Chapter III

Fusion with anticodon binding domain of GluRS is not sufficient to alter the substrate specificity of a chimeric Glu-Q-RS
3.1 Introduction

Aminoacyl tRNA synthetases (aaRSs) are modular multi-domain proteins containing several domains including the catalytic domain that are responsible for cognate tRNA binding and catalysis. The aaRSs are thus necessary for maintaining the fidelity of translation during faithful protein synthesis in cellular context [1]. The main function of aaRSs consists in catalyzing the charging of the amino acid to the acceptor end of their cognate tRNA [1]. Based on the catalytic core structure, signature sequences, regiochemistry and interaction with their tRNA, the aaRSs are divided into two structural and functional classes, class I and class II [2]. The catalytic domains of the aaRSs are believed to belong to the most ancient macromolecules [3,4]. It has been postulated that during evolution, aaRSs acquired additional domains to improve catalytic efficiency, to increase specificity for their cognate substrates and also to promote hydrolysis of false end-products resulting from catalytic errors [5,6]. However, beside of their housekeeping function, aaRSs also acquired many non-canonical cellular functions [7-9].

Many organisms contain duplicated genes of a given full-length aaRS [6]. *E. coli* contains two LysRSs and *B. subtilis* has two ThrRSs that allow adaptation of the organism to distinct growth conditions [10]. However in several cases, organisms contain “aaRS like” genes encoding a protein homologous to the protein sequence of the catalytic core of the aaRS, deprived from anticodon binding domain, and which acquired a novel function during evolution [10]. A recently described example of aaRS paralog is glutamyl-Q-tRNA\(^{\text{Asp}}\) synthetase (Glu-Q-RS), a paralog of glutamyl-tRNA synthetase (GluRS) belonging to class I and present in the genome of more than forty species of actinobacteria, cyanobacteria and proteobacteria. Interestingly like GluRS this paralog activates glutamate and transfers it to a tRNA [11]. Glu-Q-RS exhibits striking structural similarity with the catalytic core of GluRS [12, 13]. The amino acid sequence of *E.coli* Glu-Q-RS shares 29 to 38% identity with bacterial GluRS [14]. Like GluRS, Glu-Q-RS contains an ATP binding domain in the Rossmann-fold including “KMSKS” and “HIGH” motifs separated by the connective peptide (CP) and the fold contacting the tRNA acceptor stem [15]. These
domains are conserved in the catalytic core of all class I aaRSs. However, in contrast to GluRS, Glu-Q-RS lacks the anticodon-binding domain [12] and despite of significant homologies with the catalytic core of GluRS, it exhibits distinct functional properties [16]. Whereas GluRS transfers the activated L-glutamate (L-Glu) on the 3’ accepting end of the cognate tRNA\textsubscript{Glu}, Glu-Q-RS transfers the activated L-Glu on queuosine (Q34) located in the first anticodon position of the tRNA\textsuperscript{Asp} generating a hypermodified tRNA\textsuperscript{Asp} [11,17]. Further, whereas glutamate activation by GluRS is tRNA dependent, Glu-Q-RS activates glutamate in the absence of tRNA [15,18]. Despite distinct functional properties and recognition of different tRNA species, most amino acids contacting the Glu and ATP substrates are conserved in Glu-Q-RS and GluRS [16].

Recently, it was reported that in the absence of the anticodon binding domain, the N-terminal part (domain 1-3) of \textit{E. coli} GluRS (NGluRS) can specifically bind to its cognate tRNA\textsuperscript{Glu}, however the catalytic efficiency of aminoacylation is decreased 270-fold compared to the full-length GluRS [19]. Since NGluRS is able to catalyze aminoacylation of only tRNA\textsuperscript{Glu} [19-21], the glutamylation capacity of tRNA\textsuperscript{Asp} by Glu-Q-RS is surprising.

To understand the substrate specificity of Glu-Q-RS we undertook a systemic approach by investigating the biophysical and biochemical properties of the NGluRS (1-301), CGluRS (314-471) and Glu-Q-RS-CGluRS (1-298 of Glu-Q-RS fused to 314-471 from GluRS). Circular dichroism, fluorescence spectroscopy and differential scanning calorimetry analyses revealed absence of N-terminal domain (1-298 of Glu-Q-RS) and C-terminal domain (314-471 from GluRS) communication in the chimera, in contrast to the native full length GluRS. Further, we found that the chimeric Glu-Q-RS-CGluRS conserved the affinity for the tRNA\textsuperscript{Glu} substrate but is unable to bind it functionally probably as a consequence of the lack of domain-domain communication.
3.2 Materials and Methods

Restriction enzymes were purchased from Fermentas. HIS-Select HF Nickel Affinity Gel column material was purchased from Sigma-Aldrich. The radioactive L-Glu was purchased from the PerkinElmer. All other materials were of analytical grade from Sisco Research Laboratory Pvt. Ltd., India and Merck, India. The in-vitro transcribed jack bean tRNA^A^ has been kindly provided by Pr Gabor Igloi, University of Freiburg, Germany.

