Aminoacyl-tRNA Synthetases Optimize Both Cognate tRNA Recognition and Discrimination against Noncognate tRNAs†

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ABSTRACT: Specific protein−nucleic acid interactions are usually the product of sequence-dependent hydrogen bonding. However, in the crystal structure of *Escherichia coli* glutaminyl-tRNA synthetase (GlnRS) in complex with $tRNA^{Gln}$, leucine 136 (Leu136) stabilizes the disruption of the weak first (U1-A72) base pair in $tRNA^{Gln}$ by stacking between A72 and G2. We have demonstrated, by a combined *in vivo* and *in vitro* mutational analysis, that Leu136 is important for tRNA specificity despite making no hydrogen bonds with $tRNA^{Gln}$. Both more (L136F) and less (L136V, L136M, L136A, and L136T) mischarging mutants of GlnRS have been identified. GlnRS(L136F) is more mischarging and less specific than wild-type GlnRS *in vivo*, due not to an increased affinity for the noncognate tRNAs but to a decreased affinity for $tRNA^{Gln}$. Also, unlike other mischarging mutants of GlnRS that have been characterized, it does not exhibit generally relaxed tRNA specificity *in vivo* and mischarges only a subset of the tRNAs tested. A possible sequence preference for a Py1-Pu72/Pu2-Py71 combination is suggested. The L136A/M/T/V mutants are the first GlnRS variants, including wild-type, expressed on pBR322 which no longer mischarge *tyrT(UAG) in vivo*. We have shown that, while the L136A mutant is less mischarging than wild-type both *in vivo* and *in vitro*, it is not more specific as it also exhibits reduced affinity for its cognate glutamine tRNA. On the basis of these results, we suggest that the aminoacyl-tRNA synthetases have evolved to balance cognate tRNA recognition and discrimination against noncognate tRNAs.

The accuracy of protein translation depends on the fidelity with which the correct amino acids are esterified to their cognate tRNA molecules by aminoacyl-tRNA synthetases (AARSs). *In vivo*, a synthetase is presented with the challenge of selecting its cognate tRNA from among approximately 60 different tRNA species. In essence then, the tRNA recognition problem is as follows. How does an AARS specifically recognize its cognate tRNA and discriminate against noncognate tRNAs when the secondary and tertiary structures of tRNAs are so well-conserved?

While a great deal is known about tRNA identity and recognition elements and the competition *in vivo* between AARSs for a given tRNA, relatively little is known about the tRNA specificity determinants which reside on the AARS due to its relative complexity and large size (Schulman, 1991; Giege et al., 1993). The solution of numerous AARS crystal structures has facilitated the understanding of AARS specificity determinants and has also led to a more direct approach to designing AARS mutants (Carter, 1993; Delarue, 1995).

The relaxed tRNA specificity of *Escherichia coli* GlnRS makes it an ideal enzyme for the study of tRNA recognition determinants (Sherman et al., 1995). Expressed in single copy, GlnRS mischarges $tRNA^{Trp}$(UAG), and overexpressed, GlnRS mischarges *tyrT(UAG)* (Yaniv et al., 1974; Swanson et al., 1988). In addition, many tRNA mutants and artificial amber suppressors are recognized by GlnRS (Normanly & Abelson, 1989; Sherman et al., 1995). Although the mutations which confer glutamine identity on tRNAs are located mainly in the anticodon, a number also weaken the acceptor stem (Rogers & Söll, 1988; Seong et al., 1989; Varshney et al., 1991a; Normanly et al., 1992). The crystal structure of the GlnRS/$tRNA^{Gln}$/ATP ternary complex has provided an explanation for this sequence preference (Rould et al., 1989). In the complex, the U1-A72 base pair of $tRNA^{Gln}$ is broken and the CCA end bent back toward the anticodon. Similar disruption of the acceptor stem has been observed for $tRNA^{fMet}$ and has been predicted to occur in precursor tRNA interacting with M1 RNA of RNase P (Ferguson & Yang, 1986; Westhof & Altman, 1994). In $tRNA^{Gln}$, this conformation is stabilized by a hydrogen bond between the N2 of G73 (the discriminator) and the phosphate oxygen of A72 (hence the need for a weak first base pair and a guanine discriminator) as well as by the hydrophobic stacking of the leucine 136 (Leu136) side chain between G2 and A72 (Figure 1; Rould et al., 1989). Leu136 has been implicated in tRNA specificity, as mutations in amino acids whose side chains are likely to affect the position of Leu136 result in generally relaxed tRNA specificity (Weygand-Durasevic et al., 1993).

