CHAPTER 4

TERNARY COMPLEX OF AMINOACYL-tRNA SYNTHETASES WITH COGNATE AND NON-COGNATE tRNAs: IMPLICATION FOR PROTEIN SYNTHESIS
Abstract

Recognition of cognate tRNA and rejection of non-cognate tRNAs by aminoacyl-tRNA synthetases play a crucial role in reducing error in protein synthesis. It is generally believed that non-cognate tRNAs bind to the same active site, although with modestly weaker affinity. We show here by NMR, fluorescence quenching and capillary electrophoresis that non-cognate tRNAs bind to two aminoacyl-tRNA synthetases, Glutamyl- and Glutaminyl-tRNA synthetase, at a site different from the active site. We also demonstrate that tight ternary complex between cognate, non-cognate tRNA and aminoacyl-tRNA synthetases can be formed. This separate binding of the non-cognate tRNA does not inhibit aminoacylation of the cognate tRNA. We propose that binding of non-cognate tRNAs to a separate site lower the free intracellular concentration of tRNAs leading to lowered mis-acylation and increased fidelity of protein synthesis.
Introduction

Recognition of cognate tRNA and rejection of non-cognate tRNA by aminoacyl-tRNA synthetases (aaRSs) play the most vital role in reducing error in translation. The discrimination of the cognate tRNA occurs at several steps including binding and aminoacylation. In addition, the aaRSs have evolved many mechanisms to ensure that formation of incorrectly aminoacylated tRNAs are kept to a minimum. Editing and negative discrimination are two of these mechanisms. Apart from these inherent factors, other external forces strongly influence the mis-incorporation rates. Soll and co-workers (1) have demonstrated that overproduction of Glutaminyl-tRNA synthetase leads to dramatic increase in protein synthesis error rate, suggesting that relative levels of aminoacyl tRNA synthetase and its cognate tRNA are important for minimizing error rate. Intracellular levels of tRNAs may also have significant influence on translation rate and such responses as stringent response (2,3,4,5,6,7,8,9).

Although, much has been learned about cognate tRNA/aaRS interaction in the past decade, little is known about the interaction of non-cognate tRNAs with the synthetases. In vivo, however, the aaRS always aminoacylates in the presence of high levels of non-cognate tRNAs. It is generally assumed that non-cognate tRNAs bind to the same site as the cognate tRNA. Several studies had reported non-cognate/aaRS dissociation constant to be close to the cognate/tRNA pair. Given that the non-cognate tRNA concentration is more than an order of magnitude higher than the cognate one, this would imply significant competitive inhibition or misacylation. How the synthetases have evolved to cope with this problem is an interesting question. Glutaminyl- and Glutamyl-tRNA
synthetase are two evolutionarily close aaRSs. We have studied the interaction of non-cognate tRNA\textsuperscript{Glu} with GlnRS and tRNA\textsuperscript{Glu} with GluRS in order to understand how two closely related synthetases discriminate between the cognate and a non-cognate but evolutionarily related tRNA (10,11).
Experimental Procedures

Materials

Bis-tris, ampicilin, HEPES, acrylamide, ATP, PMSF, Poly ethylene glycol – 8000 (PEG-8000), Dextran T-500, BSA and L-glutamic acid were purchased from Sigma Chemical Co. (St. Louis, MO). 2-Mercaptoethanol was from Aldrich Chemical Co. Tryptone, agar and yeast extract were purchased from Hi-media (India). All other chemicals were of analytical grade. L-\[^{14}\text{C}\] glutamic acid was purchased from New England Nuclear.

Methods

Fluorescence Methods

All the fluorescence spectra were measured in a Hitachi F3010 spectrofluorimeter. The excitation and emission bandpasses were 5 nm unless stated otherwise. All the fluorescence values were corrected for volume change and inner filter effect according the equation:

\[ F_{\text{corr}} = F_{\text{obs}} \times \text{antilog} \left[ \frac{(A_{\text{ex}} + A_{\text{em}})}{2} \right] \]

Where \(A_{\text{ex}}\) is the absorbance at the excitation wavelength, \(A_{\text{em}}\) is the absorbance wavelength, \(F_{\text{corr}}\) is the corrected fluorescence and \(F_{\text{obs}}\) is the observed fluorescence.

Binding of tRNA\(^{\text{Glu}}\) and tRNA\(^{\text{Gln}}\) to GluRS were determined by fluorescence quenching at 25\(^{\circ}\) C. The protein concentration was 0.2 \(\mu\)M. Excitation was at 295 nm and the emission was measured at 340 nm. The experiment was carried out in 10 mM HEPES buffer, pH 7.2 containing 5 mM MgCl\(_2\). The data were fitted to a single site binding equation, using Kyplot. Both the initial fluorescence value and fluorescence at infinite
tRNA concentration were kept as floating parameter. Each point is an average of four independent titrations.

