CHAPTER 2

ION AND WATER RELEASE DURING SUBSTRATE BINDING BY GLUTAMYL-tRNA SYNTHETASE: IMPLICATION FOR DISCRIMINATION
Abstract

Discrimination of cognate and non-cognate tRNA by aminoacyl-tRNA synthetases occurs at several steps during the catalytic cycle. The initial binding step certainly discriminates between cognate and non-cognate tRNAs, although the structural and molecular basis of such discrimination is not completely understood. We have studied the effect of ionic strength on association constants of cognate and non-cognate tRNAs by glutamyl-tRNA synthetase from *E. Coli*. The slope of the log $K_a$ versus log [KCl] plot indicated that the number of ion release is less than 2 in case of cognate tRNA<sub>Glu</sub> / GluRS interaction and 0.5 in case of non-cognate tRNA<sub>Ala</sub> / GluRS interaction. Water release stiochiometry was calculated from the effect of tri-ethylene glycol on the association constants using the method of Rau and Garner (1). In GluRS, cognate tRNA<sub>Glu</sub> binding leads to release of about 2 water molecules, while non-cognate tRNA<sub>Glu</sub> binding leads to release of 8 water molecules. This result is in sharp contrast to specific and non-specific protein-DNA interactions. Unexpectedly, non-cognate tRNA<sub>Glu</sub> binding to GluRS, leads to larger water release stiochiometries, suggesting additional interactions between non-cognate tRNAs and the synthetases. It appears that although the non-cognate tRNAs bind to the synthetases with only a modest decrease in affinity, when compared to the cognate ones, the nature of the interactions are significantly different. Implication of these results for the discrimination process is discussed.
Introduction

Correct aminoacylation of cognate tRNAs by aminoacyl-tRNA synthetases is perhaps the most important step in transition of mRNA code to the linear sequence of amino acids in proteins. *In vivo*, the aminoacyl-tRNA synthetases have to achieve a high degree of specificity in aminoacylation in the presence of many non-cognate tRNAs presenting similar conformational features (1). This high degree of specificity is a result of direct interactions and indirect conformational preferences of different nucleo-bases present in different tRNAs. It is likely that all the bases do not play an important role in imparting an "identity" on a tRNA. Those bases, which are important in determining the identity of a tRNA in terms of correct aminoacylation have been termed "identity elements" and have been mapped for several tRNAs (2,3).

Mechanically, how these identity elements increase aminoacylation rate, is not well understood. Their influence may occur at several stages (1) at the initial binding level (2) at the catalysis level or (3) at the post catalysis step, i.e. editing. Although editing is probably an important feature of some synthetases (4,5,6), it is unlikely to be of general importance. The binding and catalysis steps can be separated in a formal sense and importance of each step can be elucidated in the process. However, in analogy with other enzymes, it is likely that part of the binding energy is utilized in the catalysis step through conformational changes. Evidence for such a mechanism is mounting (7,8) and a major future challenge is to elucidate the structural and physico-chemical nature of such a mechanism.
One of the ways to investigate the nature of transition-states and ground states is to study the effect of ionic strength and hydration on different steps of aminoacylation. A number of workers have investigated ion and water release that occurs upon protein-DNA interaction (9,10,11). In case of regulatory proteins, ion and water release upon binding of a protein to specific DNA sequence constitutes a major driving force. On the other hand, very little water release occurs upon binding of these proteins to non-specific DNA sequences, which is driven primarily by ion release. It has been suggested that the water release in the specific complexes may occur because of direct, specific hydrogen bonding of protein side-chains with the polar atoms in the major and minor grooves of DNA, which does not occur in the non-specific complexes. In addition, catalytic steps of restriction endonucleases have been probed for water release using neutral osmolytes. Sliger and co-workers have looked at the dependence of $k_{cat}$ upon osmotic pressure to infer hydration changes in the transition states (12).

