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# A Functional Loop Spanning Distant Domains of Glutaminyl-tRNA Synthetase Also Stabilizes a Molten Globule State

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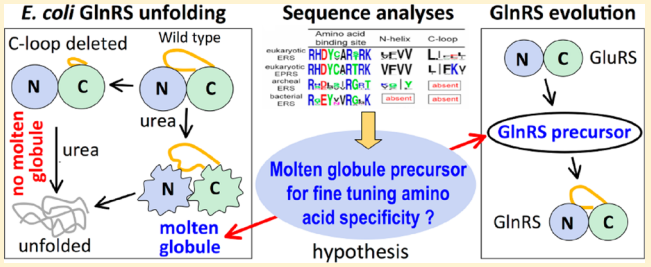
Supporting Information

**ABSTRACT:** Molten globule and other disordered states of proteins are now known to play important roles in many cellular processes. From equilibrium unfolding studies of two paralogous proteins and their variants, glutaminyl-tRNA synthetase (GlnRS) and two of its variants [glutamyl-tRNA synthetase (GluRS) and its isolated domains, and a GluRS–GlnRS chimera], we demonstrate that only GlnRS forms a molten globule-like intermediate at low urea concentrations. We demonstrated that a loop in the GlnRS C-terminal anticodon binding domain that promotes communication with the N-terminal domain and indirectly modulates amino acid binding is also responsible for stabilization of the molten globule state. This loop was inserted into GluRS in the eukaryotic branch after the archaea–eukarya split, right around the time when GlnRS evolved. Because of the structural and functional importance of the loop, it is proposed that the insertion of the loop into a putative ancestral GluRS in eukaryotes produced a catalytically active molten globule state. Because of their enhanced dynamic nature, catalytically active molten globules are likely to possess broad substrate specificity. It is further proposed that the putative broader substrate specificity allowed the catalytically active molten globule to accept glutamine in addition to glutamic acid, leading to the evolution of GlnRS.




Many functional proteins fold into a well-defined three-dimensional structure. However, it is now clear that not all proteins fold into a uniquely defined native state conformation. Many are intrinsically unfolded, while others can be partially folded or present in a molten globule-like structure in which the fold is compact but the internal mobility is significantly enhanced.[1] The molten globule class of compact states is ubiquitous in nature, and in many cases, they are produced under mildly denaturing conditions.[2] However, for many proteins, a molten globule state has not yet been detected under several denaturing conditions, indicating that they are energetically far removed from the native state. The physicochemical properties that stabilize a molten globule state with respect to the native state have not been fully elucidated, except in a few cases. The most important insight has come from pairs of paralogs in which one protein forms the molten globule state under mild denaturing conditions and the other does not.[3] A classic example is that of the lysozyme–lactalbumin pair. It has been concluded that non-native interactions of a small part of bovine $\alpha$-lactalbumin play a crucial role in the stabilization of the molten globule state.[4] It is not known whether non-native contacts of a small region in a protein play important roles in the stabilization of the molten globule state of other proteins as well. If this turns out to be a general mechanism, then insertion of a suitable peptide sequence into the protein structure may produce the molten globule state during evolution. Such stabilization of the molten globule state, if true, may be significant for the evolution of protein folding, transport, and other cellular processes.

It was believed that the enhanced mobility of a molten globule state does not favor enzyme activity. However, Hilvert and co-workers have recently described a catalytically active molten globule state of an enzyme, chorismate mutase, suggesting that enhanced mobility of a protein does not contraindicate enzyme activity.[5] Enhanced dynamicity of the molten globule state may even be an advantage under certain conditions, because of its potential for broader substrate specificity.[6] Binding of disordered proteins to different ligands followed by remodeling of the protein around it has been found.[7] The recognition of different substrates by a disordered protein depends on the highly flexible nature of the disordered

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state. In a similar vein, the enhanced dynamic nature of the molten globule state may allow different substrates to bind and create complementary binding sites without incurring a heavy energetic penalty. Broader substrate specificity of the molten globule state, thus, may create conditions for switching over to new substrate specificity during enzyme evolution.

This article reports a comparative study of a pair of paralogs, GlnRS and GluRS of *Escherichia coli*; GlnRS forms the molten globule state under mild denaturing conditions, whereas GluRS does not. A loop spanning the anticodon binding domain and the catalytic domain of GlnRS plays a crucial role in the stabilization of the molten globule state. The same loop was previously reported to be involved in transmitting the anticodon binding information to the active site.[8] This loop was inserted into GluRS just around the time of evolution of GlnRS from GluRS.[9] We hypothesize that the stabilization of the molten globule state played an important role in the switch of substrate specificity from glutamic acid to glutamine.

## EXPERIMENTAL PROCEDURES

**Materials.** Restriction enzymes were purchased from either New England Biolabs (NEB) or Promega. The polymerase chain reaction (PCR) kit (containing high-fidelity polymerase, dNTPs, assay buffers, and $MgCl_2$) was purchased from Promega. Ultrapure urea was purchased from Sigma. All other chemicals were of analytical grade.

**Methods.** *Construction of NGluRS, CGluRS, cGluGlnRS, and delGlnRS.* Construction of the isolated N-terminal domain (residues 1−314; NGluRS) and the C-terminal domain (residues 314−471; CGluRS) of *E. coli* GluRS has been described previously.[10] The NGluRS of *E. coli* was fused with the C-terminal domain (residues 335−554) of *E. coli* GlnRS to construct the chimeric protein cGluGlnRS, as described previously.[9] For the construction of six-His-tagged *E. coli* GlnRS, plasmid pMN20, carrying the wild-type *E. coli* GlnRS gene, was used as a template. The gene was amplified via PCR using the forward and reverse primers, P1 and P4, respectively (the primer sequences are provided in Table S1 of the Supporting Information). The resultant PCR product was subsequently cloned within the NdeI−BamHI restriction sites of vector pET-28a (Novagen). To construct the version of *E. coli* GlnRS from which the C-terminal loop (474−495) has been deleted (delGlnRS), one forward primer (P3) and a reverse primer (P2) were designed along with primers P1 and P4. Primer P3 and primer P2 carried sequences for the PANK sequence at their 5′ ends (the primer sequences, sequence of the PANK motif, and construction details are provided in Figure S1 of the Supporting Information). Two separate sets of PCRs were performed by taking the wild-type *E. coli* GlnRS gene (pMN20) as a template with the following combination of primers: (a) with P1 and P4 and (b) with P3 and P2. The two PCR products were used to perform a PCR-based overlap extension using two end primers (P1 and P2). The resultant PCR product was cloned within the NdeI and BamHI restriction sites of vector pET-28a (Novagen). Therefore, the C-terminal loop$^{474-495}$ of *E. coli* GlnRS was replaced with a four-residue flexible sequence (Table S1 of the Supporting Information). This construct of *E. coli* GlnRS is designated as delGlnRS. All gene constructs were confirmed by DNA sequencing of the entire gene insert with T7 forward and T7 reverse primers (Novagen), and internal primers when required.

