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Effects of Surface Charge on Denaturation of Bovine Carbonic Anhydrase

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This work compares the denaturation of two proteins—bovine carbonic anhydrase II (BCA) and its derivative with all lysine groups acetylated (BCA-Ac_{18})—by urea, guanidinium chloride (GuHCl), heat, and sodium dodecyl sulfate (SDS). It demonstrates that increasing the net negative charge of the protein by acetylation of lysines reduces its stability to urea, GuHCl, and heat, but increases its kinetic stability (its thermodynamic stability cannot be measured) towards denaturation by SDS. Increasing the ionic strength of the buffer improves the stability of BCA-Ac_{18} to urea and heat, but still leaves it less stable than unacetylated BCA to those denaturants. In urea, the large change in electrostatic interactions not only modifies the free energy of denaturation, but also introduces a stable intermediate into the unfolding pathway. This work shows that modifications of charges on the surfaces of proteins can have a large effect—positive or negative, depending on the denaturant—on the stability of the proteins despite the exposure of these charges to high dielectric solvent and buffer ions.

Introduction

Do electrostatic interactions on the surface of a protein stabilize or destabilize it to unfolding? Often, changing electrostatic interactions on the surfaces of proteins (by site-directed mutagenesis or by chemical modifications) results in minimal changes in stability of those proteins;[1,2] this insensitivity is usually rationalized by using the argument that surface charges on proteins are highly shielded by water and by buffer ions.[3] Occasionally, however, individual charged amino acids can contribute several kcal mol^{-1} to the stability of proteins through specific electrostatic interactions;[4–6] the surrounding solvent does not screen those interactions.

Site-directed mutagenesis is the tool most often used to probe the influence of electrostatic interactions on the stability of proteins.[2,7,8] Site-directed mutagenesis conveniently introduces one or two changes to the charged groups in a protein. The difference in stability between the mutant and the wild-type mainly reflects the contribution of the electrostatic interaction between the residue of interest and other charged groups to the free energy of unfolding. It is usually impractical to use this approach to make large changes in the number of charged groups on the protein (and thus in net charge), since multiple changes require multiple rounds of mutagenesis.

Modified proteins with large changes in net charge sometimes do show interesting biological behavior. For example, erythropoietin with all of its lysine groups chemically modified by carbamylation exhibited only neuroprotective activity, without unwanted erythropoeitic activity.[9] A derivative of TNF-α with all of its lysines removed by mutagenesis remained biologically active; this derivative was used to study the effects of site-specific PEGylation of the protein on the bioactivity of TNF-α.[10] The derivative, lacking all reactive amino groups except the N terminus, and thus specifically PEGylated only at the N terminus, showed greater antitumor potency than the randomly PEGylated wild-type.

Several groups have investigated the effects of large changes in the net charge of proteins on the stability of proteins using chemical modifications. Hollecker and Creighton modified the lysine residues of several proteins—cytochrome *c*, ribonuclease, and β-lactoglobulin—using succinic anhydride; this modification reversed the charge on the lysine residues from positive to negative.[11] The authors used urea-gradient electrophoresis to probe the effects of these modifications on stability of the proteins and found that, in general, the effect of charge reversal was small. All of the amino groups of ribonuclease, 18 out 19 amino groups of cytochrome *c*, and up to 10 amino groups of β-lactoglobulin could be modified with marginal changes in stability, but the low resolution of urea-gradient electrophoresis did not allow for more quantitative measurements of stability. Loladze and Makhatadze tested the effects of electrostatic interactions in ubiquitin by first replacing the arginine residues with lysine residues by mutagenesis, and then carbamylating all lysine residues.[12] They measured the stability towards urea of the starting and carbamylated derivatives in a buffer of pH 2 in which all carboxylic acid residues are protonated. The authors found that the carbamylated derivative (with a net charge of ~0 at pH 2) was more stable to urea than the starting ubiquitin (with positive net charge at pH 2) and concluded that surface charges are not required for the stability and folding of ubiquitin. Hagihara et al. used circular dichroism to compare the stabilities of horse cytochrome *c* and its acetylated derivatives to urea, heat, and guanidinium

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chloride (GuHCl).[13] The authors found that acetylation, while reducing the net positive charge of cytochrome *c*, decreased its stability to urea and heat, but did not have an effect with GuHCl. The authors concluded that local electrostatic interactions, rather than the net charge of the protein, influence the stability of cytochrome *c*, and that these interactions are screened in ionic guanidinium chloride.

We were interested in exploring the effects of *large* changes to the network of electrostatic interactions on the surface of a protein on its stability to common denaturants (heat, GuHCl, urea) and to a denaturing surfactant—sodium dodecyl sulfate (SDS). We also wished to explore the effect of screening these interactions by high concentrations of salt in the buffer. We chose to conduct the study on carbonic anhydrase—a protein commonly used as a model for biophysical and physical-organic studies by us[14–16] and others.[17,18]

Experimental design

We have developed acylation of the lysine ε-NH_3^+ groups of proteins as a convenient procedure with which to make large changes in charge.[19] We have studied the behavior of bovine carbonic anhydrase II (BCA) and its peracetylated derivative (BCA-Ac_{18}) in detail.[15] At room temperature, both BCA and BCA-Ac_{18} are stable and similar in tertiary structure. Both have similar affinity towards sulfonamide inhibitors, similar activity in catalytic hydrolysis of *p*-nitrophenylacetate, and thus, we infer, similar structures of the active site.[15] These properties, and the extensive literature characterizing denaturation of different isozymes of carbonic anhydrases,[20–24] make BCA and BCA-Ac_{18} a convenient model system with which to study the effects of large changes in the surface charges in proteins on the properties of those proteins. Here, we compare the stability of these two proteins to GuHCl, urea, and heat (under conditions of low and high ionic strength in the case of the last two denaturants). We have previously described studies of the denaturation of BCA and BCA-Ac_{18} with SDS.[15,25] In these studies, we found that BCA-Ac_{18} was kinetically more stable to denaturation by SDS than BCA.

