Discrimination among tRNAs Intermediate in Glutamate and Glutamine Acceptor Identity†

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ABSTRACT: The set of nucleotides in *Escherichia coli* $tRNA^{Gln}$ which facilitate aminoacylation by glutaminyl-tRNA synthetase (GlnRS) has been defined [Hayase et al. (1992), *EMBO J. 11*, 4159–4165]. To determine whether the glutamine "identity set" is sufficient to confer acceptance on a noncognate tRNA, we constructed $tRNA^{Glu}$ mutants with the set of glutamine recognition elements. These mutants were examined for aminoacylation *in vitro* with GlnRS and also with glutamyl-tRNA synthetase (GluRS) to correlate gains in glutamine acceptance with losses of glutamate acceptance. Incorporating glutamine recognition elements in only the acceptor stem or anticodon loop of $tRNA^{Glu}$ improved the specificity constant (k_{cat}/K_M) for aminoacylation by GlnRS. However, the introduction of all defined glutamine recognition elements in $tRNA^{Glu}$ resulted in a substrate with a specificity constant 100-fold below that for aminoacylation of $tRNA^{Gln}$. Including the tertiary framework of $tRNA^{Gln}$ (in addition to the glutamine recognition elements) in the $tRNA^{Glu}$ context further improved aminoacylation by GlnRS, but the specificity was still reduced compared with that of $tRNA^{Gln}$. The increase in glutamine acceptance was correlated for all mutants with a decrease in glutamate acceptance, indicating that GluRS also recognizes acceptor stem and anticodon sequences in cognate tRNA. The inability to completely convert $tRNA^{Glu}$ to glutamine acceptance with these mutations suggests that $tRNA^{Glu}$ contains antideterminants to glutamine identity. The analysis of these mutants with both enzymes revealed that there is a strong element of discrimination between glutamate and glutamine tRNAs associated with the anticodon. To test this dependence, mutants of both tRNAs were made to effect anticodon switches to the possible glutamate and glutamine isoacceptors. The kinetic evaluation of the anticodon switch mutants suggests that overlap in anticodon recognition is avoided through specificity for the third anticodon position coupled with divergent preferences for the wobble base.

The fidelity of protein synthesis is dependent on the correct aminoacylation of the cognate tRNA by each aminoacyl-tRNA synthetase. The successful identification of the cognate tRNA is due to both specific interaction with sequences in the cognate tRNA (termed recognition elements) and nonproductive interaction with noncognate substrates [reviewed in Pallanck and Schulman (1992)]. Disrupting either the productive interactions or the nonproductive interactions can lead to misacylation, which in turn leads to misincorporation of amino acids during translation.

For the interaction of *Escherichia coli* $tRNA^{Gln}$ and glutaminyl-tRNA synthetase (GlnRS),[1] the X-ray structure of the GlnRS·$tRNA^{Gln}$ complex (Rould et al., 1989) allows the base-specific contacts suggested by many genetic and biochemical studies [reviewed in Rogers et al. (1993)] to be directly visualized. All of the $tRNA^{Gln}$ sequences which are seen to be in contact with GlnRS in the X-ray crystal structure have been tested for the base specificity of each interaction. This has allowed the definition of a set of bases which specify glutamine acceptor identity (Hayase et al., 1992). These studies identify the discriminator base (G73) and three base pairs at the top of the acceptor stem, G10 in the D-stem, and anticodon loop sequences in $tRNA^{Gln}$ as critical for productive interaction with GlnRS (Figure 1). Specifically, the contact of the 2-amino groups of G2, G3, and G10 in the tRNA with protein side chains has been demonstrated by using inosine-substituted $tRNA^{Gln}$ transcripts as substrates for aminoacylation (Hayase et al., 1992).

The structure of the GlnRS·$tRNA^{Gln}$ complex also reveals a number of backbone contacts between the enzyme and the tRNA which are not sequence-specific (Rould et al., 1989). It is likely that these nonspecific contacts provide a necessary structural context for the placement of the identity elements. We have examined this possibility by testing the ability of the defined glutamine identity set to confer glutamine acceptance on a noncognate tRNA. As GlnRS misacylates $tRNA^{Glu}$ *in vitro* better than it misacylates any other noncognate *E. coli* tRNA (Hoben, 1984), this background was chosen for conversion to glutamine acceptance. A sequence comparison between $tRNA^{Glu}$ and $tRNA^{Gln}$ reveals no greater similarity than that for any other pair of tRNA acceptor types (Figure 1; Sprinzl et al., 1989). However, it was hoped that the $tRNA^{Glu}$ context would prove to be favorable for conversion to glutamine acceptance due to the evolutionary relationship between the two aminoacylation systems, as evidenced by the sequence similarities between GluRS and GlnRS (Breton et al., 1986).

This work describes the kinetic analysis of a number of tRNA mutants intermediate in glutamate and glutamine acceptor identities. The glutamine identity elements have been introduced in a synthetic gene for $tRNA^{Glu}$, allowing *in vitro* transcription to produce substrates for aminoacylation assays with GlnRS. In light of the sequence homology between

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[1] Abbreviations: Aminoacyl-tRNA synthetases are abbreviated by the three-letter amino acid code followed by "RS" (i.e., GlnRS and GluRS). Modified nucleosides are abbreviated as follows: mnm^5s^2U, 5-[(methylamino)methyl]-2-thiouridine; s^2U, 2-thiouridine; D, dihydrouridine; Ψ, pseudouridine.

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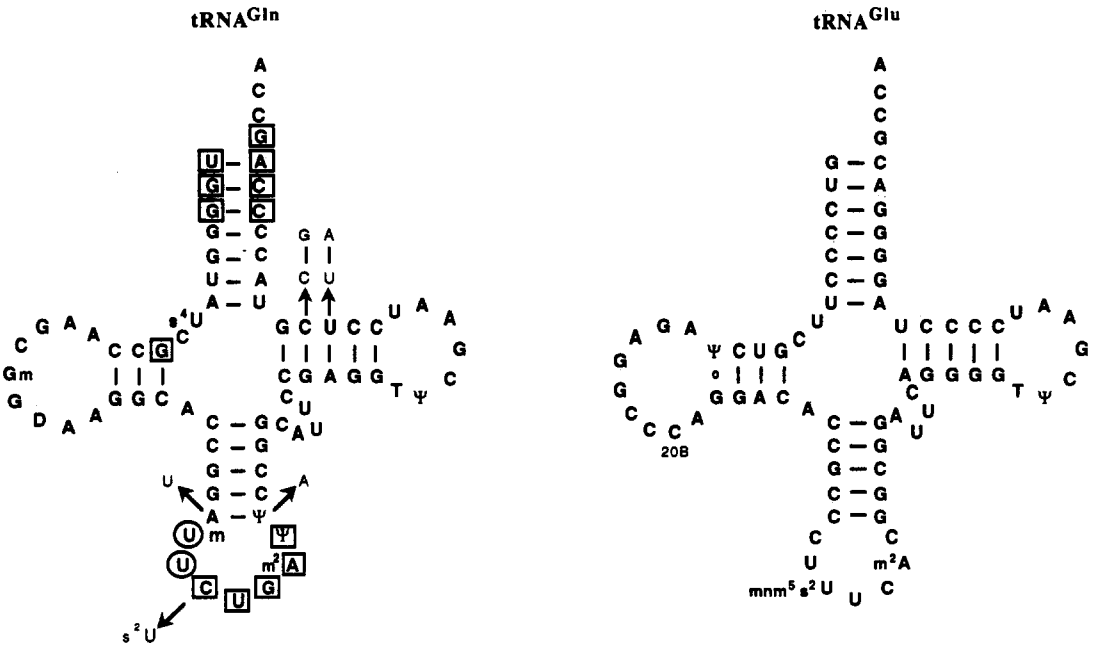

FIGURE 1: $tRNA^{Gln}$ and $tRNA^{Glu}$ of *Escherichia coli*. The sequence for the major $tRNA^{Gln}$ isoacceptor (Gln_2) is shown, with arrows indicating sequence differences in the minor isoacceptor (Gln_1). Nucleosides in $tRNA^{Gln}$ that have been identified as recognition elements for GlnRS are boxed; circled positions may contribute to glutamine identity by stabilizing the proper anticodon loop conformation (Rould et al., 1989; Hayase et al., 1992).

E. coli GluRS and GlnRS (Breton et al., 1986), we also have examined the mutants for aminoacylation by GluRS to determine if increases in glutamine acceptance necessarily correspond to decreases in glutamate acceptance.

