The Terminal Adenosine of $tRNA^{Gln}$ Mediates tRNA-Dependent Amino Acid Recognition by Glutaminyl-tRNA Synthetase†

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ABSTRACT: Sequence-specific interactions between *Escherichia coli* glutaminyl-tRNA synthetase and $tRNA^{Gln}$ have been shown to determine the apparent affinity of the enzyme for its cognate amino acid glutamine during aminoacylation. Specifically, structural and biochemical studies suggested that residues Asp66, Tyr211, and Phe233 in glutaminyl-tRNA synthetase could potentially facilitate cognate amino recognition through their specific interactions with both A76 of $tRNA^{Gln}$ and glutamine. These residues were randomly mutated and the resulting glutaminyl-tRNA synthetase variants were screened in vivo for changes in their ability to recognize noncognate tRNAs and retention of tRNA-glutaminylation activity. When the variants selected in this way were characterized in vitro, they all showed dramatic decreases in apparent affinity (K_M) for glutamine but little or no change in cognate tRNA affinity. Conservative replacements such as Y211F, F233L, and D66E resulted in 60-, 19-, and 18-fold increases compared to wild-type in the K_M for glutamine, respectively, but had little effect on the turnover number (k_{cat}). Nonconservative replacements affected both K_M for glutamine and k_{cat}; Y211S, F233D, and D66F displayed 1700, 3700, and 1200-fold decreases in k_{cat}/K_M for glutamine compared to wild-type. Double mutant cycle analysis indicated that Tyr211, and Phe233 interact strongly to enhance glutamine binding. These data now show that Asp66, Tyr211 and Phe233 mediate tRNA-dependent cognate amino acid recognition via the invariant 3′-terminal adenosine of $tRNA^{Gln}$.

Aminoacyl-tRNA synthetases (aaRSs) catalyze the esterification of amino acids with their cognate tRNAs and thus are essential in maintaining fidelity during protein biosynthesis. Aminoacylation is achieved in two steps, first the amino acid is activated to form an aminoacyl adenylate in the presence of aaRS and ATP, then the activated amino acid is transferred to the 3′-terminus of its cognate tRNA. There are 20 aaRSs which have been organized into two groups, class I and II, of 10 enzymes each based on short consensus amino acid (*1*) and structural motifs (*2*). The active site in class I aaRSs is built in the N-terminal domain around a Rossmann nucleotide-binding domain (alternating parallel β-strand and α-helices) (*3*) which is flanked by the characteristic signature sequences KMSKS and HIGH. Class II aaRSs do not contain a Rossmann fold with the active site instead composed of an antiparallel β-fold.

The fidelity of protein synthesis depends on an amino acid being paired accurately with its cognate tRNA to produce aminoacyl-tRNA, which may be achieved by an aaRSs alone or acting in concert with a post charging conversion mechanism (*4*). While in many cases, differences in amino acid structure are sufficient to allow accurate discrimination, some aaRSs have editing activities which ensure that misactivated amino acids are edited. These editing reactions may occur either in the absence [e.g., methionyl-tRNA synthetase, (*5*)] or the presence of tRNA. For example, $tRNA^{Ile}$ induces hydrolysis of enzyme-bound valyl-adenylate which is misactivated by isoleucyl-tRNA synthetase (*6, 7*). Thus, tRNA plays a critical role in the discrimination of noncognate amino acids. More recently, it has been shown that tRNA is also important in ensuring accurate cognate amino acid recognition by glutaminyl-tRNA synthetase [GlnRS (*8, 9*)]. The aim of this work was to characterize the point of interaction between GlnRS and $tRNA^{Gln}$, which optimizes glutamine binding in the active site.

Among the 20 known aaRSs, glutamyl-, glutaminyl, and arginyl-tRNA synthetases are the only three known examples where the presence of tRNA is required for amino acid activation (*10*). The crystal structure of *Escherichia coli* GlnRS complexed with $tRNA^{Gln}$ and ATP (*11*) defined four functional domains and provided detailed information on the amino acid residues of GlnRS involved in the interactions between the synthetase and its cognate tRNA. The residues responsible for determining tRNA specificity during interaction with the acceptor stem and anticodon binding domains and the connectivity subdomain have subsequently been studied systematically (*9, 12*−*15*). The two halves of the dinucleotide-binding domain of GlnRS (amino acid residues 25−100 and 210−260), which are interrupted by the insertion of the tRNA acceptor stem-binding domain, provide major contacts for ATP and glutamine as well as making sequence-specific interactions with the acceptor stem of

