, R: GCCAACCGCAGTCGATTGGT; *SRS1*, F: TCAAGGCATTGTGCGTGACC, R: TGTCCACTCACGTACCGAAA; *SRS2*, F: CGAGAATGGCGACGCGTGCGTCTT, R: TTCCCCACTCAATAGGCAAGT; *SRS3*, F: CACAACGCGAAATGCGCTTA, R: CACATATTGCCATCACGCAT; *SRS4*, F: CTTTCTGGCCTGGTGTTTGT, R: TGCGGATCCCGTCTGGACAGCTGAAAATT; *BSR4*, F: GACTACTCGAGGGACGGT, R: CCCAAGGAACTAACAATGA; *GRA1*, F: CGGTTTGCTTGTGTTGTTTG, R: CATGGGGTACGATCACAACA; *GRA2*, F: CCTGCGAACTGATGACAGAA, R: CGGCTTTGTAGACCTTCAGC; *GRA3*, F: TACGCGTCGAGTAACCAGTG, R: AGAGACTGGCACGATGCTTT; *GRA4*, F: GGAACATGTAGCGTCCACTG, R: AATCGCATGCAACGTAACAG; *ROP1*, F: GCGATATGGCTTGTCGTCAG, R: TTAACTTCGGAGGACCCCGC.
39. PCR products were gel-purified from low-melt agarose gels followed by recovery on glass beads using the UltraClean 15 DNA Purification Kit (MoBio Labs, Inc.). Sequencing was performed at the Stanford PAN Facility on 0.5 to 1 μg of purified DNA using 2 pmol of the forward or reverse PCR primers.

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Uniform Binding of Aminoacyl-tRNAs to Elongation Factor Tu by Thermodynamic Compensation

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Elongation factor Tu (EF-Tu) binds all elongator aminoacyl–transfer RNAs (aa-tRNAs) for delivery to the ribosome during protein synthesis. Here, we show that EF-Tu binds misacylated tRNAs over a much wider range of affinities than it binds the corresponding correctly acylated tRNAs, suggesting that the protein exhibits considerable specificity for both the amino acid side chain and the tRNA body. The thermodynamic contributions of the amino acid and the tRNA body to the overall binding affinity are independent of each other and compensate for one another when the tRNAs are correctly acylated. Because certain misacylated tRNAs bind EF-Tu significantly more strongly or weakly than cognate aa-tRNAs, EF-Tu may contribute to translational accuracy.

Elongation factor Tu (EF-Tu) is a guanine nucleotide binding protein that, when complexed with guanosine 5′-triphosphate (GTP), binds elongator aminoacyl-tRNAs (aa-tRNAs) and participates in the early steps of codon-directed peptide bond formation catalyzed by the ribosome. Tight binding of a tRNA by EF-Tu requires the presence of a cognate amino acid esterified to its 3′ terminus by the appropriate aminoacyl–tRNA synthetase (aaRS) (*1*). EF-Tu is generally thought of as a nonspecific binding protein because it binds every elongator aa-tRNA with approximately the same affinity (*2–4*), despite a wide diversity of tRNA sequences, as well as substantial differences in the size, charge, and hydrophobicity of the esterified amino acid. However, EF-Tu binds certain tRNAs esterified with a noncognate amino acid quite differently than it binds the corresponding correctly aminoacylated tRNA. For example, the Su^{+7} suppressor tRNA binds *Escherichia coli* EF-Tu about threefold as tightly when misacylated with glutamine than when correctly acylated with tryptophan, possibly explaining why glutamine is introduced at amber codons more efficiently than tryptophan (*5*). Another notable example comes from the large number of microorganisms that lack either AsnRS or GlnRS and instead use a nondiscriminating AspRS or GluRS to misacylate $tRNA^{Asn}$ or $tRNA^{Gln}$ followed by a tRNA amidotransferase to synthesize Asn-$tRNA^{Asn}$ and Gln-$tRNA^{Gln}$ (*6*). Two groups have shown that the misacylated Asp-$tRNA^{Asn}$ and Glu-$tRNA^{Gln}$ intermediates in this pathway do not bind to EF-Tu, potentially explaining why misincorporation of aspartate and glutamate does not occur in these organisms (*7*, *8*). These results suggested that the correct combination of amino acid and tRNA body are required for efficient EF-Tu binding, although how this specificity is achieved remained unclear.

