

Structural elements defining elongation factor Tu mediated suppression of codon ambiguity

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ABSTRACT

In most prokaryotes Asn-tRNA^{Asn} and Gln-tRNA^{Gln} are formed by amidation of aspartate and glutamate mischarged onto tRNA^{Asn} and tRNA^{Gln}, respectively. Coexistence in the organism of mischarged Asp-tRNA^{Asn} and Glu-tRNA^{Gln} and the homologous Asn-tRNA^{Asn} and Gln-tRNA^{Gln} does not, however, lead to erroneous incorporation of Asp and Glu into proteins, since EF-Tu discriminates the misacylated tRNAs from the correctly charged ones. This property contrasts with the canonical function of EF-Tu, which is to non-specifically bind the homologous aa-tRNAs, as well as heterologous species formed *in vitro* by aminoacylation of non-cognate tRNAs. In *Thermus thermophilus* that forms the Asp-tRNA^{Asn} intermediate by the indirect pathway of tRNA asparaginylation, EF-Tu must discriminate the mischarged aminoacyl-tRNAs (aa-tRNA). We show that two base pairs in the tRNA T-arm and a single residue in the amino acid binding pocket of EF-Tu promote discrimination of Asp-tRNA^{Asn} from Asn-tRNA^{Asn} and Asp-tRNA^{Asp} by the protein. Our analysis suggests that these structural elements might also contribute to rejection of other mischarged aa-tRNAs formed *in vivo* that are not involved in peptide elongation. Additionally, these structural features might be involved in maintaining a delicate balance of weak and strong binding affinities between EF-Tu and the amino acid and tRNA moieties of other elongator aa-tRNAs.

INTRODUCTION

In all living organisms, the translational machinery requires a minimum set of at least 20 aminoacyl-tRNAs (aa-tRNAs), one for each of the standard amino acids (aa) found in proteins. Most aa-tRNAs are formed by direct

attachment of aa to the homologous tRNA by the cognate aminoacyl-tRNA synthetase (aaRS) (1,2). However, asparaginylation of tRNA^{Asn} (Asn-tRNA^{Asn}) and glutaminylation of tRNA^{Gln} (Gln-tRNA^{Gln}) can be formed via an alternate route in which tRNA^{Asn} and tRNA^{Gln} are mischarged with aspartate (Asp) and glutamate (Glu), respectively, prior to amidation of the aa by a tRNA-dependent amidotransferase (AdT) (3–5). Mischarged Asp-tRNA^{Asn} and Glu-tRNA^{Gln} are unable to bind *in vitro* elongation factor Tu (EF-Tu), which delivers aa-tRNAs to the ribosome A-site during peptide elongation (5,6). Nevertheless, poor binding of the mischarged Asp-tRNA^{Asn} and Glu-tRNA^{Gln} was reported *in vivo* when the mischarged aaRS is overexpressed (7,8). It is proposed that because of the weak affinity of these mischarged aa-tRNAs for EF-Tu, the binding is low enough to prevent effective competition with the homologous aa-tRNAs under physiological conditions. Thus, these non-cognate aa-tRNAs escape protein synthesis, and therefore do not lead to misreading of codons. This property contrasts with the ability of EF-Tu to bind non-specifically to most aa-tRNAs including some non-cognate species formed *in vitro* (9,10) and tRNAs acylated with non-natural aa (11).

Crystal structures of the EF-Tu•GTP•aa-tRNA ternary complexes (12,13) show that the protein recognizes aa-tRNA through non-specific contacts with the backbone of the tRNA and the aa moiety explaining, at the structural level, the wide specificity of EF-Tu for aa-tRNAs. To be delivered to the ribosome, aa-tRNAs must bind EF-Tu with an appropriate affinity (14). Recently, it has been shown that EF-Tu binds different cognate aa-tRNAs with a similar affinity that is maintained through a balance of strong and weak interactions with the aa and tRNA moieties of the aminoacylated tRNA (15). This suggests that the poor affinity of some misacylated tRNAs for EF-Tu results from weak binding interactions at both the aa and tRNA contact sites. However, the structural determinants that modulate EF-Tu binding affinity for the aa and tRNA moieties are not well understood. Asp-tRNA^{Asn} and Glu-tRNA^{Gln} are two physiologically relevant mischarged aa-tRNAs that are

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unable to bind EF-Tu. This feature makes these aa-tRNAs well suited for investigations into structural determinants that modulate EF-Tu binding capacity.

Here, we identify the tRNA and protein structural elements that determine the inability of Asp-tRNA^{Asn} to bind EF-Tu. We show that two base pairs in the T-arm of the tRNA, which distinguish tRNA^{Asn} and tRNA^{Asp}, and a single residue in the aa binding pocket of EF-Tu are responsible for the loss of binding capacity. Surprisingly, these structural elements are conserved even in organisms that do not form Asp-tRNA^{Asn}. Further, the tRNA elements that prevent binding of Asp-tRNA^{Asn} to EF-Tu are present in other tRNA species that are excluded from protein synthesis as well as in some tRNAs involved in canonical aa-tRNA pairs, that do participate in peptide elongation. This suggests that the tRNA structural elements that are involved in the discrimination between Asp-tRNA^{Asn} and Asp-tRNA^{Asp} are likely also involved in the balance of binding interactions between tRNA and aa moieties of other cognate aa-tRNAs and EF-Tu (15).

MATERIALS AND METHODS

Strains, plasmids and proteins

Thermus thermophilus EF-Tu and elongation factor Ts (EF-Ts), and *Escherichia coli* EF-Tu were overexpressed in *E. coli* (strains kindly provided by M. Sprinzl, University of Bayreuth and B. Kraal, University of Leiden) and purified as previously described (16,17). Mutagenesis of *T. thermophilus* EF-Tu was performed by PCR using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) with the pQE12 vector containing the EF-Tu ORF (18) and two complementary 35-mer oligonucleotides containing the desired mutations. Mutations were confirmed by DNA sequencing. The His-tagged EF-Tu variants were purified by precipitation of the thermolabile proteins from *E. coli* followed by chromatography on a Ni⁺⁺ substituted Sepharose matrix, a procedure that prevents contamination by endogenous *E. coli* EF-Tu.

tRNA^{Asn} and tRNA^{Asp} from *T. thermophilus* were purified from *E. coli* strain JM103 transformed with the pKK expression vector encoding the tRNA genes (19). The post-transcriptional modifications of the two tRNAs expressed in *E. coli* have not been characterized, but both are charged as efficiently and with the same rate by the homologous aaRS as the tRNAs isolated from *T. thermophilus* (not shown). Transcripts of wild-type and mutant *T. thermophilus* tRNAs and *S. epidermidis* tRNA^{GlyIA} were obtained from reconstructed genes cloned into pUC18 as described (20).

