Effect of Salt on the Urea-Unfolded Form of Barstar Probed by *m* Value Measurements†

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ABSTRACT: To probe for residual structure present in the urea-unfolded form of the small protein barstar, to determine how salt might modulate such structure, and to determine how such structure might affect the stability of the protein, mutant variants that display *m* values different from that of the wild-type protein have been studied. The mutant proteins were obtained by site-directed mutagenesis at residue positions located on the surface of the folded protein. The *m* value, which represents the preferential free energy of interaction of urea with the unfolded form in comparison to that with the folded state, was determined from equilibrium urea-induced unfolding curves. Mutant proteins for which the *m* values were significantly greater than (m^+ mutant forms), significantly smaller than (m^- mutant forms), or similar to (m^0 mutant forms) the *m* value determined for the wild-type protein were studied. The unfolded forms of the m^0, m^+ and m^- mutant proteins represent different components within the unfolded form ensemble, which differ from each other in their solvent-exposed surface areas. Hence, the *m* value has been used as a measure of residual structure in the unfolded form. To further understand the nature of structures present in the unfolded form ensemble, the effects of the salt KCl on the stabilities of the wild-type and the mutant proteins, as well as on the structures present in the unfolded form ensemble, were also studied. It was found that the *m* values of the m^0, m^+ and m^- mutant proteins all converge to the wild-type *m* value in the presence of KCl. This result indicates that the salt modulates residual structure in the unfolded form by screening electrostatic interactions that maintain compact and expanded components in the unfolded protein ensemble. The use of free energy cycles has allowed the effect of salt on the structure and free energy of the unfolded protein to be related to the stability of the protein.

To understand how a protein folds, it is important to understand the nature of the unfolded form from which folding commences. For a long time, the unfolded form of a protein under extreme denaturing conditions, such as in the presence of 8 M urea, was considered to be a random coil, to be expanded, highly solvated, and unstructured, and to lack any side-chain interactions (*1*, *2*). Recent data suggest, however, a different picture: the unfolded form not only comprises random coil-like states but also comprises compact states in which parts of the protein possess fluctuating structures that are not fully accessible to the solvent (*3*, *4*). Residual structures in unfolded forms of proteins have been detected by small-angle X-ray scattering studies (*5*, *6*) and, in a few cases, have been characterized at high resolution by nuclear magnetic resonance spectroscopy (*4*, *7*). Such structures usually appear to be either hydrophobic clusters (*8*−*11*) or fluctuating secondary structures (*12*, *13*) and may be stabilized by either native-like (*14*) or non-native interactions (*15*), which could be hydrophobic (*8*, *9*) or electrostatic (*16*, *17*) in origin. Experimental studies that indicate that unfolded proteins possess residual structures have been complimented by computational studies that indicate that unfolded forms are complex with size changing continuously with a change in denaturant condition (*18*), that the conformation of a residue in an unfolded protein is not always independent of neighboring residues as expected for true random coils (*19*), and that much backbone surface in unfolded proteins may be within local structure (*20*). Both experimental and computational studies suggest that the unfolded form is an ensemble with differently structured and unstructured components (*21*) and that different components may predominate under different conditions. Characterization of residual structure in the unfolded form is expected to provide insights on the mechanism of protein folding (*4*, *22*−*24*), especially in cases where multiple pathways are available for folding (*25*−*27*).

A practical measure of structure in the unfolded form of a protein is the *m* value obtained from an equilibrium unfolding study (*28*), which defines how the free energy of unfolding changes as a function of denaturant concentration. The *m* value has been correlated with the difference between accessible surface areas in the unfolded and folded states (*28*, *29*), and it is expected to be proportional directly to the change in accessible surface area upon unfolding; that is, $m \propto \Delta A$, where $\Delta A = A_U - A_N$ (*30*). The *m* value characterizing the unfolding transition of a protein can be altered by a change in the conditions of unfolding or by mutation. Such perturbations are unlikely to change the accessible surface area of the native state, A_N, and are more likely to change

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the accessible surface area of the unfolded form, A_U. Indeed, small changes in the *m* value have been accounted for by significant changes in the area exposed in the unfolded form (*31*). Thus, an increase or decrease in the *m* value is taken to reflect a change in the compactness and, hence, structure of the unfolded form (*21*, *28*).

In the case of m^+ mutant proteins, the *m* value exceeds the wild-type *m* value; for m^- mutant proteins, the *m* value is lower (*32*). The m^+ mutant proteins must reflect an increase in ΔA upon unfolding, which could result either from an increase in A_U or a decrease in A_N. Many m^+ mutant proteins are known to display greater than 30% increases in the *m* value, suggesting a shift in the distribution of the mutant unfolded form to less structured or more expanded components or both. Several wild-type proteins show increases in the *m* value at low pH, which have been explained on the basis of the residual structure in the unfolded form getting disrupted by electrostatic repulsions (*33*). On the other hand, m^- mutant proteins must reflect a decrease in ΔA upon unfolding, which can result either from a decrease in A_U or an increase in A_N. A decrease in A_U is the more likely explanation: even in the most pronounced m^- mutant form of staphylococcal nuclease, no significant changes in the accessible surface area in the native state (*34*) were seen in a high-resolution structure. The most conclusive evidence in support of *m* value effects being the consequence of changes in the unfolded form and not in the native state has come from a study of the hydrogen exchange kinetics of the of wild-type and mutant forms of staphylococcal nuclease (*35*). These studies have demonstrated clearly that the unfolded form of an m^+ mutant protein is more expanded and that the unfolded form of an m^- mutant protein is more compact than the wild-type unfolded form.

