CHAPTER 3

UREA INDUCED EQUILIBRIUM UNFOLDING OF GLUTAMYL-tRNA SYNTHETASE
Abstract

The glutamyl-tRNA synthetase (GluRS) and glutaminyl-tRNA synthetase (GlnRS) are closely related and belong to the same subclass of class I. The amino-terminal half of *Thermus thermophilus* GluRS shows a sequence similarity with that of *Escherichia coli* GlnRS, while the carboxy terminal half of GluRS displayed an all a-helix architecture compare to all b-barrel structure of GlnRS carboxy terminal. Extensive studies has been done previously in our laboratory on the unfolding behavior of GlnRS and it has been found that GlnRS have a stable "molten globule" like intermediate at low urea concentration. (23). We have studied the urea induced unfolding behavior of GluRS, a multidomain protein, by equilibrium method mainly using fluorescence and circular dichroism (CD). The far UV CD and fluorescence studies demonstrate that GluRS may have an intermediate nearly at 3M urea concentration. But near UV CD and Stoke's radius measurement clearly showed that the intermediate is not "molten globule" like. So, we have isolated both N- and C-terminal together after proteolysis with thermolysin to see whether domain-domain interactions is the primary reason for the different urea denaturation pattern between GlnRS and GluRS. But FPLC gel filtration have shown that domain-domain interaction is not so strong. So, it may be the architectural difference in the C-terminal of GluRS and GlnRS plays the major role for the differences in the nature of intermediate of these two very closely related proteins.
Introduction

One of the most important problems in physical biochemistry is how the information present in the linear amino acid sequence of a protein is translated into a unique three-dimensional structure (1). It is generally believed that, the unique three-dimensional structure of a protein having specific function, to be the thermodynamically most stable conformation that can be adopted by a polypeptide chain (1), with important exceptions (2). Folding and unfolding behavior of smaller proteins are often characterized by two-state behavior (3). In contrast, it has been observed that refolding of large multi-domain proteins is characterized by an initial rapid phase in which large changes in several physical parameters (fluorescence, CD and hydrogen exchange) are seen (4). This is followed by a much slower phase, characterized by regaining of biological activity (5). This has been interpreted as initial rapid folding of domains to a compact domain-unpaired state, followed by slow domain pairing. Whether this domain pairing leads to a global energy minimum is unknown at the present. The denaturation studies on large multi-domain proteins have also lagged behind, although it has been suggested that majority of the proteins in the cell belong to this class (6). Compact denatured states in proteins can be generated by varying pH, temperature, and concentrations of various kinds of denaturants, such as, urea and guanidine hydrochloride (7,8,9). The characterization of these compact denatured states in proteins had a tremendous impact on folding studies (10,11,12).

Aminoacyl-tRNA synthetases (aaRS) strictly recognize and ligate their cognate tRNA and amino acid, thus contributing to the fidelity of translation of genetic information. In spite of the common features of the aminoacylation reaction, the 20 aaRSs exhibit broad
structural diversity. In 1990, Eriani et al proposed, on the basis of adenosine triphosphate (ATP)-binding motifs, that the 20 aaRS are divided into two classes, each consisting of 10 members (13). The members of class I and II have been subdivided into three and four subclasses, respectively (14,15). The glutaminyl- and glutamyl-tRNA synthetase (GlnRS and GluRS, respectively) are closely related and show some sequence similarity (16). Moreover they belong to the same subclass of class I. Gram-negative bacteria and the cytoplasm of eukaryotes have GlnRS which aminoacylates glutamine transfer RNA (tRNA^{Gln}) with glutamine. In contrast, when GlnRS is missing, GluRS aminoacylates both tRNA^{Glu} and tRNA^{Gln} with glutamate, and an amidotransferase converts the Glu-tRNA^{Gln} to Gln- tRNA^{Gln}, in other system (17,18). Recently, as a result of primary structure analysis, it was proposed that the prokaryotic GlnRS of Gram-negative bacteria was of eukaryotic origin and was acquired by horizontal gene transfer (19).

