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The role of the catalytic domain of *E. coli* GluRS in $tRNA^{Gln}$ discrimination

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ABSTRACT

Discrimination of $tRNA^{Gln}$ is an integral function of several bacterial glutamyl-tRNA synthetases (GluRS). The origin of the discrimination is thought to arise from unfavorable interactions between $tRNA^{Gln}$ and the anticodon-binding domain of GluRS. From experiments on an anticodon-binding domain truncated *Escherichia coli* (*E. coli*) GluRS (catalytic domain) and a chimeric protein, constructed from the catalytic domain of *E. coli* GluRS and the anticodon-binding domain of *E. coli* glutaminyl-tRNA synthetase (GlnRS), we show that both proteins discriminate against *E. coli* $tRNA^{Gln}$. Our results demonstrate that in addition to the anticodon-binding domain, $tRNA^{Gln}$ discriminatory elements may be present in the catalytic domain in *E. coli* GluRS as well.

1. Introduction

High fidelity of protein translation is maintained in cells by a family of multi-domain enzymes known as aminoacyl-tRNA synthetases (aaRSs) [1]. While aminoacylation of tRNA is the primary function of the catalytic domain, the anticodon-binding domain recognizes the correct tRNA with additional contributions to optimize the aminoacylation step [2]. Glutamyl-tRNA synthetase (GluRS) and glutaminyl-tRNA synthetase (GlnRS), two members of this family, are grouped in the GlxRS subclass because of the shared evolutionary pathway of their catalytic domains [3,4]. The catalytic domain of Glx subfamily is believed to be more ancient, having evolved from a common GluRS ancestor that contained only the catalytic domain [3,4]. Anticodon binding domains of extant bacterial and eukaryotic/archeal GlxRS appeared independently at a later stage, with the anticodon-binding domain of bacterial GlnRS being acquired by an unique horizontal gene transfer event from the eukaryotic kingdom [3,4].

Bacterial GluRS comes in two flavors, discriminatory GluRS (D-GluRS) and non-discriminatory GluRS (ND-GluRS). While D-GluRS exclusively catalyses the transfer of Glu to $tRNA^{Glu}$, the ND-GluRS can also glutamylate $tRNA^{Gln}$ forming Glu-$tRNA^{Gln}$. The misacylated product is then transformed to Gln-$tRNA^{Gln}$ by an enzyme known as glutamyl-$tRNA^{Gln}$ amidotransferase [5,6]. Identification of key residues (identity elements) in GluRS, GlnRS and the corresponding tRNAs that play a crucial role in aminoacylation showed that the participation of both domains is required for efficient aminoacylation of $tRNA^{Glu}$ by D-GluRS and $tRNA^{Gln}$ by GlnRS [7–10]. However, tRNA recognition pattern of these two enzymes to their cognate tRNAs are different. For GluRS–$tRNA^{Glu}$ interaction, the identity elements were mainly clustered in the catalytic domain of GluRS and on the acceptor arm and augmented D-helix of $tRNA^{Glu}$ [8]. For GlnRS–$tRNA^{Gln}$ interaction, the identity elements were widely spread in both the domains of GlnRS and in $tRNA^{Gln}$ [9].

In contrast to the wide range of data available for cognate tRNA interaction [7–10], information about non-cognate tRNA discrimination is scanty and is focused only on the role of the anticodon-binding domain [11–13]. Sekine et al. [11] showed that for *Thermus thermophilus* GluRS (*Tt*GluRS), Arg358 (at position R) is responsible for $tRNA^{Gln}$ discrimination, because an Arg358Gln mutation resulted in a GluRS that showed relaxed specificity towards the

Abbreviations: GluRS, glutamyl-tRNA synthetase; GlnRS, glutaminyl-tRNA synthetase; NGluRS, N-terminal catalytic domain of GluRS; CGluRS, C-terminal anticodon-binding domain of GluRS; cGluGlnRS, chimera of NGluRS and the anticodon-binding domain of GlnRS; ASA, solvent accessible surface area; aaRS, aminoacyl-tRNA synthetase; D-GluRS, discriminatory GluRS; ND-GluRS, non-discriminatory GluRS

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anticodons of $tRNA^{Glu/Gln}$ ($^{34}CUC^{36}/^{34}CUG^{36}$). Although this Arg residue is conserved in D-GluRS, several ND-GluRSs are also known to contain an Arg residue at this position. Based on limited sequence alignment it was suggested that an Arg at position R is allowed in ND-GluRS provided there is no Glu at another upstream position (Glu443 in *Tt*GluRS) [12]. However, this rule is not universal (GluRS in *Thermotogae petrophila*, non-discriminatory due to the absence of GlnRS in the genome and the concomitant presence of GatCAB genes, contains an Arg-Glu pair at positions 358/443 corresponding to *Tt*GluRS). That the Arg residue is not the sole player in tRNA discrimination is also evident from the work of Lee and Hendrickson [13]. A mutation at position R (Arg350Glu) in *Helicobacter pylori* GluRS1, a canonical D-GluRS, did not affect discrimination against $tRNA^{Gln}$ and produced Glu-$tRNA^{Glu}$ like the wild type protein. On the other hand, introduction of an Arg residue at position R in *H. pylori* GluRS2 (Glu334Arg), a non-canonical GluRS that produces misacylated Glu-$tRNA^{Gln}$ but does not produce Glu-$tRNA^{Glu}$, did not nullify its discrimination against $tRNA^{Glu}$ but showed weak glutamylation of $tRNA^{Gln}$. Another single mutation (Gly417Thr) in *H. pylori* GluRS2 produced weak activity towards $tRNA^{Glu}$ while a double mutant (Glu334Arg, Gly417Thr) more robustly glutamylated $tRNA^{Glu}$ instead of $tRNA^{Gln}$, with undetectable production of Glu-$tRNA^{Gln}$ for both cases.