*Expression and Purification of Enzymes.* All constructs (NGluRS, CGluRS, cGluGlnRS, delGlnRS, and the two wild-type enzymes, *E. coli* GlnRS and *E. coli* GluRS) were expressed separately in *E. coli* strain BL21(DE3). The expression and purification of NGluRS, CGluRS, cGluGlnRS, and *E. coli* GluRS were conducted as described previously.[10] For the six-His-tagged *E. coli* GlnRS and delGlnRS, the transformed cells were grown at 37 °C. The six-His-tagged *E. coli* GlnRS was induced at the same temperature (at $A_{595} \sim 0.6$) with 0.5 mM IPTG for 4 h, while delGlnRS was induced at 16 °C (at $A_{595} \sim 0.2$) with 0.5 mM IPTG for 12 h. For both enzymes, the induced cells were pelleted down and the cell pellets were lysed in buffer A (buffer compositions in Table S2a of the Supporting Information). The suspended cells were then subjected to sonication and were centrifuged at 14000*g* for 45 min. The supernatant obtained from centrifugation was loaded on a Ni-NTA agarose column (pre-equilibrated with buffer B at a flow rate of 0.5 mL/min). After the loading was complete, the column was washed with 20 column volumes of buffer C, followed by 10 column volumes of buffer D. Finally, both *E. coli* GlnRS and delGlnRS were eluted with buffer E. All the eluted fractions were checked by 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis. The major eluted fractions of both *E. coli* GlnRS and delGlnRS were pooled and dialyzed against buffer F. *Methanothermobacter thermautotrophicus* GluRS was expressed and purified from plasmid pTYB1-GluRS in *E. coli* strain BL21(DE3) (with pSJS1240) as described in ref 11. The purified fractions were concentrated with Amicon Ultra 30 K molecular weight cutoff centrifugal filters (Millipore). Purified protein was kept in an appropriate buffer (see Table S2b of the Supporting Information) for further use.

*Urea-Induced Unfolding Experiments.* For urea-induced unfolding experiments, all enzymes (*E. coli* GlnRS, delGlnRS, *E. coli* GluRS, NGluRS, CGluRS, cGluGlnRS, and *M. thermautotrophicus* GluRS) were dialyzed in the respective working buffers (see Table S2b of the Supporting Information for the buffer compositions); 0−9 M stock solutions of ultrapure urea (Sigma) were prepared in the respective buffers. The concentrations of the urea solutions were confirmed by measuring the corresponding refractive index. Appropriate concentrations of enzymes (Table S2b of the Supporting Information) were added in each case, and the sets were incubated for 16 h. The final urea concentrations after additions of protein were recalculated.

*Circular Dichroism Spectroscopy.* The urea-induced unfolding profiles of the enzymes were monitored by far-UV (210−260 nm) circular dichroism (CD) spectroscopy at 25 °C in a JASCO J-815 spectropolarimeter in a cuvette with a path length of 2 mm. For each spectrum, at least three accumulations were recorded for both far-UV and near-UV studies. The spectra of appropriate buffers were subtracted from each enzyme spectrum. Near-UV CD spectra were recorded at 25 °C in a JASCO J-815 spectropolarimeter with a 10 mm path-length cuvette.

*Fluorescence Spectroscopy Methods.* Steady state fluorescence (300−500 nm, excitation wavelength of 295 nm) was measured in an LS-45 fluorescence spectrometer (Perkin-Elmer). The excitation and emission band-pass was kept at 5 nm. Spectra of appropriate buffers were subtracted from the fluorescence spectra. For the ANS binding experiments, freshly prepared ANS was added to urea buffer containing protein to a final concentration of 30 $\mu$M. The mixture was incubated at

room temperature for 30 min prior to the fluorescence measurements. The samples were excited at 420 nm, and the emission was monitored at 482 nm after the appropriate subtraction of blank values.

*Curve Fitting.* Urea denaturation curves were analyzed as described previously.[12] Specifically, eqs 1 and 2, corresponding to two- and three-state models, respectively, were used to fit urea-induced equilibrium denaturation data, monitored by CD and fluorescence (signal $S$).

$$S = \left[S_N^\circ + S_U^\circ e^{-(\Delta G_{U/N}^\circ + m_{U/N}X)/RT}\right] \Big/ \left[1 + e^{-(\Delta G_{U/N}^\circ + m_{U/N}X)/RT}\right] \quad (1)$$

$$S = \left[S_I^\circ + S_N^\circ e^{-(\Delta G_{N/I}^\circ + m_{N/I}X)/RT} + S_U^\circ e^{(\Delta G_{I/U}^\circ + m_{I/U}X)/RT}\right] \Big/ \left[1 + e^{-(\Delta G_{N/I}^\circ + m_{N/I}X)/RT} + e^{+(\Delta G_{I/U}^\circ + m_{I/U}X)/RT}\right] \quad (2)$$

where subscripts N, U, and I correspond to the native, unfolded, and intermediate states, respectively, $S^0$ corresponds to the signal of the pure states, $\Delta G^\circ_{Y/X}$ corresponds to the standard free energy for the X → Y transition, $m_{Y/X}$ is the proportionality constant relating $\Delta G^\circ_{Y/X}$ in the absence and presence of $X$ molar urea, $T$ is the absolute temperature, and $R$ is the universal gas constant.

