Denaturation of Proteins by SDS and Tetraalkylammonium Dodecyl Sulfates

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Supporting Information

ABSTRACT: This article describes the use of capillary electrophoresis (CE) to examine the influence of different cations (C⁺; C⁺ = Na⁺ and tetra-α-alkylammonium, NR₄⁺, where R = Me, Et, Pr, and Bu) on the rates of denaturation of bovine carbonic anhydrase II (BCA) in the presence of anionic surfactant dodecylsulfate (DS⁻). An analysis of the denaturation of BCA in solutions of Na⁺DS⁻ and NR₄⁺DS⁻ (in Tris-Gly buffer) indicated that the rates of formation of complexes of denatured BCA with DS⁻ (BCA⁻DS⁻ₙₛₐ𝑡) are indistinguishable and independent of the cation below the critical micellar concentration (cmc) and independent of the total concentration of DS⁻ above the cmc. At concentrations of C⁺DS⁻ above the cmc, BCA denatured at rates that depended on the cation; the rates decreased by a factor >10⁴ in the order of Na⁺ ≈ NMMe₄⁺ > NNet₄⁺ > NR₄⁺ > NBu₄⁺, which is the same order as the values of the cmc (which decrease from 4.0 mM for Na⁺DS⁻ to 0.9 mM for NBu₄⁺DS⁻ in Tris-Gly buffer). The relationship between the cmc values and the rates of formation of BCA⁻DS⁻ₙₛₐ𝑡 suggested that the kinetics of denaturation of BCA involve the association of this protein with monomeric DS⁻ rather than with micelles of (C⁺DS⁻)ₙ. A less-detailed survey of seven other proteins (α-lactalbumin, β-lactoglobulin A, β-lactoglobulin B, carboxypeptidase B, creatine phosphokinase, myoglobin, and ubiquitin) showed that the difference between Na⁺DS⁻ and NR₄⁺DS⁻ observed with BCA was not general. Instead, the influence of NR₄⁺ on the association of DS⁻ with these proteins depended on the protein. The selection of the cation contributed to the properties (including the composition, electrophoretic mobility, and partitioning behavior in aqueous two-phase systems) of aggregates of denatured protein and DS⁻. These results suggest that the variation in the behavior of NR₄⁺DS⁻ with changes in R may be exploited in methods used to analyze and separate mixtures of proteins.

INTRODUCTION

This article analyzes the interaction between proteins and anionic surfactants (C⁺DS⁻) that consist of dodecylsulfate (DS⁻) paired with cation Na⁺, tetramethylammonium (NMMe₄⁺), tetraethylammonium (NNet₄⁺), tetra-α-propylammonium (NR₄⁺), or tetra-α-butylammonium (NBu₄⁺). Using capillary electrophoresis (CE), we analyzed the formation of complexes of DS⁻ with bovine carbonic anhydrase II (BCA; E.C. 4.2.1.1) in detail. This reaction involves the association of n equiv of DS⁻ with BCA and results in complexes of denatured protein and DS⁻ (BCA⁺−DS⁻ₙₛₐ𝑡), where nₛₐ𝑡 is the stoichiometry of DS⁻ bound to BCA at saturation in solutions containing excess DS⁻, as observed when BCA is analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis; nₛₐ𝑡 is approximately 150 for BCA.

\[
\text{BCA} + n\text{DS}^- \rightarrow \text{BCA}^+ - \text{DS}^-_{n_{sat}} \quad (1)
\]

The rates of denaturation of BCA depended on C⁺. At concentrations of C⁺DS⁻ below the critical micellar concentration (cmc), the rates were independent of the cation and increased with the concentration of DS⁻ (log \( k \) was approximately proportional to \([\text{DS}^-]\) below the cmc). At concentrations above the cmc, however, the rates of formation of BCA⁺−DS⁻ₙₛₐ𝑡 were independent of increases in the total concentration of C⁺DS⁻ and depended on the cation, decreasing in the order of Na⁺ ≈ NMMe₄⁺ > NNet₄⁺ > NR₄⁺ > NBu₄⁺. This order is the same as the order of values of the cmc and suggests that the rates of formation of BCA⁺−DS⁻ₙₛₐ𝑡 above the cmc depend on the concentration of monomeric DS⁻. This order is also, of course, the order of increasing size and hydrophobicity (described by increasing values of log \( P \) for the water–octanol partitioning of NR₄⁺: \(-2.7, -2.1, -1.3, 0.4\)).

We also surveyed, albeit in less detail, seven other proteins (α-lactalbumin, β-lactoglobulin A, β-lactoglobulin B, carboxypeptidase B, creatine phosphokinase, myoglobin, and ubiquitin) for their interactions with C⁺DS⁻. The influence of the cation on the formation of aggregates between protein and DS⁻ varied with the protein. For some (e.g., ovalbumin), the rate of formation of protein−DS⁻ was indistinguishable in solutions of Na⁺DS⁻ and NR₄⁺DS⁻. For others (e.g., carboxypeptidase B, CPB), the cation influenced the kinetics of formation and the composition of aggregates of protein and DS⁻.

Motivation. Treatments of proteins with surfactants are widely used in biotechnology, analytical biochemistry, and medicine. Examples include the ubiquitous application of SDS in SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), the use of surfactants in formulations of...
biopharmaceuticals (to prevent the aggregation of proteins and the adsorption of proteins to surfaces), and the use of surfactants in cleaning, disinfecting, and sterilizing.\textsuperscript{12–16} Our initial interest was in SDS-PAGE. This technique remains an integral part of protein biochemistry, but obvious questions—why dodecyl sulfate rather than some other surfactant? why sodium ions?—seem never to have been systematically addressed. We hoped that such an examination would increase the number of knobs that could be turned by biochemists in manipulating the interactions among proteins and surfactants.

In the preparation of samples for SDS-PAGE, the combination of temperature (100 °C), surfactant (≈70 mM SDS \( \gg 4.0 \text{ mM} \)), the cmc in Tris-Gly buffer, one that is commonly used in SDS-PAGE), and disulfide reducing agent unfolds most globular proteins and generates aggregates of denatured protein and \( \text{DS}^- \) (which we write as protein\(^D\text{−} \text{DS}^-\)\text{sat}.\text{)}\text{).17,18 The stoichiometry of binding is roughly independent of the amino acid sequence and structure of the native protein (≈1.4 g of SDS per 1 g of protein or approximately 1 equiv of \( \text{DS}^- \) for every 2 amino acid residues).\textsuperscript{19,20} This feature enables the resolution of proteins by PAGE according to molecular weight. Exceptions that show anomalous migration include post-translationally modified proteins, membrane-associated, or membrane-bound proteins and proteins with very high or low isoelectric points.\textsuperscript{21–24} Many elements of the interaction of \( \text{DS}^- \) with proteins remain unclear. Details about the sites of association, the relative importance of hydrophobic and electrostatic contributions to binding, and the mechanism by which \( \text{DS}^- \) induces conformational change are incompletely understood despite extensive investigation.\textsuperscript{10,20,24–28}

Whether or how \( \text{Na}^+ \) (or other cations in buffer solutions of \( \text{Na}^+\text{DS}^- \) and protein) participates is not known.

Our primary objective in this study was to compare the influence of cations having a range of hydrophobicities (\( \text{C}^+ = \text{Na}^+, \text{NR}_{4}^{+}; \text{R} = \text{Me, Et, Pr, Bu}) to identify the effect of \( \text{NR}_{4}^{+} \) on the formation of protein−\( \text{DS}^- \text{ sat} \) we analyzed BCA and several additional proteins using CE in solutions of \( \text{C}^+ \text{DS}^- \). Zana et al. compared the properties (cmc, aggregation number (\( N \)), degree of ionization (\( \alpha \)), microviscosity, and micropolarity) of \( \text{Na}^+\text{DS}^- \) and \( \text{NR}_{4}^{+} \text{DS}^- \) in buffered aqueous solution; the results suggested that the assembly of \( \text{DS}^- \) into micelles was more favorable for \( \text{NR}_{4}^{+} \text{DS}^- \) than for \( \text{Na}^+\text{DS}^- \). Trends in the values for cmc and \( \alpha \) implicated the binding of \( \text{NR}_{4}^{+} \) to micelles with an affinity greater than that for \( \text{Na}^+ \).\textsuperscript{29–33} We anticipated that the variation of the cation from \( \text{Na}^+ \) to \( \text{NR}_{4}^{+} \) would provide a way to manipulate interactions between \( \text{DS}^- \) and proteins and to probe the reaction of \( \text{DS}^- \) with BCA.

