Retracing the evolution of amino acid specificity in glutaminyl-tRNA synthetase

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Received 28 July 1998

Abstract Molecular phylogenetic studies of glutaminyl-tRNA synthetase suggest that it has relatively recently evolved from the closely related enzyme glutamyl-tRNA synthetase. We have now attempted to retrace one of the key steps in this process by selecting glutaminyl-tRNA synthetase mutants displaying enhanced glutamic acid recognition. Mutagenesis of two residues proximal to the active site, Phe-90 and Tyr-240, was found to improve glutamic acid recognition 3–5-fold in vitro and resulted in the misacylation of $tRNA^{Gln}$ with glutamic acid. In vivo expression of the genes encoding these misacylating variants of glutaminyl-tRNA synthetase reduced cellular growth rates by 40%, probably as a result of an increase in translational error rates. These results provide the first biochemical evidence that glutaminyl-tRNA synthetase originated through duplication and consequent diversification of an ancestral glutamyl-tRNA synthetase-encoding gene.

Key words: Glutamic acid; Glutamine; Glutaminyl-tRNA synthetase; tRNA; Evolution; *Escherichia coli*

1. Introduction

The aminoacyl-tRNA synthetases are a family of enzymes whose structure and function are highly conserved in the living kingdom [1]. They are largely responsible for the synthesis of correctly charged aminoacyl-tRNAs, the only known substrate for ribosomal mRNA translation. This function necessitates that they display a high degree of accuracy in identifying their cognate tRNA and amino acid substrates and an ability to discriminate against non-cognates. For the tRNA substrate this is achieved through a series of sequence specific protein-RNA interactions between tRNA and synthetase. The exchange of these recognition elements between different tRNAs or AARSs results in corresponding changes in substrate specificity [2,3]. The discrimination of different amino acids is potentially more problematic as these molecules are far smaller and may differ by as little as a single methyl group. These small differences, as for example between isoleucine and valine, cannot be adequately differentiated by AARSs to reduce potential miscoding errors during translation to a level tolerable during normal cell growth. Thus, the synthetases responsible for synthesizing the corresponding aminoacyl-tRNAs posses distinct editing activities which serve to eliminate non-cognate reaction intermediates and products [4].

It is becoming increasingly apparent that direct aminoacylation, where an aminoacyl-tRNA is made by a single enzyme able to recognize the correct amino acid and tRNA moieties, is not the only route for aminoacyl-tRNA synthesis. Gln-$tRNA^{Gln}$, selenocysteinyl-$tRNA^{Sec}$ (Sec-$tRNA^{Sec}$) and Asn-$tRNA^{Asn}$ are all synthesized via mischarged tRNA intermediates in certain organisms [5]. Sec-$tRNA^{Sec}$ is only known to be made via the mischarging of $tRNA^{Sec}$ with Ser, possibly because of the inability of cysteinyl-tRNA synthetase to discriminate selenocysteine and cysteine [6] which precludes the existence of a 'selenocysteinyl-tRNA synthetase'. In contrast, mischarging pathways for the formation of Gln-$tRNA^{Gln}$ and Asn-$tRNA^{Asn}$ are limited to certain organisms/organelles, raising the question of why two pathways have persisted through evolution. The prevailing hypothesis for Gln-$tRNA^{Gln}$ formation, is that the mischarging pathway represents the ancestral route to this aminoacyl-tRNA, which has subsequently been replaced by a distinct glutaminyl-tRNA synthetase (GlnRS) enzyme which evolved from glutamyl-tRNA synthetase (GluRS; see e.g. [7]). This hypothesis suggests that it should be possible to reverse this process, and thus improve Glu recognition by GlnRS, via discrete amino acid replacements in GlnRS. The experimental rationale was based upon the well-documented ability of aminoacyl-tRNA synthetases to mischarge non-cognate suppressor tRNAs in vivo (missense suppression [8]) and the capacity of *Escherichia coli* to tolerate the low level of translational errors associated with this process.

