Predicting the Binding Affinities of Misacylated tRNAs for *Thermus thermophilus* EF-Tu·GTP

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ABSTRACT: The free energies for the binding of 20 different unmodified *Escherichia coli* elongator aminoacyl-tRNAs to *Thermus thermophilus* elongation factor Tu (EF-Tu) were determined. When combined with the binding free energies for the same tRNA bodies misacylated with either valine or phenylalanine determined previously [Asahara, H., and Uhlenbeck, O. C. (2002) *Proc. Natl. Acad. Sci. U.S.A. 99*, 3499−3504], these data permit the calculation of the contribution of each esterified amino acid to the total free energy of binding of the complex. The two data sets can also be used to calculate the free energy of binding of EF-Tu to any misacylated *E. coli* tRNA, and the values agree well with previously published experimental values. In addition, a survey of active misacylated suppressor tRNAs suggests that a minimal threshold of binding free energy for EF-Tu is required for suppression to occur.

Elongation factor Tu (EF-Tu) binds GTP and aminoacyl-tRNA (aa-tRNA) to form a ternary complex that subsequently binds ribosomes. Experiments analyzing the thermodynamics of the binding of *Thermus thermophilus* EF-Tu·GTP to four tRNAs each acylated with their cognate and three noncognate amino acids established that the total free energy of binding ($\Delta G°$) was equal to the sum of independent contributions of the esterified amino acid and the tRNA body (*1*). Interestingly, the contribution of the different amino acids and the different tRNA bodies to $\Delta G°$ varied significantly in magnitude, but were arranged in such a way that when tRNAs were esterified with their cognate amino acid, the value of $\Delta G°$ was constant. Thus, when a tRNA body was "tight", its cognate amino acid was "weak", and vice versa. In contrast, misacylated tRNAs varied greatly in binding affinity, with binding ranging from as much as 13-fold weaker to more than 400-fold tighter than that of the average cognate aa-tRNA. This specificity of EF-Tu·GTP for its cognate amino acid and tRNA body was proposed to have evolved to improve the accuracy of translation (*1*).

Two types of experiments were subsequently performed to corroborate that the contributions of the esterified amino acid and the tRNA body to $\Delta G°$ were variable and compensatory for cognate pairs. In the first, we showed that the $\Delta G°$ for EF-Tu·GTP binding to 20 *Escherichia coli* tRNAs esterified with either Val or Phe varied by 3.6 kcal/mol (*2*). These experiments established a hierarchy of affinities of tRNA bodies, varying from $tRNA^{Glu}$ as the tightest to $tRNA^{Tyr}$ as the weakest. Second, the converse experiment was performed by measuring $\Delta G°$ values for binding of EF-Tu·GTP to yeast $tRNA^{Phe}$ esterified with 13 different amino acids (*3*). In this case, the $\Delta G°$ values varied by 2.5 kcal/mol, and the resulting hierarchy of affinities of amino acids was roughly the opposite of that of their cognate tRNAs, with Gln being the tightest amino acid and Asp the weakest. However, the latter experiments were incomplete since the affinity of Asp-$tRNA^{Phe}$ was too weak to measure accurately and it was not possible to misacylate $tRNA^{Phe}$ with the remaining seven amino acids.

In this paper, an alternate approach was used to obtain the relative contributions of 19 of the 20 esterified amino acids to the total free energy of binding of aa-tRNAs to EF-Tu·GTP. The affinities of *T. thermophilus* EF-Tu·GTP for 20 *E. coli* elongator tRNAs esterified within their cognate amino acids were determined. Since the contributions of the amino acid and tRNA body to $\Delta G°$ are independent, these data could be combined with our previous data for the same tRNAs esterified with Val to extract the binding energies of each amino acid relative to Val. The two data sets also permit the calculation of $\Delta G°$ for each of the hundreds of possible misacylated *E. coli* tRNAs. These calculated values could then be compared to previous experimental values for $\Delta G°$ of misacylated tRNAs and provide insight into the ability of misacylated tRNAs to function inside cells.

MATERIALS AND METHODS

Aminoacylation Reactions. Unmodified *E. coli* tRNA transcripts were prepared as described previously (*2*). The numbering of isoacceptors was that from ref *4*. In the case of $tRNA^{Thr}$, the transcript with a GAC anticodon (which corresponds to Val) was used instead of that with the normal sequence.

Aminoacylation reactions were performed with 2 μM tRNA, 4 mM ATP, 30 mM KCl, 15 mM $MgCl_2$, 5 mM DTT, 20 μM [^{3}H]amino acid, and 0.2−1 μM purified aminoacyl-tRNA synthetase (aaRS), except that $tRNA^{Tyr}$ and $tRNA^{Trp}$ were aminoacylated with a mixture of *E. coli* aaRSs (Sigma).

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Thr-tRNA^{Thr} was formed with tRNA^{Thr} with a GAC anticodon, [^{3}H]Thr, and the (*5*) T222P mutant ValRS lacking an editing function. For aminoacylation with Cys and Asn, the tRNAs with [^{3}H]adenine at the 3′ end were aminoacylated with unlabeled amino acids. After incubation for 30 min at 37 °C, 0.1 volume of 3 M NaOAc (pH 5.3) was added and the reaction mixture was subjected to phenol/chloroform extraction and ethanol precipitation. aa-tRNAs were dissolved in 5 mM NaOAc (pH 5.3) and stored at −80 °C.

Protein Purification. Histidine-tagged *E. coli* GluRS, GlyRS, ArgRS, LeuRS, and SerRS were purified from plasmids provided by T. Yokogawa and K. Nishikawa (Gifu University, Gifu, Japan) as described previously (*3*). Yeast PheRS and *E. coli* AlaRS were purified as described previously (*6*). Purified *E. coli* GlnRS and ValRS were provided by J. Perona (University of California, Santa Barbara, CA). Purified *Deinococcus radiodurans* AspRS, AsnRS, and *Chlamydia trachomatis* LysRS were gifts from D. Söll and G. Raczniak (Yale University, New Haven, CT). P. Schimmel and L. Nangle (Scripps Institute) provided purified *E. coli* IleRS, MetRS, and misacylating T222P mutant ValRS (*5*). Purified *E. coli* CysRS and ProRS were provided by Y. M. Hou and V. Scully (Thomas Jefferson University, Philadelphia, PA). Purified *E. coli* HisRS was a gift from C. Francklyn (University of Vermont, Burlington, VT). *T. thermophilus* EF-Tu was overexpressed in *E. coli* and purified as described previously (*7*). EF-Tu was stored in its GDP-bound form, and its concentration was determined with a Bradford assay.