Preliminary experiments with mutant $tRNA^{Glu}$ transcripts suggested that the anticodon sequence was the major site of discrimination between glutamate and glutamine tRNAs for both GluRS and GlnRS. For this reason, the ability of the anticodon sequences alone to effect glutamate or glutamine acceptance was investigated by *in vitro* aminoacylation by GluRS and GlnRS of anticodon switch mutants corresponding to all possible permutations of glutamate and glutamine isoacceptors in both tRNA contexts. The importance of the anticodon in $tRNA^{Gln}$ identity has long been established (Rogers et al., 1993). However, isoacceptors for glutamate (anticodons UUC and CUC) and glutamine (anticodons UUG and CUG) have two of the three anticodon bases in common, although the wobble base is differently modified (Sprinzl et al., 1989). The results presented in this study suggest that GluRS and GlnRS avoid overlap in recognition of anticodon sequences not only by tight specificity for the third anticodon position but also by divergent preference for the different isoacceptor sequences and tolerance for wobble base modification.

MATERIALS AND METHODS

General. Uniformly labeled L-[^{3}H]glutamic acid (56 Ci/mmol) and L-[^{3}H]glutamine (55 Ci/mmol) were purchased from Amersham. Purified T7 RNA polymerase was a gift from D. Jeruzalmi, and purified GlnRS was a gift from J. Sherman and U. Thomann. Oligonucleotides were provided by G. O'Neill or were purchased from Oligos, etc. or from the William M. Keck Foundation for Biochemical Research (Yale University). *E. coli in vivo* $tRNA^{Gln}_1$ and $tRNA^{Glu}$ were purchased from Subriden RNA (Rolling Bay, WA), and purified *in vivo* $tRNA^{Gln}_2$ was a gift from D. Jeruzalmi and J. Sherman.

GluRS Purification. E. coli GluRS was purified from DH5α transformed with pLQ7611ΔNru1 containing the *gltX* gene (Breton et al., 1986) in an FPLC (Pharmacia) adaptation of the published purification (Lapointe et al., 1985). The DEAE-cellulose column was replaced by a MonoQ FPLC column equilibrated in 10 mM Tris-HCl, pH 7.0, 20 mM 2-mercaptoethanol, 10 mM KCl, 1 mM $MgCl_2$, and 10% glycerol, with an elution gradient to 500 mM KCl over 12 min, at a flow rate of 1 mL/min. GluRS elutes from this column as reported for the DEAE-cellulose column (Lapointe et al., 1985). The Blue-Sepharose column was run as described, but FPLC was used with the same gradient and flow rate as for the MonoQ step. The purified enzyme (specific activity of 2000 units/mg, 3.6 mg/mL) was stable in 50 mM K-HEPES (pH 8.6), 0.2 mM dithiothreitol, 10 mM 2-mercaptoethanol, and 20% glycerol at −20 °C. GlnRS is separated from GluRS during the MonoQ purification step, and the purified GluRS preparation showed no GlnRS activity in aminoacylation assays (data not shown).

Construction of a Synthetic $tRNA^{Glu}$ Gene for in Vitro Transcription. The phosphorylation and ligation of the three pairs of complementary and overlapping oligonucleotides including the T7 promoter and the $tRNA^{Glu}$ sequence was as described (Sampson & Uhlenbeck, 1988). To obtain a restriction site at the 3′-end of the tRNA gene sequence to provide the 3′-CCA end of the tRNA by run-off transcription, the synthetic gene fragment (*Eco*RI–*Bam*HI) was ligated to a processing deoxyoligonucleotide (sequence 5′-GGATTC-CGGACATCCTGCAG-3′; A. Schön, personal communication) with an internal *Fok*I site positioned for cleavage upstream at the 3′-end of the tRNA gene. This synthetic $tRNA^{Glu}$ gene was inserted as an *Eco*RI–*Pst*I fragment into pUC2119 to produce plasmid pKR320. *E. coli* strain DH5α was used for cloning. Constructs for mutants E→QSL and E→QID were made in a similar manner, with all other mutants being made by site-directed mutagenesis of pKR320. The sequence of each construct was confirmed by dideoxy sequencing of the plasmid DNA.

In Vitro Transcription and Purification of $tRNA^{Glu}$ and $tRNA^{Gln}$ Species. Plasmid preparations for transcription of the tRNA genes were made by standard techniques using

cesium chloride gradient purification (Maniatis et al., 1982), and the tRNA gene sequences were confirmed after large-scale preparations by dideoxy sequencing. Template DNA for transcription by T7 RNA polymerase was digested with *Fok*I for $tRNA^{Glu}$ templates and *Bst*NI for $tRNA^{Gln}$ templates. Transcriptions were as described (Sampson & Uhlenbeck, 1988), using 0.5 mg/mL template DNA and 0.8 mg of T7 RNA polymerase per milligram of template DNA. Typical yields of transcription were 300–350 mol of transcript per mole of template. Full-length transcripts were purified from shorter products by electrophoresis on 12% polyacrylamide gels containing 7 M urea. Transcript bands were visualized in preparative gels by UV shadowing. The purified transcripts were eluted for 48 h in 0.5 M Tris-HCl (pH 7.0), 10 mM $MgCl_2$, 0.1 mM EDTA, and 0.1% SDS. The eluted transcripts were phenol extracted, ethanol precipitated, and renatured by heating at 65 °C for 5 min, followed by slow cooling to room temperature. Wild-type transcripts and control *in vivo* tRNAs prepared in this manner could be aminoacylated to >1200 pmol of aminoacyl-tRNA per A_{260} unit of RNA.

Site-Directed Mutagenesis of Synthetic $tRNA^{Glu}$ and $tRNA^{Gln}$ Genes. Oligonucleotides used for mutagenesis of U-containing template DNA (Kunkel, 1985) were 20–22 nucleotides in length, directed to the tRNA-like strand. Mutants of $tRNA^{Glu}$ were made on the pKR320 construct described above, and mutants of $tRNA^{Gln}$ were made from the pGlnG1 construct (Jahn et al., 1991). To obtain U-containing DNA as a template for mutagenesis, CJ236 (Kunkel, 1985) was transformed with the plasmid construct, and helper phage M13K07 was used to induce single-stranded DNA production as described (Viera & Messing, 1987).

In Vitro Aminoacylation Assays. Aminoacylation assays were performed with [^{3}H]glutamate or [^{3}H]glutamine. To correct for the low counting efficiency of free ^{3}H-labeled amino acids in comparison to that of [^{3}H]aminoacyl-tRNA, total charging assays with either ^{3}H- or ^{14}C-labeled amino acids were performed in parallel so that the conversion factors for correcting the counting efficiency of the free [^{3}H]amino acids could be calculated.

The same assay conditions were used for both GluRS and GlnRS assays. Assays were performed as described (Lapointe et al., 1985) in 50-μL reaction mixtures containing 50 mM K-HEPES (pH 7.2), 16 mM $MgCl_2$, 2 mM ATP, and a total of 400 μM amino acid, with 5 μM ^{3}H-labeled amino acid and 395 μM unlabeled amino acid. For all kinetic assays, the concentration of tRNA varied over at least a 10-fold range (around 0.1–5 μM for good substrates, 2–50 μM for intermediate substrates, and 10-100 μM for poor substrates), with an enzyme:tRNA molar ratio always lower than 1:20. All assays were performed in duplicate trials using five tRNA concentrations with at least four time points taken in the linear range of the assay. Kinetic constants were calculated by using both Lineweaver–Burk and Eadie–Hofstee plots.

RESULTS

Construction of Glutamate to Glutamine Conversion Mutants and Tertiary Domain Mutants. To attempt the conversion of $tRNA^{Glu}$ to glutamine acceptance, mutations were made by multiple rounds of oligonucleotide-directed mutagenesis in the $tRNA^{Glu}$ gene construct for *in vitro* transcription. Mutations were introduced to match $tRNA^{Gln}$ sequences at positions in the acceptor stem and the anticodon loop that were previously identified as glutamine recognition elements (Figure 2; Hayase et al., 1992). The changes in the anticodon loop and acceptor stem regions were tested for effects