To further explore the contribution of the amino acid and the tRNA body to EF-Tu binding, variants of *E. coli* $tRNA^{Ala}$, $tRNA^{Val}$, $tRNA^{Gln}$, and yeast $tRNA^{Phe}$, lacking modified nucleotides, were each aminoacylated with alanine, valine, glutamine, and phenylalanine, creating 4 cognate aa-tRNAs and 12 misacylated aa-tRNAs (*9*). Because

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tRNAs are generally quite difficult to misacylate in vitro, one or more identity nucleotides for noncognate aaRSs were introduced into each tRNA to facilitate misacylation. Mutations were limited to regions of the tRNA known not to affect the interaction with EF-Tu, but allowed the tRNAs to be misacylated, in some cases by two or even three different aaRSs (Table 1).

Two versions of a ribonuclease (RNase) protection assay were used to determine equilibrium dissociation constants (K_d's) for the 16 different aa-tRNAs to *Thermus thermophilus* EF-Tu · GTP (*10*). First, the fraction of [^{3}H]aa-tRNA protected from rapid RNase A digestion was measured as a function of EF-Tu concentration and the K_d was determined (Fig. 1A). Second, the fraction of [^{3}H]aa-tRNA protected was measured as a function of time after RNase A addition, allowing the determination of k_{off}, the rate of release of aa-tRNA (Fig. 1B). An excellent correlation between the two assays was observed for many aa-tRNAs, suggesting a constant association rate constant, k_{on}, and allowing calculation of K_d's from k_{off} measurements (*11*). In agreement with earlier studies (*12*, *13*), the four unmodified tRNAs esterified with their cognate amino acid bound EF-Tu with K_d values similar to those of the corresponding fully modified tRNAs [Web table 1 (*14*)]. Control experiments also confirmed that the mutations introduced to facilitate misacylation did not affect the K_d for binding to EF-Tu when the tRNA was aminoacylated with the cognate amino acid [Web table 2 (*14*)]. Thus, the absence of modified nucleotides and the presence of identity mutations do not influence the comparison of EF-Tu binding to four different tRNAs, each esterified with four different amino acids.