**Interaction of Proteins with Na\(^+\)DS\(^−\): Concentrations Below or Near the cmc.** Isomers describing the binding of Na\(^+\)DS\(^−\) to proteins have identified two types of association between protein and \( \text{DS}^- \) the binding of Na\(^+\)DS\(^−\) at low stoichiometric equivalencies at concentrations below the cmc and the cooperative binding of Na\(^+\)DS\(^−\) at high equivalencies at concentrations near or above the cmc. At concentrations ≤1 mM, Na\(^+\)DS\(^−\) binds to BSA (≈6 equiv) and cutinase (≈14 equiv) without a significant change in the structure of the protein; mushroom tyrosinase, creatine phosphokinase, ubiquitin, and soybean peroxidase are other examples of proteins that show this type of binding.\textsuperscript{34–37} Discrete stoichiometries of binding, at concentrations below the cmc, suggest the binding of individual molecules of Na\(^+\)DS\(^−\) (rather than micelles) with high affinity. At concentrations of Na\(^+\)DS\(^−\) near the cmc, the cooperative binding of large numbers of Na\(^+\)DS\(^−\) is accompanied (for most proteins) by the loss of native tertiary structure and by changes in the secondary structure of the native protein.\textsuperscript{20,40}

The concentration of Na\(^+\)DS\(^−\) that causes denaturation varies with the protein. For myoglobin\textsuperscript{37,41} and ferrocytochrome \( \text{C} \), the binding of monomeric Na\(^+\)DS\(^−\) at concentrations (≤1 mM) below the cmc is sufficient to cause denaturation. Proteins such as BCA and creatine phosphokinase, however, do not bind Na\(^+\)DS\(^−\) and remain folded until concentrations are near or above the cmc (4.0 mM in Tris-Gly).\textsuperscript{25,38} Rapid denaturation, at concentrations just below the cmc, suggests a mechanism in which the protein serves as a template for the condensation of \( \text{DS}^- \) and a site of nucleation for micelles of Na\(^+\)DS\(^−\). This hypothesis is consistent with the adsorption of Na\(^+\)DS\(^−\) onto self-assembled monolayers of CH\(_3\)-terminated alkanethiols on Au at concentrations below the cmc (by a factor of ∼10).\textsuperscript{43,44} Factors that may contribute to the resistance of some proteins (e.g., superoxide dismutase and avidin) to denaturation at 22 °C, even at concentrations well above the cmc, include the electrostatic repulsion of highly negatively charged proteins (e.g., pepsin) or structural features of the native proteins (e.g., extensive β-sheet structure).\textsuperscript{22,45}

We have studied the association of Na\(^+\)DS\(^−\) to ubiquitin (UBI), an 8.6 kDa single-chain protein, in detail.\textsuperscript{46} Upon equilibration in solutions of Na\(^+\)DS\(^−\) (0.05–10.00 mM in Tris-Gly buffer, 22 °C), UBI forms six groups of complexes (UBI-SDS\(_p\)) distinguishable by CE. Stoichiometries were approximately defined for several complexes (UBI-SDS\(_{−1}\), UBI-SDS\(_{−2}\), and UBI-SDS\(_{−3}\)).\textsuperscript{47} The identification of these complexes suggested that the denaturation of UBI (ultimately leading to UBI-SDS\(_{−4}\)) proceeds through several stable intermediates. UBI-SDS\(_{−1}\) retained the secondary structure of native UBI, and the stoichiometry of binding was approximately equivalent to the number of cationic residues (UBI has seven lysine and four arginine residues). This observation was consistent with the hypothesis that Na\(^+\)DS\(^−\) binds to proteins initially at the sites of cationic residues (inferred from similar observations in other proteins, e.g., lysozyme).\textsuperscript{5,10,34,48,49} An investigation of UBI-SDS\(_{−1}\) by NMR, however, found that SDS associates primarily at residues with hydrophobic side chains located near positively charged amino acid residues but not at the lysine and arginine residues themselves.\textsuperscript{50}

**Interactions of Proteins with Micelles of Na\(^+\)DS\(^−\):** Solutions of Na\(^+\)DS\(^−\) above the cmc contain both monomeric Na\(^+\)DS\(^−\) and micelles of Na\(^+\)DS\(^−\). Above the cmc, an increase in the concentration of Na\(^+\)DS\(^−\) leads to greater numbers of micelles (as well as changes in the size and shape of the micelles, as discussed below), but the concentration of monomeric Na\(^+\)DS\(^−\) remains constant.\textsuperscript{51–55} Interactions between native proteins and micelles of Na\(^+\)DS\(^−\) have been inferred from the analysis of proteins at concentrations of Na\(^+\)DS\(^−\) above the cmc. Rates of unfolding for S6 (a 101-residue polypeptide)\textsuperscript{28} increased (from \( 10^2 \) to \( 10^4 \text{ s}^{-1} \)) with concentrations of Na\(^+\)DS\(^−\) in the range of 10–100 mM, a range well above the cmc, and supported the conclusion that S6 unfolds upon direct association with a micelle of Na\(^+\)DS\(^−\).\textsuperscript{28,37,41} At concentrations of Na\(^+\)DS\(^−\) ≥ 200 mM (at which rodlike micelles begin to predominate), the rates of unfolding increased with a power-law dependence on the concentration of Na\(^+\)DS\(^−\) (\( k_{\text{obs}} \propto [\text{Na}^+\text{DS}^-]^{1.5} \)), suggesting that rodlike micelles are more reactive than spherical micelles in denaturing S6.\textsuperscript{58,59}

**Influence of Cations on the Formation of Micelles of \( \text{DS}^- \):** The assembly of anionic surfactants into micelles is opposed
by the electrostatic repulsion of negatively charged headgroups. The participation of cations, by association with the surface of the micelles and the screening of negatively charged headgroups, is essential to the formation of micelles of DS. The association of Na+ with micelles of DS has been inferred from the conductivity of solutions of Na(DS)2. Data from this method suggest a value of 0.23 for the degree of ionization (αD) for micelles of Na(DS)2 and therefore suggest a value of 0.77 for the degree of association (1 − αD) of Na+ with micellar DS (the degree of association is the fraction of electrostatic charge of the surfactant headgroup neutralized by counterions bound at the surface of the micelle). Other techniques corroborate the binding of Na+ to micelles of DS. Increasing concentrations of Na+ (e.g., by the addition of NaCl) result in lower cmc values; the cmc is inversely proportional to \(c_{\text{cmc}} + [\text{Na}^+]_{\text{added}}\), where \(c_{\text{cmc}}\) is the cmc in the absence of additional Na+.

The association of cations also influences the size and shape of micelles. With increasing [Na+] (from 20 to 140 mM), values of the aggregation number \(N\), the average number of surfactant molecules comprising an individual micelle) for micelles of Na(DS) increase from 60 to 90 and suggest a tighter packing of DS+, enabled by the screening of the SO4− headgroups by Na+. Rodlike micelles require tighter packing of the headgroups and greater screening by Na+ than in spherical micelles; the transition from spherical to rodlike micelles is observed at \(140 - 200 \text{ mM Na(DS)}_{2} \). Properties of Na(DS) and Na+(DS) prepared by ion-exchange chromatography. Values of the cmc in unbuffered aqueous solution decreased from 8.0 to 1.2 mM in the order of Na+ ≈ NMe4+ > NET4+ > NPr4+ > NBu4+. This order was also found in the values for the degree of ionization (αD) for micelles of Na(DS) and NR4+DS-, decreasing from 0.23 for Na(DS) to 0.17 for NBu4DS-. These trends suggested that the binding of NR4+ to micelles of DS− is more favorable than the binding of Na+ (presumably due to hydrophobic interactions). Values of the aggregation number \(N\) for Na(DS) and for each NR4+DS− were in the range of 60−100, indicating micelles that were spherical and approximately the same size, regardless of the cation. These data indicated that micelles of NPr4+DS− and NBu4+DS− differ from micelles of Na(DS) in three ways: (i) the surface density of the net negative charge, due to differences in the density of cations bound to the surfaces of micelles; (ii) hydrophobic interactions between NR4+ and DS− at the surfaces of micelles; (iii) hydrophobic interactions among NR4+. We chose to study Na+(DS)− and NR4+(DS)−, rather than the series of surfactants consisting of DS− and alkali metal ions, because differences in the properties of C(DS)− (C = Li+, Na+, K+, or Cs+) are relatively small as C+ is varied. For example, as C+ is changed from Li+ to Cs+, the cmc values decrease from 8.9 to 6.1 mM (in unbuffered solution at 25°C). The range of cmc values is broader for NR4+(DS)− and Na+(DS)− (1.2−8.0 mM).