3.2.1 Construction of chimera protein Glu-Q-RS-CGluRS

For construction of the chimera protein, the *yadB* gene encoding Glu-Q-RS (298 amino acids) and the part of the *gluS* gene encoding the CGluRS (amino acids 314-471) were amplified by polymerase chain reaction. As the crystal structure of *E. coli* GluRS was not available, the construct was based on the crystal structure of *Tth* GluRS, whose sequence is homologous to that of *E. coli* GluRS. The *yadB* gene was amplified using the following forward and reverse primers: 5’-CATGCCATGGGCATGACAGACACACAG-3’ and 5’-CGCGGATCCGCATGACGCATTTGAGAA-3’, containing Neol and BamHI restriction sites respectively. The CGluRS ORF was amplified by the following forward and reverse primers: 5’-CGCGGATCCCTGCCGCCGGAGTATG-3’ and 5’-CCCAAGCTTCTGCTGATTTTCGCGTTC-3’ harboring respectively the BamHI and HindIII restriction sites. The two amplicons were cloned together in frame in the pET28a(+) vector and the resulting chimeric C-terminal His-tagged protein consisting of the *E. coli* Glu-Q-RS (1-298)-GS- *E. coli* GluRS (314-471) residues, was named Glu-Q-RS-CGluRS (Fig.1). The two amino acids Gly-Ser (GS) were incorporated at the interface of the chimera to introduce a BamH1 site during primer designing. The ORF encoding the N-terminal His-tagged CGluRS was cloned in the same vector.
3.2.2 Expression and Purification of the proteins tRNA^{Glu} and tRNA^{Asp}

The *E. coli* DH5α strains overproducing *E. coli* tRNA^{Glu} and the *E. coli* GluRS were kindly provided by Pr J. Lapointe, (University of Laval, Quebec, Canada). *E. coli* GluRS(1-471), NGluRS(1-301), CGluRS(314-471) and Glu-Q-RS-CGluRS were expressed in the BL21(DE3) strain transformed by the recombinant pET-EcERSXT [19], pTYB11, and pET28a(+) vectors and Glu-Q-RS in the BL21(DE3) Rosetta II strain containing the recombinant pDEST17 vector [12]. NGluRS was expressed as a fusion with an intein-chitin binding domain. The strains were grown overnight at 37°C (except that expressing NGluRS which was grown at 25°C) and protein expression was induced at logarithmic-phase (A_{585 nm} 0.5-0.8) by addition of 1 mM of IPTG. For CGluRS and Glu-Q-RS-CGluRS purification, the cells were centrifuged, resuspended in lysis buffer (100 mM Tris-HCl pH 8.0, 5 mM β-mercapto-ethanol, 150 mM NaCl, 20% glycerol and 1 mM phenylmethanesulfonyl fluoride (PMSF)) and sonicated intermittently on ice by 8 pulses of 30 s each with 60 s intervals using a 130 Watt ultrasonic processor. The extracts were then centrifuged at 12000 xg for 30 min and loaded on a His-Select HF Nickel Affinity Gel column equilibrated with the lysis buffer. After washing with two volumes of lysis buffer containing 5 mM...
imidazole, the proteins were eluted with the same buffer containing 250 mM imidazole. The eluted fractions were analyzed by SDS-PAGE and those containing the protein of interest were dialyzed against 100 mM Tris-HCl buffer pH 8.0, containing 150 mM NaCl and 20% glycerol. The NGluRS expressing cells were sonicated in the binding buffer (50 mM Tris-HCl pH 8.0, 500 mM NaCl, 0.1 mM β-mercapto-ethanol, 0.5 mM Na2EDTA, 10% glycerol and 1 mM PMSF), the extract centrifuged for 90 min at 105000 xg and then loaded onto a chitin column equilibrated with the same buffer. After washing with ten volumes of binding buffer, the column was incubated for 48 hours at 4°C in the cleavage buffer (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 50 mM β-mercapto-ethanol, 0.5 mM Na2EDTA, 10% glycerol and 1 mM PMSF) before elution of the protein with the binding buffer. The eluted fractions were analyzed by SDS-PAGE and those containing the protein were dialysed against 100 mM Tris-HCl buffer pH 8.0 containing 10% glycerol. Purification of Glu-Q-RS and GluRS were performed as described [17,21]. Modified E. coli tRNAAsp (accepting capacity 32 nmol/mg) was purified from an overexpressing JM103 E. coli strain as described [17] and E. coli tRNAGlu (accepting capacity 35 nmol/mg) was isolated from overproducing DH5α (pKR15) strain as described [22].

### 3.2.3 Circular Dichroism

Far UV-CD (190-250nm) study of proteins was monitored in a JASCO J-815 spectropolarimeter at 25°C using a 1 mm path-length cuvette. For each sample 5 scans were performed with the scan speed of 20nm/min. The final scan was the average of 5 scans. The percentage of α-helix of proteins was calculated from the Mean Residue Ellipticity (MRE) value at 222 nm using Eqn. 3.1:

\[
MRE = \frac{[100 \times \text{Observed} \, (\theta) \times \text{MW}]}{[10 \times C \times n \times l]} \text{ in deg cm}^2 \text{dmol}^{-1} \text{......... (Eqn.3.1)}
\]

where \( \theta \) is the observed ellipticity in millidegrees, MW the molecular weight in kiloDalton (kDa), C the concentration in mg/ml, n the number of amino acids and l the pathlength in cm [23,24].
3.2.4 Fluorescence spectroscopy

Equilibrium unfolding of proteins was carried out in a HITACHI F-7000 fluorescence spectrometer at 25 °C. The excitation wavelength was 295 nm and the emission spectra were recorded from 310 to 410 nm. The excitation and emission slits width were 5 and 10 nm respectively. For denaturation studies, 2 µM of proteins were incubated with increasing concentrations of guanidine chloride (GdnHCl, 0-5 M) for 24h at room temperature to attain thermodynamic equilibrium. The emission maxima (λ_max) values were calculated from the first-order derivative of emission spectra of individual protein samples. The GdnHCl denaturation data were analyzed assuming a two-state mechanism of unfolding where the native protein cooperatively unfolds to denatured state as shown:

Native (N) ⇌ Denatured (D)

In the two-state mechanism native and denatured protein exist in the absence of well-populated intermediate conformations at equilibrium [25-28].