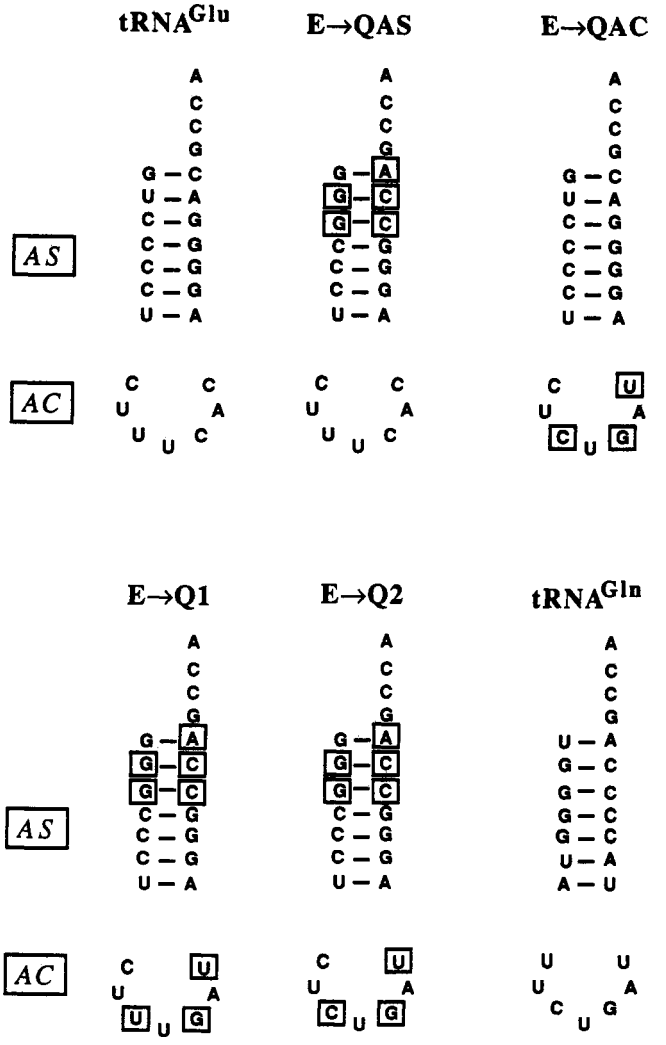

FIGURE 2: $tRNA^{Glu}$ mutants for conversion to glutamine acceptance. Boxed nucleotides represent mutations made to the defined set of glutamine recognition elements (Figure 1; Hayase et al., 1992) on the glutamate tRNA. For clarity, only acceptor stem and anticodon loop sequences are shown to represent each tRNA construct; "*AS*" and "*AC*" indicate acceptor stem and anticodon sequences, respectively.

on aminoacylation separately ($tRNA^{Glu}$ mutants E→QAS and E→QAC) and jointly (mutants E→Q1 and E→Q2). As the studies to identify the glutamine recognition elements were conducted with a $tRNA^{Gln}$ transcript carrying a U→G1 mutation to improve transcription (Jahn et al., 1991), the corresponding C→A72 change was made in $tRNA^{Glu}$ to yield the same unpaired G1/A72 sequence at the top of the acceptor stem in all mutants. The other acceptor stem changes preserved base pairing, with mutation of U2·A71 to G2·C70 and the reversal of the C3·G70 pair to match the G3·C70 of $tRNA^{Gln}$.

The anticodon loop was also targeted for mutations of $tRNA^{Glu}$. The $tRNA^{Gln}_2$ isoacceptor (anticodon CUG) was used for the mutational analysis identifying GlnRS recognition elements (Jahn et al., 1991); therefore, U→C34 and C→G36 mutations were introduced in the anticodon of $tRNA^{Glu}$ for conversion to the CUG glutamine anticodon. One additional anticodon loop mutation (C→U38) was included, as the kinetic analysis of the $tRNA^{Gln}$ transcript showed that a change to a guanosine at this position greatly affected aminoacylation by GlnRS, indicating that there might be some specificity in interaction with this position. During the final round of mutagenesis to combine acceptor stem and anticodon loop mutations, the other glutamine isoacceptor (anticodon se-

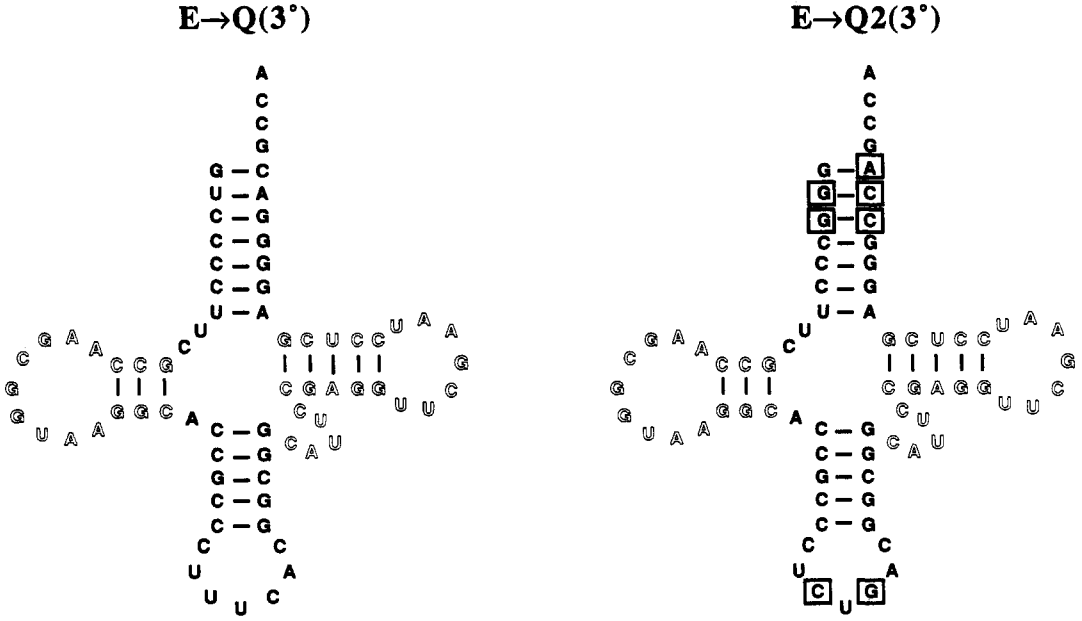

FIGURE 3: $tRNA^{Glu}$ tertiary domain mutants. Bases in outline font represent sequences of $tRNA^{Gln}$ constructed in the $tRNA^{Glu}$ framework. The E→Q(3°) construct is $tRNA^{Glu}$ with Gln D-stem/loop, V-loop, and TΨC-stem/loop sequences. Construct E→Q2(3°) is the same construct with additional mutations (boxed nucleotides) to include the identified set of bases which confer recognition by GlnRS (Figure 1; Hayase et al., 1992).

quence UUG) was isolated, providing the opportunity to examine both glutamine anticodons in combination with the acceptor stem changes (Figure 2, mutants E→Q1 and E→Q2).

As the glutamine recognition elements introduced on the $tRNA^{Glu}$ background failed to completely convert the tRNA to glutamine acceptance (see below), two more $tRNA^{Glu}$ constructs were made to mimic the $tRNA^{Gln}$ tertiary structure. There are unusual features of the D-loop sequence of $tRNA^{Glu}$ which could indicate a difference in the L-shaped structures of $tRNA^{Glu}$ and $tRNA^{Gln}$. Among all *E. coli* tRNAs, $tRNA^{Glu}$ is the only short variable loop tRNA with a 20B position in the bottom of the D-loop (Figure 1; McClain & Nicholas, 1987), and $tRNA^{Glu}$ shares with *E. coli* $tRNA^{Tyr}$ (which has a long variable loop) the unique absence of any dihydrouridines (or uridine residues), for which the D-loop is named (Sprinzl et al., 1989). The dihydrouridines in the D-loop are thought to decrease stacking interactions between bases of the loop, with other loop sequences interacting with sequences in the variable loop to form tertiary interactions that stabilize the L-shaped structure of the tRNA (Kim et al., 1974; Robertus et al., 1974). Thus, it is possible that the unique features of the $tRNA^{Glu}$ D-loop in combination with the short variable loop could alter the conformation of the "variable pocket" formed by the two loops (Ladner et al., 1975) in a way that negatively impacts aminoacylation by GlnRS. To address this possibility, the $tRNA^{Gln}$ sequences in the "tertiary domain" (D-stem/loop, variable loop, and TΨC-stem/loop; Komatsoulis & Abelson, 1993; McClain, 1993) were introduced in the $tRNA^{Glu}$ context, with [E→Q2(3°)] and without [E→Q-(3°)] the set of glutamine recognition elements (Figure 3). As the major glutamine isoacceptor (anticodon CUG) with a G1/A72 mispair was used for earlier mutational studies (Jahn et al., 1991; Hayase et al., 1992), these same sequences were included in the E→Q2(3°) mutant for comparison. However, for the E→Q2(3°) mutant, U38 was not included as a glutamine recognition element, as it could be inferred from a comparison of the aminoacylation of E→QAC to that of the $tRNA^{Glu}$(CUG) anticodon mutant (see below) that the U38 mutation on the $tRNA^{Glu}$ background actually had a deterimental effect on aminoacylation by GlnRS.

