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Functional Connectivity Between tRNA Binding Domains in Glutaminyl-tRNA Synthetase

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The structure of *Escherichia coli* glutaminyl-tRNA synthetase (GlnRS) in complex with $tRNA^{Gln}$ and ATP has identified a number a sequence–specific protein–tRNA interactions. The contribution to glutamine identity has previously been determined for the nucleotides in $tRNA^{Gln}$. Here, we report the mutational analysis of residues in all three tRNA recognition domains of GlnRS, thus completing a survey of the major sequence–specific contacts between GlnRS and $tRNA^{Gln}$. Specifically, we analyzed the GlnRS determinants involved in recognition of the anticodon which is essential for glutamine identity and in the communication of anticodon recognition to the acceptor binding domain in GlnRS. A combined *in vivo* and *in vitro* approach has demonstrated that Arg341, which makes a single sequence-specific hydrogen bond with U35 in the anticodon of $tRNA^{Gln}$, is involved in initial RNA recognition and is an important positive determinant for this base in both cognate and non-cognate tRNA contexts. However, Arg341, as well as Arg402, which interacts with G36 in the anticodon, are negative determinants for non-cognate nucleotides at their respective positions. Analysis of acceptor-anticodon binding double mutants and of a mutation of Glu323 in the loop-strand-helix connectivity subdomain in GlnRS has further implicated this domain in the functional communication of anticodon recognition. The better than expected activity (anticooperativity) of these double mutants has led us to propose an ''anticodon-independent'' mechanism, in which the removal of certain synthetase interactions with the anticodon eliminates structural constraints, thus allowing the relaxed specificity mutants in the acceptor binding domain to make more productive interactions.

Keywords: aminoacyl-tRNA synthetase; tRNA specificity; mutation; anticodon

Introduction

Transfer tRNAs have well-conserved secondary and tertiary structures. How an aminoacyl-tRNA synthetase (AARS) specifically recognizes its cognate set of tRNA species and discriminates against non-cognate tRNAs is critical for the accuracy of aminoacylation and thus of protein biosynthesis. As predicted by early experiments, the anticodon is an important tRNA recognition determinant for almost all *Escherichia coli* AARSs (Schulman, 1991). However, tRNA identity elements are also found in the acceptor stem, which is more than 60 Å away from the anticodon in uncomplexed tRNA (Giegé *et al*., 1993) and more than 35 Å away in the GlnRS:$tRNA^{Gln}$ complex (Rould & Steitz, 1992). While tRNA identity elements have been well studied, it is only with the solution of a number of AARS crystal structures and the discovery of an AARS classification system that similarly detailed experiments have been undertaken for synthetases (Carter, 1993).

The anticodon is an essential determinant of glutamine identity for the small monomeric, class I glutaminyl-tRNA synthetase from *E. coli* (Rogers *et al*., 1992; Rould & Steitz, 1992; Sherman *et al*., 1995). However, glutaminyl-tRNA synthetase (GlnRS) also specifically recognizes the acceptor stem / discriminator base domain of the tRNA (Rogers & Söll, 1988; Sherman *et al*., 1992; Weygand-Durasevic *et al*., 1993). This enzyme requires tRNA to catalyze aminoacyl-adenylate formation, the first step in the aminoacylation reaction (Kern *et al*.,

Abbreviations used: AARS, aminoacyl-tRNA synthetase; GlnRS, glutaminyl-tRNA synthetase.

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1980). Thus, in the glutamine system, anticodon recognition is closely coupled to acceptor stem specificity and to catalysis. We wanted to study the determinants in GlnRS which are responsible for anticodon recognition and for communicating anticodon recognition to the acceptor binding domain and active site.

The crystal structure of the GlnRS:tRNA^{Gln} complex has identified three regions of the protein involved in tRNA binding: the acceptor and anticodon binding domains and a connectivity subdomain. These regions interact with the acceptor stem / discriminator base, the anticodon and the D-stem / loop of the tRNA, respectively (Rould *et al*., 1989, 1991). The loop-strand-helix connectivity subdomain also forms a bridge between the anticodon and acceptor binding domains (Perona *et al*., 1993) and in genetic studies has been demonstrated to transmit the anticodon recognition signal to the acceptor binding domain and thus to the active site (Rogers *et al*., 1994). This is one of two routes by which this signal is transmitted, the second being the ''broccoli'' loop in the proximal β-barrel (Perona, 1990; Weygand-Durasevic *et al*., 1994).

In the course of this study, mutations in all three tRNA recognition domains have been characterized, thus completing a survey of the major sequence-specific contacts between GlnRS and tRNA^{Gln} (Rould & Steitz, 1992; Sherman *et al*., 1995; Sherman & Söll, 1996). In the anticodon binding domain, both Arg341 and Arg402 have been mutated (Figure 1). In the crystal structure, Arg341 makes a sequence-specific contact *via* its guanidinium group with the oxygen at position 4 of U35, which has been shown to be the single most important glutamine identity element (Jahn *et al*., 1991; Rould *et al*., 1991; Rogers *et al*., 1992; Sherman *et al*., 1995). On the other hand, G36 which, with Arg402, makes two sequence-specific hydrogen bonds, has been demonstrated to be important for tRNA^{Gln} recognition *in vitro*, but not for glutamine identity *in vivo* (Jahn *et al*., 1991; Rould *et al*., 1991; Sherman *et al*., 1995). In the acceptor binding domain (Figure 1), substitutions for leucine at position 136 and for aspartate at position 235 have been analyzed (Inokuchi *et al*., 1984; Swanson *et al*., 1988; Perona *et al*., 1989; Schwob & Söll, 1993; Sherman & Söll, 1996). Leu136 stabilizes the disruption of the weak first base-pair of tRNA^{Gln} and supports the peculiar acceptor end conformation which is important for glutamine identity (Rould *et al*., 1989; Weygand-Durasevic *et al*., 1993; Sherman *et al*., 1995). Asp235 makes three sequence-specific hydrogen bonds with three of the four nucleotides in the G2·C71 and G3·C70 base-pairs, which are critical identity elements in the acceptor stem (Perona *et al*., 1989; Jahn *et al*., 1991). Finally, in the connectivity subdomain (Figure 1), Glu323 makes a direct hydrogen bond with the nitrogen at position 2 of the well-conserved G10 in tRNA^{Gln} and a water-mediated hydrogen bond with its base-pairing partner C25. Mutations in G10·C25 impair GlnRS recognition *in vitro* (Rould *et al*., 1991; Hayase *et al*., 1992). Here, we describe the characterization of Asp235, Leu136, Arg341, Arg402 and Glu323 mutants of GlnRS alone and combined to form double mutants; these mutants have been analyzed both *in vitro* for their effects on cognate tRNA and amino acid kinetics and *in vivo* for their impact on the ability of GlnRS to discriminate against non-cognate and mutant cognate tRNAs.

