

Biochemistry. Author manuscript; available in PMC 2011 August 10.

Published in final edited form as:

Biochemistry. 2010 August 10; 49(31): 6727–6736. doi:10.1021/bi100886z.

Synthesis of Glu-tRNA^{GIn} by engineered and natural aminoacyl-tRNA synthetases[†]

Annia Rodríguez-Hernández, Hari Bhaskaran, Andrew Hadd, and John J Perona^{*} Department of Chemistry & Biochemistry, and Interdepartmental Program in Biomolecular Science and Engineering, University of California Santa Barbara, CA 93106-9510

Abstract

A protein engineering approach is described to delineate which distinct elements of homologous tRNA synthetase architectures are responsible for divergent RNA-amino acid pairing specificities. Previously, we constructed a hybrid enzyme in which 23 amino acids from the catalytic domain of E. coli glutaminyl-tRNA synthetase (GlnRS) were replaced with the corresponding residues of human glutamyl-tRNA synthetase (GluRS). The engineered hybrid (GlnRS S1/L1/L2) synthesizes Glu-tRNA^{Gln} over 10⁴-fold more efficiently than GlnRS. Detailed comparison of kinetic parameters between GlnRS S1/L1/L2 and the naturally occurring M. thermautotrophicus GluRSND, which is also capable of Glu-tRNA^{Gln} synthesis, now shows that both k_{cat} and K_{m} for glutamate are recapitulated in the engineered enzyme, but that $K_{\rm m}$ for tRNA is 200-fold higher. Thus, the simultaneous optimization of paired amino acid and tRNA binding sites found in a naturally occurring enzyme is not recapitulated in a hybrid that is successfully engineered for amino acid complementarity. We infer that the GlnRS architecture has differentiated to match only cognate amino acid-RNA pairs, and that the substrate selection functions do not operate independently of each other. Design and characterization of four additional hybrids identifies further residues involved in improving complementarity for glutamate, and in communicating between amino acid and tRNA binding sites. The robust catalytic function demonstrated in this engineered system offers a novel platform for exploring the stereochemical origins of coding as a property of the ancient Rossmann fold.

Aminoacyl-tRNA synthetases translate the genetic code by catalyzing the specific pairing of amino acids and tRNAs (1). In a common two-step reaction pathway, an activated aminoacyl adenylate is first synthesized from amino acid and ATP. In the second step, one of the hydroxyl groups located on the 3'-terminal ribose sugar of the tRNA attacks the carbonyl carbon of the mixed anhydride linkage of the adenylate intermediate, generating aminoacyl-tRNA with release of AMP. The tRNA synthetases specific for glutamine, glutamate, lysine, and arginine (GlnRS, GluRS, class I LysRS, ArgRS)1 form a subgroup that function only as ribonucleoprotein (RNP) catalysts. These four enzymes, which possess a common Rossmann fold catalytic domain, cannot detectably synthesize the aminoacyl adenylate intermediate in the absence of tRNA.

[†]Supported by grant RO1GM063713 from the National Institutes of Health (to J.J.P.)

^{*}To whom correspondence should be addressed. Department of Chemistry & Biochemistry, University of California, Santa Barbara CA 93106-9510. Tel. 805-893-7389; FAX. 805-893-4120; perona@chem.ucsb.edu.

¹Abbreviations: GlnRS, glutaminyl-tRNA synthetase; GluRS, glutamyl-tRNA synthetase; LysRS, lysyl-tRNA synthetase; ArgRS, arginyl-tRNA synthetase; IleRS; isoleucyl-tRNA synthetase; WT, wild-type; RNP, ribonucleoprotein; SDS, sodium dodecyl sulfate; $K_m(AA)$, K_m value for the amino acid; $K_m(Glu)$, K_m value for the amino acid; $K_m(AA)$, K_m value for the tRNA

The glutamyl and glutaminyl-tRNA synthetase families share a common evolutionary history. Phylogenetic analysis has shown that an ancestral GluRS enzyme possessing a catalytic Rossmann fold acquired distinct tRNA binding modules upon separation of the bacterial and archaeal domains (2,3). Bacterial GluRS acquired an α -helical anticodon-binding domain, as exemplified by crystal structures of the *Thermus thermophilus* enzyme (4). In contrast, archaeal GluRS acquired anticodon domains that form β -barrel structures. After the divergence of the eukaryal lineage, duplication of the gene encoding GluRS allowed differentiation of GlnRS for the first time. Thus, cytoplasmic GluRS and GlnRS from eukaryotes are of the β class. Subsequently, horizontal transfer of eukaryotic GlnRS to a number of bacterial phyla established the enzyme in that domain (5). In these bacteria, then, α -class GluRS coexist with β -class GlnRS, and the enzymes are relatively distantly related. The β -barrel anticodon domain structures of β -class enzymes was first revealed by the crystal structure of *E. coli* GlnRS (6).

Most GluRS enzymes in organisms that lack GlnRS are nondiscriminating (GluRSND): they are able to synthesize both Glu-tRNA^{Glu} and Glu-tRNA^{Gln} (7,8). The Glu-tRNA^{Gln} is then converted to Gln-tRNA^{Gln} by ATP and tRNA-dependent amidotransferase enzymes (9). Thus, these organisms are able to use glutamine as an encoded amino acid despite the absence of GlnRS. GluRSND are found in all archaea, in bacteria that lack GlnRS, and in eukaryotic organelles (10,11). A GluRSND enzyme may be either of the α -class (bacteria) or β -class (archaea): crystal structures of both varieties have been reported (12,13). In some bacteria, GluRS gene duplication has also occurred. One GluRS enzyme in these organisms appears to be dedicated to Glu-tRNA^{Gln} synthesis alone, and hence may represent a third class of GluRS specificity beyond the discriminating (GluRS^D) and nondiscriminating (GluRSND) enzymes (14,15). This more recently recognized GluRS variety, characterized from *Helicobacter pylori* and *Acidothiobacillus ferrooxidans*, has been termed GluRS2.

We have been interested in the origins of amino acid specificity in the related GluRS and GlnRS enzyme families (referred to jointly as the GlxRS family). Because substantial evidence for the well-studied E. coli GlnRS suggests that the tRNA and amino acid binding are interdependent (16–19), and because all GlxRS are thought to function as RNP enzymes, we have also developed the notion that certain tRNA nucleotides may assist the protein active site in selecting the proper amino acid (20). To examine the origins of amino acid discrimination among GlxRS enzymes, we introduced amino acids from the primary amino acid binding site of the evolutionarily related β-class human GluRS, into E. coli GlnRS. The resulting enzyme (GlnRS C229R/Q255I/S227A/Y233F) catalyzes Glu-tRNA^{Gln} synthesis 20-fold more efficiently than WT GlnRS, as judged from measurements of k_{cat} and K_{m} for glutamate [K_m(Glu)] (20). Further introduction of two distal surface loops bridging core secondary structural elements of the Rossmann fold then produced a hybrid enzyme (GlnRS S1/L1/L2) that synthesizes Glu-tRNA^{Gln} 1.6×10^4 -fold faster than WT GlnRS (21), with $[K_{\rm m}({\rm Glu})]$ reduced by at least 130-fold (Fig. 1A). However, $k_{\rm cat}/K_{\rm m}$ with respect to glutamate, for aminoacylation by this hybrid enzyme, remains 800-fold reduced as compared with cognate aminoacylation by WT GlnRS. The hybrid enzyme is completely inactive in the synthesis of Gln-tRNA^{Gln}, indicating that a bona fide switch in specificity has occurred.

The GlnRS S1/L1/L2 enzyme provides an experimental platform for exploring how the evolutionarily related GlnRS and GluRS sequences have become differentiated for selective pairing of cognate amino acid and tRNA. One important question concerns whether amino acid selectivity in the GlxRS family is associated entirely with the protein component of the RNP. Given the requirement for tRNA in aminoacyl adenylate synthesis, a plausible alternative hypothesis is that certain tRNA nucleotides function to help exclude noncognate

amino acid substrates. The idea that particular tRNA nucleotides may assist amino acid discrimination has also been considered in the context of amino acid editing by IleRS (22).