The experimental data show that tRNA discrimination by the anticodon-binding domain of GluRS is complex and operates in synergy among more than one residue positions. It is also likely that the catalytic domain of GluRS plays a role in tRNA discrimination, since $tRNA^{Glu}$ as well as $tRNA^{Gln}$ contain identity elements not only in the anticodon loop but also in the augmented D-loop and the acceptor arm [7–10]. The contribution of the D-GluRS catalytic domain in discrimination against $tRNA^{Gln}$ is an open question that needs to be addressed. We have compared the properties of *Escherichia coli* GluRS (*Ec*GluRS; D-GluRS) and its two domains, the N-terminal catalytic domain of GluRS (NGluRS) and the C-terminal anticodon-binding domain of GluRS (CGluRS), to probe the contributions of the two domains in terms of cognate (*Ec*$tRNA^{Glu}$) and non-cognate (*Ec*$tRNA^{Gln}$) glutamylation. In addition, $tRNA^{Gln}$ discrimination by a previously reported chimeric protein, chimera of NGluRS and the anticodon-binding domain of GlnRS (cGluGlnRS) [14], constructed from NGluRS and the anticodon-binding domain of *Ec*GlnRS was also studied for comparison. Our results show that the N-terminal catalytic domain discriminates against $tRNA^{Gln}$ demonstrating that in addition to the anticodon-binding domain, anti-determinants are also present in the catalytic domain of *Ec*GluRS.

2. Materials and methods

2.1. Materials

BSA, ATP, L-Glutamate, D-Glutamate, L-Glutamine were purchased from Sigma. *Ec*$tRNA^{Glu}$ was purchased from Sigma (accepting capacity: 1.4 nmol/OD_{260nm}) while *Ec*$tRNA^{Gln}$ was purified from an overexpressing strain pRS3 as described earlier [15]. The accepting capacity of the eluted fractions that were used in assay experiments was: 1.6 nmol/OD_{260nm}. [^{3}H]L-Glu (specific activity: 42.9 Ci/mmol) was purchased from NEN™ Life Science Products, Boston, USA and [^{3}H]L-Gln (specific activity: 52 Ci/mmol) was purchased from GE Healthcare. tRNA concentrations were determined by assuming 1.6 nmol/ml/OD_{260} for 100% aminoacylation [16].

2.2. Cloning of N-terminal and C-terminal domain of E. coli GluRS

Due to the unavailability of the crystal structure of *Ec*GluRS, residues defining the N-terminal and C-terminal domains were determined indirectly from the known crystal structure of *Tt*GluRS [17], a close sequence homolog of *Ec*GluRS (pdb code: 1J09). Accordingly the NGluRS was defined by residues 1–314 and the CGluRS was defined by residues 318–471 (Fig. 1a). The design and construction of the NGluRS plasmid DNA in pET28a (+) (Novagen) has been described in a previous report [14]. For the cloning of CGluRS, the forward and reverse primers were designed according to the multiple cloning sites present in the pET28a (+) (Novagen) expression vector and the known sequence of the *Ec*GluRS gene. Using GluRS gene (strain PLQ7612) as the template, the CGluRS gene were PCR-amplified using the primers 5′-GGCCCATATGGCTACTCACTTAC-3′ (forward primer) and 5′-GAAAATCAGCAGTAAGGATCCCGG-3′ (reverse primer) with *NdeI* and *BamHI* restriction sites. The PCR product was digested with corresponding enzymes and was ligated into the *NdeI* and *BamHI* digested pET28a (+) vector. The recombinant colonies obtained were checked for the presence of the insert by Agarose gel electrophoresis and also by restriction digestion and subsequently confirmed by DNA sequencing.

2.3. Protein expression and purification

Plasmid DNA of NGluRS and CGluRS were transformed in *E. coli* strain BL21 (DE3) followed by the inoculation in Luria–Bertani medium. The cultures were grown at 37 °C containing 50 μg/ml of kanamycin up to an OD of 0.4 (for CGluRS) and 0.2 (for NGluRS). All bacterial cultures were induced by 0.5 mM IPTG (isopropyl-1-thio-β-D-galactoside) at 16 °C for overnight. Cell pellets were kept at −20 °C after harvesting. NGluRS and CGluRS were purified from cell pellets by Ni-NTA column chromatography protocol (Qiagen) as described earlier [14]. *Ec*GluRS, cGluGlnRS and *Ec*GlnRS were purified as previously described [14,15]. Plasmid DNA of *Ec*GluRS, NGluRS, CGluRS and cGluGlnRS were also transformed into a thermo-sensitive strain JP1449 (DE3) [18,19] and the respective proteins were isolated and purified as described before [14]. Additionally the JP1449 (DE3) strains was separately inoculated and parallel mock NGluRS-like isolation and purification steps were performed. The elute fractions were analyzed by 16% SDS–PAGE.