*Analyses of Sequence and Structure.* A total of 35 GlnRS (15 bacterial and 20 eukaryal), 37 GluRS (15 bacterial, 10 eukaryal, and 12 archeal), and 10 (eukaryal) glutamyl-prolyl tRNA-synthetase (EPRS) sequences were downloaded from the KEGG genome database.[13] Detailed information about the sequence database is given in Tables S3−S6 of the Supporting Information. Multiple-sequence alignments were performed using MUSCLE[14] and manual curating. The amino acid conservation patterns were visualized by Sequence-logo plots.[15] Using the structure of *Thermosynechococcus elongatus* GluRS [Protein Data Bank (PDB) entry 2cfo], a homology-modeled structure of *E. coli* GluRS was constructed using SWISSMODEL.[16] Structural alignments were performed using MATRAS,[17] and all structural analysis molecular graphics images were produced using UCSF Chimera.[18]

## RESULTS

It was shown previously that GlnRS forms a molten globule state under mildly denaturing conditions.[19] The studied protein, however, had no affinity tags. Because many of the variants studied here are relatively unstable, six-His-tagged versions of both GluRS and GlnRS were used in this study, which allowed rapid purification of the variants. Thus, the unfolding behavior of His-tagged GlnRS was performed initially to compare with the previous work and to standardize protocols used in this work.

**The His Tag Has No Significant Effect on the Stability of the Molten Globule State of GlnRS.** Urea-induced equilibrium unfolding of GlnRS has been reported previously.[19] From fluorescence and CD experiments, it was shown that the unfolding profile of GlnRS fit best with a model with an intermediate. The intermediate state was further characterized as a molten globule state from its characteristic ANS binding profile, separated from the native state by a high activation energy barrier (96 kJ mol$^{-1}$). GlnRS is a multidomain protein. As shown in Figure 1, like the untagged protein, His-tagged GlnRS exhibits a transition at low urea concentrations

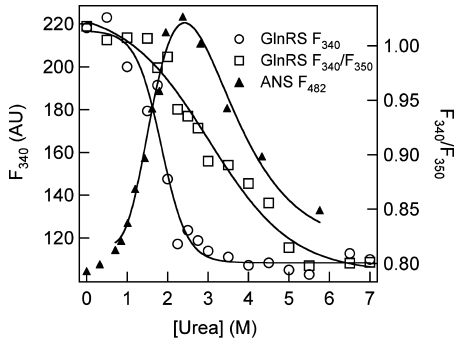


**Figure 1.** Urea-induced unfolding profiles of GlnRS (His-tagged) monitored by the intrinsic Trp fluorescence intensity at 340 nm ($F_{340}$), the ratio of fluorescence intensities at 340 and 350 nm ($F_{340}/F_{350}$), and the ANS fluorescence at 480 nm (ANS Fl) as a function of urea concentration in the presence of GlnRS at 23 °C. A two-state fit yielded a $\Delta G^\circ$ of 4.07 kcal mol$^{-1}$ and an $m$ of 2.2 kcal mol$^{-1}$ M$^{-1}$ for $F_{340}$ and a $\Delta G^\circ$ of 2.03 kcal mol$^{-1}$ and an $m$ of 0.67 kcal mol$^{-1}$ M$^{-1}$ for $F_{340}/F_{350}$.

(monitored by the fluorescence intensity at 340 nm, $F_{340}$) followed by another transition at higher urea concentrations (monitored by $F_{340}/F_{350}$). A two-state fit to the unfolding events yielded a $\Delta G^\circ$ of 4.07 kcal mol$^{-1}$ and an $m$ of 2.2 kcal mol$^{-1}$ M$^{-1}$ for the N-to-I transition ($F_{340}$-monitored) and a $\Delta G^\circ$ of 2.03 kcal mol$^{-1}$ and an $m$ of 0.67 kcal mol$^{-1}$ M$^{-1}$ for the I-to-U transition ($F_{340}/F_{350}$-monitored), matching well with the earlier report. More importantly, ANS fluorescence (480 nm), in the presence of GlnRS, monitored as a function of urea concentration, showed an initial rise followed by a fall; the peak follows the difference between the $F_{340}$- and $F_{340}/F_{350}$-monitored transitions, indicating preferential binding of ANS to the GlnRS intermediate along its urea-induced equilibrium unfolding pathway. The ANS fluorescence profile and its correlation with the growth and decay of the GlnRS intermediate clearly identify the intermediate as a molten globule, as was observed earlier for the GlnRS that was not His-tagged. In other words, His tagging of GlnRS has a minimal effect on the stability of the molten globule state of GlnRS at low urea concentrations.

**Urea-Induced Unfolding of GluRS, NGluRS, and CGluRS Shows No Molten Globule-like Intermediates.** Having established the presence of a molten globule state in the His-tagged GlnRS, we focused on GluRS, a paralog of GlnRS. The N-terminal catalytic domain of GluRS is homologous to the N-terminal catalytic domain of GlnRS. However, the respective C-terminal anticodon binding domains are non-homologous and have very different three-dimensional folds. The idea behind studying GluRS unfolding was to determine if the N-terminal domain of GluRS, structurally similar to the N-terminal domain of GlnRS, is primarily responsible for formation of the molten globule state under mild denaturing conditions. In addition to GluRS, its isolated N-terminal (NGluRS) and C-terminal (CGluRS) domains were also studied. The preparation and characterization of NGluRS, CGluRS, and GluRS have been reported previously.[10] From CD and fluorescence experiments, it has been demonstrated that NGluRS and CGluRS maintain the backbone structural integrity of the parent GluRS, with some loss of tertiary structure originally present in GluRS.[10] Here we report the urea-induced unfolding behavior of GluRS, NGluRS, and


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CGluRS, as monitored by CD ($\theta_{222}$) and fluorescence spectroscopy ($F_{340}/F_{350}$).