3′ End Labeling of tRNAs. Terminal tRNA nucleotidyltransferase (TNTase) was used to label the 3′-terminal adenosine of tRNA. *E. coli* TNTase was purified from a plasmid provided by C. McHenry (University of Colorado Health Science Center, Denver, CO). First, the 3′-terminal adenosine was removed from tRNA by incubating 2 μM tRNA, 100 μM pyrophosphate, 10 μM [2,8-^{3}H]ATP, and 0.4 μM TNTase in a buffer containing 50 mM glycine-HCl (pH 9.0) and 10 mM $MgCl_2$ at 37 °C for 20 min. Then 1 unit of inorganic pyrophosphatase (Sigma) was added to the reaction to start the reverse reaction. After incubation at 37 °C for 10 min, the reaction mixture was subjected to phenol/chloroform extraction and desalted twice with a Micro BioSpin 6 column (Bio-Rad).

EF-Tu Assays. EF-Tu·GTP was prepared immediately prior to use by incubating 4 μM EF-Tu·GDP in buffer A [50 mM K-Hepes (pH 7.0), 0.6 mM phosphoenolpyruvate, 10 mM DTT, 20 μM GTP, 20 mM $MgCl_2$, and 0.5 M NH_4Cl] with 30 μg/mL pyruvate kinase at 37 °C for 3 h. Prior to measurement of the equilibrium dissociation constant (K_D), the fraction of active EF-Tu·GTP in the total EF-Tu preparation was determined by a stoichiometric titration. The very tight binding Thr-tRNA^{Thr} (K_D = 0.04 nM) was used for these experiments to ensure that the titration was carried out at saturation. Eleven EF-Tu·GTP concentrations between 1 μM and 1 nM were prepared by 2-fold serial dilution using buffer A, and then 25 μL of each diluted EF-Tu·GTP was mixed with 25 μL of 20 nM [^{3}H]Thr-tRNA^{Thr}. After incubation for 20 min on ice to form a ternary complex, 5 μL of 0.8 mg/mL RNase A was added which is sufficient to degrade all the aa-tRNA in a few seconds. After incubation for 20 s, reactions were quenched by the addition of 10 μL of 1 mg/mL unfractionated tRNA and 70 μL of 10% TCA and filtered through a nitrocellulose membrane. Samples were washed and counted as described previously (*7*). The [^{3}H]Thr-tRNA^{Thr} concentration was calculated from the specific activity of [^{3}H]Thr. Control experiments showed that negligible (<3%) deacylation had occurred during the experiment. By plotting the concentration of complex as a function of EF-Tu concentration added, we determined that 5% of the EF-Tu·GTP bound aa-tRNAs (data not shown). This fraction of active EF-Tu·GTP for the preparation used in this work is at the lower end of the range of 5−25% typically observed for this protein (*1*−*3*, *6*, *7*). This low activity is not manifest in the binding of [^{32}P]GTP or GDP where >80% binding is observed (L. Sanderson, unpublished experiments). The fraction of active EF-Tu in a given preparation is stable over several months and is observed in a range of assay buffers and temperatures with several different aa-tRNAs. Low activities of EF-Tu·GTP for binding aa-tRNA have been noted previously (*8*).

Table 1: K_D and k_{off} Values for Binding of Unmodified *E. coli* aa-tRNAs to *T. thermophilus* EF-Tu·GTP[a]

aa-tRNA[b]	measured K_D (nM)	measured k_{off} ($\times 10^{-3}$ s^{-1})	calculated K_D[d] (nM)	[NH_4Cl] slope
Ala-tRNA$_2^{Ala}$	2.3	2.4[c]	2.2	0.99
Arg-tRNA$_2^{Arg}$	4.1	3.1	2.8	1.2
Asn-tRNA^{Asn}	1.0	–	–	–
Asp-tRNA$_1^{Asp}$	1.0	2.5	2.3	1.0
Cys-tRNA^{Cys}	–	0.13[c]	0.12	2.7
Gln-tRNA$_2^{Gln}$	0.37	0.82[c]	0.74	1.6
Glu-tRNA$_2^{Glu}$	2.3	3.5	3.2	0.93
Gly-tRNA$_3^{Gly}$	–	0.49[c]	0.45	1.7
His-tRNA^{His}	–	1.6[c]	1.5	1.3
Ile-tRNA$_1^{Ile}$	1.6	3.9	3.6	0.56
Leu-tRNA$_1^{Leu}$	4.6	4.8	4.4	1.0
Lys-tRNA^{Lys}	7.3	5.0	4.5	0.67
Met-tRNA^{Met}	2.7	3.2	2.9	0.75
Phe-tRNA^{Phe}	3.3	2.1[e]	1.9	1.1
Pro-tRNA$_3^{Pro}$	0.8	1.5	1.3	1.1
Ser-tRNA$_1^{Ser}$	–	1.1[c]	1.0	1.3
Thr-tRNA$_3^{Thr}$	–	0.04[c]	0.04	2.9
Trp-tRNA^{Trp}	–	0.94[c]	0.86	1.6
Tyr-tRNA$_2^{Tyr}$	1.4	3.3	3.0	1.2
Val-tRNA$_1^{Val}$	–	9.2[e]	8.4	0.69

[a] All values are in buffer A (see Materials and Methods) at 2 °C. A dash indicates the measurement was not made. [b] tRNA isoacceptor numbers and sequences are according to ref *4*. [c] k_{off} values extrapolated from higher NH_4Cl concentrations. [d] K_D calculated from experimental k_{off} and a k_{on} of 1.1 × 10^6 M^{-1} s^{-1} (see the text). [e] Data from ref *2*.

The procedure for measuring K_D is described in ref *7*. Errors in measured K_D values shown in Table 1 are within 30%. Dissociation rates (k_{off}) were determined as described previously (*2*). A typical experiment included a ternary complex formed between 50 nM active EF-Tu·GTP and 10−30 nM aa-tRNA, ensuring nearly complete binding of the aa-tRNA. The ribonuclease concentration was sufficiently high to degrade the dissociated aa-tRNA in a few seconds, which is much faster than the time it would take for rebinding (*9*).

RESULTS

Binding of EF-Tu·GTP to Cognate aa-tRNAs. The ribonuclease protection assay (*10*) was used to determine the K_D for binding of *E. coli* aa-tRNAs to *T. thermophilus* EF-Tu·GTP. In this assay, a low concentration of radiolabeled aa-tRNA was first incubated with a series of EF-Tu·GTP

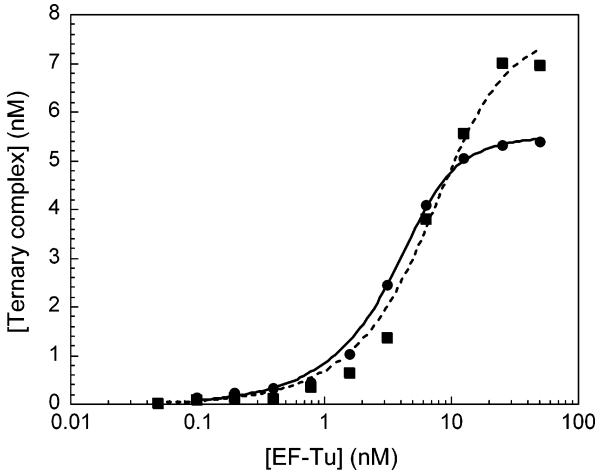

FIGURE 1: RNase protection assay for measuring the K_D for binding of *T. thermophilus* EF-Tu·GTP to Pro-tRNA^{Pro} (●) and Met-tRNA^{Met} (■) in buffer A at 2 °C. Lines are best fit curves using the complete binding equation (*7*) with K_D values of 0.8 and 2.7 nM, respectively.