Unfolding and refolding of BCA

Many groups have used carbonic anhydrase—both bovine and human—as a model protein for studies of folding and unfolding. Henkens et al.[26] studied equilibrium unfolding of BCA in GuHCl using circular dichroism and fluorescence spectroscopy, and found that BCA undergoes a three-state (native, intermediate, and unfolded) denaturation in GuHCl that proceeds through a stable intermediate conformation. The first transition from the native to the intermediate state, detected by binding of an inhibitor dansyl amide (DNSA), corresponded to a change in the near-UV circular dichroism (CD) signal. DNSA is an inhibitor of carbonic anhydrase that is strongly fluorescent when bound in the active site (quantum yield $\phi = 0.84$, $\lambda_{em} =$ 460 nm), but negligibly fluorescent ($\phi = 0.055$, $\lambda_{em} = 580$ nm) when free in solution.[27] The near-UV CD signal is a measure of the tertiary structure of a protein.[28] Thus, the similarity in the concentration of GuHCl at which the DNSA fluorescence and near-UV CD signal decreased suggested that the loss of the structure of the active site and the loss of the overall tertiary structure occurred simultaneously. Further characterization by CD measurements showed that the intermediate possessed most of the secondary structure of the native state and was more compact than a fully denatured protein. These characteristics describe a particular type of an intermediate—a molten globule.[29] In a study of folding of BCA, Dolgikh et al. observed the kinetic equivalent of a molten globule.[30] In contrast to denaturation with GuHCl, no one has observed intermediates during the denaturation of BCA with urea.[22,31]

Assays

We used intrinsic fluorescence of the tryptophan residues, as a probe that is sensitive to the conformational state of the protein, to monitor denaturation by GuHCl and urea. Mårtensson et al.[24] carefully examined the contributions of individual Trp residues to the total fluorescence of human carbonic anhydrase II (HCA). They showed that, upon denaturation with GuHCl and exposure to solvent, the emission of Trp residues of HCA red-shifts and decreases in intensity; these changes in spectra are reflected in the ratio of fluorescence intensities at 332 and 352 nm. Using this ratio, Mårtensson et al.[24] showed that denaturation of HCA with GuHCl proceeds through a stable intermediate. The first transition corresponded to a transition also detected in enzymatic activity. The second transition corresponded to a transition observed with absorbance at 292 nm.[17]

HCA has seven Trp residues, six of which are conserved in the cytosolic isoenzymes of mammalian species;[24] all seven Trp residues are conserved between HCA and BCA. Given the similarity in sequence and structure between HCA and BCA, intrinsic fluorescence should be a sensitive probe of the conformational states of BCA and BCA-Ac_{18} as well. In addition, Bushmarina et al.[22] used fluorescence intensities and lifetimes to study the denaturation of BCA in GuHCl and also observed three-state denaturation.

Figure 1 A shows BCA in a representation with a transparent molecular surface. The lysine (blue) residues are solvent-accessible, while tryptophans (green) are mostly buried. Figure 1 B shows the shift in the Trp emission upon denaturation with urea for BCA and BCA-Ac_{18}. Small differences in the emission spectra between the native BCA and BCA-Ac_{18} might result from changes to the environment of the tryptophans caused by acetylation of lysines that are nearby. The differences in emission spectra between the denatured states of BCA and BCA-Ac_{18} could indicate differences in the structure of the denatured state and the solvent accessibility of the tryptophans.

In agreement with Mårtensson et al.,[24] we found that the ratio of fluorescence intensities at 332 and 352 nm (I_{332}/I_{352}), with an excitation wavelength of 295 nm, depended on the state (native, intermediate, or denatured) of BCA and BCA-Ac_{18}. This relation allowed us to follow the equilibrium denaturation of these proteins in urea and GuHCl. We supplemented the intrinsic fluorescence experiments with studies of the fluores-

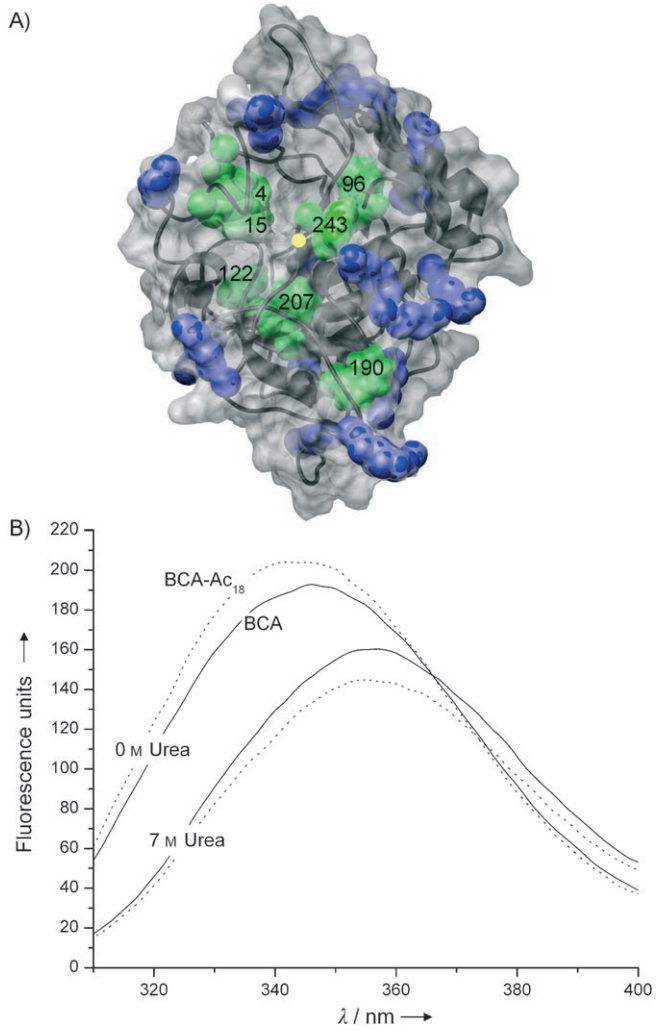

Figure 1. A) Molecular surface of BCA (PDB ID: 1V9E), shown transparently, with lysine residues in blue, tryptophan residues in green, and the Zn^{2+} ion in the active site in yellow. Denaturation exposes the buried tryptophans to the solvent and changes their fluorescent properties. B) Fluorescence spectra of BCA (——) and BCA-Ac_{18} (····) in the native and denatured states in 10 mM phosphate buffer, pH 8. The excitation wavelength is 295 nm, and the protein concentration is 1 μM.

cence of dansyl amide and circular dichroism (CD). The decrease in fluorescence of DNSA with increasing concentration of denaturant indicates loss of the structure of the active site and, we presume, of the tertiary structure of the protein.[26] The CD signal in the far-UV monitors changes in the secondary structure of the protein.