We have performed a number of experiments to further test the notion that differentiation of the GluRS and GlnRS protein structures alone may provide selectivity for the amino acid substrate, in the context of efficient aminoacylation. Mutations were introduced at four further positions within the second half of the Rossmann fold of the GlnRS S1/L1/L2 hybrid, to examine the contribution of additional amino acids, and to assess whether noncomplementarity with adjacent regions of GlnRS might mask higher catalytic efficiency. The ability of the hybrid enzymes to provide effective complementarity for tRNA was assessed by measuring tRNA $K_{\rm m}$ values, and by examining the role of GlnRS Thr231 in a structural element bridging the amino acid and tRNA binding sites. Finally, we address the important question of how to benchmark the activities of the hybrid enzymes, by measuring steady-state kinetic parameters for Glu-tRNA $^{\rm Gln}$ synthesis by a naturally occurring GluRS $^{\rm ND}$ from the β class, and by comparing these values to those of the hybrids and of other GluRS enzymes.

EXPERIMENTAL PROCEDURES

Enzyme construction and purifications

Mutations in the gene encoding GlnRS S1/L1/L2 containing a C-terminal hexahistidine affinity tag were constructed using the Quikchange protocol (Stratagene), and were confirmed by DNA sequencing. Expression of all *E. coli* GlnRS mutants studied here was achieved in the context of an N-terminal signal sequence tag directing the protein to the periplasm; this added tag is without effect on the steady-state kinetic parameters of WT GlnRS (20). Mutants were expressed as N-terminal fusions with the leader sequence of the bacterial fd gene III protein (Invitrogen). As described for GlnRS S1/L1 and GlnRS S1/L1/L2, high-level expression of the mutants was achieved by addition of 1 mM IPTG and 10 mM β -mercaptoethanol to the culture at the time of induction (A₆₀₀ ~ 0.4). Purification of the mutants by affinity chromatography on a nickel resin yielded enzyme preparations at better than 98% purity, as judged by SDS polyacrylamide gel electrophoresis. The hybrid enzymes were stored at high concentration at -20° C, in a buffer containing 50 mM NaCl, 50 mM potassium phosphate (pH 7.2), 5 mM β -mercaptoethanol, and 50% glycerol. Enzymes were quantitated based on E₂₈₀ [1 mg/ml] = 1.06.

The Methanothermobacter thermautotrophicus GluRSND gene cloned into a pCYB1 vector (New England Biolabs) was the generous gift of Kelly Sheppard and Dieter Söll. The enzyme was expressed in Rosetta cells pLysS and purified as described (23). Briefly, the cells were grown at 37°C to A_{600} of 0.8, induced with 0.5 mM IPTG, and allowed to grow overnight at 25°C. The cells from 1 liter of saturated culture were centrifuged and resuspended in 35 mL of lysis buffer containing 20 mM sodium Hepes (pH 8.5) and 500 mM NaCl, followed by addition of 150 μg DNAse I, 50 mg lysozyme, and 1 pellet protease inhibitor (Complete Mini, EDTA-free protease inhibitor cocktail tablets - Roche Diagnostics, GmbH, Germany). The cells were sonicated for 5 min with an alternating 2 sec pulse on and pulse off mode using a Branson 450 digital sonicator (Danbury, CT). The cellfree lysate was then centrifuged at 15,000 × rpm for 45 min and the supernatant applied to a pre-equilibrated Chitin resin. GluRSND was cleaved from the column by overnight incubation with 50 mM DTT at 37°C; recovered material was dialyzed against and stored in a buffer containing 25 mM HEPES-KOH pH 7.2, 500 mM NaCl, 1 mM DTT, and 50% glycerol. Yield of purified enzyme from one liter of cell culture is about 2 mg. The purity is estimated to be greater than 95% as judged by SDS polyacrylamide gel electrophoresis.

Preparation of tRNA substrates

Duplex DNA templates for *in vitro* transcription of *E. coli* tRNA^{Gln} (CUG) and *M. thermautotrophicum* tRNA^{Gln} (CUG) were synthesized from two single-stranded oligodeoxynucleotides containing a complementary overlap duplex region, as described (24). For the *M. thermautotrophicum* tRNA, the oligonucleotides used were:

5' AATTCCTGCAGTAATACGACTCACTATAAGTCCCGTGGGGTAATGGCAATCC TGAT

s5'
mUmGGTAGTCCCGAGCGGAGTCGAACCGCTATCGATGGATCCAGAGTCCAT
CA

GATTG where the underlined region represents the T7 RNA polymerase promoter sequence. For both tRNAs, the two 3'-terminal deoxynucleotides on the noncoding strand incorporated 2'-O-methyl sugars (mU and mG in the sequences), to improve the fidelity of transcription termination by T7 RNA polymerase. This thereby generates a high proportion of enzymatically active tRNA transcripts. The *E. coli* tRNA^{Gln} gene was synthesized with a catalytically neutral U1G mutation to promote efficient transcription initiation (24).

Milligram quantities of each tRNA were transcribed with the Del(172–173) variant of T7 RNA polymerase, as described (24,25); we found that the presence of A1 in *M. thermautotrophicus* tRNA^{Gln} reduced yields by only two-fold. For each tRNA, the transcription reactions were loaded on 5 mL DE-52 (Whatman) columns, eluted, precipitated with ethanol, dried, and stored either dried (*E. coli* tRNA^{Gln}) or in 10 mM Tris (pH 8.0), 1 mM EDTA (*M. thermautotrophicum* tRNA^{Gln}) at –20 °C. Prior to use the tRNAs were resuspended in highly purified water or in 10 mM Tris, 1 mM EDTA.

Enzyme kinetics

The aminoacylation assays were performed using E. coli or M. thermautotrophicum tRNA^{Gln} transcripts ³²P-labeled at the 3'-terminal internucleotide linkage using the exchange reaction of tRNA nucleotidyltransferase, as described (19,21,26). For E. coli tRNA^{Gln}, labeled tRNA was purified on a Biorad P30 spin column, and was then mixed with unlabeled tRNA to the desired final concentration. The tRNA was then refolded by heating to 80°C for 2-3 min., followed by addition of MgCl₂ to a final concentration of 10mM, and slow-cooling to ambient temperature over a period of 15–30 min. For M. thermautotrophicum tRNA^{Gln}, labeled tRNA was also purified on a Biorad P30 spin column, but was then further purified on a 20% native polyacrylamide gel prior to mixing with unlabeled tRNA and refolding as above. All steady-state aminoacylation reactions were quenched by the addition of 400 mM sodium acetate (pH 5.2). Single-turnover reactions performed for the M. thermautotrophicum GluRSND were carried out using a Kintek RQF-3 rapid chemical-quench kinetics apparatus, and were quenched in a solution containing 400 mM sodium acetate (pH 5.2) and 0.1% SDS. P1 nuclease digestions were performed by adding 1-5 µl. of the quenched reaction mixture to a microtiter well containing 3-5 µl. of 0.01 – 0.1 mg/mL P1 nuclease (Fluka) in quenching buffer. After incubation for 8–10 min., the reactions were spotted on prewashed PEI cellulose TLC plates (SIGMA), and developed in 100 mM ammonium acetate, 5% (v/v) acetic acid. Dried TLC plates were quantitated by phosphorimaging analysis. Single-turnover reactions were fit to a single exponential function to derive k_{obs} . Initial velocities in multiple turnover reactions were fit to the Michaelis-Menten equation and analyzed with Eadie-Hofstee plots.

