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# Glutamate Counteracts the Denaturing Effect of Urea through Its Effect on the Denatured State*

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**The urea induced equilibrium denaturation behavior of glutaminyl-tRNA synthetase from *Escherichia coli* (GlnRS) in 0.25 M potassium L-glutamate, a naturally occurring osmolyte in *E. coli*, has been studied. Both the native to molten globule and molten globule to unfolded state transitions are shifted significantly toward higher urea concentrations in the presence of L-glutamate, suggesting that L-glutamate has the ability to counteract the denaturing effect of urea. D-Glutamate has a similar effect on the equilibrium denaturation of glutaminyl-tRNA synthetase, indicating that the effect of L-glutamate may not be due to substrate-like binding to the native state. The activation energy of unfolding is not significantly affected in the presence of 0.25 M potassium L-glutamate, indicating that the native state is not preferentially stabilized by the osmolyte. Dramatic increase of coefficient of urea concentration dependence (*m*) values of both the transitions in the presence of glutamate suggests destabilization and increased solvent exposure of the denatured states. Four other osmolytes, sorbitol, trimethylamine oxide, inositol, and triethylene glycol, show either a modest effect or no effect on native to molten globule transition of glutaminyl-tRNA synthetase. However, glycine betaine significantly shifts the transition to higher urea concentrations. The effect of these osmolytes on other proteins is mixed. For example, glycine betaine counteracts urea denaturation of tubulin but promotes denaturation of S228N λ-repressor and carbonic anhydrase. Osmolyte counteraction of urea denaturation depends on osmolyte-protein pair.**

Folding of a polypeptide chain into a precise three-dimensional structure has been a subject of intense study over the past several decades (1, 2). Despite much progress, a complete understanding still eludes us. Much attention is now devoted to protein folding *in vivo*, where the cellular environment profoundly influences folding (3, 4). This has led to the discovery of the chaperones. Another aspect of *in vivo* environments that differ significantly from *in vitro* environments normally used for protein folding studies is the presence of osmolytes. Osmolytes are small molecules that accumulate inside the cell at relatively high concentrations and protect the intracellular proteins against environmental stress. Thus, they play a crucial role in protein stabilization.

Although some efforts have been directed toward understanding the effects of osmolytes on protein stability, little is known about their effect on folding intermediates and partially unfolded states of proteins. Partially unfolded states are not only of crucial importance in understanding the folding processes but may play a crucial role in many diseases that involve extracellular protein aggregation and amyloid fibril formation, such as Alzheimer's disease and Scrapie (5, 6). Similar intracellular protein aggregates (Lewy bodies) are also known to play an important role in other neurodegenerative diseases, such as Parkinson's disease (7). Very little is known about the role of osmolytes on such processes, although it is generally recognized that cells in the brain accumulate high concentrations of several osmolytes (8, 9).

Many different types of osmolytes have been identified: amino acids, sugars, polyhydroxy compounds, and amine derivatives (10). Despite the varied chemical nature of different osmolytes, it is generally thought that osmolytes do not bind to proteins. A common mechanism of stabilization is the osmophobic effect that is the preferential destabilization of the unfolded state due to unfavorable transfer free energy of the peptide backbone from water to osmolyte solution (11). However, it has been pointed out that the effect of an osmolyte may vary from protein to protein, suggesting the presence of other types of interactions (12). A comparison of actions of different osmolytes on unfolding behavior of different proteins may yield valuable clues regarding the mechanism of action of different osmolytes.

In this article we have chosen to study the ability of several osmolytes to counteract urea denaturation of several proteins. In particular, we have studied in detail the effect of L-glutamate, a naturally occurring osmolyte (12–14), on urea denaturation of GlnRS.[1] We have also investigated the effect of different osmolytes on urea-induced equilibrium denaturation of GlnRS and other unrelated proteins and found that the osmolyte effect is dependent on the nature of the osmolyte and the denatured states of the protein.

## EXPERIMENTAL PROCEDURES

### *Materials*

Bovine erythrocyte carbonic anhydrase, potassium L-glutamate, and potassium D-glutamate were purchased from Sigma. Ultrapure urea was purchased from Spectrochem. All other chemicals were of analytical grade. Tubulin was purified according to Bhattacharya *et al.* (15).

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[1] The abbreviations used are: GlnRS, glutaminyl-tRNA synthetase; TMAO, trimethylamine *N*-oxide; CD, circular dichroism; ANS, 1-anilino-8-naphthalenesulfonate; *m*, coefficient of urea concentration dependence.

## *Methods*

*Protein Purification*—Glutaminyl-tRNA synthetase was purified according Bhattacharyya *et al.* (16). S228N λ repressor was a gift from Prof. N. C. Mandal and was purified according to Jana *et al.* (17).

*Equilibrium Denaturation Experiments*—All experiments were carried out in 0.1 M Tris-HCl buffer, pH 7.5, at 25 °C unless specifically mentioned otherwise. The equilibrium denaturation experiments were performed after incubating the protein in the desired urea concentration for 18 h at room temperature. Fluorescence measurements were carried out in a Hitachi F3010 spectrofluorometer. The excitation and emission band passes were 3 nm each. For measurement of ANS fluorescence, 30 μM ANS was mixed with the denatured protein, and sample fluorescence was measured. A 30 μM concentration was chosen because binding isotherms indicated that beyond this concentration some degree of saturation may occur. The excitation wavelength was 420 nm, and the emission wavelength was 482 nm. Emission wavelength was fixed at 482 because this is around the emission maximum of ANS bound to molten globule states of several proteins. Far UV CD spectra were measured in a Jasco J-600 spectropolarimeter using a 1-mm path length cuvette. Five spectra were signal-averaged for improvement of the signal-to-noise ratio. GlnRS concentrations were 7.5 μM for CD experiments and 2 μM for fluorescence experiments.

*Kinetics of Unfolding*—Kinetics of unfolding was measured at 25 °C at different urea concentrations. Glutaminyl-tRNA synthetase was diluted to a given urea concentration (either in the presence of 0.25 M potassium L-glutamate or 0.25 M potassium chloride) at a final protein concentration of 2 μM and immediately transferred to a Hitachi F3010 spectrofluorometer. Fluorescence intensity at 340 nm was measured as a function of time. The excitation wavelength was 295 nm. For measurement of CD kinetics, a similar protocol was followed. The final protein concentration was 7.5 μM. Urea concentrations of 5, 6, 6.5, 7, and 8 M were used for CD, and 4.5, 5, 5.5, 6, 6.5, and 7 M were used for fluorescence.

*Limited Proteolysis and Trypsin Assay*—GlnRS was incubated at 37 °C in the presence of 25:1 w/w L-1-tosylamido-2-phenylethyl chloromethyl ketone-trypsin in 50 mM potassium phosphate buffer, pH 7.5, containing either no osmolytes or 0.25 M osmolyte. The reaction was quenched with phenylmethylsulfonyl fluoride at the designated times at a final concentration of 1 mM and electrophoresed on an SDS-polyacrylamide gel using standard protocols (16). A trypsin assay was conducted using benzoyl arginyl ethyl ester as the substrate, which was monitored at 253 nm.