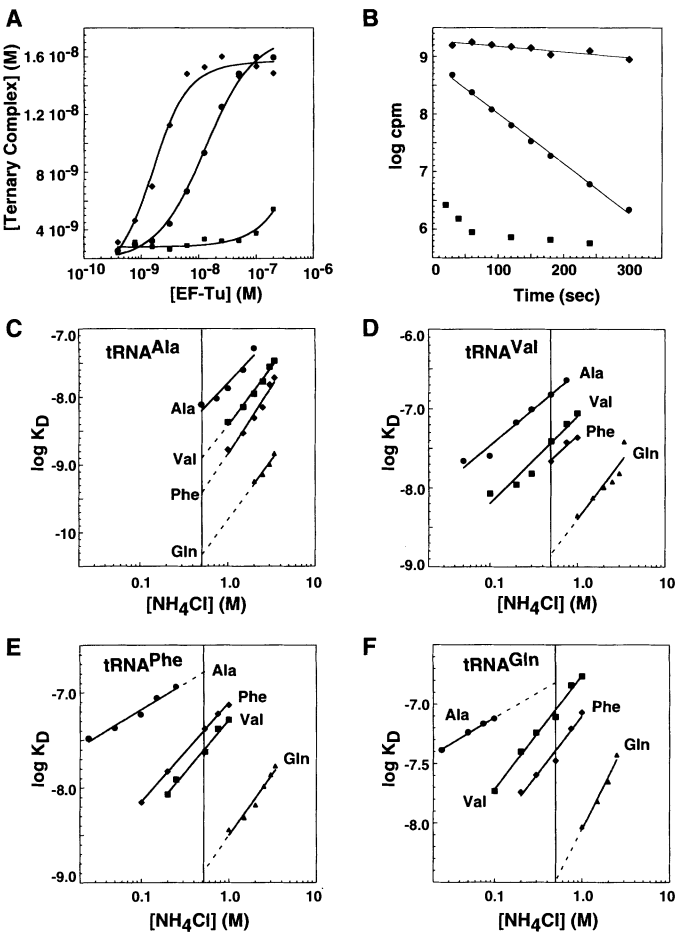

Fig. 1. Representative equilibrium binding curves (**A**) and representative dissociation rate curves (**B**) for *T. thermophilus* EF-Tu to (◆) Phe-CAF, (●) Ala-CAF, or (■) Ala-YFA2 in 0.5 M NH_4Cl at 4°C and pH 7.0. Plots of log[NH_4Cl] versus log[K_d] for *T. thermophilus* EF-Tu at 4°C with (**C**) $tRNA^{Ala}$, (**D**) $tRNA^{Val}$, (**E**) $tRNA^{Phe}$, and (**F**) $tRNA^{Gln}$, each aminoacylated with alanine, valine, phenylalanine, and glutamine, as indicated. Dashed lines indicate extrapolation to 0.5 M NH_4Cl.

When the 12 misacylated tRNAs were assayed under standard conditions, many of the affinities were either too weak or too tight to be accurately determined (Fig. 1, A and B). To address this problem, we determined K_d values for all 16 aa-tRNAs as a function of NH_4Cl concentration. In each case, log K_d increased linearly with increasing log[NH_4Cl] over a range of ionic strengths where accurate data could be obtained (Fig. 1, C to F). The slopes of log K_d versus log[NH_4Cl] plots were similar for each tRNA, regardless of the esterified amino acid, as expected for competition between the protein and the NH_4^+ ions for the same tRNA body (*15*). To compare the affinities of the 4 cognate and 12 misacylated tRNAs under a single set of conditions, we obtained the K_d for each aa-tRNA in 0.5 M NH_4Cl at 4°C by extrapolation of its log K_d versus log[NH_4Cl] plot. For several of the aa-tRNAs that bound tightly, K_d values were also determined at a series of higher temperatures in 0.5 M NH_4Cl. The resulting linear van't Hoff plots permitted extrapolation of a K_d at 4°C [Web fig. 1 and Web table 3 (*14*)].

K_d values for the binding of EF-Tu to each of the 16 aa-tRNAs are presented in Fig. 2. As had been previously observed for the modified tRNAs (*3*), the four cognate aa-tRNAs bound EF-Tu within a relatively narrow, 10-fold range of affinities. A much larger, 5000-fold range of affinities was observed among the 12 misacylated tRNAs, including aa-tRNAs that bind much more tightly or much more weakly than the cognate aa-tRNAs. Such a broad range is striking, because it even exceeds the 1000-fold difference in K_d between Phe-$tRNA^{Phe}$ and deacylated $tRNA^{Phe}$ (*1*). Furthermore, the contributions of the amino acid side chain

Table 1. Summary of tRNAs used in this study.