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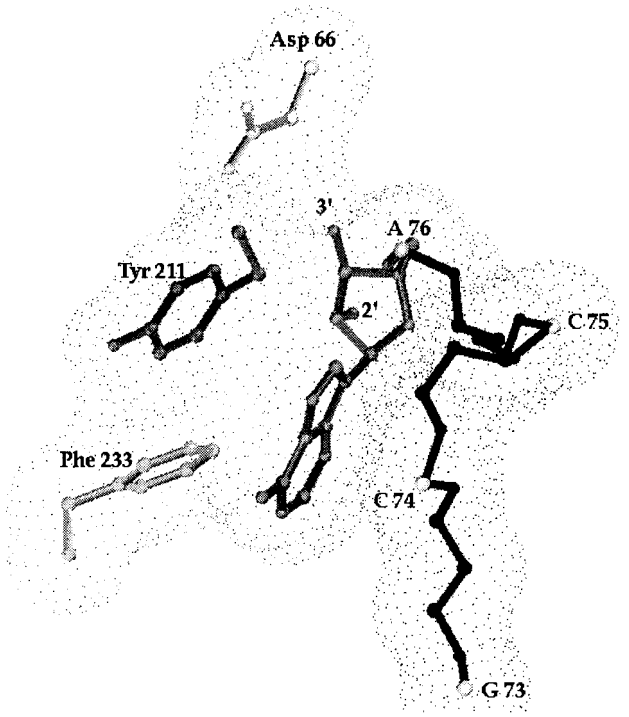

FIGURE 1: Detail of the interaction between the 3′ end of $tRNA_2^{Gln}$ and residues Asp66, Tyr211, and Phe233 of GlnRS (*16)*. The amino acid side chains and the phosphodiester backbone of the tRNA are shown (P is shown as white spheres), except for A76 where the ribose and base moieties are also shown. Prepared with RIBBONS (*35*).

tRNA. Investigation of Asp235, which is located in the second half of this domain and is involved in pairing with G2 and G3 in $tRNA^{Gln}$ (*11*), showed amino acid recognition to be tRNA-mediated (*8*). Mutation of Asp235 to His resulted in an elevated K_M for glutamine when noncognate $tRNA_{CUA}^{Tyr}$ is used instead of cognate $tRNA^{Gln}$ as a substrate for aminoacylation. This showed that cognate amino acid recognition is tRNA dependent (*8*) and suggested that GlnRS employs a mechanism which is similar to the tRNA-dependent editing process found in other aaRSs. However, the interactions between $tRNA^{Gln}$ and GlnRS which mediate this process were not resolved.

The crystal structure of GlnRS indicates that three residues, Asp66, Tyr211, and Phe233, contact the 3′-terminus of $tRNA^{Gln}$ (see Figure 1). Model building of the complex with glutamine suggested that the positively charged NH_3^+ group of glutamine forms a salt bridge to the side-chain carboxylate of Asp66 (*16*). It was also suggested that Tyr211 and Phe233 form a hydrophobic "lid" to the amino acid binding site and provide stacking interactions with the adenine ring of the 3′-terminal A76 of $tRNA^{Gln}$. The main-chain carbonyl group of Phe233 serves to orient the carboxyl-amide as a hydrogen bond acceptor from the glutamine substrate (*16*). Glutamyl-tRNA synthetase (GluRS) is another aaRS which requires the presence of tRNA for the activation of its amino acid (*17*). Comparison of a number of predicted GlnRS and GluRS amino acid sequences indicated that Asp66 and Tyr211, but not Phe233, are highly conserved between these enzymes (*18*). In an earlier study of the catalytic core of *E. coli* GlnRS, variants with deletion of the acceptor-binding domain (defined as residues 110−210) sustained the growth of a strain thermosensitive for GlnRS at a nonpermissive temperature. Small deletions extending beyond the acceptor binding domain (114−215), including the deletion of Tyr211, led to loss of complementation (*19*). This indicated the importance of Tyr211 in maintaining the function of GlnRS. More recent studies showed that the nucleotide sequence of $tRNA^{Gln}$ exerts an influence on the efficiency of cognate amino acid recognition and suggested that Asp66, Tyr211, and Phe233 could potentially facilitate the accurate attachment of glutamine through their interaction with A76 of $tRNA^{Gln}$ (*8, 9*). The apparent importance of these residues led us to probe their possible roles during tRNA-dependent glutamine recognition. Our strategy was to randomly mutate the three positions and select the resulting variants for changes in tRNA specificity. This strategy allowed the selection of mutants with dramatically decreased apparent affinity for glutamine, indicating that these three residues mediate tRNA-dependent amino acid recognition.

MATERIALS AND METHODS

Strains and Plasmids. The *E. coli* strains BT3213 (*supF, glnS*^{ts}; derived from BT32) and KL2576 have been described (*8*). Opal suppressors [GLNA3U70 and GLNG3U70, encoding $tRNA_{UCA}^{Gln}$ A3U70 and $tRNA_{CUA}^{Glu}$ U70, respectively; (*7*)] and the two amber suppressors *gluA*-A73 and *ser1* (which encode $tRNA_{CUA}^{Glu}$ A73 and $tRNA_{CUA}^{Ser1}$, respectively) cloned into the plasmid pACYC184 have previously been reported (*14*).