The crystal structure of GluRS from Thermus thermophilus was solved and refined at 2.5Å resolution (20). The amino terminal half of GluRS shows a structural similarity with that of Escherichia coli GlnRS, while the carboxy terminal half of GluRS displayed an all α-helix architecture compare to all β-barrel structure of GlnRS carboxy terminal (20). Although GluRS shows several similarities with GlnRS regarding structure and catalytic properties (21), but the most striking structural difference between GluRS and GlnRS of Escherichia coli is that GluRS of Escherichia coli contains one atom of zinc (Zn) essential for its catalytic activity (22). In comparison, neither GluRS of Thermus thermophilus nor GlnRS of Escherichia coli have any zinc atom in their structures.

Previously in our laboratory extensive studies has been done on unfolding behavior of relatively large protein, GlnRS, in presence of urea (23). GlnRS, with a molecular weight
of approximately 64500 kDa, is a large monomeric protein having four domains found to have a stable molten globule-like intermediate at low urea concentration (23).

A crystal structure of *Thermus thermophilus* GluRS shows that it has four domains (20, 24). Domains-I and II belong to the N-terminal half and Domains-III and IV belong to C-terminal half of GluRS and studies showed that N- and C-terminal of GluRS are possibly tightly bound and can’t be separated easily (25), i.e., their interaction may be strong. But the nature of interaction is still unknown although partial proteolysis with thermolysin gives two distinct bands corresponding to nearly 29.5 and 31 kDa on SDS-PAGE (26). The apparent molecular weight of the two fragments suggest that thermolysin has cleaved native GluRS at a single site, Leu238 (26).

We have studied the denaturation profile of *Escherichia coli* GluRS to observe where there is any specific difference between *Escherichia coli* GlnRS, which belong to the same subclass of aminoacyl-tRNA synthetase. We also observed how domain-domain interactions play a role in the protein unfolding.
Experimental Procedures

**Materials**

Ultrapure urea was purchased from E. Merck (Germany). HEPES, BSA, ovalbumin, α-lactalbumin and lysozyme were purchased from Sigma Chemical Co. (St. Louis, MO). ANS was purchased from Molecular Probes Inc. (Eugene, OR). All other chemicals were of analytical grade.

**Methods**

**Fluorescence Methods**

Tryptophan fluorescence emission spectra were measured at various urea concentrations in 10 mM HEPES pH 7.2 containing 1 mM MgCl₂. The GluRS concentration was 1 μM. The excitation wavelength was at 295 nm and the emission wavelength was at 340 nm. The bandpasses were 5 nm for both excitation and emission unless otherwise mentioned. The temperature was controlled by circulating water through the cell holder. For measurements containing ANS (10 μM), the excitation wavelength was 420 nm to avoid inner filter effect and the emission was monitored at 482 nm. Appropriate blank values were always subtracted from all measurements. Urea denaturation experiments were performed by diluting the stock enzyme solution with different volumes of buffer and a standard urea solution in the same buffer in such a way so as to attain the desired final enzyme and urea concentrations. The solutions were then incubated overnight to attain complete equilibrium.
Circular dichroism Study

Circular dichroism (CD) spectral measurements in the near UV and far UV regions were made on a JASCO J-600 spectropolarimeter. We have used 0.1 cm and 10 cm pathlength cuvettes for far and near UV CD measurements respectively. GluRS concentration was typically 1-2 μM for the far UV CD measurements and 3 μM for the near UV CD measurements.

Size Exclusion Chromatography

To determine the compactness of the intermediate state, Stokes radii of the native, the intermediate and the denatured states were determined by size exclusion chromatography (FPLC) on Superose 12 (1.0 X 30 cm) at 25° C. The column was equilibrated with 0.1 M Tris-HCl, pH-7.5, buffer containing different concentrations of urea. The glutamyl-tRNA synthetase was incubated overnight at a final concentration of 2 μM at a given urea concentration and 100 μL was loaded onto the Superose 12 column equilibrated with the same buffer, containing the same concentrations of urea. The column was calibrated with proteins of known Stokes radii in buffer containing no urea. Uversky (27) has shown that a Superose 12 column elution property shows no significant change in a wide range of urea concentrations. Bovine serum albumin, ovalbumin, α-lactalbumin and lysozyme were used for calibration. The Stokes radii of glutamyl-tRNA synthetase under different conditions were determined from a plot of Stokes radius of known proteins versus the elution volume.