In order to both address the question of GlnRS evolution and to gain insights into the rationale underlying the phylogenetic distribution of the direct and indirect pathways for Gln-$tRNA^{Gln}$ formation, a genetic screen was established for amino acid mischarging using a non-essential target protein. This allowed the isolation of GlnRS mutants which were able to form Glu-$tRNA^{Gln}$ both in vivo and in vitro. Biochemical and physiological analysis of these variants suggested that the indirect pathway for Gln-$tRNA^{Gln}$ formation is essential for maintaining the fidelity of protein synthesis under certain cellular conditions and that this may provide a basis for the occurrence of the two pathways.

2. Materials and methods

2.1. General

Wild-type [9] and mutant [10] GlnRS proteins, as well as dihydrofolate reductase (DHFR [11]), were produced and purified as previously described. $tRNA_2^{Gln}$ was a gift from J.M. Sherman, $tRNA_1^{Gln}$ and $tRNA^{Glu}$ were purchased from Subriden RNA (Rolling Bay, WA). Media for bacterial growth and molecular biology protocols were standard unless otherwise noted [12].

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PII: S0014-5793(98)00968-5

2.2. Strains and plasmids

The *E. coli* strains CC101 (*lacZ* UAG_{461}; [13]), HAPPY101 [10] and UT172 [14] have been described previously. The 2 kb *Dra*I fragment containing the *glnS* gene (which encodes GlnRS) with *Eco*RI linker [15] was cloned into the *Eco*RI site of the pBluescript vectors pKS^- and pKS^+ to prepare single-stranded DNA for enzymatic mutagenesis. To construct compatible plasmids containing the amber suppressor tRNA genes, the *Pvu*II fragments of pGFIB-*supF* G73 [16] or pGFIB-*supE* [17] which carry the $tRNA^{Tyr}_{CUA}$ G73 and $tRNA^{Gln}_{CUA}$ genes respectively, were subcloned into the *Hin*cII site of pACYC184. Plasmid pD3am contains the coding region of the *E. coli* DHFR gene *fol* with an amber mutation at residue 3, under control of the *tac* promoter [18]. The pD3am-*supF*G73 plasmid was constructed by subcloning the *Pvu*II fragments of pGFIB-*supF*G73, which carry the gene encoding $tRNA^{Tyr}_{CUA}$ G73, into pD3am which had been digested with *Bam*HI and filled using Klenow fragment.

2.3. In vitro mutagenesis and screening of glnS

To allow screening for GlnRS mutants which mischarge $tRNA^{Tyr}_{CUA}$ with glutamic acid in vivo, the two dinucleotide binding domains [19] encoded in the *glnS* gene were randomly mutagenized using enzymatic methods [20]. For the enzymatic misincorporation reaction, two synthetic primers (5′-CGATGAAGATTTGGCCAGTGGTAAG-3′ and 5′-GTGTAACCACGTCGACGCAGACC-3′) complementary to regions 5′ of the first and 3′ of the second dinucleotide binding domains respectively, were hybridized with single-stranded *glnS* DNA separately. The primer was extended with Klenow fragment in four separate sets of reactions, each of which generates a population of molecules terminating just before a given type of base, due to limiting amounts of that nucleotide. After removal of free nucleotides, the four molecular populations were misincorporated with three wrong nucleotides by reverse transcriptase under conditions where proofreading does not occur. The four mutagenized molecules were mixed and completed with excess amounts of dNTPs by polymerase and ligase. The ligation mixture and pACYC-*supF*G73 DNA were cotransformed into strain CC101 and plated on MacConkey plates containing ampicillin and chloramphenicol. After 1 or 2 days of incubation at 30°C plasmid DNA was isolated from pink colonies, retested for the phenotype and identified by sequencing. Site-directed mutagenesis was carried out by PCR as previously described [21].

2.4. Chemicals

All chemicals were of analytical quality. L-Glutamic acid (Sigma, St. Louis, MO) contained no detectable glutamine as determined by anion exchange amino acid analysis (M. Crawford, Keck Biotechnology Laboratory, Yale University, New Haven, CT). L-[U-^{14}C]Glutamic acid was from NEN-DuPont (Boston, MA) and contained no glutamine as assessed by thin layer chromatography (A.W. Curnow, personal communication). Methotrexate resin for the affinity purification of DHFR was from Sigma (St. Louis, MO).