*Curve Fitting*—The data fitting was done using Kyplot (Koichi Yoshioka, 1997–1999, version 2.0 beta 4). The equilibrium profiles were fitted as described in Das *et al.* (18). For two-state equilibrium, the following equation was used.

$$S = \frac{S_0 + S_\infty \times \exp((-\Delta G^0 + (m \times [\text{urea}]))/RT)}{1 + \exp((-\Delta G^0 + (m \times [\text{urea}]))/RT)} \quad \text{(Eq. 1)}$$

For $F_{340}/F_{350}$, a correction was introduced in the equation,

$$S = \frac{S_0 + (1 - (F_{350}^{\infty}/F_{350}^{0})) \times S_\infty \times \exp((-\Delta G^0 + (m \times [\text{urea}]))/RT)}{1 + (1 - (F_{350}^{\infty}/F_{350}^{0})) \times \exp((-\Delta G^0 + (m \times [\text{urea}]))/RT)} \quad \text{(Eq. 2)}$$

For the three-state equilibrium, the equation,

$$S = \frac{S_0 + S_I..\exp((-\Delta G_1^0 + (m_1.[\text{urea}]))/RT) + S_\infty.\exp((-\Delta G_2^0 + (m_2.[\text{urea}]))/RT)}{1 + \exp((-\Delta G_1^0 + (m_1.[\text{urea}]))/RT) + \exp((-\Delta G_2^0 + (m_2.[\text{urea}]))/RT)} \quad \text{(Eq. 3)}$$

was used, where $S_0$, $S_I$, and $S_\infty$ are signal intensities for 100% reactant, 100% intermediate, and 100% product, respectively. $F_{350}^{\infty}/F_{350}^{0}$ refers to the ratio of fluorescence intensity at 350 nm for 100% product and 100% reactant, respectively. $\Delta G^0$ refers to the free energy at zero urea concentration (the corresponding equilibrium constant defined as [product]/[reactant]). In the second equation, $\Delta G_1^0$ and $\Delta G_2^0$ refer to the free energy at zero urea concentration with the corresponding equilibrium constants defined as [intermediate]/[reactant] and [product]/[reactant], respectively. $R$, $T$, and $m$ carry their usual meaning.

The kinetics of fluorescence or CD change was fitted to the equation,

$$S(t) = \frac{k_1(S_0 - S_\infty)}{(k_1 + k_1)} \times [k_1/k_1 + \exp(-(k_1 + k_1)t)] + S_\infty \quad \text{(Eq. 4)}$$

whereas the $F_{340}/F_{350}$ ratio change was fitted to the equation,

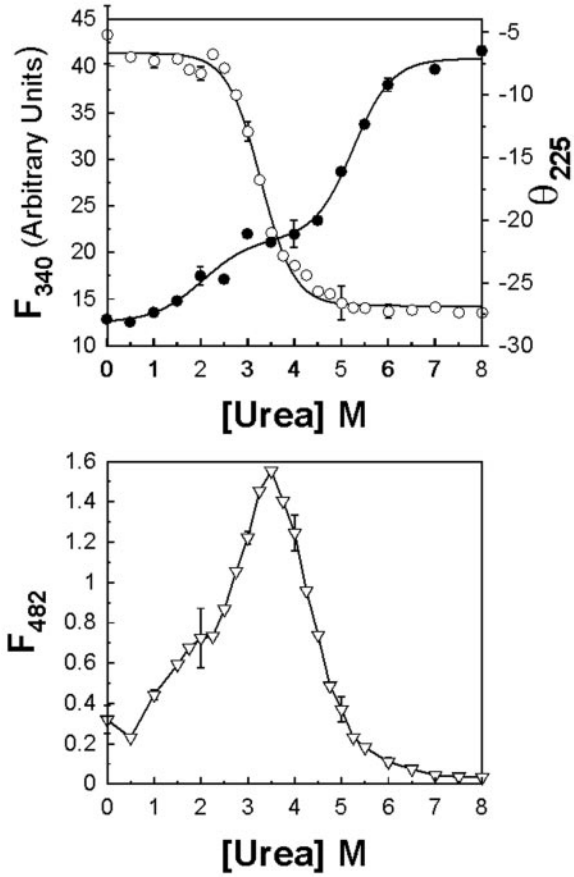


FIG. 1. **Change of spectroscopic signals of GlnRS as a function of urea concentration under equilibrium conditions in the presence of 0.25 M potassium L-glutamate.** *Upper panel*, ○, fluorescence intensity at 340 nm when excited at 295 nm; 340 nm was chosen as native emission maximum is close to this wavelength. ●, the CD signal at 225 nm; 225 nm was chosen as urea absorption at lower wavelengths may interfere with measurements. *Lower panel*, ▽, ANS fluorescence at 482 nm when excited at 420 nm The *solid lines* represent the best fit to either a two-state equilibrium (fluorescence intensity) or to a three-state equilibrium (CD signal) in the *upper panel*. In *lower panel* the *solid lines* are merely used to connect the symbols. Protein concentrations were 2 and 7.5 μM for fluorescence and CD experiments, respectively. The solution conditions were 0.1 M Tris-HCl buffer, pH 7.5. The temperature was 25 °C. *Error bars* indicate S.E. obtained from three independent measurements.

$$S(t) = \frac{(k_1/(k_1 + k_{-1})).(S_0 - (F_{350}^{\infty}/F_{350}^{0}).S_\infty).[k_{-1}/k_1 + \exp(-(k_1 + k_{-1})t)] + (F_{350}^{\infty}/F_{350}^{0}).S_\infty}{\{(k_1/(k_1 + k_{-1})).(1 - (F_{350}^{\infty}/F_{350}^{0})).[k_{-1}/k_1 + \exp(-(k_1 + k_{-1})t)]\} + (F_{350}^{\infty}/F_{350}^{0})} \quad \text{(Eq. 5)}$$

where $S(t)$ is the spectral signal at time $t$, $k_1$ is the forward rate constant, $k_{-1}$ is the backward rate constant, $S_0$ is the signal at time 0, and $S_\infty$ is the signal if all the starting material was converted to the product. $F_{350}^{0}$ and $F_{350}^{\infty}$ refer to fluorescence values at 100% reactant and 100% product concentrations.