concentrations until equilibrium was reached. In most cases, ^{3}H-labeled amino acids were used; however, in those cases where no ^{3}H-labeled amino acid was available or its specific activity was too low, the terminal adenosine of tRNA was labeled with [^{3}H]ATP using tRNA nucleotidyl transferase (*11*). After a high concentration of pancreatic RNase had been added to each mixture and the mixture incubated for 20 s, carrier RNA was added and each mixture was acid precipitated, filtered, and counted using a phosphorimager. Since control experiments showed that free aa-tRNA is degraded in 20 s, acid precipitable radioactivity reflected the fraction of aa-tRNA that was bound to protein. Sample binding curves for two aa-tRNAs are shown in Figure 1. Since the concentration of aa-tRNA approached that of protein for some of the data points, the value of K_D was obtained by fitting the data to the complete binding equation (*7*).

As described in greater detail elsewhere (*9*), the assay described above is only suitable over a narrow range of K_D values. If the K_D is too tight, it was not possible to obtain a sufficiently high specific activity for the aa-tRNA throughout the binding curve. While the lower limit for K_D varies depending upon the available specific activity of each amino acid, K_D values of less than 0.3 nM are not reliable. In contrast, if the K_D is too weak (>7 nM), too much of the aa-tRNA dissociates from the protein during the 20 s of incubation with RNase. Because of these constraints on the assay, the K_D values for only 13 of the cognate aa-tRNAs could be obtained with the standard 0.5 NH_4Cl buffer chosen for these experiments (Table 1).

To obtain the K_D values for the remaining aa-tRNAs and confirm the experiments described above, the dissociation rate constant (k_{off}) of each complex was determined using a variation of the RNase protection assay (*12*). In this assay, a saturating concentration of EF-Tu·GTP was mixed with each [^{3}H]aa-tRNA sample, RNase was added, and aliquots were removed at various times and filtered. The time course of the decrease in precipitable counts reflects the rate of dissociation of the aa-tRNA from the protein. While the assay can also only access a limited range of k_{off} values (0.02−0.002 s^{-1}), its simplicity makes determining k_{off} at a series

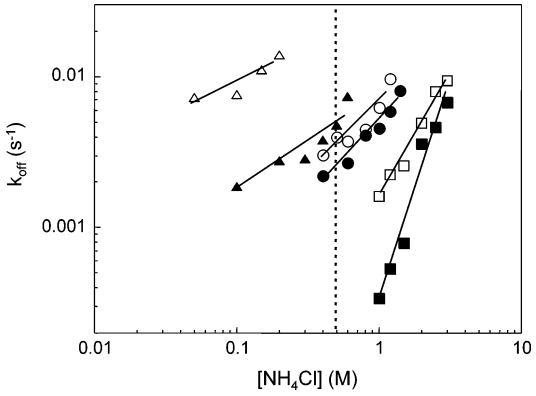

FIGURE 2: NH_4Cl concentration dependence of k_{off} for deacylated tRNA^{Asp} (△), Lys-tRNA^{Lys} (▲), Glu-tRNA^{Glu} (○), Asp-tRNA^{Asp} (●), Gly-tRNA^{Gly} (□), and Thr-tRNA^{Thr} (■). Slopes of plots and the extrapolated or interpolated k_{off} value at 0.5 M NH_4Cl (vertical dashed line) are given in Table 2.

of different NH_4Cl concentrations straightforward. As shown in Figure 2 for five aa-tRNAs, k_{off} increases with an increase in ionic strength for each tRNA and the slopes vary from 0.6 to 2.7 (Table 1). For those aa-tRNAs that dissociate too rapidly or too slowly at the reference 0.5 M NH_4Cl, a value of k_{off} can be obtained by extrapolation. Values of k_{off} for 19 elongator aa-tRNAs are reported in Table 1.

For the 13 aa-tRNAs for which experimental K_D and k_{off} values were both determined, the apparent association rate constant k_{on} ($=k_{off}/K_D$) was in the narrow range of 0.7−2.4 × 10^6 M^{-1} s^{-1}. An additional set of nine *E. coli* tRNAs misacylated with either Phe or Val also gave experimental k_{off}/K_D values from 0.5 to 1.3 × 10^6 M^{-1} s^{-1} (data not shown). In other words, the values of k_{off} and K_D correlated, suggesting that the different aa-tRNAs all exhibited the same rate of binding. However, since the value of k_{on} was considerably lower than that for a diffusion-controlled reaction, the rate constant may also reflect an isomerization step. In any case, since the value for k_{off} could be obtained for all the tRNAs and its determination was somewhat more reproducible, we used an average k_{on} value of 1.1 × 10^6 M^{-1} s^{-1} to calculate K_D values from k_{off} for all the aa-tRNAs in this study (Table 1).

The free energies for binding of the *E. coli* aa-tRNAs to EF-Tu·GTP were calculated from the K_D values in Table 1 and are reported in Table 2. The $\Delta G°$ values are also shown graphically in Figure 3 in order of their magnitude. Eighteen of the elongator aa-tRNAs have $\Delta G°$ values within a range of 1.6 kcal/mol (19-fold in K_D) from Gly-tRNA^{Gly} to Val-tRNA^{Val}. The experimental error of an individual determination is ±0.4 kcal/mol. Thus, in agreement with previous work (*12*), most of the elongator aa-tRNAs bind EF-Tu·GTP with similar affinities. However, two aa-tRNAs, Thr-tRNA^{Thr} and Cys-tRNA^{Cys}, bind substantially tighter than the others. As will be further discussed below, this result is probably an artifact of using *E. coli* tRNAs with *T. thermophilus* EF-Tu.

Binding of EF-Tu to Deacylated tRNAs. The esterified amino acid makes an important thermodynamic contribution to the binding affinity of aa-tRNA for EF-Tu·GTP. For example, the affinity of deacylated yeast tRNA^{Phe} for *E. coli* EF-Tu·GTP was 2250-fold weaker than for Phe-tRNA^{Phe} (*13*). However, we have shown that certain tRNA bodies contribute much more to protein binding than tRNA^{Phe} (*2*).