**Nuclear Magnetic Resonance**

The NMR spectra were measured in a 500 MHz Bruker DRX-500 NMR spectrometer in an 8-mm proton probe. WATERGATE water suppression pulse (using Z-pulsed field gradient) was used to suppress water signal. Temperature was 27° C. line broadening of 0.5 Hz was used for processing. The experiments were conducted in 20 mM potassium phosphate buffer, pH 7.2 containing 1 mM EDTA and 16 mM MgCl₂. The concentration of GluRS, tRNA⁶⁶ and tRNA⁶⁶³ are 80 μM respectively. For each spectrum, 512 scans were averaged.

**Assay Procedure**

GluRS was assayed by measuring the rate of formation of [¹⁴ C] glutamyl-tRNA. The final reaction mixture contains 50mM sodium HEPES buffer pH 7.2, 16 mM MgCl₂, 2 mM ATP, 0.8 mM 2-mercaptoethanol, 0.1mM L-[¹⁴ C] glutamate and 3.6mg/ml unfractionated tRNA. The final concentration of wild type GluRS in the reaction mixture was about 0.1 μg/ml to ensure the initial velocity condition. The enzymes were always diluted in 50 mM HEPES pH 7.2, containing 1mg/ml BSA, 1 mM 2-mercaptoethanol and 10% glycerol. After incubation at 37° C for various times (usually four time points for precise measurement), 10 μl aliquots were withdrawn and applied to Whatman 3 MM filter paper. After washing with cold trichloro acetic acid and isopropanol, the papers were dried and placed in a scintillation liquid and the precipitated radioactivity was determined by scintillation counting. Kinetic data were analyzed according to Lineweaver and Burk (12).
The kinetic constants $K_m$ and $K_i$ of GluRS for tRNA$\text{Glu}$ and tRNA$\text{Gln}$ were determined under similar conditions. Special care was taken to select GluRS concentration (0.85 nM) and incubation times to maintain linearity of incorporation of glutamate into tRNA over the observed time period. For the determination of $K_m$, tRNA$\text{Glu}$ concentrations were varied from 0.1 $\mu$M – 5 $\mu$M and for $K_i$ tRNA$\text{Gln}$ concentrations were varied from 10 $\mu$M – 50 $\mu$M.

Capillary Electrophoresis

Capillary Electrophoresis of tRNA$\text{Glu}$, tRNA$\text{Gln}$, tRNA/GluRS (cognate and non-cognate) complexes and that of ternary complexes were carried out in ds-DNA capillary cartridge in Beckman P/ACE 5010 system, under non-denaturing conditions. The electrophoresis was carried out in 100 mM Tris-HCl buffer pH – 7.5 containing 5 mM MgCl$_2$. The concentrations of tRNA$\text{Glu}$, tRNA$\text{Gln}$, tRNA/GluRS (cognate and non-cognate) complexes and that of ternary complexes were 50$\mu$M for each component. The data were analyzed using Beckman Gold software.
Fluorescence quenching or enhancement has been widely used to study ligand binding to proteins. In cases where the ligands do not fluoresce, quenching of protein internal tryptophan fluorescence upon ligand binding has been used to study protein-ligand interactions. Lin et al (13) have used both fluorescence and equilibrium dialysis to study the ligand binding in *E. coli* ArgRS and obtained similar results. Fluorescence quenching is one of the popular methods to study ligand binding to protein and has been used to study tRNA/synthetase interaction. Figure 1 shows the fluorescence quenching that occurs upon binding of tRNA$^{Glu}$ to GluRS and tRNA$^{Glu}$ to GluRS. With the cognate tRNA the extent of fluorescence quenching is significantly higher and occurs at lower total tRNA concentration than that of the non-cognate one. The profile was fitted to a single site binding equation using kyclot (Kyclot, version 2.0, beta 9 (32 bit) Koichi Yoshioka, 1997-2000) and the extracted dissociation constants are $1.07 \times 10^{-7}$ and $1.86 \times 10^{-6}$ for the cognate and non-cognate tRNA, respectively.