The small protein barstar has been used extensively as a model protein for protein folding studies (*25*−*27*, *36*−*38*). Very little is known, however, about the nature of its unfolded form. Although electrostatic repulsion can unfold barstar, either partially or completely, at extreme values of pH (*36*, *37*), the role of electrostatic interactions in determining residual structure of the unfolded form at neutral pH is not known. Salts do not alter the *m* value for the equilibrium unfolding of wild-type barstar (*38*). Thus, if residual structure stabilized by favorable electrostatic interactions or residual structure destabilized by unfavorable electrostatic interactions is present in the unfolded form ensemble, it must be populated so sparsely that its disruption by addition of salt has no measurable effect on the accessible surface area of the unfolded form. However, different salts do stabilize, differentially, different structural components of the early folding intermediate ensemble, I_E (*38*). It was therefore of considerable interest to identify the presence of electrostatically stabilized compact residual structure, as well as electrostatically destabilized expanded residual structure, in the unfolded ensemble, and to determine whether salt can stabilize, similarly, such structures. To perturb structurally the unfolded form of barstar to preferentially populate these otherwise sparsely present structures, residue positions corresponding to the surface of the folded protein were mutated. Figure 1 shows the positions of the mutated residues. The charged surface residues that were chosen for mutation did not appear to be involved in any stabilizing interactions in the native state; hence, mutating them was not expected to have any effect on the native-state structure. Since their side-chains are mobile and largely solvent-exposed (*39*), the positions occupied by these residues were expected to easily tolerate substitutions. Exposed charged residues of helices 2, 3, and 4 of barstar (*40*) were mutated (Figure 1, Table 1). Those in the barnase-binding site were not mutated because that would affect the activity of the protein. The strategy used here was that of either charge reversal or charge neutralization. In this way, a set of mutant forms of barstar were generated.

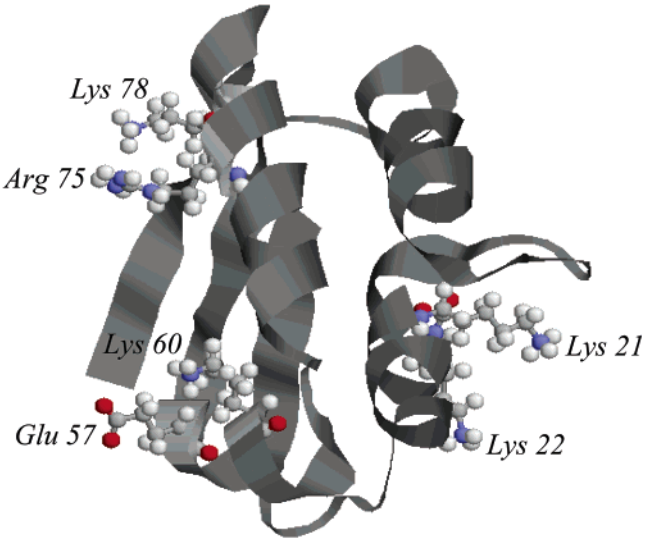

FIGURE 1: Ribbon diagram of the solution structure of wild-type barstar. The sites of the various mutations studied here are shown. The drawing was generated from the Protein Data Bank file 1btb (*40*) using Rasmol. The main chain and side chain atoms of all sites mutated in this study are shown as ball-and-stick drawings and each mutation site is labeled. In the native protein, the side chain of any of the six mutated residues is at least ~0.6 nm away from any other charged side chain. The carbonyl oxygen of the peptide bond and the oxygen atoms in the side chain (of glutamate) are in red; the amide nitrogen of the peptide bond and the nitrogen atoms in the side chains (of arginine and lysine) are in blue.

Table 1: Mutation Sites in Barstar and Their Structural Features[a]

mutation site	secondary structure	residue(s) mutated to	nonpolar side-chain accessibility (%)	polar side-chain accessibility (%)	total side-chain accessibility (%)
Lys21	helix 2	Ala and Gln	27.6	57.1	34.9
Lys22	helix 2	Gln	67	92.9	3.4
Glu57	helix 3	Lys	38.9	96	6.8
Lys60	helix 3	Glu and Leu	14.7	73.4	29.3
Arg75	helix 4	Leu and Gln	37	60.8	50.9
Lys78	helix 4	Ala and Gln	34.5	47	37.6

[a] Solvent accessibility was determined using the program Access (*39*) with the PDB file 1btb.

To determine whether these mutant proteins are m^+ or m^-, as well as to determine their stabilities, equilibrium urea-induced unfolding studies were carried out. The m^+, m^-, and m^0 mutant forms could be identified, indicating that the unfolded form of wild-type barstar contains both compact and expanded components that are normally too sparse to be detected, in addition to the predominant unfolded component. To determine whether these sparsely populated members of the unfolded ensemble are compact and expanded because of stabilizing electrostatic interactions and destabilizing electrostatic repulsions, respectively, the effects

of salt on *m* values as well as on stability were studied. In the presence of 0.5 M KCl, which screens all electrostatic interactions, the compact and expanded components are no longer detected: the *m* values of the m^+ and the m^- mutant proteins become the same as that of the wild-type protein, indicating that the accessible surface areas of all unfolded forms are identical. Upon a further increase in KCl concentration from 0.5 to 1 M, the only effect is the further strengthening of hydrophobic interactions. No further change in *m* values is observed for any of the mutant proteins, indicating that hydrophobic interactions have little role to play in stabilizing the structures apparent in the unfolded forms of the mutant proteins in the absence of salt. The effects of salt, at both concentrations, on the stabilities of the wild-type and mutant proteins can be rationalized in terms of the effects on the *m* values.

EXPERIMENTAL PROCEDURES

Bacterial Strain and Plasmid and Protein Purification. E. coli strain MM294 was used for protein expression. The expression plasmid for wild-type barstar was pMT316. The method used to purify barstar has been described in detail (*36*). Protein concentrations were calculated using an extinction coefficient of 23 000 M^{-1} cm^{-1}.

The mutant proteins were generated by site-directed mutagenesis (*41*). The mutant proteins K21A, K21Q, K22Q, K60E, K60L, R75L, R75Q, K78A, and K78Q were purified from the soluble fraction of the cell lysate (*36*). Only E57K had to be purified from inclusion bodies. The inclusion body pellet was washed twice with Triton-X 100 to remove membrane proteins and other soluble proteins, followed by solubilization in lysis buffer (50 mM Tris HCl, 1 mM EDTA, 100 mM NaCl, 0.1 mM PMSF (added fresh), 8 M urea (deionized), pH 8.5) After ultrafiltration, the solubilized inclusion bodies were subjected to gel exclusion chromatography using a G-75 Sephadex column. The appropriate fractions were collected and subjected to ion exchange chromatography using DEAE Sephadex. It was essential for the pH to be kept at 9.2 during every step; visible aggregates appeared below a pH of 9. The eluted fraction was subjected to ultrafiltration; ammoniated water at pH 9.2 was used for six cycles of ultrafiltration. After a 10^6 dilution was achieved, the solution was lyophilized.