The CGluRS on the other hand followed a three-state unfolding mechanism where the native, partially unfolded (intermediate) and denatured forms of protein exist in significant population at equilibrium [29]:

Native (N) ⇌ Intermediate (I) ⇌ Denatured (D)

The fraction of protein unfolded at any denaturant concentration (f_D) was calculated [30,31] using Eqn. 3.2:

\[ f_D = \frac{Y - Y_N}{Y_D - Y_N} \]  

(Eqn.3.2)

where Y is the observed variable parameter and Y_N, and Y_D the baseline values for folded or native and unfolded or denatured conformations. The free energy for unfolding (ΔG^0), and the slope of the transition (m), were obtained from the non-linear least square fitted curves assuming a two-state unfolding mechanism of the proteins (except for CGluRS ) using Kyplot (Eqn. 3.3) [32]. The Eqn. for the two-state unfolding mechanism (Eqn. 3.3) [32]:

\[ f_D = \frac{Y - Y_N}{Y_D - Y_N} \]  

(Eqn.3.3)
\[
S = \frac{S_0 + S_\infty \exp \left( (-\Delta G^0 + (m \cdot [\text{GdnHCl}]))/RT \right)}{1 + \exp \left( (-\Delta G^0 + (m \cdot [\text{GdnHCl}]))/RT \right)} \quad (\text{Eqn. 3.3})
\]

However, unlike fluorescence intensity, the emission maxima of proteins are not directly related to denaturant concentration and thus a correction term introduced and the corrected Eqn. for the two-state unfolding mechanism (Eqn. 3.4) [32]:

\[
S = \frac{S_0 + (1-f_D)S_\infty \exp \left( (-\Delta G^0 + (m \cdot [\text{GdnHCl}]))/RT \right)}{1 + (1-f_D)\exp \left( (-\Delta G^0 + (m \cdot [\text{GdnHCl}]))/RT \right)} \quad (\text{Eqn. 3.4})
\]

Similarly, the corrected Eqn. for the three-state equilibrium unfolding (Eqn. 3.5) [32]:

\[
S = \frac{S_0 + (1-f_D)S_1\exp \left( (-\Delta G^0_1 + (m_1 \cdot [\text{GdnHCl}]))/RT \right) + (1-f_D)S_\infty \exp \left( (-\Delta G^0_2 + (m_2 \cdot [\text{GdnHCl}]))/RT \right)}{1 + (1-f_D)\exp \left( (-\Delta G^0_1 + (m_1 \cdot [\text{GdnHCl}]))/RT \right) + (1-f_D)\exp \left( (-\Delta G^0_2 + (m_2 \cdot [\text{GdnHCl}]))/RT \right)} (\text{Eqn. 3.5})
\]

Here, \( S_0, S_1, \text{ and } S_\infty \) are the signal intensities for 100% reactant, intermediate and product respectively. \( \Delta G^0 \) was the free energy at zero GdnHCl concentration, \( \Delta G^0_1 \) and \( \Delta G^0_2 \) the standard free energy differences between native and intermediate states and between native and unfolded states respectively, \( R \) the gas constant, \( T \) the absolute temperature and \( m_1 \) and \( m_2 \) the slopes of the first and second transitions.

The binding of tRNA\(^{\text{Asp}}\) and tRNA\(^{\text{Glu}}\) to GluRS, Glu-Q-RS-CGluRS and Glu-Q-RS was determined by measuring the intrinsic tryptophan fluorescence of the proteins kept at a concentration of 0.5 µM in 100 mM Tris-HCl buffer pH 8.0 containing 150 mM NaCl and 10% glycerol. The binding isotherm was determined by single point titration as described [33]. The inner-filter correction was performed using Eqn. 3.6 [34]:

\[
F_{corr} = F_{obs} \cdot 10^{\left( A_{ex}+A_{em}\right)L/2} \quad \text{.......................... (Eqn. 3.6)}
\]
where $F_{\text{corr}}$ the corrected fluorescence intensity, $F_{\text{obs}}$ the observed fluorescence intensity, $A_{\text{ex}}$ and $A_{\text{em}}$ the absorbance at the excited and emitted wavelength, and $L$ the pathlength of the cuvette. The dissociation constant ($K_d$) was determined from binding isotherm using Eqn. 3.7 [35]:

$$F_{\text{ratio}} = 1 - \Phi \left( (K_d + nP_0 + L_T) - \sqrt{(K_d + nP_0 + L_T)^2 - (4nP_0L_T)} \right) / \left( 2nP_0 \right) \ldots\ldots\ldots\ldots\ldots (\text{Eqn. 3.7})$$

where, $F_{\text{ratio}} (F/F_0)$ is the corrected fluorescence ratio, $\Phi$ the fluorescence ratio change amplitude, $P_0$ the protein concentration, $K_d$ the dissociation constant, $n$ the stoichiometry of binding sites and $L_T$ the total ligand concentration.

3.2.5 Differential scanning calorimetry method

The thermal stability of NGluRS, CGluRS, GluRS, Glu-Q-RS and Glu-Q-RS-CGluRS was investigated using a VP-DSC Microcalorimeter (differential scanning calorimetry). The experiments were done in 100 mM Tris-HCl buffer pH 8.0 containing 150 mM NaCl and 20% glycerol in the presence of 15 µM of enzyme. The heating rate was 1°C per min in the temperature range from 25 to 70°C. Buffer scan was subtracted from protein scan. After baseline correction the midpoint of transition ($T_m$) was calculated from the maximum peak value [36-38].