For *M. thermautotrophicum* GluRSND, reactions were carried out at 37°C in 50–100 mM Hepes-KOH (pH 7.0), 10 mM MgCl₂, 5 mM DTT, unless otherwise noted. For the hybrid

enzymes derived from $E.\ coli$ GlnRS, reactions were performed at 37°C in 50 mM Tris (pH 7.4), 10 mM MgCl₂, and 5 mM DTT. The concentrations of enzymes and substrates used in each experiment are provided together with the results below. For both the hybrid enzymes and $M.\ thermautotrophicum\ GluRS^{ND}$, active-site titrations were not performed because burst kinetics at the aminoacylation step were not indicated, and because the product-release limited adenylate formation reaction cannot be performed in the absence of tRNA. The k_{cat} values reported assume that the enzyme preparations are fully active, and thus represent lower bounds (Tables 1 and 2).

RESULTS

Amino acid complementarity in hybrid GlnRS enzymes synthesizing Glu-tRNA^{Gln}

Our previous work has demonstrated that amino acid specificity is a distributed property of the GlxRS Rossmann fold domain (20). The WT E. coli GlnRS enzyme, which serves as the target platform for the protein engineering experiments, exhibits 10⁷-fold specificity for glutamine over glutamate, as measured by $k_{\text{cat}}/K_{\text{m}}$ with respect to the amino acid $(k_{\text{cat}}/K_{\text{m}})$ [AA]), for synthesis of Gln-tRNA^{Gln} versus the misacylated Glu-tRNA^{Gln} (21). Replacement of Cys229, Gln255, Ser227 and Phe233 in the primary glutamine binding site of E. coli GlnRS, with their equivalents in the evolutionarily related β -class human GluRS, improves $k_{\text{cat}}/K_{\text{m}}$ for Glu-tRNA^{Gln} synthesis by just 20-fold. The very high K_{m} of 230 mM for glutamate in this quadruply-mutated enzyme further underscores that the local environment of the amino acid binding pocket functions in synergy with more distal regions to generate selectivity against glutamate. Further replacement of the L1 loop from human GluRS (amino acids Thr214-Leu231 in E. coli GlnRS) reduces K_m[Glu] to 44 mM, while replacement of the L2 loop as well (amino acids Val243-Tyr256 in E. coli GlnRS) reduces $K_{\rm m}[{\rm Glu}]$ to 5.8 mM (20). The $k_{\rm cat}$ of this hybrid enzyme (GlnRS S1/L1/L2) is measured at 0.09 s^{-1} , about 35-fold lower than k_{cat} of WT GlnRS for Gln-tRNA^{Gln} synthesis. Based on comparison with WT GlnRS, the hybrid remains 800-fold reduced in k_{cat}/K_{m} [AA] for aminoacyl-tRNA synthesis. GlnRS S1/L1/L2 is constructed from 22 amino acid substitutions and one deletion in WT GlnRS, and is completely inactive in cognate GlntRNA^{Gln} synthesis ((20); Fig. 1A). This hybrid enzyme represents a significant achievement in the reengineering of amino acid specificity in a tRNA synthetase.

All of the replaced amino acids in GlnRS S1/L1/L2 are from the second half of the catalytic Rossmann fold, which in total comprises amino acids 211–260 of the enzyme. Inspection of the cocrystal structure shows that the replaced portion of GlnRS constitutes a fairly compact subdomain that extends to solvent at the surface opposite to the active site cleft, and makes interactions with surrounding protein structure at a number of positions (Fig. 1A). Although there is some conservation of sequence between GlnRS and GluRS at these interfaces, at many positions non-native contacts must occur between introduced GluRS amino acids and the surrounding GlnRS structure. We thus systematically examined these regions with a view toward identifying whether any of the new interactions might disrupt the protein structure on electrostatic or steric grounds. We reasoned that correcting such unfavorable contacts by further mutagenesis could unmask a more efficient Glu-tRNA^{Gln} synthesis activity.

Two positions were identified in this manner. First, GlnRS Tyr256 within loop L2 is a partially solvent-accessible residue that also packs on Ile14 from the short, partly helical N-terminal peptide (residues 1–25) that precedes the first half of the Rossmann fold (Fig. 1B). In GlnRS S1/L1/L2, Tyr256 is substituted with tryptophan. The bulkier indole side chain at Trp256 in the hybrid could clearly disrupt packing with the N-terminal segment. Second, the hydroxyl group of GlnRS Ser218 within loop L1 donates a hydrogen bond to the backbone amide at position Thr214 within L1, and also packs on residues Arg64 from the first half of

the Rossmann fold (residues 26–99), and Tyr102 from the inserted domain (residues 100– 210) (Fig. 1C). In GlnRS S1/L1/L2, Ser218 is substituted with valine. Again, removal of an internal hydrogen bond together with potential steric clashes with surrounding protein could disrupt the structure. Accordingly, to restore the native interactions, we carried out sitedirected mutagenesis using the GlnRS S1/L1/L2 gene as template, to separately revert Trp256 of GlnRS S1/L1/L2 to tyrosine (W256Y), and Val218 of GlnRS S1/L1/L2 to serine (V218S). The enzymes were targeted to the periplasm for expression, as described for the parent hybrid (20). Steady-state kinetic analysis of the two new enzymes was carried out with 250 nM or 500 nM enzyme at 10 mM ATP and 30 μM tRNA $^{\mbox{Gln}},$ while varying the concentration of glutamate between 0.1 and 30 mM. The catalytic efficiency of V218S is decreased by three-fold compared with GlnRS S1/L1/L2, with the effects divided roughly equally between k_{cat} and K_{m} . However, in W256Y, $K_{\text{m}}[\text{Glu}]$ is decreased two-fold while k_{cat} remains unchanged (Table 1; Fig. 2). Thus, each of these third-shell packing interactions, found at an interface between E. coli GlnRS and human GluRS sequences, exerts a modest influence on the efficiency of the enzyme. The lower $K_m[Glu]$ in W256Y reflects an improved hybrid enzyme design; this mutant possesses $k_{\text{cat}}/K_{\text{m}}[AA]$ that is reduced 260-fold compared with WT GlnRS.

In addition to unmasking an inherent catalytic efficiency by correcting distal packing defects, we also considered whether further, thus-far unconsidered substitutions adjacent to the amino acid pocket might also improve activity. The capacity for further improvement was suggested by the lower amino acid $K_{\rm m}$ values (100–200 $\mu {\rm M}$) that have been reported for members of the GlxRS enzyme family (19,27,28). While nearly all of the amino acids in and around the GlnRS amino acid binding pocket have already been substituted in GlnRS S1/L1/ L2, a prominent exception is Tyr240, a third-shell residue that donates a hydrogen bond from its phenolic hydroxyl group to the side-chain carboxylate of Asp219. Asp219, in turn, is connected via a water-mediated hydrogen-bonding network to substrate glutamine (Fig. 1D). In the context of WT GlnRS, mutations of Tyr240 to glutamate or glycine decreased $K_{\rm m}$ for glutamine by two to-three fold, with no effect on $k_{\rm cat}/K_{\rm m}$ for aminoacylation (29). The adjacent amino acid, Asp241 of E. coli GlnRS, was also not substituted in the construction of GlnRS S1/L1/L2. Its side-chain carboxylate group accepts hydrogen bonds from the imidazole and guanidinium moieties of His252 and Arg254, respectively, within loop L2. Inclusion of Tyr240 and Asp241 in the hybrid enzyme design, then, would appear to have the capacity to influence the detailed structure and/or dynamics of the glutamate binding pocket, with possible effects on catalytic function.