Table 1: Kinetic Analysis of Aminoacylation for Glutamate→Glutamine Conversion Mutants[a]

tRNA transcript	aminoacylation by GlnRS			aminoacylation by GluRS		
	K_M (μM)	k_{cat} (s^{-1})	rel k_{cat}/K_M	K_M (μM)	k_{cat} (s^{-1})	rel k_{cat}/K_M
$tRNA^{Gln}_2$ (G1)[b]	0.13	3.4	1.0	n.d.	n.d.	n.d.
E→QAS	35	6.2×10^{-2}	6.8×10^{-5}	26	4.9×10^{-2}	2.0×10^{-2}
E→QAC	2.2	0.15	2.6×10^{-3}	52	4.4×10^{-2}	8.9×10^{-3}
E→Q1	8.3	0.41	1.9×10^{-3}	35	2.0×10^{-3}	6.0×10^{-4}
E→Q2	0.89	0.26	1.1×10^{-2}	130[c]	3.0×10^{-3} [c]	2.4×10^{-4} [c]
$tRNA^{Glu}$	51	1.1×10^{-2}	8.2×10^{-6}	20	1.9	1.0[d]

[a] Mutants are designated as in Figure 2. n.d. indicates that aminoacylation was not detected. [b] $tRNA^{Gln}$ constructs transcribed *in vitro* contain a U→G1 mutation to facilitate transcription (Jahn et al., 1991). [c] K_M values above 100 μM are estimates, as substrate concentrations used in assays were limited by practical considerations. [d] It should be noted that the specificity constant of aminoacylation for the *in vitro* transcript of $tRNA^{Glu}$ is reduced 200-fold relative to wild-type *in vivo* $tRNA^{Glu}$ (Sylvers et al., 1993).

Kinetic Analysis of Glutamate→Glutamine Conversion Mutants and Tertiary Domain Mutants. The kinetic parameters for aminoacylation by both GluRS and GlnRS for the $tRNA^{Glu}$ mutants with glutamine recognition elements are shown in Table 1. The relative specificity constants are reported relative to the wild-type $tRNA^{Glu}$ transcript for GluRS and to the $tRNA^{Gln}_2$ transcript (anticodon CUG) including the U→G1 mutation for GlnRS. However, it should be noted that the wild-type $tRNA^{Glu}$ transcript is already decreased 200-fold in specificity constant for aminoacylation by GluRS relative to wild-type *in vivo* $tRNA^{Glu}$, due to the missing wobble base modification, 5-[(methylamino)methyl]-2-thiouridine (mnm^5s^2U), which is a recognition element for GluRS (Sylvers et al., 1993).

The E→QAS transcript containing the glutamine acceptor stem recognition elements is only slightly better than the wild-type $tRNA^{Glu}$ transcript in aminoacylation with GlnRS, with a 10-fold increase in specificity constant due to equivalent effects on K_M and k_{cat}. In aminoacylation by GluRS, this mutant is reduced 50-fold in relative specificity constant, suggesting that the enzyme has some recognition of the wild-type acceptor stem sequences. This decrease in glutamate

acceptance is almost entirely due to a decrease in k_{cat}, as the K_M for this mutant is only marginally increased from 20 to 26 μM. The absolute specificity constants (k_{cat}/K_M) determined for this mutant are similar for aminoacylation by GluRS and GlnRS, suggesting that the mutant transcript is equally determined for glutamate and glutamine identities. However, the absence of the U34 wobble modification present in the anticodon of $tRNA^{Glu}$ *in vivo* provides an inherent bias against recognition by GluRS (Sylvers et al., 1993).

The effect of the anticodon loop mutations (mutant E→QAC) was larger than that of the acceptor stem changes for aminoacylation by both GluRS and GlnRS (Table 1). For GlnRS, E→QAC was a better substrate than the wild-type $tRNA^{Glu}$ transcript by greater than 3 orders of magnitude, with a K_M of 2.2 μM and a k_{cat} of 0.15 s^{-1}. However, in comparison to aminoacylation of the $tRNA^{Gln}_2$ transcript, this mutant is still decreased in relative specificity constant by three orders of magnitude. For GluRS, the E→QAC transcript showed the same decreased k_{cat} as measured for the acceptor stem mutant (E→QAS) but also exhibited an increased K_M of 52 μM. These results indicate that the anticodon sequence has a greater influence than the first three acceptor stem base pairs on recognition of the $tRNA^{Glu}$ variants by both GlnRS and GluRS.

The two "total conversion mutants", E→Q1 and E→Q2 (Figure 2), revealed a difference in aminoacylation by GlnRS (Table 1). While the two substrates show similar k_{cat} values (0.41 and 0.26 s^{-1}, respectively), there is a 10-fold difference in K_M leading to an order of magnitude difference in relative specificity constant. The 8.3 μM K_M of the E→Q1 isoacceptor (anticodon UUG) is higher than that measured for the construct with anticodon mutations only (2.2 μM for E→QAC), although the higher turnover of E→Q1 results in these two substrates having only minor differences in relative specificity constant. This suggests that the unmodified UUG anticodon sequence is less preferable than the CUG anticodon in the $tRNA^{Glu}$ context in binding by GlnRS.

The two mutants E→Q1 and E→Q2 with both acceptor stem and anticodon loop mutations (Figure 2) were similar in overall effect on specificity of aminoacylation by GluRS, with a decrease in relative k_{cat}/K_M of 3–4 orders of magnitude. However, there was a significant difference in K_M for the two different mutant isoacceptors. The K_M for the E→Q1 (anticodon UUG) transcript was elevated less than 2-fold to 35 μM (compared to 20 μM for the wild-type $tRNA^{Glu}$ transcript), while the K_M for the E→Q2 mutant was estimated to be 130 μM. In fact, the K_M for E→Q2 is within experimental error of the 150 μM K_M for the noncognate $tRNA^{Gln}_1$ transcript (Table 1), as the high K_M precludes precise measurement in this substrate range. This apparent preference shown by GluRS for the two different glutamine isoacceptors was addressed by the analysis of the anticodon switch mutants presented below.

The inability to complete the conversion of $tRNA^{Glu}$ to glutamine acceptance *in vitro* by the imposition of the glutamine recognition elements suggested either that a neccessary recognition element was still absent or that the $tRNA^{Glu}$ context was unfavorable for recognition by GlnRS. The influence of the $tRNA^{Glu}$ context in hindering GlnRS recognition could be due to a specific base (or bases) which directly blocks interaction or to a general structural incompatibility between $tRNA^{Glu}$ and GlnRS which prevents efficient binding or catalysis. To examine the possibility that the $tRNA^{Glu}$ structural context presents obstacles to productive interaction with GlnRS, we preserved the tertiary interactions of $tRNA^{Gln}$ by including glutamine D-stem/loop, variable loop, and TΨC-stem/loop sequences in $tRNA^{Glu}$ (Figure 3). The results of *in vitro* aminoacylation with GlnRS and GluRS are shown in Table 2, with the kinetic parameters for $tRNA^{Glu}$, $tRNA^{Gln}$, and mutant E→Q2 shown for reference.

Table 2: Kinetic Analysis of Aminoacylation for $tRNA^{Glu→Gln}$ Tertiary Domain Mutants[a]

tRNA transcript	aminoacylation by GlnRS			aminoacylation by GluRS		
	K_M (μM)	k_{cat} (s^{-1})	rel k_{cat}/K_M	K_M (μM)	k_{cat} (s^{-1})	rel k_{cat}/K_M
$tRNA^{Glu}$ [b]	51	1.1×10^{-2}	8.2×10^{-6}	20	1.9	1.0
E→Q(3°)	13	1.7×10^{-2}	5.0×10^{-5}	20	2.5×10^{-2}	5.0×10^{-5}
E→Q2[b]	0.89	0.26	1.1×10^{-2}	130[c]	3.0×10^{-3} [c]	2.4×10^{-4} [c]
E→Q2(3°)	0.22	0.26	4.0×10^{-2}	130[c]	2.5×10^{-4} [c]	1.9×10^{-5} [c]
$tRNA^{Gln}_2$ (G1)[b,d]	0.13	3.4	1.0	n.d.	n.d.	n.d.

[a] Mutants are designated as in Figures 2 and 3. n.d. indicates that aminoacylation was not detected. [b] Data reprinted from Table 1 for convenience of reference. [c] K_M values above 100 μM are estimates, as substrate concentrations used in assays were limited by practical considerations. [d] $tRNA^{Gln}$ constructs trascribed *in vitro* contain a U→G1 mutation to facilitate transcription (Jahn et al., 1991).

The replacement of this tertiary domain of $tRNA^{Glu}$ with $tRNA^{Gln}$ sequences had opposite kinetic effects on aminoacylation by the two enzymes (Table 2). For GlnRS, there was about a 4-fold reduction in K_M associated with the imposition of the glutamine tertiary domain, which was consistent between the mutants with and without the set of glutamine recognition elements. Mutant E→Q(3°), corresponding to $tRNA^{Glu}$ with just the glutamine tertiary domain, was improved in K_M with a decrease from 51 to 13 μM for aminoacylation by GlnRS, with no significant effect on k_{cat} (Table 2). The mutant containing both the glutamine recognition elements and the tertiary domain [E→Q2(3°), Figure 3] showed an improvement in K_M to 0.22 μM (compared to 0.89 μM for mutant E→Q2), again with no measurable effect on turnover. This suggests that the $tRNA^{Glu}$ context does hinder productive interaction with GlnRS. However, although the mutant E→Q2(3°) is improved in K_M to approach that of $tRNA^{Gln}$, it is still impaired in turnover by greater than 10-fold.