General. β-Galactosidase activity was used to estimate the suppression level of $tRNA_{CUA}^{Tyr}$. All the procedures were similar to those described (*8, 20*) with cell disruption by SDS and chloroform. To investigate other possible changes in the spectrum of tRNA recognition, the two opal suppressors GLNA3U70 and GLNG3U70 or one of the two amber suppressors *gluA*-A73 or *ser1* were cotransformed with plasmids encoding GlnRS mutated at positions Asp66, Tyr211, and Phe233 into the strains KL2576 or BT3213. The resulting transformants were plated and screened for recognition of suppressor tRNAs by synthetase mutants as previously described (*13, 21*). Functional complementation of a temperature-sensitive *glnS* gene was carried out in the strain UT172 (*22*). Purification of wild-type and mutant GlnRS was as described (*23*) except that a HiTrap Blue affinity column (Pharmacia) was used as the first step instead of ammonium sulfate precipitation. A buffer of 25 mM Tris-HCl, 2 mM $MgCl_2$, and 10% glycerol was used to load the supernatant from an S-100 extract. GlnRS was eluted with the above buffer in the presence of 0.6 M NaCl.

Saturation Mutagenesis of Asp66, Tyr211, and Phe233. The template for PCR mutagenesis was double-stranded DNA containing the *glnS* gene cloned into the vector pBR322. Two 51-mer oligonucleotide primers corresponding to the complementary DNA strands encoding amino acids 198−214 were synthesized, in which each of the three nucleotides of the Tyr211 codon was randomized. These primers contained one silent mutation designed to remove a *Bst*XI restriction site. In a similar way, two 24-mer oligonucleotide primers corresponding to amino acids 229−236 were synthesized, in which each of the nucleotides of the Phe233 codon was randomized. A new *Xho*I restriction site was introduced in these two primers. Two 47-mer oligonucleotide primers corresponding to amino acids 63−

78 were also synthesized and included a silent mutation to remove an *Eco*RV restriction site. Another two 24-mer primers complementary to the 5′- and 3′-ends of the *glnS* gene were synthesized, and then combined with one mutated primer from each pair and used for PCR. Full length *glnS* genes were then prepared by overlap extension using the mutant products from the previous reactions as templates and the 5′ and 3′ terminal oligonucleotides as primers. These fragments were then digested with restriction enzymes and cloned into pBR322 (*8*). Construction of the double mutant Y211F/F233Y was carried out in a similar way except that the template contained the single mutation Y211F and the primers contained a TTC→TAC change in the Phe233 codon.

Genetic Selection. Genetic selection was based on the ability of the protein encoded by a mutated *glnS* gene to mischarge the amber suppressor $tRNA^{Tyr}_{CUA}$ (encoded by the *supF* gene) with glutamine in vivo. As previously described, the overproduction of wild-type GlnRS results in the mischarging of $tRNA^{Tyr}_{CUA}$ with glutamine in vivo (*21*,*24*). The ligation mixtures containing the Asp66, Tyr211, or Phe233 mutants were used to transform strain BT3213 and plated on MacConkey plates containing ampicillin. After 1 day of incubation at 30 °C, the colonies were restreaked at 30 and 42 °C, incubated for 1 day, and plasmid DNAs isolated, screened by restriction analysis, and sequenced. White colonies were derived from GlnRS mutants which no longer mischarge $tRNA^{Tyr}_{CUA}$ with glutamine, red colonies from wild-type, or mutant GlnRSs which mischarge $tRNA^{Tyr}_{CUA}$ with glutamine. Pink colonies showed intermediate characteristics. Subsequent sequence analysis was limited to genes encoding functional GlnRS as evidenced by the ability of colonies to grow at 30 and 42 °C on MacConkey plates.

Enzyme Kinetics. Aminoacylation assays were performed as previously described (*8*) except as detailed below. For the measurement of kinetic parameters for glutamine, 0.025−10 mM [^{3}H]glutamine (48−50Ci/mmol) was used. Normally, this glutamine concentration corresponded to a range between 0.2 and 5 times K_M, except for the GlnRS variants Y211F, Y211S, and F233D whose high K_M prevented the use of saturating [^{3}H]glutamine concentrations of sufficient specific activity. For the measurement of kinetic parameters for ATP, glutamine was maintained at 5 times K_M (except 15 mM for Y211F, 10 mM for Y211S, and F233D), and ATP concentrations of 0.2−5 times K_M were used. For all the above measurements, the GlnRS concentration was 10−100 nM. Kinetic parameters were calculated from Eadie−Hofstee plots. The values were from the average of at least two independent determinations within 15% error. The association of GlnRS and $tRNA^{Gln}$ was monitored by stopped-flow fluorimetry as previously described (*8*).

