Rational design and directed evolution of a bacterial-type glutaminyl-tRNA synthetase precursor

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ABSTRACT

Protein biosynthesis requires aminoacyl-transfer RNA (tRNA) synthetases to provide aminoacyl-tRNA substrates for the ribosome. Most bacteria and all archaea lack a glutaminyl-tRNA synthetase (GlnRS); instead, Gln-tRNAGln is produced via an indirect pathway: a glutamyl-tRNA synthetase (GluRS) first attaches glutamate (Glu) to tRNAGln, and an amidotransferase converts Glu-tRNAGln to Gln-tRNAGln. The human pathogen Helicobacter pylori encodes two GluRS enzymes, with GluRS2 specifically aminoacylating Glu onto tRNA Gln. It was proposed that GluRS2 is evolving into a bacterial-type GlnRS. Herein, we have combined rational design and directed evolution approaches to test this hypothesis. We show that, in contrast to wild-type (WT) GlnRS2, an engineered enzyme variant (M110) with seven amino acid changes is able to rescue growth of the temperature-sensitive Escherichia coli glnS strain UT172 at its non-permissive temperature. In vitro kinetic analyses reveal that WT GluRS2 selectively acylates Glu over Gln, whereas M110 acylates Gln 4-fold more efficiently than Glu. In addition, M110 hydrolyzes adenosine triphosphate 2.5-fold faster in the presence of Glu than Gln, suggesting that an editing activity has evolved in this variant to discriminate against Glu. These data imply that GluRS2 is a few steps away from evolving into a GlnRS and provides a paradigm for studying aminoacyl-tRNA synthetase evolution using directed engineering approaches.

INTRODUCTION

Translation of the genetic information from DNA to protein is a central process in all three domains of life (bacteria, archaea and eukaryotes). Protein synthesis on the ribosome requires aminoacyl-transfer RNA (aa-tRNA) substrates, which are formed by aminoacyl-tRNA synthetases (aaRSs) and delivered to the ribosome by elongation factors (1–3). Except for selenocysteine, each of the 22 proteinogenic amino acids has a cognate aaRS and delivers its corresponding tRNA to the ribosome (1–3). For example, the legume Medicago truncatula encodes a urocysteinyl-tRNA synthetase that ligates urocanic acid to tRNA Cys (4). Many bacteria and archaea lack a glutaminyl-tRNA synthetase (GlnRS) and use a Glu-AdT to produce Gln-tRNAGln from Glu-tRNAGln, which is synthesized by a glutamyl-tRNA synthetase (GluRS) (4,6) (Figure 1). In contrast, eukaryotes have evolved a GlnRS to directly synthesize Gln-tRNAGln (1). In most bacteria and archaea, glutamate (Glu) is attached to both tRNAGlu and tRNAGln by a ND-GluRS. Certain bacteria (e.g. Helicobacter pylori) encode two GluRS enzymes: GluRS1 that ligates Glu to tRNAGlu and GluRS2 that specifically attaches Glu to tRNAGln (7,8) (Figure 1). Phylogenetic and structural studies suggested that modern GluRSs and GlnRSs evolved from a common ancestral ND-GluRS (9). GlnRS evolved from ND-GluRS after the split between archaea and eukaryotes, and the gene encoding eukaryotic GlnRS was later transferred horizontally to certain bacterial species (e.g. Escherichia coli) (10–12). In this work, we have combined rational design and directed evolution approaches to obtain a H. pylori GluRS2 variant that is capable of rescuing a glnS temperature-sensitive E. coli strain (13,14), and aminoacylating Gln to tRNA Gln in vitro. We have thus identified an important precursor that could further evolve into a bacterial-type GlnRS.
MATERIALS AND METHODS

Strains, plasmids and protein purification

Escherichia coli UT172 glnS temperature-sensitive strain was used for selection of the H. pylori GluRS2 mutation library carried on pERS2 vector, which was derived from pUC18. Escherichia coli total tRNA used in this study was purchased from Roche. Wild-type (WT) and mutant H. pylori GluRS2 were cloned into pET20b vector for overexpression in the BL21(DE3) E. coli strain. Expression of WT GluRS2 was induced in Luria–Bertani (LB) broth with 1 mM isopropyl \(\beta\)-D-1-thiogalactopyranoside for 1 h at 37°C when \(A_{600}\) reached 1.2. M110 GluRS2 formed inclusion bodies when overexpressed at 37°C. Its expression was thus induced at 30°C with 0.1 mM isopropyl \(\beta\)-D-1-thiogalactopyranoside for 5 h at \(A_{600} \sim 1.2\). The proteins were purified with Ni-NTA affinity resin (Qiagen) according to the standard procedures and dialyzed against a storage buffer containing 50 mM Hepes (pH 7.5), 100 mM NaCl, 10 mM 2-mercaptoethanol, 3 mM MgCl\(_2\) and 50% glycerol.