Mass spectroscopy using a Micromass Q-TOF Ultima showed all proteins to be pure. The measured masses (10 300.33 Da for R75L, 10 315.01 Da for R75Q, 10 285.11 Da for K21A, 10 342.20 Da for K22Q, 10 342.24 Da for K21Q, 10 285.54 Da for K78A, 10 341.84 Da for E57K, 10 343.65 Da for K60E, 10 327.89 Da for K60L, and 10 342.73 Da for K78Q) were consistent with the N-terminal methionine residue remaining uncleaved during synthesis. Purity was also checked by gel electrophoresis (16% SDS PAGE), and all proteins showed >95% purity.

Buffers and Solutions. A mixture of 30 mM Tris-HCl (pH 8) (ultrapure, 99.9% from GibcoBRL), 250 μM EDTA (disodium salt, dihydrate, 99+% from SIGMA), and 250 μM DTT (ultrapure from GibcoBRL) constituted the native buffer used for all equilibrium and kinetic experiments. Native buffer containing 9−10 M urea (ultrapure, 99.9% from USB) constituted the unfolding buffer. The concentrations of stock solutions of urea were determined by measurement of the refractive index using an Abbe 3L refractometer from Milton Roy. For studies in the presence of 0.5 or 1.0 M KCl (minimum 99% from SIGMA), the salt was present in the refolding as well as unfolding buffer. All buffers and solutions were filtered through 0.45 μm filters before use.

Spectroscopic Characterization. Fluorescence spectra were collected on a SPEX DM 3000 spectrofluorimeter. The excitation wavelength was 280 nm, and the emission was monitored between 300 and 400 nm with a bandwidth of 0.37 nm for excitation and 10 nm for emission. Each spectrum was an average of three scans. The protein concentration was typically 2−4 μM, and the path length of the cuvette used was 1 cm.

Equilibrium Unfolding Studies. Protein stability at equilibrium was determined from urea-induced unfolding studies using fluorescence emission at 320 nm as the probe, as described above. Prior to fluorescence measurements, the samples were equilibrated for at least 4 h. Identical results were obtained if the time of incubation was 24 h.

Data Analysis. The change in free energy, ΔG_{UN}, that occurs upon unfolding of a protein in the presence of urea and salt can be expressed as a function of urea and salt concentration as follows:

$$d\Delta G_{UN} = \frac{\delta \Delta G_{UN}}{\delta[\text{urea}]} d[\text{urea}] + \frac{\delta \Delta G_{UN}}{\delta[\text{salt}]} d[\text{salt}] = m_{UN}\, d[\text{urea}] + m_S^{UN}\, d[\text{salt}] \quad (1)$$

m_{UN} or the *m* value $= (\delta \Delta G_{UN}/\delta[\text{urea}])_{salt}$ is the change in free energy associated with the preferential interaction of the denaturant with unfolded protein. $m_S^{UN} = (\delta \Delta G_{UN}/\delta[\text{salt}])_{urea}$ is the change in free energy associated with the preferential interaction of the salt with the folded protein.

It follows from eq 1, as the condition for an exact differential, that

$$\frac{\delta m_{UN}}{\delta[\text{salt}]} = \frac{\delta^2 \Delta G_{UN}}{\delta[\text{urea}]\delta[\text{salt}]} = \frac{\delta m_S^{UN}}{\delta[\text{urea}]} \quad (2)$$

Hence the dependence of m_{UN} on salt concentration is the same as that of m_S^{UN} on urea concentration. For the wt protein, as well as for m^0 mutant proteins at all salt concentrations, and for the m^+ and m^- mutant proteins at KCl concentrations greater than 0.5 M, this cross-derivative is found to be zero. For the wt protein, ΔG_{UN} has already been shown to be dependent linearly on both urea and salt concentration (*38*). On the other hand, the cross-derivative is found here to be positive for m^- mutant proteins and negative for m^+ mutant proteins at low salt concentration.

Since the free energy of unfolding of barstar is dependent linearly on urea concentration (*38*,*42*), the equilibrium data (in the absence and presence of KCl) for the unfolding of N as a function of urea concentration were fit to a two-state U ⇌ N model according to the equation:

$$Y_O = \frac{Y_N + m_N[\text{urea}] + (Y_U + m_U[\text{urea}])\, e^{-(\Delta G_{UN} + m_{UN}[\text{urea}])/(RT)}}{1 + e^{-(\Delta G_{UN} + m_{UN}[\text{urea}])/(RT)}} \quad (3)$$

where Y_O is the value of the spectroscopic property being

Table 2: Thermodynamic Parameters from Urea-Induced Equilibrium Unfolding Studies of Wild-Type and Mutant Forms of Barstar in the Presence of 0.5 and 1.0 M KCl[a]