Table 2: Calculation of Binding Energies of Esterified Amino Acids Relative to That of Val[a]

$tRNA^X$	$\Delta G°$(X-$tRNA^X$) (kcal/mol)	$\Delta G°$(Val-$tRNA^X$) (kcal/mol)	$\Delta\Delta G°$(X-Val) (kcal/mol)
$tRNA_2^{Ala}$	−10.8	−11.8	1.0
$tRNA_2^{Arg}$	−10.7	−10.4	−0.3
$tRNA^{Asn}$	−11.3	−10.2	−1.1
$tRNA_1^{Asp}$	−10.8	−12.3	1.5
$tRNA^{Cys}$	−12.4	−10.9	−1.5
$tRNA_2^{Gln}$	−11.4	−9.6	−1.8
$tRNA_2^{Glu}$	−10.6	−13.0	2.4
$tRNA_3^{Gly}$	−11.7	−12.0	0.3
$tRNA^{His}$	−11.0	na[b]	na[b]
$tRNA_1^{Ile}$	−10.6	−10.0	−0.6
$tRNA_1^{Leu}$	−10.5	−10.8	0.3
$tRNA^{Lys}$	−10.4	−10.4	0
$tRNA^{Met}$	−10.7	−10.7	0
$tRNA^{Phe}$	−10.9	−10.5	−0.4
$tRNA_3^{Pro}$	−11.1	−10.6	−0.5
$tRNA_1^{Ser}$	−11.2	−10.3	−0.9
$tRNA_3^{Thr}$	−13.0	−12.5	−0.5
$tRNA^{Trp}$	−11.3	−9.8	−1.5
$tRNA_2^{Tyr}$	−10.7	−9.4	−1.3
$tRNA_1^{Val}$	−10.1	−10.1	0

[a] All values are in buffer A at 2 °C. $\Delta G° = RT \ln K_D$. The calculated values of K_D from Table 1 were used for $\Delta G°$(X-$tRNA^X$), except for Asn-$tRNA^{Asn}$ where the experimental value was used. The $\Delta G°$(Val-$tRNA^X$) values were calculated from experimental k_{off} values from ref *2* and a k_{on} of 1.1×10^6 M^{-1} s^{-1}. Corrected data for Val-$tRNA^{Thr}$ are included. [b] Not available.

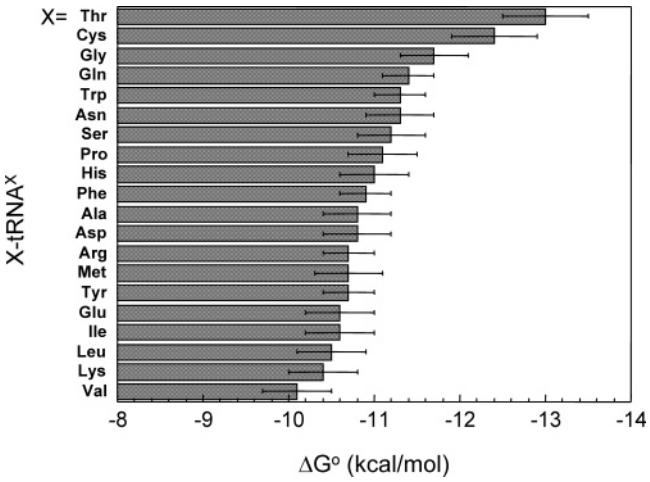

FIGURE 3: Free energies for binding of *T. thermophilus* EF-Tu·GTP to *E. coli* aa-tRNAs.

This prompted the measurement of k_{off} for the deacylated forms of the four tRNA bodies expected to make the greatest contributions ($tRNA^{Glu}$, $tRNA^{Asp}$, $tRNA^{Thr}$, and $tRNA^{Gly}$). This was accomplished using the RNase protection assay with tRNAs labeled at their 3′-terminus with [^{3}H]adenosine. Of the four tRNAs that were tested, reliable k_{off} values could be obtained for only $tRNA^{Asp}$ at lower ionic strengths (Figure 2). Extrapolation to 0.5 M NH_4Cl gave a K_D of 18 nM for deacyl-$tRNA^{Asp}$ which is 145-fold tighter than that for deacyl-$tRNA^{Phe}$, confirming the view that tRNA bodies show sequence specificity for EF-Tu·GTP. Interestingly, deacyl-$tRNA^{Asp}$ binds protein only 9-fold weaker than Asp-$tRNA^{Asp}$, supporting the conclusion that the esterified Asp does not contribute substantially to protein binding (*3*).

Prediction of the Amino Acid Hierarchy. The independent thermodynamic contributions of esterified amino acid and tRNA body to $\Delta G°$ (*1*) permit the calculation of the relative binding energy of each amino acid with respect to Val. This is done by subtracting the $\Delta G°$ of each tRNA body misacylated with Val determined previously (*2*) from the $\Delta G°$ of the corresponding tRNA body esterified with the cognate amino acid in Table 2. Thus, for each amino acid X

$$\Delta\Delta G°(\text{X-Val}) = \Delta G°(\text{X-tRNA}^X) - \Delta G°(\text{Val-tRNA}^X)$$

For this calculation, the values of $\Delta G°$ for the Val-$tRNA^X$ were recalculated from the experimental k_{off} values determined previously (*2*) and the k_{on} of 1.1×10^6 M^{-1} s^{-1} determined in this work. The resulting 19 values of $\Delta\Delta G°$-(X-Val) are given in Table 2 and presented graphically in Figure 4. The absence of a value for Val-$tRNA^{His}$ prohibited the calculation of $\Delta\Delta G°$(His-Val). The total range of binding energies is 4.2 kcal/mol (2300-fold in K_D) ranging from Gln which binds 1.8 kcal/mol tighter than Val to Glu which binds 2.4 kcal/mol weaker than Val. This range is significantly greater than the 2.5 kcal/mol range that was determined previously (*3*) among a set of misacylated yeast $tRNA^{Phe}$. The unavailability of a value for Glu in the earlier work can primarily account for this discrepancy.

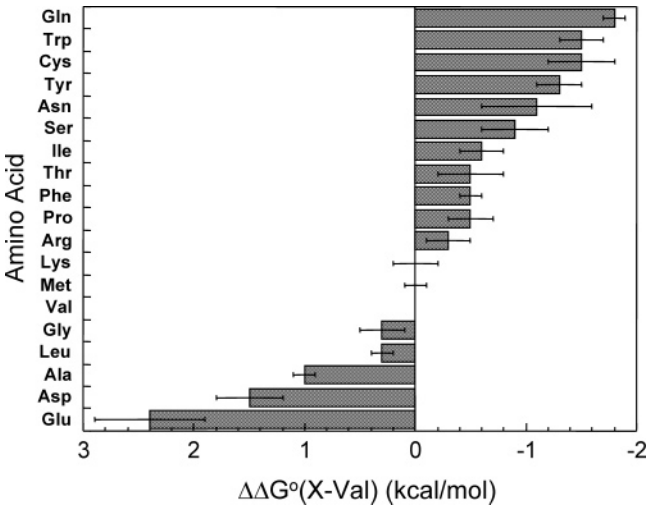

FIGURE 4: Free energies of binding of amino acids relative to that of Val calculated from the $\Delta G°$ of cognate aa-tRNAs and Val-tRNAs in Table 2.