We have examined the impact of mutations of Leu136 on the specificity of GlnRS. We were interested in determining whether GlnRS could be made more mischarging and less specific by substituting leucine for a potentially more stabilizing phenylalanine (or the chemically different methionine) or less mischarging and more specific by substituting progressively smaller side chains. *In vivo* and *in vitro* analyses of the effects of the Leu136 mutants on cognate tRNA recognition and on discrimination against noncognate

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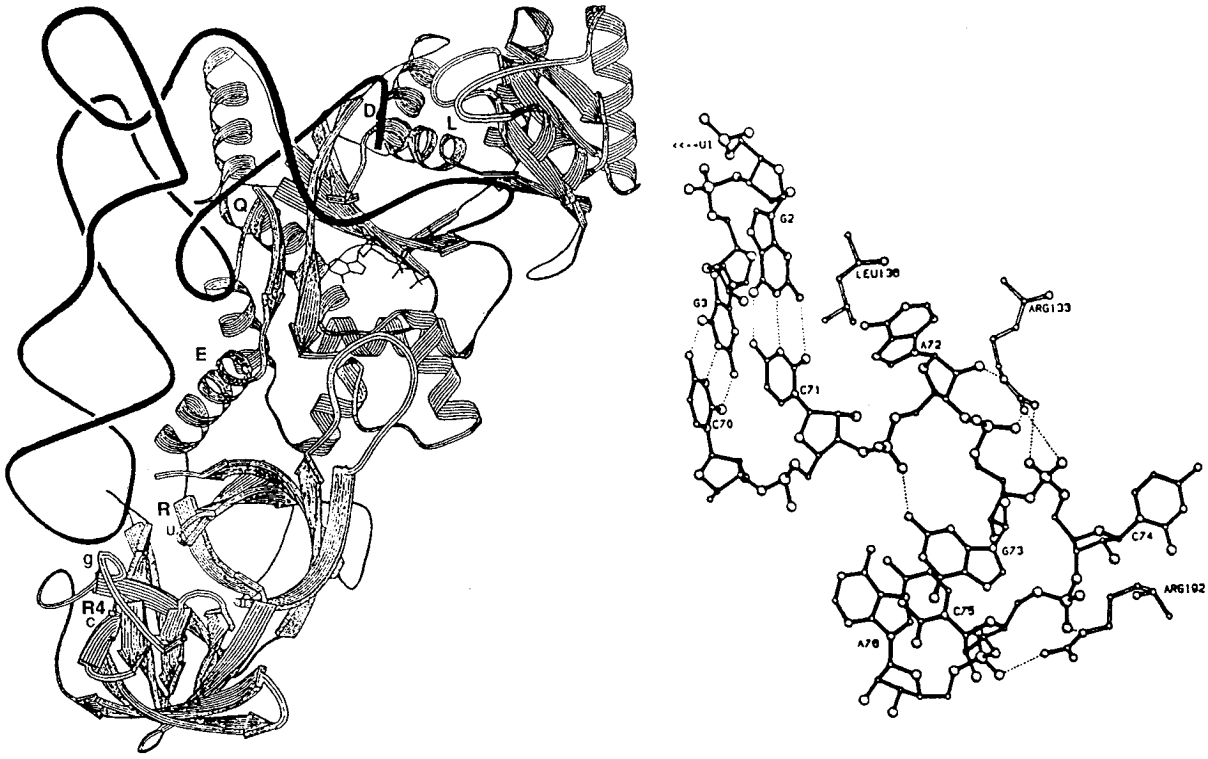

FIGURE 1: Leucine 136 stabilizes the conformation of the acceptor end of $tRNA^{Gln}$. In the crystal structure of GlnRS in complex with $tRNA^{Gln}$ and ATP, Leu136 (L) is located in the acceptor binding domain of GlnRS (left) (Rould et al., 1989). The acceptor end of $tRNA^{Gln}$ assumes a hairpin conformation in which the U1-A72 base pair is disrupted and the CCA end bent back toward the anticodon by an intramolecular hydrogen bond between the N2 of the discriminator G73 and the phosphate oxygen of A72. Leu136 stabilizes the disruption of the first base pair by stacking between A72 and G2 (right). The detail (right) was taken from Perona (1990).

tRNAs have identified both more (L136F) and less (L136A) mischarging mutants of GlnRS. These mutants exhibit a number of unusual properties and suggest that AARSs balance the need for cognate tRNA recognition with the need to discriminate against noncognate tRNAs.

MATERIALS AND METHODS

General. Uniformly labeled L-[^{3}H]glutamine was purchased from Amersham and NEN. Pure $tRNA_2^{Gln}$ (specific activity of approximately 1500 pmol/A_{260}) was the generous gift of D. Jeruzalmi. All protein purification steps were carried out at 4 °C unless otherwise noted. Media for bacterial growth and the molecular biology protocols were both standard (Maniatis et al., 1982).

Site-Directed Mutagenesis and Constitutive Expression of Leu136 Mutants. The Leu136 mutants of GlnRS, with the exception of L136V, were all made by oligonucleotide-directed mutagenesis using an M13mp19 clone of *glnS* (the gene encoding GlnRS), including its promoter and terminator, as the template, and the mutants were screened for by dideoxy sequencing. The mutant (L136F/M/A/T) *glnS* genes were then recloned for *in vivo* expression as *Eco*RI-*Hin*dIII fragments into pBR322. The L136V mutant was isolated during random mutagenesis of the codons for amino acids 126−138 of the *glnS* gene cloned on Bluescript pKS$^-$ (Weygand-Durasevic et al., 1993). A *Csp*45I-*Cla*I fragment containing the L136V mutant was used to replace the wild-type fragment on pBR*glnS* for *in vivo* experiments. Reclones were screened for initially by restriction enzyme analysis; the mutant fragment contains an additional translationally silent *Kpn*I site as a result of the saturation mutagenesis. Potential positive clones were then sequenced to confirm the presence of the L136V mutation.

