Recoding Aminoacyl-tRNA Synthetases for Synthetic Biology by Rational Protein-RNA Engineering

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Supporting Information

ABSTRACT: We have taken a rational approach to redesigning the amino acid binding and aminoacyl–tRNA pairing specificities of bacterial glutaminyl–tRNA synthetase. The four-stage engineering incorporates generalizable design principles and improves the pairing efficiency of noncognate glutamate with tRNA glutamate by over 10⁵-fold compared to the wild-type enzyme. Better optimized designs of the protein–tRNA complex include substantial reengineering of the globular core region of the tRNA, demonstrating a role for specific tRNA nucleotides in specifying the identity of the genetically encoded amino acid. Principles emerging from this engineering effort open new prospects for combining rational and genetic selection approaches to design novel aminoacyl–tRNA synthetases that ligate noncanonical amino acids onto tRNAs. This will facilitate reconstruction of the cellular translation apparatus for applications in synthetic biology.

The expansion of genetic codes through incorporation of noncanonical amino acids (ncAAs) offers substantial potential to develop designer proteins capable of yielding new insights into cellular processes.¹,² Engineering of the translational apparatus is also a key foundational technology in synthetic biology, with possible applications to metabolic pathway reconstruction, design of antimicrobial resistance, cancer therapeutics, and other areas.³ These aspirations depend on the creation of novel aminoacyl–tRNA synthetases (aaRS) capable of ligating one or more ncAAs to a new tRNA that reads an unused codon triplet or quadruplet—most commonly the UAG amber stop codon.⁴–⁷ A directed evolution approach involving both positive and negative selections, applied to libraries targeting an aaRS active site, has allowed incorporation of over 100 ncAAs in bacterial or eukaryotic cells.⁵,⁸,⁹ The choice of which aaRS scaffold to employ in these selections has been largely dictated by the requirement for orthogonality: neither the new aaRS nor the new tRNA may substantially cross-react with endogenous parallel aaRS–tRNA systems. In some cases, orthogonality may also require that similar positive and negative selections be carried out to optimize the tRNA sequence.¹⁰,¹¹

Although this directed evolution approach has been successful in producing ncAA-containing proteins in vivo, better integration of the new aaRS–tRNA pairs into the translational apparatus requires further efforts. Very high concentrations of ncAAs and overexpression of tRNAs and engineered aaRS are presently needed for efficient protein synthesis, but these features diminish cellular fitness by increasing competitions with endogenous aaRS for both amino acid and tRNA pools.⁶,¹²,¹³ Further, limits to the number of amino acid positions that can be sampled in libraries have generally confined explorations of aaRS structures to the immediate amino acid binding site environments. The development of multiplex automated genome engineering (MAGE) greatly streamlines library constructions and allows for introduction of mutations at many loci but does not solve the problem of where to target.¹⁴ Limitation of the selections to a small number of aaRS scaffolds, primarily methanogen tyrosyl– and pyrrolysyl–tRNA synthetases (TyrRS; PylRS), likely further limits the range of ncAAs that can ultimately be incorporated.⁸,¹⁵,¹⁶

Weak activities of genetically selected aaRS in amino acid activation and tRNA transfer,⁶,⁸,¹⁷ and susceptibilities of enzyme-bound noncanonical aminoacyl adenylates to competitive attack by water,¹⁸,¹⁹ underlie the requirements for high concentrations of ncAAs and orthogonal tRNAs in selections. We suggest that these deficiencies in the catalytic performance of aaRS enzymes emerging from directed evolution may be remediated through methodologies that incorporate rational design.⁶ This approach is facilitated by reliable crystal structures bound to cognate amino acid for nearly all of the 24 phylogenetically independent families of aaRS.²⁰ The aaRS families are partitioned into two structural classes (class I and...
class II), possessing class-specific homologous catalytic domains. Within each class, the exhaustive sequence information for aaRS (and tRNAs) then allows construction of structure-based alignments and predictions of selectivity determinants for coded amino acids. An early application of this rational design approach to a different enzyme family was the successful identification of chymotrypsin specificity determinants by transplantation of amino acids into the homologous trypsin scaffold. We posited that rational design could be similarly effective in elucidating how homologous aaRS architectures have differentiated to select among the large number of amino acids present in the cell. This approach is analogous to the transplantation of proposed tRNA identity nucleotides into noncognate tRNA frameworks, which is long established in the aaRS field. Insights derived from this rational engineering should then assist in formulating new approaches to obtain novel aaRS capable of encoding ncAAs for synthetic biology.