## RESULTS

*Influence of L-Glutamate on Equilibrium Unfolding of GlnRS*—The most ubiquitous unfolding intermediates belong to the molten globule class and are produced under a variety of conditions (19–21). In a previous study we detected an intermediate of molten globule class by equilibrium urea denaturation (18). Fig. 1 shows the plot of fluorescence intensity, far UV circular dichroism, and ANS fluorescence in the presence of 0.25 M potassium L-glutamate as a function of urea. It is clear from this curve that a two-state approximation cannot be

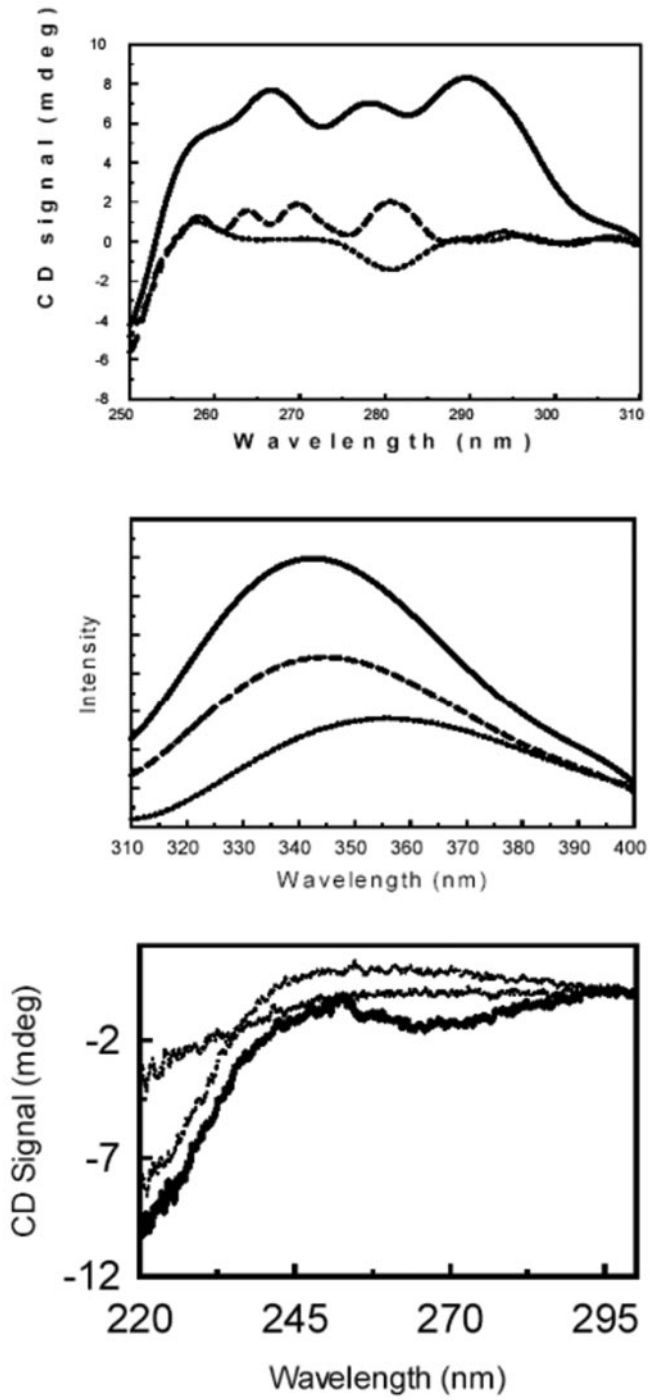


FIG. 2. **Representative spectra of the native, molten globule, and denatured states of glutaminyl-tRNA synthetase in 0.25 M glutamate.** The *solid lines* are the spectra of the native state, the *dashed lines* are the spectra of the molten globule, and the *dotted lines* are the spectra of the denatured state. *Upper panel*, near UV CD spectra of no urea, 3.5 M urea, and 8 M urea. The path length for CD measurements was 1 cm, and the protein concentrations were 3 $\mu$M. The *middle panel* is the fluorescence emission spectra at the same urea concentrations as mentioned above. The *lower panel* is the far UV CD spectra at 0, 3.15, and 7.16 M urea. The other solution conditions were the same as in Fig. 1.

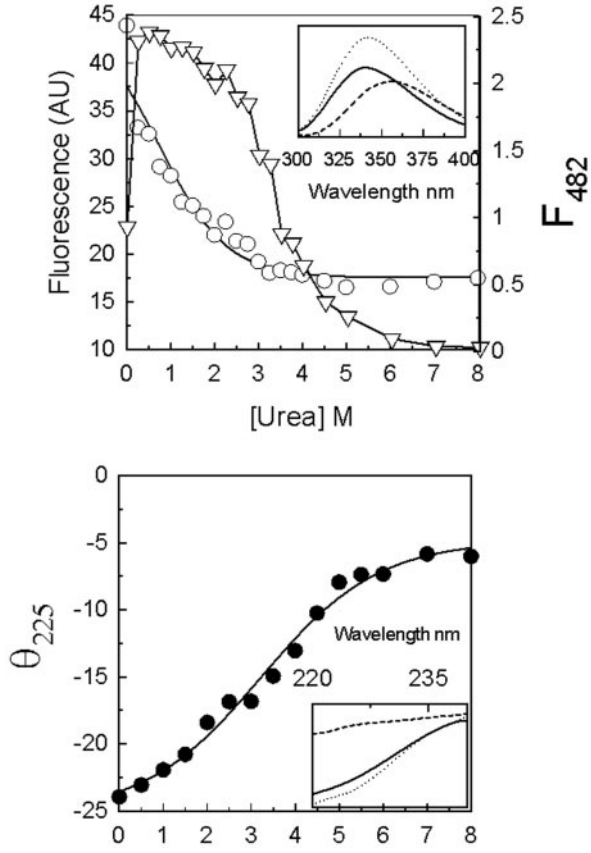


FIG. 3. **Change of spectroscopic signals of GlnRS as a function of urea concentration under equilibrium conditions in the presence of 0.25 M potassium chloride.** *Upper panel*, ○, fluorescence intensity at 340 nm when excited at 295 nm; ▽, ANS fluorescence at 482 nm when excited at 420 nm. The *inset* shows the emission spectra of the native (*dotted*; 0 M), molten globule(*solid*; 1 M), and denatured state (*dashed*; 8 M). *Lower panel*, ●, the CD signal at 225 nm. The *inset* shows the CD spectra of the three states described above. The *lines* have the same meaning. The *solid lines* in the figures represent the best fit to either a two-state equilibrium (fluorescence intensity) or to a three-state equilibrium (CD signal). The *solid line* in ANS data is merely used to connect the symbols. The other solution conditions and the methodology are identical to the legend of Fig. 1.

made. Fluorescence intensity declines rapidly with a midpoint around 3.25 M urea. The far UV CD spectral intensity declines with approximately two phases. The major phase has the midpoint of transition around 5.5 M urea. It was shown previously that in the presence of 0.1 M KCl, the native to molten globule transition occurred with very significant loss of fluorescence intensity and relatively small far UV CD change (18). Based on these criteria, we suggest that the first transition, characterized by a major loss of fluorescence intensity, represents the previously characterized native to molten globule transition. It is now well documented that anilinonaphthalenesulfonates bind to molten globule states with higher fluorescence yields and are often used to verify the presence of molten globule states (22, 23). ANS fluorescence increase peaks around 3.5 M urea followed by a rapid decline, supporting the argument that this is the previously characterized native to molten globule transition. It is likely that the transition represented by an almost total loss of far UV CD represents the intermediate to unfolded state transition.