Cloning of the Mutant Glutamine Opal Suppressor and $tRNA^{Phe}$(UAG) Genes. For the purpose of the *in vivo* experiments, the mutant opal suppressor derived from $tRNA^{Gln}$ (GLNA3U70) and the amber suppressor derived from $tRNA^{Phe}$ [$tRNA^{Phe}$(UAG)] were recloned onto pACYC184. The $tRNA^{Phe}$(UAG) gene was recloned from pGFIB as described previously (Weygand-Durasevic et al., 1993), while the gene encoding GLNA3U70 was recloned as a *Pvu*II fragment into the *Eco*RV site of pACYC184. DNA sequence analysis confirmed the presence of the tRNA genes and of the appropriate mutations.

In Vivo Recognition of Noncognate Amber Suppressors and of a Mutant Glutamine Opal Suppressor. The ability of the Leu136 mutants of GlnRS expressed on pBR322 to recognize a battery of amber suppressor tRNAs (Figure 2) was assessed using the *lacZ(UAG1000)* glutamine-specific marker in *E. coli* strains BT235 [*su*°, *lacZ(UAG1000)*] transformed with pACYC clones of the amber suppressor tRNAs, and RS109 [*tyrT(UAG), lacZ(UAG1000)*] (Uemura et al., 1988; Weygand-Durasevic et al., 1993). Each of the BT235/pACYCtRNA(UAG) strains and RS109 was also transformed with the pBR*glnS* mutant clones. Recognition of the coexpressed amber suppressor tRNA by the GlnRS-(L136X) mutants results in a Lac$^+$ phenotype (growth on appropriately supplemented lactose minimal plates) at 30 °C. The ability of the GlnRS mutants expressed on pBR322 to recognize *tyrT(UAG)* and *proH(UAG)* was quantitated using β-galactosidase assays. Six different transformants were picked for each and grown in appropriately supplemented glucose minimal broth at 30 °C. The β-galactosidase activity of each culture was assayed in triplicate using 0.5 mL of culture. Incubation at 28 °C was terminated at between 90 and 120 min.

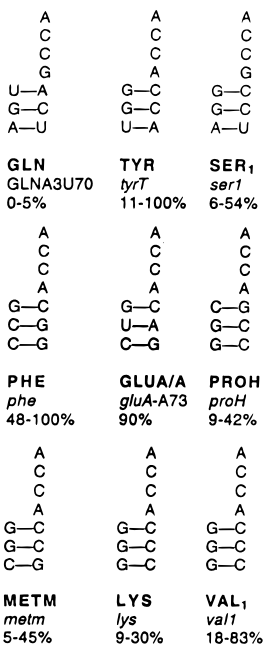

FIGURE 2: Acceptor ends of *E. coli* tRNAs used for *in vivo* specificity assays. The range of suppressor efficiencies indicated below the tRNA acceptor types (bold) are for the amber (UAG) suppressors except for the glutamine tRNA where the suppressor efficiency is that of the A3-U70 mutant opal (UGA) suppressor. The glutamate acceptor stem is that of *gluA(UAG)*-A73 which no longer inserts glutamine.

The mutant opal suppressor derived from $tRNA^{Gln}$, GLNA3U70, no longer inserts a sufficient amount of glutamine to suppress the glutamine-specific *trpA(UGA15)* marker in *E. coli* strain KL2576 (Rogers et al., 1992). KL2576 was transformed with pACYCGLNA3U70 and then with the pBR*glnS*(L136X) clones. A Trp^+ phenotype resulted in growth on glucose minimal plates in the absence of indole and tryptophan at 30 °C. Due to the lower suppressor efficiency of the opal suppressor tRNA, growth was slower than for suppression of the *lacZ(UAG1000)* marker by the amber suppressor tRNAs.