Substitution of Tyr240 and Asp241 with their equivalents in human GluRS (Phe and Tyr, respectively) was carried out by site-directed mutagenesis using the GlnRS S1/L1/L2 gene as template, and the new hybrid enzyme (Y240F/D241Y) containing 25 substitutions toward human GluRS was expressed and purified to homogeneity. Steady-state kinetics with respect to glutamate was performed at 250 nM enzyme, 10 mM ATP and 30 µM tRNA^{Gln}, with concentrations of glutamate in the range of 1–150 mM. Although we anticipated that these additional substitutions might improve performance, we found instead that $K_m[Glu]$ is increased four-fold, while k_{cat} remains unchanged (Table 1). Because both k_{cat} and complementarity for tRNA (see below) are maintained in Y240F/D241Y, it appears most likely that the decreased complementarity for glutamate arises from a more local disruption in the structure or dynamics of the binding pocket. However, all of the interactions anticipated to be made by Phe240 are with residues imported from human GluRS (Fig. 1C), while Tyr241 occupies a solvent-accessible position at which it also appears to be easily accommodated. The four-fold decrease in $k_{\text{cat}}/K_{\text{m}}$ by Y240F/D241Y may then reflect dynamic interactions either with more distal regions of the protein that are retained as GlnRS sequences, or with the nearby acceptor stem of the tRNA.

Relationship between tRNA and amino acid binding sites in the hybrid enzymes

The 16000-fold enhancement of $k_{\rm cat}/K_{\rm m}$ for Glu-tRNA Gln synthesis by GlnRS S1/L1/L2 represents an evaluation with respect to the binding of amino acid. A full assessment of function must also examine the efficiency with which the hybrid interacts with ATP and tRNA Gln. To address this, we first measured $K_{\rm m}$ for ATP in the aminoacylation reaction at 250 nM enzyme, 30 µM tRNA Gln and 100 mM glutamate, using ATP concentrations from 0.1–16 mM. The $K_{\rm m}$ value is 620 \pm 10 µM, which is elevated by only three-fold as compared with WT GlnRS ($K_{\rm m}$ of WT GlnRS for ATP is 210 µM (30) (Table 1)). Thus, it appears that the integrity of the ATP binding site in GlnRS S1/L1/L2 is largely preserved. Although we have not measured $K_{\rm m}$ [ATP] for the other hybrids, the relatively similar values obtained for $k_{\rm cat}$ and $K_{\rm m}$ [Glu] suggest that the ATP binding site remains intact in these enzymes as well.

We next measured $K_m[tRNA]$ for GlnRS S1/L1/L2 and for its Y240F/D241Y, W256Y and V218S derivatives, at 100 nM (Y240F/D241Y, W256Y, V218S) or 250 nM (GlnRS S1/L1/L2) enzyme, saturating levels of glutamate (200 mM) and ATP (10 mM), and tRNA concentrations ranging from 1–80 μ M (Y240F/D241Y, W256Y, V218S) or 2–80 μ M (GlnRS S1/L1/L2). In all four hybrids, $K_m[tRNA]$ is elevated by 15–30 fold, as compared with the value of 0.3 μ M measured in the cognate GlnRS reaction (Table 1). These findings demonstrate that the engineered specificity for glutamate has been achieved at significant cost to the complementarity of the enzyme-tRNA interface. The new data provide additional support for the notion that the amino acid and tRNA binding sites in GlnRS are interdependent (16,20).

The elevated tRNA K_m value in GlnRS S1/L1/L2 prompted us to reexamine its sequence with a view toward elucidating whether any of the introduced residues might function to directly couple the amino acid and tRNA binding sites. Five consecutive α-helical residues, located at positions 234-238 in the second half of the GlnRS Rossmann fold, each make either direct or water-mediated interactions with the tRNA acceptor stem (Fig. 1A). None of these residues were substituted in GlnRS S1/L1/L2, since the aim of the design was to test the extent to which the amino acid and tRNA selectivities are separable in GlxRS enzymes (20). However, a short loop preceding these amino acids, at positions Thr230-Phe233, makes diverse interactions. Among these four residues, Thr230 and Glu232 are conserved in human GluRS and have roles in ATP binding, while Phe233 was substituted (with tyrosine) because it forms part of the amino acid pocket. The role of the partly solvent-accessible Leu231, which was converted to Thr in GlnRS S1/L1/L2, has been unclear. In WT GlnRS, Leu231 contacts main-chain and side-chain atoms of Gln234, Phe258, Ser259, and Lys317, and approaches to 4.3 – 4.5 Å of the 2'-OH ribose moiety of C5 in the tRNA acceptor stem (Fig. 1E). Therefore, while Leu231 does not directly contact tRNA, it may stabilize the direct tRNA interactions made by Gln234 and Lys317. Further, the equivalent residue in T. thermophilus GluRS, Glu207, directly accepts a hydrogen bond from the 2'-OH moiety at C5 of tRNA^{Glu} (31). It appears then that the amino acid at Rossmann fold position 231 could provide tRNA complementarity or could link the amino acid and tRNA binding sites in the hybrid enzymes.

To isolate the amino acid selectivity function of the protein portion of the GlxRS RNP as clearly as possible, we thus generated a new GlnRS S1/L1/L2 hybrid containing the T231L reversion, and analyzed its steady-state kinetic properties with respect to glutamate and tRNA Gln, using enzyme and tRNA levels identical to those of the other hybrids. We find that $k_{\rm cat}$ and $K_{\rm m}$ [tRNA] are unchanged, but that $K_{\rm m}$ [Glu] is elevated by four-fold as compared with GlnRS S1/L1/L2 (Table 1). Thus, including the Leu231 substitution in the original design improves complementarity for glutamate, even though the position of this

residue implies a more direct role in tRNA binding. It appears then that the short bridging peptide at positions 230–233 plays a critical role in bridging all three substrate binding sites.

Benchmarking the activities of the hybrid enzymes

An important benchmark for the efficiency of the reconstructed enzymes is comparison with a naturally occurring enzyme that catalyzes Glu-tRNA^{Gln} synthesis. For these measurements we chose the evolutionarily related M. thermautotrophicum GluRSND, which possesses the β-type anticodon binding domain found in E. coli GlnRS. This enzyme has been previously expressed in E. coli (23), and a structure of the apo-enzyme refined at 1.65 Å resolution has recently been reported (12). We purified recombinant enzyme from E. coli to homogeneity using the previously reported expression construct. tRNA^{Gln}(CUG) from M. thermautotrophicum was generated as a T7 RNA polymerase in vitro transcript without mutation of the A1 residue, since high levels of RNA were produced despite the absence of 5'G in the construct (see Experimental Procedures). After refolding, 3'-end labeling, and gel purification of the tRNA, we first tested aminoacylation capacity using single-turnover kinetics with 2 nM $- 2 \mu M$ GluRSND and less than 1 nM labeled tRNA^{Gln}, at 10 mM ATP and 50 mM glutamate (Figure 4). At saturating levels of enzyme (250 nM and above), the observed rate k_{obs} reaches a maximum of 0.12 $\sec^{-1}(k_{\text{max}})$ (Table 2). This value represents either the rate of the chemical steps on the enzyme, or that of a closely linked first-order rearrangement of the complex before initiation of the bond-breaking and bond-making steps. Plateau aminoacylation levels were consistently in the 60%-75% range for all the experiments reported here (Figure 4).

We next determined $K_{\rm m}({\rm ATP})$, $K_{\rm m}$ (Glu), and $K_{\rm m}$ (tRNA^{Gln}) under multiple turnover conditions. $K_{\rm m}$ [ATP] and $K_{\rm m}$ [Glu] were measured using 500 nM tRNA^{Gln} and 10 nM GluRSND, with constant levels of 50 mM glutamate and 10 mM ATP in reactions where the concentrations of ATP and glutamate, respectively, were varied. $K_{\rm m}$ [tRNA] was measured using 2 nM GluRSND, 10 mM ATP, 50 mM glutamate and 0.02–1.0 μ M tRNA. The $k_{\rm cat}$ values determined in these three experiments are identical within experimental error, providing an internal check on the precision of the measurements (Table 2). The value of $k_{\rm cat}$ is also very similar to the single-turnover $k_{\rm max}$, indicating that the rate-limiting step corresponds to the first-order conformational rearrangement, or to the chemical steps. $K_{\rm m}$ [tRNA] is relatively low compared with other tRNA synthetases, suggesting good complementarity at the enzyme-tRNA interface. This also suggests that post-transcriptional modification is unlikely to be important to aminoacylation, consistent with the presence of C34 rather than U34 in the 5'-anticodon position. Although the importance of U34 modification to glutamylation in archaea is not known, 2-thiolation of U34 in bacterial tRNA^{Glu} lowers $K_{\rm m}$ [tRNA] for glutamylation (32,33).

