Peracetylated Bovine Carbonic Anhydrase (BCA-Ac_{18}) Is Kinetically More Stable than Native BCA to Sodium Dodecyl Sulfate

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Bovine carbonic anhydrase (BCA) and its derivative with all lysine groups acetylated (BCA-Ac_{18}) have different stabilities toward denaturation by sodium dodecyl sulfate (SDS). This difference is *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. The rates of renaturation of BCA-Ac_{18} are greater than those of BCA, when these proteins are allowed to refold from a denatured state ([SDS] = 10 mM) to a folded state ([SDS] = 0.1 to 1.5 mM). On renaturation, the yields of the correctly folded protein (either BCA or BCA-Ac_{18}) decrease with increasing concentration of SDS. At intermediate concentrations of SDS (from 0.7 to 2 mM for BCA, and from 1.5 to 2 mM for BCA-Ac_{18}), both unfolding and refolding of the proteins are too slow to be observed; an alternative process—probably aggregation—competes with refolding of the denatured proteins at those intermediate concentrations. Because it is experimentally impractical to prove equilibrium, it is not possible to establish whether there is a difference in the *thermodynamics* of unfolding/refolding between BCA and BCA-Ac_{18}.

Introduction

Although polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) is a technique that is ubiquitous in biochemistry, the details of the interaction of SDS with proteins are poorly understood. Under the conditions normally used for SDS-PAGE (concentration of SDS = 0.1%, ~3.5 mM),[1] globular proteins associate, on average, with one molecule of SDS per two amino acid residues (or 1.4 g of SDS per 1 g of protein),[2] and upon binding, denature and lose their three-dimensional structure. This broad (although not universal) invariance of the stoichiometry of SDS to protein in the protein·SDS aggregates has the useful consequence that the rate of electrophoretic migration through a matrix of polyacrylamide gel depends only on molecular weight. Membrane proteins, proteins with posttranslational modifications, and proteins with extremely low or high isoelectric points interact atypically with SDS; these irregularities can contribute to difficulties in characterization of complex mixtures of proteins.[3] Better understanding of the interactions involved between the protein and surfactant, the mechanism and kinetics of denaturation of proteins by surfactants, and the structure of the final protein−surfactant complex should improve the application of SDS-PAGE to the characterization of proteins, and perhaps suggest new methods of separation of proteins.

Denaturation of proteins by SDS is believed to involve both electrostatic and hydrophobic interactions.[2] The fact that most nonionic surfactants do not denature proteins, while ionic surfactants—both positively and negatively charged—with structurally similar hydrophobic tails do, points to the critical role of the electrostatic interactions in denaturation of proteins by surfactants.[4] Nelson[5] observed that the particularly negatively charged proteins pepsin and papain (isoelectric point <1) were more resistant to binding SDS than other proteins, and bound at saturation <0.2 g of SDS/g of protein (vs an average value of 1.4 g).

Manning and Colón[6] identified a group of proteins (superoxide dismutase, avidin, papain, and others) that resisted denaturation by SDS at concentrations as high as 1% (35 mM) at room temperature. The authors attributed the observed stability of these proteins to particularly slow kinetics of denaturation (i.e., kinetic stability), rather than to the thermodynamic stability of the native conformation of the proteins in SDS. They suggested that the important feature of those proteins was extensive β-sheet structure, and not necessarily the high net negative charge.

Experimental Design. We investigated the role of electrostatics in the interaction of a protein and SDS using native carbonic anhydrase (BCA) and its charge-modified derivative (BCA-Ac_{18}) having all of its lysine groups acetylated (ϵ-NH_3^+ → ϵ-$NHCOCH_3$). Carbonic anhydrase is a 30 kDa, single-chain protein that is often used as a model in protein biophysics.[7,8] It does not contain any disulfide bonds that complicate denaturation and renaturation experiments. It possesses a mix of α-helical and β-sheet structural elements, with 10 β-strands forming the core of the protein.[9] Carbonic anhydrase contains a Zn(II) cofactor in the active site; this cofactor (as Zn(II)-OH) is necessary for its catalytic activity and for binding arylsulfonamide inhibitors.[10] Many inhibitors of CA are commercially available and their binding is well characterized.[11]

In an earlier paper, we described the refolding of BCA and BCA-Ac_{18} from their denatured states in SDS.[12] Our objective in that work was to investigate the effect of large perturbations in charge, and therefore in electrostatic interactions, on the surface of the protein on its ability to fold into an active form. We characterized the folded BCA and BCA-Ac_{18} by circular dichroism, activity as an esterase, and binding of an inhibitor, and showed that a large change in charge on the surface did not perturb the three-dimensional structure of BCA-Ac_{18}. We found that both BCA and BCA-Ac_{18} refolded with similar kinetics into the native conformation when the solutions of denatured protein were rapidly diluted to 0.1 mM SDS. Both proteins also refolded when SDS was slowly removed by dialysis. Thus, elimination of a large number of positively

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charged surface groups by acetylation of lysines did not influence the ability of the protein to refold.