2.5. Aminoacylation assays

Aminoacylation assays to determine the kinetic parameters for glutamine were performed as described before [9]. Inhibition constants (K_i) for glutamic acid were determined by standard methods [22].

2.6. Electrophoresis of aminoacylated tRNAs

Aminoacylation of tRNAs with Glu by GlnRS was performed in 100 mM HEPES, pH 7.2, 10 mM $MgCl_2$, 5 mM ATP, 0.1 mg/ml bovine serum albumin, 5 μM tRNA, 500 nM GlnRS and 1–8 mM L-[^{14}C]glutamic acid. Following incubation at 20°C for 1 h, aminoacylated tRNAs were prepared as previously described [23] except that they were finally resuspended in sample buffer and electrophoresed using the method of Varshney et al. [24] modified as described [25].

3. Results

3.1. Isolation of GlnRS variants with improved Glu recognition

The regions of the *glnS* gene mutated correspond to residues 24 to approximately 124 and 194 to approximately 294, which includes all the domains of GlnRS implicated in amino acid binding from structural studies [26]. Sequencing of mutant *glnS* genes revealed two changes which gave rise to improved Glu recognition, as assessed by their ability to actively suppress the $lacZ_{461}$ mutation in *E. coli* strain CC101 in the presence of both a cognate and a non-cognate amber suppressor tRNA. These were C-U at nucleotide 352 which results in a Phe-Leu change at residue 90 (F90L), and C-A at nucleotide 804 which results in the replacement of Tyr-240 with an in-frame ochre stop codon (Y240taa; Table 1). To determine the effect of the second mutation on GlnRS production, a *glnS* gene was constructed which lacked the nucleotides encoding the residues between Tyr-240 and the C-terminus. Expression of this truncated gene in *E. coli* strain HAPPY101 gave rise to a stable protein of the expected molecular weight (determined by SDS-PAGE and immunoblotting, data not shown) but did not cause active suppression of $lacZ_{461}$. Further investigation of Y240taa production showed the majority of GlnRS was full length rather than truncated, indicating that the ochre stop codon is efficiently suppressed. Thus it seems likely that active suppression of $lacZ_{461}$ results from substitution of Tyr-240 with another amino acid in full length GlnRS rather than by production of a truncated protein. This was later confirmed by in vitro experiments (see below).

To further investigate the effect of mutating Tyr-240 on glutamic acid recognition, the codon for this residue was randomly mutagenized and the resulting *glnS* variants screened for their ability to actively suppress $lacZ_{461}$. Sequencing of positive clones from this screen showed that substitution of Tyr-240 by either Glu or Gly led to improved glutamic acid recognition in vivo. Both these and the other GlnRS variants which showed improved recognition of glutamic acid were than further analyzed in vitro.

3.2. Kinetic characterization of GlnRS variants with improved Glu recognition

All the GlnRS variants were purified from strain HAPPY101 as previously described. For the Y240taa mutant a homogeneous protein of the same molecular weight as wild-type was isolated. The yield of protein was approximately five-fold lower than for wild-type in all cases, except for Y240taa whose yield was ten-fold reduced, indicating that overproduction of these mischarging mutants is toxic to *E. coli*.

The steady state kinetic parameters with respect to Gln were investigated using the aminoacylation reaction (Table 2). All mutants showed 2–4-fold reduced K_Ms for Gln compared to wild-type GlnRS, indicating that all the replacements lead to an apparent increase in the affinity for Gln. k_{cat} values were 2–3-fold lower for the mutants than for wild-type and consequently their catalytic efficiencies (as estimated by k_{cat}/

Table 1
Ability of *glnS* mutants to suppress $lacZ_{461}$[a]

glnS isolate $tRNA^{Gln}_{CUA}$[a]	*glnS*[ts] complementation[b]	$tRNA^{tyr}_{CUA}$[a]
Wild-type	+	− −
F90L	+/−	+ +
Y240taa	+/−	+ +
Y240E	+/−	+ n.d.
Y240G	+/−	+ n.d.

[a]Suppression was assayed by the ability of transformants to produce pink colonies due to amber suppression of $lacZ_{461}$ in strain CC101 at 30°C. +, pink colonies after 2 days; −, white colonies after 2 days.
[b]Complementation of *E. coli* UT172 (*ts*) tested after overnight incubation on complete medium at 42°C. n.d., not determined.