RESULTS

Selection of Active GlnRS Mutants with Altered Recognition of the 3′-Terminal Adenosine of tRNA. It has been proposed that Tyr211 and Phe233 participate in the aminoacylation of $tRNA^{Gln}$ with glutamine through stacking interactions with A76 of $tRNA^{Gln}$. To probe this interaction, these residues and Asp66, which also interacts with A76, were randomly mutated, and the resulting GlnRS variants screened for changes in tRNA specificity. The substrate of choice was $tRNA^{Tyr}_{CUA}$, which has previously been shown to form productive interactions with GlnRS despite an altered positioning of its 3′-end compared to $tRNA^{Gln}$ (*8*). The genetic selection described above allowed rapid screening of complete populations of GlnRS variants. Of all colonies grown at 30 °C from transformants containing Tyr211 variants, 75% showed a white color indicating they would no longer aminoacylate $tRNA^{Tyr}_{CUA}$. These colonies were restreaked at 30 and 42 °C, and only those which maintained growth at 42 °C were subjected to sequence analysis. Four mutants were identified in this way, Y211L, Y211F, Y211G, and Y211S. In addition, a number of red colonies were scanned for the introduction of a silent mutation which removed a *Bst*XI restriction site during PCR mutagenesis. Of these 86 red colonies, only 10 contained the silent mutation site. Sequence analysis showed that the Tyr211 codon had been changed to a stop codon (1 example, UAG; charging of $tRNA^{Tyr}_{CUA}$ with its cognate amino acid tyrosine would result in the translation of this codon as Tyr, which could explain the observed phenotype), a different Tyr codon (3) and in 6 cases still contained the wild-type Tyr codon. A similar scanning procedure was performed on transformants from mutagenesis of Phe233. In 13 DNA sequences of plasmids isolated from white colonies, only the two GlnRS mutants F233Y and F233L were found. From five red colonies which contained plasmids with the silent mutation, all the mutated *glnS* genes still encoded wild-type protein. In addition, 10 pink colonies were found. Sequence analysis of plasmids isolated from these colonies showed them to contain a mixture of wild-type and mutant codons at residue 233, giving rise to an intermediate phenotype. These mixed plasmids were then retransformed into BT3213, and a white colony subsequently isolated. Sequence analysis showed that the plasmid-encoded GlnRS contained the replacement F233D. The colonies from transformants containing Asp66 variants showed a completely white color, except in some cases where a central red color was surrounded by white. In the DNA sequences of plasmids from 11 complete white colonies and 12 central red ones, nine GlnRS mutants were found. They were GlnRS containing the replacements D66P, D66R, D66L, D66G, D66V, D66E, D66S, D66F, and D66H.

When the same sets of mutants were tested for their recognition and charging of cognate $tRNA^{Gln}$, they showed varied growth rates at 42 °C when expressed in strain UT172 (Table 1). Y211G and Y211S grew very poorly compared with Y211L and Y211F, which had the same growth rate as that of wild-type GlnRS. Similar results were found for changes in positions Asp66 and Phe233. D66E, D66S, D66F, F233L, and F233Y grew at the same rate as wild-type, while other mutants of Asp66 and F233D grew very poorly at 42 °C.

tRNA Substrate Specificity of GlnRS Mutants. The GlnRS mutants isolated above were tested for their ability to recognize a broad spectrum of noncognate suppressor tRNAs in vivo. Quantification of the in vivo recognition of $tRNA^{Tyr}_{CUA}$ by GlnRS, assayed by suppression of the $lacZ_{1000}$ allele, indicated that in all cases mischarging of this tRNA was reduced by 7−17-fold for the Tyr211 and Phe233 variants and 4−10-fold for the Asp66 variants compared to wild-type (Table 1). These values correlate with the white color observed when transformants producing these mutant proteins were plated on MacConkey indicator plates. These

Table 1: Characterization of GlnRS Mutants

gln*S* isolate	*glnS*^{ts} complementation[a]	β-galactosidase activity (R)[b]	amber suppressor[c] $tRNA^{Tyr}_{CUA}$	amber suppressor[c] $tRNA^{Ser1}_{CUA}$	amber suppressor[c] $tRNA^{Gln}_{UCA}$ A73	opal suppressors[c] $tRNA^{Gln}_{CUA}$ A3U70	opal suppressors[c] $tRNA^{Glu}_{UCA}$ U70
WT	+++	1	+				
Y211G	+	0.06					
Y211L	+++	0.11					
Y211F	+++	0.06					
Y211S	++	0.12					
F233L	+++	0.10					
F233Y	+++	0.08					
F233D	++	0.14					
Y211F/F233Y	++	0.14	nd	nd	nd	nd	nd
D66P	++	0.23					
D66R	+	0.10					
D66L	++	0.19					
D66G	+	0.10					
D66V	++	0.11					
D66E	+++	0.26					
D66S	+++	0.24					
D66F	+++	0.27					
D66H	+	0.15	+				
D235A	+	8.9	+	+	+	+	+
D486R/L488Q	++	nd	nd	nd	nd	+	+

[a] Complementation of *glnS172* (*ts*) was tested after incubation on complete medium at 42 °C; +++, normal growth after 16 h; ++, weaker growth after 1 day; +, marginal growth after 2 days. nd, not determined. [b] Activity relative to BT3213 transformed with pB*R*322 containing the wild-type *glnS* gene (*8*). [c] Suppression was assayed by growth of transformants on minimal medium due to opal suppression of *trpA* (UGA15) or by the appearance of red colonies due to the amber suppression of *lacZ* (UAG_{1000}). As controls for the various in vivo activities, the GlnRS mutants D235A (*8*) and D486R/L488Q (*13*) were used.