Partial Proteolysis of GluRS by thermolysin and purification of GluRS fragments by FPLC
GluRS (0.5 mg/ml) in 100 mM Tris-HCl pH - 7.5, 10% glycerol and 0.5 mM β-mercaptoethanol was partially digested with thermolysin (25:1 w/w) for 1 hour at 37°C. The lysate was then applied to Mono-Q HR 5/5 column using a 0 to 0.6 M NaCl gradient in a buffer of 20 mM Tris-HCl pH 7.9, containing 10% glycerol and 20 mM β-mercaptoethanol. Two very close peaks were eluted at around 0.45 M NaCl. The peaks were collected and the fractions were analyzed on SDS-PAGE gel.
Results

Figure 1 shows the plot of far UV circular dichroism, ratio of fluorescence intensity at 340 nm to 350 nm, emission maximum shift and ANS fluorescence as a function of urea concentration. It is now well documented that ANS binds to molten globule states with higher fluorescence yields and often used to verify the presence of molten globule states. In order to see whether the intermediate is molten globule or not, ANS binding to GluRS have been observed at different urea concentration. The fluorescence value increases slowly at lower urea concentrations, peaks around 3 M urea and then declines slowly with mid point around 4 M urea. The increased fluorescence value may be due to increased quantum yield or increased affinity for binding or increased number of binding sites.

The fluorescence intensity values at different urea concentration of GluRS was fitted in two-state transition model using Kyplot and it fitted well giving ΔG value of 2.65 KCal/mole and m value of 909.69 Cal/(mole.M).

The emission maximum of native GluRS is 337.4 nm, changes little (339.2) upto 2 M urea, beyond that, there is a significant red shift of emission maximum, which finally levels off beyond 6 M urea. A similar trend is observed for the F340/F350 ratio, which also reflects the emission maximum shift. The approximate mid point of transition is around 3 M urea in both cases.

The far UV CD intensity shows little change upto 2 M urea and then declines very rapidly to approximately 15% of the initial value at 8 M urea and the approximate mid point of transition is again around 3 M urea.
Figure 3.1. Equilibrium urea denaturation profile of GluRS. The fluorescence measurements were performed in HITACHI F3010 spectrofluorometer. The study was conducted in 0.01 M potassium HEPES buffer pH 7.2 containing 1 mM MgCl₂. The concentration of GluRS was 1 μM at 25°C. The excitation wavelength was 295 nm and the emission spectra was taken from 300-400 nm at scan speed of 60 nm/min. The bandpasses were 5 nm for both excitation and emission. For measurements containing ANS (10 μM), the excitation wavelength was 420 nm and the emission spectra was taken from 450-520 nm and the value at 482 nm was monitored. For CD measurements we have used JASCO J-600 spectropolarimeter. For far UV CD 0.1 cm pathlength cuvette was used. The concentration of GluRS was 1 μM. The temperature and buffer was same as fluorescence measurements. The CD spectrum was taken from 200-250 nm and molar ellipticity values at 222 nm was monitored. (---o---) represents F₃₄₀/F₃₅₀ of tryptophan fluorescence when excited at 295 nm. (---Δ---) represents ANS fluorescence at 482 nm when excited at 420 nm. (---■---) represents shifting of wavelength maxima (λ_max) as a function of urea concentration. (---▽---) represents the CD signal at 222 nm.
It is well established that if different unfolding probes, fluorescence or absorbance measurements, circular dichroism and viscosity gives the same unfolding curve then the unfolding transition of a protein is a cooperative two-state transitions. So we had used far-UV CD to see whether there is any change in secondary structure at 3 M urea. The CD values obtained at different urea concentrations were fitted in two-state transition model using Kyplot. The plot was fitted well using two-state transition model having ΔG value of 7.99 Kcal/mole and m value 2228.84 Cal/ (mole.M).