The unfolding profiles of GluRS, CGluRS, and NGluRS are shown in Figure 2, where fluorescence intensity and ellipticity

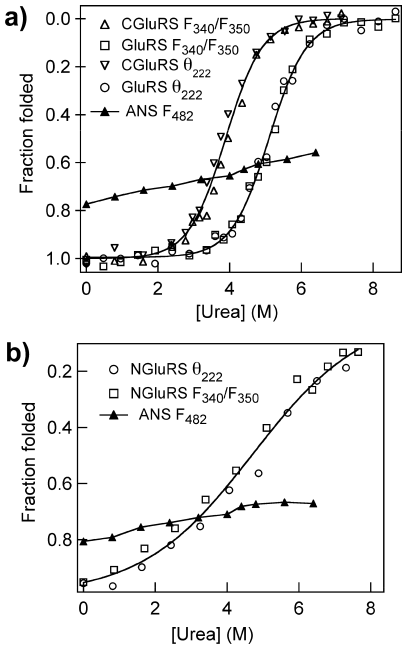


**Figure 2.** Urea-induced unfolding profiles of (a) GluRS and CGluRS and (b) NGluRS, monitored by the Trp fluorescence intensity ratio at 340 and 350 nm ($F_{340}/F_{350}$), ellipticity $\theta_{222}$, and ANS fluorescence at 480 nm (ANS Fl) as a function of urea concentration in the presence of GluRS (a) and NGluRS (b) at 23 °C. A two-state fit yielded a $\Delta G^\circ$ of 4.07 kcal mol$^{-1}$ and an $m$ of 2.2 kcal mol$^{-1}$ M$^{-1}$ for $F_{340}$ and a $\Delta G^\circ$ of 2.03 kcal mol$^{-1}$and an $m$ of 0.67 kcal mol$^{-1}$ M$^{-1}$ for $F_{340}/F_{350}$.

data are converted to fraction folded. For both GluRS and CGluRS, the individual CD- and fluorescence-monitored unfolding profiles were superimposable (Figure 2a). A global fit using a two-state equation yielded the following parameters: $\Delta G^\circ$ = 5.75 kcal mol$^{-1}$ and $m$ = 1.13 kcal mol$^{-1}$ M$^{-1}$ for GluRS, and $\Delta G^\circ$ = 4.48 kcal mol$^{-1}$ and $m$ = 1.15 kcal mol$^{-1}$ M$^{-1}$ for CGluRS, indicating that CGluRS is somewhat less stable than GluRS but unfolds with a very similar cooperativity. Unfolding profiles for NGluRS and the corresponding two-state global fit are shown in Figure 2b ($\Delta G^\circ$ = 1.81 kcal mol$^{-1}$ and $m$ = 0.38 kcal mol$^{-1}$ M$^{-1}$). The midpoint of NGluRS unfolding is very close to what was observed for GluRS. However, the folding is much less cooperative, and the $\Delta G^\circ$ is lower by ~4 kcal mol$^{-1}$.

Although GluRS exhibited an apparent two-state unfolding profile (without any intermediate), to compare with the GlnRS data, ANS fluorescence (482 nm) was monitored as a function of urea concentration in the presence of GluRS (Figure 2a). Unlike what was observed for GlnRS, the ANS fluorescence did not show any characteristic rise and fall that are typically associated with the appearance of a molten globule state. By the same criterion, no molten globule state could be detected during urea-induced unfolding of NGluRS either (Figure 2b). In summary, unfolding experiments with GluRS, CGluRS, and NGluRS indicated the absence of a molten globule state, implying that the fold of the N-terminal domain is not a determining factor alone for the formation of the molten globule state.

**Unfolding of the Chimeric GluRS–GlnRS Species Also Shows No Evidence of a Molten Globule.** A possible source of a stabilized molten globule in GlnRS could be its C-terminal domain. Efforts to clone and express the isolated C-terminal domain of GlnRS failed. Instead, a chimeric protein, cGluGlnRS, in which the N-terminal domain of GluRS was fused with the C-terminal domain of GlnRS was produced. Details of the expression, purification, and characterization of cGluGlnRS have been reported previously.[9] Here we report the urea-induced unfolding studies of cGlnGluRS, as summarized in Figure 3. Both the fluorescence-monitored ($F_{340}/F_{350}$) and

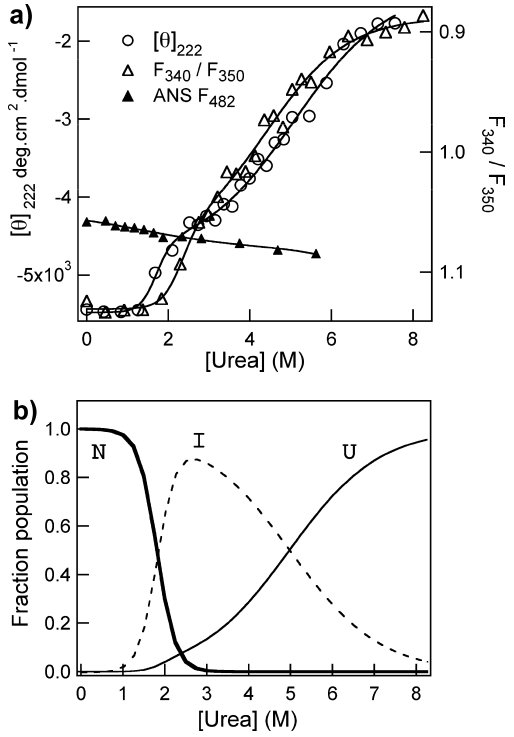


**Figure 3.** (a) Urea-induced unfolding profiles of cGluGlnRS monitored by Trp fluorescence ($F_{340}/F_{350}$) and CD spectroscopy ($[\theta]_{222}$). The solid lines represent the global three-state fits. The ANS fluorescence at 482 nm (ANS Fl), in the presence of cGluGlnRS, is also shown as a function of urea concentration. (b) Fraction population of the native (N), intermediate (I), and unfolded (U) states of cGluGlnRS as a function of urea concentration, as determined from the global fit parameters.