Analysis of denaturation curves

We analyzed all denaturation curves by fitting Equation (1) (given in the Experimental Section) to the single transition or, if there are two transitions, to the two transitions separately. From Equation (1), we could determine the values of C_m [M]—the concentration of the denaturant at the midpoint of the transition—and m ($=-\partial\Delta G^0/\partial[D]$, kcal mol^{-1} M^{-1})—the slope of the plot of ΔG^0 versus concentration of denaturant D.[28] The value of m correlates with the amount of surface area exposed upon denaturation.[32] It also indicates the degree of cooperativity of the unfolding process.[28] From the linear extrapolation model (LEM, Eq. (2), below)[33] and the values of C_m and m, we determined the values of $\Delta G^0_{N\rightarrow D}$ (or $\Delta G^0_{N\rightarrow I}$ and $\Delta G^0_{I\rightarrow D}$ if there are two transitions). The values of $\Delta G^0_{N\rightarrow D}$ from LEM are only approximate because deviations from linearity can occur at concentrations of denaturant close to zero.[28]

We used differential scanning calorimetry (DSC) to study the heat denaturation of these proteins. In previous studies,[14,25] we used the fluorescence of DNSA and capillary electrophoresis to examine denaturation in SDS.

Results and Discussion

Preparation of BCA-Ac_{18}

Acetylating the lysine ε-NH_3^+ residues of BCA with acetic anhydride yielded BCA-Ac_{18} in ~90% yield.[15,19] The other ~10% of the protein was BCA-Ac_{17} (17 out of 18 Lys groups acetylated). We monitored the progress of the acetylation and the composition of products using capillary electrophoresis, which separates the acetylated derivatives of BCA on the basis of charge.[34,35]

Charged groups and net charge of BCA and BCA-Ac_{18}

Native BCA contains 18 positively charged lysine residues, nine positively charged arginines, and 29 negatively charged residues (glutamic and aspartic acids) Histidines, with average pK_a of 6.5 are largely neutral at pH 8. The net charge of BCA is about −3, as measured by capillary electrophoresis[35] and calculated by using the Linderstrøm-Lang model of colloids (which assumes a spherical protein with uniform charge distribution).[36] The peracetylated BCA, with all lysine -NH_3^+ groups modified to electrically neutral -$NHCOCH_3$ groups, has a large imbalance between the numbers of positively and negatively charged residues, and a net charge of about −19.[15] Examination of the crystal structure of BCA indicates that none of the lysines is involved in local interactions, such as salt bridges.

Of course, each acetylation introduces additional hydrophobicity simultaneously with the removal of charge. The change in the value of log P—a parameter often used to quantify changes in hydrophobicity[37]—on conversion of -NH_3^+ groups (log $P=-2.12$) to -$NHCOCH_3$ groups (log $P=-1.21$) is +0.9 per group.[15] The destabilization of a protein due to additional exposed hydrophobic surface from an acetyl group is, we believe, small compared to the destabilization due to charge. In related work, we found that the hydrophobic contribution to destabilization of BCA-Ac_{18} is less than 1 kcal mol^{-1}.[14]

Denaturation with urea

We used the intrinsic fluorescence of Trp residues to study the denaturation of BCA and BCA-Ac_{18} in the presence of urea in a buffer of low ionic strength (10 mM phosphate, pH 8). We incubated the protein (1 μM) in solutions of urea for at least 24

hours. Figure 2A shows the denaturation profiles of BCA and BCA-Ac_{18} in urea. In addition to using intrinsic fluorescence, we examined the stability of the proteins in urea using the DNSA binding assay (Figure 2C) and circular dichroism (Figure 2E). In the DNSA assay, the inhibitor was added to the preincubated samples of protein (0.1 μM) in urea immediately before the measurement to minimize the possible stabilization of the protein by the inhibitor.