**Solutions of C(DS)− Tris-Gly Buffer.** Values of the cmc or NR4+(DS)− in buffered solutions have not been reported. We determined these values for Na+(DS)− and NR4+(DS)− in Tris-Gly buffer with established methods using fluorescent probes (pyrene and 8-anilinonaphthalenesulfonate) or UV-absorbing probes (e.g., 2-naphtalenecarbinol) in CE experiments. CE was also useful for characterizing the electrophoretic mobility of micelles of Na+(DS)− and NR4+(DS)−. The method used to analyze the partitioning of UV-absorbing probes into micelles is described in the Supporting Information.

Concentrations of C(DS)− in the range of 0.0−10.0 mM allowed us to analyze proteins in solutions below and above the cmc. Previous work showed that this range is convenient for analyzing reactions of Na+(DS)− with proteins at 22°C; for many proteins, the formation of proteinNa+(DS)− = DS− n,sat is rapid at concentrations of Na+(DS)− within this range. Concentrations of Na+(DS)− and NR4+(DS)− were also low (≪200 mM) to justify the assumption that micelles of Na+(DS)− and NR4+(DS)− were spherical and approximately the same size (values of \(N\) in the range of 60−100).

We analyzed proteins in solutions of C(DS)− in Tris-Gly buffer (25 mM Tris−192 mM glycine, pH 8.4), a buffer that is typically used in SDS-PAGE. The ions in Tris-Gly buffer are the cation (HOC(CH2)3CH2)NH+, 8.3 mM) and a mixture of the anions of glycine (H2NCH2CO2−, 8.3 mM) and the zwitterion of glycine (H2NCH2CO2H, 184 mM) (concentrations were determined by calculation, using pK\(_a\) values reported for Tris of 8.06 and for glycine of 2.3, 4.3, 7.7, and 9.7). It is possible that buffer cations participate in the interactions between protein and DS−. The buffer cations, however, are not responsible for data distinguishing the influence of C+ (Na+ and NR4+) upon the formation of protein−DS− because all of our experiments used Tris-Gly at the same concentration. We did not test buffers other than Tris-Gly (e.g., phosphate, carbonate, or borate).

**BCA as a Model Protein.** We and others have used BCA extensively as a model protein in biophysical studies aimed at protein−surfactant interactions, and rational ligand design. BCA is monomeric and has no disulfide bonds. Native BCA has both α-helical and β-sheet elements; a nine-stranded β-sheet forms the core of the protein. The electrophoretic migration of BCA in SDS-PAGE is consistent with its molecular weight (29.1 kDa). On the basis of stoichiometries of binding that are typical of proteins analyzed in SDS-PAGE, we assume that the formation of BCA−DS− n,sat involves the association of ~150 equiv of DS− (i.e., \(n \approx 150\), estimated from the binding of ~1.4 g of Na(DS)− for every 1 g of protein).

**Analysis of the Formation of BCA−DS− n,sat by CE.** We used CE for its ability to detect and characterize the association of DS− with proteins. UV absorbance (214 nm) of the amides of the polypeptide backbone allowed us to detect BCA in all possible forms (i.e., native or denatured in complexes with DS−), without complications from DS− and C+, which are transparent in the UV.

CE resolves analytes according to their electrophoretic mobility—the ratio of electrostatic charge to hydrodynamic drag—in free solution. Equation 2 is an expression for the mobility of a native protein (\(\mu_n\)) having net electrostatic charge \(Q_n\) and mass \(M_G\): \(\mu_n\) is a constant that depends on the shape of the analyte, often estimated to be \(2/3\) for...
The binding of DS⁻ (and the formation of protein−DS⁻ₙ) results in values of electrophoretic mobility that are higher than μN. Equation 3 shows the dependence of the mobility of protein−DS⁻ₙ (μden) on the electrostatic charge and mass of DS⁻ (ΔZ_{DS}− and M_{DS}−) and a correction factor (C_p) for the nonlinear dependence of μ on Z at high surface potentials (>25 mV).

$$\mu_{den} = C_pC_v \frac{Z_0 + m \Delta Z_{DS}−}{(M_0 + nM_{DS}−)}$$

Values of μ_{den} are greater than μN because the increase in negative charge is significant (upon the binding of DS⁻ at a ratio of 1 DS⁻ for every 2 amino acid residues) and is only partially compensated for by an increase in the hydrodynamic drag (due to an increase in mass by a factor of ~2.4). The amount of negative charged contributed by the binding of each equivalent of DS⁻ (ΔZ_{DS}−) is, however, less than one unit, and probably ~0.9, as a result of charge regulation. Furthermore, eq 3 does not include contributions from cations associated with protein−DS⁻ₙ; the association of Na⁺ has typically been ignored in the analysis of μ_{den}. (Expressions that include contributions from C⁺ are discussed in the Supporting Information.) In practice, values of μ_{den} for protein−DS⁻ₙ are typically 18−22 cm² V⁻¹ s⁻¹⁻¹.

### Table 1. Values of the cmc of n-C_{12}H_{25}SO_{4}⁻ as a Function of the Cation

<table>
<thead>
<tr>
<th>Cation</th>
<th>cmc (mM)</th>
</tr>
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<tbody>
<tr>
<td>Na⁺</td>
<td>C⁻</td>
</tr>
<tr>
<td>Na⁺</td>
<td>IE²</td>
</tr>
<tr>
<td>NMe₄⁺</td>
<td>IE</td>
</tr>
<tr>
<td>NEt₄⁺</td>
<td>IE</td>
</tr>
<tr>
<td>NPr₄⁺</td>
<td>IE</td>
</tr>
<tr>
<td>NBu₄⁺</td>
<td>IE</td>
</tr>
</tbody>
</table>

¹Tris-Gly buffer: 25 mM Tris−192 mM glycine, pH 8.4. ²Determined by analyzing the fluorescence spectrum of pyrene in solutions of surfactant (data in the Supporting Information, Figure S1). ³Determined by analyzing the mobility of 2-naphthalencarbinol in solutions of surfactant, by CE (data in Figure S1D). ⁴Values taken from ref 29. ⁵Recrystallized twice from ethanol. ⁶Prepared by ion exchange (as described in the text) from a single batch of recrystallized Na⁺DS⁻.

### RESULTS AND DISCUSSION

**Preparation of NR₄⁺DS⁻ from Na⁺DS⁻ by Cation Exchange.** We prepared surfactants Na⁺DS⁻, NMe₄⁺DS⁻, NEt₄⁺DS⁻, NPr₄⁺DS⁻, and NBu₄⁺DS⁻ by using a three-step procedure: (i) recrystallization of Na⁺DS⁻ from ethanol (twice); (ii) the replacement of Na⁺ with H₂O⁺ by cation-exchange chromatography; and (iii) the reaction of H₂O⁺DS⁻ with standard solutions of C⁺OH⁻ (NaOH, NMe₄OH, NEt₄OH, NPr₄OH, or NBu₄OH). We used this procedure to prepare samples of Na⁺DS⁻ and NR₄⁺DS⁻ derived from a single batch of recrystallized Na⁺DS⁻. Samples of Na⁺DS⁻ prepared by two different
methods—recrystallization (step 1 only) or by ion-exchange (steps i–iii)—showed agreement with respect to cmc values, 1H NMR spectra, and data characterizing the interaction of Na⁺DS⁻ with proteins. This agreement suggested that the procedure for replacing Na⁺ with C⁺ did not alter samples of DS⁻ in unintended ways (e.g., by hydrolyzing C₁₂H₂₅−OSO₃⁻ groups or adding impurities from the resin). We prepared stock solutions of each surfactant in Tris-Gly buffer (25 mM Tris−25 mM glycine, pH 8.4).