The effect of replacement of the tertiary domain of $tRNA^{Glu}$ on aminoacylation by GluRS is the mirror image of that for GlnRS. Mutant E→Q(3°) is equal in K_M to the $tRNA^{Glu}$ *in vitro* transcript but shows a 100-fold reduction in k_{cat} (Table 2). In a parallel fashion, mutant E→Q2(3°), containing both the glutamine recognition elements and the tertiary domain, is approximately equal in K_M to mutant E→Q2 containing only the glutamine recognition elements. The measured difference in turnover between E→QID and E→Q2 is 10-fold in this case. Although the effect on turnover is significant in magnitude, the aminoacylation by GluRS of E→QID is near the limit of detectability. These results imply, however, that the contribution of the tertiary domain to recognition of tRNA by GluRS is at the level of catalysis and not at the level of binding.

Construction and Kinetic Analysis of $tRNA^{Glu}$ and $tRNA^{Gln}$ Anticodon Switch Mutants. The results of the kinetic assays with the glutamate→glutamine conversion mutants suggested that it would be of interest to examine the degree of glutamate versus glutamine identity conferred by the anticodon alone. Therefore, mutants were made in both $tRNA^{Gln}$ and $tRNA^{Glu}$ sequences to effect anticodon switches of all potential glutamate and glutamine isoacceptors (Table 3). Specifically, this involved making mutations in the $tRNA^{Gln}$ transcript to change the CUG anticodon of the major (Gln_2) acceptor to UUG (the Gln_1 isoacceptor), UUC (the existing glutamate isoacceptor, lacking modification at U34), and CUC, the other

Table 3: Kinetic Analysis of Aminoacylation for Anticodon Switch Mutants[a]

tRNA substrate	anticodon specificity	aminoacylation by GlnRS			aminoacylation by GluRS		
		K_M (μM)	k_{cat} (s^{-1})	rel k_{cat}/K_M	K_M (μM)	k_{cat} (s^{-1})	rel k_{cat}/K_M
$tRNA^{Gln}$ substrates							
(*in vivo*) s^2UUG	Gln	0.20	5.8	1.0	190[b]	7×10^{-5} [b]	10^{-8} [b]
(*in vivo*) CUG	Gln	0.36	3.3	0.35	n.d.	n.d.	n.d.
(*in vitro*) UUG[c]	Gln	2.2	1.5	0.026	150[b]	5×10^{-4} [b]	10^{-7} [b]
(*in vitro*) CUG[c]	Gln	0.13	3.4	0.90	n.d.	n.d.	n.d.
(*in vitro*) UUC[c]	Glu	66	0.23	1.7×10^{-4}	78[d]	0.016[d]	2.0×10^{-5} [d]
(*in vitro*) CUC[c]	Glu	31	1.8	2.0×10^{-3}	150[b]	6.0×10^{-5} [b]	10^{-8} [b]
$tRNA^{Glu}$ substrates							
(*in vivo*)[e] mnm^5s^2UUC	Glu	160[b]	0.022[b]	5.2×10^{-6} [b]	0.36	3.4	1.0
(*in vitro*) UUC	Glu	51	0.011	7.4×10^{-6}	20	1.9	1.0×10^{-2}
(*in vitro*) CUC	Glu	30	0.050	1.9×10^{-5}	37	0.65	1.9×10^{-3}
(*in vitro*) UUG	Gln	14	0.17	4.2×10^{-4}	44	0.16	3.9×10^{-4}
(*in vitro*) CUG	Gln	0.43	0.080	7.2×10^{-3}	63	0.060	1.0×10^{-4}

[a] n.d. indicates that aminoacylation was not detected. [b] K_M values above 100 μM are estimates, as substrate concentrations used in assays were limited by practical considerations. [c] $tRNA^{Gln}$ constructs transcribed *in vitro* contain a U→G1 mutation to facilitate transcription (Jahn et al., 1991). [d] This mutant did not obey Michaelis–Menten kinetics with GluRS, although the GlnRS data is consistent with Michaelis–Menten predictions. [e] *In vivo* $tRNA^{Glu}$ was purified from an overexpression construct (Sylvers et al., 1993).

potential glutamate isoacceptor, which is not found in *E. coli* (Komine et al., 1990; Tremblay & Lapointe, 1986) but is a functional glutamate tRNA in many eukaryotes (Sprinzl et al., 1989). For the $tRNA^{Glu}$ transcript, the UUC anticodon was changed to CUC, UUG, and CUG.

Aminoacylation by GlnRS. GlnRS showed different affinities for the two glutamine anticodons on both tRNA backgrounds. *In vivo* $tRNA^{Gln}_1$ (anticodon s^2UUG) appears to be a slightly better substrate for GlnRS than *in vivo* $tRNA^{Gln}_2$ (anticodon CUG) with respect to both k_{cat} (5.8 versus 3.3 s^{-1}) and K_M (0.20 versus 0.36 μM). These modest differences in the two substrates may reflect the different methods of isolation, however, as $tRNA^{Gln}_1$ was purified from natural abundance, while the $tRNA^{Gln}_2$ used in these assays was prepared from a $tRNA^{Gln}_2$ overexpression strain (Perona et al., 1988). The analysis of the *in vitro* transcripts of the two isoacceptors of $tRNA^{Gln}$ reveals variation in aminoacylation by GlnRS; while the *in vitro* $tRNA^{Gln}_2$ transcript, with a K_M of 0.13 μM and a k_{cat} of 3.4 s^{-1}, is aminoacylated almost as efficiently as *in vivo* $tRNA^{Gln}_1$, the same is not true for the transcript of the other isoacceptor. The $tRNA^{Gln}_1$ transcript shows a 10-fold increase in K_M, with a decrease in k_{cat} of 4-fold contributing to a reduction in relative specificity constant of 50-fold.

Similar differences in aminoacylation by GlnRS were obtained with the UUG and CUG glutamine anticodons on the noncognate $tRNA^{Glu}$ background. The $tRNA^{Glu}$(UUG) transcript is aminoacylated with an increase in K_M to 14 μM, compared with a K_M of 0.43 μM for the $tRNA^{Glu}$(CUG) transcript. The turnover rates are similar for the two substrates with only a 2-fold difference in k_{cat} values. In terms of specificity constant for aminoacylation, this corresponds to a decrease of 10^{-4} for the $tRNA^{Glu}$(UUG) transcript and of 10^{-3} for the $tRNA^{Glu}$(CUG) transcript, relative to that for *in vivo* $tRNA^{Gln}_1$. These results indicate that the unmodified UUG anticodon is suboptimal for aminoacylation in comparison with both CUG and s^2UUG glutamine anticodons.

The introduction of the glutamate anticodons on the $tRNA^{Gln}$ background had dramatic effects on binding by GlnRS, with an increase in K_M from 0.2 μM for *in vivo* $tRNA^{Gln}_1$ to 66 μM for $tRNA^{Gln}$(UUC) and to 31 μM for $tRNA^{Gln}$(CUC). The increase in K_M seen for the UUC anticodon is matched by a decrease in k_{cat}, leading to an overall decrease in relative k_{cat}/K_M of 10^{-4} for this substrate. As the $tRNA^{Gln}$(CUC) transcript is less affected in turnover, the relative specificity constant is only 1000-fold below that for *in vivo* $tRNA^{Gln}_1$.

Greater than 5 orders of magnitude in specificity separate cognate and noncognate aminoacylation by GlnRS (Table 3). Misacylation of *in vivo* $tRNA^{Glu}$ by GlnRS occurs with an 800-fold increase in K_M (from 0.2 to 160 μM) coupled with a 250-fold reduction in k_{cat} relative to aminoacylation of *in vivo* $tRNA^{Gln}_1$. The misacylation by GlnRS of the *in vitro* $tRNA^{Glu}$(UUC) isoacceptor was similar to that measured for *in vivo* $tRNA^{Glu}$, but with an improved K_M of the *in vitro* transcript (K_M = 51μM). For the other potential glutamate isoacceptor tRNA, $tRNA^{Glu}$(CUC), misacylation by GlnRS was improved over that of the UUC isoacceptor (both *in vivo*- and *in vitro*-made), with a 10-fold increase in specificity constant (from 10^{-6} to 10^{-5}) due to balanced effects on K_M and k_{cat}. This preference by GlnRS for the CUC glutamate anticodon over the unmodified UUC glutamate anticodon is consistent with the bias for the wobble position nucleotide demonstrated with the glutamine anticodons above.