CD-monitored ($\theta_{222}$) unfolding profiles clearly indicated that the unfolding process is not a simple two-state process. Accordingly, the fluorescence and the CD data were simultaneously fit with a three-state equation, yielding the following thermodynamic parameters: $\Delta G^\circ_{NI}$ = 5.0 (CD) and 5.37 (Fl) kcal mol$^{-1}$, $m_{NI}$ = 2.95 (CD) and 2.52 (Fl) kcal mol$^{-1}$ M$^{-1}$, $\Delta G^\circ_{IU}$ = 2.65 (CD) and 2.70 (Fl) kcal mol$^{-1}$, and $m_{IU}$ = 0.52 (CD) and 0.66 (Fl) kcal mol$^{-1}$ M$^{-1}$. The intermediate state in the three-state model starts to appear shortly beyond 1 M urea, peaks around approximately 3 M urea, and then disappears to produce the unfolded state (Figure 3b). Despite exhibiting an intermediate over the same range of urea concentrations that were observed for GlnRS, ANS fluorescence, monitored as a function of urea concentration, was featureless up to ~6 M urea (Figure 3a), indicating that the intermediate does not possess the characteristics of a molten globule state.


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**A C-Terminal Domain Loop Stabilizes the Molten Globule State in GlnRS.** Experiments described until now indicate that the likely stabilizer of the molten globule state may lie in the localized differences of structural elements, rather than in the presence or absence of whole domains. What structural elements could play the critical role in GlnRS? Given the lack of molten globule formation in any of GluRS domains and in the GluRS−GlnRS chimera, one possibility could be that non-native interactions between some elements of the C-terminal and N-terminal domains may be responsible.

Functional connectivity (via structural cross talk) between the N- and C-terminal domains plays an important role in maintaining the tRNA specificity for both bacterial GluRS and GlnRS.[10,20,21] Two alternative signaling pathways between N-terminal and C-terminal domains have been implicated in *E. coli* GlnRS, one through a helix−loop−helix motif (residues 310−338) and another through a loop (residues 472−495, called the C-loop hereafter), inserted between two antiparallel $\beta$-strands.[8] Structural comparison between *E. coli* GlnRS and NGluRS (model) showed that the helix−loop−helix motif of *E. coli* GlnRS is structurally conserved in *E. coli* GluRS. Interestingly, the C-loop as well as its interacting loop−helix region in the N-terminal domain (N-helix, residues 265−285), encircled in Figure 4a, is missing in *E. coli* GluRS. The C-loop

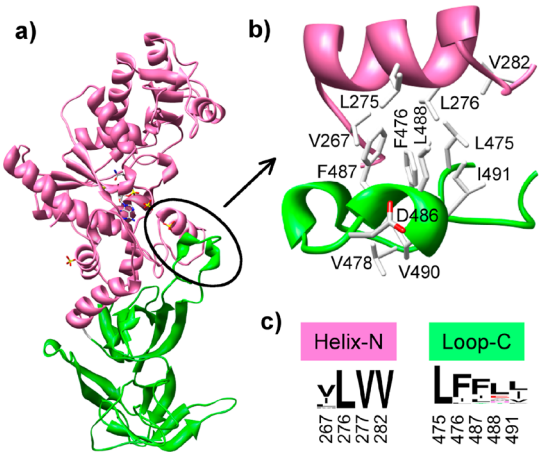


**Figure 4.** (a) Structure of *E. coli* GlnRS (PDB entry 1gts). The N-terminal catalytic domain and C-terminal anticodon binding domains are colored magenta and green, respectively. The N-helix (residues 265−285) and C-loop (residues 472−495) are encircled. (b) Close-up of the C-loop−N-helix interaction highlighting the hydrophobic nature of the interface. (c) Sequence logo plot depicting the conservation of the interacting sites of the C-loop and N-helix in a 96-bacterial GlnRS sequence database.

architecture shows the loop to contain two (residues 480 and 481 and residues 492 and 493) Asn-Pro-mediated $\beta$-turns, two short helical stretches, and an intramolecular hydrophobic staple between V478 and V490 at its base. As shown in Figure 4b, the unique backbone architecture juxtaposes five hydrophobic C-loop residues (L475, F476, F487, L488, and I491) toward the N-terminal domain interface, specifically facing four hydrophobic residues (V267, L276, V277, and V282) in the N-helix. In other words, the connectivity between the N-terminal and C-terminal domains of *E. coli* GlnRS is maintained by specific hydrophobic interactions between the C-loop and N-helix.

Sequence analysis of a large number of bacterial species (95) that contain both GluRS and GlnRS showed that both the N-helix and the C-loop are present in all bacterial GlnRS forms, where the hydrophobic residues are conserved (Figure 4c). In some bacterial GlnRS forms (for example, *Deinococcous radiodurans*, PDB entry 2hz7), the C-loop has large insertions compared to that in *E. coli* GlnRS, without affecting the core structure of the loop (Figure S3 of the Supporting Information). On the other hand, the corresponding bacterial GluRS sequences are devoid of the N-helix (Figure S2 of the Supporting Information). In summary, the C-loop−N-helix interaction, absent in bacterial GluRSs, is unique to all bacterial GlnRS enzymes. Interestingly, non-native hydrophobic interactions have been implicated to play an important role in the stability of the molten globule state in $\alpha$-lactalbumin.[22] In this context, the C-loop−N-helix interaction in GlnRS is a potential candidate for the stabilization of the molten globule state of GlnRS at low urea concentrations.

**GlnRS with Its C-Loop Deleted Does Not Show Any Evidence of the Molten Globule State.** To probe if indeed the C-loop is responsible for the stabilization of the molten globule in GlnRS, a version of *E. coli* GlnRS with its C-loop (residues 474−495) deleted (delGlnRS) was constructed, and its urea-induced unfolding profiles were studied, as shown in Figure 5. The CD-monitored ($\theta_{222}$) and fluorescence-

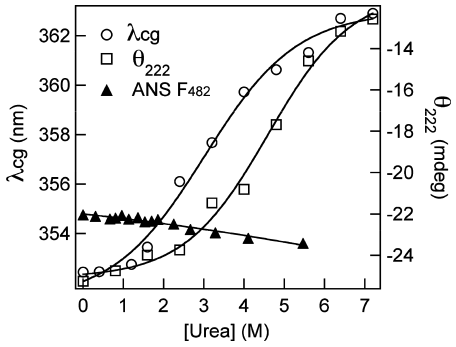


**Figure 5.** Urea-induced unfolding profiles of delGlnRS, monitored by CD [(□) $\theta_{222}$] and fluorescence [(○) $\lambda_{cg} = \sum F_i\lambda_i/\sum F_i$, where $F_i$ represents the fluorescence intensity at $\lambda_i$]. The lines correspond to two-state fits to the data ($\Delta G^\circ$ = 3.06 kcal mol$^{-1}$ and $m$ = 0.67 kcal mol$^{-1}$ M$^{-1}$ for $\theta_{222}$ data, and $\Delta G^\circ$ = 1.84 kcal mol$^{-1}$ and $m$ = 0.61 kcal mol$^{-1}$ M$^{-1}$ for $\lambda_{cg}$-monitored unfolding). The ANS fluorescence intensity (482 nm) is also shown as a function of urea concentration (in the presence of delGlnRS).