3.2.6 Aminoacylation assays

For all enzyme assay experiments, reaction mixtures containing standard aminoacylation buffer (50mM HEPES (pH 7.5), 16mM MgCl$_2$, 2mM ATP, 0.8mM β-mercaptoethanol, 0.1mM unlabelled L-Glu with trace amount of radioactive L-Glu (>1000 fold lower than unlabelled L-Glu) and 5µM tRNA$^{\text{Glu}}$ or 10 µM tRNA$^{\text{Asp}}$ respectively) was used. The enzymes assays of Glu-Q-RS-CGluRS and E. coli GluRS with E. coli tRNA$^{\text{Glu}}$ were conducted with L-$[^{3}\text{H}]$ Glu (42.9Ci mmol$^{-1}$) and respectively 0.4 and 0.2 µM of enzyme [21] and those with tRNA$^{\text{Asp}}$ with L-$[^{14}\text{C}]$ Glu (0.25 Ci mmol$^{-1}$) using 7.8 and 2 µM of enzyme respectively [17]. The assay experiments were performed at 37 °C and at each time point 10 µl of aliquot
were withdrawn, spotted on 3-MM Whatman filter paper, washed thrice with TCA (Trichloro acetic acid) and finally twice with absolute alcohol. The filter papers were then dried for 15 minutes in an oven, transferred to vial and cocktail-O was added to each vial to measure the remaining radioactivity on the filter paper using liquid scintillation counter.

3.2.7 Complementation assay

The JP1449 DE3 pLysS strain was a kind gift from Pr Jacques Lapointe (University Laval, Québec). This strain is unable to grow at 42°C due to an endogenous thermosensitive mutation in the gltX gene [39]. To test in vivo complementation by Glu-Q-RS-CGluRS, the strain was transformed by the plasmids containing GluRS (positive control) and Glu-Q-RS-CGluRS and allowed to grow at 32°C. The transformed colonies of the respective plasmids were then grown in a fresh solid medium with appropriate antibiotics at 42°C. Negative control was performed with the JP1449 DE3 pLysS strain transformed with the empty pET28a(+) plasmid.

3.2.8 Homology Modeling

The overall sequence from the Glu-Q-RS-CGluRS chimeric protein was submitted to the SWISS-MODEL server [40a]. A first homology model was generated using the Tih GluRS structure [PDB code 1j09; 41]. The N-terminal domain corresponding to the Glu-Q-RS sequence was then replaced by the structure of the E. coli Glu-Q-RS [PDB code 4a91; 50]. The model was then adjusted manually with COOT [42] to improve the Ramachandran plot and remove clashes. A final step of geometry optimization was performed using the phenix.pdbtools from the PHENIX package [43]. Finally the geometry and quality of the model was checked with MOLprobity [44].

In this context, I greatly acknowledge Professor Daniel Kern and Dr. Mickeal Blaise for helping me by constructing the above model.
3.3 Results

3.3.1 The domains of chimeric Glu-Q-RS-CGluRS are correctly folded

Circular dichroism is a very well recognized technique for studying secondary and tertiary structures of proteins in solution [23,24]. Variations of the conformation and secondary structures of the proteins can be monitored by far-UV CD spectra measurements between 200 to 250 nm. To determine whether the fusion of CGluRS with Glu-Q-RS alters the conformation and the secondary structure of the proteins the far-UV CD spectra of Glu-Q-RS, NGluRS, CGluRS, GluRS and Glu-Q-RS-CGluRS were compared (Fig. 3.2).

Fig. 3.2 Far-UV CD of E. coli Glu-Q-RS ( ), NGluRS ( ), CGluRS ( ), GluRS ( ) and Glu-Q-RS-CGluRS ( ) shown as mean residue elipticity (MRE) at 25°C. The concentrations of proteins were kept at 5µM in 100 mM Tris-HCl buffer (pH 8.0), 150 mM NaCl and 10% glycerol. The bandwidth was 1 nm. The pathlength of the cuvette for CD measurement was 0.1 cm. The arrows indicate the position of characteristic \( \alpha \)-helical minimum at 222 nm.
The CD spectra of NGluRS, CGluRS, GluRS and Glu-Q-RS show negative curves between 200-250 nm, indicating that all of them have formed a well-defined secondary structure. The percent of $\alpha$-helix present in NGluRS, CGluRS, GluRS and Glu-Q-RS as calculated using Eqn. 3.1 agreed well with previous reports [15,21]. However, whereas the CD spectra of Glu-Q-RS, NGluRS and Glu-Q-RS-CGluRS present a minimum at 208 nm, those of GluRS and CGluRS present additionally a shoulder at 222 nm (indicated by arrow in Fig. 3.2). This shoulder constitutes a signature for the presence of alpha-helices [45]. The CD spectra of Glu-Q-RS-CGluRS showed an increase in the content of $\alpha$-helices compared to Glu-Q-RS due to the high $\alpha$-helix content of CGluRS.

3.3.2 Glu-Q-RS is significantly more stable than NGluRS

The resistance of the proteins to unfolding was investigated by GdnHCl treatment. The denaturation curves of Glu-Q-RS, NGluRS, CGluRS, Glu-Q-RS-CGluRS and GluRS are shown in Fig 3.3. The fraction of unfolded protein ($f_u$) is calculated according to Eqn. 3.2 and plotted as a function of GdnHCl concentration. Table 3.1 shows the thermodynamic parameters of unfolding of the proteins derived from the curves.
Fig. 3.3 Fractional denaturation ($f_D$) curves of Glu-Q-RS ( ), NGluRS ( ▲ ), CGluRS ( ▼ ), Glu-Q-RS-CGluRS ( ● ) and GluRS ( ○ ) as a function of GdnHCl concentration. The unfolding profile was monitored by Trp fluorescence intensity at 340 nm and 350 nm ($F_{340}/F_{350}$) at 25°C. The excitation wavelength was 295 nm. The concentration of proteins were kept at 2 µM in 100 mM Tris-HCl buffer (pH 8.0), 150 mM NaCl and 10% glycerol. The data ($f_D$) were analyzed assuming a two-state mechanism of unfolding, except CGluRS that followed a three-state unfolding mechanism (see Materials and Methods).