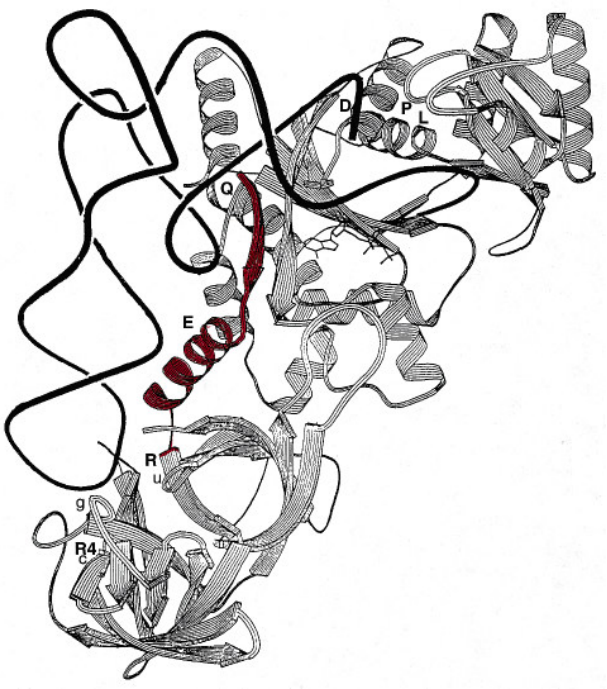

Figure 1. Anticodon recognition and functional and structural connectivity in *E. coli* GlnRS. The connectivity subdomain (colored red on this ribbon diagram of the GlnRS:tRNA^{Gln}:ATP complex) is proposed to connect the acceptor and anticodon binding domains of GlnRS and to transmit anticodon recognition to the active site (Rould & Steitz, 1992; Rogers *et al*., 1994). Also marked are the approximate positions of residues implicated in anticodon recognition, acceptor stem recognition and / or functional communication: Arg342 (R), Arg402 (R4), Glu323 (E), Gln318 (Q), Asp235 (D), Pro181 (P) and Leu136 (L) (Rould *et al*., 1989, 1991; Sherman *et al*., 1995). Finally, the approximate positions of the anticodon bases of tRNA^{Gln} (C34, U35, G36) are indicated with lower case letters (c, u, g).

Results

Site-directed mutagenesis and construction of the double mutants

The contribution of Arg341 and Arg402 to anticodon recognition in GlnRS was determined by changing Arg341 to alanine (R341A) and proline (R341P), and Arg402 to glutamine (R402Q) and alanine (R402A). In order to study the functional communication of anticodon recognition to the acceptor binding domain and thus to the active site, Glu323 was replaced by glycine (E323G) in the

Table 1. *In vitro* kinetic parameters for $tRNA_2^{Gln}$ of GlnRS single and double mutants

	tRNA^{Gln2}			
GlnRS	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1} s^{-1}$)	Relative k_{cat}/K_m
GlnRS+	0.27	3.83	14.20	1.00
L136F	0.40	0.36	0.90	6.3×10^{-2}
E323G	1.17	0.17	0.15	1.1×10^{-2}
R341A	15.80	2.07	0.13	9.2×10^{-3}
R341P	11.50	5.05	0.44	3.1×10^{-2}
D235N	0.20	0.45	2.25	1.6×10^{-1}
L136F/R341A	5.41	5.52	1.02	7.2×10^{-2}
D235N/R341A	2.79	4.24	1.52	1.1×10^{-1}

connectivity subdomain of GlnRS (Rould & Steitz, 1992; Rogers *et al*., 1994) and mutations in the acceptor (L136F, D235N) and anticodon binding domains have been combined to create double mutants (L136F/R341A, D235N/R341A, L136F/R402A, L136F/R402Q). However, we were limited experimentally both in the substitutions we could make at positions 323 and 341 and the mutations we could combine, possibly because the resulting enzymes are toxic to the cell. Mutants of this essential enzyme have been known to give rise to toxic proteins due to the misincorporation of amino acids through misacylation of tRNA (Schwob & Söll, 1993). This toxicity may only be exacerbated when multiple specificity determinants are lost in the same region (e.g. in double mutants in the anticodon binding domain).