**Measurement of the cmc.** Methods for estimating the cmc, by detecting micelles with pyrene or ANS as fluorescent probes, have been described in detail previously.⁷⁷,⁷⁹ We analyzed the fluorescence spectrum of pyrene (2 μM) in solutions of Na⁺DS⁻ and NR₄⁺DS⁻ in Tris-Gly buffer to determine the cmc of each surfactant. Values of the cmc are reported in Table 1. (Examples of fluorescence spectra and analysis are in the Supporting Information, Figure S1A-B.)

We confirmed these values with a second method. We analyzed the partitioning of 2-naphthalencarbinol (naph) into micelles of C⁺DS⁻ by CE. Electrophoretic mobility values for naph (μnaph) indicated the rapid equilibration of naph between aqueous buffer and negatively charged micelles of DS⁻. Estimates of the cmc, obtained by extrapolating curves relating μnaph to [C⁺DS⁻], were within 0.3 mM of values determined by the fluorescence of pyrene (also in Table 1).⁹³

**Replacing Na⁺ with NR₄⁺ Decreases the cmc of DS⁻ in Tris-Gly.** Values of the cmc in Tris-Gly buffer (Table 1) follow the order Na⁺ > NMe₄⁺ > NEt₄⁺ > NPr₄⁺ > NBu₄⁺. The trend is the same as the trend in unbuffered aqueous solution (last column in Table 1, taken from ref 29). It suggests that the binding of cations to micelles of DS⁻ is more favorable for increasing hydrophobic cations because the formation of micelles depends upon the screening of negatively charged R−OSO₃⁻ headgroups by cations. Values of the cmc are lower for each surfactant in Tris-Gly than in unbuffered solution and are probably the result of additional screening of DS⁻ by the buffer ions in Tris-Gly (ionic strength ~10 mM) upon micelle formation.

Zana also identified trends in θ and N for NR₄⁺DS⁻ in unbuffered solution,⁵⁸ these trends probably hold in solutions of Tris-Gly as well. We assumed that micelles of Na⁺DS⁻ and NR₄⁺DS⁻ are spherical and approximately the same size (aggregates of 60−90 equiv of DS⁻)⁵⁸ at concentrations through 10.0 mM and that the quantity of cations associated with micelles of DS⁻ increases in the order of Na⁺ < NMe₄⁺ < NEt₄⁺ < NPr₄⁺ < NBu₄⁺.

**Hypotheses Rationalizing the Influence of C⁺ on Reactions of DS⁻ with Proteins.** The trend in the cmc of C⁺DS⁻ describes the influence of C⁺ on the formation of micelles of DS⁻; it also suggests several hypotheses for how C⁺ may influence the interaction of DS⁻ with proteins. We considered three cases:

(i) If denaturation is induced by the association of monomeric DS⁻ to proteins, then the rates of denaturation in solutions below the cmc should increase with the concentration of DS⁻ but should be independent of C⁺. Equation 4a describes a process in which α equiv of monomeric DS⁻ (DS⁻<sub>mon</sub>) associates with the protein in the initial step, causing the protein to unfold partially, to the cooperative association of DS⁻ and denaturation. Rates of denaturation should not increase with the concentration of C⁺DS⁻ above the cmc because additional DS⁻ above the cmc is present only in micelles. Because cmc values indicate the maximum possible concentration of monomeric DS⁻ in a solution of C⁺DS⁻, the hypothesis of eq 4a and the cmc values in Table 1 predict the rates of denaturation in the order of Na⁺ > NMe₄⁺ > NEt₄⁺ > NPr₄⁺ > NBu₄⁺ in solutions above the cmc.

\[
\text{protein} + \alpha \text{DS}^-_{\text{mon}} \rightleftharpoons [\text{protein} - (\text{DS}^-)_{\alpha}]^+ \\
\text{DS}^- \rightarrow \text{protein} + (\text{DS}^-)_{\alpha,\text{sat}} \tag{4a}
\]

(ii) If denaturation is induced by the cooperative condensation of DS⁻ onto the protein (eq 4b), in a process similar to the formation of C⁺DS⁻ micelles, then rapid denaturation should occur at concentrations of C⁺DS⁻ near the cmc. According to this hypothesis, denaturation would be rapid near the cmc because C⁺ would promote

![Figure 1.](image-url)
the aggregation of DS$^-$ onto the protein (the protein provides a template) in the same way that C$^+$ promotes the aggregation of DS$^-$ into micelles. In eq 4b, \([\text{protein}-(\text{DS}_{\text{mic}},b)]\) is the structure of the protein as it nucleates the condensation of \(b\) equiv of DS$_{\text{mon}}$ and the formation of a micelle of DS$^-$. This hypothesis predicts rapid denaturation at concentrations of C$^+$DS$^-$ that follow the trend in the cmc values and therefore at concentrations that increase in the order of NBU$_4^+ < \text{NPr}_4^+ < \text{NEt}_4^+ < \text{NMe}_4^+ < \text{Na}^+$. 

\[
\begin{align*}
\text{protein} + b - \text{DS}_{\text{mon}} & \rightarrow [\text{protein} - (\text{DS}_{\text{mic}},b)]^+ \\
& \rightarrow \text{protein}^D - (\text{DS}^-)_{n,\text{sat}} 
\end{align*}
\tag{4b}
\]

(iii) If denaturation is induced by interactions with preformed micelles of C$^+$DS$^-$ (eq 4c), then the kinetics of denaturation should characterize the influence of C$^+$ bound to micelles of DS$^-$. In eq 4c, \([\text{protein}-(\text{DS}_{\text{mic}},-60-100)]\) is a structure involving the direct association of protein with a preformed micelle. We could not, however, predict whether NR$_4^+$ bound to the surfaces of micelles would increase the rates of reaction (through favorable hydrophobic interactions of proteins with both DS$^-$ and NR$_4^+$) or decrease the rates (by blocking the interaction of micelles of DS$^-$ with proteins).

\[
\begin{align*}
\text{protein} + (\text{DS}_{\text{mic}},-60-100) & \rightarrow [\text{protein} - (\text{DS}_{\text{mic}},-60-100)]^+ \\
& \rightarrow \text{protein}^D - (\text{DS}^-)_{n,\text{sat}} 
\end{align*}
\tag{4c}
\]

Testing Hypotheses by Analyzing the Denaturation of BCA. The mechanism of denaturation of BCA in Na$^+$DS$^-$ is not known. Previous kinetic studies showed that the rate of denaturation increases by a factor of $10^6$ with a small increase in the concentration of Na$^+$DS$^-$ (2.5–4.0 mM) just below the cmc (4.0 mM in Tris-Gly buffer). The rates also increase with the concentration of Na$^+$DS$^-$ beyond the cmc, but only by a factor of less than 10 in the range of 4.0–10.0 mM Na$^+$DS$^-$. These data are ambiguous with regard to the mechanism of denaturation of BCA. The rates are sensitive to concentrations of Na$^+$DS$^-$ near the cmc and are therefore consistent with the idea that denaturation is induced by the assembly of DS$^-$ into micelles, templated by BCA (i.e., hypothesis ii). They do not, however, rule out hypothesis i, which proposes that several equivalents of monomeric DS$^-$ interact with BCA to induce denaturation but that these interactions are not related to micelle formation. By examining the effects of replacing Na$^+$ with NR$_4^+$ and testing the hypotheses of eqs 4a and 4b, we hoped to shed light on the mechanism of the reaction.

Analysis by SurfCE: Formation of BCA$^D$–DS$^-_{n,\text{sat}}$ in Solutions of Na$^+$DS$^-$. We used SurfCE to carry out and analyze the reactions of BCA with Na$^+$DS$^-$. Traces obtained by injecting and analyzing samples of native BCA (8 $\mu$M) in capillaries with 0–10.0 mM Na$^+$DS$^-$ showed peaks for either native BCA or BCA$^D$–DS$^-_{n,\text{sat}}$ (Figure 1A). SurfCE showed that the generation of BCA$^D$–DS$^-_{n,\text{sat}}$ is rapid at concentrations $\geq$ 6.0 mM; the reaction is complete within the interval of time that native BCA takes to reach the detector ($\sim$2.6 min.), under the conditions used to collect the data in Figure 1A. The broadness of the peak in 4.0 mM Na$^+$DS$^-$ indicated that the time required for conversion to BCA$^D$–DS$^-_{n,\text{sat}}$ is 2 to 3 min at the cmc.