We have chosen Escherichia coli glutaminyl-tRNA synthetase (GlnRS) as a model class I enzyme for rational design based on extensive structural and mechanistic studies, and on its function as a relatively small 553 amino acid monomer (Figure 1A). GlnRS requires tRNA^{Glu} as a cofactor for synthesis of the activated glutaminyl adenylate intermediate and is thus properly considered as a ribonucleoprotein (RNP) enzyme. Previously, we reported early steps in a multistage design process in which amino acids derived from human glutamyl-tRNA synthetase (GluRS) were introduced into the GlnRS scaffold, and GlnRS originated in early eukaryotes from the duplication and subsequent differentiation of a gene encoding a nondenaturing archaean GluRS (GluRS^{arch}) capable of aminoacylating both tRNA^{Glu} and tRNA^{Gln} with glutamate (Glu). The presence of GlnRS in some contemporary bacterial taxa is solely a consequence of horizontal gene transfer from eukaryotes, and the enzyme is more closely related to eukaryotic than bacterial GluRS.

All GluRS possess two arginine (Arg) residues that bind the Glu substrate carboxylate group, while all GlnRS retain only one Arg (Figure 1B; Supporting Information Figure 1). We previously showed that introduction of the second Arg into GluRS (GlnRS C229R) lowers $K_m$(Glu) from >750 mM to 240 mM, but without improving $k_{cat}/K_m$ for Glu-tRNA^{Glu} synthesis (which is diminished by 10^{5}-fold compared to Gln-tRNA^{Gln} synthesis by wt GlnRS (Table 1)). Most GlnRS and GluRS also possess three additional distinguishing residues in their homologous primary amino acid binding sites (S1 sites). Further introduction of these three GluRS residues into GlnRS generated the S1 GlnRS enzyme (Table 1; Figure 1B), which exhibited 20-fold improved $k_{cat}/K_m$ for Glu-tRNA^{Glu} synthesis compared to WT GlnRS. However, S1 GlnRS remains highly inefficient, with $k_{cat}/K_m$ still reduced by nearly 10^{4}-fold compared to Gln-tRNA^{Gln} synthesis by wt GlnRS. This full replacement of all first-shell protein residues represents the first stage of the rational design effort. It demonstrated that determinants of amino acid selectivity in GlnRS must be primarily located outside of the immediate amino acid binding pocket. Neither S1 GlnRS nor the enzymes produced in the later design stages retain any detectable activity for cognate Glu-tRNA^{Gln} synthesis.

In the second stage of design, we examined more distal elements of protein structure within the second half of the catalytic Rossmann fold (RF), which is primarily responsible for amino acid binding in class I aaRS (Figure 1C). Because protein sequence alignments did not identify significant conserved differences between GlnRS and GluRS enzymes outside of the S1 site (Supporting Information Figure 1), we swapped distal human GluRS peptides into S1 GlnRS based on structural considerations. Replacement of two surface loops bridging RF secondary structure elements (loops L1 and L2; Figure 1C) improved $k_{cat}/K_m$ for Glu-tRNA^{Glu} synthesis by 10^{5}-fold compared to S1 GlnRS and reduced $K_m$(Glu) by 40-fold (L1L2 GlnRS; Table 1). Replacement of distal surface loops in serine proteases similarly effected conversion of amino acid selectivity in the context of peptide bond hydrolysis, providing inspiration for these experiments.

In the third design stage, we examined all interactions made by the 23 replaced amino acids in L1L2 GlnRS with surrounding regions of the protein structure. This allowed assessment of the role of selected third-shell residues in amino acid substrate discrimination and of the potential for non-complementarity between the introduced mutations and surrounding regions of the protein. By this process, we found that the W256Y mutation, representing a reversion back to the GlnRS sequence, improved $K_m$(tRNA) and $K_m$(Glu) each by 2-fold compared to L1L2 GlnRS—probably by alleviating a steric clash with nearby Ile14 (Table 1; Figure 1D).
GlnRS is well below the intracellular Glu concentration in E. coli and is lower than measured for yeast GluRS and archaeal GluRS ND (Table 1).27,29 The \( k_{cat} \) in L1L2W256Y GlnRS is identical to GluRS ND, although it remains 15- to 30-fold lower than that of E. coli GlnRS or yeast GluRS. However, \( K_m(tRNA) \) is 15- to 125-fold higher than measured in cognate aminoacylation by any of the three benchmark enzymes annotated in Table 1, despite the fact that