Table 2: Aminoacylation Kinetics of GlnRS with Gln and ATP

GlnRS	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M ($min^{-1}\ \mu M^{-1}$)	$\Delta\Delta G$(kcal mol^{-1})[a]
Gln				
wild-type	114 ± 12	157 ± 7	1.38 ± 0.16	
Y211L	118 ± 11	24.1 ± 1.0	0.205 ± 0.021	
Y211F	7050 ± 910	88.7 ± 6.9	0.0126 ± 0.0019	−2.87 ± 0.12
Y211S	6050 ± 600	4.92 ± 0.25	0.000 812 ± 0.000 090	
F233Y	525 ± 81	181 ± 16	0.345 ± 0.062	−0.85 ± 0.13
F233L	2210 ± 160	97.2 ± 2.5	0.0439 ± 0.0034	
F233D	7760 ± 850	2.87 ± 0.18	0.000 370 ± 0.000 0 47	
D66E	2020 ± 180	42 ± 2.4	0.0209 ± 0.0022	
D66F	758 ± 74	0.846 ± 0.031	0.001 15 ± 0.000 12	
Y211F/F233Y	584 ± 55	33.1 ± 1.4	0.0589 ± 0.0061	−1.93 ± 0.15
ATP				
wild-type[b]	110	168	1.53	
Y211L	315 ± 17	19.1 ± 0.4	0.0606 ± 0.0035	
Y211F	740 ± 44	90.6 ± 3.1	0.122 ± 0.008	−1.54
Y211S	735 ± 78	8.16 ± 0.41	0.0111 ± 0.0013	
F233Y	420 ± 42	148 ± 6	0.352 ± 0.038	−0.90
F233L	745 ± 49	165 ± 5	0.221 ± 0.016	
F233D	538 ± 61	2.54 ± 0.14	0.004 72 ± 0.000 59	
D66E	134 ± 8	37.6 ± 1.2	0.280 ± 0.019	
D66F	593 ± 94	0.953 ± 0.091	0.001 61 ± 0.000 30	
Y211F/F233Y	246 ± 8	27.8 ± 0.4	0.113 ± 0.004	−1.59

[a] Apparent difference between mutant and wild-type in the Gibbs' free energy for Gln or ATP binding, $\Delta\Delta G = RT \ln[(k_{cat}/K_M)_{mut}/(k_{cat}/K_M)_{wild}]$. [b] Values taken from ref *8*.

data also show that perturbation of the interaction between the 3′-terminus of $tRNA^{Tyr}_{CUA}$ and GlnRS results in a higher substrate specificity with respect to discrimination of noncognate tRNA. This was supported by the observation that, in contrast to all but one previously isolated GlnRS acceptor-stem recognition mutant, none of these mutants recognize either the amber suppressors $tRNA^{Ser1}_{CUA}$ and $tRNA^{Glu}_{CUA}$ or the opal suppressors $tRNA^{Gln}_{UCA}$ A3U70 and $tRNA^{Gln}_{UCA}$ U70 (Table 1).

In Vitro Characterization of GlnRS Variants. The observation that all of the isolated *glnS* mutants complemented the *glnS* temperature-sensitive strain UT172 indicated that the resulting GlnRS variant proteins retained the ability to aminoacylate $tRNA^{Gln}$ with glutamine. The most stable of these mutants, as assessed by in vivo complementation, were then selected for further characterization in vitro. The impact of mutating residues Asp66, Tyr211, and Phe233 on the steady-state kinetic parameters for the aminoacylation reaction was examined in vitro (Table 2). The effects of mutating Tyr211 were strictly dependent on the nature of the substitution. Y211L showed no change in its K_M for glutamine and a 6-fold decrease in k_{cat} compared to wild-type, in contrast

Table 3: Equilibrium Constants and Gibbs' Free Energies for Binding of $tRNA^{Gln}$ to Wild-Type and Mutant GlnRS[a]

GlnRS	k_{on} (s^{-1} M^{-1})	k_{off} (s^{-1})	K_{tRNA} (μM)	$G_{E\cdot tRNA}$ (kcal/mol)	ΔG (kcal/mol)
WT[b]	21.2 ± 0.9	7.1 ± 1.5	0.33	−8.76	
Y211F	48.0 ± 2.2	6.5 ± 2.1	0.135	−9.28	−0.52
Y211S	58.3 ± 2.3	8.0 ± 2.5	0.137	−9.27	−0.52
Y211L	56.3 ± 3	5.8 ± 3.1	0.103	−9.44	−0.68
F233L	54.2 ± 1.4	5.4 ± 1.2	0.099	−9.46	−0.70
F233D	55.0±3.4	7.1±2	0.129	−9.31	−0.55
F233Y	52.7±2.8	4.5±1.6	0.086	−9.55	−0.79
Y211F/F233Y	50.7±1.4	7.6±2.1	0.150	−9.30	−0.54