Construction and selection of H. pylori GluRS2 mutation library

To construct a pERS2 plasmid, the full-length gene encoding GluRS2 was inserted into pUC18 plasmid between EcoRI and PstI sites. An additional G nucleotide was included after the EcoRI site to allow GluRS2 controlled by Plac promoter. Seven residues (A5, S7, E39, C178, I190, R192 and H196) located in the amino acid binding pocket of H. pylori GluRS2 were chosen based on the crystal structure of Thermus thermophilus GluRS:tRNAGlu (2CV0) to generate the mutation library. I190 and R192 were rationally mutated to serine and cysteine, respectively. A5, S7, E39, C178 and H196 were randomized. Overlap polymerase chain reaction was performed as described previously (15). 4 \(\times\) 10\(^7\) transformants were obtained for the library, which was 12 times larger than calculated library size (3.4 \(\times\) 10\(^7\)).

The mutation library was selected in E. coli UT172 strain, in which mutants charging tRNA\(^{Gln}\) with glutamine would survive on LB plate supplemented with 100 \(\mu\)g/ml ampicillin at 42°C. During the first round of selection, 1\(\mu\)g plasmid was transformed into UT172 electrocompetent cells to obtain 1 \(\times\) 10\(^9\) transformants. The transformants were recovered in 20 ml Super Optimal broth with Catabolite repression (SOC) at 30°C for 2 h, followed by the addition of 100 \(\mu\)g/ml ampicillin and 8 h of growth. 0.3 ml saturated culture was diluted into 15 ml SOC medium and grown for 5 h at 30°C. Next, 1 ml culture was spread on 20 \(\times\) 20 cm LB plate supplemented
with 100 µg/ml ampicillin and the plate was incubated at 42°C for 24 h. Colonies growing on the plate were scraped off and extracted for plasmids. The plasmids from the first round of selection were subjected to a second round of selection with the same procedure.

Enzymatic assays

Adenosine triphosphate (ATP)–pyrophosphate (PPi) exchange reaction was performed in the buffer containing 100 mM Hepes–KOH, pH 7.2, 30 mM KCl, 12 mM MgCl₂, 2 mM dithiothreitol (DTT), 2 mM KF, 5 mg/ml total E. coli tRNA, 5 mM ATP, 5 mM PPI, 0.1 µCi [γ-³²P]ATP, 9 µM enzymes and 0.24–10 mM Glu and Gln. The 1 µl reaction mixture was spotted onto thin-layer chromatography (TLC) polyethylenimine (PEI) cellulose F plates (Merck). Then, the TLC plates were developed in 1 M KH₂PO₄ and 1 M urea to separate ATP and PPI. Detection and quantification of signals were performed as described before (16).

Aminoacylation assay was essentially performed as described before (8) with slight modifications. The 40 µl reaction mixture contained 100 mM Hepes–KOH, pH 7.2, 30 mM KCl, 12 mM MgCl₂, 2 mM DTT, 5 mg/ml total E. coli tRNA, 5 mM ATP, 40 µM [¹⁴C]Glu or [³²P]Gln and 2.3 µM enzymes; 8 µl aliquots were taken out at each time point for scintillation counting.

ATP consumption was performed in the presence of 100 mM Hepes–KOH (pH 7.2), 30 mM KCl, 12 mM MgCl₂, 2 mM DTT, 5 mg/ml total E. coli tRNA, 0.1 µCi [γ-³²P]ATP, 1 mM cold ATP, 10 mM Glu or Gln and 9 µM GluRS2. At each time spot, a 2-µl aliquot was quenched with 2 µl acetic acid. The 1 µl resulting mixture was spotted on PEI cellulose plates and separated in 0.1 M ammonium acetate and 5% acetic acid. The Adenosine monophosphate (AMP)/ATP ratios were quantified with phosphorimaging. No aminoacyl adenylate spot was observed during the reaction time course.