|                      | \( k_{cat} \) (s\(^{-1}\)) | \( K_m[tRNA] \) (μM) | \( k_{cat}/K_m[tRNA] \) (s\(^{-1}\) M\(^{-1}\)) | \( K_m[GLU] \) (mM) | \( k_{cat}/K_m[GLU] \) (s\(^{-1}\) M\(^{-1}\)) |
|----------------------|--------------------------|----------------------|-----------------------------------------------|------------------|-----------------------------------------------|
| E. coli GlnRS\(^a\)  | tRNA\(^{GLN}\)           | 0.046 ± 0.013        | 19 ± 3                                        | 2.4 \times 10^3  | >750                                          | 9.5 \times 10^{-3}  |
| C229R GlnRS\(^b\)   | tRNA\(^{GLN}\)           | 3.2 ± 0.1 x10^{-4}   | 240 ± 10                                       | 1.3 \times 10^{-3} |
| S1 GlnRS\(^b\)      | tRNA\(^{GLN}\)           | 5.0 ± 0.6 x10^{-3}   | 230 ± 17                                       | 2.2 \times 10^{-3} |
| L1L2\(^b\)          | tRNA\(^{GLN}\)           | 0.09 ± 0.02          | 7.6 ± 3.0                                      | 1.2 \times 10^{4}  | 5.8 ± 0.5                                     | 15.5                  |
| L1L2 W256Y\(^c\)    | tRNA\(^{GLN}\)           | 0.10 ± 0.04          | 4.8 ± 0.2                                      | 2.1 \times 10^{3}  | 2.6 ± 1.0                                     | 38.5                  |
| L1L2 W256Y\(^d\)    | tRNA\(^{GLN}\)           | 0.014 ± 0.01         | 1.2 ± 0.4                                      | 1.2 \times 10^{3}  | 14 ± 7                                        | 1.0                   |
| L1L2 W256Y           | tRNA\(^{GLN}\)           | 0.05 ± 0.01          | 1.4 ± 0.7                                      | 3.6 \times 10^{3}  | 53 ± 22                                       | 0.9                   |
| L1L2 W256Y           | tRNA\(^{GLN}\)           | 0.32 ± 0.05          | 2.4 ± 0.8                                      | 1.3 \times 10^{3}  | 1.9 ± 0.2                                     | 168.4                 |
| L1L2 R237D/R238E/W256Y| tRNA\(^{GLN}\)          | 0.069 ± 0.01         | 0.74 ± 0.07                                    | 9.3 \times 10^{3}  | 3.3 ± 0.6                                     | 20.9                  |
| L1L2 R237D/R238E/W256Y| tRNA\(^{GLN}\)          | 0.092 ± 0.01         | 3.4 ± 0.7                                      | 2.7 \times 10^{3}  | 12 ± 5                                        | 7.7                   |
| L1L2 R237D/R238E/W256Y| tRNA\(^{GLN}\)          | 0.29 ± 0.05          | 3.4 ± 0.5                                      | 8.5 \times 10^{3}  | 3.0 ± 0.7                                     | 97.7                  |

**Table 1. Steady State Aminoacylation Parameters**

\(^a\)Reported in ref 25. All values in this table reflect glutamylation except for the benchmark represented by E. coli GlnRS for its cognate glutaminylation reaction (E. coli GlnRS + GLN). The value 0.26 ± 0.04 represents \( K_m[GLN] \). No activity with GLN as a substrate is detectable for S1 GlnRS or for any of the L1L2 hybrids. \(^b\)Reported in ref 26. S1 GlnRS includes the following mutations: C229R/Q255I/S227A/F233Y. S1L1L2 GlnRS adds the following mutations: T214A/H215C/C216P/S218V/A220S/L221I/I224V/L231T/V243I/L244I/D245E/N246A/I247L/T248G/P250R/ΔV251/H252K/R254Y/Y256W \(^c\)Reported in ref 27. \(^d\)Sequences of hybrid Q/E tRNAs are provided in Figure 2 and the Supporting Information. \(^e\)Reported in ref 32.