So, the ΔG value and m value we obtained from fluorescence ratio and CD intensity values were different indicating the presence of an intermediate. So, the denaturation of GluRS passed through two transitions, the first one from native to intermediate and the second one from intermediate to denatured state.

Figure 2 shows far UV CD of GluRS at 0 M, 3 M and 8 M urea. CD signal of GluRS at 0 M and 3 M urea show similar negative ellipticity indicating almost no disruption of secondary structure in presence of 3 M urea, while no negative CD signal is detected in the presence of 8 M urea indicating complete disruption of secondary structure.

The above experiment showed that there is almost no change in secondary structure at 3 M urea concentration, so we had done near UV CD experiment. It is known that at "molten globule" state of a protein the tertiary interaction is disrupted and it is reflected in the reduced side chain CD signal.

We have used GlnRS as a benchmark for GluRS denaturation studies because not only that both of them belong to same subclass of aaRS but also the denaturation of GlnRS had been extensively studied in our laboratory (23). From that study, that is from
Figure 3.2. Far UV CD spectra of Glutamyl-tRNA synthetase (GluRS) in the presence of 0.01 M potassium HEPES buffer pH 7.2 containing 1 mM MgCl₂ in (O) no urea, (●) 3 M urea and (□) 8 M urea. The pathlength for CD measurements were 0.1 cm and the concentration of GluRS was 1 μM at 25°C. Five spectra were signal averaged to improve the signal to noise ratio.
Fluorescence, CD and ANS binding studies it became clear that GlnRS has a "molten globule" like state near 2 M urea. We choose GluRS in 3 M urea to study near UV CD spectrum as GluRS shows not only highest peak in ANS binding near 3 M urea but also other fluorescence and CD experiments shows it have a transition near 3 M urea.

Figure 3 shows near UV side chain CD of GluRS at 0 M, 3 M and 8 M urea and from the figure it is clearly observed that GluRS at 0 M and 3 M urea have positive CD signals and there is very little, almost negligible lowering of CD signal of GluRS in 3 M urea in comparison to the native. This shows there may be no change in tertiary interaction of GluRS in 3 M urea. It is also evident from the figure that all native like near UV CD spectrum vanishes in presence of 8 M urea. Thus the tertiary interaction of GluRS at 3 M urea remained intact indicating that different mid-point in ANS titration may be due to some local disruption of GluRS structure in presence of urea creating some hydrophobic patches to which ANS molecules binds to give increased fluorescence quantum yield. To further investigate whether "molten globule" state is there, we have used FPLC gel filtration as a probe and it was already known the Stokes radius of "molten globule" increases because of disruption of tertiary interaction.

Figure 4 shows the elution profiles of FPLC size exclusion column chromatography of GluRS at 0 M, 3 M and 8 M urea concentrations. The elution volumes at 0 M, 3 M and 8 M urea concentrations are 11.6, 11.6 and 9.8 ml respectively with the corresponding Stokes radii are 3.3, 3.3 and 4.8 nm. It is clear that at 3 M urea concentration GluRS has an elution volume identical to native indicating that the hydrodynamic property of GluRS
Figure 3.3. Near UV CD spectra of Glutamyl-tRNA synthetase (GluRS) in the presence of 0.01 M potassium HEPES buffer pH 7.2 containing 1 mM MgCl₂ in (O) no urea, (▲) 3 M urea and (□) 8 M urea. The pathlength for CD measurements were 10 cm and the concentration of GluRS was 3 μM at 25°C. Ten spectra were signal averaged to improve the signal to noise ratio.
Figure 3.4. Measurement of Stokes radii of Glutamyl-tRNA synthetase (GluRS) at 0 M, 3 M and 8 M urea using FPLC size exclusion chromatography on Superose 12 (1.0 X 30 cm) at 25°C. The column was equilibrated with 0.1 M tris-HCl pH 7.5 buffer containing different concentration of urea. The column was calibrated with proteins of known Stokes radii (Bovine serum albumin, Ovalbumin, α-lactalbumin and lysozyme) in the same buffer containing no urea. The Stokes radii of Glutamyl-tRNA synthetase (GluRS) under different urea concentrations were determined from a plot of Stokes radius of known proteins versus elution volume. (Q) represents elution volume of standard proteins and (■) represents elution volume of GluRS at different urea concentrations. The Stokes radius at 0 M, 3 M and 8 M urea were found to be 3.3 nm, 3.3 nm and 4.6 nm respectively.
does not change much up to 3 M urea concentration. Hydrodynamic property, i.e., Stokes radius of GluRS at 3 M urea did not change in comparison to native state indicates that the intermediate of GluRS may not be "molten globule" like, as normally "molten globules" have a higher radius in comparison to "native" state.