Table 2
Aminoacylation kinetics of GlnRS with Gln in the presence and absence of Glu[a]

GlnRS	K_M (μM Gln)	k_{cat} (min^{-1})	k_{cat}/K_M (min^{-1} μM^{-1})	K_i[b] (mM Glu)
Wild-type	193 ± 21	282 ± 24	1.5	96 ± 20
F90L	50 ± 3	121 ± 4	2.4	29 ± 2
Y240taa	59 ± 5	280 ± 14	4.8	17 ± 2
Y240E	66 ± 7	84 ± 5	1.3	21 ± 5
Y240G	117 ± 15	203 ± 16	1.7	47 ± 8

[a]Aminoacylation experiments were performed at 37°C in standard buffer with Gln levels varied over the range 0.2–5 times K_M. K_i values were determined with unlabeled Glu added at final concentrations of 20 mM and 40 mM.
[b]All enzymes tested displayed competitive inhibition by Glu. K_i was determined from the following formula $K_{Mapp} = K_{Mreal}$ (1+ [Glu]/K_i).

K_M) were little effected, the only exception being Y240taa which showed a 3-fold increase. Overall these data indicate that while the replacements affect amino acid binding they are not detrimental to catalysis.

The ability of mutant and wild-type GlnRSs to recognize glutamic acid was initially investigated by determining the kinetic parameters for glutamine in the presence of unlabelled Glu. Aminoacylation by wild-type GlnRS and all of the mutants was competitively inhibited by Glu. The inhibition constant (K_I) for the mutants was 2–5-fold lower than for the wild-type, with the largest decreases observed for Y240taa and Y240E GlnRS (Table 2). These results, which indicated an increase in the apparent affinity of the active sites of the mutants for glutamic acid, were in agreement with the in vivo data.

The ability to synthesize Glu-tRNA was then directly investigated for the two GlnRS mutants originally isolated in vivo, F90L and Y240taa. To determine whether the mutants could synthesize glutamylated tRNA, aminoacylation reactions were performed with radiolabelled glutamic acid at concentrations up to 8 mM. Despite the use of various reaction conditions, no significant level of product could be detected by scintillation counting. The reaction products were therefore analyzed by polyacrylamide gel electrophoresis (Fig. 1). The level of radiolabelled charged tRNA was shown to be dependent on the glutamic acid concentration, confirming that both wild-type and mutant GlnRS are able to synthesize Glu-$tRNA^{Gln}$. The amount of product formed was approximately 3-fold higher for the mutants which broadly correlates with the differences in K_I compared to wild-type. The tRNA substrate specificity of these mutants was also tested in the presence of glutamic acid: both were able to use $tRNA_1^{Gln}$ and $tRNA_2^{Gln}$ equally well, but neither could use $tRNA^{Glu}$ (data not shown).

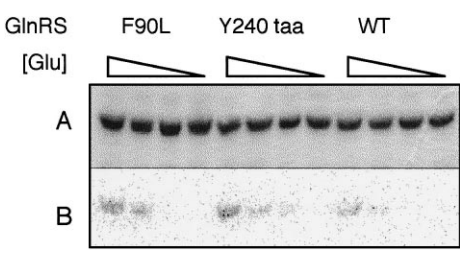

Fig. 1. In vitro aminoacylation of $tRNA_2^{Gln}$ with ^{14}C-labeled glutamic acid. Reactions were performed as indicated in the text in the presence of 8, 4, 2 and 1 mM glutamic acid and the samples then subjected to PAGE. A: Staining with methylene blue. B: Visualization of radiolabeled samples by phosphorimaging.

3.3. Effects of tRNA mischarging on protein synthesis and growth

We attempted to quantify the extent of replacement of glutamine by glutamic acid in proteins during growth in the presence of Y240taa and F90L GlnRS. This was done by analyzing the amino acid composition of the third residue of the DHFR protein expressed from the plasmid pD3am-*supF*G73, as previously described [18]. Following N-terminal sequencing of purified DHFR, glutamine and tyrosine were found at a ratio of approximately 10:1 at position 3 in both cases. This is in agreement with previously determined values for wild-type GlnRS [16]. Glutamic acid was detected at less than 10% the level of glutamine and could result from deamidation of glutamine during the sequencing reaction. Thus these experiments show that the actual level of glutamic acid misincorporation into proteins is less than 5% in these mutant backgrounds.