The $K_{\rm m}$ values determined for ATP and glutamate are higher than typically found for tRNA synthetases (Table 2). The value for $K_{\rm m}[{\rm ATP}]$ is approximately 15-fold higher than that determined for $E.~coli~{\rm GlnRS}~(26)$ or $E.~coli~{\rm GluRS}^{\rm D}~(30)$ (Table 2). Millimolar $K_{\rm m}$ for ATP has also been reported for M.~thermautotrophicum pyruvate carboxylase, suggesting compatibility with physiological function in this organism (34). The $K_{\rm m}[{\rm Glu}]$, similarly, is 60-fold higher than the 100 μ M value reported for $E.~coli~{\rm GluRS}^{\rm D}~(28)$. This may reflect the physiology of the thermophilic methanogen, or a particular feature of misacylating reactions generally. No determination of $K_{\rm m}[{\rm ATP}]$ or $K_{\rm m}[{\rm Glu}]$ for a Glu-tRNA $^{\rm Gln}$ synthesis reaction has previously been reported. To evaluate whether the unusually high $K_{\rm m}$ values might be related to the discrepancy between the 37°C temperature at which the assays are performed, and the 65°C temperature optimum for the organism, we also measured initial velocities at 52°C, at which the tRNA $^{\rm Gln}$ transcript remains folded. As expected, $k_{\rm cat}$ for Glu-tRNA $^{\rm Gln}$ synthesis increased approximately four-fold at this elevated temperature, but $K_{\rm m}[{\rm ATP}]$ and $K_{\rm m}[{\rm Glu}]$ were unchanged (data not shown).

Recently, k_{cat} and $K_m[tRNA]$ for Glu-tRNA^{Gln} synthesis at 37°C by M. thermautotrophicum GluRSND were reported by another laboratory (35). The values determined, $k_{cat} = 0.7 \text{ min}^{-1}$ and $K_m[tRNA^{Gln}] = 2.1 \, \mu\text{M}$, are 10-fold lower and 55-fold higher, respectively, than we have measured (Table 2), suggesting a much more weakly functioning enzyme. However, we anticipate that these values reflect the subsaturating level of glutamate (1 mM) and the elevated concentration of enzyme (50 nM) that were used in the experiments. It was also reported that $tRNA^{Gln}$ binds tRS^{ND} with $tR_d = 37 \, tR_d$ avalue that is identical to the $tR_m[tRNA]$ that we have measured (Table 2). A finding of identical values for tR_d and tR_d is consistent with a rate-determining step that follows substrate binding but precedes product release, as we have independently demonstrated here by the equivalence of tR_d and tR_d in the multiple-turnover and single-turnover experiments, respectively (Table 2). No explanation for the large reported discrepancy between tR_d and tR_d and tR_d was offered in the previous work. Measurements of tR_d and tR_d and tR_d were not reported (35).

DISCUSSION

Coevolution of tRNA and tRNA synthetase sequences

We have demonstrated that engineered E. coli GlnRS-derived hybrid enzymes catalyze GlutRNA^{Gln} synthesis with efficiencies comparable to that of a naturally occurring enzyme, the M. thermautotrophicum GluRSND. Both enzymes exhibit k_{cat} of approximately 0.1 sec⁻¹, while K_{m} [Glu] in the W256Y revertant is two-fold lower than in the naturally occurring enzyme. However, the naturally-occurring and engineered enzymes diverge markedly in their respective capacities to provide a binding interface for $tRNA^{Gln}$: K_{m} [tRNA] for the methanogen-derived enzyme is 200-fold lower than in the hybrids (Tables 1, 2).

These findings provide evidence that the tRNA and amino acid selection functions in these tRNA synthetases do not operate independently. With the possible exception of Thr231, none of the 23 replaced amino acids in GlnRS S1/L1/L2 interact with tRNA (Figs. 1A, 1E). Accordingly, if the binding sites for amino acid and tRNA functioned independently, it would be expected that $K_m[tRNA]$ would more closely match that of the parent WT *E. coli* GlnRS. That it does not is a clear demonstration that the GlnRS protein architecture has differentiated to provide a matching function: the tRNA Gln binding site is not properly configured in the absence of cognate glutamine, even though an apparently well-formed glutamate binding site replaces the WT glutamine pocket. The occurrence of enzyme homologs such as the β -class GlnRS/GluRS $^{\rm ND}$ pair, which differ in amino acid specificity in the context of the same tRNA isoacceptor specificity, demonstrates that across evolutionary time the common Rossmann fold protein scaffold has the capacity to differentiate in such a way as to match either glutamate or glutamine with tRNA $^{\rm Gln}$. The very high $K_m[tRNA]$ values of the engineered hybrids show that this matching function has not been reproduced in the engineered enzymes.

The notion that tRNA synthetase and tRNA sequences have coevolved suggests a process of optimization by which acquired mutations in either macromolecule improve catalytic efficiency for cognate but not noncognate amino acid pairing. This coevolution occurs within the context of a particular amino acid specificity (15,36). The implication is that certain tRNA nucleotides function together with key residues in the enzyme, to optimize the formation of a selective amino acid pocket. If this general conceptual framework is correct, then optimizing the Glu-tRNA^{Gln} synthesis activity of the engineered hybrids will very likely require alteration of the enzyme-tRNA interface. Successful nucleotide alterations are predicted to be those that collaborate with the enzyme to optimize the tRNA/amino acid pairing. Such nucleotides might be considered as an embedded signature in the tRNA: a second identity set that functions not to select tRNA^{Gln} from the cellular tRNA pool, but

instead to assist the enzyme in forming a selective glutamate binding site. The tRNA requirement for aminoacyl adenylate synthesis gives this notion added force in the GlxRS family and in class I ArgRS and LysRS, but we suggest that tRNA collaboration in amino acid selection may be a feature of other tRNA synthetases as well. The protein engineering approach we are pursuing represents an in-depth attempt to delineate which distinct elements of homologous tRNA synthetase architectures are responsible for divergent RNA-amino acid pairing specificities. The work presented here provides crucial validation of the experimental system by clearly demonstrating which properties of naturally occurring enzymes have been recapitulated and which have not, and by demonstrating robust catalytic function in five different hybrids.

It is remarkable that the engineered hybrids match and indeed surpass $K_m[Glu]$ of M. thermautotrophicum $GluRS^{ND}$, and this finding can perhaps be taken to indicate a successful design for amino acid binding. Thus, complementarity for the amino acid perhaps could be viewed as a property of the protein alone, and not as a joint property of the RNP. However, it is premature to take this comparison as definitive. Arguably, M. thermautotrophicum $GluRS^{ND}$, a β -class GluRS capable of Glu-tRNAGli synthesis, is indeed a better model for the GlnRS hybrids than E. $coli\ \alpha$ - $GluRS^D$, for which $K_m[Glu]$ is reported as $100\ \mu M$ (28). The latter enzyme possesses a structurally distinct anticodon-binding domain, and pairs glutamate with $tRNA^{Glu}$ instead of $tRNA^{Gln}$. However, it should be noted that there is no known naturally occurring β -class GluRS that is a specific misacylator - that does not also retain the capacity to form Glu- $tRNA^{Glu}$. Possibly, the bacterial α -class GluRS2 enzymes, which may solely form Glu- $tRNA^{Gln}$, may be better models even though they diverge further from GlnRS (14,15).