From the above studies of denaturation of GluRS, it can be concluded that the denaturation profile of GluRS is different from GlnRS. It may be due to two following reasons:

1. This may be due to architectural difference in the C-terminal end of GluRS and GlnRS. Crystal structures of *E. coli* GlnRS and *Thermus thermophilus* GluRS shows that the C-terminal half of GluRS displayed an all α-helix architecture compared to all β-barrel structure of GlnRS C-terminal (22).

2. Co-elution of N- and C-terminal from FPLC column and our failure to isolate them separately in native state also indicate that the domain-domain interaction may be very strong in GluRS which may not be true in case of GlnRS.

Figure 5 shows silver stained SDS-PAGE gel of thermolysin cut GluRS after purification in FPLC using mono-Q column. First is the native GluRS, second are the markers, third and fourth lanes are the peak fractions collected during isolation. The gel shows that the combined N- and C-terminals are almost 100% pure.

We failed to isolate N- and C-terminal of GluRS separately in native state and so we thought that the interaction although non-covalent in nature between the domains must be very strong. So we did FPLC gel filtration and it was expected that if the interaction is
Figure 3.5. Silver stained SDS-PAGE gel of thermolysin cut Glutamyl-tRNA synthetase (GluRS) after purification in FPLC using mono-Q column. GluRS (0.5 mg/ml) was partially digested with thermolysin (25:1) for 1 hour at 37°C in 0.1 M Tris-HCl containing 10 % glycerol and 0.5 mM β-mercaptoethanol. The lysate was then applied to Mono-Q HR 5/5 column using a 0 to 0.6 M NaCl gradient in a buffer of 0.02 M Tris-HCl pH 7.9, containing 10 % glycerol and 20 mM β-mercaptoethanol. Two very close peaks were eluted at around 0.45 M NaCl. The peaks were collected and analyzed on SDS-PAGE gel. Lane 1 is marker, lane 2 is native GluRS, lane 3 is λ-repressor, lanes 4 and 5 are the peak fractions collected during isolation. The gel shows that the combined N- and C-terminals are almost 100 % pure.
Figure 3.6. Measurement of Stokes radii of thermolysin cut GluRS using FPLC size exclusion chromatography on Superose 12 (1.0 X 30 cm) at 25°C. The column was equilibrated with 0.1 M tris-HCl pH 7.5 buffer. The column was calibrated with proteins of known Stokes radii (Bovine serum albumin, native GluRS, Ovalbumin, carbonic anhydrase and lysozyme) in the same buffer. The Stokes radii of Glutamyl-tRNA synthetase (GluRS) cut with thermolysin were determined from a plot of Stokes radius of known proteins versus elution volume. (O) represents elution volume of standard proteins and (▲, ■) represents elution volume of N- and C-terminal of GluRS. From the plot it is evident that both N- and C-terminal eluted very closely at Stokes radius of nearly 2.4 nm.
strong then N- and C-terminal together will elute at the identical position as that of native GluRS.