Clearly, use of such techniques to study ion and water release in the ground and transition states of aminoacylation would yield important information of how the specificity of aminoacylation is achieved. The initial binding step of tRNA and synthetase interaction has been studied for several cognate and mutant tRNAs, primarily by derivation of $K_M$ through steady-state kinetics. A study of direct equilibrium binding of tRNAs with aminoacyl-tRNA synthetases in different salt/osmolyte concentrations can provide a wealth of information about ion and water release stiochiometries. This in turn can provide information on the nature of forces involved in tRNA/synthetase complexes, thus shedding light on the importance and the role of the initial binding step on the overall process of discrimination. More importantly, knowledge about the transition-state can be
derived by studying the effect of salt and osmolytes on the $k_{cat}$. Due to its transient nature, these types of indirect techniques are the only ones available for elucidation of the nature of the transition-state. In this chapter, we have studied ion and water release that occurs upon binding of tRNA$^{\text{met}}$ to its cognate synthetase, glutamyl-tRNA synthetase from E. coli.
**Experimental Procedures**

**Materials**

tRNAs_{\text{glu}}, DTT, PEG-8000, Dextran T-500, PMSF were purchased from Sigma chemical Co. (St. Louis, MO). The measured specific activity of tRNA_{\text{glu}} was around 1.5 nmoles/A_{260}\text{and was used without further purification. All other chemicals used were of analytical grade.}

**Methods**

**Purification of GluRS**

Purification of GluRS was carried out according to Lin et al., (13). DH5α cells, containing conditional runaway replication plasmid pLQ7612, were grown in LB medium, supplemented with 0.1 mg/mL ampicilin. The cells were first grown at 32°C upto an A_{595} value of 0.2, during which the plasmid is present at one copy/cell. This was followed by a 55 minute heat-shock at 43°C, which allows amplification of plasmid upto 1000 copies per cell. This was achieved by immediately mixing equal volumes of medium, one kept at 55°C without cells, but having 0.1 mg/mL ampicilin and the other containing cells grown at 32°C upto an A_{595} value of 0.2, so that the final A_{595} value became 0.1. Finally, a residual growth of 2 hours allowed the expression of the amplified genes and growth to an A_{595} value of 0.6. The cells were harvested and frozen at this point.

Frozen cells (5 g) were thawed after addition of buffer A (20 mM Tris-HCl, pH-6.8 containing 10% glycerol, 0.1 mM EDTA, 2 mM PMSF and 3 mM 2-mercaptoethanol),
disrupted by sonication in an ice bath and centrifuged at 12000 X g for 45 minutes to remove cell debris.

Partition in a Poly Ethylene Glycol – 8000 (PEG-8000) and Dextran T-500 two phase system

The partition was carried out in 20 ml buffer A (20 mM Tris-HCl, pH-7.8, 10% glycerol, 7 % PEG, 1.5 % Dextran T-500, 0.05 mM EDTA, 1 mM PMSF and 1.5 mM 2-mercaptoethanol) and the suspension was mixed for 1 hour and then the two phases was separated by centrifugation at 12000 X g for 15 minutes. The top phase aqueous layer contains most of the GluRS activity and it was taken for further purification.

Anion-exchange Chromatography on a Mono-Q HR-5/5 column

The top phase was diluted four times with buffer B (20 mM Bis-Tris, pH-6.4, 0.2 mM 2-mercaptoethanol, 10% glycerol and 0.1 mM PMSF) to allow it to be loaded directly at a flow rate of 1 ml/min on a Pharmacia Mono-Q HR-5/5 column equilibrated in the same buffer. The column was first washed with buffer B and then with a 0 to 0.6 M NaCl gradient in the same buffer (0 to 10 ml 0% NaCl, 10 to 25 ml 30% NaCl, 25 to 50 ml 50% NaCl, 50 to 60 ml 100% NaCl) (15). The GluRS activity was eluted at about 0.2 M NaCl (33%).