In vitro kinetic analysis

The steady-state aminoacylation kinetics of wild-type and mutant GlnRS were investigated (Table 1). The specificity constants ($k_s = k_{cat}/K_m$) for $tRNA_2^{Gln}$ of both GlnRS(R341A) and GlnRS(E323G) are decreased by two orders of magnitude. For GlnRS(E323G), this decrease is due mainly to an approximately 20-fold decrease in k_{cat}; however, for GlnRS(R341A), it is due almost completely to a near 60-fold increase in K_m. Similar elevations in K_m were observed for the R341P mutant (Table 1) as well as for the R402Q and R402A mutants (Thomann *et al*., 1996). Analogous to the E323G mutant, the kinetic parameter affected in both GlnRS(D235N) and GlnRS(L136F) is k_{cat}; however the overall decrease in k_s for these mutants is much less, due in part to an unchanged K_m (Table 1).

Likewise, neither of the double mutants, i.e. GlnRS(L136F/R341A) or GlnRS(D235N/R341A), had a k_s reduced as much as that of the single R341A mutant (Table 1). Instead, their specificity constants resemble those of their respective acceptor binding single mutants, L136F and D235N, suggesting that the mutations are not acting independently. The mutations in the anticodon and acceptor binding domains appear to act anticooperatively in both double mutants (Table 2); both of their differences in $\Delta\Delta G$ values (difference between the theoretical and experimental $\Delta\Delta G$) are negative values (between –2.5 and –3.0 kcal/mol). Although their $\Delta\Delta G$ values approximate those of their respective acceptor binding single mutants, both double mutants exhibit K_m increases of tenfold or more, which is similar to the impact of the R341A mutation, while the k_{cat} decreases observed for the acceptor binding single mutants, GlnRS(L136F) and GlnRS(D235N), have been eliminated.

The anticooperativity of these mutations was also confirmed for glutamine (Table 3). In fact, with glutamine as the variable substrate, GlnRS(L136F/R341A) and GlnRS(D235N/R341A) have $\Delta\Delta G$ values of less than or equal to zero, suggesting that these mutants are of approximately the same or greater efficiency than wild-type GlnRS. Also, as observed with tRNA, the differences in $\Delta\Delta G$ values for both of the double mutants are similar in magnitude, between 1.0 and 1.5 kcal/mol. The smaller differences in the $\Delta\Delta G$ values reflect the generally smaller differences in K_m and k_{cat} measured for glutamine (data not shown).

Together, these results show that mutations in the acceptor and anticodon binding domains, although

Table 2. Cooperativity analysis of $tRNA_2^{Gln}$ kinetics of acceptor and anticodon binding mutants of GlnRS

$\Delta\Delta G$(acc)[a] +	$\Delta\Delta G$(ac)[b] =	$\Delta\Delta G$(theoret.)[d]	$\Delta\Delta G$(experim.)[c]	D$\Delta\Delta G$
$\Delta\Delta G$(L136F)+	$\Delta\Delta G$(R341A) =	$\Delta\Delta G$(L136F + R341A)	$\Delta\Delta G$(L136F/R341A)	
1.70	2.89	4.59	1.62	–2.97
$\Delta\Delta G$(D235N)+	$\Delta\Delta G$(R341A) =	$\Delta\Delta G$(D235N + R341A)	$\Delta\Delta G$(D235N/R341A)	
1.13	2.89	4.02	1.37	–2.65

Data from Table 1. Calculations based on Carter *et al*., 1984:

$$\Delta\Delta G = -RT \ln \frac{k_{cat}/K_m(\text{mutant})}{k_{cat}/K_m(\text{wild-type})}$$

[a] $\Delta\Delta G$(acc) = $\Delta\Delta G$(acceptor binding domain)
[b] $\Delta\Delta G$(ac) = $\Delta\Delta G$(anticodon binding domain)
[c] $\Delta\Delta G$(experim.) = $\Delta\Delta G$(experimentally determined)
[d] $\Delta\Delta G$(theoret.) = $\Delta\Delta G$(theoretically determined)
$\Delta\Delta G$(acc + ac) = $\Delta\Delta G$(acc) + $\Delta\Delta G$(ac) = $\Delta\Delta G$(theoret.)
D$\Delta\Delta G$ = $\Delta\Delta G$(experim.) – $\Delta\Delta G$(theoret.)
Definitions from Putz *et al*., 1993:
If D$\Delta\Delta G$ = 0, then mutations are acting independently.
If D$\Delta\Delta G$ > 0, then mutations are acting cooperatively.
If D$\Delta\Delta G$ < 0, then mutations are acting anticooperatively.

Table 3. Cooperativity analysis of glutamine kinetics of acceptor and anticodon binding mutants of GlnRS

$\Delta\Delta G$(acc)	+	$\Delta\Delta G$(ac)	=	$\Delta\Delta G$(theoret.)	$\Delta\Delta G$(experim.)	D$\Delta\Delta G$
$\Delta\Delta G$(L136F)	+	$\Delta\Delta G$(R341A)	=	$\Delta\Delta G$(L136F + R341A)	$\Delta\Delta G$(L136F/R341A)	
0.71		0.82		1.53	+0.03	−1.50
$\Delta\Delta G$(D235N)	+	$\Delta\Delta G$(R341A)	=	$\Delta\Delta G$(D235N + R341A)	$\Delta\Delta G$(D235N/R341A)	
−0.25		0.82		0.57	−0.42	−0.99

Definitions as in Table 2.

more than 35 Å apart (Rould & Steitz, 1992) are not independent, but rather appear to act anticooperatively to affect the amino acid binding site as well as the extensive tRNA interface. These effects may be the direct result of altered substrate interactions or may be the indirect result of changes in solvation energy. Nevertheless, the double mutant enzymes make more productive interactions with both glutamine and tRNA substrates.