Determination of the concentration of active EF-Tu

The concentration of active EF-Tu was determined by active site titration as described previously (18,21). GDP complexed to EF-Tu was exchanged with [³H]-labeled GDP in a mix containing 50 mM Tris-HCl pH 7.4, 50 mM NH₄Cl, 10 mM MgCl₂, 2 mM [³H]GDP (500 cpm/pmol), 0.2 μM EF-Tu and 2 nM EF-Ts. After incubation for 20 min on ice, the reaction mix was filtered on

nitrocellulose discs and washed three times with 1 ml of buffer containing 10 mM Tris-HCl pH 7.4, 10 mM NH₄Cl and 10 mM MgCl₂. The % of active molecules of native and mutated EF-Tu, determined by nitrocellulose disc filtration of radioactive GDP bound on EF-Tu was evaluated to 70%. Multiple investigations have shown that molecules of *T. thermophilus* EF-Tu competent in the GDP exchange assay are active in formation of the ternary EF-Tu • GTP • aa-tRNA complex (16,18,21,22).

Preparation of tRNA transcripts

Prior to transcription, constructs were linearized by *Bst*NI digestion to yield transcripts terminated by the 3' CCA. *In vitro* transcriptions were performed in 500 μl mixtures containing 40 mM Tris-HCl pH 8.1, 22 mM MgCl₂, 1 mM spermidine, 5 mM DTE, 0.01% Triton X-100, 4 mM of each ATP, CTP, GTP and UTP, 16 mM GMP, 50 μg of linearized plasmid and 120 U of T7 RNA polymerase during 3 h of incubation at 37°C. GMP was added to obtain transcripts starting with guanosine 5' monophosphate. To overcome the poor yield of transcription for tRNA^{Asn} that starts with a U, the tRNA^{Asn} gene was flanked at the 5' end by a *cis*-acting hammerhead ribozyme starting with three G residues to facilitate T7 polymerase recognition. Self-cleavage of the hammerhead ribozyme was completed during one hour of incubation at 60°C after five-fold dilution of the transcription mix with 50 mM Tris-HCl pH 8.1 and 20 mM MgCl₂ (23). Nucleic acids were recovered by ethanol precipitation, dried, dissolved in 1 ml water and applied to a Uno-Q6 (Bio-Rad, Hercules, CA, USA) anion-exchange chromatography column. Washing was performed with 40 ml of 50 mM Tris-HCl pH 7.5 followed by elution with a gradient from 0 to 1 M NaCl (2 × 25 ml). Transcripts eluted at 400 mM salt were precipitated with ethanol and dissolved in water. Their charging capacities were determined to be 60–70%.

Preparation of aa-tRNAs

Aminoacylation of tRNAs was performed in a mixture containing 4 μM transcript, 100 mM Na-HEPES pH 7.2, 30 mM KCl, 2 mM ATP, 10 mM MgCl₂ and 20 μM of either L-[¹⁴C]Asp (270 cpm/pmol), L-[³H]Asn (500 cpm/pmol) or L-[¹⁴C]Phe (444 cpm/pmol) and 0.2 μM of *T. thermophilus* non-discriminating AspRS, AsnRS or PheRS. After 40 min incubation at 37°C the reaction was stopped by addition of 60 mM sodium acetate pH 4.5 and 250 mM KCl and the aa-tRNAs were extracted with acid-buffered phenol and chloroform, and precipitated with ethanol. Pellets were dried, dissolved in water, and stored at –20°C.

Activation of EF-Tu

Purified EF-Tu from *T. thermophilus* or *E. coli* (14 μM) were activated in a reaction mix containing 10 mM Na-HEPES pH 7.2, 10 mM NH₄Cl, 2 mM MgCl₂, 1 mM GTP, 1 mM phosphoenolpyruvate, 5 U pyruvate kinase and 0.14 μM of *T. thermophilus* EF-Ts (22). Reactions were conducted for 20 min at 37°C. Until use, the mix was stored on ice.

Filter binding assays for K_D measurements

Reaction mixtures of 400 μ l contained 20 nM [14 C]- or [3 H]-labeled aa-tRNA and EF-Tu • GTP in concentrations ranging from 0.02 to 2 μ M in 50 mM Na-Hepes pH 7.2, 5 mM NH_4Cl , 50 mM KCl, 10 mM MgCl_2 and 1 mM GTP. After 5 min incubation at 4°C, the mixture was filtered on a nitrocellulose disc presoaked in washing buffer. Filters were washed with 400 μ l of reaction buffer depleted in GTP, dried and counted in a liquid scintillation counter. K_D were determined by fitting the experimental data of concentrations of EF-Tu • aa-tRNA complex, total EF-Tu and total aa-tRNA in the second-degree equation derived from $K_D = [\text{free EF-Tu}] \times [\text{free aa-tRNA}] / [\text{EF-Tu} \bullet \text{aa-tRNA complex}]$, in which [free EF-Tu] is substituted by [total EF-Tu] – [EF-Tu • aa-tRNA complex] and [free aa-tRNA] by [total aa-tRNA] – [EF-Tu • aa-tRNA complex]. The binding assays (Figure 1) show that the aa-tRNA bound to the EF-Tu • GTP complex is totally retained on the nitrocellulose filter. Thus the efficiency of retention of the ternary complex is near 100%.

The experimental data of [RL] (concentration of EF-Tu • aa-tRNA), [Rt] (concentration of total EF-Tu) and [Lt] (concentration of total aa-tRNA) were fitted to the second degree equation whose positive solution expresses [RL] as a function of K_D , [Rt] and [Lt]: $[RL] = 1/2[(K_D + [Lt] + [Rt]) - \sqrt{(K_D + [Lt] + [Rt])^2 - 4([Lt] \times [Rt])}]$ (24,25). K_D and the correlation coefficient were calculated using the Kaleidagraph software. K_D values are the average of at least three independent determinations where the K_D of Asp-tRNA^{Asp} was determined in parallel to serve as control. The affinity constants K_A were calculated from $1/K_D$.

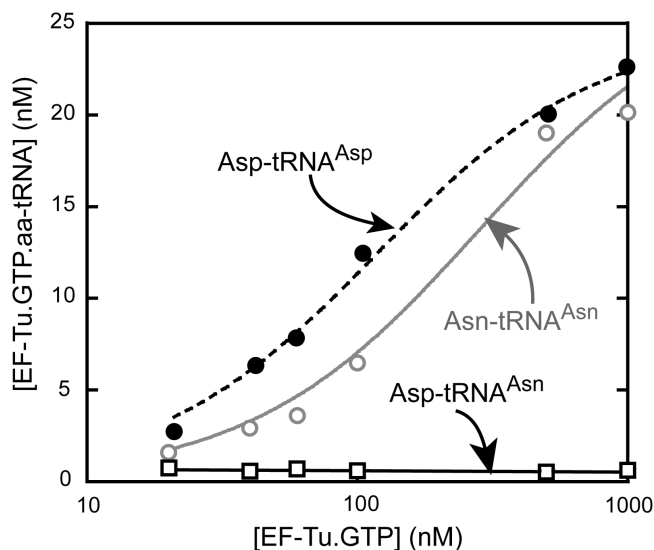


Figure 1. Binding of aa-tRNAs to EF-Tu. Twenty nM *T. thermophilus* Asp-tRNA^{Asp}, Asp-tRNA^{Asn} and Asn-tRNA^{Asn} were saturated with increasing amounts of *T. thermophilus* EF-Tu • GTP. Bound complex retained on nitrocellulose filter discs was quantitated as described (see Materials and Methods section). Binding of aa-tRNA was specific since no radioactivity was retained in the absence of protein nor in the presence of bovine serum albumin.