2.4. Aminoacylation assay experiments

Reaction mixtures (volume: 100–300 μl) for all aminoacylation experiments contained 50 mM HEPES (pH 7.5), 16 mM $MgCl_2$,

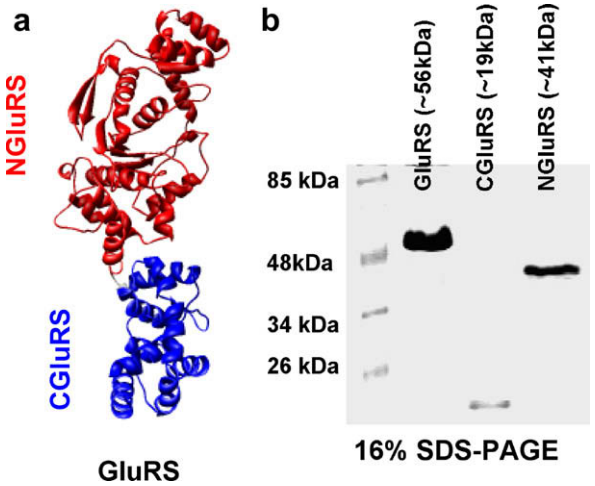

Fig. 1. (a) Homology modeled structure of *E. coli* GluRS with *T. thermophilus* GluRS crystal structure (pdb code: 1J09) as the template. The catalytic domain (NGluRS: 1–314) is shown in red (top) and the anticodon-binding domain (CGluRS: 318–471) is shown in blue (bottom). (b) 16% SDS–PAGE profiles of *E. coli* GluRS and the two isolated domains – NGluRS and CGluRS.

2 mM ATP, 0.8 mM β-mercaptoethanol, 0.1 mM unlabelled amino acid (either L-Glu or L-Gln), 4 μM purified tRNA (either *Ec*tRNA^{Glu} or *Ec*tRNA^{Gln}) with trace quantities of radioactive amino acids [^{3}H]L-Glu and [^{3}H]L-Gln (>1000-fold lower than unlabelled amino acids). The reaction mixture contained a fixed amount of protein (see legends to figures) obtained from pure elute fractions which were extensively dialyzed in 20 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 5 mM β-mercaptoethanol and 20% glycerol prior to the assay experiments. The cognate aminoacylation activities of the *Ec*tRNA^{Glu} and *Ec*tRNA^{Gln} were checked with *Ec*GluRS and *Ec*GlnRS, respectively. The assay experiments were performed at 37 °C as described in Bhattacharyya et al. [15] where 20 μl of aliquot were withdrawn at each time point for the measurement of radioactivity. In addition parallel assay experiments were also performed at 42 °C with *Ec*GluRS, NGluRS, CGluRS and cGluGlnRS expressed in the thermosensitive strain JP1449 (DE3) that is incapable of producing endogenous GluRS at 42 °C. The latter experiments were performed to eliminate potential contamination of the reaction mixture with endogenous GluRS.

2.5. Determination of k_{cat} and K_m for NGluRS

Aminoacylation reactions for determination of K_m and k_{cat} for NGluRS were performed at 37 °C in 50 mM HEPES (pH 7.5), 16 mM $MgCl_2$, 2 mM ATP, 0.8 mM β-mercaptoethanol. For the measurement of $K_m^{L\text{-}Glu}$ and $k_{cat}^{L\text{-}Glu}$, L-Glu concentration was varied from 0.1-3 mM, for the measurement of $K_m^{tRNAGlu}$ and $k_{cat}^{tRNAGlu}$, *Ec*tRNA^{Glu} concentration was varied from 0 to 8 μM. For all cases NGluRS concentration was kept at 1.5 μg/assay point (20 μl). Initial slopes of each assay were plotted against the respective ligand concentrations for obtaining K_m and k_{cat} values.

2.6. Structural studies

Circular Dichroism (CD) studies were performed on *Ec*GluRS, NGluRS and CGluRS on a Jasco J-600 spectro-polarimeter. Far UV CD was measured at 25 °C in a 0.1 cm path-length cuvette, whereas near-UV CD was measured in a cuvette of path-length 10 cm. Typical concentrations used were in the range 2–10 μM in 50 mM phosphate buffer (pH 7.5) containing 100 mM KCl and 20% glycerol. Fluorescence spectra for *Ec*GluRS, N-GluRS and C-GluRS were obtained in the same buffer in a Hitachi F-3010 spectrofluorimeter.

2.7. Binding studies

Binding of cognate and non-cognate substrates to *Ec*GluRS, NGluRS and CGluRS was followed by monitoring intensity changes in intrinsic Trp fluorescence. All binding experiments with *Ec*GluRS, NGluRS and CGluRS were performed in 20 mM HEPES buffer, pH 7.5, containing 20% glycerol and 5 mM $MgCl_2$ at 25 °C. For the binding of ATP and the amino acids the enzyme concentration in each case was taken to be 2 μM. Binding of *Ec*tRNA^{Glu} and *Ec*tRNA^{Gln} were monitored by single point titration as described before [14]. The resulting binding isotherms were analyzed using standard equations assuming a 1:1 binding stoichiometry [20].