Figure 6 shows the elution profiles of FPLC size exclusion column chromatography of BSA, native GluRS, ovalbumin, carbonic anhydrase, lysozyme and thermolysin cut GluRS to observe the domain-domain interactions between N- and C-terminal halves which apparently appear to be very tightly bound as they cannot be separated by FPLC methods using different combination of salts under native conditions. The two halves of GluRS cut with thermolysin are eluted at different position in the chromatogram which leads to the conclusion that the N- and C-terminal halves are not so tightly attached as it was thought of.
Discussion

The molten globule state of many small proteins have been studied so far, very few large multidomain proteins have been studied in this respect. Previously in our laboratory equilibrium denaturation of glutaminyl-tRNA synthetase (GlnRS), which is a monomeric protein of approximately 64.5 kDa having four distinct domains, had been extensively studied and the presence of molten globule state has been well-characterized (20). Glutamyl-tRNA synthetase (GluRS) belongs to the same subclass of aminoacyl-tRNA synthetases (aaRS) and also has four domains monomeric protein having molecular weight of 53.8 kDa. We have used several probes, such as fluorescence, far and near UV CD, size exclusion FPLC and proteolysis, to characterize the urea induced denaturation.

From fluorescence experiments, measurement of ratio of fluorescence intensity at 340 nm to 350 nm and emission maximum shift indicate midpoint of transition is at near 3 M urea and far UV CD further enhances the support. But fitting of fluorescence ratio value and far UV CD values gave different ΔG value and m values, thus clearly indicating the presence of one or more intermediates. To assess whether these intermediates are "molten globule" like or not, we tried to determine the nature of this intermediate.

ANS binding studies was performed which showed that the increase of ANS fluorescence is not as much dramatic and it may be due small opening up of the protein molecule and thus increasing the number of ANS binding sites. Size exclusion chromatography studies
of the intermediate also suggest that there is no change in the hydrodynamic property (Stokes radius) of the intermediate in comparison to the native.

The most important characteristic of the "molten globule" is the loss of tertiary interactions while almost maintaining the secondary structure of the native protein. But near UV CD spectrum shows that the ellipticity at 0 M and 3 M urea are almost identical, indicating the retention of tertiary interaction at 3 M urea concentration.

Thus it can be concluded that the intermediate is not a "molten globule" like.

So GluRS, although structurally, functionally and evolutionary closely related to GlnRS, the nature of intermediates of these two proteins are probably different and stabilities of the intermediates are also different.

After proteolysis we have tried several methods to isolate N and C terminal separately in the native state, but have failed to do so. So we thought the domain-domain interaction of N and C terminal of GluRS may be very strong. But size exclusion chromatography of GluRS cut with thermolysin shows no peak near native GluRS, but two separate peaks appear in the later half of chromatogram. From this it can be easily concluded that the two halves are not so tightly bound as previously thought of.

ANS binding studies of GluRS at different urea concentration showed that the ANS fluorescence increases upto 3 M urea, but it is only 2-3 fold increment from native GluRS ANS binding fluorescence. Actually, in practice "molten globule" like state of protein
shows 20-30 fold increment in ANS fluorescence than that of ANS fluorescence of protein in native state, as it happened in case of GlnRS near 2 M urea.

So in conclusion, it is observed that although GluRS is a large protein having four domains and belongs to the same subclass of GlnRS, but their denaturation profiles are completely different. Although, both of them have an intermediate at nearly the same molar urea concentration, the nature of intermediate is completely different. While GlnRS has shown a stable "molten globule" like intermediate, GluRS has an intermediate that is not "molten globule" like and its stability is less in comparison to GlnRS intermediate. This may be due to two possible reasons: one, due to strong domain-domain interaction or two, it may be due to architectural difference in the C-terminal domain. But FPLC studies goes against the first possibility as N- and C-terminal eluted separately through gel filtration column indicating lack of strong interactions between N- and C-terminal domains of GluRS. So the difference in denaturation profile may be due to architectural difference in the C-terminal of GluRS and GlnRS.


References