We observed that BCA-Ac_{18} denatured more slowly in high concentrations of SDS than BCA, and wished to establish whether the observed difference between the two proteins was due to the difference in the thermodynamics of native and denatured forms of the two proteins, or due to the kinetics of denaturation. This paper describes our detailed examination of the rates of unfolding and refolding of both proteins over a range of concentrations of SDS. We conclude that BCA-Ac_{18} is kinetically more stable to denaturation by SDS than BCA. We are unable to measure the thermodynamic difference between the native conformation and BCA·SDS aggregate of the two proteins: an alternative process—probably aggregation of the proteins—competes with refolding of the denatured proteins. The fact that BCA and BCA-Ac_{18} have similar three-dimensional structures, different numbers of positively charged residues on their surfaces (18 ϵ-NH_3^+ in BCA; 0 ϵ-NH_3^+ in BCA-Ac_{18}; 9 arginines in both), and different rates of denaturation by SDS is compatible with the proposals by Tanford[13] and others[2,4] that electrostatic interactions play a major role in forming the aggregates of the proteins and SDS.

Results and Discussion

Critical Micelle Concentration of SDS in Tris-Gly Buffer. The critical micelle concentration (CMC) of SDS in 25 mM Tris−192 mM Gly, pH 8.4 buffer is 4.3 mM, as measured by isothermal titration calorimetry.[10] In this work, we concentrate on the kinetics of denaturation and renaturation of proteins in concentrations of SDS below the CMC, but show that the difference in kinetics of denaturation of the two proteins also occurs in concentrations of SDS above the CMC.

Preparation of BCA-Ac_{18}. We synthesized BCA-Ac_{18} by reacting the lysine residues of BCA with acetic anhydride in 100 mM HEPES buffer, pH 8.2.[14] The N-terminus of BCA is acetylated posttranslationally. The final product contained ~90% of BCA-Ac_{18} and ~10% of BCA-Ac_{17}, as determined by peak areas in an electropherogram from separation by capillary electrophoresis. The net charge of BCA in Tris-Gly buffer is ca. −3,[15] and the net charge of BCA-Ac_{18} is ca. −19.[12,16]

Binding of DNSA to BCA and BCA-Ac_{18}. Dansyl amide (DNSA) is an inhibitor of BCA and BCA-Ac_{18} that is strongly fluorescent only when bound in the active site of the protein. In free solution, its quantum yield for fluorescence is 0.055 at λ_{em} = 580 nm; when bound to the active site of BCA, its quantum yield increases to 0.84 and the wavelength of maximum emission shifts to 468 nm.[17] We used the fluorescence of DNSA as a measure of the amount of folded protein in solution in the denaturation/renaturation experiments.[18] The presence of SDS (below or above the CMC) did not alter the fluorescence of DNSA in solution.

Unfolding Kinetics. We measured the rates of denaturation of BCA and BCA-Ac_{18} by incubating the protein (200 nM) in solutions of different concentrations of SDS. Immediately prior to the measurement, we mixed an aliquot of solution containing protein and SDS with a solution containing DNSA and the same concentration of SDS; this protocol minimized the possibility that DNSA might stabilize the native conformation of the protein, and influence the rate of its denaturation. When we denatured BCA in concentrations of SDS of 4 mM and higher, we added DNSA to the original, denaturing solution because the rate of denaturation was too fast to follow with manual mixing of aliquots of protein and DNSA.[19]

Figure 1 plots the fluorescence of DNSA, normalized to the signal at $t = 0$, as a function of time of incubation of samples