In the range of 3.00–3.40 mM Na$^+$DS$^-$, SurfCE data showed shoulders of peaks for BCA$^D$–DS$^-_{n,\text{sat}}$ (Figure 1B); the conversion of BCA to BCA$^D$–DS$^-_{n,\text{sat}}$ takes place over several minutes at these concentrations. Lower applied voltages (5–20 kV) in SurfCE provided longer windows of observation. (Retention times of native BCA were 4–16 min.) Longer periods of time allowed for complete conversion to BCA$^D$–DS$^-_{n,\text{sat}}$ in 3.00 mM Na$^+$DS$^-$ (data available in Figure S2). Data obtained by SurfCE were therefore useful for examining reactions taking place over several minutes and provided qualitative information about the rates of reactions (i.e., whether reactions take place over periods of time that are greater than, less than, or approximately the same as the time required for the migration of native BCA in CE experiments).

A further discussion of the electropherograms in Figure 1B is provided in the Supporting Information. Quantitative analysis of the absorbance over the broad range in mobility (4 $< \mu < 22$ cm$^2$ kV$^{-1}$ min$^{-1}$) shows that the shapes of the peaks are consistent with pseudo-first-order kinetics for the conversion of native BCA into the denatured state.
BCA to BCAD− during the SurfCE run (Figure S4). Rate constants determined by analyzing the peak shapes agreed with rate constants measured by an independent method (analysis of the fraction of BCA present in native form, measured using a fluorescent inhibitor of BCA, dansyl amide; Figure S4).

The formation of BCAD− at concentrations of Na+DS− below the cmc (4.0 mM) showed that interactions with monomeric DS− can lead to the formation of BCAD−, preformed micelles of Na+DS− are not required for the denaturation of BCA. An analysis of BCA in solutions of Na+DS− by SurfCE did not detect intermediates along the pathway to BCAD−.

**NR4+ Decreases the Rate of Formation of BCAD− in SurfCE.** SurfCE experiments analyzing BCA in solutions of NR4+DS− revealed that the structure of the cation influences the interaction of DS− with BCA. Figure 2 shows the analysis by SurfCE of BCA in solutions of NMe4+DS− and NEt4+DS− (0–10 mM); data for NPr4+DS− and Nu4+DS− are in the Supporting Information (Figure S7). The amount of BCAD−DS−, generated in 10.0 mM NR4+DS− decreased across the series in the order of NMe4+DS− ≈ NEt4+DS− ≈ NPr4+ ≈ Nu4+. Solutions of NEt4+DS−, even at concentrations well above the cmc, generated only small amounts of BCAD−DS− (<20% conversion within the ~3 min period of time allowed by SurfCE); only native BCA was observed in solutions of 0–10 mM NPr4+DS− and Nu4+DS−. The influence of C+ in the SurfCE experiments was incompatible with the prediction of the hypothesis of eq 4b (i.e., that the formation of micelles of DS− would induce the formation of BCAD−DS−). We concluded that the formation of BCAD−DS− in solutions of NR4+DS− is not related to the formation of micelles of NR4+DS−. The data, however, remain compatible with the prediction of eq 4a: the substitution of NR4+ for Na+ results in decreasing amounts of BCAD−DS− because the denaturation of BCA is induced by monomeric DS−. We hypothesized that the formation of smaller amounts of BCAD−DS− in NR4+DS− with increasingly hydrophobic NR4+ was the result of decreasing concentrations of monomeric DS− due to micelle formation at lower cmc values.

SurfCE data in Figure 2, however, provided a window of observation limited to reactions of BCA with DS− that take place within 2 to 3 min. From these experiments, the underlying reasons for the absence of BCAD−DS− in solutions of NPr4+DS− and Nu4+DS− are unclear. Other experiments—observation over longer periods of time and the acceleration of reactions by heating—were necessary to determine whether the SurfCE results were the outcomes of thermodynamics favoring native BCA or the slow kinetics of denaturation.

**Generation of BCAD− in Nu4+DS−.** BCA remained folded in solutions of 10 mM Nu4+DS−, kept at 22 °C, for several weeks. After 3.5 and 15 days of incubation, analysis by capillary zone electrophoresis (CZE) showed that BCA in the sample was in its native form (Figure 3). (An analysis of the samples after 100 days showed a decrease in the total absorbance;...
we did not determine the reason for the apparent loss in total protein.) We identified the small peak that developed at \( \mu \approx 17 \text{ cm}^2 \text{ kV}^{-1} \text{ min}^{-1} \) as BCA\(^{\text{D}}\)–DS\(^{-\text{n}_{\text{sat}}} \) by showing that it has the same mobility as the major peak for samples heated to 40 °C (for 32 h). These results showed that the formation of BCA\(^{\text{D}}\)–DS\(^{-\text{n}_{\text{sat}}} \) in NBu\(_4^+\)DS\(^-\) is slow and that CZE data showing native BCA in 10 mM NBu\(_4^+\)DS\(^-\) are early snapshots of a slow reaction at 22 °C (with a half-life greater than 10 days). The formation of BCA\(^{\text{D}}\)–DS\(^{-\text{n}_{\text{sat}}} \) at 22 °C is slower in 10.0 mM NBu\(_4^+\)DS\(^-\) than in 10.0 mM Na\(^+\)DS\(^-\) by a factor of >10\(^4\). (The approximation is based on reactions that require an amount of time of <1 min in 10.0 mM Na\(^+\)DS\(^-\) but >10 days in 10.0 mM NBu\(_4^+\)DS\(^-\).) We inferred that the results of SurfCE in Figure 2 were due to the kinetics of the system: decreasing amounts of BCA\(^{\text{D}}\)–DS\(^{-\text{n}_{\text{sat}}} \) in SurfCE are the result of a decrease in the rates of reaction between DS\(^-\) and BCA in solutions containing Na\(^+\) or NR\(_4^+\).

**Kinetics of Formation of BCA\(^{\text{D}}\)–DS\(^{-\text{n}_{\text{sat}}} \) at 40 °C.** To circumvent the sluggish kinetics in solutions of NBu\(_4^+\)DS\(^-\) at 22 °C, we analyzed the rate of formation of BCA\(^{\text{D}}\)–DS\(^{-\text{n}_{\text{sat}}} \) at 40 °C (a temperature still substantially below the melting temperature of BCA, which is 64 °C).\(^6^4\) Traces collected by CZE for times of up to 32 h showed the conversion of BCA to BCA\(^{\text{D}}\)–DS\(^{-\text{n}_{\text{sat}}} \) in solutions of 10 mM NBu\(_4^+\)DS\(^-\) (Figure 4A).

Peak areas for native BCA and BCA\(^{\text{D}}\)–DS\(^{-\text{n}_{\text{sat}}} \) allowed us to quantify the amount of native BCA and to estimate a value of 1.8 × 10\(^{-5}\) s\(^{-1}\) for the pseudo-first-order rate constant for the disappearance of native BCA (Figure 4B). We ignored the decrease in the concentration of NBu\(_4^+\)DS\(^-\) expected over the course of the reaction, caused by the absorption of ~150 equiv of DS\(^-\) by BCA.\(^6^4\) The rate of formation of BCA\(^{\text{D}}\)–DS\(^{-\text{n}_{\text{sat}}} \) in 10.0 mM NR\(_4^+\)DS\(^-\) at 40 °C (2.6 × 10\(^{-4}\) s\(^{-1}\)) was greater than that in 10.0 mM NBu\(_4^+\)DS\(^-\) by a factor of ~10 (Figure S8).