In vivo specificity and activity assays

Mutations of the arginines at positions 341 and 402, like those of Leu136 and Asp235, may be expected to have some effect on the ability of GlnRS to discriminate against non-cognate tRNAs *in vivo*. For the purpose of these experiments, the various GlnRS mutants are assumed to be expressed to the same level from pBR322 *in vivo*, as the yields of pure AARS have been similar for all of the purified mutants (data not shown).

Of the previously uncharacterized single mutants, only the Arg402 mutants mischarge any of the non-cognate amber suppressor tRNAs, with GlnRS(R402A) exhibiting the greatest reduction in specificity *in vivo* (Tables 4 and 5). Not only do these mutants mischarge *tyrT*(*UAG*) as does wild-type GlnRS (Swanson *et al*., 1988), but unlike wild-type GlnRS they all recognize *proH*(*UAG*) and $tRNA^{Phe}$(UAG).

In contrast, the R341A, R341P and E323G mutants no longer even mischarge *tyrT*(*UAG*) in quantities sufficient to confer a Lac^+ phenotype on strain RS109. These GlnRS mutants do not mischarge any of the other amber suppressors tested and thus are not likely to have a different, but equally relaxed tRNA specificity (Tables 4 and 5).

Of the double mutants, only L136F/R402Q and L136F/R402A mischarge any of the amber suppressors (Tables 4 to 6). Both of them mischarge *tyrT*(*UAG*), *proH*(*UAG*) and $tRNA^{Phe}$(UAG) more efficiently than the single mutants from which they are derived. In contrast, coupling of the R341A anticodon binding mutant with either L136F or D235N abolishes the mischarging of both *tyrT*(*UAG*) and *proH*(*UAG*) observed for acceptor binding single mutants.

The results obtained with the mutant opal suppressor derived from $tRNA^{Gln}$, GLNA3U70, differ markedly from those obtained with the non-cognate amber suppressors (Table 4). GlnRS(D235N) is the only single mutant which clearly aminoacylates this tRNA *in vivo* sufficiently to suppress the glutamine-specific *trpA*(*UGA15*) marker. While the R402A mutant confers a weak Trp^+ phenotype on KL2576/pACYCGLNA3U70, all of the other GlnRS single mutants exhibit at best a marginal phenotype. However, all of the double mutants recognize GLNA3U70, including the D235N/R341A and L136F/R341A mutants, which fail to recognize any of the non-cognate amber suppressors tested, and the L136F/R402Q and L136F/R402A mutants, which mischarge several of the non-cognate amber suppressors (Tables 4 and 5).

Table 4. *In vivo* specificity of single and double mutants of GlnRS for suppressor tRNAs

	tRNA(UAG) ($Lac^{+/-}$)[a]					tRNA(UGA) ($Trp^{+/-}$)[a]
GLNRs	*tyrT*	*gluA-A73*	*proH*	*phe*	*ser1*	GLNA3U70
GlnRS+	+	–	–	–	–	–
L136F	+	–	++	±	–	±
D235N	++	–	+ +	+	–	+++
E323G	–	–	–	–	–	–
R341A	–	–	–	–	–	±
R341P	–	–	–	–	–	–
R402Q	+	–	±	±	–	+
R402A	+++	–	+	++	–	+
L136F/R341A	–	–	–	–	–	++
D235N/R341A	–	–	–	–	–	+++
L136F/R402Q	+++	–	+++	+++	–	++
L136F/R402A	+++	–	+++	+++	–	++

[a] $Lac^{+/-}$ and $Trp^{+/-}$ indicate growth on appropriately supplemented lactose and glucose minimal plates respectively at 30°C relative to a given suppressor/strain.

Table 5. *In vivo* mischarging of *tyrT*(UAG) and *proH*(UAG) by GlnRS single and double mutants

GLNRS	β-Galactosidase activity (Miller units) *tyrT*	*proH*
GlnRS+	17	3
L136F	20	34
D235N	54	34
E323G	3	ND
R341A	2	< 1
R342P	3	ND
R402Q	13	5
R402A	84	14
L136F / R341A	2	< 1
D235N / R341A	3	< 1
L136F / R402Q	98	190
L136F / R402A	101	159

Cultures for the β-galactosidase assays were grown at 30°C and the assay mixes incubated for a maximum of 90 to 120 minutes at 28°C (Sherman & Söll, 1996). ND = not determined.

Discussion

The crystal structure of the GlnRS:tRNA^{Gln}:ATP complex has identified three tRNA recognition domains in GlnRS: the acceptor and anticodon binding domains and the loop-strand-helix subdomain which connects them (Rould & Steitz, 1992). We have carried out a mutational analysis of amino acid residues in GlnRS which interact sequence-specifically with tRNA^{Gln} and reside in all three domains.

Arginine 341

Arg341 is located in the anticodon binding domain and makes a sequence-specific hydrogen bond with the middle base of the anticodon, U35, which is critical for the glutamine identity of the RNA (Rould *et al*., 1991; Jahn *et al*., 1991; Rogers *et al*., 1992; Sherman *et al*., 1995). This amino acid is both a positive determinant for U35 and a negative determinant for C35 and presumably for the other non-cognate nucleotides at this position. Evidence for Arg341 specifying productive interactions with U35-containing tRNAs comes from both *in vitro* and *in vivo* experiments. *In vitro*, an alanine for arginine substitution at position 341 decreases the k_s of GlnRS for tRNA^{Gln} by two orders of magnitude; a proline substitution has a similar effect (Table 1). This decrease in k_s renders GlnRS(R341A) one of the most catalytically deficient AARS point mutants characterized to date (Schulman, 1991; Weygand-Durasevic *et al*., 1993, 1994; Rogers *et al*., 1994). Arg341 appears to be involved in initial tRNA recognition as both the alanine and proline substitutions increase the K_m for tRNA^{Gln} by more than a factor of 50. This is consistent with the observation that the tRNA^{Gln} anticodon stem-loop is a more effective competitive inhibitor of tRNA^{Gln} aminoacylation than the acceptor stem microhelix (Wright *et al*., 1993). In sharp contrast, the effect of U35 mutations in the tRNA affect mainly k_{cat} (Jahn *et al*., 1991).