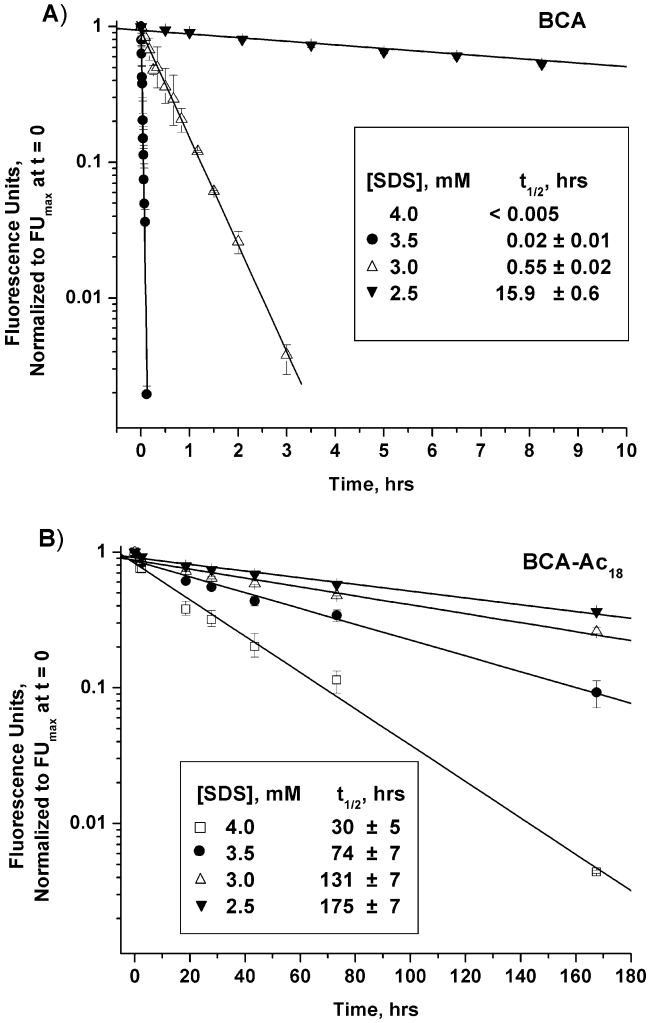

Figure 1. Unfolding of BCA (A) and BCA-Ac_{18} (B) as a function of time at various concentrations of SDS, using DNSA fluorescence to measure the concentration of correctly folded protein. The tables list the half-lives ($t_{1/2}$) for denaturation of BCA and BCA-Ac_{18} at several concentrations of SDS. The denaturation of BCA at 4 mM occurs very rapidly, and data are not shown to avoid overlap with data at 3.5 mM. The error bars represent the difference between the average and the largest and smallest values measured from at least three replicate measurements.

of BCA and BCA-Ac_{18} in various concentrations of SDS. The decrease in the intensity of fluorescence of DNSA, and thus in the amount of folded protein, followed first-order kinetics. Denaturation of proteins with surfactants probably proceeds through intermediates,[20−22] but when using a marker that is specific to the folded active site of carbonic anhydrase, we are unable to detect any transitions beyond the initial unfolding of the three-dimensional structure of the protein.[23]

BCA denatures in concentrations of SDS at and above 2.5 mM, a value that is substantially below the CMC (4.3 mM in Tris-Gly buffer). In concentrations below 2 mM, we observed no denaturation of the proteins over long (~1 month) periods of time. In high concentrations of SDS, BCA denatured very quickly: $t_{1/2}$ at 4 mM was 15 s (with DNSA present during denaturation), and $t_{1/2}$ at 10 mM was <5 s (faster than the deadtime of the conventional fluorimeter with manual mixing).

BCA-Ac_{18} denatures in concentrations of SDS at and above 2.5 mM, but with time constants greater than those of BCA by several orders of magnitude. In lower concentrations of SDS (below 2 mM), we observed no denaturation over long (~1 month) periods of time. In high concentrations of SDS, BCA-

Ac_{18} denatured significantly more slowly than BCA: $t_{1/2}$ at 4 mM was 30 h, $t_{1/2}$ at 10 mM was 160 min; BCA denatured on the time scale of seconds at those concentrations. This difference, approximately a factor of 10^4, corresponds to a difference of 5 kcal/mol in the activation energy for denaturation of these two proteins. The large differences between BCA and BCA-Ac_{18} in the rates of denaturation occurred both below and above CMC of SDS, and the presence of the micelles in solution was not necessary to induce unfolding.

At concentrations of SDS at and above 2.5 mM, there is clearly a large difference (10^1-10^4) in the kinetics of denaturation of BCA and BCA-Ac_{18}. At concentrations of SDS below 2 mM, it is unclear whether BCA and/or BCA-Ac_{18} would denature, given a sufficient amount of time, or whether the native state is the thermodynamically favored state at those SDS concentrations.

Refolding Kinetics. We measured the rates of refolding of BCA and BCA-Ac_{18} from the fully denatured states (in 10 mM SDS) by dilution of the solutions into buffers containing concentrations of SDS from 0.1 to 2 mM, again using the fluorescence of DNSA as a marker for the appearance of the folded state. At these concentrations of SDS, the proteins exist in folded form (i.e., they do not denature when SDS at these concentrations is added to the folded proteins). As in denaturation studies, we mixed an aliquot of solution of the protein and SDS with an equal aliquot of solution of DNSA with the same amount of SDS immediately prior to the measurement to eliminate the possibility of DNSA influencing refolding kinetics. The buffer also contained 100 μM $ZnSO_4$ to ensure that the folded protein can incorporate a Zn(II) cofactor.[10]