Calculating $\Delta G°$ for Misacylated tRNAs. The free energy values in Table 2 can also be used to predict a $\Delta G°$ for any misacylated tRNA, Y-$tRNA^X$, by the formula

$$\Delta G°(\text{Y-tRNA}^X) = \Delta\Delta G°(\text{Y-Val}) + \Delta G°(\text{Val-tRNA}^X)$$

To assess the accuracy of this approach, $\Delta G°$ values were calculated for the 12 misacylated aa-tRNAs measured by LaRiviere et al. (*1*) and the 12 misacylated derivatives of yeast $tRNA^{Phe}$ measured by Dale et al. (*3*). These 24 aa-tRNAs are different from the ones used to obtain the values of $\Delta\Delta G°$(Y-Val) and $\Delta G°$(Val-$tRNA^X$) in Table 2 and therefore provide a critical test of the approach. As shown in Figure 5, the agreement between experimental and predicted values was excellent over a wide range of $\Delta G°$ values. This suggests that the $\Delta G°$ of any misacylated tRNA calculated from the data in Table 2 is likely to be accurate.

Figure 6 shows the distribution of $\Delta G°$ values for the 342 possible misacylated elongator tRNAs that can be calculated from the data in Table 2. These vary from −7.0 kcal/mol

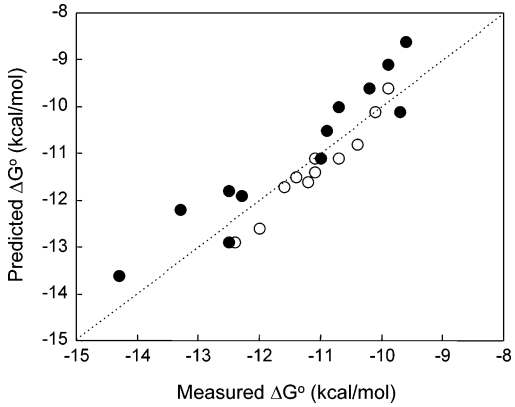

FIGURE 5: Comparison of predicted $\Delta G°$ values of misacylated tRNAs with $\Delta G°$ values calculated from experimental k_{off} values from (●) LaRiviere et al. (*1*) and (○) Dale et al. (*3*) and a k_{on} of 1.1×10^6 M^{-1} s^{-1}.

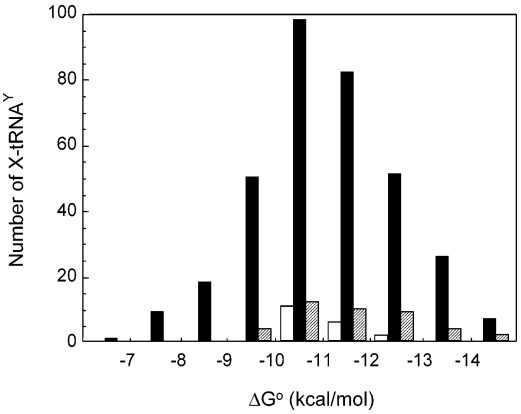

FIGURE 6: Calculated $\Delta G°$ values for binding of misacylated tRNAs (black bars), cognate aa-tRNAs (white bars), and misacylated suppressor tRNAs (hatched bars) to EF-Tu·GTP.

for Glu-tRNA^{Tyr} to −14.8 kcal/mol for Gln-tRNA^{Glu}, with the majority of species in the same range of −10 to −12 kcal/mol observed for the cognate aa-tRNAs. Nevertheless, at least 78 misacylated tRNAs are predicted to bind weaker and 33 tighter than any of the cognate aa-tRNAs.

A large number of experiments studying tRNA function in vivo have made use of suppressor tRNAs which contain point mutations in the anticodon or, rarely, in other sites in the body and that read one of the three termination codons. While most suppressor tRNAs are recognized by their cognate aaRS, some can be recognized by a noncognate aaRS and therefore insert a noncognate amino acid at the termination codon. A partial list of active misacylated suppressor tRNAs is given in Table 3. In most cases, the insertion of the noncognate amino acid into the protein was confirmed by sequencing a suppressed test protein (*14*−*21*). While the ability of these misacylated tRNAs to read a stop codon varies, many are as active as suppressor tRNAs aminoacylated with their cognate amino acid. For each of these active misacylated *E. coli* suppressor tRNAs, the $\Delta G°$ for binding EF-Tu·GTP was calculated using the data in Table 2. It is striking that in virtually every case, the calculated $\Delta G°$ exceeded −9 kcal/mol. Thus, as shown graphically in Figure 6, the active misacylated suppressor tRNAs show a distinct bias toward tighter binding to EF-Tu·GTP. This supports the view that any misacylated-tRNAs that bind EF-Tu·GTP too weakly would be unable to be translated. The data also suggest that the selective binding of certain suppressor tRNAs to EF-Tu·GTP could influence suppressor efficiency.

Table 3: Calculation of $\Delta G°$ Values for Misacylated Suppressor tRNAs

suppressor aa-tRNA	calculated $\Delta G°$ (kcal/mol)	refs
Lys-tRNA^{Ala}	−11.8	*15, 16*
Ala-tRNA^{Phe}	−9.5	*16*
Lys-tRNA^{Phe}	−10.5	*14, 16, 17*
Gln-tRNA^{Phe}	−12.3	*16, 17*
Tyr-tRNA^{Phe}	−11.8	*14, 16, 17*
Gln-tRNA^{Gly}	−13.8	*16, 19, 20*
Arg-tRNA^{Phe}	−10.8	*14*
Leu-tRNA^{Phe}	−10.2	*14*
Thr-tRNA^{Phe}	−11.0	*14, 17*
Lys-tRNA^{Arg}	−10.4	*14, 18*−*20*
Gln-tRNA^{Ala}	−13.6	*15*
Ala-tRNA^{Lys}	−9.4	*15*
Gln-tRNA^{Lys}	−12.2	*15, 18*
Trp-tRNA^{Lys}	−11.9	*15*
Gly-tRNA^{Ala}	−11.5	*15*
Met-tRNA^{Ala}	−11.8	*15*
Arg-tRNA^{Ala}	−12.1	*15*
Val-tRNA^{Phe}	−10.5	*17*
Arg-tRNA^{Lys}	−10.7	*18*
Gln-tRNA^{Arg}	−12.2	*18*
Gly-tRNA^{Phe}	−10.2	*19*
Gly-tRNA^{Arg}	−10.1	*19*
Gly-tRNA^{Lys}	−10.1	*19*
Lys-tRNA^{Gly}	−12.0	*19*
Pro-tRNA^{Gly}	−12.5	*19*
Thr-tRNA^{Gly}	−12.5	*19*
Tyr-tRNA^{Gly}	−13.3	*19*
Met-tRNA^{Gly}	−12.0	*19*
Gly-tRNA^{Gln}	−9.3	*19*
Gln-tRNA^{Trp}	−11.6	*20*
Gln-tRNA^{Ile}	−11.8	*20*
Gln-tRNA^{Glu}	−14.8	*20*
Tyr-tRNA^{Glu}	−13.3	*20*
Arg-tRNA^{Glu}	−12.0	*20*
Lys-tRNA^{Ile}	−10.0	*20*
Lys-tRNA^{Met}	−10.7	*20*
Lys-tRNA^{Asp}	−12.3	*20*
Lys-tRNA^{Thr}	−12.5	*20*
Lys-tRNA^{Val}	−10.1	*20*
Ser-tRNA^{Leu}	−11.7	*21*
Gln-tRNA^{Leu}	−12.6	*21*