RESULTS

Design and evolution of a GluRS2 variant that rescues a glnS temperature-sensitive strain

Previous studies have shown that unlike ND-GluRS that aminoacylates Glu to both tRNA^{Glu} and tRNA^{Gln}, H. pylori GluRS2 (HpGluRS2) specifically charges Glu to tRNA^{Gln}, indicating that GluRS2 may be on the way to evolve into a bacterial-type GlnRS that is distinct from the eukaryotic-type GlnRS found in modern organisms (7,8). Phylogenetic analyses have shown that GluRS2 is closely related to bacterial GlnRSs but is more distant from GlnRSs present in eukaryotes and certain bacteria (9). Our sequence alignment revealed multiple active site residues that have distinct patterns in GluRS and GlnRS proteins (Figure 2). For example, position 190 (H. pylori GluRS2 numbering) is either an isoleucine or valine in bacterial GluRSs but is changed to a conserved serine in GlnRSs, and R192 of HpGluRS2 corresponds to a cysteine in GlnRSs. The crystal structure of T. thermophilus GluRS shows that these two residues interact with the side chain of the substrate Glu in the presence of tRNA (17) (Figure 3). The structures of E. coli GlnRS also reveal that S227 and C229 (corresponding to 1190 and R192 in HpGluRS2, respectively) are located close to the side chain of Gln at the active site (18,19).

To evolve HpGluRS2 into a GlnRS, we rationally introduced mutations I190S and R192C. We further randomized five active site residues that are important for amino acid binding based on the structure of T. thermophilus GluRS (17) (Figure 3). The plasmid library expressing randomized HpGluRS2 was transformed into E. coli strain UT172, which harbored a temperature-sensitive glnS gene (13,14). This E. coli strain was not able to grow at 42°C due to a defect in Gln-tRNA^{Gln} synthesis, and as expected, WT HpGluRS2 did not complement growth at 42°C (Figure 4). Selection of the transformed library at 42°C led to one GluRS2 variant (named M110) that rescued the growth of UT172 (Figure 4). Growth analysis revealed that UT172 transformed with M110 exhibited a doubling time of 94 min at 42°C, compared with 32 min for the strain transformed with E. coli GlnRS (Figure 4B).

Sequencing results showed that M110 contained mutations A5L, S7R, E39R, C178L, I190S, R192C and H196Q. The in vivo complementation assay suggested that M110 GluRS2 likely supplied the E. coli cells with sufficient Gln-tRNA^{Gln} to support growth. Alternatively, M110 GluRS2 might possess a higher Gln-tRNA^{Gln} synthesis activity than the WT at 42°C, and Glu misincorporation at Gln codons rescued the growth of UT172. Such possibilities were further investigated using biochemical assays in vitro.

M110 GluRS2 has evolved a GlnRS activity

To probe the amino acid specificity of the evolved enzyme, we purified the WT and M110 GluRS2 variants and performed in vitro pyrophosphate exchange experiments, which measured the activation of amino acids with ATP. To our surprise, WT GluRS2 exhibited similar activation efficiencies (determined by the $k_{cat}/K_m$ value) for Glu and Gln (Table 1). Both the $k_{cat}$ and $K_m$ values for Glu were ~2-fold lower than Glu. Conversely, the M110 variant showed 2.3-fold preference for Gln over Glu. The Glu activation efficiency was 9-fold higher for M110 than WT due to increased $k_{cat}$ and decreased $K_m$ values.

Next, we measured aminoacylation activities of WT and M110 GluRS2. Despite similar amino acid activation efficiencies, WT GluRS2 only attaches Glu, but not Gln, to tRNA^{Gln} at both 37 and 42°C (Figure 5). M110 showed 4-fold increased aminoacylation efficiency for Gln than Glu, confirming that this variant had evolved a GlnRS activity. M110 still maintained the Glu charging activity, although it was reduced by 30% compared with the WT GluRS2 at 37°C. The WT and M110 variants showed almost identical Glu charging efficiency at 42°C. Given that only M110 but not the WT GluRS2 rescued the glnS temperature-sensitive strain, our aminoacylation data suggested that instead of Glu-tRNA^{Gln} production, the Gln-tRNA^{Gln} synthesis activity that had evolved in M110 was responsible for rescuing the growth phenotype of UT172.
M110 GluRS2 uses an editing mechanism to reduce aminoacylation of Glu to tRNA<sub>Gln</sub>

The discrepancies between amino acid activation and aminoacylation results led us to investigate whether a quality control mechanism might help GluRS2 variants to discriminate between Glu and Gln. It has been previously shown that <i>E. coli</i> GlnRS hydrolyzes glutaminyl adenylate (Gln-AMP) in the presence of tRNA (20), mimicking a pre-transfer editing activity discovered in other aaRSs (21–24). We measured the hydrolysis of \([\alpha-^{32}P]\)ATP by WT and M110 GluRS2 in the presence of tRNA and Glu or Gln (Figure 6). WT GluRS2 hydrolyzed ATP about 3-fold more slowly in the presence of Gln than Glu, suggesting that the WT enzyme does not use an editing activity to prevent Gln from being attached to tRNAGln. Rather, the activated Gln-AMP is likely positioned in a non-productive fashion at the active site of WT GluRS2, restricting the transfer of Gln to tRNA.