Figure 2 plots DNSA fluorescence as a function of time after dilution of denatured (10 mM SDS) protein into several concentrations of SDS. The insets show that the increase in fluorescence followed first-order kinetics. Time constants for refolding of both proteins increased with increasing concentration of SDS, but refolding of BCA-Ac_{18} proceeded faster than refolding of BCA in solutions with the same concentration of SDS. The yields of correctly folded proteins, based on the intensity of the fluorescence signal, decreased with increasing concentration of SDS in the refolding buffer. We did not observe any refolded BCA at concentrations of SDS of 0.8 mM and higher, or any refolded BCA-Ac_{18} at concentrations of SDS of 1.6 mM and higher, either due to particularly slow kinetics of refolding or to yields that were too low to be detectable.

The rate-limiting step in the folding of BCA from solutions of GuHCl occurs with a time constant on the order of 10 min, and has been attributed to proline isomerization.[24] Refolding of BCA from the SDS-denatured state into a minimal (0.1−0.2 mM) concentration of SDS occurs on a similar time scale, and thus may also be proline isomerization.[12] Because the rates of refolding decrease with increasing SDS in the buffer, it is possible that the rate-limiting step in folding switches from proline isomerization to dissociation of SDS from the polypeptide chain—a process dependent on the concentration of free SDS in the solution.

Figure 3 summarizes the rate constants of unfolding and refolding of both proteins as functions of [SDS]. For both proteins, there exists a "window" in which we were not able to measure the rates of either unfolding or refolding. It is possible that the change in the slope in the rates of refolding, which occurs at 0.3 mM SDS, indicates a change in the rate-limiting step.

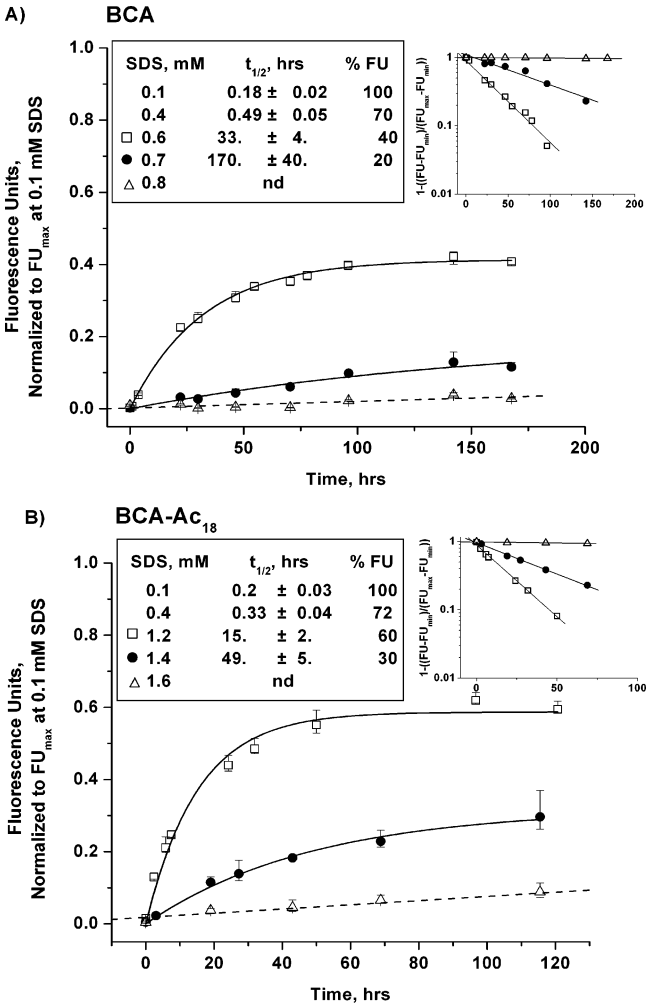

Figure 2. Refolding of BCA (A) and BCA-Ac_{18} (B) as a function of time, initiated by rapid dilution of the denatured protein in 10 mM SDS into various concentrations of SDS; the fluorescence signal from DNSA was normalized to that of refolded protein at 0.1 mM SDS. The raw data emphasize the decrease in the yield of folded protein with increasing concentration of SDS. The insets demonstrate that the data fit first-order kinetics. The half-lives ($t_{1/2}$) for renaturation were determined, where possible, by fitting the data to $FU = FU_{max}(1 - \exp(-t/t_{1/2})) + FU_{min}$; the fits are shown (solid lines). The tables provide data for refolding in 0.1 and 0.4 mM SDS as well as for concentrations on the graphs for comparison. Where the renaturation does not occur and the data cannot be fit to the function above, the symbols are connected by dashed lines. The error bars represent the difference between the average and the largest and smallest values measured from at least three replicate measurements.