Chromatography on a Hydroxyl Appetite (HAP) column

The eluted protein was pooled and dialyzed against 10 mM potassium phosphate buffer, pH-6.8 and then injected directly to a Bio-Rad FPLC HAP column equilibrated against the same dialyzed buffer. Then a linear gradient from 20 mM to 300 mM was run and the variant was eluted near 170 mM phosphate buffer (16) and was >95% pure as judged by SDS-PAGE at this stage.
Isolation of tRNA\textsubscript{Glu}

*Escherichia coli* (DH5\textalpha{}) containing pKR15 plasmid were grown overnight in Luria-Bertani (LB) broth medium supplemented with 100 µg/ml ampicillin at 37°C. Cells were pelleted down at 5000 rpm for 15 minutes. Frozen cells (12 g) were resuspended in 20 ml buffer (10 mM magnesium acetate, 10 mM Tris-HCl, and pH-7.2). To 20 ml cell suspension equal volume of phenol (saturated with 10 mM Tris-HCl, 1 mM EDTA pH-7) was added and shaken for 30 minutes in dark condition. After that the cells were centrifuged at 5000 rpm for 10 minutes at 20°C. Aqueous layer from centrifuge tube was transferred into the previous flash and 40 ml resuspension buffer was again added and again it was shaken for 30 minutes. The process was repeated thrice and then all the aqueous layers were pooled and again the same procedure was repeated using phenol saturated buffer. The aqueous layer again pooled and to that 2.5 volume ice-cold isopropanol was added and kept at -20°C overnight.

Next day a thick white precipitate was formed and was pelleted down at 4°C for 25 minutes at 14000 rpm. The pellet was resuspended in another buffer (0.2 M Tris-HCl pH-9) at room temperature under sterile condition. After that the solution was again centrifuged at 4°C for 10 minutes at 14000 rpm. Supernatant was taken in a sterile conical flask.

**Chromatography on DE-52 column**

The supernatant was loaded on a 20 ml DE-52 column which was already equilibrated with buffer (0.2 M NaCl, 0.001 M EDTA, 0.01 M magnesium chloride, 0.002 M sodium thiosulphate and 0.02 M Tris-HCl pH 7.5). After loading the column was washed with
the same buffer and then with a linear gradient from 0.2 M to 0.9 M NaCl in the same buffer at a flow rate of 1 ml/min. The tRNA\textsuperscript{Glu} activity was eluted near 0.4 M NaCl.

**Fluorescence Methods**

All the fluorescence spectra were measured in a Hitachi F3010 spectrofluorimeter. The excitation and emission bandpasses were 5 nm unless stated otherwise.

Binding of tRNA\textsuperscript{Glu} to GluRS was determined by fluorescence quenching at 25\textdegree C. The protein concentration was 0.5 µM. 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 1 mM MgCl\textsubscript{2} and different concentrations of KCl or Tri-ethylene glycol.

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.

Fluorescence at each point was measured separately. The fluorescence of the protein at a concentration of 0.2 µM was determined first. A pre-determined concentration of tRNA was added to that and after about 1 minute, the fluorescence was determined again.

The data were fitted to a single site binding equation, using Kyplot (14). 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.
Results

Ion release resulting from protein-DNA interaction has been studied from the dependence of binding equilibrium on ionic strength. Figure 1 shows plots of log association constants versus log salt concentrations for Glutamyl-tRNA synthetase/tRNA\textsuperscript{Glu} interaction. The inset of the plot shows actual quenching curves for two different salt concentrations. The plot shows monotonic decrease of affinity with increasing salt concentration. The slope of the line corresponds to a cation release stochiometry of 1.7. This value is much smaller than protein-DNA interactions. In lac repressor Record and co-workers have estimated a cation release stochiometry of approximately 8 upon lac repressor binding to operator.

Figure 2 shows plots of log association constants versus log salt concentrations for Glutamyl-tRNA synthetase/tRNA\textsuperscript{Glu} interaction. The inset of the plot shows actual quenching curves for two different salt concentrations. The plot shows monotonic decrease of affinity with increasing salt concentration. The slope of the line corresponds to a cation release stochiometry of 0.5. It is not unexpected but the number of cation release is too small in comparison to protein-nonspecific DNA interaction.