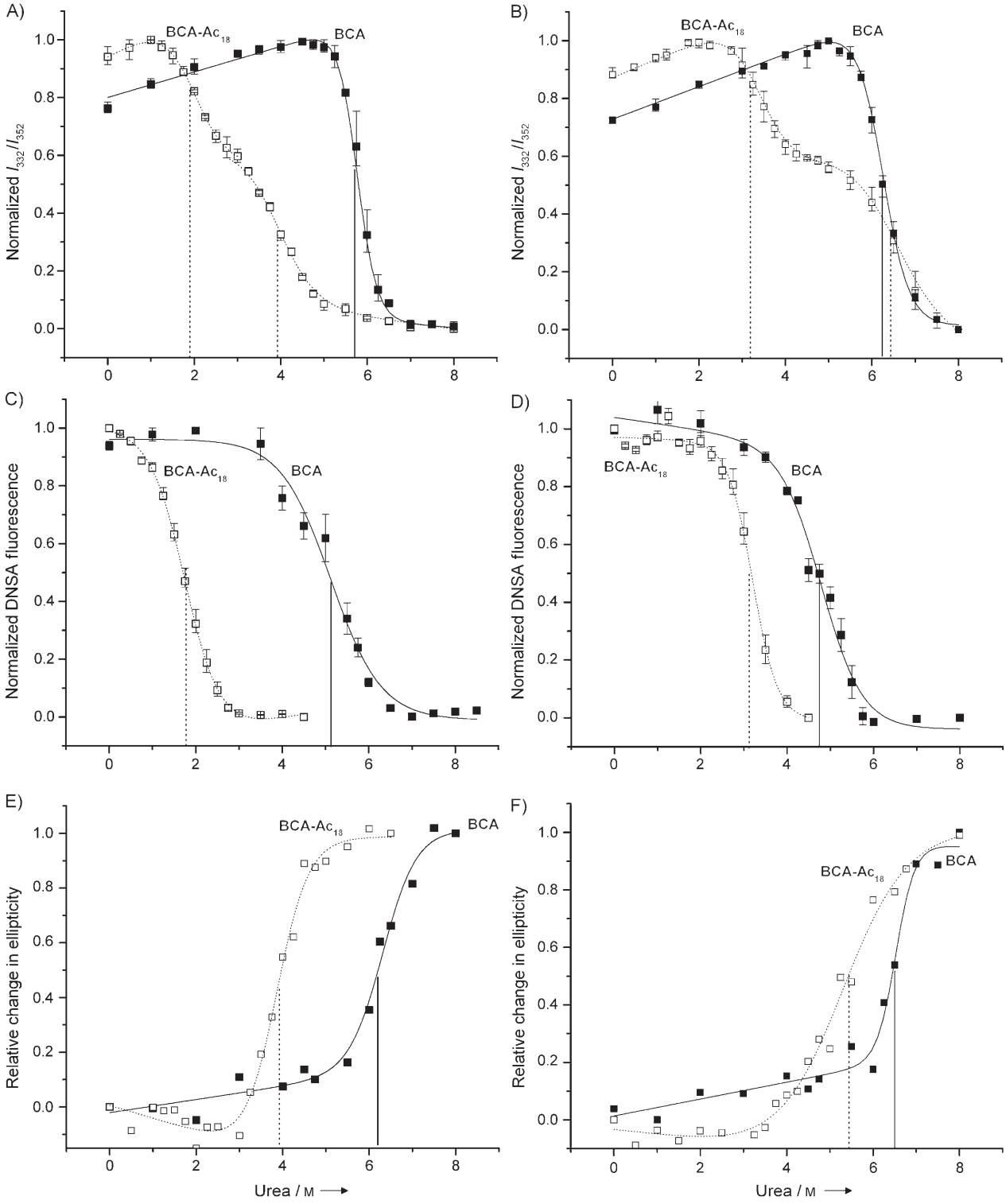

Figure 2. Urea-induced changes in intrinsic fluorescence (A, B), binding of DNSA (C, D), and ellipticity (E, F) at 222 nm of BCA (■) and BCA-Ac_{18} (□). Measurements in A, C, and E were made in 10 mM phosphate, pH 8; those in B, D, and F were in phosphate with 0.2 M Na_2SO_4. The plot also shows the least-squares fit of Equation (1) to the transitions of BCA (——) and BCA-Ac_{18} (····). The vertical lines mark the midpoints of the transitions for both proteins. The error bars, where present, represent the difference between the average and the largest and smallest values measured from at least three replicated measurements.

Table 1. Thermodynamic parameters associated with denaturation of BCA and BCA-Ac_{18} by urea in the absence and presence of 0.2 M Na_2SO_4 and in GuHCl, determined from I_{332}/I_{352} fluorescence data.

	C_{m_1} [a] [M]	m_1 [kcal M^{-1} mol^{-1}]	$\Delta G^0_{N\rightarrow I}$ [kcal mol^{-1}]	C_{m_2} [a] [M]	m_2 [kcal M^{-1} mol^{-1}]	$\Delta G^0_{I\rightarrow D}$ [kcal mol^{-1}]	$\Delta G^0_{N\rightarrow D}$ [kcal mol^{-1}]
Urea							
BCA	–	–	–	5.8 ± 0.1	2.9 ± 0.4	–	17 ± 2
BCA + SO_4^{2-}	–	–	–	6.2 ± 0.2	2.6 ± 0.4	–	16 ± 2
BCA-Ac_{18}	1.9 ± 0.1	1.2 ± 0.2	2.4 ± 0.5	3.9 ± 0.1	1.3 ± 0.1	5.1 ± 0.3	7.5 ± 0.6
BCA-Ac_{18} + SO_4^{2-}	3.4 ± 0.2	1.6 ± 0.2	5.4 ± 0.9	6.6 ± 0.2	1.2 ± 0.1	7.9 ± 0.8	13 ± 1
GuHCl							
BCA	1.6 ± 0.2	6.3 ± 0.4	10.1 ± 1.4	2.3 ± 0.1	3.4 ± 0.4	7.8 ± 1.0	18 ± 2
BCA-Ac_{18}	0.7 ± 0.1	5 ± 2	3.2 ± 1.4	2.5 ± 0.1	2.7 ± 0.4	6.8 ± 1.0	10 ± 2

[a] C_{m_1} and C_{m_2} are the midpoints of the first and second transitions. Only a single transition is observed with BCA; the parameters for that transition are listed as C_{m_2} and m_2. [b] $\Delta G^0_{N\rightarrow D} = \Delta G^0_{N\rightarrow I} + \Delta G^0_{I\rightarrow D}$ for a protein exhibiting a three-state transition.

The plot of the ratio of fluorescence at 332 and 352 nm shows that BCA exists in its native state for up to ~5 M urea (Figure 2A). Further increase in the concentration of the denaturant causes BCA to proceed, without an observable intermediate, directly to the denatured state through a sharp, cooperative transition with a midpoint at 5.8 M. The onset of the transition observed in the DNSA binding assay (Figure 2C) appears to occur at slightly lower concentrations of urea, with a midpoint of the transition at 5.1 M urea. It is possible that, starting at concentrations of ~4 M, urea affects the structure of the active site, and thus binding of an inhibitor, without causing a major disruption of the tertiary structure; this perturbation in structure might not be detected by using intrinsic fluorescence. Monitoring the disruption of secondary structure by CD at 222 nm shows a transition with a midpoint at 6.3 ± 0.3 M urea (Figure 2E).

Others have only detected a two-state denaturation (N→D) of BCA with urea,[22] but the fact that the transition detected with DNSA fluorescence occurs at slightly lower concentrations of urea than that detected with intrinsic fluorescence might be the evidence for an intermediate on the denaturation pathway. This intermediate, however, is unstable and does not accumulate in detectable concentrations.

In contrast to BCA, the unfolding of BCA-Ac_{18} with urea seems to proceed through a stable, observable thermodynamic intermediate. The first transition (N→I) observed by intrinsic fluorescence (C_{m_1} = 1.9 ± 0.1 M) corresponds exactly to the transition observed by DNSA fluorescence (C_m = 1.8 ± 0.1 M); we attribute this transition to the change in tertiary structure of the protein. The second transition (I→D), observed by intrinsic fluorescence (C_{m_2} = 3.9 ± 0.1 M) corresponds to the transition observed by CD at 222 nm (C_m = 3.8 ± 0.1 M); thus the secondary structure is present in the intermediate. Thus, for BCA-Ac_{18}, intrinsic fluorescence is a more sensitive and convenient spectroscopic probe than DNSA or CD with which to study denaturation, as intrinsic fluorescence is able to capture both transitions, while inhibitor binding and far-UV CD detect only one. The intermediate has a secondary structure similar to that of the native state, but its tertiary structure is not the same as that of native protein, as judged from DNSA binding; the intermediate thus satisfies the criteria for a molten globule. Further characterization would, however, be necessary to conclude that this intermediate is identical to the molten globule that forms in GuHCl.