DISCUSSION

In this study and a previous one (*2*), unmodified tRNAs were used to investigate the properties of binding of EF-Tu·GTP to aa-tRNA. This choice was dictated by the need to introduce the GAC anticodon into each tRNA body so it could be misacylated in vitro with ValRS or PheRS (*2*). However, provided that magnesium ion concentrations are sufficiently high to permit proper folding of tRNAs (*22*, *23*), the lack of modifications does not affect binding of EF-Tu to aa-tRNAs (*1*, *24*, *25*). These results are consistent with the X-ray crystal structures which show that modified nucleotides are not located in the part of tRNA that contacts EF-Tu (*26*, *27*). Changes in the anticodon sequence of tRNA also do not affect EF-Tu binding affinity, permitting the tRNA containing the normal codon used in this work to be compared with the previously used tRNAs containing the GAC anticodon (*2*).

This study examines the binding of *E. coli* tRNA sequences with the EF-Tu from a different organism, *T.*

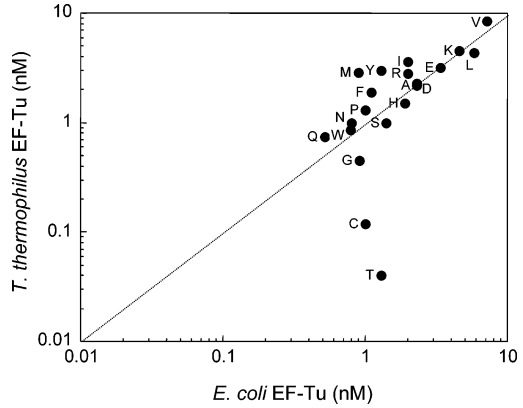

FIGURE 7: Comparison of the binding affinities of *E. coli* aa-tRNAs for *T. thermophilus* EF-Tu·GTP in this work with *E. coli* EF-Tu·GTP from ref *12*.

thermophilus. The protein was chosen because of the availability of an X-ray cocrystal structure of the nearly identical *Thermus aquaticus* EF-Tu (*26*, *27*). However, because many of the *T. thermophilus* tRNA sequences were not known, *E. coli* tRNAs had to be used instead. If the way in which aa-tRNAs recognize EF-Tu·GTP is substantially different between *T. thermophilus* and *E. coli*, the use of a heterologous system could be inappropriate. However, there are several reasons to expect that the data obtained for the heterologous interaction is not very different from those from either of the homologous interactions. First, the sequences of *T. thermophilus* and *E. coli* EF-Tu are ~70% identical (*28*), including virtually all of the residues in the amino acid binding pocket and the tRNA binding interface (*27*). Second, *T. thermophilus* EF-Tu can participate in translation on *E. coli* ribosomes both in vitro and in vivo (*29*). Finally, as shown in Figure 7, in most cases, the affinities of the unmodified *E. coli* aa-tRNAs for *T. thermophilus* EF-Tu measured here are very similar to the binding affinities of the same modified *E. coli* aa-tRNAs with *E. coli* EF-Tu determined previously (*12*). The only exceptions are that *E. coli* Cys-tRNA^{Cys} and Thr-tRNA^{Thr} bind tighter to *T. thermophilus* EF-Tu than to *E. coli* EF-Tu. Since the sequence of *T. thermophilus* tRNA^{Thr} is quite different from that of *E. coli* tRNA^{Thr} (*30*), this may mean that the two proteins have identical amino acid specificities and slightly different tRNA sequence specificities. A more extensive analysis of the tRNA sequence specificity of the two homologous EF-Tu-tRNA interactions will be published elsewhere, but it is clear that the two proteins bind most *E. coli* tRNAs similarly.

This work uses the experimental binding free energies of 38 different aa-tRNAs to EF-Tu·GTP to calculate the binding free energies of more than 340 different possible misacylated tRNAs. A critical assumption of this calculation is that the thermodynamic contributions of the esterified amino acid and the tRNA body to the total free energy of binding are completely independent. While such independence had been demonstrated in a limited number of cases (*1*), it was possible that it may not be the case with all amino acids and all tRNA bodies. Indeed, although the cocrystal structure indicates that the amino acid binding pocket and tRNA binding interface are sufficiently distant to support independence, the ability of certain amino acids to stack on the terminal A residue in the free aa-tRNA (*31*) raises the possibility that thermodynamic coupling could occur. However, the successful prediction in Figure 5 of experimental $\Delta G°$ values of 24 misacylated tRNAs that were not used for obtaining the parameters strongly supports the assumption that the amino acid and tRNA body act as independent thermodynamic units and suggests that the predicted $\Delta G°$ values of other misacylated tRNAs are accurate.

The thermodynamic contributions of the different esterified amino acids relative to Val in Figure 4 show a hierarchy of free energies that is virtually identical to the hierarchy of free energies of binding for derivatives of yeast tRNA^{Phe} esterified with 13 different amino acids (*3*). Gln, Asn, and the large aromatic amino acids Trp and Tyr bind very tightly to the pocket in EF-Tu. Phe binds somewhat weaker than Tyr, suggesting that the hydroxyl group in Tyr may make an additional hydrogen bond. On the other hand, the negatively charged amino acids Glu and Asp bind less well than Arg and Lys, presumably reflecting the fact that the amino acid pocket has a net negative charge. As might be expected, the small amino acids Gly and Ala also bind weakly. As discussed previously (*3*), it is likely that each amino acid binds in the large binding pocket in EF-Tu somewhat differently, making a detailed understanding of the hierarchy difficult to achieve in the absence of structures for each esterified amino acid.

The ability to predict the binding affinity of different aa-tRNAs for EF-Tu·GTP helps us understand the efficiency of incorporating different misacylated tRNAs into protein in vivo. Although misacylation of native tRNAs is normally a rare event, nonsense suppressor tRNAs are often misacylated, presumably because the modification of the anticodon sequence makes the tRNA a substrate for a noncognate aaRS. It is striking that all 41 misacylated tRNAs that were reported to be active suppressors had calculated $\Delta G°$ values in excess of -9 kcal/mol. This supports the view that misacylated suppressor tRNAs that bind EF-Tu·GTP at less than this threshold value are not as active in translation because their weak binding prevents them from being delivered to the A site of the ribosome. Although it is known that certain tRNAs are unable to be converted to active suppressors by introducing a nonsense anticodon (*32*, *33*), this was previously explained as the result of poor aminoacylation. From the data presented here, poor EF-Tu·GTP binding can also lead to inactive suppressor tRNAs.