Figure 2. tRNA structure and protein interactions. Left: tertiary structure of tRNA\(^{Glun}\) in the conformation bound to GlnRS. The 3–70 and 5–68 base pairs replaced in the acceptor stem are shown in green. The C16 and A59 core region nucleotides, replaced with U16 and U59 in some of the hybrid RNPs, are shown in red, while the remainder of the replaced tRNA core region is shown in green and consists of the C9G substitution together with replacements of the full D and variable loops. Upper right: Structural details of the protein–tRNA interface showing the backbone contacts made at the G5 phosphate of tRNA\(^{Glun}\) by Arg237 and A238 (dark blue). Bottom right: secondary structure depictions of human tRNA\(^{Glu}\) and E. coli tRNA\(^{Gln}\).
no mutations at the tRNA^Gln_ interface were introduced. Therefore, while alterations to protein structure alone fully recreate a functional Glu binding site in GlnRS, the resulting hybrid enzyme remains deficient in pairing amino acid with tRNA. These observations underscore the interdependence of amino acid and tRNA binding in GlnRS as previously illustrated by the sharply elevated K_m(tRNA) for Glu–tRNA^Gln by the wt enzyme (Table 1). Although comparable protein engineering experiments have not been performed with other aaRS, it is likely that such substrate interdependence is embedded in the architectures of all aaRS from both structural classes.

These observations led us to consider a fourth rational design stage to explore the possibility that RNA structure is required for amino acid coding. Sequence comparisons among bacterial tRNA^Gln_ and eukaryotic tRNA^Glu species reveal conserved differences in the acceptor arm at positions 3–70 and 5–68, and in the globular core at positions 16 and 59 in the T and D loops, respectively (Figure 2A,B). We began by pairing GlnRS L1L2W256Y with a mutant tRNA^Gln_ in which the G3–C70 and G5–C68 pairs were altered to C3–G70 and U5–A68, respectively (Q/E_tRNA1; Figure 2). Remarkably, K_m(tRNA) is reduced 4-fold by incorporation of these tRNA^Gln_ acceptor-stem base pairs, suggesting improved complementarity at the protein–RNA interface in a region close to the introduced eukaryotic GluRS peptides (Table 1; Supporting Information Figure 2). However, k_cat for Glu–tRNA^Gln_ synthesis is diminished 7-fold while K_m(Glu) is elevated 5-fold in this hybrid RNP, thus diminishing its overall catalytic performance.

These findings suggest an allosteric mechanism connecting protein–RNA contacts at base pairs 3–70 and 5–68 in the acceptor stem, with both the Glu binding site and the catalytic center where the two-step aminocacylation reaction occurs. To test this, we examined distinguishing bacterial GlnRS and eukaryotic GlnRS interactions at these acceptor-stem positions. Arg237 and Arg238 in E. coli GlnRS, which interact with the sugar–phosphate backbone at nucleotide G5 (Figure 2), are replaced by Asp237 and Glu238 in GluRS. We therefore constructed the L1L2W256Y/R237D/R238E GlnRS enzyme repressed by Asp237 and Glu238 in GluRS. We therefore constructed the L1L2W256Y/R237D/R238E GlnRS enzyme and examined its capacity to glutamylate Q/E_tRNA1. Remarkably, K_m(tRNA) is reduced 4-fold by incorporation of these tRNA^Gln_ acceptor-stem base pairs, suggesting improved complementarity at the protein–RNA interface in a region close to the introduced eukaryotic GluRS peptides (Table 1; Supporting Information Figure 2). However, k_cat for Glu–tRNA^Gln_ synthesis is diminished 7-fold while K_m(Glu) is elevated 5-fold in this hybrid RNP, thus diminishing its overall catalytic performance.

Next, we examined the role of the tRNA core domain. Introducing the C16U and A59U mutations into GlnRS, which interact with the sugar–phosphate backbone at nucleotide G5 (Figure 2), were replaced by Asp237 and Glu238 in GluRS. We therefore constructed the L1L2W256Y/R237D/R238E GlnRS enzyme and examined its capacity to glutamylate Q/E_tRNA1. k_cat for Glu–tRNA^Gln_ synthesis is fully reconstituted in this RNP, and K_m(Glu) is unchanged, but K_m(tRNA) is elevated 3-fold (Table 1). The reconstitution of k_cat indeed suggests intramolecular signaling of acceptor-stem backbone interactions to the catalytic center, but the other measurements indicate that this subregion of the protein–RNA interface does not operate as an independent module but instead depends on other portions of the RNP for its function.