The effects of F90L and Y240taa GlnRS on batch growth kinetics were investigated in the *E. coli* strain UT172. This strain contains a mutation in the chromosomal *glnS* gene which results in the production of a thermolabile GlnRS which is unable to sustain growth at 40°C. The doubling times of transformants producing wild-type, F90L and Y240taa GlnRS were measured at 40°C and 42°C on LB medium containing either 0.25% or 2.5% NaCl. The higher salt concentration would be expected to cause significant glutamate accumulation in the cells due to osmotic adaptation [27,28]. At low salt concentrations the mutant transformants showed a 40% increase in doubling time at both temperatures compared to wild-type (Table 3). Growth at higher salt did not effect the doubling time of wild-type or F90L transformants but did cause a further 20% increase in the doubling time of the Y240taa transformant at both temperatures. The level of pro-

Table 3
Batch growth parameters[a] for *glnS* mutants[b]

NaCl (w/v)	40°C		42°C	
	0.25%	2.5%	0.25%	2.5%
glnS	Doubling Time (min)			
Wild-type	33 ± 2	31 ± 1	52 ± 5	52 ± 4
F90L	46 ± 3	49 ± 3	71 ± 5	72 ± 2
Y240taa	50 ± 2	59 ± 3	64 ± 3	79 ± 6

[a]Doubling times during the logarithmic phase of batch growth were measured in triplicate for all cultures (growth medium was LB containing the indicated NaCl concentrations).
[b]The *E. coli* strain UT172 (*ts*) was transformed with plasmids containing the appropriate *glnS* gene.