Alternatively, since the gene duplication leading to GlnRS occurred after differentiation of the eukarya, a β -class eukaryotic GluRS^D might also offer a relevant comparison. These considerations highlight the need for a systematic and rigorous examination of the kinetic properties for representatives of all six GlxRS subclasses: GlnRS, α -GluRS^D, β -GluRS^D, α -GluRSND, and GluRS2. Most of this information remains unavailable; for example, to our knowledge, K_m [Glu] has only previously been reported for the relatively well-studied E. coli GluRS^D and for the α -class M. tuberculosis GluRSND (28,37).

Design of E. coli GlnRS-derived enzymes that synthesize Glu-tRNA^{Gln}

We have explored the functional characteristics of GlnRS S1/L1/L2 by measuring a full set of steady-state kinetic parameters, and by using further site-directed mutagenesis to assess the efficacy of the design. As we have noted, the still-narrow initial question has been to ask whether the amino acid selection function of GlnRS could be exchanged with that of a GluRS, by manipulating just the protein portion of the RNP. We have now shown that K_m [Glu] for the hybrids matches that of a naturally occurring GluRS enzyme, but the values measured in the millimolar range are unexpectedly high (Tables 1, 2). A minimum answer to the query would then be that a functional glutamate binding site has indeed been created, and that some further optimization may yet be possible.

The four new hybrid enzymes possess steady-state kinetic characteristics that are not markedly different from the original design: at most, four-fold changes in $K_{\rm m}({\rm AA})$ for amino acid are measured, and the $K_{\rm m}[{\rm tRNA}]$ values vary less than two-fold (Table 1). The modest kinetic effects measured for V218S and W256Y suggest that there is not a great deal of structural incompatibility at the interfaces between the human GluRS and E. coli GlnRS portions of the enzymes. No other positions along the interfaces between GluRS and GlnRS sequences appear to create unfavorable steric or electrostatic appositions. Thus, we tentatively conclude that the inserted GluRS substructure in GlnRS S1/L1/L2 contributes to a stable Rossmann fold in the hybrid enzymes.

Our findings do not yet establish a minimal component of protein structure that could suffice to bring about the amino acid specificity change that we have demonstrated. We have shown that replacement of four amino acids in the S1 site alone reduces $K_m[Glu]$ of WT GlnRS to just 230 mM, and that the addition of loop L1 to the design produces a hybrid possessing $K_{\rm m}[{\rm Glu}]$ that remains 10-fold above that of GlnRS [S1/L1/L2] (20). It is anticipated that careful structure-based analysis could lead to the design of mutants that bring about the functional change with substantially fewer than the 23 amino acid replacements we have constructed. Pursuit of this important question, which should provide detailed insight into the importance of distal structural elements in conferring substrate selectivity, remains open as a topic for future research. It is also possible that additional substitutions in the protein, in the third and fourth shells surrounding the amino acid pocket, could bring about further lowering of K_m [Glu]. For example, reverting Val218 to Ser in V218S did not improve catalytic efficiency, suggesting that the introduction of Val218 did not create an unfavorable steric clash with surrounding GlnRS residues. However, Ser218 interacts with Tyr102 of GlnRS, a position occupied by histidine in human GluRS (Fig. 1C). Possibly, then, introducing Y102H could improve the design. Many such experiments can be envisioned. Intuitively, however, it seems unlikely that further mutagenesis of distal protein residues would be likely to lower $K_{\rm m}[t{\rm RNA}]$, the major functional defect. Instead, the structural elements of the RNP that provide selective tRNA-amino acid matching should be explored directly by combined protein and tRNA mutagenesis at the macromolecular interface.

It should also be recognized that protein engineering of bacterial GlnRS to create a misacylating GluRS enzyme has features of a reverse evolutionary trajectory, since a specific Glu-tRNA^{Gln} synthesis activity exists in GluRSND enzymes that were present in the earliest common ancestor of all life (38). The mutational experiments presented here simply introduced sequences from the contemporary human GluRS^D enzyme, and do not represent the reconstruction of an ancestral GlxRS protein using phylogenetic techniques. Such reconstructions in the glucocorticoid receptor system have shown that the pathways of evolutionary development of ligand-binding specificity may sometimes not be readily reversed, because intervening epistatic mutations, which generate restrictive structural constraints, limit the capacity of the enzyme to reacquire its ancestral activity (39). It is therefore possible that the number of amino acid substitutions ultimately required to fully recreate a highly efficient Glu-tRNA^{Gln} synthesis activity in GlnRS might be quite large, because of a requirement to unravel structural constraints that are not inherently required for the new function.

Acknowledgments

The expression clone for *M. thermautotrophicum* GluRSND was a generous gift of Dieter Söll and Kelly Sheppard. A.R.H. wishes to express her thanks to the National Council for Science and Technology (CONACYT-Mexico) for financial support during the development of these experiments.

References

- Ibba M, Söll D. Aminoacyl-tRNA synthesis. Annu Rev Biochem. 2000; 69:617–650. [PubMed: 10966471]
- 2. Siatecka M, Rozek M, Barciszewski J, Mirande M. Modular evolution of the Glx-tRNA synthetase family: Rooting of the evolutionary tree between the bacteria and archaea/eukarya branches. Eur J Biochem. 1998; 256:80–87. [PubMed: 9746349]
- 3. Woese CR, Olsen GJ, Ibba M, Söll D. Aminoacyl-tRNA synthetases, the genetic code, and the evolutionary process. Microbiol Mol Biol Rev. 2000; 64:202–236. [PubMed: 10704480]
- 4. Nureki O, Vassylyev DG, Katayanagi K, Shimizu T, Sekine S, Kigawa T, Miyazawa T, Yokoyama S, Morikawa K. Architectures of class-defining and specific domains of glutamyl-tRNA synthetase. Science. 1995; 267:1958–1965. [PubMed: 7701318]

 Lamour V, Quevillion S, Diriong S, N'Guyen VC, Lipinski M, Mirande M. Evolution of the GlxtRNA synthetase family: the glutaminyl enzyme as a case of horizontal gene transfer. Proc Natl Acad Sci USA. 1994; 91:8670–8674. [PubMed: 8078941]