The formation of BCA\(^{\text{D}}\)–DS\(^{-\text{n}_{\text{sat}}} \) at 40 °C in solutions of 10.0 mM Na\(^+\)DS\(^-\), NMMe\(_4^+\)DS\(^-\), and NEt\(_4^+\)DS\(^-\) was too rapid for analysis by CZE. Instead of using CZE, we used a spectroscopic method to measure the rates of denaturation of BCA under these conditions. We analyzed the rates of denaturation of BCA by measuring the fluorescence intensity of dansyl amide (DNSA) bound to the active site of folded BCA; the fluorescence of DNSA at 460 nm increases by a factor of >100 upon binding the active site of BCA.\(^5^5,\(^9^5,\(^9^6\) By adding DNSA to aliquots taken from reaction mixtures of BCA and C\(^{3}\) at points over time and measuring the fluorescence of DNSA, we quantified the amount of BCA that remained folded and determined the rate of denaturation. Figure 5 shows rate constants for the denaturation of BCA in solutions of Na\(^+\)DS\(^-\) and NR\(_4^+\)DS\(^-\) at 40 °C. (Examples of raw data for DNSA fluorescence are available in Figure S9.)

Denaturation rates spanned factors of ~10\(^4\) in solutions of 10.0 mM C\(^{3}\) at 40 °C and decreased in the order of Na\(^+\) ~ NMMe\(_4^+\) > NEt\(_4^+\) > NPr\(_4^+\) > NBu\(_4^+\). Denaturations in solutions of either Na\(^+\)DS\(^-\), NMMe\(_4^+\)DS\(^-\), or NEt\(_4^+\)DS\(^-\) were rapid and almost complete within the time required to mix BCA with solutions of surfactant manually (~4 s, points in Figure 5 overlap).

The data in Figure 5 reveal three key features in the kinetics of denaturation of BCA: (i) Rates are similar for different C\(^{3}\) concentrations when the concentration of C\(^{3}\) is below the cmc. (ii) The increase in rate essentially stops at the cmc (rates increase by factors of ~10 as [C\(^{3}\)] rises just beyond the cmc and are indistinguishable at [C\(^{3}\)] well above the cmc).\(^5^7\) (iii) The increase in the rate constant is not a simple function of the concentration of C\(^{3}\). Below the cmc, the relationship between the rate of denaturation and the concentration of DS\(^-\) is approximately given by eqs 5a and 5b.

\[
\frac{k_{\text{den}} \propto 10^{\Delta G^T}}{k_{\text{den}} \propto \log \Delta G^T \propto [\text{DS}^-]} \quad (5a)
\]

In eq 5, \( k_{\text{den}} \) is the rate constant for denaturation (with units of s\(^{-1}\)), and [DS\(^-\)] (mM) is the concentration of C\(^{3}\) below the cmc.
Figure 6. Analysis of equilibrated samples of BCA$^{D-}\text{DS}^{-\text{n,sat}}$ by CZE. Samples of BCA$^{D-}\text{DS}^{-\text{n,sat}}$, prepared by heating BCA in either 10.0 mM NBu$_4^+\text{DS}^-$ or 10.0 mM Na$^+\text{DS}^-$ for 56 h, were diluted 10-fold in solutions of 10.0 mM DS$^-$ and mixtures of NBu$_4^+$ and Na$^+$. After equilibration for 20 min at 22 °C, samples were analyzed by CZE. Traces are shown for the analysis of BCA$^{D-}\text{DS}^{-\text{n,sat}}$ prepared initially in NBu$_4^+\text{DS}^-$ (peaks up) or in Na$^+\text{DS}^-$ (peaks down). Samples analyzed in [NBu$_4^+'] = [\text{Na}^+] = 5.0 mM (marked by *) were equilibrated at 40 °C for 6 h.

Scheme 1

According to this interpretation of Figure 5, NBu$_4^+$ affects the reaction of DS$^-$ with BCA by setting an upper limit on the concentration of monomeric DS$^-$. In any solution of C'DS$^-$, the formation of micelles at the cmc limits the concentration of monomeric DS$^-$/C0 that can be reached in the presence of C'. We conclude that the rate of denaturation is determined by the concentration of monomeric DS$^-$/C0 and not by micelles of C'DS$^-$/C0. The kinetics indicate a rate-limiting step that involves monomeric DS$^-$/C0. Values for the rate constant $k_{\text{den}}$ do not have a linear dependence on [DS$^-$/C0]; the rate-limiting step therefore involves the interaction of BCA with many equivalents of DS$^-$/C0. (We do not know how many equivalents of DS$^-$/C0 from these data.) Although the interactions of monomeric DS$^-$/C0 with BCA may be cooperative, the data in Figure 5 suggest that these interactions are not the same as those involved in the self-association of monomeric DS$^-$/C0 to form micelles of DS$^-$/C0. Instead, the initial step in the denaturation may be the association of monomeric DS$^-$/C0 (possibly several equivalents) with native BCA that induces local unfolding and the exposure of the hydrophobic surface area; the subsequent association of more monomeric DS$^-$/C0 to partially unfolded protein may cause the denaturation of BCA and the formation of BCA$^{D-}\text{DS}^{-\text{n,sat}}$. Because we do not observe shifts in $\mu$ in SurfCE to suggest stable complexes of BCA with a small number of equivalents of DS$^-$/C0, we conclude that the unfolding of BCA is highly cooperative and rapid when the concentration of C'DS$^-$ reaches an appropriate value.

Values of Mobility for BCA$^{D-}\text{DS}^{-\text{n,sat}}$ Suggest That NBu$_4^+$ and Na$^+$ Associate with BCA$^{D-}\text{DS}^{-\text{n,sat}}$. Values of $\mu$ for BCA$^{D-}\text{DS}^{-\text{n,sat}}$ were lower in 10.0 mM NBu$_4^+\text{DS}^-$ (16.8 cm$^2$ kV$^{-1}$ min$^{-1}$) than in 10.0 mM Na$^+\text{DS}^-$ (22.0 cm$^2$ kV$^{-1}$ min$^{-1}$) (Figure 6). The shift in $\mu$ suggested the influence of the cation on the composition of BCA$^{D-}\text{DS}^{-\text{n,sat}}$. To demonstrate that the mobility of BCA$^{D-}\text{DS}^{-\text{n,sat}}$ depends on the cation (Na$^+$ or NBu$_4^+$) in solution but does not depend on how BCA$^{D-}\text{DS}^{-\text{n,sat}}$ is prepared, we carried out the reactions in Scheme 1.

We generated two samples of BCA$^{D-}\text{DS}^{-\text{n,sat}}$—one in NBu$_4^+\text{DS}^-$ and another in Na$^+\text{DS}^-$—by heating BCA (8 μM) in a solution of 10.0 mM NBu$_4^+\text{DS}^-$ or 10.0 mM Na$^+\text{DS}^-$ (40 °C, 56 h). We analyzed BCA$^{D-}\text{DS}^{-\text{n,sat}}$ from both preparations after treating the samples to a three-step procedure: (i) concentration of the protein to ~80 μM using a membrane with a 10 kDa molecular weight cutoff; (ii) 10-fold dilution into 10.0 mM NBu$_4^+\text{DS}^-$ (and, in parallel, 10-fold dilution into 10.0 mM Na$^+\text{DS}^-$); and (iii) incubation for 20 min at 22 °C. Both preparations of BCA$^{D-}\text{DS}^{-\text{n,sat}}$, after equilibration in 10.0 mM NBu$_4^+\text{DS}^-$, gave CZE traces showing agreement in $\mu$ (~16.8 cm$^2$ kV$^{-1}$ min$^{-1}$, top set of traces in Figure 6; running buffer contained 10.0 mM NBu$_4^+\text{DS}^-$). CZE traces also agreed for BCA$^{D-}\text{DS}^{-\text{n,sat}}$ that had been equilibrated in 10.0 mM Na$^+\text{DS}^-$ ($\mu \approx 22.0$ cm$^2$ kV$^{-1}$ min$^{-1}$, bottom set of traces in Figure 6).

We drew three conclusions from the results: (i) Conversion between BCA$^{D-}\text{DS}^{-\text{n,sat}}$ with $\mu = 16.8$ and BCA$^{D-}\text{DS}^{-\text{n,sat}}$ with $\mu = 22.0$ is reversible at 22 °C (t ≈ 20 min; intervals of time allowed by SurfCE were not sufficient to equilibrate the sample). (ii) The value of $\mu$ for BCA$^{D-}\text{DS}^{-\text{n,sat}}$ depends on the cation, suggesting that Na$^+$ and NBu$_4^+$ associate with BCA$^{D-}\text{DS}^{-\text{n,sat}}$. 