tRNA	Mutations	Amino acid specificity
CA0	(*E. coli* $tRNA_2^{Ala}$)	Ala
CA2	G4C, C69G	Ala
CAF*	G35A, C36A	Ala, Phe
CAV*	G34U, G35A	Ala, Val
CAQ*	G34C, G35U, C36G, A73G	Gln
YF0	(Yeast $tRNA^{Phe}$)	Phe
YF90	A73G	Phe
YFA2	C2G, G4C, U69G, C70U, G71C	Phe, Ala
YFV†	A36C	Val, Ala
YFQ†	G34C, A35U, A36G, G37A, A38U, A73G	Gln
CV0	(*E. coli* $tRNA_1^{Val}$)	Val
CVA	C70U	Val, Ala
CVF	U34G	Val, Phe
CVQ	U34C, A35U, C36G, A38U, A73G	Gln
CQ0‡	(*E. coli* $tRNA_2^{Gln}$)	Gln
CQX‡	C34G, U35A, G36C, C70U, G73A	Ala, Phe, Val

*Mutations made in CA2 tRNA background. †Mutations made in YFA2 tRNA background. ‡tRNAs constructed by ligation of chemically synthesized tRNA half molecules (*35*).

and the tRNA body to the overall binding affinity are independent of one another. As shown in Table 2, for each pair of amino acids and tRNA bodies, the sum of the binding free energies of the cognate and noncognate tRNAs were identical. Thus, for each tRNA, binding by an esterified glutamine is the tightest, followed by phenylalanine, valine, and alanine. Likewise, for a given esterified amino acid, binding by $tRNA^{Ala}$ is the tightest, followed by $tRNA^{Val} \cong tRNA^{Phe} > tRNA^{Gln}$. A similar independence of thermodynamic effects for singly deoxy-substituted versions of Ala-YFA2 and Phe-YFA2 binding to EF-Tu was recently demonstrated (*16*). The unexpectedly large 5000-fold range of affinities indicates that EF-Tu displays a substantial specificity for both the esterified amino acid and the tRNA body.

The data in Fig. 2 and Table 2 demonstrate that the nearly uniform binding of the four correctly acylated tRNAs arises from the thermodynamic contributions of the amino acid and the tRNA body compensating one another. Thus, a "tight" amino acid such as glutamine is correctly esterified to the comparatively "weak" $tRNA^{Gln}$, whereas a "weak" amino acid such as alanine is correctly esterified to the comparatively "tight" $tRNA^{Ala}$. As a result, both Gln-$tRNA^{Gln}$ and Ala-$tRNA^{Ala}$ show similar K_d values, whereas the misacylated Gln-$tRNA^{Ala}$ binds much more strongly and Ala-$tRNA^{Gln}$ binds much more weakly. In other words, rather than being a nonspecific aa-tRNA binding protein, EF-Tu instead exhibits considerable specificity for both the amino acid and the tRNA portions of the aa-tRNA. Moreover, the specificities are arranged in a way that "weak" binding of certain tRNAs is compensated by a "tight" binding amino acid, and vice versa, resulting in uniform binding of cognate aa-tRNAs. It should be noted that the data are currently limited to four tRNAs and four amino acids. An important goal is to investigate the remaining 16 amino acids and their corresponding tRNAs with a similar approach.

An initial understanding of the amino acid specificity of EF-Tu is obtained from an examination of the x-ray cocrystal structures of Phe-$tRNA^{Phe}$ and Cys-$tRNA^{Cys}$ bound to *Thermus aquaticus* EF-Tu · GDPNP (*17*, *18*). The esterified amino acid is located in a spacious pocket containing six highly conserved amino acid side chains, several main-chain groups, and the phosphodiester backbone of the 3′ terminus of tRNA. Both the esterified phenylalanine and cysteine side chains are stacked with His-67, potentially explaining why tRNAs aminoacylated with phenylalanine bind better than valine or alanine. The observed tight binding of glutamine may be due to the formation of a hydrogen bond within the amino acid binding pocket. The overall negative charge of the amino acid binding pocket, as determined by the program GRASP (*19*), predicts weaker binding for aspartate and glutamate and tighter binding for lysine and arginine. An understanding of how distinct tRNA sequences contribute differently to the overall binding energy is less clear. The crystal structures indicate that EF-Tu interacts with tRNA exclusively through backbone contacts with phosphates and 2′-hydroxyl groups of the acceptor and T helices, which are present in all cytoplasmic tRNAs (*17*, *18*). Presumably, the differences in affinity among these four tRNAs are the result of variations in the structure and dynamic properties of the tRNA backbone that arise from the different nucleotide sequences of the acceptor and T helices. A comparison of the $tRNA^{Phe}$ and $tRNA^{Cys}$ cocrystal structures (*17*, *18*) reveals several differences in the contacts made between EF-Tu and the two tRNAs that may result in different affinities. However, because some of the protein · tRNA contacts do not contribute to the overall binding affinity (*16*), it is still unclear which, if any, of these structural differences are important. It is clear that EF-Tu