	0 M KCl		0.5 M KCl		1.0 M KCl		$\Delta\Delta G_{UN}$	
protein	ΔG_{UN} (kcal mol^{-1})	m_{UN} (kcal mol^{-1} M^{-1})	ΔG_{UN} (kcal mol^{-1})	m_{UN} (kcal mol^{-1} M^{-1})	ΔG_{UN} (kcal mol^{-1})	m_{UN} (kcal mol^{-1} M^{-1})	$\Delta\Delta G_{UN}^{(0-0.5\ M)}$ (kcal mol^{-1})	$\Delta\Delta G_{UN}^{(0.5-1.0\ M)}$ (kcal mol^{-1})
wt	4.8	−1.16	5.8	−1.12	6.9	−1.19	1.0	1.1
R75L	5.5	−1.13	6.5	−1.13	7.6	−1.15	1.0	1.1
R75Q	4.5	−1.17	5.4	−1.15	6.5	−1.17	0.9	1.1
K78Q	5.2	−1.14	6.1	−1.14	7.2	−1.14	0.9	1.1
K21A	3.4	−1.02	5.0	−1.10	6.1	−1.11	1.6	1.1
K78A	4.4	−1.02	6.0	−1.18	7.0	−1.16	1.6	1.0
K21Q	4.1	−1.32	4.6	−1.18	5.7	−1.12	0.5	1.1
K22Q	4.3	−1.22	4.7	−1.12	5.8	−1.14	0.4	1.1
E57K	4.4	−1.25	4.9	−1.14	6.0	−1.16	0.5	1.1
K60E	4.2	−1.44	4.5	−1.19	5.5	−1.13	0.3	1.0
K60L	5.8	−1.30	6.2	−1.17	7.3	−1.16	0.4	1.1

[a] All data were obtained at 25 °C, pH 8.0 in 30 mM Tris-HCl, 250 μM EDTA, 250 μM DTT. The m^+ mutant proteins are shown in bold, and the m^- mutant proteins are shown in italics.

measured as a function of urea concentration, Y_N and Y_U represent the intercepts and m_N and m_U the slopes of the native protein and unfolded protein baselines, respectively. Thus, fits of denaturant-induced equilibrium unfolding data (in the absence and presence of KCl) to eq 3 yield values for ΔG_{UN} and m_{UN}.

Raw equilibrium unfolding data of N as a function of urea concentration were also analyzed in an alternative way (*42*). They were first converted to plots of fraction unfolded (f_U) versus urea concentration, using eq 4:

$$f_U = \frac{Y_O - (Y_N + m_N[\text{urea}])}{(Y_U + m_U[\text{urea}]) - (Y_N + m_N[\text{urea}])} \quad (4)$$

The f_U values were then fit to eq 5:

$$f_U = \frac{e^{-(\Delta G_{UN} + m_{UN}[\text{urea}])/(RT)}}{1 + e^{-(\Delta G_{UN} + m_{UN}[\text{urea}])/(RT)}} \quad (5)$$

In eq 5, f_U is related to ΔG_{UN} by a transformation of the Gibbs−Helmholtz equation in which the equilibrium constant for unfolding in the transition zone, K_{UN}, is given by $K_{UN} = f_U/(1 - f_U)$ for a two-state transition.

RESULTS

Urea-Induced Unfolding Studies of Barstar Variants. The urea-induced equilibrium unfolding of the barstar variants was monitored using fluorescence. Data were analyzed according to the two-state N ⇌ U model, which has been validated (*42*) for describing the unfolding of barstar (see Data Analysis in the Experimental Procedures section). The values determined for ΔG_{UN} and m_{UN} for wild-type barstar (Table 2) agree well with values reported earlier. The urea-induced unfolding of wild-type barstar in the absence of salt was repeated many times, and the standard deviation of the m value was determined. The average m value for wild-type barstar was −1.16 kcal mol^{-1} M^{-1}, and the standard deviation determined was 0.015 kcal mol^{-1} M^{-1}, or 1.3%. Barstar variants were classified based on m value deviations. Any deviation beyond 3 standard deviations of the wild-type m value was considered to be significant. Mutant proteins that had m values within ±3 standard deviations of the wild-type m value were classified as m^0, those with m values outside the ±3 standard deviation range were taken to be either m^- or m^+. Accordingly, mutant proteins with m values between −1.11 and −1.21 kcal mol^{-1} M^{-1} were classified as m^0, those with m values $\leq|-1.10|$ kcal mol^{-1} M^{-1} were classified as m^-, and those with m values $\geq|-1.22|$ kcal mol^{-1} M^{-1} were termed m^+. In the detailed study done on staphylococcal nuclease variants (*32*), the error in the determination of the m value was ±2%. In that study, those mutant proteins for which the m value exceeded the wild-type m value by a factor of 1.05 were designated as m^+ mutant proteins, those for which m value was lower than the wild-type m value by a factor of 0.95 were designated as m^-, and those with m values between 0.96 and 1.04 were labeled m^0 mutant proteins. It is evident that the criteria used here for the classification of the barstar variants as m^+, m^-, or m^0 are very similar to those used previously in the case of staphylococcal nuclease.

Table 2 shows the values of the thermodynamic parameters obtained for the variants of barstar from the two-state analysis. Mutant proteins R75L, R75Q, and K78Q have m values similar to that of wild-type barstar and belong to the m^0 class of mutant proteins. K21A and K78A show decreases in m values relative to the wild-type and fall in the m^- class, and K21Q, K22Q, E57K, K60E, and K60L show enhanced m values and belong to the m^+ class of mutant proteins. Force-fitting the urea-induced unfolding curves for the m^+ and m^- mutant proteins using the wild-type m value did not yield satisfactory fits. Figure 2 shows representative ΔG_{UN} versus urea concentration plots of m^+ and m^- mutant proteins. The slope of the linear fit yields the m value. Overall, the effect of a surface mutation on ΔG_{UN} ranges from −1.3 to +1.0 kcal mol^{-1}, which is of the magnitude expected for mutations on the surface of a protein (*43*).