In vivo experiments provide additional evidence that Arg341 is a positive determinant for the glutamine identity of U35-containing tRNAs. Mutants in this position, either alone or combined to form double mutants expressed on pBR322, do not mischarge any of the non-cognate amber suppressors tested, including *tyrT*(*UAG*). The latter is even recognized by similarly expressed wild-type GlnRS (Tables 4 and 5). While it is possible that the R341A and R341P single mutants are simply not active enough to display any mischarging *in vivo*, a decrease in aminoacylation efficiency *in vitro* (Table 1) does not necessarily correlate with a

Table 6. Cooperativity analysis of *in vivo* mischarging of acceptor and anticodon binding mutants of GlnRS

β-gal(acc)	+	β-gal(ac)	=	β-gal(theoretical)	β-gal(experimental)	More / less mischarging
tyrT(UAG) mischarging						
L136F	+	R341A	=	L136F + R341A	L136F / R341A	
20		2		22	2	Less
D235N	+	R341A	=	D235N + R341A	D235N / R341A	
54		2		56	2	Less
L136F	+	R402Q	=	L136F + R402Q	L136F / R402Q	
20		84		104	102	Equal
L136F	+	R402A	=	L136F + R402A	L136F / R402A	
20		13		33	98	More
proH(UAG) mischarging						
L136F	+	R341A	=	L136F + R341A	L136F / R341A	
34	<1			34–35	< 1	Less
D235N	+	R341A	=	D235N + R341A	D235N / R341A	
34		< 1		34–35	< 1	Less
L136F	+	R402Q	=	L136F + R402Q	L136F / R402Q	
34		14		48	159	More
L136F	+	R402A	=	L136F + R402A	L136F / R402A	
34		5		39	190	More

Data taken from Table 5.

decrease of specificity *in vivo* (Jahn *et al*., 1991; Meinnel *et al*., 1991; Despons *et al*., 1992; Pallanck *et al*., 1992; Komatsoulis & Abelson, 1993; Sherman & Söll, 1996). For example, the L136F/R341A and D235N/R341A double mutants both have higher specificity constants with respect to $tRNA_2^{Gln}$ than GlnRS(L136F) which mischarges both *tyrT*(*UAG*) and *proH*(UAG), yet they do not mischarge any of the amber suppressors tested (Tables 1, 4 and 5). These observations justify the conclusion that Arg341 is an important positive determinant for U35, a nucleotide critical for the mischarging of U35-containing tRNAs by GlnRS (Table 1; Sherman *et al*., 1995). Disruption of the Arg341:U35 interaction abolishes the recognition of non-cognate amber suppressor tRNAs.

Although the L136F/R341A and D235N/R341A double mutants no longer recognize the non-cognate U35-containing amber suppressor tRNAs, both recognize the mutant opal suppressor derived from $tRNA^{Gln}$, GLNA3U70 with its C35 (Table 4). As neither the L136F nor the R341A single mutants aminoacylate this tRNA *in vivo*, these results suggest that Arg341 may be a negative determinant for tRNAs containing a C35. Our failure to isolate mutants in the U35 binding pocket which allow GlnRS to recognize the UCA anticodon of GLNA3U70 is consistent with this conclusion (unpublished results). Thus, the L136F and/or D235N mutation(s) may be required, in the absence of the Arg341:U35 interaction, to make a more productive interaction with the acceptor end of the tRNA, one that is sufficient to overcome the low specificity constant of the R341A mutant and the high apparent threshold for recognition of the opal suppressor (Tables 1 and 2; Rogers *et al*., 1992, 1994).

The role of Arg341 and the kinetic properties of its mutants are analogous to those of Trp461, Lys732 and possibly Arg402 in *E. coli* MetRS, IleRS and GlnRS, respectively (Ghosh *et al*., 1990; Shepard *et al*., 1992). Trp461 in IleRS and Lys732 in MetRS are thought to make sequence-specific contacts with the anticodon, a critical identity element for their respective tRNAs. Mutations of these amino acids affect K_m to about the same extent as the R341A, R402Q and R402A mutations of GlnRS (Brunie *et al*., 1990; Ghosh *et al*., 1990; Shepard *et al*., 1992; Thomann *et al*., 1996). As already discussed above, Arg341 in GlnRS is both a positive and a negative determinant of glutamine identity (Table 3); a similar role can be attributed to Trp461 in MetRS (Ghosh *et al*., 1990). Finally, the involvement of all of these amino acids mainly in the binding step of aminoacylation lends support to the hypothesis derived from mini-/microhelix and AARS deletion experiments that regions of the AARS outside the acceptor binding domain and the active site contribute mostly binding energy and increased efficiency to aminoacylation (Francklyn *et al*., 1992; Martinis & Schimmel, 1993; Buechter & Schimmel, 1993; Schmidt & Schimmel, 1993; Schwob & Söll, 1993).