monitored (center of gravity of emission, $\lambda_{cg} = \sum F_i\lambda_i/\sum F_i$, where $F_i$ represents the fluorescence intensity at $\lambda_i$) unfolding profiles are nonsuperimposable, although individually both fit well with a two-state model ($\Delta G^\circ$ = 3.06 kcal mol$^{-1}$ and $m$ = 0.67 kcal mol$^{-1}$ M$^{-1}$ for $\theta_{222}$ data, and $\Delta G^\circ$ = 1.84 kcal mol$^{-1}$ and $m$ = 0.61 kcal mol$^{-1}$ M$^{-1}$ for $\lambda_{cg}$-monitored unfolding). The difference in the unfolding profiles indicates the presence of an intermediate in the urea-induced unfolding path of delGlnRS, peaking around 3.5 M urea. However, the ANS fluorescence is insensitive to the appearance of this intermediate as shown in Figure 5. The featureless variation of ANS fluorescence showed that by deleting the C-loop we have essentially destabilized the molten globule state in GlnRS. Although the lack of enhanced ANS binding is a good indicator of the absence of a molten globule-like intermediate, it can be argued that in the absence of urea delGlnRS is already a molten globule. To rule out that


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possibility, we measured the near-UV CD spectra of delGlnRS. Figure 6 shows the near-UV CD spectra of GlnRS and

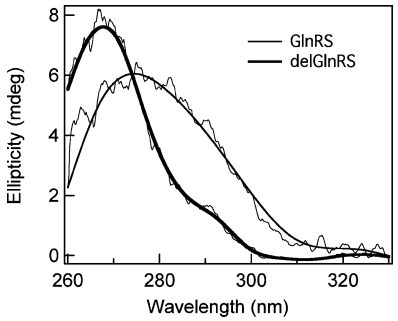


**Figure 6.** Near-UV CD spectra of 12 $\mu$M GlnRS and delGlnRS [in 100 mM Tris-HCl (pH 7.5) containing 10 mM glycerol and 10 mM $\beta$-mercaptoethanol]. The spectra were recorded in a cuvette with a path length of 10 mm at 23 °C.

delGlnRS. Clearly, the intensities of near-UV CD bands of delGlnRS and GlnRS are comparable, indicating no loss of tertiary structure upon deletion of the loop.

**Unfolding Behavior of a C-Loop Mutant of GlnRS.** Although the deletion of the C-loop of GlnRS indicated the disappearance of the molten globule in the urea-induced unfolding profile of GlnRS, complete deletion of a loop from a structurally evolved protein can be a major perturbation to its folded structure and dynamics. A more subtle way to study the effect of the C-loop is its selective mutation followed by unfolding studies. With this goal in mind, a GlnRS C-loop double mutant (D486R/L488Q) was purified.[23] This particular double mutant causes a relaxed tRNA anticodon specificity and replaces a key hydrophobic residue, L488. The CD- and fluorescence-monitored urea-induced unfolding profiles of the loop mutant are shown in Figure 7. The unfolding profiles of the loop mutant and wild-type GlnRS share a common feature. For both proteins, the fluorescence intensity at 340 nm ($F_{340}$) decreases at a much lower urea concentration than the $F_{340}/F_{350}$ profile (the $F_{340}/F_{350}$ profile of the loop mutant is very similar to the $\theta_{222}$ profile). For all other proteins studied here (GluRS and its variants, cGluGlnRS and delGlnRS), $F_{340}$ and $F_{340}/F_{350}$

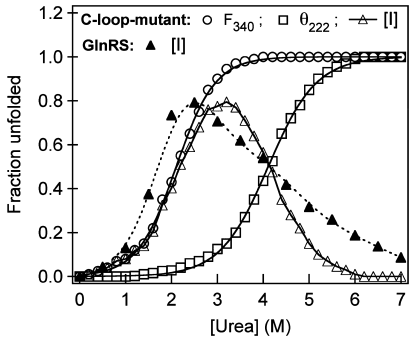


**Figure 7.** Urea-induced unfolding profiles of the GlnRS C-loop double mutant (D486R/L488Q), monitored by CD [(□) $\theta_{222}$] and fluorescence [(○) $F_{340}$]. The lines correspond to two-state fits to the data ($\Delta G^\circ$ = 4.65 kcal mol$^{-1}$ and $m$ = 4.10 kcal mol$^{-1}$ M$^{-1}$ for $\theta_{222}$ data, and $\Delta G^\circ$ = 2.93 kcal mol$^{-1}$ and $m$ = 2.1 kcal mol$^{-1}$ M$^{-1}$ for fluorescence-monitored unfolding). The filled triangles and the thick broken line correspond to the difference between the fits of CD and fluorescence data for wild-type GlnRS[19] and the GlnRS C-loop double mutant, respectively.

are superimposable. While $F_{340}/F_{350}$ reflects the degree of solvent exposure of the tryptophan side chains, thus reporting on the unfolding of the core protein structure, $F_{340}$ can change if there is a change in rate processes that quench tryptophan fluorescence. Such rate processes can be sensitive to subtle changes in the side chain orientations of tryptophan as well as its neighbors, which can occur if the protein structure loses some or all of its tertiary interactions. In other words, a change in $F_{340}$ at lower denaturant concentrations followed by a change in the $F_{340}/F_{350}$ profile at a higher denaturant concentration indicates the presence of a molten globule state at low urea concentrations. This was confirmed by the ANS fluorescence profile for GlnRS. However, when compared to the wild-type GlnRS, the intermediate state in the loop mutant appears at a somewhat higher urea concentration. This can be seen if one compares the urea concentrations corresponding to the peaks of the difference between the CD and fluorescence unfolding profiles for wild-type GlnRS and the loop mutant (Figure 7). Thus, the C-loop through its interaction with the N-helix stabilizes the molten globule state at low urea concentrations, presumably through non-native interactions.