Effect of KCl on m Values. Figure 3 shows the effect of salt on the m values of representative m^+ and m^- mutant proteins. The m^0 mutant proteins have a wild-type m value, which is not affected in the presence of salt, as seen in Figure 3a for a representative m^0 mutant protein, R75L. For m^+ mutant proteins, the slope of the unfolding transition (ΔG_{UN} vs urea concentration plot) is higher than that for the wild-type protein in the absence of any salt and becomes wild-type-like in the presence of 0.5 M KCl, as shown in Figure 3b for a representative m^+ mutant protein, K60E. Similarly, the slope of the urea-induced unfolding transition (ΔG_{UN} vs urea concentration plot) is less for m^- mutant proteins than

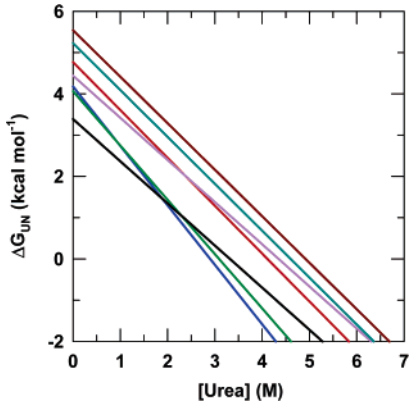

FIGURE 2: Dependence of stability on urea concentration. Linear fits of ΔG_{UN} versus urea concentration for some representative m^+, m^-, and m^0 mutant proteins, as well as wild-type barstar, are shown. The representative proteins are as follows: m^0 mutant proteins, wild-type (red), R75L (brown), and K78Q (cyan); m^+ mutant proteins, K60E (blue) and K21Q (green); m^- mutant proteins, K21A (black) and K78A (purple). The slope of the linear fit in each case gives the *m* value for the urea-induced unfolding of the respective mutant protein, and the *y*-intercept yields ΔG_{UN}.

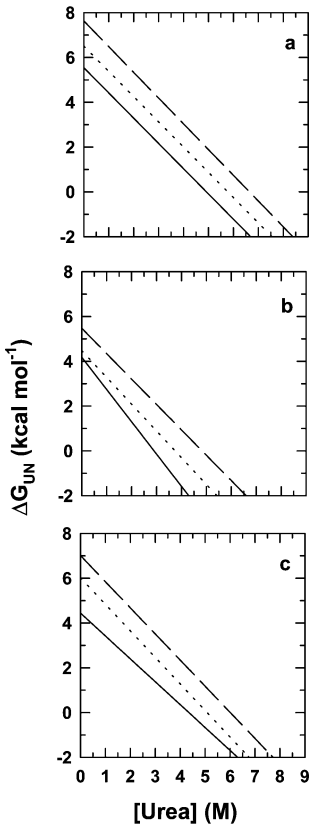

FIGURE 3: Salt effects on *m* values. Linear fits of ΔG_{UN} versus urea concentration for representative m^0, m^+, and m^- mutant proteins in the absence of KCl (—), presence of 0.5 M KCl (···), and presence of 1.0 M KCl (− − −) are shown: (a) m^0 mutant protein R75L; (b) m^+ mutant protein K60E; (c) m^- mutant protein K78A.

for the wild-type protein and converges to the wild-type *m* value in the presence of 0.5 M KCl, as is shown for the representative m^- mutant protein, K78A, in Figure 3c. Table 2 shows the thermodynamic parameters for urea-induced unfolding of all the mutant proteins in the absence of salt and in the presence of 0.5 and 1.0 M KCl. The value of $\delta m_{UN}/\delta$[salt] (eq 2) is negative for the m^+ mutant proteins, positive for the m^- mutant proteins, and zero for the m^0 mutant proteins, when the KCl concentration is <0.5 M. Thus, the *m* values of the m^+ and m^- mutant proteins converge to the *m* value of m^0 mutant proteins upon addition of 0.5 M KCl. At higher KCl concentrations (0.5−1 M), the value of $\delta m_{UN}/\delta$[salt] (eq 2) appears to be zero for all mutant proteins. Thus, upon further addition of KCl, at 1 M concentration, no further changes are seen for the *m* values for any of the mutant proteins: in 1 M KCl, the *m* values for all the mutant proteins are like those of the wild-type protein. Except in the case of K21A, the effect of KCl on the *m* value is seen in 0.5 M KCl itself.

Effect of KCl on ΔG *Values.* Upon increase of the KCl concentration from 0 to 0.5 M, the difference in free energy of unfolding, $\Delta\Delta G_{UN}^{(0-0.5M)}$, for wild-type barstar is approximately 1.0 kcal mol^{-1}, which is the same as that seen for all m^0 mutant proteins (Table 2). $\Delta\Delta G_{UN}^{(0-0.5M)}$ for m^+ mutant proteins is ≤ 0.5 kcal mol^{-1} and for m^- mutant proteins is ≥ 1.6 kcal mol^{-1} (Table 2). Upon increase of the KCl concentration from 0.5 to 1.0 M, the difference in free energy of unfolding, $\Delta\Delta G_{UN}^{(0.5-1.0M)}$, for all the mutant proteins is essentially the same and is wild-type-like, approximately 1.1 kcal mol^{-1} (Table 2). Upon increase of the KCl concentration from 0.5 to 1 M, the primary effect is stabilization of the native state of the mutant and wild-type proteins, which occurs to the same extent, due to preferential hydration of the protein, and there seems to be no further effect on the compactness of their unfolded states.

Effect of KCl on the Spectral Properties of the Native and Unfolded Forms of the Mutant Proteins. The wavelength of maximum fluorescence emission, λ_{max}, of the native state as well as of the unfolded form of the wild-type protein and of the mutant proteins in the absence of KCl and in the presence of 0.5 and 1.0 M KCl was determined. There was essentially no difference in the λ_{max}, either for the N state or for the U form, for the different mutant proteins. Similarly, there was no difference in the fluorescence intensity, either for the N state or for the U form. Moreover, these parameters did not change upon addition of salt (data not shown).

DISCUSSION

Point mutations in a protein can cause unpredictable changes to overall stability and very often cause unpredictable changes in apparent *m* value (*44*). Pronounced *m* value effects were reported first for mutant forms of staphylococcal nuclease (*32*), and such effects have also been studied in the case of T4 lysozyme (*45*), dihydrofolate reductase (*46*), T1 RNase (*47*), gene V protein of f1 phage (*48*), apomyoglobin (*49*), calbindin (*50*), cytochrome *c* (*51*), and the amino terminal fragment of λ repressor (*52*). The one consistent trend that has emerged from these data is that there is almost always a decrease in the *m* value when a protein is constrained by covalent cross-links, suggesting that the unfolded forms of those mutant proteins are more compact than the unfolded form of the corresponding wild-type proteins (*28*). While the effects of mutations on stability are explained generally in terms of changes in the native state interactions, their effects on *m* values can only be explained on the basis of major changes in the physical interactions stabilizing the unfolded form. So far, it has usually been difficult to rationalize changes in *m* values with changes in

stability. In this study, *m* value effects seen upon mutation of barstar have been correlated with changes in the surface area exposed in the unfolded form and also with changes observed in protein stability.