Aminoacylation by GluRS. The results of the analysis of $tRNA^{Glu}$ and $tRNA^{Gln}$ anticodon mutants with GluRS are presented in Table 3. As both *in vivo*- and *in vitro*-made tRNAs were studied, the specificity constants are reported relative to *in vivo* $tRNA^{Glu}$ (anticodon mnm^5s^2UUC). In both $tRNA^{Glu}$ and $tRNA^{Gln}$ contexts, the UUC glutamate anticodon was favored over the CUC anticodon, although both substrates are clearly impaired when compared to *in vivo* $tRNA^{Glu}$ with the modified uridine in the wobble position (Sylvers et al., 1993). While the difference in overall specificity constants for the two anticodons in the $tRNA^{Glu}$ context is only five-fold, there is a dramatic difference in the two anticodons on the $tRNA^{Gln}$ background. The relative specificity constant for the $tRNA^{Gln}$(CUC) mutant is at the limit of detection, with a decrease of 8 orders of magnitude relative to *in vivo* $tRNA^{Glu}$, while the UUC anticodon on $tRNA^{Gln}$ is down by a factor of 10^{-5}. Although the two substrates demonstrate a 2-fold difference in apparent K_M, the major difference is a one 1000-fold better turnover rate for the $tRNA^{Gln}$(UUC) substrate. Replacement of the UUC anticodon of the $tRNA^{Glu}$ transcript with the glutamine anticodons (UUG and CUG) resulted in substrates which were only 10–100-fold down in specificity constant for aminoacylation by GluRS in comparison with the two $tRNA^{Glu}$ transcripts (Table 3). An effect was observed with the UUG and CUG glutamine anticodons parallel to that seen for the UUC and CUC glutamate anticodons; the UUG sequence was better by a factor of 10 than the CUG anticodon, with small effects on both K_M (44 versus 63 μM) and k_{cat} (0.16 versus 0.06 s^{-1}) contributing to this difference. This was consistent with the

analysis of both glutamine anticodons in the $tRNA^{Gln}$ context, as it was possible to measure misacylation of the UUG isoacceptor of both *in vivo*- and *in vitro*-made $tRNA^{Gln}$, while there was no measurable misacylation of the CUG isoacceptors under identical conditions of aminoacylation with GluRS. There was an apparent difference in k_{cat} for *in vivo* and *in vitro* $tRNA^{Gln}_1$ substrates (anticodons s^2UUG and UUG), with the *in vitro* substrate being better in turnover by 10-fold. However, both substrates are extremely poor substrates for aminoacylation by GluRS, and the difference between them is close to the limit of detection of these assays.

There is a difference in specificity constant of 10^8 exhibited by GluRS in aminoacylation of the cognate *in vivo* $tRNA^{Glu}$ and the noncognate *in vivo* $tRNA^{Gln}$, compared to a difference of only about 5 orders of magnitude between cognate aminoacylation and misacylation of $tRNA^{Glu}$ by GlnRS (Table 3). These data support the *in vivo* observation that, while GlnRS is rather indiscriminate in the ability to misacylate noncognate tRNAs (Sherman et al., 1992; Normanly et al., 1990; Swanson et al., 1988), GluRS has not been determined to date to misacylate any noncognate tRNA.

DISCUSSION

The experiments presented here investigate the interaction of GlnRS and GluRS with tRNA substrates which are intermediate in glutamine and glutamate acceptance. Due to the close evolutionary relationship between *E. coli* GluRS and GlnRS (Breton et al., 1990), it is of interest to examine the similarities and differences in the methods of distinguishing cognate from noncognate substrates. The results of this study allow the expansion of our understanding of glutamine identity to encompass discrimination between glutamate and glutamine aminoacylation systems. It is clear from these experiments that both enzymes, while specifically interacting with the same regions of their cognate tRNAs, also exploit the sequence differences within these regions to achieve specificity.

Recognition Domains for GluRS and GlnRS Are Overlapping. Prior to this work, the only data available on the recognition of $tRNA^{Glu}$ by GluRS indicated interaction in the anticodon region, with little information on other regions of the tRNA that might also be involved (Kisselev, 1985). While it could be predicted, on the basis of the sequence homology identified in GluRS and the sequences coding for the acceptor stem binding domain of GlnRS (Breton et al., 1990), that GluRS might also recognize the acceptor stem of $tRNA^{Glu}$, there was little biochemical or genetic evidence to support this prediction. Analysis of the $tRNA^{Glu}$ transcript carrying acceptor stem mutations to match glutamine recognition elements (construct E→QAS, Figure 2) demonstrates that these mutations affect aminoacylation by GluRS as well as recognition by GlnRS. In fact, the impact of these mutations is to create a tRNA transcript which is aminoacylated with very similar kinetic parameters by both enzymes (Table 1), indicating a tRNA with equal degrees of glutamate and glutamine identity.

The fact that all of the glutamine identity elements located in the acceptor end of $tRNA^{Gln}$, with the exception of the discriminator nucleotide (position 73), differ in $tRNA^{Glu}$ provides an opportunity for discrimination between these two tRNAs. The evidence that GluRS recognizes sequence elements in the acceptor stem is clear, but as all of the acceptor stem changes were made on one construct, it is possible that the overlap in acceptor stem recognition includes only a subset of those positions recognized by GlnRS. Nonetheless, the recognition of acceptor stem sequences by GluRS coupled with dependence on anticodon sequences reveals that GluRS and GlnRS share similar recognition patterns. However, as increases in glutamine acceptance were accompanied by decreases in glutamate acceptance for all mutants examined, there is an apparent incompatibility between the sets of recognition elements specifying the two acceptor identities.

The Major Discriminants for Glutamate and Glutamine Identity Are in the Anticodon. Previous studies to delineate glutamine recognition elements utilized point mutations of the acceptor stem and anticodon of $tRNA^{Gln}$ to identify sequences which interact with GlnRS (Jahn et al., 1991; Hayase et al., 1992; Rogers et al., 1992). The largest reductions in aminoacylation were associated with point mutations in the anticodon sequence, but the extensive interaction with the acceptor stem sequences made it difficult to assess which region of the tRNA was more important in conferring glutamine identity. By examination of the effects of the anticodon and acceptor stem recognition elements separately on the noncognate $tRNA^{Glu}$ background, it is evident that the anticodon sequence has a greater capacity to impose glutamine acceptance in the $tRNA^{Glu}$ context. The $tRNA^{Glu}$ construct containing only acceptor stem changes (E→QAS) is reduced 40-fold in specificity constant for aminoacylation by GlnRS relative to that of the construct with only the anticodon loop changes (E→QAC), with the primary difference being in K_M. In fact, the $tRNA^{Glu}$ construct containing only acceptor stem changes is less than 10-fold better in aminoacylation by GlnRS than the wild-type $tRNA^{Glu}$ transcript, with a K_M that is 250 times higher than that for the wild-type $tRNA^{Gln}$ transcript. It should be noted, however, that the $tRNA^{Glu}$ background provides the G73 discriminator base, which has been indentified as an important glutamine recognition element (Jahn et al., 1991; Sherman et al., 1992). For this reason, it is predicted that the glutamine acceptor stem recognition elements, including G73, would be much more effective in imposing glutamine acceptance on another tRNA background in which the wild-type discriminator base was not guanosine.

The fundamental influence of the anticodon sequence on recognition of tRNA by GlnRS is also apparent from the kinetic analysis of $tRNA^{Glu}$ and $tRNA^{Gln}$ anticodon switch mutants (Table 3). The $tRNA^{Glu}$ constructs with the two glutamine anticodons differ in K_M from wild-type *in vivo*- and *in vitro*-made $tRNA^{Gln}$ by less than 10-fold, while the $tRNA^{Gln}$ mutants with glutamate anticodons show increases in K_M of 30- and 300-fold. It has previously been reported that GlnRS demonstrates specificity in aminoacylation primarily on the level of turnover rather than binding (Jahn et al., 1991). However, these results suggest a significant level of discrimination against the tRNA mutants in this study based on binding, as the variation in k_{cat} for all mutants examined with GlnRS was over a 300-fold range, while K_M values differed by greater than 1000-fold.

The analysis of the $tRNA^{Glu}$ acceptor stem mutant (E→QAS, Table 3) in aminoacylation with GluRS suggests that the modified wobble base, mnm^5s^2U34, makes a contribution to interaction with the enzyme that is greater than that of the bases at the top of the acceptor stem, considering that the impairment measured for the acceptor stem mutant includes the effect of the missing modification in the anticodon. However, the contribution of the entire anticodon to glutamate identity is difficult to estimate by transplanting the unmodified glutamate anticodon onto $tRNA^{Gln}$. While mutation of the $tRNA^{Gln}$ anticodon to UUC does result in an *in vitro* transcript which is measurably misacylated by GluRS (Table 3), the lack of the U34 modification is almost certain to limit the

effectiveness of the anticodon in conferring glutamate acceptance on the noncognate tRNA.