Purification of Wild-Type and Mutant E. coli GlnRS. Wild-type GlnRS (specific activity of ~1000 nmol/mg min) was purified as described (Hoben & Söll, 1985). In order to eliminate contamination of the mutant protein preparations with wild-type GlnRS activity, the Leu136 mutants were purified from the *glnS* temperature-sensitive *E. coli* strain UT172 (Englisch-Peters et al., 1991), transformed with pBR322 clones of *glnS*(L136F) and *glnS*(L136A). For each mutant, six 1 L cultures were grown in LB medium containing ampicillin (0.1 mg/mL) at 37 °C to saturation. As a control, cultures of UT172 transformed with a Bluescript clone (pESQ19, gift of E. Schwob) of *glnS*(T266P), the temperature-sensitive *glnS* mutation in UT172, were also prepared; however, these cultures were grown at 30 °C. In all cases, the cells were harvested, washed in phosphate-buffered saline, repelleted, resuspended at a concentration of 1−2 g of cells/mL in buffer A [20 mM HEPES (pH 7.9), 20 mM KCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 2.5 mM dithiothreitol (DTT), and 0.2 mM phenylmethanesulfonyl fluoride (PMSF)], subjected to a quick freeze−thaw, and then disrupted by sonication. An S100 was prepared, chromatographed on a 100−150 mL (diethylamino)ethyl cellulose column, and eluted with a 20 to 500 mM KCl gradient in buffer A at a flow rate of 4−6 mL/min. The fractions which contained GlnRS activity and were the most enriched for a 65 kDa protein [analyzed using sodium dodecyl sulfate (SDS)-containing polyacrylamide gels] were pooled and dialyzed against buffer C [20 mM HEPES (pH 7.9), 10 mM KCl, 5% glycerol, and 0.5 mM DTT]. The dialysate was chromatographed on a MonoQ 10/10 (Pharmacia) column at room temperature and eluted with a 10 mM to 1 M KCl gradient in buffer C at a flow rate of 2 mL/min. The fractions were placed on ice as they were collected. The single most active and most pure GlnRS fraction (2 mL volume) was dialyzed against buffer E [50 mM Tris (pH 8.0), 150 mM NaCl, and 5% glycerol] and chromatographed on a polyclonal anti-GlnRS FPLC column (U. Thomann, unpublished results) at room temperature. The GlnRS variant was eluted at a flow rate of 0.5 mL/min with 50 mM triethylamine (pH 11.5) directly into 0.5 M HEPES (pH 7.0) to neutralize it (pH 7.5−8.0), and the fractions were immediately placed on ice. To maximize the recovery of GlnRS, the flow through was rechromatographed on the antibody column. Those fractions which contained GlnRS were pooled, immediately dialyzed against buffer C, and chromatographed on a MonoQ 5/5 column at room temperature. Pure GlnRS elutes at approximately 235 mM KCl. Approximately 1−2 mg of each Leu136 mutant was purified in this way. No GlnRS(T266P) protein or activity was detected in the MonoQ 5/5 eluent. This is consistent with the loss, in all of the GlnRS preparations from UT172 [which contain GlnRS(T266P)], of a small amount of activity between the MonoQ 10/10 and MonoQ 5/5 columns. As a control, pure wild-type GlnRS [i.e. no contaminating GlnRS-(T266P)] was loaded onto the antibody column, eluted at high pH, neutralized, and run over the MonoQ 5/5 column. Little or no loss of activity was observed. All of the purified mutant GlnRS preparations appeared as one band (0.8−3.1 μg applied) on a Coomassie blue-stained SDS-containing polyacrylamide gel following electrophoresis.

Aminoacylation Assays. After preincubation of the 50 μL assay mixes [100 mM HEPES (pH 7.2), 10 mM magnesium acetate, 2 mM ATP, a [^{3}H]glutamine/glutamine mixture, and tRNA] at 37 °C, the reactions were initiated by the addition of GlnRS and the mixture incubated at 37 °C. The amount of AARS added never exceeded 5 μL, and the enzymes were diluted in enzyme dilution buffer [20 mM β-mercaptoethanol, 10% (v/v) glycerol, 1 mg/mL bovine serum albumin (BSA), 50 mM HEPES (pH 7.2), and 0.2 mM DTT]. At various time points, an aliquot of the reaction mixture was removed and spotted onto 3MM filters. The filters were washed extensively in 5% trichloroacetic acid (TCA), soaked in 95% ethanol, dried, and analyzed by liquid scintillation counting. The conversion factor for correcting counting efficiency of free [^{3}H]amino acids was 3.62 pmol of [^{3}H]glutamine/[^{14}C]-glutamine (Rogers & Söll, 1993).

When the steady-state kinetic parameters for $tRNA^{Gln}$ were determined, glutamine and ATP were present in the assay mix at concentrations which are saturating for wild-type GlnRS, 680 μM and 2 mM, respectively (Kern et al., 1980). The tRNA concentration was varied over a 10-fold range, and tRNA was present in at least a 165−fold molar excess over GlnRS. Assays were performed in duplicates using a minimum of five tRNA concentrations and four time points in the linear range of initial rate, and errors were consistently less than 10%. The kinetic parameters were calculated using both Lineweaver−Burke and Eadie−Hofstee plots.

Table 1: *In Vivo* Specificity of Leu136 Variants of GlnRS for Suppressor tRNAs

	tRNA(UAG) (Lac$^{+/-}$)[a]								tRNA(UGA) (Trp$^{+/-}$)[a]
GlnRS	*tyrT*	*gluA-A73*	*proH*	*phe*	*ser1*	*metm*	*lys*	*val1*	GLNA3U70
GlnRS$^+$	+	−	−	−	−	−	−	−	−
L136F	+	−	++	±	−	−	−	−	±
L136A	−	−	−	−	−	−	−	−	−
L136M	−	−	−	−	−	−	−	−	−
L136T	−	−	−	−	−	−	−	−	−
L136V	−	−	−	−	−	nd	nd	nd	−

[a] Lac$^{+/-}$ and Trp$^{+/-}$ indicate growth on lactose minimal plates containing cysteine and ampicillin and glucose minimal plates containing methionine, ampicillin, and chloramphenicol at 30 °C, respectively, relative to others in the same strain with the same suppressor. nd = not determined.

Table 2: *In Vivo* Mischarging of *tyrT(UAG)* and *proH(UAG)* by Leu136 Variants of GlnRS

	β-galactosidase activity (Miller units)	
GlnRS	*tyrT*	*proH*
GlnRS$^+$	17	3
L136F	20	34
L136A	4	nd[a]
L136M	2	nd
L136T	3	nd
L136V	4	nd

[a] nd = not determined.

Table 3: *In Vitro* Kinetic Parameters for tRNA^{Gln2} of Leu136 Variants of GlnRS[a]

	tRNA^{Gln2}			
GlnRS	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (μM^{-1} s^{-1})	relative k_{cat}/K_M
GlnRS$^+$	0.27	3.83	14.2	1.00
L136F	0.40	0.36	0.90	0.06
L136A	0.79	3.14	3.97	0.28

[a] Kinetic constants with respect to tRNA^{Gln2} were determined with 680 μM glutamine present in the assays. This is less than the measured K_M for glutamine of GlnRS(L136A) (data not shown).