- Rould MA, Perona JJ, Söll D, Steitz TA. Structure of glutaminyl-tRNA synthetase complexed with tRNA^{Gln} and ATP at 2.8 Å resolution. Science. 1989; 246:1135–1142. [PubMed: 2479982]
- 7. Wilcox M, Nirenberg M. Transfer RNA as a cofactor coupling amino acid synthesis with that of protein. Proc Natl Acad Sci USA. 1968; 61:229–236. [PubMed: 4972364]
- 8. LaPointe J, Duplain L, Proulx M. A single glutamyl-tRNA synthetase aminoacylates tRNA^{Glu} and tRNA^{Gln} in *Bacillus subtilis* and efficiently misacylates *Escherichia coli* tRNA₁^{Gln}*in vitro*. J Bacteriol. 1986; 165:88–93. [PubMed: 3079749]
- Curnow AW, Hong K, Yuan R, Kim S, Martins O, Winkler W, Henkin TM, Söll D. Glu-tRNA^{Gln} amidotransferase: A novel heterotrimeric enzyme required for correct decoding of glutamine codons during translation. Proc Natl Acad Sci USA. 1997; 94:11819–11826. [PubMed: 9342321]
- 10. Frechin M, Duchene AM, Becker HD. Translating organellar glutamine codons: a case by case scenario? RNA Biol. 2009; 6:31–34. [PubMed: 19106621]
- 11. Sheppard K, Yuan J, Hohn MJ, Jester B, Devine KM, Söll D. From one amino acid to another: tRNA-dependent amino acid biosynthesis. Nucl Acids Res. 2008; 36:1813–1825. [PubMed: 18252769]
- 12. Nureki O, O'Donoghue P, Watanabe N, Ohmori A, Oshikane H, Araiso Y, Sheppard K, Söll D, Ishitani R. Structure of an archaeal non-discriminating glutamyl-tRNA synthetase: a missing link in the evolution of Gln-tRNA Gln formation. Nucleic Acids Res. 2010 July 3.10.1093/nar/gkq605
- 13. Schulze JO, Masoumi A, Nickel D, Jahn M, Jahn D, Schgubert WD, Heinz DW. Crystal structure of a non-discriminating glutamyl-tRNA synthetase. J Mol Biol. 2006; 361:888–897. [PubMed: 16876193]
- 14. Skouloubris S, Ribas de Pouplana L, de Reuse H, Hendrickson TL. A noncognate aminoacyltRNA synthetase that may resolve a missing link in protein evolution. Proc Natl Acad Sci USA. 2003; 100:11297–11302. [PubMed: 13679580]
- Salazar JC, Ahel I, Orellana O, Tumbula-Hansen D, Krieger R, Daniels L, Söll D. Coevolution of an aminoacyl-tRNA synthetase with its tRNA substrates. Proc Natl Acad Sci USA. 2003; 100:13863–13868. [PubMed: 14615592]
- Uter NT, Gruic-Sovulj I, Perona JJ. Amino acid-dependent transfer RNA affinity in a class I aminoacyl-tRNA synthetase. J Biol Chem. 2005; 280:23966–23977. [PubMed: 15845537]
- 17. Gruic-Sovulj I, Uter NT, Bullock TL, Perona JJ. tRNA-dependent aminoacyl-adenylate hydrolysis by a nonediting class I aminoacyl-tRNA synthetase. J Biol Chem. 2005; 280:23978–23986. [PubMed: 15845536]
- Sherlin LD, Perona JJ. tRNA-dependent active-site assembly in a class I aminoacyl-tRNA synthetase. Structure. 2003; 11:591–603. [PubMed: 12737824]
- Uter NT, Perona JJ. Long-range intramolecular communication in a tRNA synthetase complex examined by chemical quench-flow kinetics. Proc Natl Acad Sci USA. 2004; 101:14396–14401. [PubMed: 15452355]
- Bullock T, Rodriguez-Hernandez A, Corigliano E, Perona JJ. A rationally engineered misacylating aminoacyl-tRNA synthetase. Proc Natl Acad Sci USA. 2008; 105:7428–7433. [PubMed: 18477696]
- 21. Bullock T, Uter NT, Nissan TA, Perona JJ. Amino acid discrimination by a class I aminoacyltRNA synthetase specified by negative determinants. J Mol Biol. 2003; 328:395–408. [PubMed: 12691748]
- 22. Hale SP, Auld DS, Schmidt E, Schimmel P. Discrete determinants in transfer RNA for editing and aminoacylation. Science. 1997; 276:1250–1252. [PubMed: 9157882]
- Tumbula DL, Becker HD, Chang WZ, Söll D. Domain-specific recruitment of amide amino acids for protein synthesis. Nature. 2000; 407:106–110. [PubMed: 10993083]
- 24. Sherlin LD, Bullock TL, Nissan TA, Perona JJ, LaRiviere F, Uhlenbeck OC, Scaringe S. Chemical and enzymatic synthesis of tRNAs for high-throughput crystallization. RNA. 2001; 7:1671–1678. [PubMed: 11720294]

 Lyakhov DL, He B, Zhang X, Studier FW, Dunn JJ, McAllister WT. Mutant bacteriophage T7 RNA polymerases with altered termination properties. J Mol Biol. 1997; 269:28–40. [PubMed: 9192998]

- 26. Wolfson AD, Uhlenbeck OC. Modulation of tRNA^{Ala} identity by inorganic pyrophosphatase. Proc Natl Acad Sci USA. 2002; 99:5965–5970. [PubMed: 11983895]
- 27. Ibba M, Hong KW, Sherman JM, Sever S, Söll D. Interactions between tRNA identity nuclotides and their recognition sites in glutaminyl-tRNA synthetase determine the cognate amino acid affinity of the enzyme. Proc Natl Acad Sci USA. 1996; 93:6953–6958. [PubMed: 8692925]
- 28. Kern D, LaPointe J. Glutamyl transfer ribonucleic acid synthetase from *Escherichia coli*. Study of the interactions with its substrates. Biochemistry. 1979; 18:5809–5818. [PubMed: 229901]
- 29. Hong K-W, Ibba M, Söll D. Retracing the evolution of amino acid specificity in glutaminyl-tRNA synthetase. FEBS Lett. 1998; 434:149–154. [PubMed: 9738468]
- 30. Kern D, Potter S, Lapointe J, Boulanger Y. The glutaminyl-transfer RNA synthetase of *Escherichia coli*. Purification, structure and function relationship. Biochim Biophys Acta. 1980; 607:65–80. [PubMed: 6989402]
- 31. Sekine S, Nureki O, Shimada A, Vassylyev DG, Yokoyama S. Structural basis for anticodon recognition by discriminating glutamyl-tRNA synthetase. Nat Struct Biol. 2001; 8:203–206. [PubMed: 11224561]
- 32. Madore E, Florentz C, Giege R, Sekine S, Yokoyama S, Lapointe J. Effect of modified nucleotides on *Escherichia coli* tRNA ^{Glu} structure and on its aminoacylation by glutamyl-tRNA synthetase. Eur J Biochem. 1999; 266:1128–1135. [PubMed: 10583410]
- 33. Sylvers LA, Rogers KC, Shimizu M, Ohtsuka E, Söll D. A 2-thiouridine derivative in tRNA^{Glu} is a positive determinant for aminoacylation by *Escherichia coli* glutamyl-tRNA synthetase. Biochemistry. 1993; 32:3836–3841. [PubMed: 8385989]
- 34. Mukhopadhyay B, Stoddard SF, Wolfe RS. Purification, regulation, and molecular and biochemical characterization of pyruvate carboxylase from *Methanobacterium thermautotrophicum* strain ΔH. J Biol Chem. 1998; 273:5155–5166. [PubMed: 9478969]
- Rampias T, Sheppard K, Söll D. The archaeal transamidosome for RNA-dependent glutamine biosynthesis. Nucl Acids Res. 201010.1093/nar/gkq336
- 36. Lipman RS, Hou YM. Aminoacylation of tRNA in the evolution of an aminoacyl-tRNA synthetase. Proc Natl Acad Sci USA. 1998; 95:13495–13500. [PubMed: 9811828]
- 37. Paravisi S, Fumagalli G, Riva M, Morandi P, Morosi R, Konarev PV, Petoukhov MV, Bernier S, Chenevert R, Svergun DI, Curti B, Vanoni MA. Kinetic and mechanistic characterization of *Mycobacterium tuberculosis* glutamyl-tRNA synthetase and determination of its oligomeric structure in solution. FEBS J. 2009; 276:1398–1417. [PubMed: 19187240]
- 38. Sheppard K, Söll D. On the evolution of the tRNA-dependent amidotransferases, GatCAB and GatDE. J Mol Biol. 2008; 377:831–844. [PubMed: 18279892]
- 39. Bridgham JT, Ortlund EA, Thornton JW. An epistatic ratchet constrains the direction of glucocorticoid receptor evolution. Nature. 2009; 461:515–520. [PubMed: 19779450]
- Uter NT, Perona JJ. Long-range intramolecular signaling in a tRNA synthetase complex revealed by pre-steady state kinetics. Proc Natl Acad Sci USA. 2004; 101:14396–14401. [PubMed: 15452355]
- 41. Rath VL, Silvian LF, Beijer B, Sproat BS, Steitz TA. How glutaminyl-tRNA synthetase selects glutamine. Structure. 1998; 6:439–449. [PubMed: 9562563]

A.

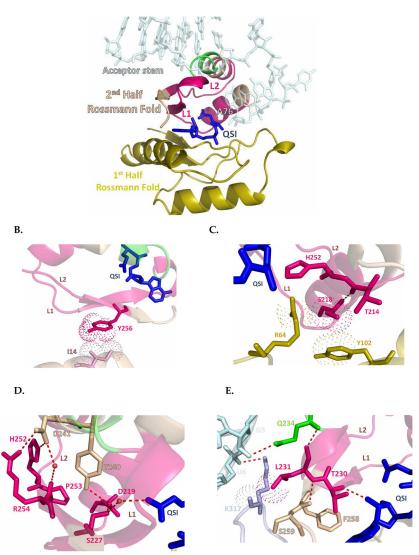


FIGURE 1.