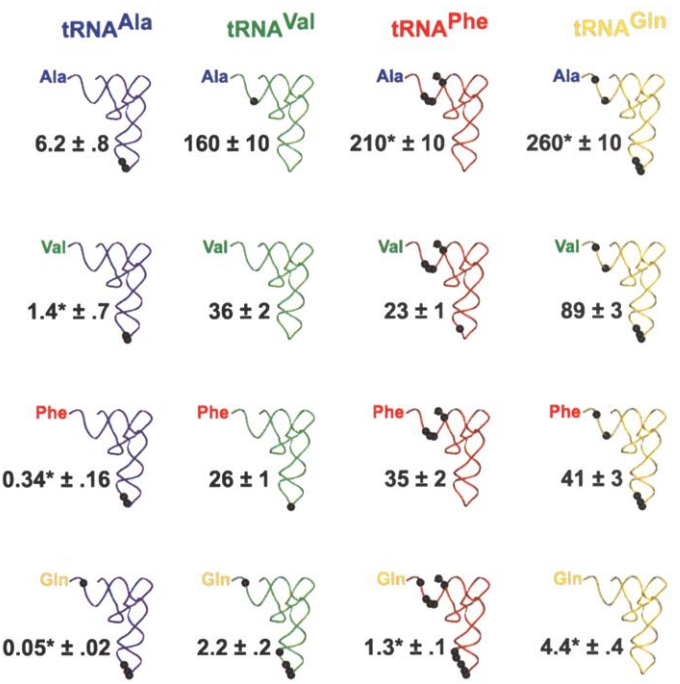

Fig. 2. Equilibrium dissociation constants (10^{-9} M) for 16 aa-tRNAs binding to EF-Tu·GTP in 0.5 M NH_4Cl at 4°C and pH 7.0. tRNAs and amino acids are colored blue (alanine), green (valine), red (phenylalanine), and yellow (glutamine). Black dots indicate positions of identity nucleotide mutations introduced to facilitate misacylation (see Table 1). Asterisks indicate constants determined from extrapolation of salt dependencies in Fig. 1. K_d's are presented as means ± SD from at least three independent experiments.

Table 2. Independent thermodynamic contributions of amino acid and tRNA body. $\Delta G° = -RT\ln(1/K_d)$.

Cognate aa-tRNAs			Misacylated aa-tRNAs		
aa-tRNA	$\Delta G°$ (kcal/mol)	Sum (kcal/mol)	aa-tRNA	$\Delta G°$ (kcal/mol)	Sum (kcal/mol)
Phe-$tRNA^{Phe}$	−9.4		Ala-$tRNA^{Phe}$	−8.4	
		−19.8			−20.4
Ala-$tRNA^{Ala}$	−10.4		Phe-$tRNA^{Ala}$	−12.0	
Val-$tRNA^{Val}$	−9.4		Phe-$tRNA^{Val}$	−9.6	
		−18.8			−19.3
Phe-$tRNA^{Phe}$	−9.4		Val-$tRNA^{Phe}$	−9.7	
Phe-$tRNA^{Phe}$	−9.4		Phe-$tRNA^{Gln}$	−9.4	
		−20.0			−20.6
Gln-$tRNA^{Gln}$	−10.6		Gln-$tRNA^{Phe}$	−11.2	
Val-$tRNA^{Val}$	−9.4		Ala-$tRNA^{Val}$	−8.6	
		−19.8			−19.8
Ala-$tRNA^{Ala}$	−10.4		Val-$tRNA^{Ala}$	−11.2	
Ala-$tRNA^{Ala}$	−10.4		Ala-$tRNA^{Gln}$	−8.3	
		−21.0			−21.4
Gln-$tRNA^{Gln}$	−10.6		Gln-$tRNA^{Ala}$	−13.0	
Val-$tRNA^{Val}$	−9.4		Val-$tRNA^{Gln}$	−8.9	
		−20.0			−19.9
Gln-$tRNA^{Gln}$	−10.6		Gln-$tRNA^{Val}$	−11.0	