Effects of Mutations on Stability and the m Value. Mutations at exposed sites on the protein surface do not usually affect the stability and function of the protein. Thus, for example, residues on the surface of T4 lysozyme (*53*) could be replaced routinely with very little change in stability and structure. Similarly, mutations involving the solvent-exposed residues of λ repressor did not show any measurable changes in T_m (*54*), and over 100 multiple substitutions within an eight-residue segment of λ repressor retained repressor activity (*55*). For a few proteins, surface mutations have been observed to increase stability (*56*−*59*), but drastic increases in stability have been observed only in very few cases (*60*, *61*). Table 2 indicates that the surface mutations of barstar that are reported here do not have major effects on stability: the changes in the free energy of unfolding depend on the site of the mutation and range from −1.3 to 1.0 kcal mol^{-1} in the absence of salt. As in the case of other proteins, the effects of surface mutations on the stability of barstar are difficult to rationalize because it is not possible to predict whether the mutations also perturb interactions that stabilize the unfolded form, in addition to perturbing interactions that stabilize the native state. For the same reason, it is difficult to rationalize the effects of the mutations on *m* values. In this study, the purpose has been to correlate the effects of the mutations on the stability and on the *m* value to use these effects to identify and describe compact and expanded components in the unfolded form ensemble.

Effect of Salt on Structure in the Unfolded Form and on Stability. Salts can affect the stabilities, solubilities, and biological activities of proteins (*62*). They are known to affect native states of proteins (*62*, *63*), molten globule forms (*64*), equilibrium intermediates (*65*), and kinetic intermediates on folding and unfolding pathways (*38*, *66*, *67*). At low concentrations, salts stabilize proteins through nonspecific electrostatic interactions, which depend only on the ionic strength of the medium (*68*). These nonspecific interactions are brought about by either Debye screening of electrostatic interactions in the protein (*69*) or weak binding of the ions to the protein (*70*). Debye theory cannot account for the large differences in salting-out and salting-in effectiveness of different salts at high concentrations. At high concentrations, salts exert a specific effect on proteins, referred to as the Hofmeister effect (*71*), which arises from an increase in the strength of hydrophobic interactions on account of weak interactions of the protein with the salt (*72*). The surface tension of water is increased due to a change in its hydrogen bonding properties (*73*), and the Hofmeister effect is specific in that it depends not only on the concentration but also on the nature of the salt. The effect of salt on the structure of the unfolded form, as reflected in how it changes the *m* value, as well as on the stability of barstar can be explained by invoking Debye screening of electrostatic interactions at low salt concentrations and by invoking the Hofmeister effect at high salt concentration.

To bring out the correlation between *m* value changes and changes in stability upon addition of KCl, free energy cycles were constructed as shown in Figure 4. Since the mutations are not expected to have any significant effect on the exposed

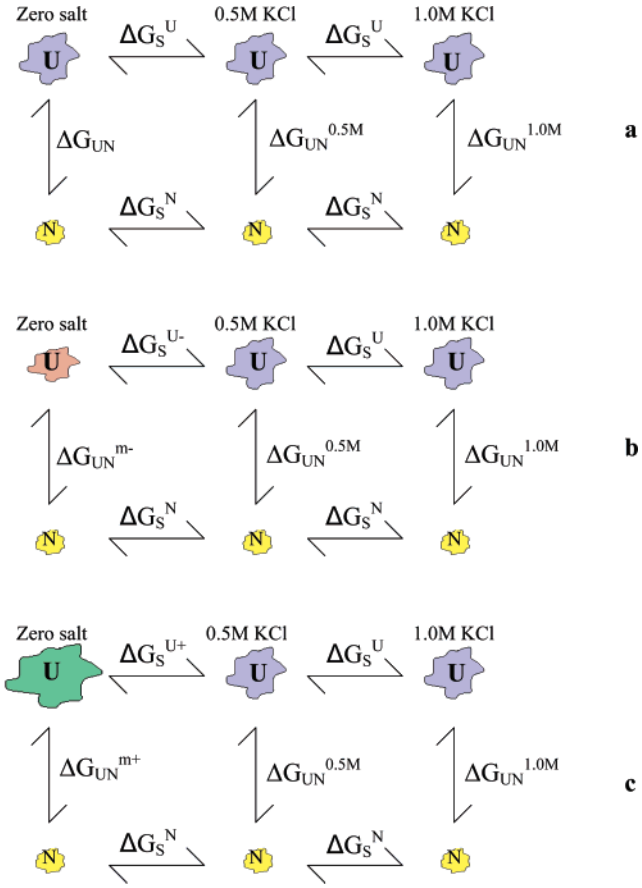

FIGURE 4: Free energy cycles correlating the effect of salt on stability for (a) m^0 mutant proteins, (b) m^- mutant proteins, and (c) m^+ mutant proteins. The free energy of unfolding of m^0, m^-, and m^+ mutant proteins is given by ΔG_{UN}, $\Delta G_{UN}^{m^-}$, and $\Delta G_{UN}^{m^+}$, respectively, in the absence of salt. $\Delta G_{UN}^{0.5M}$ and $\Delta G_{UN}^{1.0M}$ represent the free energy of unfolding in the presence of 0.5 and 1.0 M KCl, respectively, for all the three classes of mutant proteins, and their values vary from one protein to the next. ΔG_S^N is the free energy of transfer of the N state from 0 to 0.5 M KCl or from 0.5 to 1.0 M KCl, and is assumed to have the same value for the m^0, m^-, and m^+ mutant proteins (see Discussion). ΔG_S^U is the free energy of transfer of the U form from 0.5 to 1.0 M KCl, and it is assumed to have the same value for the m^0, m^-, and m^+ mutant proteins (see Discussion). It is also the free energy of transfer of the U form of an m^0 mutant protein from 0 to 0.5 M KCl. $\Delta G_S^{U^-}$ is the free energy of transfer of the U form of an m^- mutant protein from 0 to 0.5 M KCl, and $\Delta G_S^{U^+}$ is the free energy of transfer of the U form of an m^+ mutant protein from 0 to 0.5 M KCl.