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the fact that the aaRS scaffolds were not under selective pressure to resist these ncAAs. However, our experiments suggest that directed evolution will not succeed in generating highly efficient enzymes unless the second/third shell protein residues and the allostery between amino acid and tRNA substrates are also considered. All aaRS are large multidomain proteins that bind tRNA in a mutual induced fit process that likely proceeds by formation of an initial encounter complex, followed by first-order rearrangements that may also be affected by the binding of ATP and amino acid. These common features observed in X-ray structures predict that allosteric functional linkages between tRNA and amino acid binding, and the broad dispersal of amino acid specificity determinants, will also be prevalent if not universal among the aaRS.

Although the design of ncAA-coding aaRS cannot benefit from sequence databases, one approach that may allow generation of more catalytically proficient enzymes could be to rationally engineer sets of hybrid methanogen TyrRS and PylRS that incorporate distal structural elements from other homologous class 1c and class 2c aaRS, respectively. This would substantially expand the number of subtly distinct scaffolds for use in genetic selections (Figure 3). For example, structural elements from the closely homologous class 1c TrpRS could be swapped into the TyrRS scaffold to generate hybrids capable of Trp-tRNA<sub>Tyr</sub> synthesis; these hybrids would then provide a starting point for directed evolution. The exquisite sensitivities of naturally occurring aaRS to mutational perturbation suggests that even relatively small differences in active site structures and dynamics, within families of such rationally engineered hybrid enzymes, could generate significant differences in capacities for ncAA incorporation when subjected to directed evolution. Systematic rational engineering of methanogen TyrRS and PylRS along the lines described here would allow assessment of the nature and spatial distribution of amino acid specificity determinants, and the extent to which they may reside in the RNA. Precise choices of residues to be randomized, within the context of rationally engineered scaffolds, will clearly best be made when a quantitative database of structure–function information is available.

A second insight arising from these experiments is that directed evolution strategies that include selections for optimizing orthogonality in the tRNA should account for the possibility that the derived tRNAs may acquire nucleotides that render them less efficient for incorporation of a desired ncAA. This notion emerges from our finding that tRNA<sub>Glu</sub> core elements are positive determinants for encoding Glu. Careful measurements of kinetic parameters for aminoacylation of ncAAs by selected RNPs are required to evaluate this and other detailed properties of ncAA-encoding aaRS. RNA and protein sequences in ncAA-encoding RNPs should be optimized for both orthogonality and efficiency of aminoacylation.

Finally, it is worth noting that none of the efficiently functioning hybrid RNPs constructed in our studies alter any part of the tRNA anticodon arm, despite the important role for tRNA anticodon loop nucleotides in specifying tRNA<sub>Glu</sub> and tRNA<sub>Glu</sub> identities. Although further experiments are required to fully evaluate this notion, it appears that anticodon recognition by GlnRS may be less fully integrated into the protein–tRNA allosteric network than we have previously suggested. Perhaps either GlnRS-like or GluRS-like anticodon recognition may be substantially compatible with coding of either amino acid. The relative independence of anticodon recognition is beneficial to the further development of orthogonal translation systems.

**METHODS**

Construction and expression of mutant enzymes was performed as described previously. tRNAs were generated by in vitro transcription from templates assembled from overlapping nucleotides and were refolded and further purified as described. Modified nucleotides do not have kinetic effects in this system. All enzyme assays utilized tRNAs that are <sup>32</sup>P-labeled at the 3′-internucleotide linkage via the exchange activity of tRNA nucleotidyltransferase. Plateau aminoacylation values were 65% or better for all measurements. Details of experimental approaches and representative primary data, protein sequence alignments, discussions of the rationale for the kinetics methodology chosen, and calculations of free energy additivities may be found in the associated Supporting Information.

**ASSOCIATED CONTENT**

* Supporting Information

Details of experimental approaches and representative primary data, protein sequence alignments, discussions of the rationale for the kinetics methodology chosen, and calculations of free energy additivities. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**

The authors declare no competing financial interest.

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DEDICATION

This paper is dedicated to the memory of Martin John Rogers, outstanding scientist and public servant, who made major contributions to our understanding of bacterial GlnRS.

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