The K_D values we determined for Asp-tRNA^{Asp} and Asn-tRNA^{Asn} using the nitrocellulose filter-binding assay are one to two orders of magnitude higher than those determined by Ulenbeck's group using the RNase A protection assay (25). These variations may be related to the distinct methodologies used, or to subtle differences in the experimental conditions, since it has been shown that the K_D values are strongly dependent upon the temperature and the ionic strength (15,25,26). To obtain comparable K_D values each measurement involving tRNA and/or EF-Tu variants was conducted in parallel to determination of the K_D of native EF-Tu • GTP • Asp-tRNA^{Asp} and EF-Tu • GTP • Asn-tRNA^{Asn} complexes. Therefore, absolute K_D values obtained by our method might be less accurate than those obtained using the RNase assay; however, using our comparative method, the losses or gains in binding efficiency for the various mutants that we measured are accurate.

Kinetics of deacylation of aa-tRNA in the absence and in the presence of EF-Tu

The reaction mixture of 100 μ l contained: 25 mM Tris-HCl pH 7.4, 10 mM MgCl_2 , 35 mM KCl, 1 μ M [14 C] aa-tRNA and 5 or 15 μ M activated *T. thermophilus* or *E. coli* EF-Tu (22). After 10 min of incubation at 4°C, the mix was transferred to 37°C and the aa-tRNA was determined after incubation times ranging from 0 to 60 min by trichloroacetic acid precipitation of 15 μ l aliquots. The half-lives of aa-tRNA ($\text{Ln } 2/k$) were determined from first order kinetics $\text{Ln}(S_t/S_0) = -kt$, where S_t and S_0 are the aa-tRNA at times t and t_0 and k the rate constant of hydrolysis. Half-lives of 20 and 7 min were determined for free Asp-tRNA^{Asp} and Asn-tRNA^{Asn}, respectively. When the amount of aa-tRNA hydrolyzed in 60 min in the presence of EF-Tu could not be evaluated (less than 5%), its half-life was estimated to be increased more than 40-fold.

RESULTS

Base pairs G49-C65 and A51-U63 in the T-arm of tRNA^{Asn} prevent binding of the aspartylated tRNA to EF-Tu

In the bacterium *T. thermophilus*, EF-Tu encounters two aspartylated tRNA species. The cognate species, Asp-tRNA^{Asp}, is able to bind *T. thermophilus* EF-Tu while the mischarged Asp-tRNA^{Asn} cannot (5). In this study, we investigated the structural elements of the protein and of the tRNAs involved in the discrimination by EF-Tu between the two aspartylated tRNAs. Nitrocellulose filter binding assays were used to determine the binding affinities of Asn-tRNA^{Asn}, Asp-tRNA^{Asp}, and of the mischarged Asp-tRNA^{Asn} for EF-Tu (see Materials and Methods section). K_D values of 270 and 110 nM were determined for Asn-tRNA^{Asn} and Asp-tRNA^{Asp}, respectively. However, the non-cognate Asp-tRNA^{Asn} was deprived of significant affinity for EF-Tu (Figure 1). Since the same results were obtained with native purified tRNA^{Asp} and tRNA^{Asn}, the post-transcriptional modifications of these tRNAs do not contribute significantly to the discrimination of the aa-tRNAs by EF-Tu (data not shown).