3. Results

In order to address the role played by the catalytic and the anticodon-binding domains of *E. coli* GluRS in cognate and non-cognate aminoacylation, the two domains (NGluRS: 1–314; CGluRS: 318–471) of *E. coli* were expressed and purified (Fig. 1b). Prior to assessing the aminoacylation properties, structural and cognate and non-cognate binding studies were performed on the isolated domains.

3.1. Structural integrity of NGluRS and CGluRS

For isolated NGluRS and CGluRS to act as independent functional units, the overall structure of the domains should be similar to what is found in the wild type *Ec*GluRS. Two complementary techniques, Circular Dichroism (CD) and fluorescence spectroscopy, were used to assess the structural integrity of the domains and their similarity with GluRS.

3.1.1. Circular Dichroism studies

Far-UV CD spectrum is an excellent reporter of the overall folding pattern (secondary structure) of peptide backbone. In Fig. 2a the far-UV CD spectra of NGluRS, CGluRS and *Ec*GluRS are shown. Assuming that the backbone secondary structure of *Ec*GluRS is very similar to its homolog, *Tt*GluRS (pdb code: 1J09), the expected α-helical content of *Ec*GluRS, NGluRS and CGluRS are 51%, 43% and 67%, respectively. The corresponding CD-derived α-helical content, as estimated from deconvolution of the far-UV CD spectra [21], are 57%, 52% and 64%, respectively. A good match between the observed and the expected fraction α-helical contents demonstrated structural integrity of the isolated domains. As opposed to far-UV spectrum, near-UV CD spectrum arises primarily due to differential absorption of right/left circularly polarized light by aromatic amino acids (Trp, Tyr and Phe). The near-UV CD spectra of *Ec*GluRS, NGluRS and CGluRS are shown in the inset to Fig. 2a. The nature and intensities of near-UV CD spectra of NGluRS and CGluRS were different than that of *Ec*GluRS. Near-UV CD spectrum is sensitive to small changes in tertiary structure due to protein–protein interactions and/or changes in solvent conditions indicating that that upon isolation, NGluRS and CGluRS lose tertiary structure present in *Ec*GluRS. In summary the CD data showed that the isolated do-

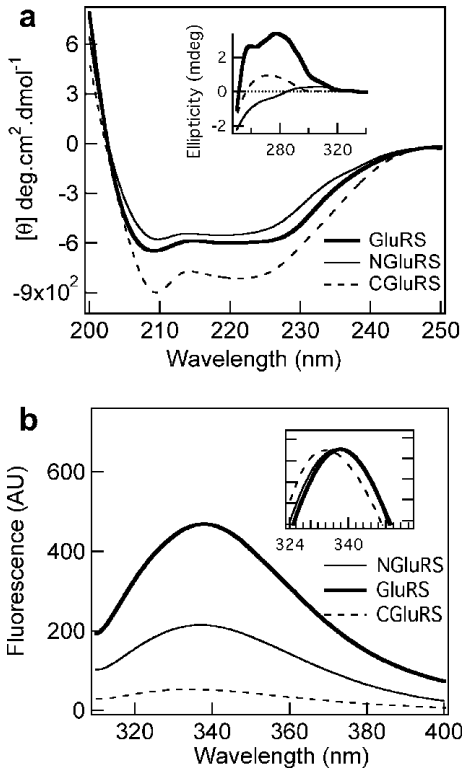

Fig. 2. (a) Far-UV CD spectra of *E. coli* GluRS (bold line), NGluRS (thin line) and CGluRS (broken line) shown as mean residue ellipticity. The near-UV spectra are shown in the inset as raw ellipticity. (b) Fluorescence spectra (λ_{ex} = 295 nm) of GluRS (bold line), NGluRS (thin line) and CGluRS (broken line). The spectra are normalized in the inset to emphasize the change in λ_{max}.

mains maintained structural integrity but lost some tertiary structure originally present in *Ec*GluRS.

3.1.2. Intrinsic fluorescence study

*Ec*GluRS has a total of eight Trp residues (six in NGluRS and two in CGluRS) that exhibit a range of solvent accessible surface area (ASA) computed from DSSP [22] program (3–8%: W27, W269, W401; 17–24%: W63, W68; 43%: W190, W305; 70%: W325; ⟨ASA⟩ ~ 26%) in a homology modeled [23] structure of *Ec*GluRS (Fig. 1a). The emission maximum (λ_{max}) of Trp fluorescence is a good indicator of Trp solvent accessibility with $\lambda_{max} \sim 350$ nm indicating solvent exposed Trp while a blue shifted λ_{max} indicating decreasing solvent accessibility. The fluorescence spectra of *Ec*GluRS, NGluRS and CGluRS are shown in Fig. 2b. *Ec*GluRS λ_{max} (338 nm; see inset to Fig. 2b) indicated buried Trp residues, compatible with the computed average ASA values. Upon domain separation, DSSP [22] calculations indicated a slight increase in ASA at one Trp residue, each in NGluRS (W305: 47%; ⟨ASA⟩ ~ 23%) and CGluRS (W325: 81%; ⟨ASA⟩ ~ 44%). The slightly blue shifted λ_{max} (337 nm) of NGluRS is compatible with a slight decrease in the computed ⟨ASA⟩. However the 4 nm blue shift in CGluRS λ_{max} (334 nm) is not compatible with the computed increase in ⟨ASA⟩. The origin of the blue shift could be a readjustment of local folding around W325 in CGluRS making it more buried than that that found in *Ec*GluRS. The observed λ_{max} can also be sensitive to the relative fluorescence yields of the Trp residues. So, if W325 in CGluRS has much diminished fluorescence yield compared to the other Trp residue (W401: ASA ~ 8%), it would also lead to a blue shifted λ_{max} in CGluRS. Despite the complications of interpreting λ_{max}, caused by differential fluorescence yields, the fluorescence data clearly showed that the domains are folded (buried Trp) maintaining considerable structural integrity.