In our laboratory, Similar fluorescence quenching studies using GlnRS has been done and the results suggest that binding of tRNA$^{Glu}$ is also tight (Amit Kumar Mandal: personal communication). Due to smaller degree of quenching by non-cognate tRNA in this system, it was difficult to obtain quantitative results (Amit Kumar Mandal: personal communication). For this reason, we have decided to estimate the dissociation constant of GlnRS/tRNA$^{Glu}$ using NMR. GlnRS is a protein of molecular weight 64 Kda and binding of tRNA$^{Glu}$ (molecular weight of approximately 25 Kda) to this large protein is expected
Figure 4.1. Quenching of fluorescence of tryptophans upon binding of GluRS tRNA^{glu} (—O—) and tRNA^{glu} (—•—). The experiments were conducted in 10 mM HEPES buffer, pH 7.2 containing 5 mM MgCl₂ at 25°C. The excitation wavelength was 295 nm and the emission wavelength was 340 nm. The protein concentration in both cases were 0.2 μM. The profile was fitted to a single site binding equation using kymplot (Kyplot, version 2.0, beta 9 (32 bit) Koichi Yoshioka, 1997-2000) and the extracted dissociation constants are $1.07 \times 10^{-7}$ and $1.86 \times 10^{-6}$ for the cognate and non-cognate tRNA, respectively.
to lead to significant line broadening due to slower tumbling rate. Imino proton region (13.3-15 ppm) is generally free of protein resonances and can be used to assess line broadening of imino resonances. Figure 2A shows the imino region spectra of tRNA\textsuperscript{Glu}, and figure 2B shows a 1:1 mixture of tRNA\textsuperscript{Gln} and tRNA\textsuperscript{Glu} complex at a concentration of 100 µM.

Given that it is possible to detect complex formation of non-cognate tRNA and synthetases using line broadening as criterion, it should be possible to detect ternary complex formation, particularly if the imino proton spectra of the two tRNAs do not overlap. Figure 3A shows the imino region spectra of a 1:1 mixture of GlnRS/tRNA\textsuperscript{Glu} at a concentration of 80 µM each. Even at this low concentration there is no significant change in linewidth is seen when compared to higher complex concentration, indicating little dissociation, if any. We estimate that the dissociation constant is well below 10\textsuperscript{-5} M. Similar results were obtained with GluRS/ tRNA\textsuperscript{Gln}. Figure 3B shows the imino region spectra of 1:1:1 mixture of tRNA\textsuperscript{Gln}, tRNA\textsuperscript{Glu} and GlnRS at concentration of 80 µM each. There are several resonances in the imino proton region of tRNA\textsuperscript{Gln} and tRNA\textsuperscript{Glu}, which do not overlap. These resonances can be used to detect ternary complex formation. If resonances from both tRNAs are broadened simultaneously in a 1:1:1 mixture of the cognate, non-cognate tRNA and the synthetase, then it is likely that a ternary complex has formed. Clearly resonances from both the tRNAs are broadened and approximately to the same extent. This is strongly suggestive of a ternary complex formation.
Figure 4.2. NMR spectra of imino region spectra of (A) tRNA$^{Glu}$ and (B) 1:1 mixture of tRNA$^{Glu}$ and tRNA$^{Glu}$ complex at a concentration of 100 μM. In both cases the experiments were done in 10 mM potassium phosphate buffer, pH 7.5, containing 100 mM KCl, 10 mM MgCl$_2$ and 1 mM EDTA. Imino proton region (13.3-15 ppm) is generally free of protein resonances and can be used to assess line broadening of imino resonances.
Figure 4.3. NMR spectra of imino region spectra of (A) 1:1 mixture of tRNA$^{\text{Glu}}$ and GlnRS and (B) 1:1:1 mixture of tRNA$^{\text{Glu}}$ and tRNA$^{\text{Glu}}$ and GlnRS complex at a concentration of 80 µM. In both cases the experiments were done in 10 mM potassium phosphate buffer, pH 7.5, containing 100 mM KCl, 10 mM MgCl$_2$ and 1 mM EDTA. Imino proton region (13.3-15 ppm) is generally free of protein resonances and can be used to assess line broadening of imino resonances. Clearly resonances from both the tRNAs are broadened and approximately to the same extent. This is strongly suggestive of a ternary complex formation.
Electrophoretic methods, primarily electrophoretic mobility shift assay (EMSA) have been used to detect complex formation between protein and nucleic acids. A modern version of the EMSA can be carried out in capillary, the advantage being speed and online non-radioactive detection. Figure 4 shows the capillary electrophoresis pattern of tRNA\textsuperscript{Gln}, tRNA\textsuperscript{Glu}, GluRS and 1:1 mixtures of GluRS/ tRNA\textsuperscript{Glu}, GluRS/tRNA\textsuperscript{Gln} and 1:1:1 mixture of GluRS/ tRNA\textsuperscript{Glu} / tRNA\textsuperscript{Gln}. The two tRNAs move roughly at about the same position, showing slight heterogeneity. Addition of GluRS to these tRNAs cause retardation as expected. Interestingly, in the GluRS/ tRNA\textsuperscript{Glu} / tRNA\textsuperscript{Gln} mixture, there is only one major peak, slightly behind the peak of the binary complex. More importantly there is no free tRNA peak, indicating that 50 µM of GluRS is capable of complexing 50 µM of tRNA\textsuperscript{Gln} and 50 µM of tRNA\textsuperscript{Glu}. This putative ternary complex has a mobility slight slower than the binary complexes which may not be unexpected as e/m ratio goes down upon ternary complex formation (compared to free tRNA assuming little charge addition from the protein).