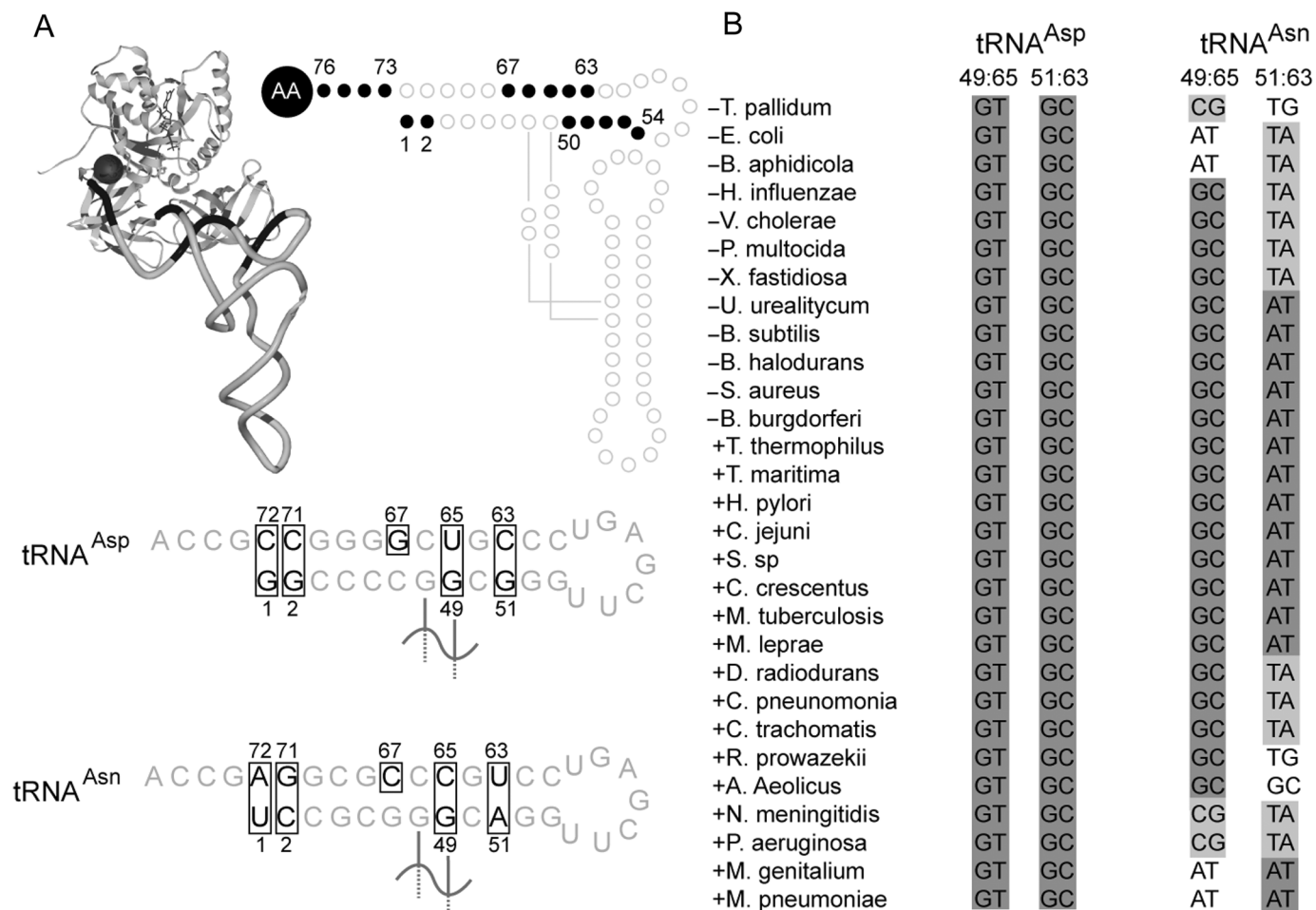


Figure 2. Strategy to identify nucleotides involved in Asp-tRNA^{Asn} discrimination by EF-Tu. (A) Top left is the 3D structure of the EF-Tu • GTP • Phe-tRNA^{Phe} complex (10) showing points of contact between tRNA^{Phe} and EF-Tu. The secondary structure of Phe-tRNA^{Phe} is shown at top right with tRNA nucleotides in the acceptor- and T-arms that contact EF-Tu represented by black spheres and numbered. A comparison of the acceptor and T-arms of tRNA^{Asp} and tRNA^{Asn} from *T. thermophilus* is shown at the bottom with nucleotides selected for mutational analysis boxed. (B) Comparison of base pairs 49–65 and 51–63 in tRNA^{Asp} and tRNA^{Asn} from various bacteria (44). The (+) or (–) in front of the organism indicates whether the species uses (+) the tRNA-dependent transamidation pathway for Asn-tRNA^{Asn} formation or not (–).

To identify the tRNA^{Asn} structural elements that determine the loss of binding of Asp-tRNA^{Asn} to EF-Tu, we investigated the nucleotides that distinguish tRNA^{Asn} (27) from tRNA^{Asp} (28). Additionally, based on available crystal structure data, the nucleotides identified as candidates were restricted to those contacting the protein (12,13). According to these criteria, we identified five base pairs located within the acceptor- and T-stem regions of tRNA^{Asp} and tRNA^{Asn} (Figure 2A). We focused our analysis on base pairs 49–65 and 51–63 in the T-stem since: (i) structural elements of the T-stem were previously found to promote EF-Tu rejection of Sec-tRNA^{Sec} (29) and yeast Met-tRNA^{Met} (30); (ii) the crystal structure of the Cys-tRNA^{Cys} • EF-Tu • GTP complex shows that nucleotide G63 establishes the unique specific interaction with residue E391 of the protein (13); and (iii) these two base pairs are well conserved in tRNA^{Asp} and tRNA^{Asn} throughout bacterial species (Figure 2B).

Using site-directed mutagenesis we swapped the base pairs at positions 49–65 and 51–63, individually and in

combination, between tRNA^{Asp} and tRNA^{Asn}. Purified transcripts of the tRNA^{Asp} and tRNA^{Asn} variants were aspartylated and the dissociation constants (K_D) of the aa-tRNAs • EF-Tu • GTP complexes were determined using the filter-binding assays. Introduction in tRNA^{Asn} of the G51-C63 base pair of tRNA^{Asp} resulted in binding of mischarged Asp-tRNA^{Asn} to EF-Tu, but with an affinity two orders of magnitude lower than Asp-tRNA^{Asp} (Figure 3A). Similarly, introduction of the G49-U65 base pair of tRNA^{Asp} into tRNA^{Asn} also restored the EF-Tu binding capacity of Asp-tRNA^{Asn} but with an affinity 50-fold lower than that of Asp-tRNA^{Asp} (Figure 3A). Introduction of the two base pairs together in tRNA^{Asn} promoted binding of Asp-tRNA^{Asn} to EF-Tu with an affinity only 4-fold lower than that of Asp-tRNA^{Asp}. Conversely, introduction in tRNA^{Asp} of A51-U63 or G49-C65 from tRNA^{Asn} led to a decreased affinity of Asp-tRNA^{Asp} for EF-Tu by 3- and 6-fold, respectively, whereas introduction of both base pairs together decreased EF-Tu affinity by one order of magnitude.