Is the Native State of BCA or BCA-Ac_{18} the Thermodynamically Favored One at Intermediate Concentrations of SDS? The way to prove that a protein is in thermodynamic equilibrium between the native and denatured states at a condition of interest is to approach this condition from both native and fully denatured states. Our examination of refolding of BCA and BCA-Ac_{18} into their native conformations after complete denaturation showed that BCA did not refold at an observable rate in 0.8−2 mM SDS—a range of concentrations in which it also did not unfold at an observable rate. BCA-Ac_{18} did not refold in 1.6−2 mM SDS—a range of concentrations in which it also did not unfold. In these "windows", the equilibration between the native and denatured states could not be reached in any practical experiment (time scales of a month). Equilibrium between native and denatured states was not reversible at those intermediate concentrations of SDS, and we

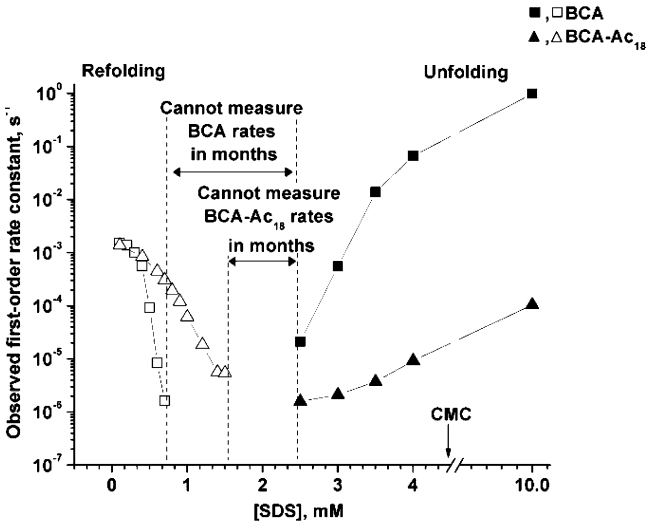

Figure 3. Summary of the rate constants for unfolding and refolding of BCA (squares) and BCA-Ac_{18} (triangles). Closed symbols refer to rate constants for unfolding, open symbols refer to rate constants for refolding. Dashed vertical lines mark the windows in the concentration of SDS where the rates of both unfolding and refolding are not measurable.

are unable to conclude that the native conformation of the proteins is the thermodynamically favored state at concentrations of SDS below 2 mM.

We also conducted the unfolding and refolding experiments at 37 °C in an attempt to speed up both rates, and to reach equilibrium. Elevated temperature only shifted the "window", in which we could not observe equilibration, to lower concentrations of SDS (see the figure in the Supporting Information); we could not eliminate the window of no equilibration entirely.

The observation that the rate of unfolding of BCA-Ac_{18} is slower than that of BCA, while the rate of refolding is faster, suggests that the thermodynamic stability of BCA-Ac_{18} in SDS is greater than that of BCA (i.e., the equilibrium constant for denaturation K_{N-D} of BCA is less than K_{N-D} of BCA-Ac_{18}, based on $K_{N-D} = k_{unf}/k_{ref}$). We cannot, however, conclude unambiguously that BCA-Ac_{18} is thermodynamically more stable than BCA in intermediate concentrations of SDS.

Why Do We Not Achieve Equilibration between Native and Denatured States at Intermediate Concentrations of SDS? The lack of equilibration between native and denatured states of the protein suggests that some process other than unfolding and refolding becomes competitive at intermediate concentrations of SDS. Aggregation is a process that often interferes with folding of proteins because it occurs between partially folded intermediates. Aggregation is a common feature of β-sheet proteins,[25] and carbonic anhydrase, in particular, is a protein that is prone to aggregation. It aggregates during renaturation when denatured with heat[26] or acid,[27] or when incubated in intermediate concentrations (1−3 M) of guanidinium chloride (GuHCl).[28−30] For example, Hammarström et al. observed low (<10%) recovery of activity of human carbonic anhydrase (HCAII) when it was renatured by dilution from 2 to 0.3 M GuHCl, but high (>80%) recovery of activity when diluted from 6 M GuHCl to 0.3 M.[30] McCoy et al.[26] also observed aggregation of BCA in refolding of the protein, denatured in a solution of 0.1% SDS and renatured with dialysis.[31]