Both the first (N→I) and second (I→D) transitions of BCA-Ac_{18} occur at lower concentrations of urea than the single transition of BCA. Thus, the changes in the surface charge of BCA not only destabilize the protein, but also modify its pathway of denaturation from an apparent two-state to a three-state one. While it is possible that denaturation of BCA kinetically proceeds through an intermediate, this intermediate does not accumulate and cannot be observed at any concentration of urea.

Table 1 lists the concentrations of urea at the midpoints of the transitions C_m, values of m, and the free energies of denaturation for individual transitions. The total value of m ($m_1 + m_2$) for BCA-Ac_{18} is similar to that of BCA; this suggests that that the surface area exposed upon denaturation is similar for both proteins. We can speculate that, since the two proteins are structurally similar in the folded states and the values of m are similar, their denatured forms are similar as well.

From a linear extrapolation to 0 M urea (Eq. (2) in the Experimental Section), we can calculate the free energy of denaturation in the absence of denaturant, $\Delta G^0_{N\rightarrow D}$, of BCA to be 17 kcal mol^{-1} and of BCA-Ac_{18} to be 7.5 kcal mol^{-1}. We propose that the difference of ~10 kcal mol^{-1} reflects the unfavorable electrostatic interactions created by elimination of 18 positively charged lysine ε-NH_3^+ groups from the surface of the protein. Determining whether this difference is evenly distributed between all lysine groups, with each acetylation of a lysine group reducing the stability of the protein by ~0.5 kcal mol^{-1} ($\Delta\Delta G^0_{N\rightarrow D}/18$), or whether this difference is attributable to specific lysines would require either multiple site-directed mutageneses or calculations similar to those described by Caravella et al.[38]

Denaturation with urea at high ionic strength

To investigate whether the stabilities of BCA and BCA-Ac_{18} are affected by high ionic strength in the buffer, we also studied the denaturation of these proteins by urea in the presence of 0.2 M Na_2SO_4 (ionic strength = 0.6 M). We chose sodium sulfate

to increase the ionic strength of the buffer, rather than salts containing monovalent anions, because several monovalent anions are known inhibitors of carbonic anhydrases[39] and thus might stabilize the protein by binding in the active site. Figure 2B shows the denaturation curves of BCA and BCA-Ac_{18} in urea with 0.2 M Na_2SO_4 constructed by using the intrinsic fluorescence of Trp residues.

The additional ionic strength of 0.6 M contributed by Na_2SO_4 does not change the apparent two-state denaturation pathway of BCA and does not significantly affect the midpoint of its transition or the value of $\Delta G^0_{N\rightarrow D}$ ($C_m = 5.8 \pm 0.1$ M, $\Delta G^0_{N\rightarrow D} = 17 \pm 2$ kcal mol^{-1} at low ionic strength, and $C_m = 6.2 \pm 0.2$ M, $\Delta G^0_{N\rightarrow D} = 16 \pm 2$ kcal mol^{-1} at high ionic strength). The unfolding of BCA-Ac_{18} with urea in 0.2 M sodium sulfate proceeds through an intermediate; this intermediate is significantly populated at around 4 M urea. Additional sodium sulfate stabilizes the native state of BCA-Ac_{18} by shifting the midpoint of the first transition from 1.9 ± 0.1 M to 3.4 ± 0.2 M urea; this transition corresponds to the loss of binding activity of DNSA (Figure 2D). Additional salt also stabilizes the intermediate state by shifting the midpoint of the second transition from 3.9 ± 0.1 M to 6.6 ± 0.2 M urea and bringing the C_m of the second transition of BCA-Ac_{18} close to that of BCA. Thus, the electrostatic destabilization of the secondary structure introduced by acetylation of lysine residues can be efficiently screened by buffer ions, while the destabilization of the tertiary structure cannot be screened completely. At high ionic strength, BCA is more stable than BCA-Ac_{18} by only ~3 kcal mol^{-1}; this number is significantly lower than the difference in stability at low ionic strength (~10 kcal mol^{-1}). Thus, screening of the unfavorable electrostatic interactions by high concentrations of buffer salts can partially reduce those interactions.

Several studies have shown that ions can influence the properties[40,41] and stabilities of proteins[42,43] through specific association (probably to side chains of opposite charge) or a Hofmeister effect in addition to Debye screening of electrostatic interactions. Ramos and Baldwin showed that a sulfate ion, in particular, stabilized ribonuclease A more than did a chloride ion.[43] The authors observed the largest difference in stabilization by the two anions at low pH, when the net charge on the protein was positive. In order to establish whether the observed stabilization of BCA-Ac_{18} to urea by sodium sulfate is predominantly due to Debye screening or to preferential adsorption of the sulfate ion to the surface of the protein (either electrostatically or through a Hofmeister effect), we measured the stability of BCA and BCA-Ac_{18} in the presence of 0.6 M NaCl (corresponding ionic strength to 0.2 M Na_2SO_4). Sodium and chloride ions are usually considered neutral in Hofmeister series and tend not to preferentially adsorb to the surfaces of proteins.[44] Qualitatively, the effects of chloride resembled those of sulfate, with the N→I transition of BCA-Ac_{18} being more affected by the additional salt than the I→D transition. BCA-Ac_{18} displayed a stable intermediate in urea with chloride as well as with sulfate, with $C_{m_1} = 2.9$ M and $C_{m_2} = 5.9$ M in chloride and $C_{m_1} = 3.4$ M and $C_{m_2} = 6.6$ M in sulfate. BCA exhibited $C_m = 5.5$ M in chloride and $C_m = 6.2$ M in sulfate. We conclude that the stabilization of BCA-Ac_{18} in a solution of 0.2 M Na_2SO_4 occurs *primarily* through Debye screening of unfavorable electrostatic interactions, but small differences in the values of the C_m's could indicate specific ion effects.