For the time courses of misaminoacylation, 6.8 μM [^{3}H]-glutamine (260 cpm/pmol) in a total glutamine concentration of 680 μM was used. The amber suppressor tRNAs, *tyrT-(UAG)* and *tyrT(UAG)*-G73, were produced *in vivo* and purified as described (Sherman et al., 1992a). For aminoacylation of *tyrT(UAG)*, the tRNA concentration was 4 μM while the concentrations of wild-type GlnRS and GlnRS-(L136A) were 0.5 μM, and that of GlnRS(L136F) was 0.26 μM. The concentration of *tyrT(UAG)*-G73 was 1.0 μM and that of all three variants of GlnRS was 0.1 μM. These tRNA concentrations were in the linear range for initial velocity (data not shown).

The assay conditions used for the *in vitro* competition experiments (Sherman et al., 1992a) between the Leu136 variants of GlnRS and purified TyrRS were very similar to those used for the time courses of aminoacylation. The concentration of *tyrT(UAG)* was 1.0 μM, the GlnRS:*tyrT-(UAG)* molar ratio was approximately 1:2, and TyrRS, when present, was added at a concentration half that of GlnRS. Unlabeled tyrosine (120 μM) was added to all the reaction mixtures. Total levels of Gln-*tyrT(UAG)* produced by GlnRS in the presence and absence of TyrRS were measured after incubation for 30 min at 37 °C.

RESULTS

In Vivo Specificity and Activity Assays. A weak first base pair is important for glutamine identity (Hooper et al., 1972; Shimura et al., 1972; Sherman et al., 1995). Leu136 stabilizes the disruption of the U1-A72 base pair and thus the conformation assumed by the acceptor end of tRNA^{Gln} (Rould et al., 1989). In order to determine the contribution of this side chain to both cognate and noncognate interactions, Leu136 was changed to phenylalanine (L136F), methionine (L136M), valine (L136V), alanine (L136A), and threonine (L136T). The effect of these mutations on the ability of GlnRS to discriminate against noncognate amber and mutant cognate opal suppressor tRNAs (Figure 2) *in vivo* was determined using the glutamine-specific markers, *lacZ*(UAG1000) and *trpA(UGA15)* (Tables 1 and 2). The results of the plate and β-galactosidase assays are consistent. *In vivo*, only the L136F mutant expressed on pBR322 still mischarges *tyrT(UAG)* as well as wild-type GlnRS. However, unlike wild-type, GlnRS(L136F) also mischarges the amber suppressor *proH(UAG)*, a hybrid proline-phenylalanine tRNA (Kleina et al., 1990), and as such is less specific *in vivo*. In contrast, none of the other Leu136 mutants (L136M, A/T/V) expressed on pBR322 even recognize *tyrT-(UAG)* well enough to confer a Lac$^+$ phenotype on RS109 and thus appear to be more specific *in vivo* than wild-type GlnRS.

Kinetic Characterization. In order to rule out the possibility that the differences in specificity observed *in vivo* are due to differences in the level of expression, overall AARS activity, or general tRNA affinity, the steady-state kinetic parameters for tRNA^{Gln} were measured for both the more mischarging GlnRS(L136F) and the less mischarging GlnRS(L136A) (Table 3). In fact, the relative specificity constant (k_{cat}/K_M) for tRNA^{Gln} of the L136A mutant is higher than that of GlnRS(L136F); for L136F, k_{cat}/K_M is reduced by a factor of 17, whereas for GlnRS(L136A), the reduction is only by a factor of 3.5. However, the k_{cat} and K_M are affected differently in the two mutants. Most of the defect in the L136F mutant is in k_{cat}, which is reduced by 1 order of magnitude, while almost the entire decrease in the specificity constant for the L136A mutant is due to an increase in K_M. In addition, the L136A mutant also exhibits an approximately 4-fold increase in its K_M for glutamine (data not shown) above the already high (~200 μM) K_M of wild-type GlnRS for its cognate amino acid (Kern et al., 1980). Overall, the magnitudes of the effects of mutating Leu136 are comparable to those observed for base pair 1−72 mutants in tRNA^{Gln} (Jahn et al., 1991).

In Vitro Mischarging. Overall, it is clear that the altered tRNA specificities of GlnRS(L136F) and GlnRS(L136A) are not due simply to changes in AARS activity and tRNA affinity (Tables 1−3). However, we were interested in determining whether the alanine substitution makes GlnRS a more accurate AARS and conversely whether the phenylalanine substitution makes GlnRS less specific. In order to conclude that a mutant AARS is more or less specific *in vitro*, the ratio of its specificity constant with respect to the noncognate tRNA [e.g. *tyrT(UAG)*] relative to that for