It is more revealing to examine the effect of transplanting the glutamine CUG anticodon on the *in vitro* transcript sequence of $tRNA^{Glu}$. The loss of the modified UUC by replacement by the noncognate anticodon sequence results in a 10 000-fold reduction in specificity constant for aminoacylation by GluRS relative to wild-type *in vivo* $tRNA^{Glu}$. The foremost effect of the noncognate CUG anticodon in the $tRNA^{Glu}$ background on aminoacylation by GluRS is due to a 600-fold increase in K_M. Thus it is likely that both GluRS and GlnRS achieve fidelity in tRNA recognition to a large extent on the basis of anticodon binding. The specificity for the individual anticodon positions will be discussed below.

$tRNA^{Glu}$ Contains Antideterminants for Recognition by GlnRS outside of the Defined Set of GlnRS Recognition Elements. Kinetic analysis of $tRNA^{Glu}$ mutants containing the set of glutamine recognition elements in aminoacylation assays with GlnRS reveals that the conversion of $tRNA^{Glu}$ to glutamine acceptance is incomplete. The $tRNA^{Glu}$ mutant with all of the acceptor stem and anticodon recognition elements for GlnRS (mutant E→Q2, Figure 2) is improved by 1200-fold compared to misacylation of the $tRNA^{Glu}$ transcript by GlnRS (Table 1). However, it is still 100-fold down in specificity constant of aminoacylation compared to the $tRNA^{Gln}$ transcript. Replacement of the $tRNA^{Glu}$ tertiary domain with $tRNA^{Gln}$ sequences (mutant E→QID, Figure 3) in addition to imposition of the glutamine recognition elements results in a tRNA substrate which is only 25-fold worse in glutamine acceptance than $tRNA^{Gln}$, but is still not totally converted to glutamine acceptance.

There are two possible explanations for this observation: either there are recognition elements for glutamine identity that have not yet been identified or the glutamate tRNA background contains sequences which hinder aminoacylation by GlnRS outside of the acceptor stem and anticodon sequences mutated in this study. This second possibility is most probable, as the identical set of glutamine recognition elements is successful in imposing glutamine acceptance when placed on the context of the yeast aspartate tRNA (M. Frugier, personal communication). It is difficult to speculate on specific sequences in $tRNA^{Glu}$ which might act as antideterminants to glutamine identity, as a comparison between yeast $tRNA^{Asp}$ and *E. coli* $tRNA^{Glu}$ sequences reveals no obvious dissimilarities to explain why GlnRS would prefer the $tRNA^{Asp}$ mutant to the $tRNA^{Glu}$ mutant. However, the probable location(s) for potential antideterminants to glutamine identity would be in the lower acceptor stem or in the anticodon stem/loop, as glutamine sequences in all other regions of the tRNA were present on the E→Q2(3°) mutant.

GlnRS Shares with GluRS a Preference for a Modified Uridine in the Anticodon Wobble Position of Cognate tRNA. Aminoacylation by GlnRS of $tRNA^{Gln}$ species containing the UUG anticodon reveals a 10-fold difference in K_M for the *in vitro* transcript relative to *in vivo* $tRNA^{Gln}_1$, with a 4-fold reduction in turnover (Table 3). This difference in K_M values for the substrates with the unmodified anticodon sequence is also evident in the two glutamate→glutamine conversion mutants (E→Q1 and E→Q2, Table 1), where the difference of U34 instead of C34 results in a 10-fold elevation in K_M, while there is little difference in the K_M values of the two *in vivo* $tRNA^{Gln}$ isoacceptors. The efficient aminoacylation of the Gln_2 transcript indicates that the modified bases outside of the anticodon of $tRNA^{Gln}$ have little detectable effect on aminoacylation by GlnRS. Therefore, the increased K_M associated with the Gln_1 transcript must be the result of the absence of the only modified base which is unique to the Gln_1 isoacceptor, the 2-thiouridine in the anticodon wobble position. While the apparent preference of GlnRS for the thiolated U34 is not as dramatic as that for mnm^5s^2U34 by GluRS (Sylvers et al., 1993), the wobble modification is contributing to the recognition of both glutamate and glutamine tRNA isoacceptors.

The refinement of the structure of the GlnRS·$tRNA^{Gln}_2$ complex revealed a highly ordered binding pocket for each anticodon nucleotide (Rould et al., 1991). The binding pocket for position 34 must accommodate both the CUG and the s^2UUG glutamine isoacceptor, and as the defined C34 binding pocket presents peptide hydrogen-bonding groups for the three Watson–Crick cytosine bonds, the same amino acids cannot bind the s^2U34 in an identical manner. Rather, a shift in both RNA and localized peptide sequences is necessary to model the proposed interaction with s^2U34. The interaction with the 2-thio group could occur by the same peptide hydrogen bonding observed for the 2-keto group of C34, but alternate peptide side chains are needed to interact with the 4-keto group of s^2U34 instead of the 4-amino group present in C34 (Rould et al., 1991). The 10-fold difference in K_M values observed for the transcript of $tRNA^{Gln}_1$ relative to *in vivo* $tRNA^{Gln}_1$ could be due to the capability of the larger 2-thio group to hydrogen bond with the side chain responsible for the interaction with the 2-keto of the C34 present in $tRNA^{Gln}_2$, in spite of the distance created by shifting the peptide to accommodate the 4-keto group.

Discrimination in Vitro between Glutamate and Glutamine tRNAs Is Less Exact for GlnRS Than for GluRS. The relaxed specificity of GlnRS observed *in vivo* for noncognate tRNA substrates has been previously reported (Swanson et al., 1988; Sherman et al., 1992). Nonetheless, the *in vitro* studies of glutamine recognition elements revealed that recognition of $tRNA^{Gln}$ by GlnRS is well-defined, pointing to the extreme sensitivity of *in vivo* suppression assays in detection of misacylation (Jahn et al., 1991). In this study, GlnRS was found to misacylate noncognate $tRNA^{Glu}$ with a specificity constant reduced by 50 000-fold relative to cognate $tRNA^{Gln}$ (Table 3). While this difference is impressive, the opal suppressor derived from $tRNA^{Gln}$ is aminoacylated *in vivo* by GlnRS even though misacylation *in vitro* is below the limit of detectability (Rogers et al., 1992). This suggests that detectable misacylation of $tRNA^{Glu}$ by GlnRS *in vivo* would be possible if the only consideration were the extent of interaction between $tRNA^{Glu}$ and GlnRS.

In contrast, the range in specificity constants for aminoacylation of $tRNA^{Glu}$ and the $tRNA^{Gln}_1$ isoacceptor by GluRS is greater than 7 orders of magnitude, with no detected misacylation of the $tRNA^{Gln}_2$ isoacceptor (Table 3). It is interesting to note that the higher level of discrimination against the noncognate tRNA exhibited by GluRS correlates in magnitude with the specificity gained through recognition of the wobble base modification (Sylvers et al., 1993). Since competition among aminoacyl-tRNA synthetases has been shown to influence tRNA identity *in vivo* (Swanson et al., 1988), the recognition of mnm^5s^2U34 by GluRS probably serves not only to decrease the possibility of misacylation of $tRNA^{Gln}$ by GluRS but also to ensure that GluRS competes effectively with GlnRS for recognition of $tRNA^{Glu}$.

The in Vitro Anticodon Preferences for GluRS and GlnRS Suggest an Evolutionary Pathway for Divergence of Glutamine and Glutamate Aminoacylation Systems. The results of the kinetic analysis of glutamate and glutamine anticodon switch mutants (Table 3) demonstrate the different proclivities of