In a recent paper, it was shown that archaeal non-discriminating GluRSs may be the immediate precursor of the GlnRS. To reinforce the idea that insertion of the loop may be the cause for stabilization of a molten globule state, we have examined the unfolding behavior of the *M. thermautotrophicus* GluRS. Figure 8 shows the urea unfolding profiles of *M.*

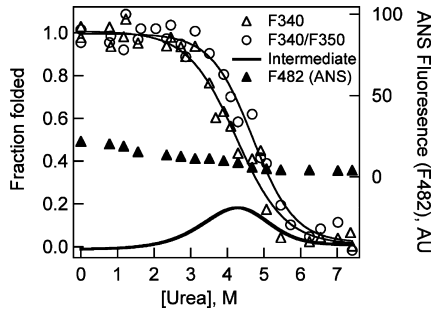


**Figure 8.** Fraction folded population of *M. thermautotrophicus* GluRS, monitored by Trp fluorescence [$F_{340}/F_{350}$ (○) and $F_{340}$ (△)] and ANS fluorescence at 482 nm (▲) as a function of urea concentration at 23 °C. The lines correspond to two-state fits to $F_{340}$ and $F_{340}/F_{350}$ data ($\Delta G^\circ$ = 3.8 ± 0.5 kcal mol$^{-1}$ and $m$ = 0.9 ± 0.1 kcal mol$^{-1}$ M$^{-1}$ for $F_{340}$-monitored data, and $\Delta G^\circ$ = 4.8 ± 0.6 kcal mol$^{-1}$ and $m$ = 1.0 ± 0.1 kcal mol$^{-1}$ M$^{-1}$ for $F_{340}/F_{350}$-monitored data). The solid line at the bottom corresponds to the population of the equilibrium intermediate (difference between the two fits).

*thermautotrophicus* GluRS as monitored by fluorescence intensity at 340 nm ($F_{340}$) and the ratio of fluorescence intensities at 340 and 350 nm ($F_{340}/F_{350}$). The unfolding profiles of *M. thermautotrophicus* GluRS monitored by these two parameters are somewhat different, indicating the presence of an intermediate. The concentration of this putative intermediate as a function of urea concentration is shown at the bottom of the figure, the peak of which occurs just above 4 M urea. To determine if this putative intermediate belongs to the molten globule class, we have measured the fluorescence of added ANS as a function of urea concentration. The fluorescence of added ANS decreases monotonically throughout the urea concentration range, indicating that the intermediate does not belong to the molten globule class.


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Clearly, the GluRS, which is the immediate evolutionary precursor of GlnRS, also shows no evidence of molten globule stabilization, suggesting that the C-loop insertion and molten globule stabilization may have occurred just before the evolution of GlnRS.

## DISCUSSION

Many proteins form molten globule states when subjected to mild denaturing conditions.[2] Quantitative analysis suggests that in such situations, the molten globule states are energetically close to the native state in the absence of the denaturants. The significance of the molten globule state for protein folding has been discussed extensively.[24] However, little is known about its role in enzymology. Enzymes are inherently dynamic in character as movements of protein segments are often a necessary part of the catalytic process. Recent work suggests that in many cases the substrate-free native state has access to most, if not all, states required for catalysis. Binding of substrates selects a particular conformation.[25]

Molten globule states are inherently more disordered than the native states while the compact character of the native state is maintained. The enhanced dynamic character of the state allows a larger number of conformations to be accessed than in a more compact native state. Conventional wisdom is that a relatively rigid active site is a prerequisite for high catalytic efficiency, a corollary being a fully folded state, sampling only a few conformational states, is required for high catalytic efficiency. Several reports in the past few years, however, indicate that disordered states can catalyze enzymatic reactions with high efficiency.[26−29] Many other cases of disordered proteins that function in the disordered state are known.[30,31] One such well-known class of proteins consists of the transcription factors. It is known that a disordered transcription factor binds to different DNA sequences and specifically recognizes these sequences with consequent conformational remodeling. The inherent flexibility allows these disordered transcription factors to recognize multiple target sites with high specificity.[32]

Thus, it may be hypothesized that molten globule states, in some cases, should have the ability to bind a larger repertoire of related molecules, provided the energy penalty is not too high. Previous experimental work has shown that the molten globule state binds substrates in some situations. Recent work by Hilvert, Warshel, and co-workers suggests that the molten globule state may possess catalytic activity, as well.[5,6] One of the possible characteristics of a catalytically active molten globule state may be a broad substrate specificity because of its enhanced dynamicity. It is possible that these catalytically active proteins may possess broader substrate specificity than the more conformationally restricted native state. Thus, this class of states, under certain conditions, may play a role in proteins evolving to achieve a different substrate specificity, as intermediate states with a broad substrate specificity.

An evolutionarily recent change in substrate specificity involves evolution of GlnRS from GluRS. How this change in substrate specificity occurred is not at all clear. The recognition of cognate amino acids and rejection of noncognate amino acids is a complex process and may involve issues of the structural complementary and dynamic nature of the active site.[33,34] A recent attempt to convert the substrate specificity of GlnRS from glutamine to glutamic acid has shed some light.[35] It appears that amino acids distant from the active site play important roles in the determination of the substrate specificity.[35] If we consider point mutations and indels only, many changes must have been needed to convert the ancestral misacylating GluRS from using glutamic acid to glutamine. These distant changes are necessary to produce a conformationally altered active site, more complementary to the alternate substrate. It is possible that the evolutionary process involved accumulation of small changes producing a very inefficient Gln-tRNA$^{Gln}$ synthesizing enzyme at the beginning and slowly evolving to a more efficient enzyme. However, there is another interesting possibility. Because the C-loop is crucial for formation of the molten globule, the insertion of the C-loop into the putative precursor may have produced a catalytically active molten globule state with enhanced dynamic character. When glutamine binds, this would allow the enzyme to convert to a conformation that is more complementary to glutamine without any significant sacrifice of binding energy. Although the molten globule state in the extant GlnRS is somewhat more unstable than the native state, the ancestral GluRS in which the loop insertion had occurred may have had a more stable molten globule state.