achieves specificity for tRNAs through an "indirect readout" mechanism proposed for many DNA-binding proteins (*20*–*22*).

Why has such an elaborate thermodynamic compensation mechanism evolved for the binding of aa-tRNAs by EF-Tu · GTP? Why does EF-Tu not interact equally well with all amino acid side chains and all tRNA bodies? One possibility is that the latter alternative cannot be readily achieved owing to the very different chemical and physical properties of the 20 amino acids and the different tRNA sequences. Thus, the simplest way for a protein to achieve uniform binding is to balance out the inevitably different contributions from distinct parts of the protein·aa-tRNA interface. An additional possibility is that the observed 10-fold difference in K_d values between the four aa-tRNAs is of physiological importance, and thermodynamic compensation has evolved to ensure it. Similar K_d differences observed for modified aa-tRNAs binding to *E. coli* EF-Tu (*3*) are inversely correlated to the relative abundance of aa-tRNAs in *E. coli* (*23*). Because aa-tRNA abundances are in turn correlated with amino acid abundances in proteins, the small K_d differences among aa-tRNAs to EF-Tu may be required to ensure uniform and efficient delivery of all amino acids to the ribosome.

Finally, the substantial specificity of EF-Tu for the amino acid and the tRNA body may have evolved to improve translational accuracy by reducing the delivery of certain misacylated tRNAs to the ribosome. Misacylated tRNAs arise when either a noncognate amino acid or a noncognate tRNA is mistakenly used by an aaRS, although the latter pathway is considered less common owing to competition between aaRSs for tRNAs in vivo (*24*, *25*). Misacylation levels are generally very low because amino acids larger than the correct one are sterically excluded from binding the active site of an aaRS, and amino acids smaller than the correct one are hydrolyzed by a second "editing" site present on many aaRSs (*26*, *27*). From the available data, it appears that those misacylated tRNAs where a small amino acid is esterified to a tRNA that normally carries a larger amino acid (such as Ala-tRNA^{Val} or Ala-tRNA^{Phe}) tend to bind EF-Tu poorly and therefore would rarely be delivered to ribosomes. This would allow EF-Tu to discriminate against those aa-tRNAs that have escaped editing by aaRSs. In addition, the naturally occurring misacylated Glu-tRNA^{Gln} intermediate in the tRNA transamidation pathway may not bind EF-Tu because both tRNA^{Gln} and presumably glutamate are "weak" and binding to EF-Tu is observed only after the amino acid has been converted to the "tight" glutamine. One problem with proposing that EF-Tu has evolved to improve translational accuracy is that a substantial number of misacylated tRNAs bind EF-Tu very tightly and thus should be efficiently recruited to ribosomes and misincorporated into protein. However, because such "tight" misacylated tRNAs generally involve a large amino acid esterified onto a tRNA meant for a small amino acid (such as Phe-tRNA^{Ala}), they are much less likely to be formed by an aaRS. It is also possible that such "tight" misacylated tRNAs are less active in translation because they may release from EF-Tu on the ribosome more slowly after GTP hydrolysis, resulting in less efficient accommodation into the ribosomal A site. Therefore, aa-tRNAs may function effectively in translation only if their affinity to EF-Tu is adjusted to be within a certain range.