High concentrations of salts in buffers have the ability to change the folding and unfolding pathways of proteins by stabilizing the intermediates.[45,46] Borén and co-workers showed that addition of 1.5 M NaCl causes the unfolding of human carbonic anhydrase (HCA) with urea to proceed through an equilibrium intermediate; without NaCl, HCA undergoes a two-state transition in urea.[31] Acid-denatured cytochrome *c*, apomyoglobin, and several other proteins form a compact molten globule when additional electrolyte is added to the buffer;[47–49] the additional electrolyte shields unfavorable electrostatic interactions between the positive charges on the protein that are not compensated for by negative charges at low pH. Here, we demonstrated that the denaturation pathway, or the accumulation of a stable intermediate, can also be affected by changing the charged groups on the protein itself and increasing unfavorable electrostatic interactions, rather than by shielding the electrostatic interactions through addition of electrolyte to the buffer, as in the case of pH-denatured proteins. This example shows that, in the denaturation of proteins with urea—a denaturant that does not directly contribute to ionic strength—electrostatic interactions can play significant stabilizing or destabilizing roles. These electrostatic interactions are not fully screened by additional salt in the buffer. Comparison of BCA and BCA-Ac_{18} indicates that the introduction of unfavorable electrostatic interactions to the surface of the protein destabilizes its native state (i.e., the tertiary structure) even under high-salt conditions. In the intermediate state, which contains most of the secondary structure of the native protein but lacks the necessary contacts for the tertiary structure and is thus less compact, these electrostatic interactions are more efficiently screened by high concentrations of salt.

Denaturation with GuHCl

In the denaturation with GuHCl, we observed two transitions (N↔I↔D) for both BCA and BCA-Ac_{18} by intrinsic fluorescence (Figure 3). Thus, studying the denaturation of these proteins in GuHCl allowed us to compare the two transitions directly to each other. Table 1 summarizes the thermodynamic parameters for the denaturation of BCA and BCA-Ac_{18} with GuHCl.

The first transition in denaturation occurs at 1.6 M GuHCl for BCA and at 0.7 M for BCA-Ac_{18}. These transition midpoints correspond to those observed with the DNSA binding assay, and thus represent unfolding of the active site (Figure S1 in the Supporting Information). Henkens et al. showed that this transition also includes the loss of the tertiary structure.[26] We also observed this transition for BCA by monitoring the signal at 270 nm, which is specific to tertiary structure, using near-UV CD. We were unable to use CD for BCA-Ac_{18} because the protein precipitated in GuHCl concentrations between 0.5 and 2 M. The difference in value of C_m and in the value of $\Delta G^0_{N\rightarrow I}$ reflects the destabilization of the tertiary structure of BCA-Ac_{18} due to unfavorable electrostatic interactions from the large imbalance of charge on the surface of the protein. GuHCl is an

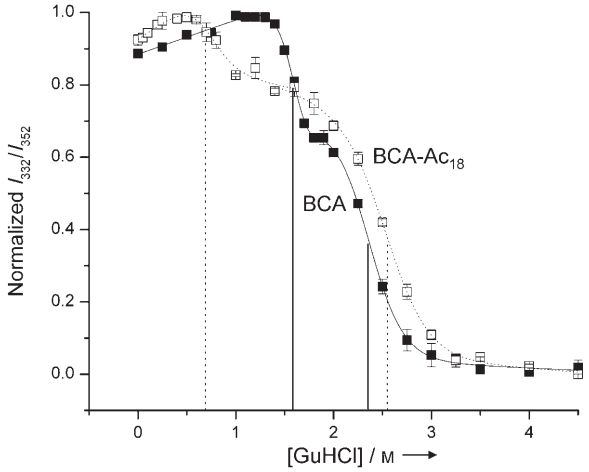

Figure 3. GuHCl-induced changes in the intrinsic fluorescence of BCA (■) and BCA-Ac$_{18}$ (□), measured by the ratio of intensities at 332 and 352 nm. The excitation wavelength is 295 nm. The plot also shows the least-squares fit of Equation (1) to the two transitions of BCA (——) and BCA-Ac$_{18}$ (····). The vertical lines mark the midpoints of the transitions for both proteins.

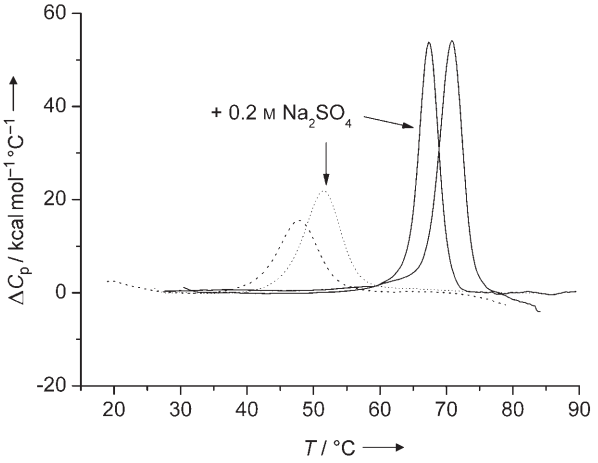

Figure 4. Thermal denaturation of BCA (——) and BCA-Ac$_{18}$ (····) measured by differential scanning calorimetry in 10 mM phosphate buffer, pH 8 with and without 0.2 M Na_2SO_4.

ionic denaturant, but additional ionic strength introduced by GuHCl did not shield these interactions.

The second transitions from the intermediate to the denatured state in the denaturation of BCA and BCA-Ac$_{18}$ occurred at similar concentrations of GuHCl (2.3 ± 0.1 and 2.5 ± 0.1 M, respectively). Others have attributed this transition to changes in the secondary structure of the polypeptide chain;[26] we also observed this transition using CD at 222 nm (Figure S1 in the Supporting Information). Comparison of the values of $\Delta G^0_{N\rightarrow I}$ and $\Delta G^0_{I\rightarrow D}$ for both proteins indicates that most of the destabilization from electrostatic interactions occurred in the N→I transition. We interpret this result to mean that the electrostatic interactions between charged groups are less important to the stability of the more extended intermediate and denatured states then to that of the more compact native state.

Thermal denaturation

We used differential scanning calorimetry (DSC) to monitor the thermal unfolding of BCA and BCA-Ac$_{18}$ (Figure 4). The melting temperature of BCA-Ac$_{18}$ in 10 mM phosphate buffer was 47.7 °C; that for BCA was 70.9 °C. Additional ionic strength in the buffer from 0.2 M Na_2SO_4 slightly lowered the melting temperature of BCA to 67.3 °C, but raised the melting temperature of BCA-Ac$_{18}$ to 51.3 °C. The enthalpy of melting ΔH_m for BCA-Ac$_{18}$ was only approximately half that of BCA (Table 2). The calculation of standard free energy of unfolding from DSC data showed that BCA is more stable than BCA-Ac$_{18}$ by ~22 kcal mol^{-1} at low ionic strength, and by ~17 kcal mol^{-1} at high ionic strength. The stabilization of BCA-Ac$_{18}$ to heat by additional salt was small.