We believe that irreversible aggregation interferes with refolding of BCA (and BCA-Ac_{18}) in intermediate concentrations of SDS and prevents equilibration (eq 1). In this equation,

$$\mathrm{BCA} + \mathrm{nSDS} \underset{k_{ren}}{\overset{k_{den}}{\rightleftharpoons}} \mathrm{I} \rightleftharpoons \mathrm{BCA}\cdot\mathrm{nSDS} \qquad (1)$$

$$\mathrm{I} \xrightarrow{k_{agg}} \text{Aggregation}$$

we cannot say anything specific about the nature of the intermediate (I) encountered on the refolding pathway, except that the intermediate is the polypeptide chain that is not fully saturated with molecules of SDS; such a conformation probably exposes some hydrophobic surface and renders the protein prone to aggregation. We suggest that as the rates of renaturation, k_{ren}, begin to decrease with increasing concentrations of SDS for BCA and BCA-Ac_{18}, aggregation and renaturation begin to compete. The drop in the yield of refolded proteins with increasing concentrations of SDS, as judged by the intensity of the fluorescence of bound DNSA, also indicates the intervention of another process that competes with refolding.

We tested for aggregation by renaturing the proteins from a denatured state at 10 mM SDS to 0.1 mM SDS, but through an incubation step at intermediate concentrations of SDS, instead of renaturing by rapid dilution from 10 mM SDS to 0.1 mM SDS directly (i.e., 10 mM SDS → 2.5 mM SDS → 0.1 mM SDS instead of 10 mM SDS → 0.1 mM SDS). For incubation, we chose concentrations of SDS, in which, we believe, aggregation occurred on a similar or shorter time scale as refolding. We diluted the protein−SDS complex from 10 mM SDS to 2.5−0.7 mM SDS, incubated the proteins for 1 week at those concentrations, and diluted the samples further to 0.1 mM SDS to initiate fast refolding. Figure 4 shows that only a fraction of total protein, as judged by the fluorescence intensity of DNSA, was recovered upon dilution of the incubated samples to 0.1 mM SDS. The reduced yield of the active protein after incubation at intermediate concentrations of SDS suggests irreversible aggregation, where the aggregating species are protein molecules that are not saturated with SDS.

We also used dynamic light scattering to measure the sizes of the folded and unfolded protein and of the presumably aggregated sample. We measured the hydrodynamic radius of the native BCA (2.7 ± 0.7 nm), and of BCA, refolded in 0.1 mM SDS (2.6 ± 0.6 nm). We denatured BCA in 10 mM SDS, and diluted to 1.8 mM SDS—a concentration at which we observe no refolding—immediately before the measurement. The denatured protein resulted in a hydrodynamic radius of 2.9 ± 0.6 nm. Denatured BCA, incubated in 1.8 mM SDS for one week, resulted in the radius of 3.6 ± 0.9 nm. The small increase in the hydrodynamic radius of the incubated sample is indicative of the formation of low-order aggregates (e.g., dimers or trimers), but the resolution of DLS measurements is not sufficient to observe each species individually. Cleland and Wang characterized aggregation of BCA at intermediate concentrations of GuHCl (0.4−1 M) and observed a regime in which inactive dimers and trimers formed, but did not proceed to form larger aggregates.[28] Our measurements suggest that multimers formed on refolding of BCA in intermediate concentrations of SDS are similar in size to those forming in GuHCl.

The yields of refolded BCA-Ac_{18} after incubation were higher than the yields of BCA. Combined with the fact that refolding of BCA-Ac_{18} proceeded to higher concentrations of SDS than refolding of BCA, these observations may indicate differential propensities for aggregation between the two proteins. BCA-