surface area of the native state (see above) because there is no evidence for any specific ion binding sites on the surface of folded barstar (*38*) and because there is no evidence that the surface area of the N state changes upon addition of salt, for any of the mutant proteins, it has been assumed that (1) the free energy of transfer of the native state of any mutant protein from 0 to 0.5 M KCl has the same value as the free energy of transfer from 0.5 to 1.0 M KCl and (2) this transfer free energy (ΔG_S^N) has the same value for all proteins. Since the exposed surface area of the unfolded form of any m^0 protein is not affected by the presence of KCl, as reflected in the *m* value being essentially independent of KCl concentration (Table 2), and because there is no evidence for specific ion binding sites on the unfolded form, the value of the free energy of transfer of the unfolded form of an m^0 protein from 0 to 0.5 M KCl is expected to be the same as

that for transfer from 0.5 to 1.0 M KCl. It is also expected to be the same as the value of the free energy of transfer of the unfolded form of an m^+ or m^- protein from 0.5 to 1 M KCl because the m values of the m^+ and m^- mutant proteins are the same in 0.5 and 1 M KCl and are the same as the m values of the m^0 proteins. Since the exposed surface area of the unfolded form is altered in the m^+ and m^- mutant proteins when no salt is present, the value of the free energy of transfer of the unfolded form of an m^+ and m^- mutant protein from 0 to 0.5 M KCl is expected to depend on the class of mutation, whether m^+ or m^-. The values of ΔG_{UN}, $\Delta G_{UN}^{0.5M}$, and $\Delta G_{UN}^{1.0M}$ are different from one protein to another, as expected, (Table 2), and the free energy cycles highlight the reason the values of $\Delta\Delta G_{UN}^{(0.5-1.0M)}$ are virtually identical (1−1.1 kcal mol^{-1}) for all proteins and they are nearly identical to the values (∼1 kcal mol^{-1}) of $\Delta\Delta G_{UN}^{(0-0.5M)}$ for the m^0 mutant proteins.

m^0 Mutant Proteins. Upon addition of 0.5 M KCl, the increase in the free energy change, $\Delta\Delta G_{UN}^{(0-0.5M)}$, of all the m^0 mutant proteins is similar in value (0.9−1 kcal mol^{-1}) to the $\Delta\Delta G_{UN}^{(0-0.5M)}$ of the wild-type protein (Table 2). The same holds good when the KCl concentration is increased from 0.5 to 1.0 M: $\Delta\Delta G_{UN}^{(0.5-1.0M)} \approx 1.1$ kcal mol^{-1}. From the free energy cycle in Figure 4a, the following relationships emerge for the m^0 mutant forms:

$$\Delta G_{UN}^{0.5M} - \Delta G_{UN} = \Delta G_S^N - \Delta G_S^U \approx 1 \text{ kcal mol}^{-1} \quad \text{(i)}$$

$\Delta G_S^N - \Delta G_S^U$ represents the stabilization of the N state by preferential hydration. Also,

$$\Delta G_{UN}^{1.0M} - \Delta G_{UN}^{0.5M} = \Delta G_S^N - \Delta G_S^U \approx 1.1 \text{ kcal mol}^{-1} \quad \text{(ii)}$$

This value is nearly identical to that of $\Delta G_{UN}^{0.5M} - \Delta G_{UN}$, which is expected because the free energy of unfolding, ΔG_{UN}, has a linear dependence on the concentration of KCl (*38*).

m^- Mutant Proteins. For the m^- mutant proteins, the value of $\Delta\Delta G_{UN}^{(0-0.5M)}$ is ∼1.6 kcal mol^{-1}, which is greater than the value of $\Delta\Delta G_{UN}^{(0-0.5M)}$ of the wild-type or m^0 mutant proteins (Table 2). Upon an increase in the KCl concentration from 0.5 to 1.0 M, the free energy change, $\Delta\Delta G_{UN}^{(0.5-1.0M)} \approx$ 1.1 kcal mol^{-1}, is similar to that seen for the wild-type or the m^0 mutant proteins, even when the individual values of $\Delta G_{UN}^{0.5M}$ and $\Delta G_{UN}^{1.0M}$ vary so much from one protein to another (Table 2). In Figure 4b, the following relationships emerge from the free energy cycle for the m^- mutant proteins:

$$\Delta G_{UN}^{0.5M} - \Delta G_{UN}^{m^-} = \Delta G_S^N - \Delta G_S^{U^-} \approx 1.6 \text{ kcal mol}^{-1} \quad \text{(iii)}$$

$$\Delta G_{UN}^{1.0M} - \Delta G_{UN}^{0.5M} = \Delta G_S^N - \Delta G_S^U \approx 1.0 \text{ kcal mol}^{-1} \quad \text{(iv)}$$

Thus,

$$\Delta G_S^U - \Delta G_S{}^{U^-} \approx 0.6 \text{ kcal mol}^{-1}$$

Thus, the unfolded form of any one of the m^- mutant proteins studied here is stabilized relative to the unfolded form of the wild-type or m^0 protein by ∼0.6 kcal mol^{-1} on the average. The free energy of stabilization of the unfolded form by salt of an m^- mutant protein is lower than that of unfolded form of an m^0 mutant protein: a stabilizing interaction present in the unfolded form of the m^- mutant protein is responsible for the relative compactness seen (from the m value) in the structure of the unfolded form. It is likely that the favorable contribution to the free energy of the unfolded form arises from an attractive long-range electrostatic interaction that brings different segments of the chain together in the unfolded form of the m^- mutant protein and makes it more compact than the unfolded form of the wild-type protein. In 0.5 M KCl, the electrostatic interaction is screened fully by the mobile charges, and the unfolded form of the m^- mutant protein expands to the size of the unfolded form of the wild-type protein, as seen from the change in the m value. Once the unfolded form of the m^- mutant protein has expanded to the size of the unfolded form of the m^0 protein, further stabilization of the m^- mutant protein upon increasing KCl concentration from 0.5 to 1 M occurs by preferential hydration and is the same as that of an m^0 protein, as expected.