Fig. 2 shows the near UV CD, the far UV CD, and the fluorescence emission spectra of GlnRS native state, molten globule state (when the first transition is nearly complete but the transition to unfolded state has not taken place), and native state, all in the presence of 0.25 M potassium L-glutamate. It is clear that almost all native-like near UV CD spectrum disappears in 3.5 M urea, where much of the far UV CD and, hence, the secondary structure is preserved. This strongly supports the idea that the intermediate is the previously characterized molten globule. Interestingly the magnitude of near UV CD intensity loss is significantly greater than the situation in the

TABLE I
*Extracted free energies from equilibrium urea denaturation*

Folding free energies ($\Delta G_f$) were extracted by fitting profiles separately, either to two-state no intermediate equilibrium (fluorescence intensity and $F_{340}/F_{350}$) or to a three-state equilibrium with one intermediate ($\theta_{225}$) using KyPlot.

| Osmolyte | Observed Signal(s) | Fitted model | $\Delta G_f$ (KCal/mole) | m (KCal/(mole.M) |
|---|---|---|---|---|
| Potassium | $F_{340}$ | Two-state | 5.5±0.5 | 1.7±0.1 |
| L-glutamate | $\theta_{225}$ | Three-state | 2.4±1.2 | 1.2±0.6 |
| 0.25 M | | | 10±1.7 | 2.6±0.5 |
| Potassium D-glutamate | $F_{340}$ | Two-state | 5±0.6 | 1.6±0.2 |
| 0.25 M | | | | |
| Potassium Chloride | $F_{340}$ | Two-state | 0.6±0.6 | 0.8±0.27 |
| 0.25 M | $\theta_{225}$ | Two-state | 1.45±0.3 | 0.45±0.07 |

absence of glutamate. Thus, the native to molten globule transition is distinctly shifted to higher urea concentrations in the presence of 0.25 M potassium L-glutamate, signifying either relative stabilization of native state or destabilization of the molten globule state.

Because part of the effect of L-glutamate can originate from electrostatic effects of L-glutamate, we have measured the different spectroscopic signals as a function of urea concentration in the presence of 0.25 M KCl. A plot of different signals *versus* urea concentration is shown in Fig. 3. The *insets* show the fluorescence emission and far UV CD spectra of the native, molten globule, and denatured state. It is quite clear that the loss of fluorescence intensity occurs at a much lower urea concentration when compared with the profile in the presence of potassium L-glutamate (incidentally, the midpoint is also at a lower urea concentration than in the presence of 0.1 M KCl). This transition is centered around 1 M urea. The ANS binding peaks at very low urea concentrations (around 0.5 M urea), remains stable, and then finally declines with the midpoint centered around 3.75 M urea. The decline of the far UV CD is centered approximately around 3.75 M urea. All transitions are fitted as described before, and the extracted free energies are reported in Table I. An interesting observation is that in the presence of L-glutamate, both the $\Delta G$ and $m$ values increase very significantly. The implication of this observation is discussed later. To clarify the relationships of different states in 0.25 M L-glutamate and KCl, we have analyzed ANS binding in detail. Fig. 4 shows the isotherms of ANS binding to native, molten globule, and denatured states of GlnRS in the presence of 0.25 M L-glutamate and KCl along with emission spectra in the *inset*. The molten globule and denatured state binding isotherms are very similar in L-glutamate and KCl. In the presence of glutamate, the native state binds ANS weakly, whereas in the presence of KCl (0.25 M) a fairly high degree of ANS binding is observed. Because in 0.1 M KCl, ANS binding is low (18), the higher ANS binding at 0.25 M KCl is consistent

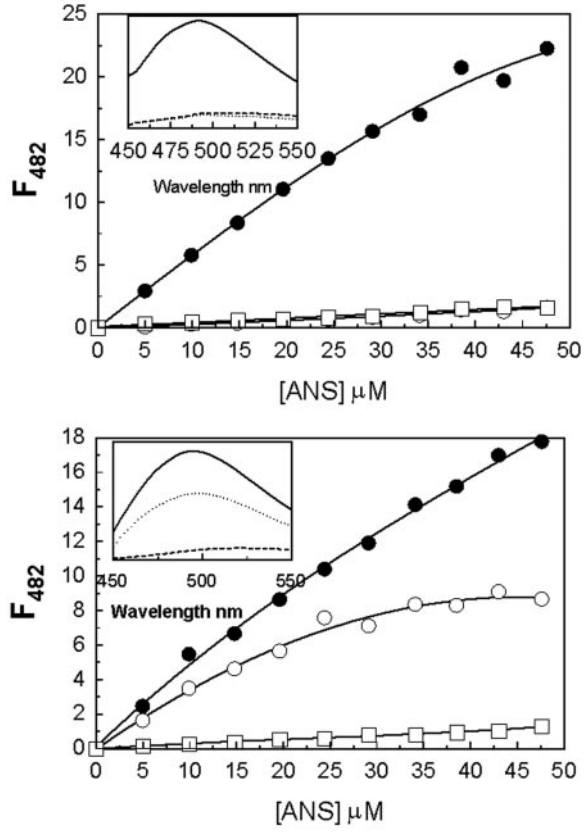


FIG. 4. **ANS binding to native (○), molten globule (●), and denatured state (□).** *Upper panel*, 0.25 M L-glutamate. *Lower panel*, 0.25 M KCl. *Insets* show the corresponding emission spectra of native (*dotted*; 0 M), molten-globule (*solid*; 3.15 M), and denatured (*dashed*; 8 M). The solution conditions are same as in Fig. 1, and the *inset lines* have same meaning as in the *inset* of Fig. 3.

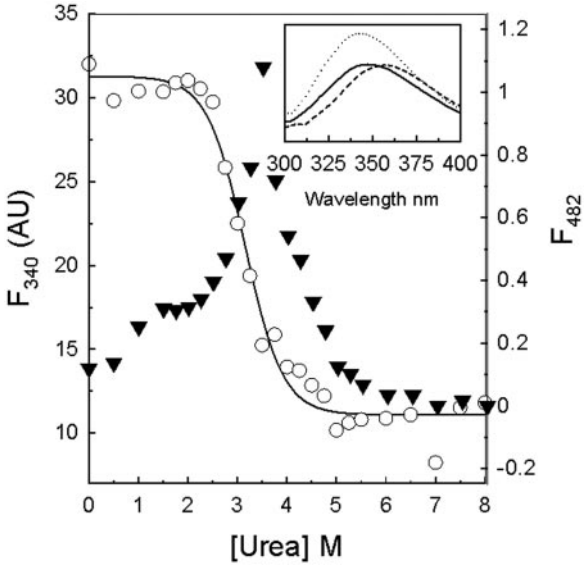


FIG. 5. **Change of spectroscopic signals of GlnRS as a function of urea concentration under equilibrium conditions in the presence of 0.25 M potassium D-glutamate.** ○, fluorescence intensity at 340 nm when excited at 295 nm; ▼, ANS fluorescence at 482 nm when excited at 420 nm. The solution conditions and the methodology are identical to the legend of Fig. 1. The *inset* shows the emission spectra of the native (*dotted*; 0 M), molten globule (*solid*; 3.15 M), and denatured state (*dashed*; 7.16 M). *AU*, arbitrary units.