3.2. Binding of NGluRS and CGluRS to cognate and non-cognate partners

Productive glutamylation of $tRNA^{Glu}$ by GluRS requires close proximity and binding of GluRS and its interacting cognate partners: $tRNA^{Glu}$, ATP and L-Glu. The first step in aminoacylation reaction is the formation of the complex aaRS-aminoacyl-adenylate, and the second step is the formation of aa-$tRNA^{aa}$. Although the first step is tRNA-independent for most aaRSs, except for a naturally occurring truncated GluRS variant called YadB that activates Glu in a tRNA-independent manner [24], amino acid activation is tRNA-dependent for GluRS [25]. In other words, the binding of one cognate ligand to GluRS is modulated by the presence of the other. Trp fluorescence quenching was used to study the binding of ATP, cognate (L-Glu, *Ec*$tRNA^{Glu}$) and non-cognate (L-Gln, D-Glu, *Ec*$tRNA^{Gln}$) ligands to *Ec*GluRS, NGluRS and CGluRS, in pairs or in isolation.

Fluorescence titrations for *Ec*$tRNA^{Glu}$ and *Ec*$tRNA^{Gln}$ binding to *Ec*GluRS, NGluRS and CGluRS are shown in Fig. 3a and the corresponding dissociation constants are shown in Table 1. Dissociation constants for *Ec*$tRNA^{Glu}$ binding to wild type *Ec*GluRS and NGluRS have been reported to be very similar with $K_d \sim 40$ nM [14]. As shown in Table 1, the K_d values measured by us match the previous values for cognate tRNA–GluRS/NGluRS interaction. The K_d values for non-cognate tRNA interaction increased by 10-fold for both *Ec*GluRS (K_d = 235 nM) and NGluRS (K_d = 499 nM). This demonstrates that the cognate as well non-cognate tRNA binding of the whole protein is qualitatively unaltered in isolated NGluRS. In contrast to the 10-fold decrease in non-cognate tRNA binding of NGluRS and *Ec*GluRS, binding of *Ec*$tRNA^{Glu}$ and *Ec*$tRNA^{Gln}$ to CGluRS were comparable (K_d = 87 and 116 nM).

Similar to the trend observed for *Tt*GluRS [26], *Ec*GluRS showed weak binding ($K_d \sim$ mM range) to cognate L-Glu, non-cognate D-Glu and L-Gln in absence of $tRNA^{Glu}$, but in presence of the cognate $tRNA^{Glu}$, the binding of the cognate amino acid L-Glu increased by 100-fold (K_d = 31 μM). This is compatible with the known $tRNA^{Glu}$-dependence of glutamylation by GluRS [25]. Presence of the non-cognate $tRNA^{Gln}$ did not significantly change the weak binding of L-Glu to GluRS. Weak binding of cognate and non-cognate amino acids and specific increase of L-Glu binding by about 100-fold in presence of *Ec*$tRNA^{Glu}$ was also observed for NGluRS implying a similar $tRNA^{Glu}$-dependent L-Glu binding as was observed for GluRS. Like *Ec*GluRS, the presence of *Ec*$tRNA^{Gln}$ did not affect the weak binding of L-Glu to NGluRS. The presence or absence of *Ec*$tRNA^{Glu}$ did not affect ATP binding to neither *Ec*GluRS (K_d = 49 and 58 nM) nor NGluRS (K_d = 91 and 64 nM). In summary, binding studies showed that NGluRS behaves very similarly to wild type

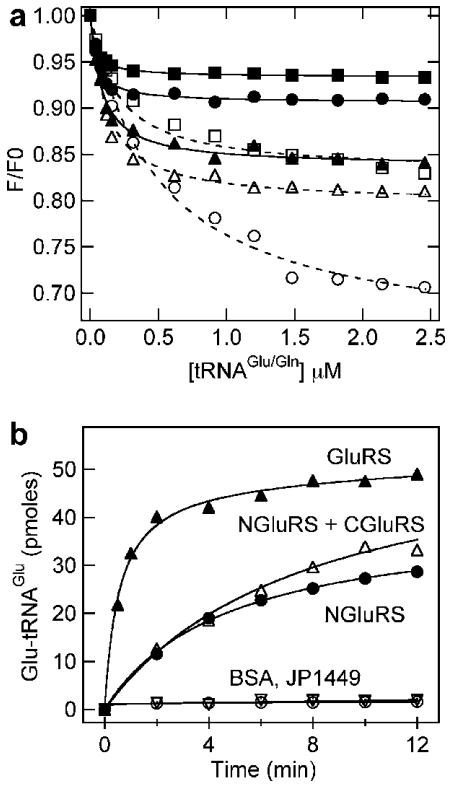

Fig. 3. (a) Trp fluorescence quenching in *Ec*GluRS (squares), NGluRS (circles) and CGluRS (triangles) as a function of added *Ec*$tRNA^{Glu}$ (filled symbols) and *Ec*$tRNA^{Gln}$ (empty symbols). The corresponding dissociation constants are given in Table 1. (b) Glutamylation assay curves of *Ec*GluRS (3 ng/assay point) (▲), NGluRS (1.5 μg/assay point) (●), 1:1 molar NGluRS + CGluRS (1.5 μg NGluRS/assay point; added in trans) (△), JP1449 (DE3) (▽) and BSA (1 μg/assay point) (○) with *Ec*$tRNA^{Glu}$.