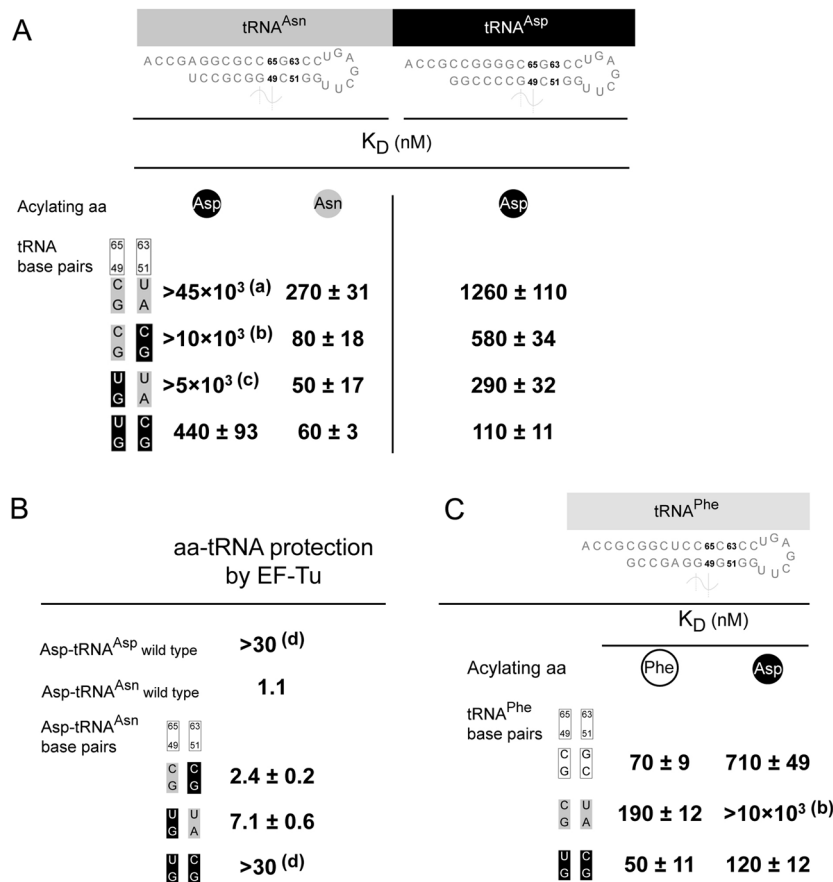


Figure 3. Effects of mutation of base pairs 49–65 and 51–63 of tRNA^{Asp}, tRNA^{Asn} and tRNA^{Phe} on affinity of aa-tRNAs for EF-Tu. (A) K_D values of *T. thermophilus* EF-Tu for Asp- or Asn- tRNA^{Asn}, Asp-tRNA^{Asp} and variants. The acylating aa and nucleotides tested at positions 49–65 and 51–63 are indicated. Gray boxes represent nucleotides found in tRNA^{Asn} and black boxes in tRNA^{Asp}. (B) Ester-bond protection of wild-type Asp-tRNA^{Asp} and Asp-tRNA^{Asn} and variants in the presence of *T. thermophilus* EF-Tu • GTP. Aa-tRNA protection is defined as the ratio of the half-life of aa-tRNA in the presence of EF-Tu • GTP over that in its absence. (C) K_D values of EF-Tu for wild-type Asp-tRNA^{Phe} and variants. Base pairs in gray, black and white boxes represent those seen in wild-type tRNA^{Asn}, tRNA^{Asp} and tRNA^{Phe}, respectively. (a),(b) Minimal value of K_D determined with 340 nM of Asp-tRNA^{Asn} and 10 μM of activated EF-Tu where 15%^(a) or 30%^(b) of aa-tRNAs were saturated. (c) Minimal value of K_D determined with 20 nM of aa-tRNA and 2 μM of EF-Tu where 30% of aa-tRNA were saturated. (d) Less than 5% of the aa-tRNA ester bonds were hydrolyzed in 60 min (see Materials and Methods section).

Surprisingly, the binding of Asn-tRNA^{Asn} was mildly affected (3- to 5-fold) when the two base pairs (49–65 and 51–63) of tRNA^{Asp} were introduced one at a time or in combination into tRNA^{Asn}. This observation indicates that the effect of tRNA base pairs 49–65 and 51–63 on aa-tRNA binding by EF-Tu is strongly dependent on the aa acylating the tRNA.

The importance of base pairs 49–65 and 51–63 of tRNA in binding of aa-tRNA on EF-Tu was also investigated by measuring the protection promoted by the protein of the aminoacyl ester bond of aa-tRNA against spontaneous hydrolysis (22,29). The half-life ($t_{1/2}$) of aa-tRNA increases with the amount of aa-tRNA complexed with EF-Tu. Thus, protection of the aspartylated tRNA^{Asn} variants was investigated by comparing the $t_{1/2}$ in the presence and absence of EF-Tu (Figure 3B). EF-Tu binding to each aa-tRNA was estimated using the ratio of $t_{1/2}$ in the presence of EF-Tu over that in its absence. We observed that while EF-Tu increases the half-life of Asp-tRNA^{Asp} more than 30-fold, it has no effect on $t_{1/2}$ of Asp-tRNA^{Asn}. However, EF-Tu increased the half-life

of the Asp-tRNA^{Asn} variants containing the G49-U65 or the G51-C63 base pair by 2.4- and 7-fold, respectively, and that of the variant containing both base pairs by more than 30-fold. These results correlate with those obtained by direct binding measurements and establish the validity of the filter binding assays. Taken together, the earlier experiments demonstrate that for the tested aa-tRNAs, a G49-U65 base pair increases the affinity for EF-Tu while an A51-U63 base pair decreases it.

Base pairs 51–63 and 49–65 of tRNA are sufficient to modulate the affinity of aa-tRNA for EF-Tu

To determine whether base pairs 49–65 and 51–63 of tRNA^{Asp} and tRNA^{Asn} are sufficient to modulate the affinity of aspartylated tRNA for EF-Tu, we transplanted them into a different tRNA framework. Transplantation was performed into a *T. thermophilus* tRNA^{Phe} variant that already contains aspartate identity elements for aminoacylation (20), and thus is able to be either phenylalanylated or aspartylated. Substitution of

the base pairs 49–65 and 51–63 of tRNA^{Phe} by the corresponding base pairs from tRNA^{Asp} increased affinity of Asp-tRNA^{Phe} for EF-Tu 10-fold, whereas substitution with those from tRNA^{Asn} decreased its affinity by 12-fold (Figure 3C). Thus, the base pairs 51–63 and 49–65 modulate the affinity of aspartylated tRNAs for EF-Tu independently of the tRNA framework. Similar results were observed with phenylalanyl-tRNA, but with a less pronounced effect. Incorporation of the base pairs from tRNA^{Asp} increased affinity for EF-Tu by 1.4 fold, whereas introduction of those from tRNA^{Asn} decreased the affinity by 2.5-fold. These results indicate that the magnitude of the observed effect, in response to 51–63 and 49–65 base pairs substitutions, is dependent on the nature of the acylating aa. The observed effects from nucleotide substitutions in tRNA acylated with Phe were weaker than those for tRNA acylated with Asp. This agrees with previous studies showing that the binding of aa-tRNAs by EF-Tu involves a synergistic recognition of both the aa and tRNA moieties (15).

E. coli EF-Tu discriminates Asp-tRNA^{Asn} from Asp-tRNA^{Asp}

Similar to what was observed with the protein from *T. thermophilus*, EF-Tu from *E. coli* did not bind Asp-tRNA^{Asn} nor protect the aminoacyl ester bond of this aa-tRNA from spontaneous hydrolysis (Figure 4). This result is of particular interest, because it demonstrates the conservation of the discrimination properties of EF-Tu in *E. coli*, even though this organism does not use the indirect pathway of Asn-tRNA^{Asn} formation and therefore is not expected to form Asp-tRNA^{Asn} (5). Moreover, analysis of tRNA^{Asp} and tRNA^{Asn} sequences from various bacterial species reveals that the 49–65 and 51–63 base pairs are conserved with few exceptions, regardless of whether the tRNA-dependent transamidation pathway is used or not (Figure 2B). Consequently, the ability of EF-Tu to discriminate Asp-tRNA^{Asn} from Asp-tRNA^{Asp} and Asn-tRNA^{Asn} is conserved even in organisms that do not require this property.

EF-Tu residue E227 is responsible for discrimination of naturally mischarged Asp-tRNA^{Asn}

The observation that aa-tRNA^{Asn} binds EF-Tu when the esterifying aa is Asn and not Asp, suggests that the aa binding-pocket of the protein displays structural elements that selectively discriminates Asp when bound to tRNA^{Asn}. Asp contains a negative charge within its side-chain that is not present in Asn. One possibility is that an acidic residue within EF-Tu in close proximity to the aa acylating the tRNA, may exert electrostatic repulsion on the negatively charged side-chain of Asp resulting in a weak interaction at the aa moiety. In the context of a weakly binding tRNA moiety, this repulsion could result in loss of binding to EF-Tu as seen in Asp-tRNA^{Asn}. Sequence alignments of EF-Tu aa-binding pockets revealed two negatively charged residues (Figure 5A). These residues, Glu and Asp (E227 and D228 in *T. thermophilus* EF-Tu) are strictly conserved in all

bacterial elongation factors and are located 3.7 and 5.4 Å, respectively, from the aa side-chain in the crystal structure of the Phe-tRNA^{Phe} • EF-Tu • GTP ternary complex (Figure 5B) (12). To analyze their role in discrimination of Asp-tRNA^{Asn} by EF-Tu, both residues were replaced independently by Ala. The EF-Tu D228A mutant binds both Asp-tRNA^{Asp} and Asn-tRNA^{Asn} with affinities similar to wild type EF-Tu, but is still unable to bind Asp-tRNA^{Asn} (Figure 5C). However, the E227A mutant binds mischarged Asp-tRNA^{Asn} with an affinity only 5- and 2-fold lower than that of the wild-type EF-Tu for Asp-tRNA^{Asp} and Asn-tRNA^{Asn}, respectively. This indicates that the conserved E227 in the aa-binding pocket of EF-Tu is sufficient to promote rejection of Asp-tRNA^{Asn}. Interestingly, the E227A mutation also slightly increases the affinity of EF-Tu for Asp-tRNA^{Asp} and Asn-tRNA^{Asn}, but does not significantly affect the affinity for Phe-tRNA^{Phe}, indicating that the observed effect depends on the nature of the tRNA and the aa moieties.