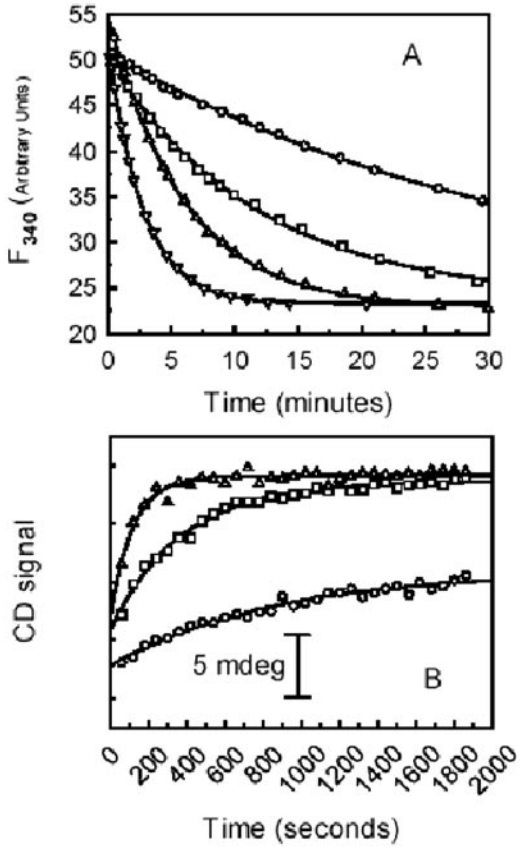


FIG. 6. *A*, unfolding kinetics of glutaminyl-tRNA synthetase as monitored by the loss of tryptophan fluorescence intensity at 4.5 M (○), 5 M (□), 6 M (△), and 7 M (▽) urea. The excitation wavelength was 295 nm, and emission was monitored at 340 nm. *B*, change in CD signal at 230 nm at 5 M (○), 6.5 M (□), and 8 M (△) urea. The other solution conditions were the same as in Fig. 1. *mdeg*, millidegree.

with significant destabilization of the native state at higher KCl concentration.

*L-Glutamate Effect Is Not Due to Substrate-like Native State Binding*—The natural substrate of glutaminyl-tRNA synthetase is L-glutamine, which is structurally similar to L-glutamate. To see whether such a dramatic effect of potassium L-glutamate is at least partly due to the substrate-like properties of potassium L-glutamate or the preferential binding to the native state, we have measured the denaturation profile in potassium D-glutamate. Because D-glutamine is not a substrate of GlnRS, it is unlikely that potassium D-glutamate will have any substrate-like properties. Fig. 5 shows the urea denaturation profile as measured by fluorescence intensity loss in the presence of 0.25 M potassium D-glutamate. The *inset* shows the fluorescence emission spectra of the native, molten globule and denatured states. As in potassium L-glutamate, the transition measured by the loss of fluorescence intensity is centered around 3.25 M urea. The ANS fluorescence peak also occurs around 3.5 M urea. The declining edge of ANS fluorescence occur around 4 M urea, suggesting that the urea denaturation in potassium D-glutamate is very similar to that in potassium L-glutamate. Thus, the effect of potassium L-glutamate on the urea denaturation profile is unlikely to be due to any putative substrate-like properties of potassium L-glutamate. This is consistent with the fact that L-glutamate accumulates at very high concentrations presumably without any effect of GlnRS activity. We have also attempted to measure the binding of glutamate to GlnRS by NMR by observing the effect of GlnRS on T1. We chose D-glutamate because it has a very similar effect on equilibrium denaturation profile. The longitudinal relaxation rates of $\alpha$-proton of 1.5 mM D-glutamate were determined in the absence and in the presence of 50 $\mu$M GlnRS. The observed T1 values are 1.43 ± 0.03 s and 1.55 ± 0.05 s in the absence and presence of GlnRS, respectively. In a previous study we determined the binding of galactose using similar techniques and found that under similar conditions binding of galactose to Gal repressor led to T1 lowering of almost 2-fold (24). This suggests no significant binding of D-glutamate in a substrate-like manner to the native state and that the counteraction effect of glutamate is not due to substrate-like binding to the native state.

One of the possibilities mentioned above is that somehow glutamate selectively stabilizes the native state (although we have ruled out the possibility of substrate-like binding). If the native state is selectively stabilized, the activation energy of unfolding would increase due to increasing energy difference between the native state and the succeeding transition state. Kinetics of unfolding has been used to study the activation energy barriers between the unfolding intermediates. Activation energies at several different denaturant concentrations may be obtained and extrapolated to zero denaturant concentration to obtain the activation energy in the absence of the denaturant. Fig. 6 shows the kinetics of denaturation of GlnRS at several urea concentrations as measured by fluorescence intensity and far UV CD. The curves can be fitted well to a first order rate equation. Fig. 7, *A* and *B*, shows the extrapolation of activation energy thus obtained to zero urea concentrations in the presence of 0.25 M potassium L-glutamate and in the presence of 0.25 M KCl. In contrast to the dramatic effect of potassium L-glutamate on the stability of the equilibrium intermediates, none of the rate constants is significantly affected when potassium L-glutamate replaces KCl. In 0.25 M KCl, the activation energies extrapolated to 0 M urea are 22.68 ± 0.16 and 23.6 ± 0.55 kcal/mol when unfolding was measured by $F_{340}$ and far UV CD, respectively. In 0.25 M potassium L-glutamate, the corresponding activation energies extrapolated to 0 M urea, are

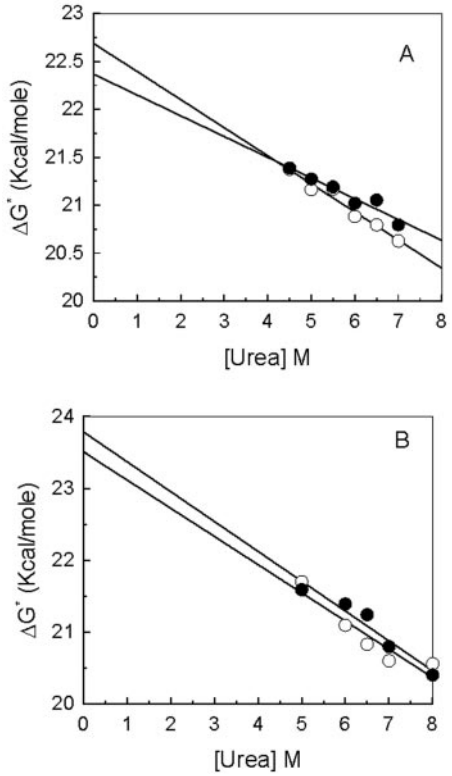


FIG. 7. **Extrapolation of activation energies obtained at different urea concentrations to zero urea concentrations.** ○, 0.25 M KCl; ●, 0.25 M potassium L-glutamate. *A*, plot as obtained from measurement of $F_{340}$. *B*, plot as obtained from measurement of CD signal at 230 nm. The solution conditions are given in the legends of Figs. 1 and 6.