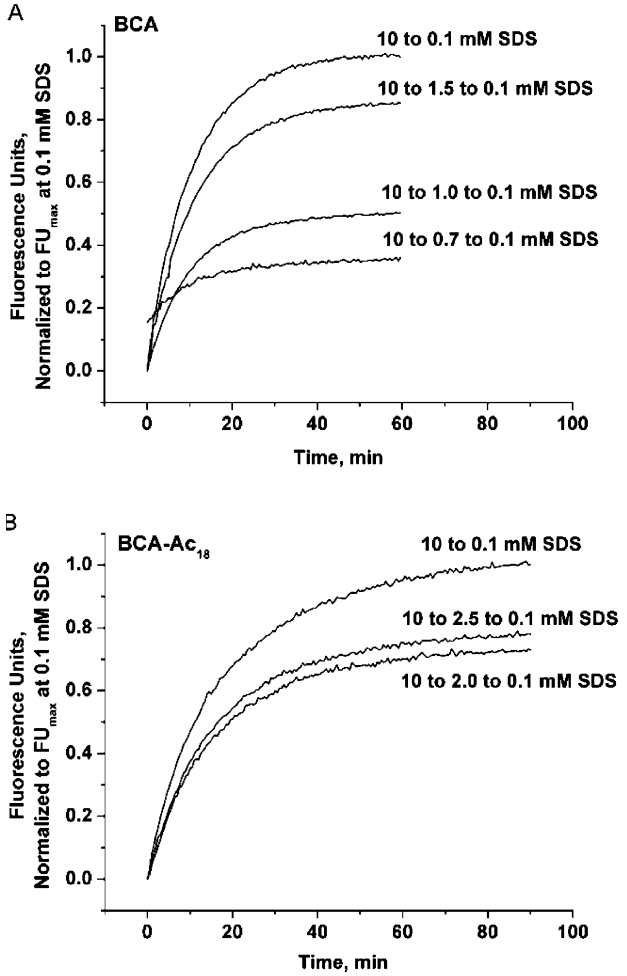

Figure 4. Refolding of BCA (A) and BCA-Ac_{18} (B) after denaturation in 10 mM SDS, incubation of the denatured proteins at intermediate concentrations of SDS (marked for each curve) for 1 week, and dilution into 0.1 mM SDS. The fluorescence signal from DNSA was normalized to that of refolded protein by rapid dilution from 10 to 0.1 mM SDS. All traces show similar half-lives for refolding ($t_{1/2}$(BCA) = 10.8 ± 0.6 min; $t_{1/2}$(BCA-Ac_{18}) = 17.5 ± 1.4 min), but the yield of folded protein varied with the concentration of SDS in which the samples were incubated.

Ac_{18} may aggregate less due to electrostatic repulsion of highly negatively charged molecules.

Since we do not have *direct* evidence of aggregation, it remains possible that another process competes with refolding of the protein. Our indirect evidence, however, along with reports from literature on the propensity of BCA to aggregate, strongly suggests that denaturation of BCA and BCA-Ac_{18} is not reversible at intermediate concentrations of SDS due to aggregation.

The Use of Additives To Prevent Aggregation. We tried to prevent aggregation of BCA and BCA-Ac_{18} with additives that are commonly used to prevent aggregation of carbonic anhydrases and other proteins. The additives included polymers (PEG),[29,32] other surfactants (CHAPS),[29] "salting-in" Hofmeister salts (tetrabutylammonium thiocyanate and sodium thiocyanate),[33,34] osmolytes (betaine),[35,36] and organic solvents (dimethyl sulfoxide and *N*-methylpyrrolidone).[37] These additives shifted the "window" in which neither unfolding nor refolding was observed, but did not help achieve equilibration at all concentrations of SDS; the shift in the "window" appeared to be linked to the shift of critical micellar concentration of SDS due to the additives. For example, in 10% DMSO, we observed refolding of BCA up to 1.2 mM SDS (vs 0.7 mM SDS without a cosolvent), but did not observe unfolding up to 2.5 mM SDS. In the presence of 1 mM CHAPS, the refolding of BCA occurred up to 1.8 mM SDS, but unfolding did not occur below 2.5 mM SDS. Representative figures of the experiment with additives are in the Supporting Information.

Conclusion

We conclude that either the net charge of BCA or the number of positively charged residues influences the *kinetics* of its denaturation with SDS. The example of BCA (net charge ca. −3, 18 lysine $-NH_3^+$ groups) and peracetylated BCA (net charge ca. −19, all lysines converted to $-NHCOCH_3$ groups) shows that the rates of denaturation with SDS of these proteins can change by ~3 orders of magnitude as a result of a change in the charge of the protein by a factor of 5. From these data, we cannot determine whether the rate of denaturation changes due to the electrostatic repulsion of the highly negatively charged protein species and negatively charged molecules of SDS, or due to the reduced number of cationic side chains available for nucleation of unfolding. The change in hydrophobicity constant (the Hansch π parameter)[38] for the conversion of the $-NH_3^+$ group ($\pi = -2.12$) to a $-NHCOCH_3$ ($\pi = -1.21$) is +0.9;[12,39] it is unlikely that this small change is responsible for the observed effect.

This work shows that structural features of proteins, as proposed by Manning and Colón,[6] are not the only determinants of kinetic stability of proteins to SDS. BCA and BCA-Ac_{18} have similar core structures based on CD spectra, yet the large negative charge of BCA-Ac_{18} relative to BCA renders the protein kinetically more stable than BCA to negatively charged SDS by reducing the rate of denaturation.

This and other studies[4,6] demonstrate that equilibration in protein/surfactant systems may occur on long times scales (days to months); this phenomenon can occur both below and above the CMC of the surfactant. Proteins and their derivatives (and we presume, mutants) may behave differently toward SDS, especially if the derivative differs from the native protein in charge.

Future studies that involve proteins and surfactants should differentiate between systems in thermodynamic equilibrium and systems with particularly slow kinetics of equilibration. We believe that kinetic stabilization of proteins by surface modifications may find applications in biotechnology as an alternative to engineering proteins with high free energy of denaturation via mutations of core residues.