It is also interesting that certain active misacylated suppressor tRNAs are predicted to bind EF-Tu·GTP much tighter than a typical cognate aa-tRNA. This suggests that tight binding of an aa-tRNA to EF-Tu·GTP does not inhibit release of aa-tRNA from EF-Tu·GDP during the accommodation step in translation. The exceptionally tight binding of esterified Gln to the amino acid binding pocket of EF-Tu may explain why so many of the active suppressor tRNAs are misacylated by Gln. Even if misacylation of a suppressor tRNA by GlnRS is very poor, EF-Tu·GTP would be able to bind the small amount of glutaminylated tRNA with high affinity and thus lead to efficient incorporation into protein.

There are several examples in the literature where poor EF-Tu·GTP binding may explain the lack of activity of a misacylated tRNA. One example involves Glu-tRNA^{Gln} and Asp-tRNA^{Asn} that are intermediates in the transamidation pathway used in the synthesis of Gln-tRNA^{Gln} and Asn-tRNA^{Asn} in many eubacteria and archaebacteria (*34*−*38*). These two misacylated tRNAs combine a weak amino acid

and a weak tRNA body so that the calculated $\Delta G°$ values (−7.2 and −8.7 kcal/mol) fall below the −9.0 kcal/mol threshold needed for an active aa-tRNA. This presumably explains why Glu and Asp are not misinserted at Gln and Asn codons in these organisms.

In some *Candida* species, the CUG codon is translated as Ser instead of the more common Leu (*39*, *40*). The *Candida zeylanoides* $tRNA^{Ser}$(CAG) responsible for this nonuniversal decoding is aminoacylated by Ser and, to a lesser extent, Leu both in vitro and in vivo (*41*). It was proposed that the CUG codon may encode both Ser and Leu in *Candida*. However, we would predict that Ser-$tRNA^{Ser}$ binds EF-Tu·GTP tighter than Leu-$tRNA^{Ser}$. Thus, the small amount (3%) of Leu-$tRNA^{Ser}$ observed in vivo is unlikely to be incorporated into protein. This conclusion is consistent with the fact that insertion of Leu at CUG codons could not be detected in two *Candida* species (*41*).

E. coli YadB is a GluRS-like protein which lacks the anticodon-binding domain and aminoacylates $tRNA^{Asp}$ with Glu (*42*). The function of the resulting Glu-$tRNA^{Asp}$ is unknown, but it is reported not to bind *E. coli* EF-Tu·GTP and thus is not misincorporated into protein (*42*). While the predicted $\Delta G°$ value of −9.9 kcal/mol for Glu-$tRNA^{Asp}$ is somewhat weaker than that of Val-$tRNA^{Val}$ ($\Delta G° = -10.1$ kcal/mol), the weakest cognate aa-tRNA, it is tighter than the −9.0 kcal/mol threshold defined by the suppressor tRNAs and thus would be expected to bind EF-Tu reasonably well. This disagreement between experimental and calculated data may reflect a difference between the specificity of *T. thermophilus* and *E. coli* EF-Tu for $tRNA^{Asp}$.

Finally, on a historical note, the success of the classic 1962 experiment (*43*) demonstrating the adaptor role of tRNA can be viewed in a new light. In those experiments, Cys-$tRNA^{Cys}$ was chemically converted to Ala-$tRNA^{Cys}$ and assayed by in vitro translation. Since it was found that Ala was incorporated at Cys codons, it was concluded that the specificity of aa-tRNA in translation was entirely dictated by the codon−anticodon interaction. In other words, the translation machinery used tRNA as a passive adaptor between the amino acid and codon. We now know that this experiment was successful only because misacylated Ala-$tRNA^{Cys}$ binds EF-Tu·GTP well enough ($\Delta G° = -9.9$ kcal/mol) to be incorporated into protein. If Chapeville and co-workers had used certain other misacylated tRNAs in their experiments, they would not have been active and tRNA would not have been considered a nonspecific adaptor!

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REFERENCES

1. LaRiviere, F. J., Wolfson, A. D., and Uhlenbeck, O. C. (2001) Uniform binding of aminoacyl-tRNAs to elongation factor Tu by thermodynamic compensation, *Science 294*, 165−8.
2. Asahara, H., and Uhlenbeck, O. C. (2002) The tRNA specificity of *Thermus thermophilus* EF-Tu, *Proc. Natl. Acad. Sci. U.S.A. 99*, 3499−504.
3. Dale, T., Sanderson, L. E., and Uhlenbeck, O. C. (2004) The affinity of elongation factor Tu for an aminoacyl-tRNA is modulated by the esterified amino acid, *Biochemistry 43*, 6159−66.
4. Komine, Y., Adachi, T., Inokuchi, H., and Ozeki, H. (1990) Genomic organization and physical mapping of the transfer RNA genes in *Escherichia coli* K12, *J. Mol. Biol. 212*, 579−98.
5. Doring, V., Mootz, H. D., Nangle, L. A., Hendrickson, T. L., de Crecy-Lagard, V., Schimmel, P., and Marliere, P. (2001) Enlarging the amino acid set of *Escherichia coli* by infiltration of the valine coding pathway, *Science 292*, 501−4.
6. Pleiss, J. A., Wolfson, A. D., and Uhlenbeck, O. C. (2000) Mapping contacts between *Escherichia coli* alanyl tRNA synthetase and 2′-hydroxyls using a complete tRNA molecule, *Biochemistry 39*, 8250−8.
7. Pleiss, J. A., and Uhlenbeck, O. C. (2001) Identification of thermodynamically relevant interactions between EF-Tu and backbone elements of tRNA, *J. Mol. Biol. 308*, 895−905.
8. Gulewicz, K., Faulhammer, H. G., and Sprinzl, M. (1981) Properties of native and nicked elongation factor Tu from *Thermus thermophilus* HB 8, *Eur. J. Biochem. 121*, 155−62.
9. Vorstenbosch, E. L., Potapov, A. P., de Graaf, J. M., and Kraal, B. (2000) 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 42*, 1−14.
10. Knowlton, R. G., and Yarus, M. (1980) Discrimination between aminoacyl groups on su+ 7 tRNA by elongation factor Tu, *J. Mol. Biol. 139*, 721−32.
11. Wolfson, A. D., and Uhlenbeck, O. C. (2002) Modulation of tRNAAla identity by inorganic pyrophosphatase, *Proc. Natl. Acad. Sci. U.S.A. 99*, 5965−70.
12. Louie, A., and Jurnak, F. (1985) Kinetic studies of *Escherichia coli* elongation factor Tu-guanosine 5′-triphosphate-aminoacyl-tRNA complexes, *Biochemistry 24*, 6433−9.
13. Janiak, F., Dell, V. A., Abrahamson, J. K., Watson, B. S., Miller, D. L., and Johnson, A. E. (1990) Fluorescence characterization of the interaction of various transfer RNA species with elongation factor Tu·GTP: Evidence for a new functional role for elongation factor Tu in protein biosynthesis, *Biochemistry 29*, 4268−77.
14. McClain, W. H., and Foss, K. (1988) Changing the acceptor identity of a transfer RNA by altering nucleotides in a "variable pocket", *Science 241*, 1804−7.
15. McClain, W. H., Chen, Y. M., Foss, K., and Schneider, J. (1988) Association of transfer RNA acceptor identity with a helical irregularity, *Science 242*, 1681−4.
16. McClain, W. H., and Foss, K. (1988) Changing the identity of a tRNA by introducing a G-U wobble pair near the 3′ acceptor end, *Science 240*, 793−6.
17. McClain, W. H., and Foss, K. (1988) Nucleotides that contribute to the identity of *Escherichia coli* tRNA(Phe), *J. Mol. Biol. 202*, 697−709.
18. McClain, W. H., Foss, K., Jenkins, R. A., and Schneider, J. (1990) Nucleotides that determine *Escherichia coli* tRNA(Arg) and tRNA(Lys) acceptor identities revealed by analyses of mutant opal and amber suppressor tRNAs, *Proc. Natl. Acad. Sci. U.S.A. 87*, 9260−4.
19. McClain, W. H., Foss, K., Jenkins, R. A., and Schneider, J. (1991) Rapid determination of nucleotides that define tRNA(Gly) acceptor identity, *Proc. Natl. Acad. Sci. U.S.A. 88*, 6147−51.
20. Normanly, J., Kleina, L. G., Masson, J. M., Abelson, J., and Miller, J. H. (1990) Construction of *Escherichia coli* amber suppressor tRNA genes. III. Determination of tRNA specificity, *J. Mol. Biol. 213*, 719−26.
21. Normanly, J., Ollick, T., and Abelson, J. (1992) Eight base changes are sufficient to convert a leucine-inserting tRNA into a serine-inserting tRNA, *Proc. Natl. Acad. Sci. U.S.A. 89*, 5680−4.
22. Sampson, J. R., DiRenzo, A. B., Behlen, L. S., and Uhlenbeck, O. C. (1989) Nucleotides in yeast $tRNA^{Phe}$ required for the specific recognition of its cognate synthetase, *Science 243*, 1363−6.
23. Hall, K. B., Sampson, J. R., Uhlenbeck, O. C., and Redfield, A. G. (1989) Structure of an unmodified tRNA molecule, *Biochemistry 28*, 5794−801.
24. Nazarenko, I. A., Harrington, K. M., and Uhlenbeck, O. C. (1994) Many of the conserved nucleotides of tRNA(Phe) are not essential for ternary complex formation and peptide elongation, *EMBO J. 13*, 2464−71.
25. Liu, J. C., Liu, M., and Horowitz, J. (1998) Recognition of the universally conserved 3′-CCA end of tRNA by elongation factor EF-Tu, *RNA 4*, 639−46.
26. Nissen, P., Kjeldgaard, M., Thirup, S., Clark, B. F., and Nyborg, J. (1996) The ternary complex of aminoacylated tRNA and EF-