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Figure 7. Continued
and that the complexes should be viewed as BCA\textsuperscript{D}−DS\textsuperscript{−}\textsubscript{\text{r, sat}} \cdot (C\textsuperscript{−}).

(iii) The influence of Na\textsuperscript{+} and NBu\textsubscript{4}\textsuperscript{+} on the mobility of BCA\textsuperscript{D}−DS\textsuperscript{−}\textsubscript{\text{r, sat}} is determined by association with BCA\textsuperscript{D}−DS\textsuperscript{−}\textsubscript{\text{r, sat}} and is independent of how BCA\textsuperscript{D}−DS\textsuperscript{−}\textsubscript{\text{r, sat}} is formed.

An analysis of the data in Figure 6 used to estimate the stoichiometry and affinity of NBu\textsubscript{4}\textsuperscript{+} and Na\textsuperscript{+} for BCA\textsuperscript{D}−DS\textsuperscript{−}\textsubscript{\text{r, sat}} is provided in the Supporting Information (Figures S10−S12).

Other Proteins. We wished to determine whether the influence of NR\textsubscript{4}\textsuperscript{−} on the formation of aggregates of protein and DS\textsuperscript{−} was similar for BCA and other proteins. We used SurfCE to survey seven additional proteins (listed in Table 2) in solutions of 0−10.0 mM Na\textsuperscript{+}DS\textsuperscript{−} and NBu\textsubscript{4}\textsuperscript{+}DS\textsuperscript{−}.

Figure 7 shows SurfCE traces for these proteins in solutions of Na\textsuperscript{+}DS\textsuperscript{−} and NBu\textsubscript{4}\textsuperscript{+}DS\textsuperscript{−}; data for analysis in solutions of NMe\textsubscript{4}\textsuperscript{+}DS\textsuperscript{−}, NEt\textsubscript{4}\textsuperscript{+}DS\textsuperscript{−}, and NPr\textsubscript{4}\textsuperscript{+}DS\textsuperscript{−} are available in Figure S13.

The results of SurfCE revealed a range of behaviors among the proteins tested. For several proteins (\(\beta\)-LacB, \(\alpha\)-Lac, MYO, and UBI), SurfCE data collected in NR\textsubscript{4}DS\textsuperscript{−} and Na\textsuperscript{+}DS\textsuperscript{−} was
similar, suggesting that the influence of the cation on the formation of protein–DS\textsuperscript{-} was relatively minor. For others (BCA, CPK,\textsuperscript{88} and β-LacA), replacing Na\textsuperscript{+} with NBu\textsubscript{4}\textsuperscript{+} decreased the amount of protein–DS\textsuperscript{-} generated in SurfCE. For CPB, the effect of the cation was distinctive; replacing Na\textsuperscript{+} with NPr\textsubscript{4}\textsuperscript{+} or NBu\textsubscript{4}\textsuperscript{+} enabled the formation of complexes of CPB with DS\textsuperscript{−} that are not observed in solutions of Na\textsuperscript{+}DS\textsuperscript{−}. NMe\textsubscript{4}\textsuperscript{+}DS\textsuperscript{−}, NEt\textsubscript{4}\textsuperscript{+}DS\textsuperscript{−}, and NPr\textsubscript{4}\textsuperscript{+}DS\textsuperscript{−} are available in Figure S13.

The influence of NPr\textsubscript{4}\textsuperscript{+} appeared to distinguish these proteins by their mechanism of denaturation. Proteins that showed only minor changes upon replacement of Na\textsuperscript{+} with NPr\textsubscript{4}\textsuperscript{+} in SurfCE were also those that denatured in solutions of Na\textsuperscript{+}DS\textsuperscript{−} at concentrations (1 to 2 mM) well below the cmc (i.e., β-LacB, α-Lac, MYO, and UBI). The denaturation of these proteins may be induced by interactions with monomeric DS\textsuperscript{−} at low concentration; we would not expect the influence of NPr\textsubscript{4}\textsuperscript{+} on the cmc or on the properties of micelles of NPr\textsubscript{4}\textsuperscript{+}DS\textsuperscript{−} to affect the denaturation of these proteins.

Proteins that did not denature in NPr\textsubscript{4}\textsuperscript{+}DS\textsuperscript{−} and NBu\textsubscript{4}\textsuperscript{+}DS\textsuperscript{−} were also those that require concentrations of Na\textsuperscript{+}DS\textsuperscript{−} (3 to 4 mM) near the cmc for denaturation (BCA and CPK). These concentrations of Na\textsuperscript{+}DS\textsuperscript{−} may be important for denaturation because the affinity of these proteins for DS\textsuperscript{−} may be low. As was the case for BCA, the influence of NPr\textsubscript{4}\textsuperscript{+} on the denaturation of CPK may be due to the concentrations of monomeric DS\textsuperscript{−} that are limited by the formation of micelles of NPr\textsubscript{4}\textsuperscript{+}DS\textsuperscript{−} at decreasing values of cmc. The formation of CPB-DS\textsubscript{−} in NBu\textsubscript{4}\textsuperscript{+}DS\textsuperscript{−} with mobilities of 6–10 cm\textsuperscript{2} kV\textsuperscript{−1} min\textsuperscript{−1} suggests a different type of involvement of NBu\textsubscript{4}\textsuperscript{+}; we do not understand the reason for the gradual shift in \(\mu\) for this protein other than it seems to involve the association of DS\textsuperscript{−} without complete denaturation.\textsuperscript{99}

Application of NBu\textsubscript{4}\textsuperscript{+}DS\textsuperscript{−} in Methods for Separating Proteins. In principle, the selective and differential formation of protein–DS\textsuperscript{−} from proteins in a mixture should be useful in the analysis or purification of proteins. Güldiken demonstrated the resolution of proteins in CE in solutions of Na\textsuperscript{+}DS\textsuperscript{−}, below the cmc, by exploiting differences in the rates of denaturation.\textsuperscript{38} The combination of aqueous two-phase partitioning (ATPP) and the selective formation of protein–DS\textsuperscript{−} in NBu\textsubscript{4}\textsuperscript{+}DS\textsuperscript{−} should also be useful for separating proteins. Most applications of ATPP in protein purification rely on differences in the partition coefficient in mixtures of aqueous dextran and poly(ethylene glycol) (PEG); the dextran-rich phase is considered to be more hydrophilic than the PEG-rich phase.\textsuperscript{100–104} We hypothesized that ATPP would be able to separate native proteins from protein–DS\textsuperscript{−}.

To show how the treatment of proteins with NBu\textsubscript{4}\textsuperscript{+}DS\textsuperscript{−} can enhance separations by ATPP, we analyzed the partitioning of MYO (a protein that denatures in NBu\textsubscript{4}\textsuperscript{+}DS\textsuperscript{−} at 22 °C in SurfCE experiments) and BCA (a protein that is kinetically stable toward denaturation in NBu\textsubscript{4}\textsuperscript{+}DS\textsuperscript{−} at 22 °C). We measured the partition coefficients for samples of MYO and BCA prepared by adding these proteins to five different solutions (all in Tris-Gly buffer at 22 °C): (i) buffer only, (ii) 16.7 mM NBu\textsubscript{4}\textsuperscript{+}Cl-, (iii) 16.7 mM Na\textsuperscript{+}DS\textsuperscript{−}, (iv) 16.7 mM NBu\textsubscript{4}\textsuperscript{+}DS\textsuperscript{−}, and (v) 20.0 mM Na\textsuperscript{+}DS\textsuperscript{−} for 5 min, followed by the addition of NBu\textsubscript{4}\textsuperscript{+}Cl-, resulting in a concentration of 16.7 mM for DS\textsuperscript{−}, Na\textsuperscript{+}, NBu\textsubscript{4}\textsuperscript{+}, and Cl-. The procedure for solution v was designed to obtain BCA–DS\textsuperscript{−} n sat and MYO–DS\textsuperscript{−} n sat in solutions containing DS\textsuperscript{−} and NBu\textsubscript{4}\textsuperscript{+} without heating. We added 360 μL of the samples in i–v to 300 μL of a stock mixture consisting of 8% PEG and 12% dextran (w/v) in Tris-Gly buffer. Final concentrations of PEG and dextran in the partitioning mixtures were 3.6 and 5.5%, respectively; the concentration of DS\textsuperscript{−} for partitioning samples iii–v was 9.1 mM. (At this concentration of surfactant, neither denatured BCA nor denatured MYO refolds.) After vortex mixing the mixture for 10 s and allowing it to equilibrate for 20 min (without further agitation), we separated the PEG-rich and dextran-rich phases by centrifugation and determined the partition coefficients by measuring the absorbance of BCA and MYO (280 nm) in each phase (data in Figure 8).