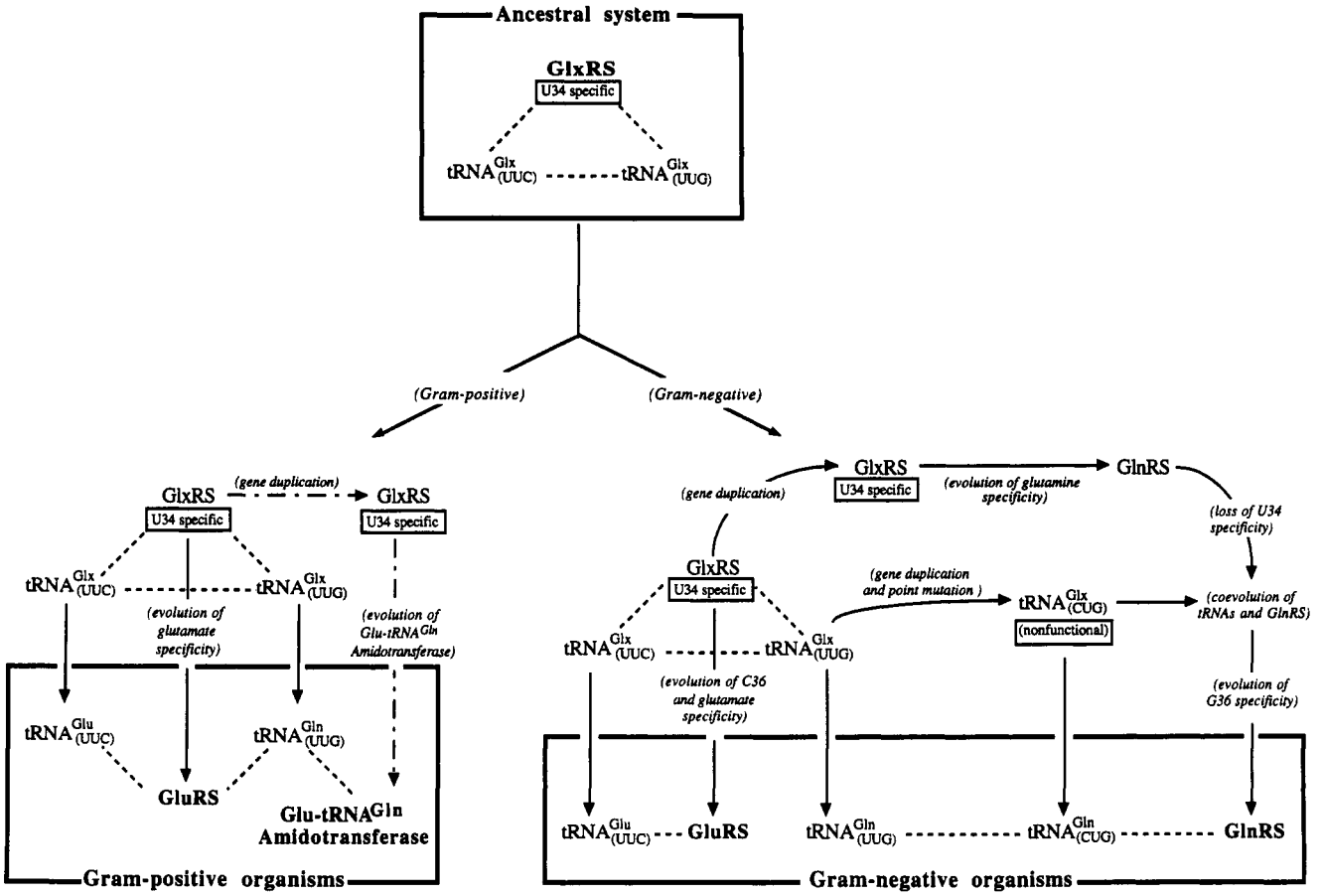

FIGURE 4: Pathway of evolutionary divergence of glutamate and glutamine aminoacylation systems. This scheme is based on the preference of GluRS for anticodons with U34 (see text). GlxRS represents an ancestral synthetase which was specific for both glutamate and glutamine. Dashed lines connect cognate tRNAs and synthetases; broken arrows represent the possible divergence of the amidotransferase from a primitive GluRS.

GluRS and GlnRS for the potential isoacceptor sequences at positions 34 and 36. It was expected that GluRS would discriminate in favor of C36 while GlnRS would prefer G36, as this is the only position which prevents total overlap in coding specificity. However, the extent to which GluRS discriminates in favor of the modified U34 over C34 was surprising, as it effectively rejects the decoding capability of this isoacceptor and implies a limited specificity for one tRNA isoacceptor by this aminoacyl-tRNA synthetase.

Recent attempts to reclone the $tRNA^{Glu}$ mutant with a UUG glutamine anticodon for *in vivo* expression have been unsuccessful. This apparent lethality probably arises because the resulting tRNA, if aminoacylated by GluRS *in vivo*, would be a missense suppressor, inserting glutamate at glutamine codons. In contrast, the $tRNA^{Glu}$ mutant with a CUG glutamine anticodon poses no apparent toxicity problem and is highly overexpressed *in vivo*, even though it is also a potential missense suppressor. The expectation that this tRNA is not aminoacylated by GluRS *in vivo* has been confirmed by the lack of suppression in a $trpA_{CAG49}$ strain (Murgola, 1985), in which glutamate insertion is required at the CAG glutamine codon for growth in the absence of tryptophan (data not shown). These observations imply that the C34 completely excludes this mutant tRNA from aminoacylation by GluRS *in vivo*, as the level of overexpression makes it improbable that strong recognition of this tRNA by GlnRS could totally remove the tRNA from competitive interaction with GluRS. The actual state of *in vivo* aminoacylation is presently under investigation for this mutant.

This inflexibility in recognition of U34 by GluRS is indirectly apparent in Gram-positive eubacterial, archaebacterial, and organellar enzymes, since only the U34 isoacceptor exists for $tRNA^{Glu}$ and for the glutamine tRNA which must be misacylated by GluRS in these systems as a first step in providing $Gln\text{-}tRNA^{Gln}$ for protein synthesis (Schön et al., 1988; Schön & Söll, 1988; Lapointe et al., 1986; Wilcox & Nirenberg, 1968). This implies that the presence of the $tRNA^{Gln}_2$ isoacceptor (anticodon CUG), which is the major glutamine isoacceptor in *E. coli*, would not provide any significant benefit for cell viability in these systems. The observation that the CUC isoacceptor of $tRNA^{Glu}$ is found primarily in the cytoplasmic systems of higher eukaryotes suggests that GluRS has only recently evolved the ability to efficiently aminoacylate the $tRNA^{Glu}$ CUC isoacceptor (Sprinzl et al., 1989).

Whether the enzyme that aminoacylates the CUC glutamate tRNA is a direct descendant of the bacterial GluRS is not clear; the only enzyme from higher eukaryotes that has been demonstrated to have GluRS activity (GluRS from *Drosophila melanogaster*) has greater homology to all known GlnRS sequences than to any GluRS sequence, allowing the possibility that this enzyme recently diverged from glutamine specificity to glutamate specificity (Cerini et al., 1991). It is likely that the common ancestor to prokaryotic GluRS and GlnRS was closer to GluRS, since the absence of GlnRS activity in Gram-positive eubacteria and archaebacteria suggests that GlnRS evolved from a GluRS gene duplication event after the split between Gram-positive and Gram-negative organisms, rather than the alternative possibility that loss of GlnRS occurred in the Gram-positive organisms subsequent to the split. This then implies that the *D. melanogaster* enzyme with GluRS activity but greater homology to bacterial and yeast GlnRS

sequences (Cerini et al., 1991) would represent another evolutionary turn in amino acid and tRNA specificity.

The specificity for U34 demonstrated by GluRS in prokaryotic and organellar systems presents a possible point of evolutionary divergence of glutamate and glutamine aminoacylation systems (Figure 4). The basic premise of the model is that a primitive "GlxRS" (where "Glx" indicates indeterminate specificity for glutamate and glutamine) aminoacylated ancestral $tRNA^{Glx}$ with a requirement for U34. Before the divergence of Gram-positive and Gram-negative eubacteria, duplication and mutation of the $tRNA^{Glx}$ genes would have provided both UUC and UUG functional anticodons. After the split, these tRNAs would have evolved separate acceptor types by coevolution with GluRS and the Glu-$tRNA^{Gln}$ amidotransferase, the enzyme responsible for conversion of Glu-$tRNA^{Gln}$ to Gln-$tRNA^{Gln}$ in Gram-positive organisms (Schön et al., 1988; Schön & Söll, 1988; Lapointe et al., 1986). Presumably, the specificity of GluRS for glutamate coevolved with the specificity of the amidotransferase for glutamine and Glu-$tRNA^{Gln}$ to provide correctly charged tRNA for translation. The functional similarity of the amidotransferase with GlnRS of Gram-negative organisms allows the speculation that the amidotransferase is also a descendant of a primitive GlxRS of an ancestral organism, although there is currently no sequence evidence to support or reject this possiblity.

Within the Gram-negative branch, gene duplication of a $tRNA^{Glx}$ (anticodon UUX) species followed by point mutation to a CUX anticodon would have produced a nonfunctional tRNA, as the only existing and functional GlxRS was specific for tRNAs with a uridine in the wobble position of the anticodon. If this event coincided with the GlxRS gene duplication, then the coevolution of the "nonfunctional" CUX tRNA species with the auxiliary GlxRS could have produced the separate glutamine aminoacylation system. If the anticodon binding capability developed by the new GlnRS enzyme was more efficient than the parental GlxRS enzyme in binding substrates with G36, GlnRS would have extended substrate specificity to include the UUG $tRNA^{Glx}$ species, while the parental GlxRS developed exclusive specificity for glutamate and $tRNA^{Glu}$(UUC). While this model is simplistic, it does provide a rationale for the two different pathways for formation of Gln-$tRNA^{Gln}$ that have been observed.

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