Table 3.1. Thermodynamic parameters obtained from equilibrium denaturation of proteins by Gdn-HCl denaturant.

<table>
<thead>
<tr>
<th></th>
<th>Glu-Q-RS</th>
<th>Nter-GluRS</th>
<th>Cter-GluRS</th>
<th>Glu-Q-RS-CGluRS</th>
<th>GluRS</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta G$ (Kcal mol$^{-1}$)</td>
<td>4.98±0.12</td>
<td>2.2±0.14</td>
<td>1.5±0.33</td>
<td>9.8±0.48</td>
<td>4.7±0.76</td>
</tr>
<tr>
<td>$m$ (Kcal mol$^{-1}$ M$^{-1}$)</td>
<td>1.98±0.05</td>
<td>1.19±0.06</td>
<td>1.9±0.3</td>
<td>3.6±0.3</td>
<td>1.8±0.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.3±0.08</td>
</tr>
</tbody>
</table>
The experimental values fit well with the curve obtained by assuming a two-state unfolding mechanism except for CGluRS, for which we assumed a three-state unfolding mechanism for the fitting. The $\Delta G^0$ for *E. coli* GluRS of 4.6 kcal mol$^{-1}$ (Fig.3.3 and table 3.1), is in good agreement with previously reported data [20]. Glu-Q-RS is significantly more stable than NGluRS as revealed by their respective $\Delta G^0$ values of 4.9 and 2.2 kcal mol$^{-1}$. The m-value is strongly dependent on the change of cooperativity of protein folding [46]. Interestingly, unfolding of NGluRS is much less cooperative compared to that of Glu-Q-RS as shown by their m values (1.9 and 1.2 kcal mol$^{-1}$ M$^{-1}$). The two $\Delta G^0$ values (1.5 and 9.8 kcal mol$^{-1}$) derived from unfolding curve of CGluRS demonstrate that this domain or part of it is more stable than NGluRS. Interestingly Glu-Q-RS-CGluRS exhibits a chemical stability and an unfolding cooperativity comparable to those of Glu-Q-RS ($\Delta G^0 = 4.7$ kcal mol$^{-1}$ and m =1.8 kcal mol$^{-1}$ M$^{-1}$ for Glu-Q-RS-CGluRS and respectively 4.9 kcal mol$^{-1}$ and 1.9 kcal mol$^{-1}$ M$^{-1}$ for Glu-Q-RS). The transition point (50% unfolded, $f_D = 0.5$) is also an index of stability of protein [47]. Fig 3.3 clearly indicates that NGluRS has the lowest transition point among the proteins investigated here.

### 3.3.3 The thermal transition of Glu-Q-RS-CGluRS is non-cooperative

Differential scanning calorimetry (DSC) is a direct and accurate method to study the thermal stability of a protein [36-38]. The DSC study reveals the thermodynamic parameters of processes such as molar heat capacity change of unfolding ($\Delta C_p$), the midpoint of transition of denaturation ($T_m$) and the change of enthalpy ($\Delta H$) during unfolding.
Fig. 3.4 Differential scanning calorimetry thermograms of (A) Glu-Q-RS, (B) NGluRS, (C) CGluRS, (D) Glu-Q-RS-CGluRS and (E) GluRS. The raw excess heat capacity (dashed line) has been fit for a single “two-state process” (solid line). The thermograms were monitored with 15μM of protein.
except Glu-Q-RS-CGluRS) present at 9 µM in 100 mM Tris-HCl buffer (pH 8.0), containing 150 mM NaCl and 20% glycerol. The thermal transitions are reported in Table 3.2.

Table 3.2 Thermal unfolding Transition temperatures ($T_m$) of proteins obtained from Differential scanning calorimetry. The experiment was carried out in 100mM Tris-HCl (pH 8.0) buffer containing 150mM NaCl, 20% glycerol. Values are the means of at least three independent experiments with standard errors indicated.

<table>
<thead>
<tr>
<th>proteins</th>
<th>Transition Temperature ($T_m$) in $^\circ$C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu-Q-RS</td>
<td>56.72 ± 0.019</td>
</tr>
<tr>
<td>Nter-GluRS</td>
<td>46.07 ± 0.039</td>
</tr>
<tr>
<td>GluRS</td>
<td>61.86 ± 0.012</td>
</tr>
<tr>
<td>Cter-GluRS</td>
<td>61.95 ± 0.11</td>
</tr>
<tr>
<td>Glu-Q-RS-CGluRS</td>
<td>56.20±0.088</td>
</tr>
</tbody>
</table>

As the unfolding scans were irreversible for all proteins studied here, the thermodynamic parameter could not be determined accurately. Therefore we compared the thermal stability of the various proteins, by comparing the midpoint of the transition temperature ($T_m$) of their denaturation curves (Fig.3.4). Table-3.2 shows that the thermal stability ($T_m$) of Glu-Q-RS was ~5 $^\circ$C lower than that of GluRS and CGluRS. Though, Glu-Q-RS shows striking structural similarities with NGluRS, it exhibits a significantly higher thermal stability (~10 $^\circ$C). Thus, both the chemical and thermal denaturation studies, suggest that Glu-Q-RS is significantly more stable than NGluRS. To elucidate the influence of CGluRS on the structural stability of Glu-Q-RS, we determined the midpoint of transition of Glu-Q-RS-CGluRS. The thermal denaturation curve of Glu-Q-RS-CGluRS shows two transition peaks, $T_{m1}$ and $T_{m2}$ corresponding to ~56 and ~61 $^\circ$C. CGluRS and GluRS have comparable $T_m$ values as shown in the denaturation curves (table 3.2).
3.3.4 Glu-Q-RS-CGluRS is unable to complement in vivo a thermosensitive E. coli GluRS strain and also cannot aminoacylate tRNA\textsubscript{Glu} in vitro