Table 2. Thermodynamic parameters for thermal denaturation of BCA and BCA-Ac$_{18}$, measured by DSC. The table lists the melting temperature, the enthalpy of melting, and the free energy of denaturation under standard conditions $\Delta G^0_{N\rightarrow D}$.

	T_m [°C]	ΔH_m [kcal mol^{-1}]	$\Delta G^0_{N\rightarrow D}$ [kcal mol^{-1}]
BCA	70.9 ± 0.1	224 ± 2	29.9 ± 0.3
BCA + SO_4^{2-}	67.3 ± 0.1	221 ± 4	27.6 ± 0.6
BCA-Ac$_{18}$	47.7 ± 0.1	113 ± 2	8.0 ± 0.3
BCA-Ac$_{18}$ + SO_4^{2-}	51.3 ± 0.1	135 ± 8	11.0 ± 1.2

Comparison of $\Delta G^0_{N\rightarrow D}$ between denaturants

The values of $\Delta G^0_{N\rightarrow D}$ from denaturation by urea match those from denaturation with GuHCl for both proteins; there is a discrepancy, however, between the values of $\Delta G^0_{N\rightarrow D}$ measured by DSC and by titration with chemical denaturants, especially for BCA. It is not unusual to observe discrepancies in the values of $\Delta G^0_{N\rightarrow D}$ from different denaturants even for a well-characterized two-state denaturation, such as that of lysozyme.[50] The discrepancies can arise from deviations from the linear dependence of the free energy of the native and denatured states of proteins on the concentration of the denaturants. It is also possible that the structure of the chemically denatured state of the protein is different from that of the heat-denatured state or that the thermogram of BCA and BCA-Ac$_{18}$ is complicated by aggregation of the denatured protein. Nonetheless, the studies with urea, GuHCl, and heat all agree that BCA-Ac$_{18}$ is less stable than BCA.

Denaturation with SDS

In previous work,[25] we studied the denaturation of BCA and BCA-Ac$_{18}$ by SDS. We observed that BCA-Ac$_{18}$ is *more* stable to denaturation with SDS than BCA. This difference is, however, *kinetic*: BCA-Ac$_{18}$ denatures more slowly than BCA by several orders of magnitude over concentrations of SDS ranging from 2.5 to 10 mM. We also measured the rates of refolding of BCA

and BCA-Ac_{18} by first denaturing the proteins in 10 mM SDS and then diluting the solutions to lower SDS concentrations. We found that BCA-Ac_{18} renatured in concentrations of SDS of up to 1.5 mM, and with faster rates than BCA, which showed refolding only up to 0.7 mM of SDS.

We could not establish whether one protein was more thermodynamically stable than another to SDS because we could not establish equilibrium between the folded and unfolded species at every concentration of SDS. There exists a range of concentrations of SDS (~0.7–2.5 mM for BCA and ~1.5–2.5 mM for BCA-Ac_{18}) in which both unfolding and refolding are too slow to be observed and characterized. A simplified analysis of the equilibrium constant as a ratio of the unfolding and refolding rate constants points towards BCA-Ac_{18} as being both kinetically and thermodynamically more stable to SDS than BCA, as the rates of unfolding are slower and the rates of refolding are faster for BCA-Ac_{18} than for BCA.[25]

We believe that the observation that BCA denatures more rapidly than BCA-Ac_{18} in SDS reflects, in part, increased electrostatic repulsion between the highly negatively charged BCA-Ac_{18} and negatively charged molecules of SDS.[14] Thus, modulation of charges on the surfaces of proteins can result in destabilization of the protein to one denaturant, but stabilization to another denaturant.

The mechanism of denaturation of proteins by chaotropic agents, such as urea and guanidinium salts, is certainly different from the mechanism of denaturation by surfactants such as SDS, although the details of both mechanisms are still unclear. Urea and GuHCl are believed to alter the structure of the network of hydrogen bonds in water and increase the solubility of nonpolar groups of proteins in water (i.e., to decrease the hydrophobic effect).[51,52] SDS denatures proteins by binding directly to the polypeptide chain and solubilizing it in micelles. Denaturation by SDS is believed to proceed through an electrostatic interaction with positively charged residues of the protein, accompanied by binding to hydrophobic regions.[53] The opposing effect on denaturation by the chaotropic denaturants and by SDS from modifications of charged groups on a protein reflects the difference in the mechanism of denaturation—indirect action of urea and GuHCl and specific binding of SDS.

Conclusion

In this work we used BCA as a model protein to examine the effects of large changes to the net charge, and thus to the network of electrostatic interactions on the surface of the protein, on stability. We used intrinsic fluorescence, CD, and ligand-binding assays to construct the equilibrium denaturation profiles of BCA and of its derivative with the lysine groups acetylated (BCA-Ac_{18}). BCA is a protein that is commonly used as a model for biophysical studies, and this work provides additional information on the role of surface-exposed lysine residues on the stability of BCA to different denaturants.

Acetylating the lysine residues on the surface of BCA, and thus increasing its net charge, decreased its stability to urea, GuHCl, and heat. In denaturation with urea, acetylation of lysines not only reduced the stability of BCA, but also introduced a stable intermediate on the denaturation pathway. In urea, BCA-Ac_{18} exhibited a stable intermediate, while BCA did not. Since urea is a nonionic denaturant, we also studied the effects of additional ionic strength (0.2 M Na_2SO_4) on the stability of BCA and BCA-Ac_{18}. In a buffer of low ionic strength, each additional acetylated lysine residue destabilized BCA by, on average, ~0.5 kcal mol^{-1} to denaturation by urea; we believe that this destabilization arose from large, uncompensated for, negative charge on the surface of the protein. In a buffer of high ionic strength, this average destabilization per lysine residue decreased to ~0.2 kcal mol^{-1}. Shielding of unfavorable electrostatic interactions by buffer ions, even if all modified groups are solvent-accessible, thus reduced, but did not completely eliminate, these interactions.