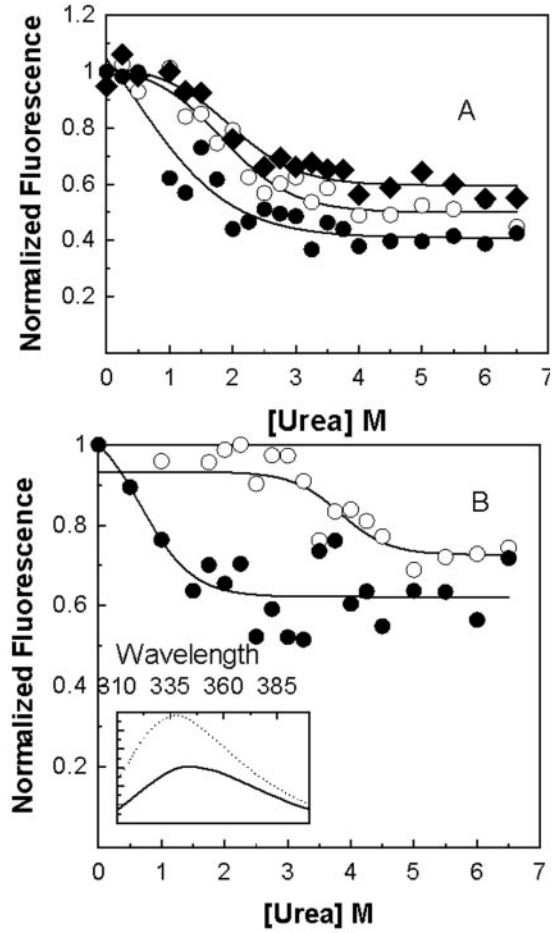


FIG. 8. **Change of fluorescence intensity of GlnRS at 340 nm as a function of urea concentration containing different osmolytes.** *A*, trimethylamine *N*-oxide (◆), inositol (○), triethylene glycol (●). *B*, sorbitol (●) and glycine betaine (○). The solution conditions were 0.1 M Tris-HCl, pH 7.5, containing 0.25 M osmolyte. The rest of the conditions are the same as in legend of Fig. 1. The lines are fit to the two-state model. The *inset* shows the fluorescence emission spectra at 0 M (*dotted*) and 3 M (*solid*) urea containing 0.25 M sorbitol.

22.36 ± 0.16, and 23.78 ± 0.41 kcal/mol, respectively. Clearly, unlike that of the stability of the intermediates derived from the equilibrium measurements, very little effect of chloride to Glu substitution is seen. The kinetics of denaturation when monitored by loss of fluorescence intensity represents native to molten-globule transition. If preferential stabilization of the native state occurred in glutamate, it is likely that the activation energy would have decreased in L-glutamate. The lack of such an effect argues against this possibility.

*Differential Effects of Osmolytes on Denatured States of Proteins*—Because L-glutamate has a very significant effect on the molten globule state of glutaminyl-tRNA synthetase, we have also explored the effects of other osmolytes. Fig. 8 shows the effect of glycine betaine, sorbitol, trimethylamine *N*-oxide, triethylene glycol, and inositol on the equilibrium denaturation profile of GlnRS. Previously we have shown that a decrease of fluorescence intensity at 340 nm occurs due to the transition from the native state to the molten globule state. Glycine betaine shows very strong counteraction ability, whereas TMAO shows only modest counteraction ability. None of the other osmolytes has a significant effect. This is somewhat surprising, because osmolytes such as trimethylamine oxide are known to have significant effects on folding equilibrium of proteins (25). We have attempted to verify this destabilizing effect of L-glutamate on the denatured states in the present system using limited proteolysis as a tool. It is well known that proteases cleave denatured proteins faster than native proteins. Higher order structures in native proteins prevent proteases from gaining access to the scissile bonds. If an osmolyte destabilizes the denatured states and, hence, drives the equilibrium toward a more folded state, it is expected that such an osmolyte would decrease the rate of proteolysis. Fig. 9 shows the rate of proteolysis of GlnRS by trypsin in the absence of any osmolyte and in the presence of 0.25 M potassium L-glutamate or inositol or tri-ethylene glycol. It is clear that 0.25 M potassium L-glutamate significantly inhibits proteolysis by trypsin, whereas no significant effect is observed on the rate of proteolysis in the presence of inositol or triethylene glycol. The rate of hydrolysis of benzoyl arginyl ethyl ester by trypsin is not significantly affected by 0.25 M potassium L-glutamate, suggesting that the glutamate effect originates from its effect on GlnRS structure (Fig. 9*D*). This supports the observation that glutamate has a strong counteraction ability of urea effect, at least in this system.

Because L-glutamate and glycine betaine has the strongest counteraction ability against urea in GlnRS system, we have attempted to see if that is true with other proteins. Table II shows the effect of osmolytes on three different proteins, carbonic anhydrase, S228N λ-repressor, and tubulin. The S228N mutant was chosen because it is known that the protein is monomeric under these conditions so that complications arising out of monomer dimer equilibrium will be avoided. In a previous study we showed that the S228N repressor denatures in two phases (there is another transition, which does not lead to any change in fluorescence spectra) if monitored by fluorescence emission maximum shift (26). We have assigned the first transition to denaturation of a part of the C-terminal domain and the transition in the higher concentrations of urea to global