Table 1
Dissociation constants (K_d) for binding of $tRNA^{Glu}$, $tRNA^{Gln}$, Glu, Gln and ATP to GluRS, NGluRS and CGluRS.[a]

Ligands	K_d (μM)						
	GluRS			NGluRS			CGluRS
	–	+$tRNA^{Glu}$	+$tRNA^{Gln}$	–	+$tRNA^{Glu}$	+$tRNA^{Gln}$	–
$tRNA^{Glu}$	0.04	–	–	0.04	–	–	0.09
$tRNA^{Gln}$	0.23	–	–	0.50	–	–	0.12
L-Glu	2310	31	881	1840	61	819	–
D-Glu	1830	3373	–	1790	909	–	–
L-Gln	3440	4380	–	4880	3530	–	–
ATP	49	58	–	91	64	–	–

[a] Glu, Gln and ATP binding was also followed in presence of $tRNA^{Glu}$ and $tRNA^{Gln}$ (only for L-Glu).

*Ec*GluRS in terms of binding cognate and non-cognate ligands and exhibiting $tRNA^{Glu}$-dependence of L-Glu binding.

Dissociation constants (K_d between GluRS variants and $tRNA^{Glu}$) reported in Table 1 exhibited an apparent inequality of dissociation free energies: ΔG° (*Ec*GluRS) < ΔG° (NGluRS) + ΔG° (CGluRS). Although this may indicate that a part of the excess free energy from binding of CGluRS is utilized in remodeling the active site via conformational changes in full length *Ec*GluRS, a quantitative comparison of binding free energies of a full length enzyme and its complementary truncated versions is not straightforward [27]. Nonetheless, as discussed in the next section, glutamylation assays showed that the effect of adding the anticodon-binding domain to the catalytic domain manifested mostly in k_{cat} values, indirectly indicating active site remodeling induced by the anticodon-binding domain.

3.3. Cognate tRNA aminoacylation

In an earlier report we showed that NGluRS was active [14]. However the aminoacylation activity of NGluRS was much diminished compared to that of wild type *Ec*GluRS. To reconfirm the earlier results, assay experiments were repeated at 37 °C (with proteins isolated from the over-expressing strain BL21 (DE3)) and at 42 °C (with proteins isolated from a thermo-sensitive strain JP1449 (DE3) containing mutant endogenous GluRS). The latter experiments were designed to eliminate any effect from background endogenous *Ec*GluRS. Additionally, the JP1449 (DE3) strain was grown without any plasmid DNA and was subjected to parallel isolation and purification procedures similar to NGluRS and treated as blank. The assay results were very similar at both temperatures. As shown in Fig. 3b, glutamylation activity of NGluRS was found to be $\sim 10^3$-fold lower than that of wild type *Ec*GluRS. Under similar conditions no detectable activity was observed for BSA (generic protein) and JP1449 (DE3) strain (blank), confirming the absence of any contamination from endogenous GluRS activity in the observed NGluRS data. The NGluRS activity remained almost same for 1:1 NGluRS:CGluRS (CGluRS added in trans) mixture. Our results confirmed an earlier report that NGluRS was active but with a diminished activity [14], and we quantified the reduced activity to be about 10^3-fold weaker than that of *Ec*GluRS. The reduction in activity is due to the absence of the anticodon-binding domain. That the reduced activity of NGluRS did not change when CGluRS was added in trans (Fig. 3b) demonstrated that the effect of the anticodon-binding domain could not be reproduced by simple diffusion mediated non-covalent domain–domain interactions. Rather, the anticodon-binding domain needed to be first covalently attached to the catalytic domain for proper manifestation of domain–domain interactions.

There are two ways the absence of the anticodon-binding domain could have affected the specific activity of NGluRS – at the *Ec*$tRNA^{Glu}$-NGluRS binding step (K_m) or at the subsequent catalytic step (k_{cat}). Kinetic parameters for glutamylation, K_m and k_{cat}, were determined for NGluRS and compared with *Ec*GluRS. K_m values, with respect to L-Glu and *Ec*$tRNA^{Glu}$, for GluRS (0.1 mM and 0.3 μM) [28] and NGluRS (0.2 mM and 1.2 μM) were very similar. This indicated that the lack of the anticodon-binding domain did not affect the $tRNA^{Glu}$ binding step. This is consistent with the fluorescence titration data (Table 1) where it was found that both *Ec*GluRS and NGluRS bound *Ec*$tRNA^{Glu}$ with similar affinities ($K_d \sim 0.04$ μM). However, the kinetic parameters for the catalytic step, with respect to L-Glu and *Ec*$tRNA^{Glu}$, were very different for *Ec*GluRS and NGluRS. For NGluRS, the k_{cat} value was $\sim 10^3$-fold lower (5.6×10^{-3} and 7.3×10^{-3} s^{-1}) than that for *Ec*GluRS (6.8 and 1.8 s^{-1}) [28,29], demonstrating the strong effect of the anticodon-binding domain even when it is physically distant from the catalytic site. This is consistent with the long-range domain–domain communication in GlnRS, a close relative of GluRS, as demonstrated by Uter and Perona [30] using pre-steady-state kinetics and by Jahn et al. [9] from k_{cat} values for the glutaminylation of synthetic *Ec*$tRNA^{Gln}$ mutants.