Figure 3 shows the crystal structure of lac repressor-operator complex in which only protein heavy atoms within 4.5 Å of the phosphorus atoms are shown. 4.5 Å was chosen as a cutoff as any group, which hydrogen bonds with the DNA phosphates will be within
Figure 2.1. Plots of log association constants versus log salt concentration for Glutamyl-tRNA synthetase (GluRS)/tRNA^{gln} interaction in 10 mM HEPES buffer, pH 7.2 containing 1 mM MgCl₂ at 25°C. The excitation wavelength was 295 nm and the emission wavelength was 340 nm. The protein concentration was 0.2 μM. Fluorescence at each point was measured separately. The fluorescence of the protein at the concentration of 0.2 μM was determined first. A pre-determined concentration of tRNA was added to that and after about 1 minute, the fluorescence was determined again. Each point is an average of four independent titrations. The inset of the plot shows actual quenching curves for 25 mM KCl (---•---) and 100 mM KCl (---Δ---). The data were fitted to a single site binding equation, using kyplot.
Plot showing the relationship between Log $[KCl]$ and Log $K_a$. The graph includes data points and curves indicating the change in $F/F_0$ with respect to [tRNA].
Figure 2.2. Plots of log association constants versus log salt concentration for Glutamyl-tRNA synthetase (GluRS)/tRNA^\text{Gln}\) interaction in 10 mM HEPES buffer, pH 7.2 containing 1 mM MgCl_2 at 25\(^\circ\)C. The excitation wavelength was 295 nm and the emission wavelength was 340 nm. The protein concentration was 0.2 \(\mu\)M. Fluorescence at each point was measured separately. The fluorescence of the protein at the concentration of 0.2 \(\mu\)M was determined first. A pre-determined concentration of tRNA was added to that and after about 1 minute, the fluorescence was determined again. Each point is an average of four independent titrations. The inset of the plot shows actual quenching curves for 25 mM KCl (—□—) and 100 mM KCl (—Δ—). The data were fitted to a single site binding equation, using kyplot.
Figure 2.3. Crystal structure of lac repressor-operator complex in which only protein heavy atoms within 4.5 Å of the phosphorus atoms are shown (in blue). 4.5 Å was chosen as a cutoff as any group, which hydrogen bonds with the DNA phosphates will be within that distance from the phosphorus atoms. As can be seen from the figure, groups from lac repressor protein closely approach approximately 13 phosphates (color yellow). Phosphates with yellow color are within 4.5 Å of protein chain while phosphorus colored red are the other one which present in the DNA molecule but not within 4.5 Å of the protein chains.
Figure 2.4. Crystal structure of *Thermus thermophilus* Glutamyl-tRNA synthetase/tRNA<sub>gIu</sub> complex in which only protein heavy atoms within 4.5 Å of the phosphorus atoms are shown (in green). 4.5 Å was chosen as a cutoff as any group, which hydrogen bonds with the DNA phosthates will be within that distance from the phosphorus atoms. As can be seen from the figure, groups from GluRS protein closely approach approximately 14 phosphates (color yellow). Phosphorus colored red are the other one which present in the RNA molecule but not within 4.5 Å of the protein chain.
Figure 2.5. Plots of log association constants versus triethylene glycol (TEG) concentration for Glutamyl-tRNA synthetase (GluRS)/tRNA\textsuperscript{glu}-interaction in 10 mM HEPES buffer, pH 7.2 containing 1 mM MgCl\textsubscript{2} at 25\textdegree{}C and different concentrations of TEG. The excitation wavelength was 295 nm and the emission wavelength was 340 nm. The protein concentration was 0.2 \textmu{}M. Fluorescence at each point was measured separately. The fluorescence of the protein at the concentration of 0.2 \textmu{}M was determined first. A pre-determined concentration of tRNA was added to that and after about 1 minute, the fluorescence was determined again. Each point is an average of four independent titrations. The inset of the plot shows actual quenching curves for 0.25M TEG (\textcircled{□}) and 1.5M TEG(\textblacktriangle{}). The data were fitted to a single site binding equation, using kyplot.
Figure 2.6. Plots of log association constants versus triethylene glycol (TEG) concentration for Glutamyl-tRNA synthetase (GluRS)/tRNA^taa interaction in 10 mM HEPES buffer, pH 7.2 containing 1 mM MgCl₂ at 25°C and different concentrations of TEG. The excitation wavelength was 295 nm and the emission wavelength was 340 nm. The protein concentration was 0.2 μM. Fluorescence at each point was measured separately. The fluorescence of the protein at the concentration of 0.2 μM was determined first. A pre-determined concentration of tRNA was added to that and after about 1 minute, the fluorescence was determined again. Each point is an average of four independent titrations. The inset of the plot shows actual quenching curves for 0.25M TEG (—□—) and 1.5M TEG(—△—). The data were fitted to a single site binding equation, using kyplot.
that distance from the phosphorus atoms. As can be seen from the figure, groups from lac repressor protein closely approach approximately 13 phosphates (Raswin, MolecularGraphics, Windows Version 2.6-ucb). This is close to the number of ion release obtained from ionic strength dependence of association constant.