The M110 variant showed 3-fold higher ATP hydrolysis rate than the WT in the presence of Glu, and the end point of hydrolyzed ATP was 15-fold higher than the available pool of tRNAGln in the reaction (Figure 6). This suggests that M110 uses an editing mechanism to selectively hydrolyze activated Glu-AMP. Collectively, our results indicate that M110 uses both kinetic discrimination and editing mechanisms to preferentially aminoacylate Gln over Glu.

DISCUSSION

Evolution of the GluRS/GlnRS family enzymes

It has been widely accepted that the indirect pathway of Gln-tRNAGln synthesis predates the direct pathway and
that GlnRS was absent in the last universal common ancestor (LUCA) (4,10,12). The GluRS in LUCA was a ND enzyme and charged Glu to both tRNA\textsubscript{Glu} and tRNA\textsubscript{Gln} (Figure 1). Glu-tRNA\textsubscript{Gln} was then converted to Gln-tRNA\textsubscript{Gln} by a Glu-AdT (GatCAB in chloroplasts and mitochondria of bacteria; GatFAB in mitochondria of yeast and GatDE in archaea) (4,25–28). GlnRS evolved in early eukaryotes from a duplicated ND-GluRS to gain

**Figure 3.** Active sites of GluRS and GlnRS. The crystal structures of (A) T. thermophilus GluRS (2CV0) and (B) E. coli GlnRS (1O0B) are shown. In parentheses are corresponding residues in H. pylori GluRS2. The rationally mutated residues are shown in purple and randomized residues are shown in blue.

**Figure 4.** Complementation of the glnS temperature-sensitive E. coli strain UT172. (A) GlnRS and M110 GluRS2, but not WT GluRS2, are able to rescue the growth of strain UT172 at 42°C on LB plates. (B) UT172 expressing M110 shows a slower growth rate compared with the strain expressing E. coli GlnRS at 42°C in LB broth. The results are the average of at least four repeats with standard deviations indicated.

**Table 1.** Pyrophosphate exchange activities of WT and M110 GluRS2 at 37°C

<table>
<thead>
<tr>
<th></th>
<th>Glu</th>
<th>Gln</th>
<th>Selectivity (Gln/Glu)</th>
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<tr>
<td></td>
<td>$k_{\text{cat}}$ ($\times 10^{-3}$ s$^{-1}$)</td>
<td>$K_m$ (mM)</td>
<td>$k_{\text{cat}}/K_m$ ($\times 10^{-3}$mM$^{-1}$ s$^{-1}$)</td>
</tr>
<tr>
<td>WT</td>
<td>18 ± 0.1</td>
<td>0.44 ± 0.07</td>
<td>41 ± 7</td>
</tr>
<tr>
<td>M110</td>
<td>18 ± 0.4</td>
<td>0.09 ± 0.02</td>
<td>190 ± 40</td>
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specificity for Gln and tRNA\textsuperscript{Gln} and was later transferred to certain bacteria (9,29). A group of proteobacteria, including \textit{H. pylori}, acquired a second GluRS (GluRS\textsubscript{2}) through horizontal gene transfer. GluRS\textsubscript{2} specifically recognizes tRNA\textsuperscript{Gln}, leading to the hypothesis that it has been evolving to become a bacterial-type GlnRS (7,8).

We show here that WT \textit{H. pylori} GluRS\textsubscript{2} already possesses the power to activate Gln, yet the resulting Gln-AMP may not be correctly positioned for the transfer of the amino acid moiety to tRNA. Our engineered GluRS\textsubscript{2} variant (M110) has gained Gln charging activity and thus represents an important precursor towards the evolution of a bacterial-type GlnRS. The aminoacylation activities of our GluRS\textsubscript{2} variants are low compared with previously published results (30), presumably because to be consistent with our \textit{in vivo} tests, we have used total \textit{E. coli} tRNA instead of purified \textit{H. pylori} tRNA\textsuperscript{Gln}. The heterologous tRNA used and the competition from non-cognate tRNAs could decrease the aminoacylation efficiency of GluRS\textsubscript{2}.