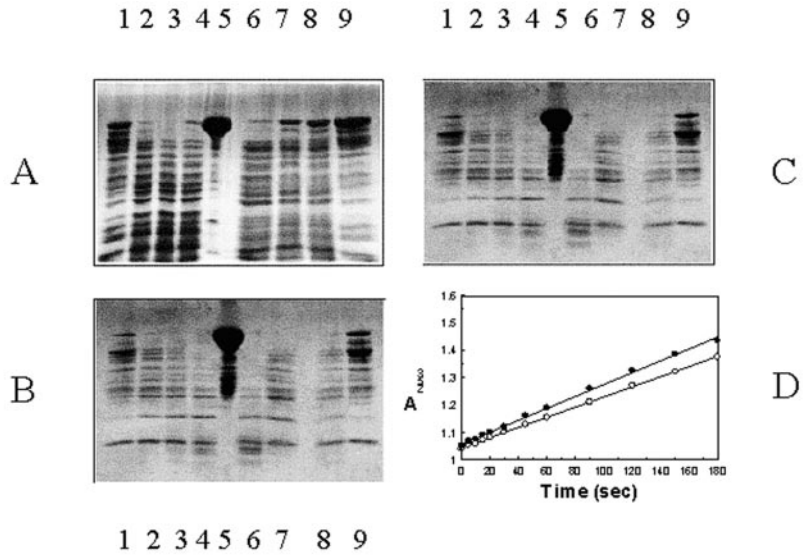


FIG. 9. **Trypsinolysis of GlnRS in the presence of 0.25 M osmolyte.** *A*, potassium L-glutamate. *B*, triethylene glycol. *C*, inositol. *D*, trypsin assay in the presence (●) and absence (○) of 0.25 M potassium L-glutamate. In all the panels *lanes 1–4* represents 15-, 30-, 45-, and 60-min time points in the absence of osmolyte. *Lane 5* represents undigested GlnRS. *Lanes 6–9* represent 60-, 45-, 30-, and 15-min time points in the presence of osmolyte. The solution conditions were 0.05 M potassium phosphate, pH 7.5, containing 0.25 M osmolyte. The details of the reaction and SDS-PAGE are given under "Experimental Procedures."

TABLE II
*Effect of different osmolytes on the urea denaturation of different proteins*

The denaturation protocol is same as described in Fig. 1 and Table I. All the osmolyte concentrations were 0.25 M. The measured parameter was $F_{340}/F_{350}$. Midpoints were determined by fitting to a two-state model in case of carbonic anhydrase and tubulin and to a three-state model in case of S228N repressor. The two values for S228N repressor represents mid points for two transitions. *TEG*, triethylene glycol.

| *Protein* | *Osmolyte* | *Mid-point of transition [Urea] M* |
|---|---|---|
| **Carbonic anhydrase** | none | 5.3 |
| | L-glutamate | 5.2 |
| | TMAO | 5.2 |
| | TEG | 5.2 |
| | Glycine betaine | 3.2 |
| **Tubulin** | None | 4.2 |
| | Glycine betaine | 8.1 |
| **S228N repressor** | None | 0.86; 6.4 |
| | TEG | 0.95; 6.4 |
| | TMAO | 1.1; 7.25 |
| | L-Glutamate | 1.53; 8.25 |
| | Glycine betaine | 0.74; 5.6 |

denaturation of the C-terminal domain. Table II shows the effect of different osmolytes on equilibrium urea denaturation profile of S228N repressor. Both the transitions are strongly counteracted by L-glutamate and relatively weakly by TMAO. Triethylene glycol has no significant effect, whereas glycine betaine shifts the transition to lower urea concentrations, thus promoting denaturation. Interestingly, L-glutamate has no significant effect on urea denaturation of carbonic anhydrase. However, glycine betaine strongly counteracts in the case of tubulin but shifts the transition to a lower urea concentration in carbonic anhydrase. We conclude that urea counteraction is osmolyte-protein pair-specific and L-glutamate counteraction of urea effect is not confined to glutaminyl-tRNA synthetase.

## DISCUSSION

Potassium L-glutamate and its close analogs are widely used in nature as osmolytes. In fact it is used as osmolytes by organisms from all three kingdoms of life (13). It is known to be a compatible solute (27, 28). It enhances protein-nucleic acid interaction and stabilizes proteins from temperature inactivation (12, 29). Several osmolytes play another important role in some organisms; that is, they counteract the effect of urea on protein structure. Trimethylamine oxide and glycine betaine belongs to this class of osmolytes (30–32). We have shown in this article that potassium L-glutamate exerts a powerful counteraction effect to the denaturing effect of urea in the glutaminyl-tRNA synthetase system. What is surprising is that this effect is more powerful than the effect of trimethylamine oxide in this system. Most interestingly, none of the counteracting osmolytes is universally effective. Their counteraction ability depends on the nature of the protein and the denatured state.

The mechanism of this powerful counteraction effect of some osmolytes is not known at this point. However, we can make some tentative conclusions. Because, L-glutamate is studied in detail here, we will focus on glutamate effect primarily. The *m* values of urea denaturation of both the native to molten globule and native to unfolded state are enhanced greatly in the presence of glutamate when compared with that obtained in the presence of potassium chloride. It was concluded that the *m* value of a transition is related to the change in the accessible surface area (33). Assuming that there is no significant change in the structure of the native state in the presence of L-glutamate (as borne out by CD and fluorescence properties), a change in accessible surface area is equivalent to a change in the structure of the denatured state. Many recent studies point out that the denatured states are not devoid of structure, as was believed earlier (34, 35). Shortle (36) and Shortle and Wrabl (37) conclude that the *m* value of a transition is related to the structure of the denatured state. The larger value of *m* implies a less structured denatured state. It is interesting to note that the near UV CD intensity of the molten globule state in the presence of glutamate is considerably less than in the absence of glutamate, perhaps indicating a more disordered structure.

The TMAO counteraction of urea effect has been studied in

most detail. It was concluded by several investigators that TMAO counteraction can be entirely accounted for by unfavorable free energy of transfer of peptide backbone from water to an osmolyte-water mixture (38). Side chains contribute little. Recent work by Murphy and coworkers (39) suggest that some of this unfavorable transfer free energy may originate from the ordering of the water structure by TMAO. In such a situation more solvent-exposed conformations will be destabilized. In the present situation, the denatured state becomes less structured in the presence of L-glutamate, and thus, it is unlikely that the glutamate counteraction is entirely due to the putative osmophobic effect, as the less structured states are likely to be more destabilized in the presence of an operative osmophobic force. A large change in $\Delta G$ between the native and the denatured states does indeed suggest destabilization of the denatured states. Because the less structured states are more prevalent in the presence of glutamate, it appears that they are destabilized the least; this is exactly the opposite of what would be expected if purely osmophobic forces are operative.

How could increased destabilization occur for denatured states that are more compact? One of the possibilities may be that the side-chain exposures of these different states are different, and increased exposure of the side chains in the more exposed states leads to a partial compensation because transfer of free energy of many of the side chains from water to osmolyte solution is favorable. Another possible origin may be from the differential interaction of glutamate with denatured states having different solvent exposure. In this situation, either the osmolyte binds to the more exposed states preferentially or it is excluded more from the less exposed state. A third possibility is that the osmophobic contribution to destabilization is not just the sum of contributions of individual side chains and peptide bonds, but the contribution changes in the context of the structure. The molecular origin of such putative effects is completely unknown at this point.

It is interesting that not all osmolytes have destabilizing effects on denatured states of GlnRS. TMAO has a modest urea counteraction effect and virtually no effect on *m* value, suggesting little effect on the nature of the denatured state. Polyols do not show any significant effect in this study. A number of other studies have previously noted this differential effect of osmolytes (13). In many situations, limited proteolysis is thought to take place from denatured states that are at equilibrium with the native state. If the denatured states are destabilized, it is expected that proteolysis under limiting conditions will decrease. It has been noted previously that although TMAO decreased the limited proteolysis rate of lactate dehydrogenase by trypsin, no significant effect is seen with glycine as an osmolyte (40). Clearly, the nature of the protein and the osmolyte pair determines the outcome. Although osmophobic force is likely to be of general importance, specific effects of different osmolytes may be superimposed to yield the differential effect of different osmolytes.