Tight binding of non-cognate tRNA to a site distinct from the active site raises the question whether such a binding has any effect on the enzymatic properties of the synthetases. We have measured the inhibition constant of the non-cognate tRNAs. Table 1 and figure 5 and 6 respectively show the $K_m$ (cognate) and $K_i$ (non-cognate) values for tRNA\textsuperscript{Glu} and tRNA\textsuperscript{Gln} using GluRS. In both cases the $K_m$ values are close to reported previously and the $K_i$ values are very high and competitive in nature, much above the binding constants of the non-cognate tRNAs. It is likely that the binding of the non-cognate tRNAs to the tight site has very little influence on the aminoacylation kinetics.
and weaker inhibition constant is due to much weaker binding to the active site. Interestingly, Soli and co-workers (14) have obtained a $K_m$ value of 50 $\mu$M for misacylation of tRNA$^{\text{Glu}}$ by GlnRS. This value is not unlike the value of $K_i$ obtained here.
Figure 4.4. Capillary electrophoresis of tRNA^Gln\(^{-}\) (---\(\vee\)--), tRNA^Glu\(^{-}\) (---\(\diamondsuit\)--), 1:1 mixtures of GluRS/ tRNA^Glu\(^{-}\) (---\(\ominus\)--), GluRS/tRNA^Gln\(^{-}\) (---\(\Delta\)--) and 1:1:1 mixture of GluRS/ tRNA^Glu\(^{-}\)/ tRNA^Gln\(^{-}\) (---\(\bullet\)--). The two tRNAs move roughly at about the same position, showing slight heterogeneity. Addition of GluRS to these tRNAs cause retardation as expected. Interestingly, in the GluRS/ tRNA^Glu\(^{-}\)/ tRNA^Gln\(^{-}\) mixture, there is only one major peak, slightly behind the peak of the binary complex. More importantly there is no free tRNA peak, indicating that 50 \(\mu\)M of GluRS is capable of complexing 50 \(\mu\)M of tRNA^Gln\(^{-}\) and 50 \(\mu\)M of tRNA^Glu\(^{-}\). This putative ternary complex has a mobility slightly slower than the binary complexes which may not be unexpected as e/m ratio goes down upon ternary complex formation (compared to free tRNA assuming little charge addition from the protein).
Figure 4.5. Lineweaver-Burk Plot for the determination of $K_m$ for Glutamyl-tRNA synthetase (GluRS). The concentrations of tRNA$^{Glu}$ used were 0.1, 0.5, 1, 2 and 5 μM. From the slope and intercept the $K_m$ value obtained was 0.327 μM for tRNA$^{Glu}$. GluRS was assayed by measuring the rate of formation of $[^{14}C]$ glutamyl-tRNA. The final reaction mixture contains 50mM sodium HEPES buffer pH 7.2 containing 16 mM MgCl$_2$, 2 mM ATP, 0.8 mM 2-mercaptoethanol, 4 μM tRNA$^{Glu}$ and variable concentration of L- $[^{14}C]$ glutamate. The final concentration of wild type GluRS in the reaction mixture was about 0.1 μg/ml. The enzymes were always diluted in 50 mM HEPES pH 7.2, containing 1mg/ml BSA, 1 mM 2-mercaptoethanol and 10% glycerol. After incubation at 37°C for various times (usually four time points for precise measurement), 10 μl aliquots were withdrawn and applied to Whatman 3 MM filter paper. After washing with cold trichloro acetic acid and isopropanol, the papers were dried and placed in a scintillation liquid and the precipitated radioactivity was determined by scintillation counting.
1 / nmoles of product formed per minute