**Structural insights into substrate recognition by WT and M110 GluRS\textsubscript{2}**

WT GluRS\textsubscript{2} specifically attaches Glu (but not Gln) to tRNA, although it also activates Gln during the pyrophosphate exchange assay (Figure 5 and Table 1). In contrast, the M110 variant acylates Gln 4-fold more efficiently than Glu (Figure 5). We propose that WT GluRS\textsubscript{2} binds activated Gln-AMP in a mode not suitable for aminoacylation. Structural studies of \textit{T. thermophilus} GluRS reveal that R205 (equivalent of R192 in \textit{H. pylori} GluRS\textsubscript{2} and C229 in \textit{E. coli} GlnRS) directly interacts with the side-chain oxygen of Glu (Figure 3). This residue might serve as a negative determinant for proper positioning of Gln-AMP during the amino acid transfer step. The evolved M110 variant contains a R192C mutation, which could be critical for glutaminylation. In line with this notion, a C229R mutation has been shown to significantly improve the \textit{K_m} of \textit{E. coli} GlnRS for Glu (31). It is interesting that the M110 variant maintains the same glutamylation activity as in the WT, presumably due to the cumulative effects of other mutations present in M110.

**Quality control mechanisms in natural and engineered aaRSs**

Selection of the correct amino acid is a big challenge for many aaRSs due to the structural and chemical similarities between amino acids. To maintain translational fidelity, such aaRSs use editing mechanisms to hydrolyze incorrect aminoacyl adenylates (pre-transfer editing) or aa-tRNAs (post-transfer editing) (32,33), whereas post-transfer editing requires a separate editing domain or a free-standing protein (34–36) and pre-transfer editing mainly occurs at the active site (21,37,38). It is intriguing that our engineered M110 GluRS\textsubscript{2} appears to have evolved an editing mechanism to discriminate against Glu in favor of Gln (Figure 6). GluRS\textsubscript{2} lacks a post-transfer editing domain, prompting us to hypothesize that M110 uses a pre-transfer editing strategy to hydrolyze activated Glu-AMP, presumably at the active site resembling \textit{E. coli} GlnRS (20). It is worth noting that a recent study shows that WT GluRS\textsubscript{2} modestly increases the hydrolysis of Glu-tRNA\textsuperscript{Gln} by an unknown mechanism (30). The fidelity mechanism for GluRS\textsubscript{2} thus remains to be addressed in future studies. Should editing occur at the active site before amino acid transfer, a water molecule is likely required for hydrolysis of Glu-AMP and Gln-AMP. We have recently shown that yeast mitochondrial threonyl-tRNA synthetase edits seryl adenylate faster than threonyl adenylate due to the minor movement of a potential catalytic water molecule (Ling \textit{et al.}, unpublished work). A similar mechanism could explain the preferential hydrolysis of Glu-AMP over Gln-AMP by M110, which needs to be clarified by future structural studies.

![Figure 5. Aminoacylation by GluRS\textsubscript{2} variants. WT GluRS\textsubscript{2} (2.3 \textmu M) charges Glu (40 \textmu M) but not Gln (40 \textmu M) to tRNA\textsuperscript{Gln} (5 mg/ml total \textit{E. coli} tRNA containing 3 \textmu M tRNA\textsuperscript{Gln} as determined by plateau charging), whereas M110 (2.3 \textmu M) preferentially aminoacylates Gln over Glu at both 37 and 42°C.](https://academic.oup.com/nar/article-abstract/40/16/7967/1031005/7972)
Several aaRSs have been successfully engineered to co-translationally incorporate a variety of unnatural amino acids into proteins in bacteria and eukaryotes (15,39–46). Both rational design and directed evolution methods have been used for unnatural amino acid incorporation, yet only a few studies have used such methods to understand the evolution of natural aaRSs. A rationally engineered *E. coli* GlnRS has obtained a misacylation activity to produce Glu-tRNAGln (31), and transplanting the GlnRS acceptor stem loop to an archaeal ND-GluRS makes it specifically recognize tRNA\(^{\text{Gln}}\) (29). In this work, we have combined rational design and directed evolution approaches to understand the evolutionary potential from GluRS2 to GlnRS. Such a strategy could be used as a model to investigate how primordial aaRSs have acquired new functions to become the enzymes present in modern organisms.

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