To determine if E227 excludes binding of Asp-tRNA^{Asn} through electrostatic repulsion of the negatively charged Asp, we analyzed the binding properties of an E227Q mutant of EF-Tu. This variant, like wild-type EF-Tu, binds Asp-tRNA^{Asp} and Asn-tRNA^{Asn}, but is unable to bind Asp-tRNA^{Asn} (Figure 5C). Since, either Glu or Gln at position 227 prevents binding of Asp-tRNA^{Asn} to EF-Tu, rejection of this aa-tRNA is probably due to steric hindrance between the two aa side chains rather than to electrostatic repulsion.

All archaea use the indirect pathway to form Gln-tRNA^{Gln} and about half of them to form Asn-tRNA^{Asn} (31,32). Alignment of EF-Tu and archaeal EF-1A sequences reveals the presence, in the archaeal

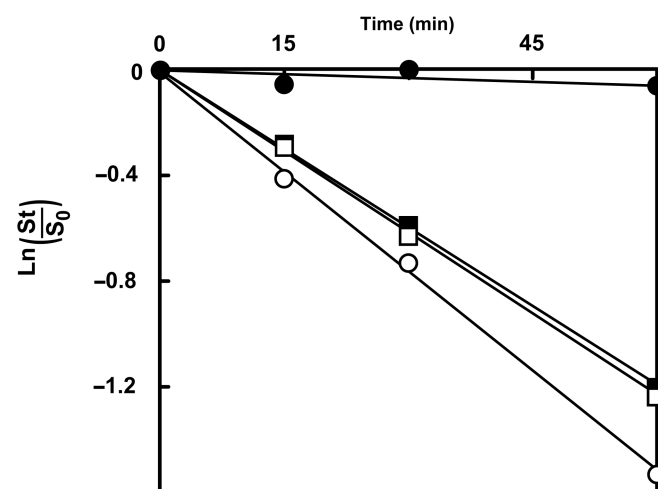


Figure 4. Protection of the ester bonds of Asp-tRNA^{Asp} and Asp-tRNA^{Asn} by EF-Tu. The kinetics of hydrolysis of the ester bond of *T. thermophilus* Asp-tRNA^{Asp} (circles) and Asp-tRNA^{Asn} (squares) were determined in the presence of *E. coli* EF-Tu • GTP (filled symbols) or in its absence (open symbols) as described in Materials and Methods section. The curves $\ln(S_t/S_0) = -kt$ are shown where S_t and S_0 are the concentrations of aa-tRNA at times t and t_0 , and k is the rate constant of hydrolysis of aa-tRNA.

factor, of a Gln residue at position corresponding to E227 in *T. thermophilus* EF-Tu (Figure 5A). This suggests that, in archaea using the tRNA-dependent transamidation pathway for Asn-tRNA^{Asn} synthesis (31,32), this residue may prevent binding of the elongation factor to the Asp-tRNA^{Asn} intermediate. Additionally, a Gln at this position is also conserved in eukaryal EF-1A. Interestingly, the tRNA-dependent transamidation pathway, and thus the necessity to discriminate between Asp-tRNA^{Asn} and Asp-tRNA^{Asp}, has so far not been shown to be necessary in eukaryotes.

EF-Tu residue E391 modulates the affinity of EF-Tu for Asp-tRNA^{Asp} and Asn-tRNA^{Asn}

Crystal structures of Cys-tRNA^{Cys} or Phe-tRNA^{Phe} in complex with *T. thermophilus* EF-Tu • GTP show that recognition of aa-tRNAs by EF-Tu is mediated through non-specific hydrogen bonds at the backbone of tRNAs. However, one specific interaction in the Cys-tRNA^{Cys} • EF-Tu • GTP complex was reported between the O_γ of EF-Tu residue E391 and the N2 of base G63 located in the minor groove of the C51-G63 base pair in the T-arm of the tRNA (13). The Glu at position 391 is strictly conserved in eubacterial EF-Tu. To investigate

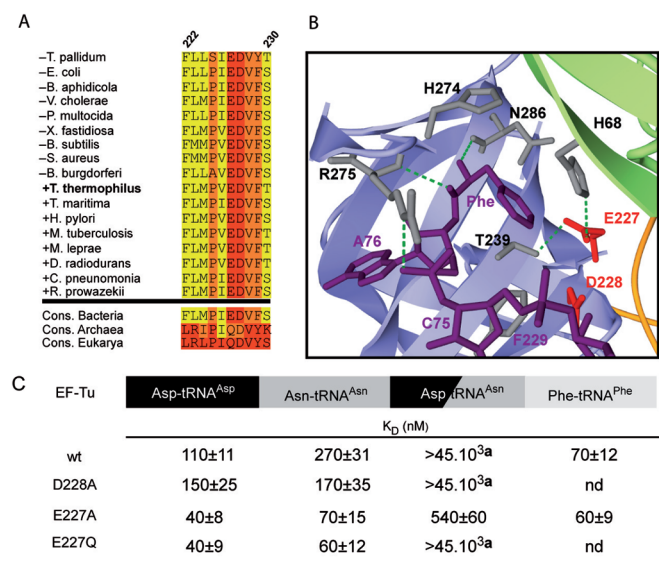


Figure 5. Identification of aa residues in the aa-binding pocket of EF-Tu potentially involved in rejection of Asp-tRNA^{Asn}. (A) Sequence alignment of the region encompassing E227 and D228 of bacterial EF-Tu and comparison with archaeal and eukaryal EF-1A. A sampling of 87 bacterial elongation factors is shown as well as the consensus sequences of bacterial EF-Tu, and archaeal and eukaryal EF-1A. The percentage of homology is symbolized by colors: red and orange denote 100 and 80% identity, respectively, and yellow indicates conservation of the chemical nature of the residue in at least 80% of the sequences. (B) Localization of E227 and D228 in the aa-binding pocket of *T. thermophilus* EF-Tu. The structure displayed is that of the EF-Tu • GTP • Phe-tRNA^{Phe} ternary complex (12). The two acidic residues that belong to the aa-binding pocket are in red and the tRNA is in purple. (C) Dissociation constants of wild-type *T. thermophilus* EF-Tu and mutants with specified aa-tRNAs. ^a Minimal value of K_D estimated with 340 nM of Asp-tRNA^{Asn} and 10 μM of activated EF-Tu where 15% of aa-tRNAs were saturated.

the importance of this residue for EF-Tu binding to Asp-tRNA^{Asp} and Asn-tRNA^{Asn}, we mutated it into Ala. The E391A EF-Tu variant displayed an increased affinity (2.8-fold) for Asn-tRNA^{Asn} and a decreased affinity (5.5-fold) for Asp-tRNA^{Asp} (data not shown). These results indicate that residue E391 modulates the affinity of EF-Tu for these aa-tRNAs.