m^+ Mutant Proteins. Upon addition of 0.5 M KCl, the values of $\Delta\Delta G_{UN}^{(0-0.5M)}$ for the m^+ mutant proteins fall in the range of 0.3−0.5 kcal mol^{-1} and are less than the values of $\Delta\Delta G_{UN}^{(0-0.5M)}$ for the wild-type or m^0 mutant proteins, by 0.5−0.7 kcal mol^{-1} (Table 2). Upon an increase in the KCl concentration from 0.5 to 1.0 M, the free energy change, $\Delta\Delta G_{UN}^{(0.5-1.0M)}$, is similar to that seen for the wild-type protein or the m^0 mutant proteins (1.0−1.1 kcal mol^{-1}). It is remarkable that these values of $\Delta\Delta G_{UN}^{(0.5-1.0M)}$ are so similar for the m^+ and m^0 proteins when the individual values of $\Delta G_{UN}^{0.5M}$ and $\Delta G_{UN}^{1.0M}$ vary so much from one protein to another (Table 2). From the free energy cycle shown in Figure 4c, the following relationships emerge for the m^+ mutant forms:

$$\Delta G_{UN}^{0.5M} - \Delta G_{UN}^{m^+} = \Delta G_S^N - \Delta G_S^{U^+} \approx 0.5 \text{ kcal mol}^{-1} \quad \text{(v)}$$

$$\Delta G_{UN}^{1.0M} - \Delta G_{UN}^{0.5M} = \Delta G_S^N - \Delta G_S^U \approx 1.0 \text{ kcal mol}^{-1} \quad \text{(vi)}$$

Thus,

$$\Delta G_S^U - \Delta G_S^{U^+} \approx -0.5 \text{ kcal mol}^{-1}$$

Thus, the unfolded form of any one of the m^+ mutant proteins studied here is destabilized relative to the unfolded form of the wild-type or m^0 protein by ∼0.5 kcal mol^{-1}. The free energy of the stabilization of the unfolded form of an m^+ mutant protein is higher than that of the unfolded form of an m^0 mutant protein because the unfolded form of the former is destabilized compared to the latter, probably because of the presence of a repulsive long-range electrostatic interaction. This destabilizing electrostatic interaction is screened in 0.5 M KCl. The m value measurements show that the unfolded form of an m^+ mutant protein in the absence of salt has greater exposure of nonpolar surface than does the unfolded form of an m^0 mutant protein but that upon addition of 0.5 M KCl, the degree of exposure of nonpolar surface becomes similar. Thus, upon addition of salt, the unfolded form of an m^+ protein contracts to the size of the unfolded form of an m^0 protein, and the stabilization of an m^+ mutant protein by preferential hydration is the same as that of the unfolded form of an m^0 protein.

The effect of 0.5 M KCl on the energetics of unfolding of the various mutant proteins can therefore be explained on the basis of electrostatic interactions in the unfolded form of an m^+ or m^- mutant protein. Upon addition of 0.5 M KCl, these electrostatic interactions are screened by the mobile charges. Since the unfolded forms of the m^+ and m^- mutant proteins differ from that of the wild-type protein in terms of their exposed hydrophobic surface area and, hence, the extent of compaction and this relative compaction or expansion no longer exists in the presence of 0.5 M KCl, it appears that 0.5 M KCl affects primarily electrostatic interactions and not hydrophobic interactions in the unfolded form. Thus, for m^+ mutant proteins, in the presence of 0.5 M KCl, the probable long-range electrostatic repulsion that caused their unfolded forms to expand relative to the unfolded forms of the wild-type protein is screened. Consequently, the unfolded form becomes wild-type-like in terms of compaction, as reflected in wild-type *m* values in 0.5 M KCl (Table 2, Figure 3b). Conversely, for the m^- mutant proteins, the probable long-range electrostatic attraction that caused their unfolded forms to become compact relative to the wild-type unfolded form is screened, such that their unfolded forms are wild-type-like in terms of surface area, as reflected in a wild-type *m* value in 0.5 M KCl (Table 2, Figure 3c).

While some of the mutations that lead to changes in *m* values are charge-reversal mutations, most are charge neutralization mutations. It is not surprising that the physical interactions responsible for the unfolded form of an m^+ mutant protein to be destabilized or for the unfolded form of an m^- mutant protein to be stabilized with respect to the unfolded form of an m^0 mutant protein are electrostatic in nature. In studies on barnase, electrostatic interactions in the unfolded form of barnase were identified and characterized extensively by studying their effects on the pK_a values of titratable groups (*16*, *17*). The results reported here suggest that complex nonnative interactions occur between residues in the unfolded form and perturbation of one interaction can have unforeseen consequences on other interactions. It is this complexity that is likely to be responsible for the apparent absence of any correlation between the type of charge change and the change in the *m* value. Another example of the complexity of interactions in the unfolded form of a protein is the network of nonnative interactions of a single tryptophan residue, which appears to stabilize a native-like core in the unfolded form of lysozyme (*15*).

CONCLUSION

In this study, *m* value measurements have been used as a measure of structure in the unfolded form of barstar. The unfolded forms of m^0, m^+, and m^- mutant proteins differ in compactness and, hence, structure. It appears that salt affects structure in the unfolded form ensemble by affecting electrostatic interactions and not hydrophobic interactions. In the case of m^+ mutant proteins, salt screens electrostatic repulsions in the unfolded form, making it more compact (m^0-like). Similarly, salt appears to exert its effect on the unfolded form of an m^- mutant protein by screening attractive electrostatic interactions, thereby making it less compact. In 0.5 M KCl, the unfolded forms of all proteins are similarly compact because all electrostatic interactions have been fully screened. Hence, no further compaction of the unfolded form occurs in 1 M KCl for any of the mutant proteins. The increase in stabilization upon increasing the KCl concentration from 0.5 to 1.0 M is accounted for by the Hofmeister effect.

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