(A) Top: Active site Rossmann fold domain structure of wild-type $E.\ coli$ GlnRS bound in a ternary complex with tRNA^{Gln} and the glutaminyl adenylate analog 5'-O-[N-(L-glutaminyl)sulfamoyl] adenosine (QSI) (41). L1 and L2 designate loops that bridge secondary structure elements in the second half of the fold, and that were replaced in the engineered enzymes. The α -helical segment depicted in green contains amino acids that directly bind the acceptor stem of the tRNA; these residues were not exchanged in the hybrids. The portion of the protein structure that was introduced from human GluRS, to produce the hybrid enzyme GlnRS S1/L1/L2, is colored pink. (B) $Center\ left$: Location of GlnRS Tyr256 in the final β -strand of the second half of the Rossmann fold, with respect to the active site (shown by the binding of QSI). Van der Waals interactions of Tyr256 with Ile14 from the N-terminal helical peptide of GlnRS (which precedes the first half of the Rossmann fold) are depicted. (C) $Center\ right$: Location of GlnRS Ser218 in the first α -helix of the second half of the Rossmann fold. Van der Waals interactions with Arg64 (first half of Rossmann fold) and Tyr102 (located in the peptide immediately following the first half of the fold) are shown. Arg64 is conserved in human GluRS, and Tyr102 is substituted with

histidine in that enzyme. (D) *Bottom left*: Interactions of GlnRS Tyr240 and Asp241 with surrounding residues in the second half of the Rossmann fold. Hydrogen bonds are depicted by dotted pink lines. All residues shown in pink were replaced in the hybrid enzymes. QSI marks the position of the active site at bottom right. (E) *Bottom right*: Interactions of GlnRS Leu231 with its surrounding environment in the RNP complex. Hydrogen bonds are depicted as dotted pink lines. Both Gln234 and Lys317 directly interact with the tRNA backbone. Interaction of the adjacent Thr230 with the sugar of QSI, an interaction preserved in the structure of the RNP bound to ATP (6), is also shown. The tRNA acceptor stem is depicted in light blue at upper left.

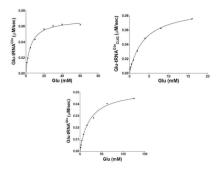


FIGURE 2. Plots of initial velocity vs. substrate concentration used for determination of $K_m[Glu]$ in GlnRS S1/L1/L2 (top left), Y240D/D241F (bottom), and W256Y (top right). In each case the data were fit to the Michaelis-Menten equation.

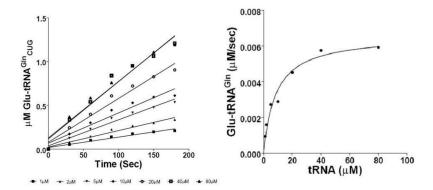


FIGURE 3. *Left*: Initial velocities for determination of steady-state kinetic parameters for the T231L hybrid enzyme, with respect to tRNA concentration (listed at bottom). *Right*: Replot of the initial velocity data to yield k_{cat} and $K_{\text{m}}[\text{tRNA}]$.

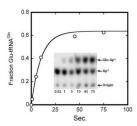


FIGURE 4.

Time course for Glu-tRNA^{Gln} formation by *M. thermautotrophicus* GluRSND. In this experiment the enzyme concentration is 500 nM, and 3' [³²P]-labeled tRNA is present at less than 1 nM final concentration. This represents saturation for tRNA binding. The inset shows a TLC plate demonstrating the separation of substrate (Ap*; center of TLC plate) and product (Glu-Ap*; top of TLC plate). The reactions were spotted at the bottom of the plate. The ratio of intensities for Glu-Ap* and Ap* yields the plateau aminoacylation value.

NIH-PA Author Manuscript

Kinetic Properties of Hybrid GlnRS Enzymes

Enzyme	Km (Glu) mM	k_{cat} (Glu) ^a sec ⁻¹	$k_{cat}/Km~(Glu)~M^{-1}sec^{-1}$	Km (ATP) mM	$k_{cat} (ATP)^{a} sec^{-1}$	k _{cat} /Km (ATP) M ⁻¹ sec ⁻¹	$Km(tRNA^{Gln})\mu M$	$k_{cat} (tRNA^{Gln})^{a} sec^{-1}$	$Km(Glu)mM-k_{cat}(Glu)^{\mathcal{U}}sec^{-1}-k_{cat}'Km(Glu)M^{-1}sec^{-1}-Km(ATP)mM-k_{cat}(ATP)^{\mathcal{U}}sec^{-1}-k_{cat}'Km(ATP)M^{-1}sec^{-1}-Km(tRNAGlu)\mu M-k_{cat}(tRNAGlu)^{\mathcal{U}}sec^{-1}-k_{cat}'Km(tRNAGlu)M^{-1}sec^{-1}$
WT GlnRS	₂ 750 <i>b</i>	0.046 ± 0.013^{b}	$(9.5 \pm 1.3) \times 10^{-4}$ C)	0.21d	1	!	0.31 ± 0.09^{e}	3.2 ± 0.5^e	$1.0 \times 10^{7}(e)$
L1L2	$5.8\pm0.5f$	$0.09 \pm 0.02 f$	$15.5^f[16,300]$	0.62 ± 0.01	0.1 ± 0.01	160	7.6 ± 3.0	0.04 ± 0.01	5.2×10^3
V218S	8.9 ± 1.0	0.06 ± 0.01	6.7 [7,000]	Q		1	4.5 ± 0.6	0.04 ± 0.005	8.8×10^3
W256Y	2.6 ± 1.0	0.1 ± 0.04	38.5 [40,500]	Q		1	4.8 ± 0.2	0.14 ± 0.01	2.9×10^4
T231L	21.2 ± 1.2	0.09 ± 0.01	4.2 [4,400]	QN	1	!	8.05 ± 0.45	0.08 ± 0.01	9.9×10^3
Y240D/D241F	21.1 ± 1.1	0.09 ± 0.01	4.3 [4,500]	ND	1	!	5.3 ± 0.3	0.04 ± 0.01	7.5×10^3

 $a_{\rm cat}$ values represent lower bounds, and assume that the enzyme preparations are fully active (see Experimental Procedures).

bre Km for glutamate in glutamylation by WT GlnRS could not be determined, because saturation is not observed. kcat was determined from reactions where the concentration of tRNAGln was varied at 10 mM ATP and 1.0 M glutamate.13

 $c_{\rm cat}/K_{\rm m}$ is derived from the linear slope of a V vs. [Glu] plot in which saturation for Glu is not observed.18

 d This value was previously reported.26

^eThese parameters represent values for cognate Gln-tRNA Gln synthesis reactions.40

freese values were previously reported.17 The numeral in brackets represents the fold improvement compared with WT GlnRS.

Table 2

Kinetic analysis of M. thermoautotrophicus GluRSND

	tRNA Gln	Gln	Glu	n	ATP	T
k_{max} (s ⁻¹)		K_M (nM)	$k_{cat} (s^{-1}) a = K_M (nM) - k_{cat} (s^{-1}) a$	K_M (mM)	K_M (mM) k_{cat} (s ⁻¹) a K_M (mM)	K_M (mM)
0.12 ± 0.01	0.12 ± 0.01 0.10 ± 0.01	38 ± 10	0.09 ± 0.05	6.2 ± 0.6	0.09 ± 0.05 6.2 ± 0.6 0.10 ± 0.05 3.1 ± 1.7	3.1 ± 1.7

 $a_{\rm Cat}$ values represent lower bounds, and assume that the enzyme preparations are fully active (see Experimental Procedures).

Page 20