[a] Parameters were derived as previously described (*8*). [b] Values for wild-type GlnRS were previously determined (*8*).

to Y211F, which was predominantly effected in its apparent affinity for glutamine, which fell 60-fold. Y211S showed both a significant increase in the K_M for glutamine and decrease in k_{cat}. In all cases, comparable but significantly less pronounced effects were seen for the ATP kinetics. With the variants of Phe233, both F233Y and F233L showed increases in K_M for glutamine but little change in k_{cat}. In contrast, F233D showed dramatic changes in both K_M and k_{cat} resulting in a 3700-fold decrease in catalytic efficiency (k_{cat}/K_M) compared to wild-type, the largest change among the mutants described in this study. As with replacements of Tyr211, substitution of Phe233 showed comparable but much smaller effects on the kinetics for ATP than observed for glutamine. The mutants of Asp66 displayed a different pattern. D66E showed a large decrease in K_M for glutamine, but not ATP, resulting in a 66-fold decrease in catalytic efficiency for glutamine. D66F led to the biggest fall in k_{cat} among all the tested mutant GlnRSs, which subsequently resulted in its very low catalytic activity. D66S was thermally unstable in the assay and lost its activity rapidly during aminoacylation, preventing its kinetic characterization.

The affinity for cognate tRNA ($tRNA_2^{Gln}$) of the various mutants involved in stacking interactions with A76 was determined by stopped-flow fluorimetry. All mutations resulted in small decreases in the dissociation constant K_{tRNA}, indicating little or no change in affinity for $tRNA_2^{Gln}$ compared to wild-type (Table 3).

Double Mutant Cycle Analysis of Tyr211 and Phe233. The double mutant Y211F/F233Y was constructed to investigate the role of the hydroxyl group of Tyr211 and the contribution of the two aromatic side chains to glutamine binding. Y211F/F233Y GlnRS showed a 5-fold increase in K_M for glutamine and 5-fold decrease in k_{cat} compared to wild-type. The double mutant also showed less dramatic changes in the kinetic parameters for ATP. The double mutant displayed lower catalytic efficiency compared to F233Y but improvement compared to Y211F. The 23-fold decreased catalytic efficiency compared with that of wild-type suggested that the correct positioning of the hydroxyl group was critical for glutamine binding. The larger difference in coupling energy for glutamine than for ATP (1.79 and 0.85 kcal/mol, respectively) derived from double mutant cycle analysis (Figure 2) indicated that the two residues, Tyr211 and Phe233, predominantly interact to optimize glutamine binding. The two residues also interact to a lesser extent to enhance tRNA binding, as evidenced by a $\Delta\Delta G$ of 0.77 kcal/mol (Table 3).

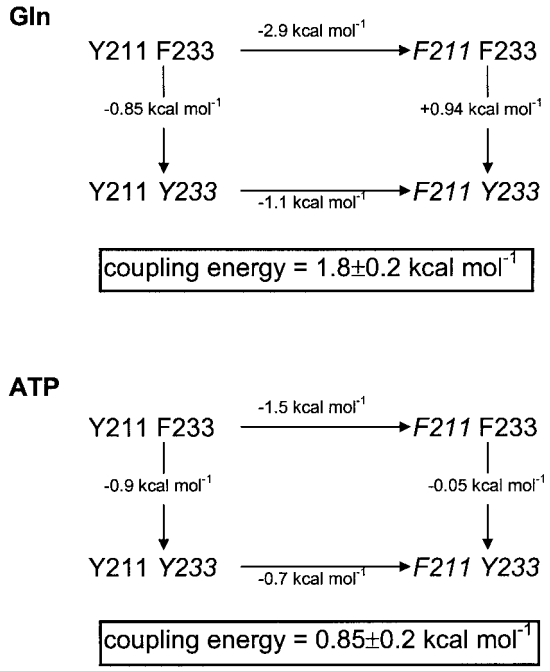

FIGURE 2: Double mutant cycle analysis (*36*) for Gln and ATP during aminoacylation by GlnRS-Y211F/F233Y.