The G51-C63 base pair of tRNA^{Asp} provides an NH₂ group in the minor groove (33) like in the Cys-tRNA^{Cys} • EF-Tu • GTP crystal structure, that may also contact residue E391 of EF-Tu by a specific hydrogen bond. Replacement of the Asp-tRNA^{Asp} base pair G51-C63 with A51-U63, removes the hydrogen bond donor group, which might explain how this substitution decreases the affinity of Asp-tRNA^{Asp} for EF-Tu. In contrast, Asn-tRNA^{Asn} already contains an A51-U63 base pair, which does not provide a hydrogen bond donor group for interaction with EF-Tu residue E391. This might explain why the E391A mutation of EF-Tu does not negatively affect binding of Asn-tRNA^{Asn}. In fact, a slight increase in affinity is observed between the E391A variant and this aa-tRNA. This structural interpretation to explain the variable affinity of the tRNA moiety of aa-tRNA for the elongation factor is also supported by the base pair swapping experiments between tRNA^{Asp} and tRNA^{Asn} described earlier. Replacement of the base pair G51-C63 by A51-U63 in tRNA^{Asp} decreases the affinity of the aspartylated tRNA^{Asp} for EF-Tu. Conversely, replacement of A51-U63 by G51-U63 in tRNA^{Asn} increases the affinity of aspartylated or asparaginylated tRNA^{Asn} for EF-Tu (Figure 3).

DISCUSSION

Base pairs 49–65 and 51–63 of tRNA modulate the affinity of aa-tRNAs for EF-Tu

We have shown that the distinct binding strengths of tRNA^{Asp} and tRNA^{Asn} for EF-Tu are promoted by base pairs 49–65 and 51–63 located in the T-arm. One question emerging from this study is whether these elements constitute the basis for EF-Tu discrimination of other aa-tRNAs escaping peptide elongation. Like Asp-tRNA^{Asn}, the Glu-tRNA^{Gln} intermediate, formed by the indirect Gln-tRNA^{Gln} transamidation pathway (4,34,35), does not bind EF-Tu (36). Comparison of consensus sequences from bacterial tRNA^{Gln} and tRNA^{Glu} shows that tRNA^{Gln} does not contain a U in the 49–65 base pair, but contains a U in the 51–63 pair (Figure 6A). In contrast, tRNA^{Glu} contains a U in the first pair but not in the second one. With only a few exceptions, these base pairs are conserved in tRNA^{Gln} and tRNA^{Glu}, and they are generally present regardless of whether or not the organism uses the indirect pathway of Gln-tRNA^{Gln} formation. Thus, EF-Tu may discriminate the mischarged Glu-tRNA^{Gln} and Asp-tRNA^{Asn} using similar mechanisms.

Our results, in combination with a previous work (26), suggest that Asp and Glu have a weak affinity for EF-Tu and that for efficient binding of aspartylated or glutamylated tRNA to the elongation factor, the tRNA moiety

should display a strong binding ability provided by the G49-U65 and G51-C63 base pairs. It is worth noting that a G-U pair provides unique structural features. This wobble base pair induces a deviation of up to 2 Å five base pairs away (37) and provides a peculiar local electrostatic environment (38). These structural properties may influence aa-tRNA binding to EF-Tu when a wobble base pair is embedded in the T-stem of the tRNA.

Base pairs G49-U65 and G51-C63 are conserved in elongator tRNA^{Gly} and absent in non-elongator tRNA^{Gly}

Staphylococcus epidermidis contains in addition to the elongator tRNA^{Gly}, the non-elongator species, Gly-tRNA^{GlyIA}, which is not involved in protein synthesis, but instead serves for synthesis of cell wall peptidoglycan (39,40). Like tRNA^{Asp}, elongator tRNA^{Gly} contains the G49-U65 and G51-C63 pairs that may confer a strong binding capacity of Gly-tRNA^{Gly} for EF-Tu, compensating the low affinity of the Gly moiety. In contrast, the affinity of the glycylylated native and transcript tRNA^{GlyIA} for EF-Tu is at least one order of magnitude lower (data not published) (41). Interestingly, tRNA^{GlyIA} contains A-U pairs at positions 49-65 and 51-63, which might explain the absence of recognition by EF-Tu (Figure 6A). This idea is strongly supported by a recent study showing that replacement of the base pair G49-U65 by G49-C65 drastically decreases the elongator capacity of *E. coli* tRNA^{Gly} *in vivo* (42). The formylated initiator Met-tRNA^{Met} (fMet-tRNA_i^{Met}), which binds to the initiator factor IF2 constitutes another example of aa-tRNA deprived of affinity for EF-Tu. It has been shown that lack of binding of fMet-tRNA_i^{Met} to *E. coli* EF-Tu is promoted by the formyl group of Met and by the base pair C1-A72, which acts as a negative determinant, since substitution of this pair by C1-G72 or U1-A72 improves binding (43). These examples and the results reported here illustrate that there might be alternative mechanisms and multiple structural determinants responsible for either strong or weak binding interactions between aa-tRNAs and EF-Tu.

The frequency of U in base pairs 49-65 and 51-63 correlates with the affinities of other tRNAs for EF-Tu

To be delivered to the ribosome, elongator aa-tRNAs must bind EF-Tu with appropriate and equivalent affinities, which are conferred by a thermodynamic compensation between a strong interaction of the aa moiety and a weak interaction of the tRNA moiety or *vice-versa*. The contributions of the aa and tRNA moieties to the overall affinity of aa-tRNA for EF-Tu are organized in a compensatory fashion such that all elongator aa-tRNAs bind with similar affinities (14,15,26,44). In a previous study, the contribution of the tRNA moieties in binding of aa-tRNAs to EF-Tu were evaluated by affinity measurements of various *E. coli* tRNAs charged with Val (26,44). To analyze whether the tRNA base pairs at positions 49-65 and 51-63 influence the binding strength of these tRNAs, we investigated the correlation between the nature of the residues at these positions in tRNAs and the affinity of the tRNAs for EF-Tu. Figure 6 shows that

E. coli tRNAs fall into two categories according to their relative affinities for EF-Tu. tRNAs exhibiting the strongest affinity ($\Delta G_0 = -11.7$ to -10.5 kcal/mol) all possess a U in the 49-65 base pair (except tRNA^{Thr}) and are deprived of a U in the 51-63 pair. The majority of tRNAs exhibiting lower affinity for EF-Tu ($\Delta G_0 = -9.6$ to -8.5 kcal/mol) do not contain a U within the 49-65 base pair but contain a U in the 51-63 base pair.