A complementation assay (Fig.3.5) was performed to study whether Glu-Q-RS-CGluRS can replace GluRS for tRNA glutamylation activity \textit{in vivo}. The JP1449 (DE3) strain carries a temperature sensitive mutation in the chromosomal copy of \textit{E. coli} GluRS and thus is unable to grow at 42\textdegree C [39]. If Glu-Q-RS-CGluRS can complement the aminoacylation activity of the endogenous GluRS, transformation of the JP1449(DE3) strain with the plasmid encoding Glu-Q-RS-CGluRS should restore the growth at 42\textdegree C.

![Fig.3.5 Ampicillin-resistant E. coli JP1449(DE3) strain was transformed with the vectors recombined with the genes of GluRS and Glu-Q-RS-CGluRS and grown on LB agar plate at non-permissive temperature of 42\textdegree C.](image)

The complementation assay clearly indicates that Glu-Q-RS-CGluRS is unable to promote tRNA glutamylation and to complement the deficient GluRS \textit{in vivo} (Fig. 3.5). The aminoacylation assays conducted in presence of \textit{E. coli} tRNA\textsuperscript{Glu} and tRNA\textsuperscript{Asp} reveal that in contrast to \textit{E. coli} GluRS the Glu-Q-
RS-CGluRS chimeric protein is unable to charge tRNA\textsuperscript{Glu} (Fig. 3.6A), whereas Glu-Q-RS-CGluRS and Glu-Q-RS both can charge tRNA\textsuperscript{Asp} (Fig. 3.6B).

3.3.5 Glu-Q-RS-CGluRS binds both tRNA\textsuperscript{Asp} and tRNA\textsuperscript{Glu}

The binding of tRNA\textsuperscript{Glu} and tRNA\textsuperscript{Asp} to Glu-Q-RS, Glu-Q-RS-CGluRS and GluRS was analyzed by fluorescence quenching of tryptophan residues (Fig. 3.7). The tryptophan fluorescence intensity or quantum yield is extremely sensitive to local protein environment [48].
Fig. 3.6 tRNA Aminoacylation activities of GluRS, Glu-Q-RS and Glu-Q-RS-CGluRS.

(A) Comparison of the tRNA^{Glu} charging activities of GluRS (●) and Glu-Q-RS-CGluRS (○). GluRS and Glu-Q-RS-CGluRS were present at 0.2 and 0.4 µM respectively and tRNA^{Glu} at 5 µM. (B) Comparison of the tRNA^{Asp} charging activities of Glu-Q-RS (○) and Glu-Q-RS-CGluRS (●). Glu-Q-RS and Glu-Q-RS-CGluRS were present at 7.8 and 2 µM respectively and tRNA^{Asp} at 10 µM.
Fig. 3.7 Quenching of Trp fluorescence of Glu-Q-RS (▲), and Glu-Q-RS-CGluRS (▼) upon binding to tRNA^{Asp} and of Glu-Q-RS-CGluRS (■) upon binding to tRNA^{Glu} and of Glu-Q-RS-CGluRS upon binding to noncognate J Bean tRNA^{Arg} (●) at 25°C. The protein concentration was 0.5 µM in 100 mM Tris-HCl buffer (pH 8.0), 150 mM NaCl and 10% glycerol. The excitation and emission wavelengths were 295 and 340 nm and the bandpass was 5 nm. The fluorescence intensity F/F₀ ratio (F₀ was fluorescence intensity of tryptophan in the absence of tRNA and F was the fluorescence intensity of tryptophan in the presence of tRNA) were plotted as a function of tRNA concentration. Three experiments were conducted under the identical condition average values and standard error were calculated. The K_d values were calculated using Kyplot.
Table 3.3. Dissociation constants (K_d) for binding of tRNA^{Asp} to Glu-Q-RS, Glu-Q-RS-CGluRS and tRNA^{Glu} to GluRS, Glu-Q-RS-CGluRS. The K_d values were determined by fluorescence quenching method (\(\lambda_{ex} = 295\) nm and \(\lambda_{em} = 340\) nm). The data was fitted to single-site binding equation using Kyplot(version 2.0 beta 15 (32 bit), Koichi Yoshioka, 1997-2001).

<table>
<thead>
<tr>
<th></th>
<th>K_d of the enzyme-tRNA^{Asp} complex (µM)</th>
<th>K_d of the enzyme-tRNA^{Glu} complex (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glu-Q-RS</td>
<td>0.87±0.21(^a)</td>
<td>N.D.</td>
</tr>
<tr>
<td>Glu-Q-RS-CGluRS</td>
<td>0.76±0.2</td>
<td>0.08±0.07</td>
</tr>
<tr>
<td>GluRS</td>
<td></td>
<td>0.09 (^b)</td>
</tr>
</tbody>
</table>

\(^a\) Values are the means of at least three independent experiments with standard errors indicated. The concentration of proteins was 0.5 µM. \(^b\) is indicated as Dasgupta et al.[21]