Experimental Procedures

Sources of Chemicals. Bovine carbonic anhydrase II was purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. SDS was purchased from J. T. Baker (Phillipsburg, NJ), recrystallized in hot ethanol three times, and stored at −20 °C. Dansyl amide, 10× Tris-Gly concentrate, zinc sulfate standard solution, HEPES, and all additives were purchased from Sigma-Aldrich. Dansyl amide (DNSA) was recrystallized once from hot ethanol. Tris-Gly concentrate was diluted 10-fold with deionized water to make 25 mM Tris−192 mM Gly, zinc sulfate (100 μM) was added, and the buffer was filtered before use. Dialysis cassettes (10 kDA cutoff) and desalting columns were purchased from Pierce (Rockford, IL). Concentrations of BCA and BCA-Ac_{18} were determined by UV spectroscopy, using $\epsilon = 57\,000\ M^{-1}\ cm^{-1}$ at 280 nm. DNSA was also quantified spectroscopically, using $\epsilon = 4640\ M^{-1}\ cm^{-1}$ at 325 nm.

Making BCA-Ac_{18}. Peracetylated BCA was prepared by the procedure of Yang et al.,[14] without denaturation of the starting

protein. Briefly, BCA (10 μM) was dissolved in 100 mM HEPES buffer. Acetic anhydride was added neat to the solution of BCA in aliquots of 100 equiv of anhydride with respect to the number of ϵ-NH_3^+ groups. The reaction mixture was stirred, and pH was actively controlled with 1 M NaOH. We monitored the extent of the reaction by capillary electrophoresis (Beckman P/ACE 5010, 40/47 cm fused silica capillary, 15 kV, Tris-Gly as running buffer) by drawing small aliquots for analysis and desalting them prior to CE. We then adjusted the pH of the reaction mixture to 10.7, incubated the mixture for 1 h at the elevated pH to deesterify any acetylated tyrosine residues, readjusted the pH to 8.4, and dialyzed the reaction mixture against Tris-Gly.

Monitoring Unfolding of BCA and BCA-Ac$_{18}$. We incubated the proteins (200 nM) in different concentrations of SDS in Tris-Gly buffer. We mixed a 100 μL aliquot of protein−SDS solution with 100 μL of DNSA solution (30 μM) with the same concentration of SDS, and measured the intensity of fluorescence (Perkin-Elmer LS50B spectrometer, $\lambda_{ex} = 280$ nm, $\lambda_{em} = 460$ nm) as a function of time. We continuously monitored denaturation of BCA in concentrations of SDS above 4 mM. We determined the rate constants for denaturation by fitting the intensity vs time data to $FU = FU_{max}e^{-kt} + FU_{min}$. Unfolding experiments at 37 °C were done similarly, with samples stored in a dry bath incubator (Fisher Scientific).

Monitoring Refolding of BCA and BCA-Ac$_{18}$. We denatured the proteins (10 μM) in 10 mM SDS for 24 h. We used CE to show that all protein was denatured.[10] Refolding was initiated by dilution of an aliquot of the denatured sample to a final concentration of protein of 200 nM, with varying concentrations of SDS in the buffer. Immediately prior to the measurement, an aliquot of renaturing protein was mixed with an equal aliquot of DNSA. The rate constants for renaturation were determined by fitting the data to $FU = FU_{max}(1 - e^{-kt}) + FU_{min}$. Refolding experiments at 37 °C were done similarly.

Additives in De-/Renaturation. We screened for the effects of the following additives on denaturation and renaturation of BCA and BCA-Ac$_{18}$: poly(ethelyneglycol) (PEG, MW = 2000), 0.1 mM, 1 mM; poly(vinylpyrrolidone), MW = 10 000, 1%, 10% w/v; 3-(3-cholamidopropyl)dimethylammoniopropane sulfonate (CHAPS), 1 mM; octanol, 1 mM; tetrabutylammonium thiocyanate, 100 mM; sodium thiocyanate, 100 mM, 1 M; sodium sulfate, 10 mM; betaine, 1 M; dimethyl sulfoxide (DMSO), 1%, 10% v/v; *N*-methylpyrrolidone, 1%, 10% v/v. The screening was done in 96-well plate format, using a SpectraMax Gemini XS spectrometer (Molecular Devices), with a protein concentration of 100 nM, a DNSA concentration of 10 μM (present in the denaturation/renaturation buffers), and variable concentrations of SDS.

Dynamic Light Scattering. DLS measurements were performed on a DynaPro light scattering device (Wyatt Technology Corp.), at protein concentrations of 10−15 μM.

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Supporting Information Available: Plots of denaturation and renaturation of BCA in the presence of additives and heat. This material is available free of charge via the Internet at http://pubs.acs.org.

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