1 / [tRNA] μM⁻¹
Figure 4.6. Lineweaver-Burk Plot for the determination of $K_i$ for tRNA$^{\text{gln}}$ using GluRS. The different concentrations of tRNA$^{\text{gln}}$ used are 10, 30 and 50 μM. The concentrations of tRNA$^{\text{Glu}}$ used were 0.1, 0.5, 1, 2 and 5 μM. From the slope and intercept the average $K_i$ value obtained was 40 μM for tRNA$^{\text{gln}}$. GluRS was assayed by measuring the rate of formation of [${}^{14}$C] glutamyl-tRNA. The final reaction mixture contains 50mM sodium HEPES buffer pH 7.2 containing 16 mM MgCl$_2$, 2 mM ATP, 0.8 mM 2-mercaptoethanol, 4 μM tRNA$^{\text{Glu}}$ and variable concentration of L- [${}^{14}$C] glutamate. The final concentration of GluRS in the reaction mixture was about 0.1 μg/ml. The enzymes were always diluted in 50 mM HEPES pH 7.2, containing 1mg/ml BSA, 1 mM 2-mercaptoethanol and 10% glycerol. After incubation at 37° C for various times (usually four time points for precise measurement), 10 μl aliquots were withdrawn and applied to Whatman 3 MM filter paper. After washing with cold trichloro acetic acid and isopropanol, the papers were dried and placed in a scintillation liquid and the precipitated radioactivity was determined by scintillation counting.
**Discussion**

The demonstration of a separate non-cognate tRNA binding site on two synthetases raises question about its importance to the function of the synthetases. Existence of relatively tight binding sites would certainly cause the free tRNA concentration to go down. Since the *in vivo* tRNA concentration is in between the binding affinity of the cognate and non-cognate tRNA to the active site, it should lead to larger lowering of aminoacylation rate of the latter than the former. This would lead to very significant lowering of misacylation and lower error rate in protein synthesis.

*In vivo* it has been estimated that there are approximately 63000-135000 tRNA molecules and 9400-16000 aaRS molecules in the cell under various growth conditions. Out of these approximately 83% of tRNA are aminoacylated. Almost all the aminoacylated-tRNAs are bound to EF-Tu. Thus, taking an average value from the above range (100,000 tRNA and 13,000 aaRS) about 17,000 molecules of tRNA are unacylated. On an average it would translate to approximately 650 aaRS and 850 unaminoacylated tRNA molecules in the cell for each tRNA-aaRS pair. Thus, an aaRS is in equilibrium with 850 cognate and 16,000 non-cognate tRNA molecule, equivalent to approximately 1.5 and 30 μM cognate and non-cognate tRNAs, respectively.

Assuming the dissociation constant of the cognate complexes to be around 1 μM under *in vivo* conditions, approximately 500 tRNA molecules would be free for each pair of tRNA-aaRS and rest bound to aaRSs as cognate complex (assuming no non-cognate binding). Thus total non-cognate concentration is approximately 10,000 molecules/cell or
20 µM. Thus, an aaRS is in equilibrium with approximately 1 µM cognate and 20 µM non-cognate tRNA.

So, aaRSs are in equilibrium with a concentration of cognate tRNA (1µM) that is much above the $K_m$ value for aaRS-tRNA pair (0.327 µM for tRNA$^{\text{Glu}}$ - GluRS pair). Again, an aaRS is in equilibrium with approximately 20 µM non-cognate tRNAs, which is much lower than the value of $K_i$ for aaRS-non cognate tRNA pair (40µM for tRNA$^{\text{Glu}}$ - GluRS pair). So, in vivo, lowering of free tRNA concentration should lead to far larger lowering of catalytically competent non-cognate complex than the cognate one. This in turn, would lead very significantly to lowering of misacylation resulting in lower error rate in protein synthesis.
Table 4.1. Determination of $K_m$ and $K_i$ for tRNA$^{\text{glu}}$ and tRNA$^{\text{gln}}$ using GluRS:

<table>
<thead>
<tr>
<th>$K_m$ for tRNA$^{\text{glu}}$ (µM)</th>
<th>$K_i$ for tRNA$^{\text{gln}}$ (µM)</th>
</tr>
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<tbody>
<tr>
<td>0.327</td>
<td>40</td>
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Kinetic constants of tRNA$^{\text{glu}}$ and tRNA$^{\text{gln}}$ using GluRS, measured by the rate of $[^{14}\text{C}]$glutamylation of tRNA$^{\text{glu}}$ in pure *E. coli* tRNA$^{\text{glu}}$ at 37°C and pH 7.2. For determination of $K_i$ for tRNA$^{\text{gln}}$ we have used three different concentrations, 10, 30 and 50 µM and the average value is given in the table. The concentrations of tRNA$^{\text{glu}}$ used are 0.1, 0.5, 1, 2 and 5 µM. In each case GluRS concentration was 0.1 µg/ml to ensure initial velocity.
References