DISCUSSION

Mischarging of Suppressor tRNAs by GlnRS Mutants. The ability of GlnRS mutants to recognize a broad range of suppressor tRNAs has been widely exploited during investigations of tRNA recognition and substrate specificity (reviewed in refs *25* and *26*). While numerous mutations of the acceptor stem-binding domain of GlnRS result in a relaxation in tRNA substrate specificity, as indicated by the ability to recognize a broadened range of suppressor tRNAs compared to wild-type (e.g., refs *12*, *24*, *27*), mutations of the residues which interact with A76 of tRNA result in an increase in tRNA specificity. Thus, GlnRS variants mutated at Asp66, Tyr211, and Phe233 and then selected for their inability to recognize $tRNA_{CUA}^{Tyr}$ are also unable to recognize other noncognate tRNAs. A comparable spectrum of suppression was previously obtained for the GlnRS mutant L136A (*14*); Leu136 stacks between A72 and G2 and stabilizes the disruption of the weak first (U1−A72) base pair of $tRNA^{Gln}$ and is thus important in determining the exact orientation of the 3′-end of $tRNA^{Gln}$. This is in contrast to mutation of Asp235, which is involved in recognition of the 2−71 and 3−70 base pairs, where similarly selected mutants displayed a reduced ability to discriminate opal suppressors derived from $tRNA^{Gln}$ (*8*). These differences suggest that the selected variants of Asp66, Tyr211, and Phe233 do not disrupt interactions distal to the active site in the GlnRS:$tRNA^{Gln}$ complex. This is consistent with the suggestion that the 3′-end of the tRNA molecule does not play a role in tRNA discrimination by an aaRS since it is common to all tRNAs, but may instead be involved in determining the rate of catalysis of aminoacylation (*28*).

Protein−RNA Interactions Optimize Glutamine Binding. Mutation of any of the three amino acid residues in direct contact with A76 of $tRNA^{Gln}$ led to significant reductions in the apparent ability of GlnRS to bind glutamine, the most pronounced changes being observed at Tyr211 and Phe233.

Mutation of Tyr211 to Phe or Ser resulted in a 60−70-fold increase in the K_M for glutamine, suggesting the importance of both the hydroxyl and aromatic moieties of Tyr211 in optimizing amino acid binding. The magnitude of $\Delta\Delta G$ for glutamine binding of the Y211F mutant compared to wild-type (−2.87 kcal mol^{-1}) suggests that the hydroxyl group may contribute to substrate binding either by direct or water-mediated hydrogen bonding interactions with glutamine. (*29*). The substitution of Tyr211 with Leu did not effect the K_M for glutamine, suggesting that in this mutant, neighboring amino acids might provide the necessary functionalities for glutamine binding. Mutation of Phe233 demonstrated the requirement for an uncharged aromatic moiety at this position; introduction of an additional hydroxyl group (F233Y) led to a 5-fold increase in K_M for glutamine, removal of the aromatic moiety (F233L), a 20-fold increase, and replacement with an acidic moiety a 75-fold increase. Taken together, the in vivo and in vitro results for the individual mutation of Tyr211 and Phe233 support the proposed role of these two aromatic residues in optimizing glutamine binding through their stacking interactions with A76 of cognate tRNAs. This was corroborated by double-mutant cycle analysis of Y211F/F233Y GlnRS (Figure 2), which showed there to be significant cooperativity between the two residues with respect to glutamine binding. This indicates that the minimum functional requirements at these positions are for two aromatic residues, presumably to allow stacking with A76 of tRNA, and a single hydroxyl group to participate in glutamine binding. This is further supported by the observation that, in the crystal structure of the complex between GlnRS:$tRNA^{Gln}$ and a glutaminyl-adenylate analogue, the hydroxyl group of Tyr211 is an obligate hydrogen bond acceptor in a network of water-mediated interactions with the amide side chain of glutamine (*30*).

tRNA-Mediated Substrate Binding in Aspartyl-tRNA Synthetase. The crystal structures of the various complexes formed by the class II aspartyl-tRNA synthetase (AspRS) and its substrates (*31*) identified a number of residues within the active site which directly contact A76 of $tRNA^{Asp}$. Mutation at three of these positions (Gln300, Ser301, and Phe304) all led to changes both in the rate of aminoacylation and the K_M for aspartic acid, although the magnitude of these changes was generally less than for the GlnRS mutants described here. Interestingly, stacking interactions between A76 and aromatic residues of AspRS contributed little to the efficiency of catalysis or substrate binding, in contrast to the dramatic effects reported here for GlnRS. This may be due to the observation that AspRS does not require the presence of tRNA for aspartyl-adenylate formation.

Stacking Interactions of RNA Bases with Aromatic Side Chains of Proteins. The data presented here clearly show the critical role of interactions between two aromatic residues, Tyr211 and Phe233, and the 3′-terminus of tRNA in optimizing the active-site conformation of GlnRS with respect to glutamine binding. The two residues perform this function by using their side chains to provide stacking interactions with the adenine ring of A76 of $tRNA^{Gln}$. Previous studies have revealed the existence of a number of other strong interactions between RNA base-amino acid pairs including aspartate-cytosine and tyrosine-cytosine (summarized in ref *32*). For example, the study of zinc-finger−RNA interactions showed that such interactions may enhance binding through increased interaction with phosphates or 2′ hydroxyls (*33*). The wide distribution of such interactions is also well illustrated by the extensive use of Phe/Tyr base stacking in the conserved RNA-binding motif of the spliceosomal protein U1A complexed with an RNA hairpin (*34*). Thus, through the use of well-established protein-RNA stacking interactions, contacts between Tyr211 and Phe233 and the invariant base A76 provide the molecular basis for tRNA-dependent amino acid recognition by GlnRS.