Figure 4 shows the picture of tRNA\textsuperscript{Glu} as approached by atoms of glutamyl-tRNA synthetase from \textit{Thermus thermophilus} using same cutoff as above. Clearly, 14 phosphates are closely approached by groups from glutamyl-tRNA synthetase. This number is far higher than ion release stiochiometry obtained from ionic strength dependence of the association constant. Possible reasons for this discrepancy is discussed below.

Neutral osmolytes have been widely used to estimate hydration changes that occur during protein-DNA complex formation. Figure 5 shows the change in association constant as a function of a neutral osmolyte, triethylene glycol (TEG). The inset shows the fluorescence quenching at two different triethylene glycol concentrations. The association constant increases modestly as the concentration of TEG is increase upto 1.5 M indicating modest amount of water release upon complex formation. The slope of the line corresponds to a water release stiochiometry of about 2 water molecules per molecule of tRNA/synthetase complex formed. The number is much smaller than water release that occurs upon specific protein-DNA complex formation. Again, we have attempted to use
lac repressor as a benchmark since its water release stoichiometry upon complex formation as well as the structure has been well investigated. It has been reported that binding of lac repressor to its operator releases about 210 water molecules. If one calculate the surface area that is buried upon complex formation (excluding any conformational change), it translates to $3600 \text{ Å}^2$ in case of lac repressor/operator interaction and $4500 \text{ Å}^2$ for GluRS/ tRNA$^{\text{Glu}}$ interaction. It would be anticipated that water release should be in the order of 200 or more for GluRS/ tRNA$^{\text{Glu}}$ interaction.

It has also been reported that binding of lac repressor to non-specific DNA sequence releases much lower amount of water than that of specific DNA sequence, i.e., operator. So we have titrated GluRS with its non-cognate tRNA$^{\text{Glu}}$ in order to observe whether there have been any change in number of water release. Figure 6 shows the change in association constant as a function of a neutral osmolyte, triethylene glycol (TEG). The inset shows the fluorescence quenching at two different triethylene glycol concentrations. The association constant increases modestly as the concentration of TEG is increase upto 1.5 M indicating modest amount of water release upon complex formation. The slope of the line corresponds to a water release stiochiometry of about 8 water molecules per molecule of non-cognate tRNA/synthetase complex formed. Surprisingly the number of water release is more in case of non-cognate tRNA/synthetase complex formation compared to cognate tRNA/synthetase complex formation. The slope of the log $K_a$ versus log [MX] plots is less than 2. This result is in sharp contrast to specific and non-specific protein-DNA interactions. Water release stiochiometries were calculated from the effect of tri-ethylene glycol using the method of Rau and Garner (1). In GluRS, cognate tRNA$^{\text{Glu}}$
binding leads to release of about 2 water molecules. Unexpectedly, non-cognate \textit{tRNA}^{\text{gin}}

binding to GluRS, lead to larger water release stiochiometries, suggesting additional interactions between non-cognate tRNAs and the synthetases. It appears that although the non-cognate tRNAs bind to the synthetases with only a modest decrease in affinity, when compared to the cognate ones, the nature of the interactions are significantly different. Implication of these results for the discrimination process is discussed.
DISCUSSION