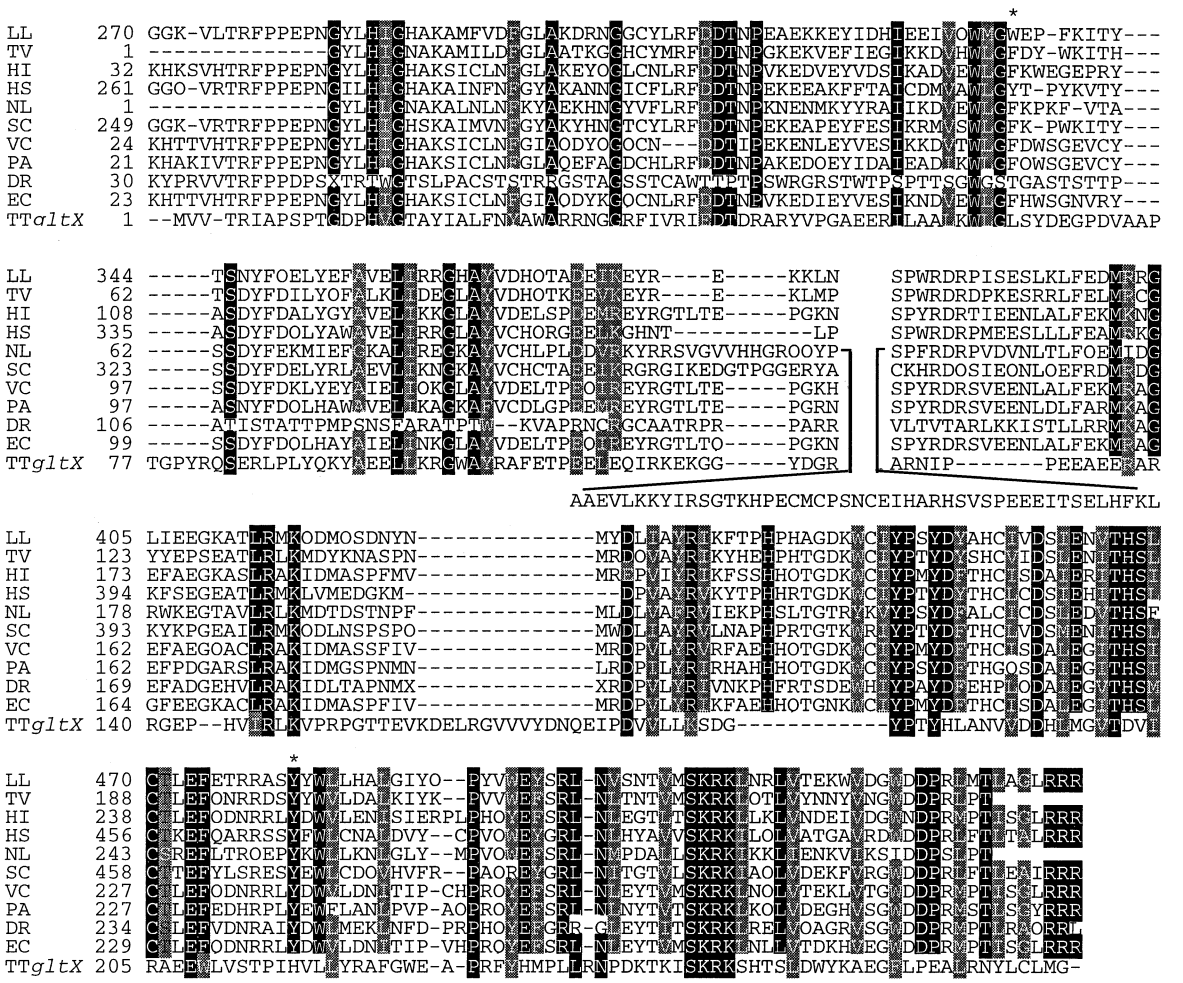

Fig. 2. Partial alignment of GlnRS amino acid sequences. The sequences were aligned using the CLUSTAL W program [36]. The sequences shown are from lupin (LL), *Trichomonas vaginalis* (TV), *Haemophilus influenzae* (HI), human (HS), *Nosema locustae* (NL), *Saccharomyces cerevisiae* (SC), *Vibrio cholerae* (VC), *Pseudomonas aeruginosa* (PA), *Deinococcus radiodurans* (DR) and *E. coli* (EC). The structure-based alignment of the *Thermus thermophilus* GluRS protein (TT*gltX* [37]) is also shown. Amino acids Phe-90 and Tyr-240 of *E. coli* GlnRS are indicated (*).

duction of full length GlnRS was comparable in all experiments as assessed by immunoblotting (data not shown).

4. Discussion

4.1. Residues proximal to the active site contribute to amino acid binding

In vitro mutagenesis of the *glnS* gene followed by in vivo screening of the encoded mutant proteins identified residues Phe-90 and Tyr-240 as being determinants of amino acid specificity. F90L GlnRS showed a 3-fold improvement compared to wild-type in its ability to recognize glutamic acid, while replacements of Tyr-240 showed up to 5-fold improvements. Examination of the kinetic parameters for Y240taa (Table 2) indicate that this protein is most likely a heterogeneous preparation containing both Y240E and wild-type GlnRS. This conclusion is based upon the observation that Y240taa displays a wild-type k_{cat} while the K_M for Gln and the K_i for Glu are nearly identical to Y240E GlnRS. This implies that the ochre stop codon UAA is suppressed by both $tRNA^{Tyr}$ and $tRNA^{Glu}$ species; while this specific mixed suppression has not been previously observed, it is consistent with established patterns of stop-codon suppression in *E. coli* [8].

The crystal structure of GlnRS complexed with $tRNA_2^{Gln}$ and the Gln-AMP analogue 5′-*O*-[*N*-(L-glutaminyl) sulfamoyl] adenosine [29] shows Tyr-240 to be close to a number of residues which determine the specificity of glutamine binding through a network of interactions with the -NH_2 group of the glutamine side chain. While not directly implicated in this network of interactions, the proximity of Tyr-240 and orientation of its sidechain suggest that it may indirectly contribute to amino acid specificity. This is supported by the observation that Tyr-240 is conserved in all known GlnRS proteins while the analogous position in GluRS is not (Fig. 2). A comparable situation has previously been observed for tyrosine binding by *Bacillus stearothermophilus* tyrosyl-tRNA synthetase (TyrRS) where a number of residues serve to position Asp-176 which acts as the hydrogen bond acceptor for the *para*-hydroxyl group of the substrate tyrosine [30]. Mutation of residues which interact with Asp-176, but not directly with tyrosine, was found to lower the ability of TyrRS to discriminate tyrosine and phenylalanine.