The binding of tRNA^{Glu} and tRNA^{Asp} to Glu-Q-RS, Glu-Q-RS-CGluRS and GluRS was analyzed by fluorescence quenching of tryptophan residues (Fig.3.7). The tryptophan fluorescence intensity or quantum yield is extremely sensitive to local protein environment [48]. The tRNA^{Asp} and tRNA^{Glu} curves do not reach the same endpoint reflects that binding of these two tRNAs had differential affects on local environment of tryptophan within the protein. This in turn indicates that the mode of binding of these two tRNAs are different. We also use in-vitro transcribed jack bean tRNA^{Arg} as a control to show that binding of tRNA^{Asp} and tRNA^{Glu} is specific, not due to any contaminants. The jack bean tRNA^{Arg} binding is very weak as evident from linear decrease in fluorescence intensity [49] and very high dissociation constant of 9.58 µM (Fig 3.7). Dissociation constants (K_d) of 0.04 and 0.08 µM were determined for binding of *E. coli* tRNA^{Glu} to GluRS [21] and Glu-Q-RS-CGluRS respectively (table 3.3). Binding of *E. coli* tRNA^{Asp} to Glu-Q-RS-CGluRS and Glu-Q-RS show comparable K_d values (around 0.8µM) (Fig. 3.7, table 3.3).
3.3.6 Structural Model of Glu-Q-RS-CGluRS

Our results indicate that tRNA\textsuperscript{Glu} binds to Glu-Q-RS-CGluRS as well as to GluRS but that it cannot be aminoacylated by the chimera. To understand these results at the structural level, we constructed a model of the Glu-Q-RS-CGluRS (Materials and Methods).

Fig. 3.8 Modeling of the complex of Glu-Q-RS-CGluRS and tRNA\textsuperscript{Glu}. A- Superposition of the Glu-Q-RS-CGluRS chimera (blue) homology model on the structure of GluRS (grey) of *Thermus thermophilus* (orange and green) bound to tRNA\textsuperscript{Glu} (PDB:1N77). B- Zoom view of the acceptor stem of tRNA\textsuperscript{Glu} fitted in the catalytic centre of the Glu-Q-RS-CGluRS chimera; the protein molecular surface is in blue and GoA (the non-hydrolyzable analog of glutamyl-AMP) the analog of the activated Glu-AMP in yellow. C- Binding of the tRNA\textsuperscript{Glu} CCA end with GluRS (white) and model of binding with Glu-Q-RS-CGluRS (blue). The dash lines indicate H-bonds.
Superposition of this model to the known structures of *Tth* GluRS [41] in the absence and presence of substrates (Fig. 3.8A) (free GluRS, GluRS:tRNA\textsuperscript{Glu}:ATP (PDB:1N77) and GluRS:tRNA:GoA (1N78) [41] complexes) shows that tRNA\textsuperscript{Glu} anticodon and acceptor stems bind the Glu-Q-RS-CGluRS without triggering any major steric hindrance (Fig. 3.8A and 3.8B) and that the activated Glu can be accommodated in the active site [16,50] of Glu-Q-RS-CGluRS (Fig. 3.8B). Indeed most residues of the GluRS involved in binding of the tRNA\textsuperscript{Glu} acceptor end are conserved in the Glu-Q-RS, except Thr43 interacting with nucleotide C75 of tRNA\textsuperscript{Glu}, substituted in Glu-Q-RS and Glu-Q-RS-CGluRS by Ile47 (Fig. 3.8C).

### 3.4 Discussion

It is believed that paralogs are evolved via gene duplication and the active site residues in paralogs diverge to acquire new function. However, the other residues may be conserved to maintain the overall structure [51]. However, in Glu-Q-RS, a paralog of GluRS, many active site residues are conserved despite acquisition by the protein of distinct functional properties [16]. This is not surprising since structurally Glu-Q-RS is strikingly similar to the catalytic domain of GluRS (NGluRS) and since both accommodate two identical ligands in their active site, namely, L-Glu and ATP. However, despite these similarities Glu-Q-RS and NGluRS, differ by their catalytic mechanisms and by the specificity for their tRNA substrate. Glu-Q-RS activates L-Glu in the absence of tRNA whereas GluRS belongs with GlnRS, ArgRS and LysRS I to the family of aaRSs that require the cognate tRNA to activate the amino acid [18]. Further, GluRS transfers the activated Glu on the amino acid accepting end of tRNA\textsuperscript{Glu} whereas Glu-Q-RS transfers it on queuosine (Q34) from the anticodon of tRNA\textsuperscript{Asp}. It was proposed by Mirande and co-workers that the ancient GluRS was formed only by a catalytic domain able to charge both tRNA\textsuperscript{Glu} and tRNA\textsuperscript{Gln} and that the capacity to discriminate the tRNAs was acquired by addition of the anticodon binding domain (ABD) to the catalytic core [52]. In this view one may postulate that Glu-Q-RS diverged from ancient GluRS deprived from ABD by acquiring a novel tRNA charging specificity. Recently, Roy
and his group reported that the chimeric NGluRS-CGlnRS formed by fusion of the N-terminal domain of GluRS with the C-terminal one of GlnRS is several-fold more active in vitro than NGluRS and is able to substitute GluRS in vivo [4]. They therefore proposed that addition of the ABD permitting interaction with the anticodon, in addition to improve the specificity for tRNA\textsuperscript{Glu} also enhances the $k_{cat}$ of GluRS [4].

Further, Lapointe and co-workers reported that domains 4 and 5 of GluRS (ABD) increase the steady-state rate of tRNA aminoacylation by facilitating end-product release [19]. To understand the role of the ABD on the stability and on the activity of GluRS we analyzed the effect of addition of this domain on Glu-Q-RS a paralog of NGluRS. We therefore fused the CGluRS domain with Glu-Q-RS and compared the stability and the functional properties of the chimera to those of NGluRS, CGluRS and Glu-Q-RS.