Contrary to the general belief, EF-Tu cannot be considered a nonspecific delivery protein because it clearly discriminates between correctly and incorrectly aminoacylated tRNAs through a complex thermodynamic compensation mechanism. Although in the classic experiment of Chapeville *et al* (*28*), alanine was successfully incorporated into globin after reduction of Cys-tRNA^{Cys} to Ala-tRNA^{Cys}, it seems apparent that not all misacylated tRNAs will be active in translation. This complicates the interpretation of many experiments that have been used to evaluate the efficiency of misacylated tRNAs by a suppression assay (*29*, *30*). For example, the tight binding by EF-Tu · GTP that is observed when any of the four tRNAs used in this study are aminoacylated with glutamine may help to explain the frequent misincorporation of glutamine with many suppressor tRNAs (*30*, *31*). In addition, because EF-Tu shows substantial specificity for the amino acid, this complicates the design of in vitro (*32*) and in vivo (*33*) systems to incorporate unnatural amino acids into proteins. Finally, it is also apparent that tRNA molecules should not be thought of as generic adapters (*34*), connecting amino acid and anticodon. Instead, the acceptor and T-stem sequences of each tRNA have evolved to ensure the uniform binding affinity of aa-tRNAs to EF-Tu. It seems quite possible that tRNA sequences are further tuned to ensure uniform use by the ribosome.

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9. CQ0 and CQX tRNAs were constructed by ligation (*35*) of chemically synthesized half tRNAs (Dharmacon Research) with T4 RNA ligase (New England Biolabs). All other tRNAs were prepared by in vitro transcription as described (*16*). Aminoacylated and misacylated tRNAs were prepared in reactions containing 1 to 2 μM tRNA, 30 mM Hepes (pH 7.5), 30 mM KCl, 15 mM $MgCl_2$, 4 mM ATP, 5 mM DTT, and inorganic pyrophosphatase (0.01 U/μl). To this, 20 μM of either [^{3}H]alanine (50 Ci/mmol), [^{3}H]phenylalanine (47 Ci/mmol), [^{3}H]valine (28 Ci/mmol), or [^{3}H]glutamine (53 Ci/mmol) was added with 50 to 500 nM of either purified *E. coli* AlaRS, ValRS, or yeast PheRS. Efficient glutaminylation required from 0.5 to 2 μM *E. coli* GlnRS. Fifteen to 80% aminoacylation was achieved for each tRNA. After aminoacylation, aa-tRNAs were extracted with acidic phenol (pH 4.5), ethanol precipitated, resuspended with 5 mM sodium acetate (pH 5.0), and stored at −70°C.
10. EF-Tu from *T. thermophilus* was overexpressed in *E. coli* and purified as described (*16*). EF-Tu stored in its GDP-bound form was converted to its GTP-bound form immediately before use as described (*12*). Equilibrium K_d's were determined with a modified version of an RNase assay (*2*, *5*) as described (*16*). Dissociation rates (k_{off}'s) were also determined with the RNase assay with the following differences: 0.5 μM EF-Tu · GTP was incubated with 0.05 μM [^{3}H]aa-tRNA in 100 μl for 20 min at 4°C to form ternary complex. After the addition of 10 μl of 0.2 mg/ml RNase A, 10-μl samples were removed at various times (0.5 to 60 min) and quenched into 100 μl of 10% trichloroacetic acid containing 0.1 mg/ml unfractionated tRNA. Samples were washed and analyzed as described (*16*). A semilogarithmic plot of the fraction of aa-tRNA bound to EF-Tu versus time yields the first-order rate constant for the dissociation of aa-tRNA from EF-Tu.
11. All K_d's were either measured directly or calculated from k_{off} measurements with the equation $K_d = k_{off}/k_{on}$, where k_{on} is 1×10^5 M^{-1} s^{-1}.
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