While mutation of both Phe-90 and Tyr-240 improves glutamic acid recognition, these changes are accompanied by concomitant improvements in the apparent affinity for the cognate substrate glutamine suggesting that only minor

changes in substrate specificity have been achieved. This reflects the limitation of the genetic approach employed in this study: any mutants selected must still retain the ability to recognize glutamine sufficiently better than glutamic acid to prevent a lethal level of translational errors arising. Site-directed mutagenesis based upon the structure of the active-site interactions with glutamine [29], in combination with a suitable expression strategy, may now provide the best means of further improving the glutamic acid substrate-specificity of GlnRS.

4.2. Physiological consequences of tRNA mischarging

The replacement of wild-type GlnRS with mischarging mutant proteins in vivo was achieved by complementation of a temperature sensitive *glnS* allele under restrictive conditions, with the resulting transformants growing approximately 40% slower than wild-type. Since the mutants have comparable catalytic efficiencies (k_{cat}/K_M) to wild-type for Gln-tRNA^{Gln} synthesis, the difference in growth rate is most likely the result of errors in translation resulting from Glu-tRNA^{Gln} synthesis. The misacylation of tRNA^{Gln} with glutamic acid only proceeds at a low level as indicated by the absence of detectable glutamic acid at a specific position in the target protein DHFR. However, increasing the cellular glutamic acid concentration by increasing the extracellular salt concentration [27,28] specifically reduced the growth rate by 20% of transformants containing GlnRS with a Tyr-240 mutation, suggesting that the level of Glu-tRNA^{Gln} synthesis, and thus translational errors, was elevated under these conditions. This observation correlates with the earlier finding that gram positive bacteria, which contain significantly larger cellular glutamic acid pools than gram negative bacteria [27,31], do not contain GlnRS and instead synthesize Gln-tRNA^{Gln} by an indirect pathway (see below). Taken together, these results suggest that the ratio of cellular glutamic acid to glutamine is a major factor in determining the potential substrate specificity of GlnRS and thus its efficiency as a component of the translational apparatus. Whether this aspect of cellular physiology has acted as an evolutionary pressure to determine the phylogenetic distribution of GlnRS remains unclear.

4.3. Evolutionary origin of glutaminyl-tRNA synthetase

GlnRS-encoding genes are not found in the majority of bacteria and organelles, and have not been detected to date in archaea [5,32]. In these cases, Glu-tRNA^{Gln} is first synthesized by GluRS and this molecule is subsequently converted to Gln-tRNA^{Gln} by a specific amidotransferase [33]. Molecular phylogenetic analyses suggest that this represents the more ancient pathway to Gln-tRNA^{Gln}, with GlnRS having recently evolved after duplication of GluRS [7,34]. This possibility, rather than separate origins of the two pathways, is supported by the recent observation that the genome of the bacterium *Deinococcus radiodurans* encodes the Glu-tRNA^{Gln} specific amidotransferase, GlnRS and GluRS (Tumbula, Curnow, Pelaschier and Söll, unpublished results).

During its molecular evolution a duplicated ancestral GluRS would only be required to lose the ability to recognize tRNA^{Glu} and gain the ability to recognize glutamine instead of glutamic acid in order to become a functional GlnRS. The necessary co-evolution of synthetase and tRNA to generate a functional, specific GlnRS:tRNA^{Gln} pair has been discussed previously [35]. The data shown here now indicate that changes in the ability to recognize glutamine and glutamic acid can be achieved by discrete mutations. While the degree of change in substrate specificity was limited by the potential toxicity of the mutant GlnRS enzymes, this would not have been the case during evolution as the 'new' product would be Gln-tRNA^{Gln}, a suitable substrate for translation.

Acknowledgements: This work was supported by a grant from NIGMS (GM22854). We thank M.J. Rogers for advice on the genetic screen and The Institute for Genomic Research and the University of Washington Genome Center for availability of sequence data before publication.

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