The identity elements of a tRNA are a set of nucleotides that positively influences the overall rate of aminoacylation. They may influence in two ways:

1. The initial binding step, that is the $K_m$ of the aminoacylation reaction

2. The rate determining step, that is the $k_{cat}$, which is the acyl transfer to the 3'-terminal adenosine

Although, the measurement is quite simple, unlike DNA-protein interaction, the interpretation is complicated by the irregularity of tRNA structure and little knowledge about its hydration properties. The interaction points between the tRNA and the cognate synthetase (in GluRS and tRNA$^{Glu}$) are largely irregular structures (loops and single stranded regions). To our knowledge, no previous water release study has been reported for RNA-protein interactions. Thus, we have attempted to correlate the ion and water release with structural information using the crystal structures of the ground state.

With respect to ion and water release, the most well characterized protein-nucleic acid interaction is perhaps lac repressor/operator complex. About 8 cations and 200 water molecules are released due to lac repressor dimer binding to lac operator. In the process, approximately 4000 Å$^2$ surface area becomes buried. However, with GluRS, similar amount of surface area buried with the release of about 2 cations and 2 water molecules.

The number of phosphate atoms in the nucleic acid that is closely approached by protein groups, for lac repressor it is 13 and for GluRS it is about 14. Clearly the difference in ion release stiochiometry has no correspondence with the structure and similarly, water
release stiochiometry also has no correlation in protein-DNA and protein-tRNA complexes. There may be several explanations for this discrepancy:

1. The counter-ion distribution (condensation) is entirely different, particularly in the vicinity around the interaction regions, due to irregular nature of tRNA structure;

2. Interactions such as hydrophobic interaction may dominate over other interactions and becoming stronger at higher ionic strength, partially compensating for the decrease of binding constant at higher ionic strength due to ionic interaction;

3. The solution structure of the and tRNA\(^{\text{Glu}}\) - GluRS complex is different from crystal structure;

4. Binding is coupled to a structural transition that compensates for some of the ion release.

Various studies suggest that there is no significant difference between solution and crystal structure. So possibility 3 can be discarded. The crystal structure of free GluRS and tRNA complexed does not have any major difference. So the fourth possibility of lowering of water molecules released can also be discounted. The first two possibilities are not mutually exclusive and both may be operative. In fact, Altman and coworkers (15) have observed virtually no ionic strength dependence of RNA-protein interaction in Ribonuclease P.

The structural origin of low water release stiochiometry is also not obvious, although the nature of hydration of tRNA is not known in great detail. One possibility that the water release is so high in lac repressor/operator interaction is that the interaction occurs primarily through the major groove of the DNA, which may be fairly polar and highly hydrated. Whereas interaction of the tRNA occurs primarily with hydrophobic bases.
which may be stacked in the free state and not significantly hydrated. Whatever the reason for such low ion and water release, it is clear that the structural correlation of DNA-protein complex has to be modified significantly in tRNA-synthetase complexes. Surprisingly, in case of GluRS, the water stoichiometry is larger in case of non-cognate tRNA\textsubscript{\text{\text{\textgln}}} /GluRS complex, which is completely different from lac repressor/operator complex. This again a strong evidence that DNA-protein interaction is different from tRNA-synthetase interaction. Not only this is true but also the larger number of water stoichiometry also a very strong indication of the fact that cognate tRNA\textsubscript{\text{\textgln}} /GluRS interaction may be completely different from that of non-cognate tRNA\textsubscript{\text{\textgln}} /GluRS interaction.
References