Table 4: *In Vitro* Specificity of Leu136 Variants of GlnRS for *tyrT* (UAG) and *tyrT*-G73 (UAG)[a]

	k_{cat}/K_M (μM^{-1} s^{-1}) ($\times 1$)	k_{cat}/K_M (μM^{-1} s^{-1}) ($\times 10^{-4}$)		k_{cat}/K_M(noncognate)/ k_{cat}/K_M(cognate) ($\times 10^{-4}$)	
GlnRS	$tRNA^{Gln}$	*tyrT*-A73	*tyrT*-G73	*tyrT*-A73	*tyrT*-G73
$GlnRS^+$	14.2	6.58	112	0.46	7.89
L136F	0.90	6.86	60.0	7.62	66.7
L136A	3.97	3.33	27.0	0.84	6.80

[a] The specificity constants for the noncognate substrates were derived from the initial rate at a single tRNA concentration which is in the linear range with respect to initial rate.

itscognate glutamine tRNA must be compared with the same ratio for wild-type GlnRS (Table 4). For this reason, the specificity constants (k_{cat}/K_M) for the mischarging of both wild-type *tyrT(UAG)* [*tyrT(UAG)*-A73] and *tyrT(UAG)*-G73 have been determined *in vitro*. The G73 mutant of *tyrT-(UAG)* was selected because it is a better substrate for recognition by GlnRS both *in vivo* and *in vitro* (Hooper et al., 1972; Shimura et al., 1972; Sherman et al., 1992a,b). Since the purified, *in vivo*-produced *tyrT(UAG)* tRNAs were only available in limited quantities (Sherman et al., 1992a), the individual kinetic parameters could not be determined. Instead, the initial rates were used to calculate the specificity constant, k_{cat}/K_M (v_0/[tRNA][E]). Table 4 shows the measured specificity constants for the amber suppressors as well as these specificity constants normalized to those for $tRNA^{Gln}$.

As predicted from the *in vivo* mischarging data for *tyrT-(UAG)*, GlnRS(L136F) mischarges *tyrT(UAG) in vitro* at the same rate and to the same level of total tRNA charging as wild-type GlnRS [and *tyrT(UAG)*-G73 and *gluA(UAG)*-G73 almost as well] (Tables 2−4). This is despite GlnRS-(L136F)'s almost 20-fold lower specificity constant for its cognate glutamine tRNA *in vitro*. Thus, the L136F mutant is clearly less specific than wild-type GlnRS, in agreement with the ability of GlnRS(L136F) to mischarge *proH(UAG) in vivo* which wild-type cannot (Tables 1 and 2). Also, as predicted from the *in vivo tyrT(UAG)* suppression results, the L136A mutant does not mischarge *tyrT(UAG)* as well as the Leu136 (wild-type) and L136F variants. In fact, it mischarges *tyrT(UAG)* at half the rate and to only 50% of the total charging of the other two AARSs (Table 4). However, the magnitude of this difference is lower than predicted from the *in vivo* results (Tables 1 and 2).

In Vitro Competition Assays. The *in vivo* and *in vitro* assays differ significantly as the *in vivo* assays are conducted in a competitive environment in which 20 AARSs, including TyrRS, are competing for *tyrT(UAG)* (Yarus, 1972; Swanson et al., 1988; Sherman et al., 1992a). In order to mimic the *in vivo* situation and to eliminate the possibility that *in vivo* GlnRS(L136A) does not compete as effectively against TyrRS for *tyrT(UAG)*, *in vitro* competition experiments have been conducted (Sherman et al., 1992a). Mischarging of *tyrT(UAG)* by the GlnRS variants was measured in the absence and presence of TyrRS, with the substrate *tyrT(UAG)* limiting to enhance competition (Figure 3). As expected, the L136F mutant competes most effectively against TyrRS. However, while GlnRS(L136A) is at least as effective as wild-type GlnRS in competing with TyrRS, it is also less mischarging, such that the overall level of Gln-*tyrT(UAG)* is lower in the reaction catalyzed by GlnRS(L136A).

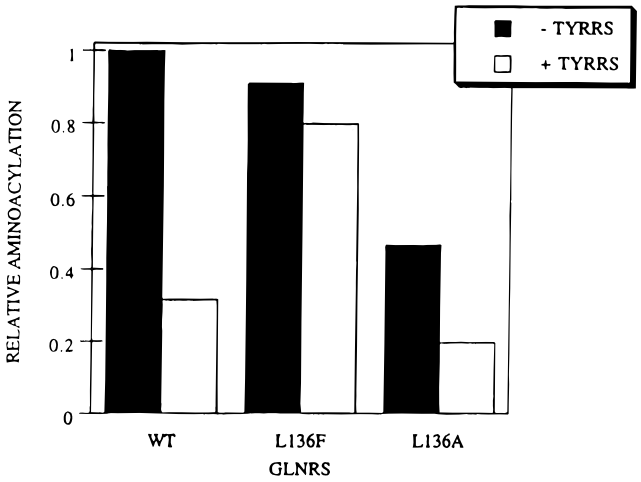

FIGURE 3: *In vitro* competition between TyrRS and Leu136 variants of GlnRS for *tyrT(UAG)*. Total levels of Gln-*tyrT* produced by each of the GlnRS variants was measured in the presence and absence of the cognate AARS for *tyrT*, TyrRS. The concentration of *tyrT* in all of the reactions was limiting as the GlnRS:tRNA ratio was 1:2. The TyrRS, when added, was present at approximately 50% of the concentration of GlnRS.