3.4. Non-cognate tRNA aminoacylation

*Ec*GluRS is a D-GluRS that does not glutamylate *Ec*$tRNA^{Gln}$. This is evident from comparative glutamylation assays of *Ec*GluRS with *Ec*$tRNA^{Glu}$ (Fig. 3b) and *Ec*$tRNA^{Gln}$ (Fig. 4a). NGluRS showed weak but detectable glutamylation of cognate $tRNA^{Glu}$ (Fig. 3a). However, similar to *Ec*GluRS, non-cognate ($tRNA^{Gln}$) glutamylation assays of NGluRS showed undetectable glutamylation of *Ec*$tRNA^{Gln}$ (Fig. 4a). For comparison, the cognate glutaminylation activity of the *Ec*$tRNA^{Gln}$ (4 μM) was followed with *Ec*GlnRS (1 ng/20 μl assay point) (Fig. 4a) to confirm that the *Ec*$tRNA^{Gln}$ used in non-cognate ($tRNA^{Gln}$) glutamylation assay experiments were active. Our results indicate that NGluRS, even without the anticodon-binding domain, known to be the primary source of $tRNA^{Gln}$ discrimination, maintains a discriminatory stand against $tRNA^{Gln}$.

Deletion of the entire anticodon-binding domain in NGluRS can drastically affect its interaction with $tRNA^{Gln}$ vis-à-vis GluRS–$tRNA^{Gln}$ interaction. Therefore, a counter explanation for this experimental data, in isolation, could be that insignificant glutamylation of $tRNA^{Gln}$ was observed not because NGluRS is inherently discriminatory but because NGluRS cannot properly orient with respect to $tRNA^{Gln}$ without the anticodon-binding domain. One way to test this hypothesis is to add a suitable anticodon-binding domain to NGluRS. A recently reported chimeric protein [14], cGluGlnRS, built by adding the anticodon-binding domain of

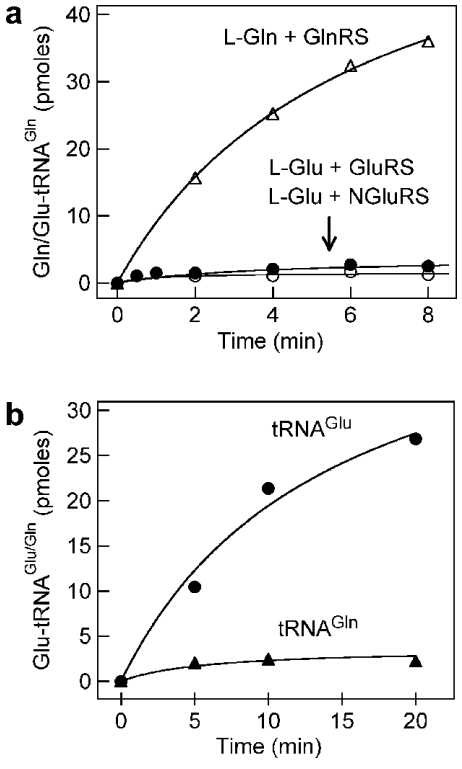

Fig. 4. (a) Non-cognate aminoacylation (glutamylation) assay curves of *Ec*$tRNA^{Gln}$ with *Ec*GluRS (3 ng/assay point) (●) and NGluRS (1.5 μg/assay point) (○). For comparison cognate aminoacylation (glutaminylation) assay curve of $tRNA^{Gln}$ with *Ec*GlnRS (1 ng/20 μl assay point) (△) is also shown. (b) Glutamylation assay curves of cognate ($tRNA^{Glu}$; (●)) and non-cognate ($tRNA^{Gln}$; (▲)) tRNA by cGluGlnRS (1 μg/assay point).

*Ec*GlnRS, was the best candidate to test since the anticodon-binding domain is optimized to interact with *Ec*tRNA^{Gln}. As reported earlier and shown in Fig. 4b, compared to *Ec*GluRS, cGluGlnRS showed a dimished (about 100-fold) *Ec*tRNA^{Glu} glutamylation activity while compared to NGluRS the activity was slightly higher (about 5–10-fold). This showed that attaching *Ec*GlnRS anticodon-binding domain to the catalytic binding domain of *Ec*GluRS (NGluRS → cGluGlnRS) as well exchanging the native anticodon-binding domain of *Ec*GluRS by the anticodon-binding domain of *Ec*GlnRS affect the cognate glutamylation efficiencies. However, the removal of anticodon-binding domain of *Ec*GluRS (*Ec*-GluRS → NGluRS) or exchanging the anticodon-binding domain of *Ec*GluRS by the anticodon-binding domain of *Ec*GlnRS (*Ec*-GluRS → cGluGlnRS) has no effect on the non-cognate glutamylation (of *Ec*tRNA^{Gln}) activity. As shown in Fig. 4, all the GluRS variants, *Ec*GluRS (Fig. 4a), NGluRS (Fig. 4a) as well as cGluGlnRS (Fig. 4b), exhibit undetectable glutamylation of *Ec*tRNA^{Gln}. The comparative cognate and non-cognate glutamylation data for the three *Ec*GluRS variants glutamylation data strongly suggest that it is not the lack of an anticodon-binding domain that makes NGluRS not glutamylate *Ec*tRNA^{Gln}, rather discrimination against *Ec*tRNA^{Gln} is an intrinsic property of the catalytic domain of *Ec*-GluRS. A quantitative comparison of the degrees of discrimination exhibited by NGluRS and *Ec*GluRS cannot be deduced since both exhibited undetectable *Ec*tRNA^{Gln} aminoacylation capacity.