What may be the effect of such strong destabilization of the partially denatured state by L-glutamate? In the presence of osmolytes such as L-glutamate, which has significant effect on stability of intermediate molten globule state, the equilibrium concentration of partially unfolded states would decrease. In this sense the osmolytes of this type may have a synergistic effect with the chaperone systems, which also lowers the free concentration of the partially folded intermediates. This may lead to inhibition of protein aggregation and enhancement of folding yields *in vivo*. Another biological milieu where concentrations of partially denatured states may exert crucial role is in amyloid fibril formation. It has been hypothesized that fibril formation proceeds through an increased accumulation of partially denatured states of a protein (6, 41). If osmolytes like L-glutamate indeed have a destabilizing role in the accumulation of such intermediates, they may play an inhibitory role in fibril formation. A complete exploration of the roles of osmolytes in protein aggregation and fibril formation may be of significant importance.

## CONCLUSION

We report that L-glutamate is a powerful counteracting agent toward denaturing effects of urea. Other osmolytes also show counteracting ability, but in all cases the effect appears to depend on the nature of the protein involved, indicating other effects beyond the osmophobic force.

## REFERENCES


1. Baldwin, R. L., and Rose, G. D. (1999) *Trends Biochem. Sci.* **24,** 77–83
2. Dobson, C. M., and Karplus, M. (1999) *Curr. Opin. Struct. Biol.* **9,** 92–101
3. Caplan, A. J. (1999) *Trends Cell Biol.* **9,** 262–268
4. Fink, A. L. (1999) *Physiol. Rev.* **79,** 425–449
5. Serpell, L. C., Sunde, M., and Blake, C. C. (1997) *Cell. Mol. Life Sci.* **53,** 871–887
6. Booth, D. R., Sunde, M., Bellotti, V., Robinson, C. V., Hutchinson, W. L., Fraser, P. E., Hawkins, P. N., Dobson, C. M., Radford, S. E., Blake, C. C., and Pepys, M. B. (1997) *Nature* **385,** 787–793
7. Conway, K. A., Harper, J. D., and Lansbury, P. T. (1998) *Nat. Med.* **4,** 1318–1320
8. Gullans, S. R., and Verbalis, J. G. (1993) *Annu. Rev. Med.* **44,** 289–301
9. Nagelhus, E. A., Amiry-Moghaddam, M., Lehmann, A., and Ottersen, O. P. (1994) *Adv. Exp. Med. Biol.* **359,** 325–334
10. Yancey, P. H., Clark, M. E., Hand, S. C., Bowlus, R. D., and Somero, G. N. (1982) *Science* **217,** 1214–1222
11. Bolen, D. W., and Baskakov, I. V. (2001) *J. Mol. Biol.* **310,** 955–963
12. Leirmo, S., Harrison, C., Cayley, D. S., Burgess, R. R., and Record, M. T., Jr. (1987) *Biochemistry* **26,** 2095–2101
13. Roberts, M. F. (2000) *Front. Biosci.* **5,** 796–812
14. Kraegeloh, A., and Kunte, H. J. (2002) *Extremophiles* **6,** 453–462
15. Bhattacharya, A., Bhattacharyya, B., and Roy, S. (1994) *J. Biol. Chem.* **269,** 28655–28661
16. Bhattacharyya, T., Bhattacharyya, A., and Roy, S. (1991) *Eur. J. Biochem.* **200,** 739–745
17. Jana, N. K., Deb, S., Bhattacharyya, B., Mandal, N. C., and Roy, S. (2000) *Protein Eng.* **13,** 629–633
18. Das, B. K., Bhattacharyya, T., and Roy, S. (1995) *Biochemistry* **34,** 5242–5247
19. Zhang, J., and Matthews, C. R. (1998) *Biochemistry* **37,** 14881–14890
20. Ohgushi, M., and Wada, A. (1983) *FEBS Lett.* **164,** 21–24
21. Kuwajima, K. (1996) *FASEB J.* **10,** 102–109
22. Poklar, N., Lah, J., Salobir, M., Macek, P., and Vesnaver, G. (1997) *Biochemistry* **36,** 14345–14352
23. Ferrer, M., Barany, G., and Woodward, C. (1995) *Nat. Struct. Biol.* **2,** 211–217
24. Chatterjee, S., Ghosh, K., Dhar, A., and Roy, S. (2002) *Proteins Struct. Funct. Genet.* **49,** 554–559
25. Gazit, E., and Sauer, R. T. (1999) *J. Biol. Chem.* **274,** 16813–16818
26. Banik, U., Saha, R., Mandal, N. C., Bhattacharyya, B., and Roy, S. (1992) *Eur. J. Biochem.* **206,** 15–21
27. Lai, M. C., Sowers, K. R., Robertson, D. E., Roberts, M. F., and Gunsalus, R. P. (1991) *J. Bacteriol.* **173,** 5352–5358
28. Chirife, J., Favetto, G., and Ferro Fontan, C. (1984) *J. Appl. Bacteriol.* **56,** 259–268
29. Jensen, W. A., Armstrong, J. M., De Giorgio, J., and Hearn, M. T. (1995) *Biochemistry* **34,** 472–480
30. Lever, M., Randall, K., and Galinski, E. A. (2001) *Biochim. Biophys. Acta* **1528,** 135–140
31. Burg, M. B., Peters, E. M., Bohren, K. M., and Gabbay, K. H. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 6517–6522
32. Lin, T. Y., and Timasheff, S. N. (1994) *Biochemistry* **33,** 12695–12701
33. Myers, J. K., Pace, C. N., and Scholtz, J. M. (1995) *Protein Sci.* **4,** 2138–2148
34. Shortle, D., and Ackerman, M. S. (2001) *Science* **293,** 487–489
35. Shi, Z., Olson, C. A., Rose, G. D., Baldwin, R. L., and Kallenbach, N. R. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 9190–9195
36. Shortle, D. (1995) *Adv. Protein Chem.* **46,** 217–247
37. Wrabl, J., and Shortle, D. (1999) *Nat. Struct. Biol.* **6,** 876–883
38. Wang, A., and Bolen, D. W. (1997) *Biochemistry* **36,** 9101–9108
39. Zou, Q., Bennion, B. J., Daggett, V., and Murphy, K. P. (2002) *J. Am. Chem. Soc.* **124,** 1192–1202
40. Yancey, P. H., and Siebenaller, J. F. (1999) *J. Exp. Biol.* **202,** 3597–3603
41. Bychkova, V. E., and Ptitsyn, O. B. (1995) *FEBS Lett.* **359,** 6–8