DISCUSSION

E. coli GlnRS exhibits relaxed tRNA specificity as shown by its mischarging of $tRNA^{Trp}$(UAG) and *tyrT*(UAG) and by the ease with which mutants of both GlnRS and tRNAs which confer glutamine identity on noncognate and mutant tRNAs have been isolated (Sherman et al., 1995). Mutational analyses of tRNAs and the GlnRS/$tRNA^{Gln}$ crystal structure indicate that GlnRS recognition requires a weak first (1−72) base pair (Hooper et al., 1972; Shimura et al., 1972; Rogers & Söll, 1988; Rould et al., 1989; Seong et al., 1989; Varshney et al., 1991a; Normanly et al., 1992). The disruption of the U1-A72 base pair in $tRNA^{Gln}$ is stabilized by the stacking of Leu136 between A72 and G2 (Figure 1; Rould et al., 1989). While the positioning of Leu136 has been implicated in specific tRNA recognition by GlnRS (Weygand-Durasevic et al., 1993), we have demonstrated directly that Leu136 is a tRNA specificity determinant (Table 1) and have identified both more and less mischarging Leu136 mutants which are significantly different from all the previously isolated GlnRS mutants.

GlnRS(L136F) Mischarges a Sequence-specific Subset of Noncognate tRNAs. GlnRS(L136F) is the first, less specific GlnRS which is not generally mischarging and which exhibits a decreased affinity for $tRNA^{Gln}$, rather than an increased affinity for noncognate tRNAs (Tables 1, 3, and 4; Inokuchi et al., 1984; Swanson, 1988; Weygand-Durasevic et al., 1993, 1994; Rogers et al., 1994). GlnRS(L136F) not only mischarges *tyrT(UAG) in vivo* but also recognizes *proH(UAG)*, an artificial proline-phenylalanine hybrid amber suppressor tRNA (Kleina et al., 1990). However, GlnRS(L136F) does not mischarge $tRNA^{Phe}$(UAG), whose suppressor efficiency is equal to or greater than that of *proH(UAG)* in almost all contexts (Figure 2), or *ser1(UAG)* and *gluA(UAG)*-A73, both of which are recognized by the GlnRS mutants which affect the positioning of Leu136 (Weygand-Durasevic et al., 1993). The L136F mutant also does not recognize $tRNA^{Metm}$(UAG) which is recognized by all of the GlnRS-mischarging mutants analyzed to date (Weygand-Durasevic et al., 1993). Thus, the failure of GlnRS(L136F) to mischarge $tRNA^{Metm}$(UAG) is not due to a context-dependent difference in suppressor

efficiency. It is tempting to speculate that the unusual Py1-Pu72 and Pu2-Py71 acceptor stem combination of *proH-(UAG)* allows GlnRS(L136F) to recognize it (Figure 2). This base pair combination maximizes hydrophobic stacking energy by allowing Leu136 or Phe136 to stack between two purines (Figure 1). Unlike the initiator methionine (C1A72/A73) and glutamine (U1-A72/G73) tRNAs which are aminoacylated by wild-type GlnRS *in vivo* and *in vitro*, GlnRS recognition of *proH(UAG)* may require the additional stabilization provided by the planar phenylalanine ring because of its strong first base pair (C1-G72) and because it lacks G73 (Figure 2; Schulman & Pelka, 1985; Rould et al., 1989; Seong et al., 1989; Varshney et al., 1991a,b). In addition to the possible sequence specificity described above, *proH(UAG)* may be a better candidate for mischarging *in vivo* because it is an artificial tRNA, and thus, the competition for *proH(UAG)* may be less than when GlnRS is competing against PheRS and LysRS, respectively, for $tRNA^{Phe}$(UAG) and $tRNA^{Met}$(UAG).

In vitro characterization of GlnRS(L136F) with both cognate glutamine and noncognate tyrosine [*tyrT(UAG)*] tRNAs has demonstrated that this mutant, unlike all of the other mischarging mutants of GlnRS characterized to date, exhibits a significant decrease in affinity for $tRNA^{Gln}$. However, GlnRS(L136F) still mischarges *tyrT(UAG)* as well and *tyrT(UAG)*-G73 almost as well as wild-type GlnRS (Tables 3 and 4; Inokuchi et al., 1984; Swanson, 1988; Weygand-Durasevic et al., 1993, 1994; Rogers et al., 1994). This phenotype has been observed in other systems, e.g. Meinnel et al. (1991). Furthermore, the reduced tRNA specificity observed *in vivo* is probably not due to an increased level of expression (see below) which could compensate for less efficient recognition and thus bias competition (Swanson et al., 1988; Hou & Schimmel, 1989; Sherman et al., 1992a), although the L136F mutant is a more effective competitor (Figure 3).

Other Leu136 Mutants Are Less Mischarging than Wild-Type. In contrast to GlnRS(L136F), all of the other Leu136 mutants (L136M/A/T/V) no longer recognize *tyrT(UAG)* and thus are less mischarging *in vivo* than wild-type GlnRS (Tables 1 and 2). These are the first mutants of GlnRS which, when expressed on pBR322, do not recognize *tyrT-(UAG)* sufficiently well to suppress the *lacZ(UAG1000)* marker (Swanson, 1988; Weygand-Durasevic et al., 1993, 1994; Rogers et al., 1994). However, two Asp235 mutants when expressed as single copy lysogens also fail to do so (Uemura et al., 1988). In addition, the L136M, L136V, L136A, and L136T mutants do not recognize any of the other amber suppressors tested and thus are not likely to have a different sequence preference. While it is possible that the inability of these Leu136 mutants to recognize *tyrT(UAG)* is due to reduced *in vivo* levels of expression (and the decreased specificity of the L136F mutant a result of elevated expression), it seems unlikely since purification of the L136F and L136A mutants yielded similar amounts of purified AARS.