Arginine 402

The third base of the anticodon in $tRNA^{Gln}$, G36, is unusual, as it seems to be important for recognition by GlnRS *in vitro*, but not for glutamine identity *in vivo* (Jahn *et al*., 1991; Sherman *et al*., 1995). In contrast, Arg402, which makes two sequence-specific hydrogen bonds with G36, is important for both cognate tRNA recognition *in vitro* (Thomann *et al*., 1996) and for discrimination against non-cognate tRNAs *in vivo* (Tables 4 and 5). Substitution of Gln and Ala for Arg402 makes GlnRS less specific. GlnRS(R402Q) and GlnRS(R402A) mischarge *tyrT*(*UAG*), *proH*(*UAG*) and $tRNA^{Phe}$(UAG) with glutamine. However, neither recognizes *gluA-A73*(*UAG*) and *serU*(*UAG*), which are recognized by many of the more mischarging mutants of GlnRS (Weygand-Durasevic *et al*., 1993). The fact that GlnRS(R402A) recognizes GLNA3U70 *in vivo* renders it the first GlnRS mutant in a position which interacts directly with the anticodon to recognize its cognate mutant opal suppressor tRNA (Rould *et al*., 1991; Rogers *et al*., 1994; Weygand-Durasevic *et al*., 1994). It is clear that GlnRS(R402A) and GlnRS(R402Q) recognize the opal anticodon (UCA) and the amber anticodon (CUA), both containing an A36, because the mutations of Arg402 to Ala402 and Gln402 results in the removal of an important A36 antideterminant.

The mischarging profiles of the double mutants L136F/R402Q and L136F/R402A were the same as those of the Arg402 single mutants for both the amber and opal suppressors. However, recognition of *tyrT*(*UAG*), $tRNA^{Phe}$(UAG), *proH*(*UAG*) and GLNA3U70 was enhanced in strains containing these double mutants (Table 6). This suggests that the mutations may be acting anticooperatively with respect to mischarging activity, analogous to the anticooperativity documented for the L136F/R341A and D235N/R341A mutants *in vitro* (Tables 2 and 3). This is similar to the observation that *E. coli* MetRS mutants with an increasing number of mutations display an enhanced recognition efficiency of $tRNA^{fMet}$(UAG) (Meinnel *et al*., 1991).

Specificity has been proposed to be derived from closely coupling acceptor binding and active site domains (Francklyn *et al*., 1992). However, since Arg402 is a negative determinant for A36 (Tables 4 and 5) and presumably of a C and U in this position as well and is involved in initial tRNA recognition (Sherman & Söll, 1996), it is clear that the binding of the anticodon is also likely to be important for tRNA specificity (Meinnel *et al*., 1993; Schwob & Söll, 1993; Wright *et al*., 1993).

Connectivity between the acceptor and anticodon binding domains

The tRNA specificity of the double mutants depends on the nature of both the anticodon and acceptor binding domain substitutions (Tables 4

and 5) and suggests that these two domains share some functional connectivity.

Glutamate 323

This residue is located in the middle of the connectivity subdomain (Figure 1) and is not likely to be involved in initial tRNA recognition. The main kinetic defect of both GlnRS(E323G) and the analogous tRNA mutations in G10:C25 are in k_{cat} (Table 1; Hayase *et al*., 1992). These observations suggest that the hydrogen bonds formed between Glu323 and G10:C25 are involved in stabilizing an alternative conformation of the AARS and/or the tRNA. This ''new'' conformation may be critical for the communication of anticodon recognition to the acceptor binding domain and active site of the enzyme. Investigations of genetically selected mutants have suggested a mechanism whereby recognition of the middle base of the anticodon by Arg341 at one end of the connectivity subdomain can be communicated through the helix-strand-loop (containing Glu323) via Gln318 to Asp235 in the acceptor binding domain and from there to the ATP binding site (Perona *et al*., 1993; Rogers *et al*., 1994). Consistent with this model is the k_{cat} defect of the D235N mutation with respect to $tRNA^{Gln}$ (Table 1).

Double mutants

Arg341 is ideally positioned to communicate U35 recognition to the acceptor binding domain. Thus, it was not unexpected when it was observed that mutations at Arg341 (R341A) and in the acceptor binding domain, L136F and D235N, were not independent. They act anticooperatively (Table 2; Carter *et al*., 1984), i.e. both GlnRS(L136F/R341A) and GlnRS(D235N/R341A) have a lower $\Delta\Delta G$ value than one would have predicted. Moreover, the differences of the theoretically and experimentally determined $\Delta\Delta G$ values for both are negative values (–2.5 and –3.0 kcal/mol, respectively). This is roughly equivalent to the energy of one hydrogen bond and is a very small amount of energy when compared to the GlnRS:$tRNA^{Gln}$ interface which buries more than 2700 $Å^2$ of GlnRS with a potential energy of 50 kcal/mol (Rould *et al*., 1991; Carter, 1993). However, these differences in $\Delta\Delta G$ become very significant when one considers that the difference in energy between interactions with cognate and non-cognate tRNAs is less than 10 kcal/mol (Ebel *et al*., 1973; Giegé *et al*., 1993). Ideally, the observed anticooperativity of the acceptor-anticodon binding double mutants would be compared to the results for double mutants in the same domain (e.g. Arg341 and Arg402 mutations in the anticodon binding domain) and to mutant combinations, which are functionally independent. However, our choice of substitutions and mutant combinations was limited experimentally by the apparent toxicity to *E. coli* of some of these mutant proteins. Nevertheless, this type of analysis has been used twice successfully with AARSs to identify independent, cooperative and anticooperative mutations (Carter *et al*., 1984; Sanni *et al*., 1991).