4. Discussion

For many bacterial species tRNA^{Gln} discrimination is an inherent function of GluRS. It was earlier shown that the anticodon-binding domain of GluRS is responsible for such discrimination [11–13]. However, despite the knowledge that identity elements in tRNA^{Glu} and tRNA^{Gln} are present both in the anticodon loop and the acceptor arm [7–10], there has been no experimental attempt to explore the contribution of the catalytic domain of GluRS in tRNA^{Gln} discrimination. How does GluRS accomplish tRNA^{Glu} glutamylation and tRNA^{Gln} discrimination in terms of contributions from its two domains? A naturally truncated GluRS variant (YadB), homologous to the catalytic domain, is capable of activating L-Glu yet unable to deliver the activated Glu to either tRNA^{Glu} or tRNA^{Gln} [24]. A number of studies on isolated catalytic domains of other aaRSs have proved to be useful in delineating the functions of the catalytic and the anticodon-binding domains of aaRSs [31–33]. Following this strategy we addressed the question of tRNA discrimination by studying the N-terminal catalytic domain (NGluRS; 1–314) and the C-terminal anticodon-binding domain (CGluRS; 318–471) of *Ec*GluRS. Although binding of the cognate substrates (L-Glu and tRNA^{Glu}) was comparable for NGluRS and GluRS, an attenuated k_{cat} substantially diminished the glutamylation activity of NGluRS. tRNA^{Glu} binding to CGluRS and GluRS were comparable. However, the addition of CGluRS to NGluRS did not significantly alter NGluRS activity. Our results show that the intact anticodon-binding domain in wild type *Ec*GluRS affects transition state energetics (k_{cat} effect) despite being distant from the catalytic site. Isolated catalytic domain of other aaRSs, like *Bacillus stearothermophilus* LysRS [31] and *Ec*CysRS [32], also showed a much diminished catalytic activity towards their cognate aminoacylation reaction, resulting mainly from the destabilization of the transition state in the cognate amino acid activation step without affecting the ground state of substrate binding. In another report it was shown that besides being active towards cognate aminoacylation, a minimalist version of *Ec*GlnRS was found to charge a non-cognate tRNA^{Tyr}-derived amber suppressor (*supF*) with glutamine [33].

Surprisingly, even with a diminished activity and absence of the anticodon-binding domain, NGluRS retained the tRNA^{Gln} discriminatory property of *Ec*GluRS. The discrimination was also shown to be present for a chimeric protein where NGluRS was attached to the anticodon-binding domain of *Ec*GlnRS [14]. The fact that the catalytic domain retains discrimination in the absence of the cognate anticodon-binding domain as well as in the presence of the non-cognate anticodon-binding domain indicates that tRNA discriminatory elements are present in the catalytic domain of *Ec*-GluRS. Therefore, both the anticodon-binding domain and the catalytic domain contribute to tRNA discrimination.

A comparison of crystal structures of GluRS in *T. thermophilus* (D-GluRS) and *T. elongatus* (ND-GluRS) did not reveal any significant difference in their catalytic domains, in terms of their differential tRNA specificity [12]. Rather, the anticodon-binding domains of the two proteins clearly showed how the presence of a conserved Arg residue in D-GluRS (Arg358 in *Tt*GluRS) and its absence in ND-GluRS (Gly366) might lead to the discrimination of tRNA^{Gln} by D-GluRS. An Arg residue is also present in the anticodon-binding domain of *Ec*GluRS at the corresponding position (Arg350 in *Ec*GluRS). In addition, the presence of Ser (Ser438) in *Ec*-GluRS at a position corresponding to Gly417 in *H. pylori* GluRS2 also suggests that the anticodon-binding domain of *Ec*GluRS is discriminatory against tRNA^{Gln}. Yet, without the anticodon-binding domain (NGluRS), *Ec*GluRS still retained the capability to discriminate against tRNA^{Gln}. Our results are consistent with the work presented by Lee and Hendrickson [13] who showed that mutating a key Arg residue in the anticodon-binding domain of GluRS1 of *H. pylori* did not affect its tRNA^{Gln} discrimination. The catalytic domain of GluRS (NGluRS in *Ec*GluRS) is considered to be the ancestral domain in GluRS, originally non-discriminatory, from which extant discriminating and non-discriminating GluRS evolved by anticodon-binding domain acquisition [3,4]. Our result, that the catalytic domain of *Ec*GluRS is inherently discriminatory against *Ec*tRNA^{Gln}, is significant and calls for a detailed bioinformatics study, focusing on the origin of this discrimination at residue level and the evolution of tRNA^{Gln} discrimination in bacteria.

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