In order to attribute the decrease in mischarging observed *in vivo* more directly to the loss of the stabilizing interaction of the leucine side chain, a representative mutant, GlnRS-(L136A), was analyzed *in vitro* with both cognate and noncognate tRNA substrates. The inability of the L136A mutant to mischarge *tyrT(UAG) in vivo* is not due to reduced tRNA affinity, as its specificity constant (k_{cat}/K_M) with respect to $tRNA^{Gln}$ is reduced by less than a factor of 4 and in fact is higher than that of GlnRS(L136F) which mischarges both *tyrT(UAG)* and *proH(UAG) in vivo* (Table 3).

Consistent with the *in vivo* results, the L136A mutant mischarges *tyrT(UAG)* with reduced efficiency *in vitro* (Table 4). However, the magnitude of this decrease in mischarging *in vitro* is less than the difference in β-galactosidase activity observed *in vivo* (Table 2). Although GlnRS(L136A) does not mischarge *in vitro* any of the amber suppressor tRNAs tested as well as wild-type GlnRS, it has a reduced affinity for its cognate glutamine tRNA such that it is of approximately the same specificity as wild-type GlnRS (Table 4). Thus, GlnRS(L136A) cannot be termed more specific, only less mischarging. Yet, the Lac^- phenotype conferred on RS109 by this mutant *in vivo* would suggest otherwise. This difference between the *in vivo* and *in vitro* results is also not due to the inability of GlnRS-(L136A) to compete effectively with TyrRS *in vivo*. In fact, the L136A mutant competes against TyrRS about as well as wild-type GlnRS, despite its elevated K_M for $tRNA^{Gln}$ (Figure 3). Thus, an elevated K_M alone does not impair the ability of an AARS to compete for a tRNA (Yarus et al., 1986; Varshney et al., 1991a). However, while GlnRS(L136A) competes slightly more effectively, it does not mischarge *tyrT(UAG)* as well, so that the total amount of Gln-*tyrT-(UAG)* produced by this mutant in the presence of TyrRS is less than that produced by wild-type GlnRS (Figure 3, Table 4). Thus, *in vitro* there is an obvious threshold which could account for the difference between Lac^+ and Lac^- phenotypes *in vivo*.

Optimization of Cognate Recognition and Discrimination against Noncognates. If GlnRS(L136A) is less mischarging than and as specific as wild-type GlnRS, then why did GlnRS not evolve to have an alanine at position 136? While an alanine at position 136 allows GlnRS to discriminate more effectively, it also decreases cognate tRNA recognition. Our results suggest that AARSs are optimized for overall specificity rather than for either cognate tRNA recognition or discrimination against noncognate tRNAs (Yarus, 1972). An analogous situation exists for the discrimination between tyrosine and phenylalanine by tyrosyl-tRNA synthetase where it is clear that the wild-type enzyme has not reached the maximum level of discrimination between the two amino acids (de Prat Gay et al., 1993). Furthermore, while GlnRS-(L136A) has relatively higher affinity for its cognate tRNA, it also has a 4-fold higher K_m for glutamine. Thus, the wild-type may in fact provide the best compromise between substrate specificity and optimal catalysis, similar to TyrRS, where the overall rate of aminoacylation is optimized by compromising between the various steps in the reaction pathway (Avis et al., 1993; Avis & Fersht, 1993). This is similar to the compromise between reaction rate and accuracy reached by the ribosome for protein biosynthesis (Yarus & Thompson, 1983; Kurland & Gallant, 1986).

The decreased efficiency with which the L136A mutant aminoacylates $tRNA^{Gln}$ could also have significant repercussions *in vivo*, as glutamine tRNAs, expressed at normal physiological levels, read through termination codons and the amber (UAG) suppressor derived from $tRNA^{Gln}$ reads the glutamine (CAG) codon in yeast (Weiss et al., 1987; Edelman & Culbertson, 1991). The chemical properties of glutamine (Gln is structurally nondisruptive and uncharged yet can act as both hydrogen bond donor and acceptor) make

it an ideal residue to insert into proteins at nonprescribed positions, and thus, a more specific GlnRS would not necessarily confer a selective advantage. Finally, the current GlnRS enzyme, with a leucine instead of an alanine at position 136, may either represent evolution in progress or reflect a lack of selection pressure to introduce such changes. In addition, it has been shown that GlnRS not only mischarges noncognate amber suppressor tRNAs but also recognizes and misaminoacylates a number of non-cognate wild-type *E. coli* tRNAs, including $tRNA^{Glu}$ (Uemura et al., 1988; Sherman et al., 1992b; Rogers & Söll, 1993). The somewhat relaxed specificity may be an artifact of evolution from an ancestral GlxRS which, in common with a number of contemporary GluRSs (Schön et al., 1988), is thought to have once recognized and aminoacylated both $tRNA^{Glu}$ and $tRNA^{Gln}$ (Lapointe et al., 1986; Breton et al., 1990; Rogers & Söll, 1993; Lamour et al., 1994).

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