The observed anticooperativity is not likely to simply be a function of the large AARS-tRNA interface, as these acceptor-anticodon binding GlnRS mutants also exhibit anticooperativity in their kinetics with respect to glutamine with its much smaller binding site (Table 3). Interestingly, GlnRS(R341A) itself has an elevated K_m for glutamine (data not shown) which may be an indirect result of altered tRNA binding. Additional support for the hypothesis that the anticooperativity of the L136F/R341A and D235N/R341A mutations is not due to separation of the determinants comes from analyzing the effects of comparable tRNA mutations; acceptor stem-anticodon mutations in $tRNA^{Gln}$ are additive and thus independent (Jahn *et al*., 1991), while distant mutations in other tRNAs tend to act cooperatively (Schulman & Pelka, 1989; Sampson *et al*., 1992; Pütz *et al*., 1993).

The fact that comparable mutations in GlnRS and $tRNA^{Gln}$ do not behave similarly suggests that this reorientation may be AARS-mediated and may be due, in part, to the nature of the GlnRS mutations which have been coupled. The R341A mutation removes a critical positive interaction with the glutamine identity element U35. Removing this interaction may reduce the constraints imposed on the orientation of the tRNA acceptor stem in the acceptor binding domain freeing the two reduced specificity mutants in this domain, L136F and D235N, to take advantage of alternative, more productive interactions with the acceptor stem (as well as with other parts) of $tRNA^{Gln}$. Lending support to this hypothesis is the correction of both the K_m defect of the R341A mutant and the k_{cat} defects of the L136F and D235N mutants with respect to glutamine in the L136F/R341A and D235N/R341A double mutants. The specificity constants of these double mutants mimic that of wild-type GlnRS (Table 3 and data not shown). This, then, suggests that even GlnRS, for which anticodon recognition is critical, can utilize an ''anticodon-independent'' reaction mechanism.

This observation is consistent with those made for both tRNA and AARS mutants in this and other systems. Recognition of the altered 3:70 base-pair in a mutant glutamine opal suppressor GLNA3U70 by the relaxed specificity mutant GlnRS(D235N) is sufficient to overcome the loss of the single most important $tRNA^{Gln}$ identity element, U35 (Rogers *et al*., 1994). Also, although the anticodons are important identity elements in the glutamine, methionine and valine systems, the microhelices derived from the corresponding tRNAs can be aminoacylated rather specifically (Frugier *et al*., 1992; Martinis & Schimmel, 1993; Wright *et al*., 1993). However, as observed for the GlnRS double mutants, glutaminylation of the $tRNA^{Gln}$ microhelix is less discriminatory (Wright *et al*., 1993). Finally, although the anticodon is an important methionine identity, the anticodon binding domain of *E. coli*

MetRS can be deleted without a complete loss of function (Kim & Schimmel, 1992). This ability to apply alternative strategies to create a productive and efficient AARS:tRNA interaction has also been noted for mutants of yeast $tRNA^{Asp}$ and for viral tRNA-like molecules (Dreher *et al*., 1992; Pütz *et al*., 1993).

Implications for enzyme mechanism

In order to facilitate recognition of both the anticodon and acceptor end of the tRNA and for the acceptor and anticodon binding domains to act anticooperatively in GlnRS, conformational changes must occur in the AARS and/or tRNA upon complex formation. This is particularly important in the *E. coli* glutamine system which 1) requires tRNA for aminoacyl-adenylate formation and 2) depends strongly on specific recognition of the anticodon, located more than 35 Å away from the active site (Sherman *et al*., 1995). There is also evidence for both structural and functional connectivity and conformational changes in the *E. coli* alanine and methionine systems (Mellot *et al*., 1989; Ghosh *et al*., 1991; Despons *et al*., 1992; Hou & Schimmel, 1992; Miller & Schimmel, 1992). Interestingly, the decreases in the specificity constants for AARS mutants are generally smaller than for comparable tRNA mutants, with the k_{cat} of the tRNA mutants accounting for the discrepancy, an effect that is enhanced in the context of unmodified transcripts (reviewed in Giegé *et al*., 1993). Thus, it is tempting to speculate that the major conformational changes occur in the tRNA and that communication occurs *via* the tRNA. This appears to be the case in the GlnRS:$tRNA^{Gln}$ system, as relatively small conformational changes have been observed in the AARS and as the addition of the anticodon stem-loop cannot stimulate aminoacylation of the microhelix derived from $tRNA^{Gln}$ (Rould *et al*., 1989; Perona, 1990; Bhattacharyya *et al*., 1991; Rould *et al*., 1991; Wright *et al*., 1993).

Materials and Methods

General

Uniformly labeled L-[^{3}H]glutamine (specific activity: 45 Ci/mmole) was from Amersham International, Buckinghamshire, UK. Pure $tRNA_2^{Gln}$ (specific activity approx. 1500 pmol/A_{260}; Perona *et al*., 1988) and purified GlnRS(D235N) (Englisch-Peters *et al*., 1991) were generously provided by D. Jeruzalmi and S. Englisch-Peters, respectively. Media for bacterial growth and molecular biology protocols were standard unless otherwise noted (Maniatis *et al*., 1982). All protein purification steps were carried out at 4°C except where indicated.

Site-directed mutagenesis and constitutive expression of mutants

The single mutants L136F, E323G, and R341A were made by site-directed mutagenesis using M13mp19*glnS* (the *glnS* gene includes its promoter and terminator) prepared from *E. coli* CJ236 as the template. While the Arg341 mutations were screened initially for loss of a unique *Bss*HII site, the mutations were confirmed by sequence analysis. Mutant *glnS* genes were recloned for *in vivo* expression as *Eco*RI-*Hin*dIII fragments into pBR322. The analogous pBR*glnS7* construct contained the D235N mutation (gift of R. Swanson). The R402Q and R402A mutants were made on pBluescriptSK$^-$ (Stratagene La Jolla, CA, USA) containing an internal *Sac*II-*Mlu*I fragment of the *glnS* gene. The mutant fragments were recloned for *in vivo* experiments and replaced the corresponding wild-type fragment in pBR*glnS*. Despite repeated attempts with degenerate oligonucleotides, it was not possible to make all desired substitutions for Glu323 and Arg341 by *in vitro* site-directed mutagenesis.