Denaturation experiments (tables 3.1 and 3.2, Fig.3, 3.4A and 3.4B) demonstrate that Glu-Q-RS is substantially more stable than NGluRS despite similar length and structure of the two proteins, suggesting that stabilization of the N-terminal domain in the full-length GluRS, is promoted by the C-terminal domain. Beside to enhance protein stability, fusion of the two domains also creates allosteric effects between the two protein parts as evidenced by the chemical and thermal denaturation experiments. Indeed the melting curves reveal cooperative melting of GluRS contrasting with that of Glu-Q-RS-CGluRS, that shows two distinct peaks corresponding to those of Glu-Q-RS and CGluRS. Therefore in Glu-Q-RS-CGluRS the fused domains are independent and probably deprived of intercommunication.

The thermal denaturation experiments show further, that the ABD of GluRS (CGluRS) exhibits higher thermal stability than NGluRS suggesting that it possesses a peculiar function independent of that of N-GluRS probably by promoting functional binding of the cognate tRNA [19]. Indeed, it has been reported that CGluRS plays a crucial role in formation of the functional complex of GluRS with tRNA\textsuperscript{Glu} [19,41] since: i) the $k_{cat}$ of tRNA glutamylation of the full length GluRS is about 2000-fold higher than that of NGluRS [19], and ii) this $k_{cat}$ is strongly reduced when identity elements of tRNA\textsuperscript{Glu} anticodon loop are mutated [53] suggesting that interaction of the tRNA anticodon with the ABD transmits information to the active site of GluRS for productive interaction with the tRNA accepting end to form the catalytically
competent complex [20,53]. This agrees with investigation of Glutaminyl-tRNA synthetase (GlnRS) which shares a common evolutionary history to GluRS, which have shown that long-range intermolecular signaling through domain-domain communication is essential for tRNA$^{\text{Glu}}$ recognition and catalysis [54-56].

In contrast the denaturation studies of Glu-Q-RS-CGluRS we here show that the sole addition of CGluRS to of Glu-Q-RS affects neither the stability nor the activity of the enzyme since the two domains of the chimeric protein unfold independently and the aminoacylation capacity and tRNA specificity of the Glu-Q-RS are not altered. Nevertheless the tRNA binding experiments, in agreement with the model of Glu-Q-RS-CGluRS complexed to tRNA$^{\text{Glu}}$ indicates that the chimera binds tRNA$^{\text{Glu}}$ with similar affinity than the native GluRS, however it cannot promote tRNA aminoacylation in vitro and in vivo. Therefore the sole fusion of the GluRS ABD with Glu-Q-RS is unable to promote the allosteric effects to transmit the signal induced by interaction of the ABD to the active site of the protein. Transmission of allosteric signals along the interface of the *E. coli* AspRS-tRNA$^{\text{Asp}}$ complex from ABD to the catalytic centre for functional complex formation was also reported for the *E. coli* aspartylation system [57, 58].

Sequence comparison reveals that tRNA$^{\text{Glu}}$ and tRNA$^{\text{Asp}}$ present a high degree of similarity since many nucleotides are conserved [53]. Further Blaise et al reported striking sequence mimicry of 6 nucleotides located respectively at the 3’-accepting end of tRNA$^{\text{Glu}}$ (69-74), and the 5’ side of the anticodon stem-loop of tRNA$^{\text{Asp}}$ (38-43) in organisms expressing Glu-Q-RS [16], and substitution of only a few nucleotides in tRNA$^{\text{Asp}}$, switches its identity to tRNA$^{\text{Glu}}$ [53] since the chimeric tRNA becomes capable to promote L-Glu activation and to be glutamylated by GluRS. Thus only a few residues of GluRS and tRNA$^{\text{Glu}}$ are involved in transmission of the signal from ABD to the catalytic centre to create the enzymatic activity. This is surprising considering that GluRS and AspRS belongs to separate classes of aaRSs and evolved from different ancestors [53]. The sequence similarities between the two tRNAs, in particular the mimicry may have been the major driving force that selected tRNA$^{\text{Asp}}$ as substrate for Glu-Q-RS.
The biochemical and biophysical characterizations of chimeric Glu-Q-RS-CGluRS demonstrate that fusion of the ABD on Glu-Q-RS is not sufficient to convert Glu-Q-RS into GluRS. Our study thus reestablished the fact that two scenario for emergence of Glu-Q-RS from GluRS are equally possible as originally proposed by Blaise et al i) Glu-Q-RS may have evolved from ancient ND GluRS by mutations of the catalytic centre to permit recognition of the tRNA\textsuperscript{Asp} anticodon and to glutamylate the Q34 nucleotide or ii) alternatively it may have evolved from modern GluRS by lack of the ABD followed by acquisition of the capacity to glutamylate tRNA\textsuperscript{Asp} anticodon [16].

### 3.5 Concluding remarks

NGluRS aminoacylates tRNA\textsuperscript{Glu} much less efficiently than the full-length GluRS. Here we have shown that the chimeric Glu-Q-RS-CGluRS can bind tRNA\textsuperscript{Glu} very tightly but in contrast to GluRS is unable to aminoacylate it. There are several possibilities as listed below for this inability of the chimeric Glu-Q-RS-CGluRS to aminoacylate tRNA\textsuperscript{Glu}; it is possible that the CGluRS domain may be incorrectly positioned when fused to GluRS for productive binding of tRNA\textsuperscript{Glu}, the lack of domain-domain communication in Glu-Q-RS-CGluRS may also be an alternative explanation or the conformational entropy of the chimera may be too high to allow tRNA\textsuperscript{Glu} to bind in a productive conformation [59].
References


95


structural elements mediating tRNA-independent activation of glutamate and glutamylation of tRNAAsp anticodon. J. Mol. Biol. 381:1224-1237.