Since both proteins exhibited a three-state denaturation profile in GuHCl, the study with this ionic denaturant allowed us to directly compare the two transitions between the two proteins. We found that most of the destabilization from acetylation occurred in the first transition, in which the tertiary structure of the protein was destroyed.

Modifying electrostatic interactions can destabilize the protein to denaturation by one denaturant, but stabilize it to denaturation by another. The example of BCA and BCA-Ac_{18} showed that the charge-modified derivative was less stable to GuHCl, urea, and heat, but more stable to SDS. Thus, each denaturant must be studied independently.

Tailoring charges on the surfaces of proteins might be one of the ways to optimize or control protein stability.[5,6,54] Unlike mutations to the interior residues, which can participate in contacts important for the tertiary structure and thus reduce the stability of the protein to all denaturants to similar extents, changes to the charged residues on the surfaces can improve stability to some denaturants, but retain the sensitivity to others.

Experimental Section

Reagents: Di- and monobasic sodium phosphate, sodium sulfate (Na_2SO_4), and urea were purchased from EM Science (Darmstadt, Germany). GuHCl was from Mallinkrodt Baker Inc. (Paris, KY). HEPES, acetic anhydride, and DNSA were purchased from Sigma–Aldrich. BCA was purchased from Sigma–Aldrich and used without further purification. DNSA was recrystallized from hot ethanol. The buffer (10 mM phosphate, pH 8) was made by mixing appropriate volumes of NaH_2PO_4 (10 mM) and Na_2HPO_4 (10 mM).

Peracetylation procedure: BCA was dissolved in HEPES buffer (100 mM; pH 8.2) to concentration of ~20 μM. Neat acetic anhydride was added to the solution of protein, in batches of 100 mol equiv with respect to the reactive amino groups on the protein. The pH of the HEPES buffer was actively adjusted during the reaction with 1 N NaOH. The progress of the reaction was monitored by using capillary electrophoresis.[19] The reaction mixture was incubated at room temperature and pH 10.5 for 1 h to de-esterify the tyrosine residues, which are also prone to acylations. The peracetylated BCA was then dialyzed extensively against phosphate buffer.

GuHCl/urea unfolding: The concentrations of the stock solutions of urea and GuHCl were determined by refractometry according to Ferreon et al.[55] The proteins were incubated in increasing concentrations of urea or GuHCl in sodium phosphate buffer (10 mM, pH 8) at 25 °C for at least 24 h. This length of time is sufficient for equilibrium to be reached. Longer incubation times resulted in indistinguishable denaturation curves. In the case of urea denaturation at high ionic strength, Na_2SO_4 (0.2 M) of was added to all concentrations of denaturant. The ionic strength (IS) of 0.2 M Na_2SO_4 is 0.6 M, calculated by $IS = 1/2\Sigma n_i z_i^2$, where n_i is the concentration of species i in the solution, and z_i is its charge.

Intrinsic fluorescence measurements: The intrinsic fluorescence measurements were made on protein samples that had been incubated for at least 24 h by using a Perkin–Elmer LS50B spectrometer, with a protein concentration of 1 μM. Spectra were taken from 300 to 400 nm, with $\lambda_{ex} = 295$ nm, 5 nm excitation slit width, and 7 nm emission slit width. The ratio of intensities at 332 and 352 nm[24] was normalized from 0 to 1.

DNSA-binding assay. Protein samples (0.1 μM) were incubated in urea or GuHCl for 24 h in 96-well plates. DNSA was added from stock solution of 1 mM in 10 mM HCl to a final concentration of 15 μM immediately before the measurement. The measurements were made by on a SpectraMax Gemini XS spectrometer (Molecular Devices), with $\lambda_{ex} = 280$ nm and $\lambda_{em} = 460$ nm. In the case of GuHCl and DNSA binding, all samples contained 2.5 M chloride ion (i.e. [GuHCl] + [NaCl] = 2.5 M) to eliminate the uneven competition of Cl^-—an inhibitor of carbonic anhydrases. The intensity of DNSA fluorescence was normalized from 0 to 1.

CD measurements: Ellipticity measurements (200–240 nm) were made on a Jasco J-715 instrument, in a quartz cuvette with 1 mm path length. The protein concentration was 5 μM. Each sample was scanned 4 times at 50 nm min^{-1}, with step size of 0.5 nm. We were unable to follow the changes in the tertiary structure of the proteins using CD in the near-UV because BCA-Ac_{18} aggregates and precipitates at the concentrations needed for the measurement (>20 μM).

Data analysis of denaturation curves: Fluorescence and CD denaturation profiles were fitted to Equation (1) (in Origin) to determine the values of C_m and m.[28]

$$F = \frac{(\alpha_N + \beta_N C) + (\alpha_D + \beta_D C)\exp(m(C - C_m)/RT)}{1 + \exp(m(C - C_m)/RT)} \quad (1)$$

In this equation, F is the spectroscopic signal, C is the concentration of the denaturant, R is universal gas constant, and T is temperature. α_N and β_N are the signal at 0 M denaturant and slope of the native state versus denaturant; α_D and β_D are the corresponding values in the denatured state. The equation accounts for the sloping baselines in the native and denatured states, often encountered with spectroscopic measurements. In the case of two transitions, each transition was fitted with Equation (1) separately.

The linear extrapolation method [Eq. (2)] was used to determine $\Delta G^0_{N\to D}$ (or $\Delta G^0_{N\to I}$ and $\Delta G^0_{I\to D}$ in the case of two transitions).

$$\Delta G^{o,C}_{N\to D} = \Delta G^{o,H_2O}_{N\to D} - mC \quad (2)$$

At midpoint of the transition, $\Delta G^{o,H_2O}_{N\to D} = 0$, and $\Delta G^{o,H_2O}_{N\to D} = mC_m$.

Differential scanning calorimetry: DSC experiments were performed on a VP-DSC instrument (Microcal, Inc., Northampton, MA) with protein concentrations of 10–15 μM and a scan rate of 90 °C h^{-1}. The enthalpy of melting ΔH_m was determined by integrating the area under the peak, and ΔC_p was determined from the pre- and post-transition baselines on the thermogram. The free energy of denaturation at a reference temperature T was calculated by assuming a two-state transition and using Equation (3):

$$\Delta G^0_{N\to D} = \Delta H_m\left(1 - \frac{T}{T_m}\right) + \Delta C_p\left(T \ln \frac{T_m}{T} - T_m + T\right) \quad (3)$$

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