To analyze whether these nucleotides are conserved in homologous tRNAs from other species (45), we compared the frequency of nucleotides which determine strong affinity of tRNA for EF-Tu to that of nucleotides which determine low affinity in other organisms. tRNAs homologous to those of *E. coli* that exhibit strong EF-Tu binding capacity combine a high frequency of U65 and a notable absence of U in position 63. Conversely, tRNAs homologous to those of *E. coli* that exhibit low EF-Tu binding capacity combine a high frequency of U in position 63 and an absence of U at position 65 (Figure 6). These observations suggest that the structural elements of tRNA that determine the strong affinity of tRNA^{Asp} and the weak affinity of tRNA^{Asn} for EF-Tu are, at least partly, conserved in other tRNAs in *E. coli* as well as in other organisms.

Discrimination of Asp-tRNA^{Asn} allows unambiguous decoding of Asn codons

Current evolutionary models agree that AsnRS and GlnRS, which promote the direct pathway for aminoacylation of tRNA^{Asn} and tRNA^{Gln}, are a recent addition to the repertoire of aaRSs (46-48). Introduction of Asn and Gln in the genetic code is believed to have occurred earlier in the course of evolution through the use of the indirect pathway of synthesis of Asn-tRNA^{Asn} and Gln-tRNA^{Gln}. This ancestral pathway relies on the tRNA-dependent amidotransferase, the enzyme that converts misacylated Asp-tRNA^{Asn} and Glu-tRNA^{Gln} into Asn-tRNA^{Asn} and Gln-tRNA^{Gln}. The tRNA-dependent biosynthesis pathway of these aa is viable only if the mischarged intermediates (Asp-tRNA^{Asn} and Glu-tRNA^{Gln}) are efficiently excluded from protein synthesis, in order to avoid ambiguous decoding of Asn and Gln codons. This suggests that the ability of EF-Tu to discriminate misacylated Asp-tRNA^{Asn} and Glu-tRNA^{Gln} preexisted or appeared concomitantly to the introduction of Asn and Gln into the genetic code.

The structural elements involved in the discrimination of Asp-tRNA^{Asn} are widely conserved in eubacterial tRNA^{Asn} and EF-Tu, even in organisms that do not use the indirect pathway of tRNA asparaginylation. In these organisms, the formation of mischarged Asp-tRNA^{Asn} is not expected to occur, even though the structural elements that allow EF-Tu to discriminate the mischarged aa-tRNA are still present. The analysis of the base pair composition of elongator tRNAs (see earlier and Figure 6), suggests that these elements may play a larger role than just discrimination of non-cognate aa-tRNAs and may be involved in the modulation of EF-Tu binding to other elongator aa-tRNAs. The maintenance of this delicate balance of interactions is essential for the

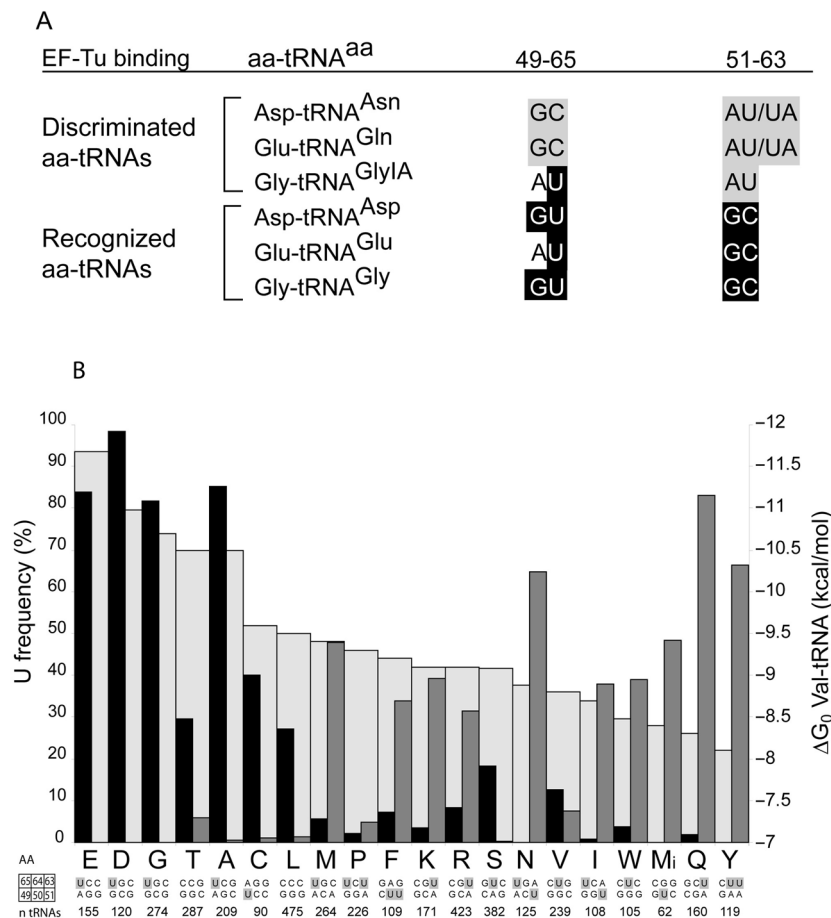


Figure 6. Analysis of base pairs 49–65 and 51–63 in the T-stem of various eubacterial tRNAs. **(A)** Comparison of base pairs 49–65 and 51–63 of aa-tRNAs that are discriminated or recognized by EF-Tu. The reported base pairs are conserved in more than 80% of specified tRNAs. tRNA^{GlyIA} base pairs are those from *S. aureus* and *S. epidermidis* tRNAs. **(B)** Correlation between the ΔG_0 of formation of EF-Tu • GTP • Val-tRNA complexes and the frequency of U in base pairs 49–65 and 51–63. The frequency of U in the 49–65 pair in combination with absence of U in the 51–63 pair is represented by black bars. The frequency of U in the 51–63 pair and absence of U in the 49–65 pair is represented by dark gray bars. The percentage of the conservation of the combinations described above is on the left axis. The ΔG_0 of formation of the complex of valylated tRNAs with EF-Tu (26,44) is represented by white bars and is indicated on the right axis. The specificities of the tRNAs are indicated by the single letter aa code. The sequences of base-pairs 49–65/50–64/51–63 of *E. coli* tRNAs and the number of eubacterial tRNA species analyzed are indicated at the bottom of the Figure. tRNA sequences were obtained from the compilation of tRNA sequences and genes (49).

role of EF-Tu to discriminate between cognate and non-cognate aa-tRNAs and properly deliver the correct substrate to the translational machinery for incorporation during protein synthesis.

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