An additional mutation (R341P) was made by random polymerase chain reaction mutagenesis. The template was a Bluescript clone of *glnS*, pJS6. The ''mutated'' PCR fragment as confirmed by sequence analysis was digested with *Bst*EII and the resulting fragment (255 bp) used to replace the corresponding wild-type fragment in pBR-*glnS*. Mutations at the Arg341 codon were screened for by loss of the *Bss*HII site and then sequenced to confirm that this was the only mutation.

Construction of GlnRS double mutants

Double mutants which combine mutations in the acceptor and anticodon binding domains of GlnRS were constructed by cloning into pBR*glnS*. In all cases, the vector-containing and internal *glnS* fragments were purified from agarose gels. For isolation of most of these double mutant clones, a temperature of 30°C for incubation of the transformation plates and initial liquid cultures was observed to be critical. Successful reclones of the R341A mutant were identified by the loss of the *Bss*HII restriction enzyme site. However, both mutations were routinely confirmed by sequencing. The Arg341 and Arg402 mutations were combined with the L136F mutation as *Csp*45I-*Hin*dIII fragments to yield the L136F/R341A, L136F/R402Q and L136F/R402A double mutants, while the L136F and D235N mutations were cloned into pBR*glnS*(R341A) as *Bgl*II fragments to give L136F/R341A and D235N/R341A. Despite repeated attempts, it was not possible to combine mutations from the same domain of GlnRS, i.e. the L136F and D235N mutants in the acceptor binding domain or the Arg341 and Arg402 mutants in the anticodon binding domain.

Purification of wild-type and mutant *E. coli* GlnRS

GlnRS mutants were purified from the temperature-sensitive *glnS E. coli* strain UT172 (Englisch-Peters *et al*., 1991), transformed with pBR*glnS* mutant clones and grown at the non-permissive temperature. Thus, mutants can be expressed and purified without being contaminated by a functional wild-type GlnRS. Routinely, S100 extracts from six liter cultures were fractionated either on DEAE-cellulose and then on MonoQ sepharose (Pharmacia, Uppsala, Sweden), or, in the case of the double mutants, directly over MonoQ sepharose. The single most pure and active GlnRS fraction was further purified by chromatography over anti-GlnRS IgG sepharose. Bound enzyme was eluted with diethylamine (pH 11) and then concentrated over MonoQ sepharose. About 1–2 mg of each GlnRS mutant enzyme was purified by this method. A control preparation of GlnRS(T266P), the temperature-sensitive GlnRS in UT172, yielded no protein or activity

after the final chromatography over MonoQ sepharose, probably due to its being denatured by the high pH conditions used for elution from the anti-GlnRS IgG sepharose (data not shown). However, similarly treated, the mutants being purified in this study and pure wild-type GlnRS as a control lost little or no activity (data not shown). All of the purified mutant GlnRS preparations appeared as one band on a Coomassie Blue stained SDS-PAGE (0.8 to 3.1 μg applied).

In vivo recognition of non-cognate and cognate suppressors

The ability of the GlnRS mutants expressed on pBR322 to recognize a number of amber suppressor tRNAs was assessed using the *lacZ*(*UAG1000*) glutamine-specific marker in *E. coli* strains RS109 [*tyrT*(*UAG*), *lacZ*(*UAG1000*)] and BT235 [*su°*, *lacZ*(UAG1000)]. The latter strain was transformed with pACYC184 clones of the other amber suppressors (Inokuchi *et al*., 1984; Weygand-Durasevic *et al*., 1993, 1994; Sherman & Söll, 1996). Each of the BT235/pACYCtRNA(UAG) strains and RS109 were then transformed with the mutant pBR*glnS* clones. Recognition of the coexpressed amber suppressor tRNA by the GlnRS 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 $tRNA^{Pro}$(UAG) was quantitated using β-galactosidase assays. The measurements represent the average activity of six different transformants, each assayed in triplicate.

The mutant opal suppressor derived from $tRNA^{Gln}$, GLNA3U70, no longer inserts a sufficient amount of glutamine to suppress the marker *trpA*(*UGA15*) in strain KL2576 (Rogers *et al*., 1992). The mutant *glnS* genes cloned on pBR322 were transformed into KL2576/pACYCGLNA3U70 and their ability to confer a Trp^+ phenotype on the strain (growth on glucose minimal plates in the absence of tryptophan) was tested at 30°C. In general, 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.

Aminoacylation assays

Glutaminylation assays were performed as described (Sherman & Söll, 1996). When $tRNA^{Gln}$ was the variable substrate, glutamine and ATP were present in the reaction at concentrations of 680 μM and 2 mM, respectively, which are saturating for wild-type GlnRS (Kern *et al*., 1980). The tRNA concentration was varied over a tenfold range and was present in at least a 60-fold molar excess over the GlnRS variant. When glutamine was the variable substrate, the concentration of ATP (2 mM) is likely to have been saturating even for the GlnRS mutants (Kern *et al*., 1980), while the tRNA concentration was two to four times the K_m value, as use of > 50 μM concentrations was prohibitive and would affect the viscosity. The glutamine concentration was varied over an eightfold range, which is the maximum possible range which could be used with [^{3}H]glutamine due to its concentration and specific activity. Assays were performed in duplicate using a minimum of five tRNA and glutamine concentrations and four time points in the linear range of initial rate. Errors were consistently less than 10%. The kinetic parameters were calculated using both Lineweaver-Burk and Eadie-Hofstee plots.

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