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REFERENCES

1. Eriani, G., Delarue, M., Poch, O., Gangloff, J., and Moras, D. (1990) *Nature 347*, 203−206.
2. Cusack, S., Berthet-Colominas, C., Härtlein, M., Nassar, N., and Leberman, R. (1990) *Nature 347*, 249−255.
3. Rossmann, M. G., Moras, D., and Olsen, K. W. (1974) *Nature 250*, 194−199.
4. Ibba, M., Curnow, A. W., and Söll, D. (1997) *Trends Biochem. Sci. 22*, 39−42.
5. Jakubowski, H., and Fersht, A. R. (1981) *Nucleic Acids Res. 9*, 3105−3117.
6. Baldwin, A. N., and Berg, P. (1966) *J. Biol. Chem. 241*, 839−845.
7. Hale, S. P., Auld, D. S., Schimdt, E., and Schimmel, P. (1997) *Science 276*, 1250−1252.
8. Hong, K.-W., Ibba, M., Weygand-Durasevic, I., Rogers, M. J., Thomann, H.-U., and Söll, D. (1996) *EMBO J. 15*, 1983−1991.
9. Ibba, M., Hong, K.-W., Sherman, J. M., Sever, S., and Söll, D. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 6953−6958.
10. Schimmel, P. R., and Söll, D. (1979) *Annu. Rev. Biochem. 48*, 601−648.
11. Rould, M. A., Perona, J. J., Söll, D., and Steitz, T. A. (1989) *Science 246*, 1135−1142.
12. Weygand-Durasevic, I., Schwob, E., and Söll, D. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 2010−2014.
13. Weygand-Durasevic, I., Rogers, M. J., and Söll, D. (1994) *J. Mol. Biol. 240*, 111−118.
14. Sherman, J. M., and Söll, D. (1996) *Biochemistry 35*, 601−607.
15. Sherman, J. M. Thomann, H.-U., and Söll, D. (1996) *J. Mol. Biol. 256*, 818−828.
16. Perona, J. J., Rould, M. A., and Steitz, T. A. (1993) *Biochemistry 32*, 8758−8771.
17. Kern, D., and Lapointe, J. (1979) *Biochemistry 18*, 5809−5818.
18. Gagnon, Y., Lacoste, L., Champagne, N., and Lapointe, J. (1996) *J. Biol. Chem. 271*, 14856−14863.
19. Schwob, E., and Söll, D. (1993) *EMBO J. 12*, 5201−5208.
20. Miller, J. H. (1992) in *A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for Escherichia and Related Bacteria*, pp 72−74, Cold Spring Harbor Laboratory Press, Plainview, NY.
21. Inokuchi, H., Hoben, P., Yamao, F., Ozeki, H., and Söll, D. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5076−5080.
22. Englisch-Peters, S., Conley, J., Plumbridge, J., Leptak, C., Söll, D., and Rogers, M. J. (1991) *Biochimie 73*, 1501−1508.
23. Thomann, H.-U., Ibba, M., Hong, K.-W., and Söll, D. (1996) *Bio/Technology 14*, 50−55.
24. Swanson, R., Hoben, P., Sumner-Smith, M., Uemura, H., Watson, L., and Söll, D. (1988) *Science. 242*, 1548−1551.
25. Ibba, M., Hong, K.-W., and Söll, D. (1996) *Genes Cells 1*, 421−427.
26. Freist, W., Gauss, D. H., Ibba, M., and Söll, D. (1997) *Biol. Chem. 378*, 1103−1117.
27. Rogers, M. J., Adachi, T., Inokuchi, H., and Söll, D. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 291−295.

28. Arnez, J. G., and Steitz, T. A. (1996) *Biochemistry 35*, 14725−14733.
29. Fersht, A. R., Shi, J.-P., Knill-Jones, J., Lowe, D. M., Wilkinson, A. J., Blow, D. M., Brick, P., Carter, P., Waye, M. M., and Winter, G. (1985) *Nature 314*, 235−238.
30. Rath, V. L., Silvian, L. F., Beijer, B., Sproat, B. S., and Steitz, T. A. (1998) *Structure 6*, 439−449.
31. Cavarelli, J., Eriani, G., Rees, B., Ruff, M., Boeglin, M., Mitschler, A., Martin, F., Gangloff, J., Thierry, J.-C., and Moras., D. (1994) *EMBO J. 13*, 327−337.
32. Lustig, B., Arora, S., and Jernigan, R. L. (1997) *Nucleic Acids Res. 25*, 2562−2565.
33. Shi, Y., and Berg, J. M. (1995) *Science 268*, 282−284.
34. Oubridge, C., Ito, N., Evans, P. R., Teo, C.-H., and Nagai, K. (1994) *Nature 372*, 432−438.
35. Carson, M. (1991) *J. Appl. Crystallogr. 24*, 958−961.
36. Carter, P. J., Winter, G., Wilkinson, A. J., and Fersht, A. R. (1984) *Cell 38*, 835−840.

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