A hypothetical scenario is proposed in which the early eukaryotes may have evolved a GluRS that mis-acylated only tRNA$^{Gln}$. Enzymes of such specificity do exist.[36,37] In addition to evolving amino acid specificity for glutamine, as GlnRS evolved from a nondiscriminating GluRS ancestor, tRNA specificity narrowed from accepting both tRNA$^{Glu}$ and tRNA$^{Gln}$ to being specific for tRNA$^{Gln}$. Recently published structural[38] and biochemical[11] data show that grafting a loop that interacts with the tRNA acceptor stem from *E. coli* GlnRS into the *M. themautotrophicus* GluRS converts the nondiscriminating archaeal enzyme to a tRNA$^{Gln}$ specific enzyme. The work indicates that for GlnRS, evolution of tRNA specificity required fewer mutational changes compared to the reassignment of amino acid specificity and supports the idea that the predecessor of GlnRS evolved tRNA$^{Gln}$ specificity before the amino acid specificity was changed from glutamate to glutamine.

Insertion of the C-loop at this evolutionary stage may have created a molten globule, which was able to form Gln-tRNA$^{Gln}$ along with Glu-tRNA$^{Gln}$ because of its conformational plasticity because of enhanced dynamic character. The latter would have transformed to Gln-tRNA$^{Gln}$ through the extant transamidation pathway. Further evolution would have led to the elimination of the glutamylation capability, resulting in a tRNA$^{Gln}$ specific GlnRS.

Both the functional and the structural importance of the C-loop led us to examine the sequence conservation of the C-loop, N-helix, and glutamine binding site (Figure 9a) in representative sets of bacterial (15), archaeal (13), and eukaryotic (20) GluRS and GlnRS sequences, as summarized in Figure 9b. The N-helix is conserved in eukaryotic GluRS and GlnRS and in bacterial GlnRS. It is absent in bacterial GluRS. Although the N-helix is present in archaeal GluRS, it shows little conservation of hydrophobic residues, at positions 267, 276, and 282 (I instead of V is conserved at position 277). Interestingly, the C-loop is absent in both bacterial and archaeal GluRSs, demonstrating the importance of the N-helix−C-loop interaction in GlnRSs. As shown in Figure 9b, the hydrophobic nature of the N-helix-interacting residues in the C-loop is also conserved in all eukaryotic GluRS and GlnRS sequences (the architecture of the C-terminal domain of bacterial GluRSs is different with no C-loop), except for eukaryotic EPRSs and ERSs where residue 488 is not always hydrophobic. This clearly

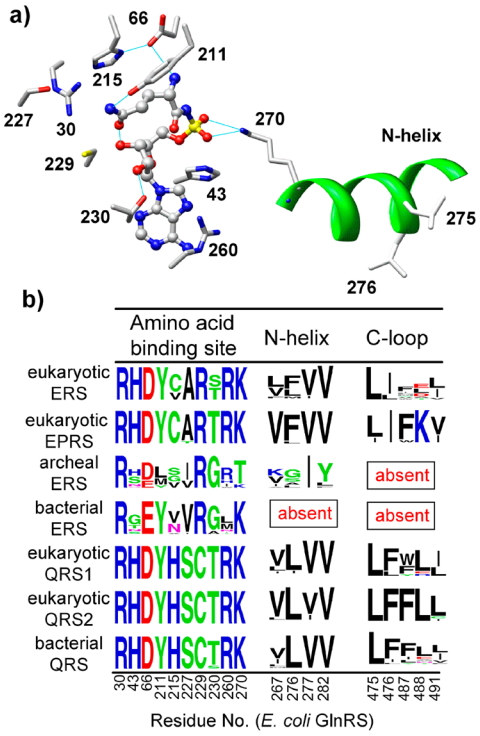


**Figure 9.** (a) Disposition of amino acids (shown as sticks) in *E. coli* GlnRS (PDB entry 1qtq) around a synthetic mimic of glutaminyl adenylate (QSI, shown as balls and sticks) and the N-helix (green). (b) Sequence logo plots of a select set of residues present in the amino acid binding site, N-helix, and C-loop in GluRS (ERS) and GlnRS (QRS) in eukaryotes (20), archaea (13), and bacteria (15). Two classes (10 of each kind) of eukaryotes were considered, one with EPRS (glutamyl prolyl-tRNA synthetase) and another with the canonical ERS.

suggests that the presence of proper C-loop−N-helix interaction is confined to the eukaryotic branch and is consistent with the idea that this insertion occurred after the split of eukarya from archaea. Amino acid binding residues (30, 43, 66, 211, 215, 227, 229, 230, 260, and 270) are mostly conserved for GlnRS (RHDYHSCTRK) and show variability for GluRS sequences. The only position that strictly changes from one amino acid to the other, in going from GlnRS to GluRS, is position 229 (Cys to Arg). This has been noted previously.[35] There are other positions (His215 and Ser227) that also change, in going from GlnRS to GluRS, the nature of the changed amino acid in GluRS being correlated with the N-helix−C-loop interaction.

In conclusion, we have demonstrated that a loop that transmits anticodon binding information and is found only in the eukaryotic domain of life (or genes that were horizontally transferred from eukaryotes) may have been inserted around the time at which GlnRS evolved from GluRS. The same loop also stabilizes a molten globule state. We suggest that the insertion of this loop may have made possible the formation of a catalytically active molten globule state that allowed the substrate specificity switch.

## ■ ASSOCIATED CONTENT

### Supporting Information

Six supplementary tables and eight supplementary figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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R.S. and S.D. contributed equally to this work.


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### Notes

The authors declare no competing financial interest.

## ■ ABBREVIATIONS

GlnRS, glutaminyl-tRNA synthetase; GluRS, glutamyl-tRNA synthetase; CGluRS, C-terminal domain of glutamyl-tRNA synthetase; NGluRS, N-terminal domain of glutamyl-tRNA synthetase; cGluGlnRS, chimeric glutamyl-glutaminyl tRNA synthetase; ANS, 1-anilinonaphthalene-8-sulfonic acid; CD, circular dichroism.

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