Tu-GTP. Recognition of a bond and a fold, *Biochimie 78*, 921−33.
27. Nissen, P., Thirup, S., Kjeldgaard, M., and Nyborg, J. (1999) The crystal structure of Cys-tRNACys-EF-Tu-GDPNP reveals general and specific features in the ternary complex and in tRNA, *Struct. Folding Des. 7*, 143−56.
28. Berchtold, H., Reshetnikova, L., Reiser, C., Schirmer, N., Sprinzl, M., and Hilgenfeld, R. (1993) Crystal structure of active elongation factor Tu reveals major domain rearrangements, *Nature 365*, 126−32.
29. Zeidler, W., Kreutzer, R., and Sprinzl, M. (1993) Participation of the overproduced elongation factor Tu from *Thermus thermophilus* in protein biosynthesis of *Escherichia coli*, *FEBS Lett. 319*, 185−8.
30. Weisshaar, M., Ahmadian, R., Sprinzl, M., Satoh, M., Kushiro, A., and Tomita, K. (1990) Sequences of four tRNA genes adjacent to the tuf2 gene of *Thermus thermophilus*, *Nucleic Acids Res. 18*, 1902.
31. Schlosser, A., Nawrot, B., Grillenbeck, N., and Sprinzl, M. (2001) Fluorescence-monitored conformational change on the 3′-end of tRNA upon aminoacylation, *J. Biomol. Struct. Dyn. 19*, 285−91.
32. Kleina, L. G., Masson, J. M., Normanly, J., Abelson, J., and Miller, J. H. (1990) Construction of *Escherichia coli* amber suppressor tRNA genes. II. Synthesis of additional tRNA genes and improvement of suppressor efficiency, *J. Mol. Biol. 213*, 705−17.
33. Raftery, L. A., and Yarus, M. (1987) Systematic alterations in the anticodon arm make tRNA(Glu)-Suoc a more efficient suppressor, *EMBO J. 6*, 1499−506.
34. Schon, A., Kannangara, C. G., Gough, S., and Soll, D. (1988) Protein biosynthesis in organelles requires misaminoacylation of tRNA, *Nature 331*, 187−90.
35. Stanzel, M., Schon, A., and Sprinzl, M. (1994) Discrimination against misacylated tRNA by chloroplast elongation factor Tu, *Eur. J. Biochem. 219*, 435−9.
36. Curnow, A. W., Ibba, M., and Soll, D. (1996) tRNA-dependent asparagine formation, *Nature 382*, 589−90.
37. Becker, H. D., and Kern, D. (1998) *Thermus thermophilus*: A link in evolution of the tRNA-dependent amino acid amidation pathways, *Proc. Natl. Acad. Sci. U.S.A. 95*, 12832−7.
38. Tumbula, D. L., Becker, H. D., Chang, W. Z., and Soll, D. (2000) Domain-specific recruitment of amide amino acids for protein synthesis, *Nature 407*, 106−10.
39. Pesole, G., Lotti, M., Alberghina, L., and Saccone, C. (1995) Evolutionary origin of nonuniversal CUGSer codon in some *Candida* species as inferred from a molecular phylogeny, *Genetics 141*, 903−7.
40. Sugita, T., and Nakase, T. (1999) Non-universal usage of the leucine CUG codon and the molecular phylogeny of the genus *Candida*, *Syst. Appl. Microbiol. 22*, 79−86.
41. Suzuki, T., Ueda, T., and Watanabe, K. (1997) The 'polysemous' codon: A codon with multiple amino acid assignment caused by dual specificity of tRNA identity, *EMBO J. 16*, 1122−34.
42. Dubois, D. Y., Blaise, M., Becker, H. D., Campanacci, V., Keith, G., Giege, R., Cambillau, C., Lapointe, J., and Kern, D. (2004) An aminoacyl-tRNA synthetase-like protein encoded by the *Escherichia coli* yadB gene glutamylates specifically tRNAAsp, *Proc. Natl. Acad. Sci. U.S.A. 101*, 7530−5.
43. Chapeville, F., Lipmann, F., Von Ehrenstein, G., Weisblum, B., Ray, W. J., Jr., and Benzer, S. (1962) On the role of soluble ribonucleic acid in coding for amino acids, *Proc. Natl. Acad. Sci. U.S.A. 48*, 1086−92.

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