Although partition coefficients for BCA and MYO were similar in solutions without surfactant or in solutions of Na\textsuperscript{+}DS\textsuperscript{−}, the partition coefficients were different by a factor of >3 when the

![Figure 8. Aqueous two-phase partitioning of BCA and myoglobin (MYO). (A) Partition coefficients for BCA or MYO that had been treated with solutions i–v described in the text. Samples of protein (0.3 mg/mL) were partitioned in mixtures of PEG (3.6% w/v, average molecular weight 20 kDa) and dextran (5.5% w/v, average molecular weight 500 kDa). Partition coefficients were determined by measuring the absorbance of protein (280 nm) in each phase. (B) CE traces analyzing mixtures of BCA and MYO prior to ATPP (bottom) and protein recovered from the dextran-rich phase after ATPP (top). After being partitioned, the dextran-rich phase was dialyzed against Tris-Gly buffer to remove NBu\textsubscript{4}+DS\textsuperscript{−}. Protein was purified from dextran by precipitating the protein by adding aqueous ammonium sulfate, collecting the precipitate by centrifugation, and dissolving the pellet in Tris-Gly. The running buffer used in CE consisted of Tris-Gly buffer containing 100 μM 4-carboxybenzenesulfonamide (I). The association of I to BCA results in a shift in mobility by ∼1 cm\textsuperscript{2} kV\textsuperscript{−1} min\textsuperscript{−1} and improves the resolution of BCA from MYO.](https://dx.doi.org/10.1021/la201832d-langmuir-2011-11560-11574)
mixtures contained NBu₄⁺DS⁻. MYO was converted to MYO−DS⁻ in solutions of NBu₄⁺DS⁻ and partitioned more favorably into the PEG-rich phase, whereas BCA remained and partitioned in its native form. These results suggested that these proteins can be separated by ATPP with the use of NBu₄⁺DS⁻.

We demonstrated the separation of BCA and MYO. We diluted a mixture of BCA and MYO (1 mg/mL in each protein) into a solution of 10.0 mM NBu₄⁺DS⁻. After partitioning the proteins in a mixture of 3.6% PEG and 5.5% dextran, we analyzed the protein recovered from the dextran-rich phase. The CZE traces in Figure 8B showed that the dextran-rich phase was enriched in BCA by a factor of ∼5 compared to the initial mixture. This demonstration suggests that the selective formation of protein₄⁺DS⁻₄,ₙ,sat in NBu₄⁺DS⁻ may, in some instances, provide a useful approach to the separation of proteins. This approach is most likely to be effective when the protein of interest is resistant to denaturation by C⁺DS⁻ (e.g., BCA in solutions of NBu₄⁺DS⁻) and impurities denature readily.

**CONCLUSIONS**

The Cation Influences the Rate of Denaturation of BCA by DS⁻ through Its Influence on the cmc of DS⁻. This study provided a set of observations demonstrating the influence of NR₄⁺ on the formation of BCA₄⁺−DS⁻₄,n,sat from DS⁻ and native BCA. SurfCE showed that the variation of the cation, from Na⁺ through increasingly hydrophobic NR₄⁺, results in decreasing amounts of BCA₄⁺−DS⁻₄,n,sat formed in reactions performed in capillaries (windows of observation of 2 to 3 min, Figures 1 and 3). The underlying reason for the SurfCE results was the influence of C⁺ on the cmc of C⁺DS⁻ and the dependence of the kinetics of denaturation of BCA on the concentration of monomeric DS⁻. At concentrations below the cmc, the rates of formation of BCA₄⁺−DS⁻₄,n,sat increased sharply with the concentration of DS⁻ (the rates of denaturation were proportional to 10¹⁺DS⁻) but were independent of C⁺; these data indicate that the cation is a spectator in the interaction of DS⁻ with BCA below the cmc. The rates of formation of BCA₄⁺−DS⁻₄,n,sat did not increase with the concentration of C⁺DS⁻ above the cmc but showed a wide range (a factor >10¹⁺) in rates at 10.0 mM C⁺DS⁻, determined by the differences in values of the cmc of C⁺DS⁻. These data show that the cation can determine whether BCA is kinetically stable in solutions of DS⁻ above the cmc (and therefore whether it remains in its native form, which is denatured by DS⁻).

The Rate-Limiting Step in the Denaturation of BCA by DS⁻ Involves an Interaction with Monomeric DS⁻. An analysis of BCA in solutions of Na⁺DS⁻ and NR₄⁺DS⁻ allowed us to characterize the importance of the cmc of C⁺DS⁻ on the kinetics of denaturation. At concentrations of C⁺DS⁻ of up to the cmc, BCA denatured at rates that were independent of C⁺ and increased with the concentration of DS⁻. Rates were, however, approximately constant at concentrations beyond the cmc. The kinetics showed that monomeric DS⁻, not micelles of C⁺DS⁻, determine the rates of denaturation for BCA.

The use of C⁺ thus provided information relevant to the mechanism of denaturation of BCA. NBu₄⁺ facilitates the assembly of DS⁻ into micelles but does not accelerate the reactions of DS⁻ with BCA. Although micelle formation is important to protein−DS⁻ interactions—complexes of denatured protein and DS⁻ consist of micellar aggregates of DS⁻—the denaturation of BCA begins with the interaction with monomeric DS⁻.

The Cation Is a Potentially Useful “Knob to Turn” in Methods for the Analysis and Purification of Proteins. The effect of NR₄⁺ on the formation of protein−DS⁻₄ in SurfCE varied among the eight proteins tested. The results in solutions of NBu₄⁺DS⁻ distinguished proteins that were kinetically stable (BCA and CPK) from those that denatured rapidly (e.g., MYO). The results identified the cation as a component of protein−DS⁻ interactions that can be exploited in methods for separating proteins, as illustrated in the use of NBu₄⁺DS⁻ in ATPP to separate MYO and BCA. Variation among proteins in the rates of denaturation by NBu₄⁺DS⁻ enables the selective denaturation of proteins in mixtures and can be used to improve the results of existing methods for purifying proteins (e.g., ATPP).

**ASSOCIATED CONTENT**

1. Supporting Information. Chemicals used, experimental protocols, and additional figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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**REFERENCES**

(1) The electrophoretic migration of BCA in SDS-PAGE is consistent with its molecular weight (29.1 kDa). On the basis of the stochiometrics of binding that are typical of proteins in SDS-PAGE, we assume that the formation of BCA₄⁺−DS⁻₄,n,sat involves the association of ∼150 equiv of DS⁻ (i.e., n = 150, estimated from the binding of 1.4 g of Na⁺DS⁻ for every 1 g of protein).


(3) Values of log P reported in ref 2 were measured by analyzing the water—octanol partitioning of NR₄⁺Cl⁻ at 37 °C; concentrations of NR₄⁺ were measured with ion-selective electrodes.


(33) Properties $\alpha$ and N are frequently discussed in the literature on ionic surfactants. For example, micelles of $C(\text{DS})^{-}$ characterized by $\alpha = 0.2$ are composed of N molecules of $\text{DS}^{-}$ and an amount of C, bound to the surface of the micelle, that neutralizes 80% of the negative charge; the binding of $C^{-}$ results in a degree of ionization of 20% for micellar $\text{DS}^{-}$. Taken together, values of $\alpha$ and N characterize the net electrostatic charge of a micelle.


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(47) These complexes were surprisingly kinetically stable in solutions of Na$^{+}$DS$^{-}$; samples of UBI-SDS, were generated by dialyzing UBI against solutions of Na$^{+}$DS$^{-}$ for 170 h at 22 °C.


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(91) A value of ~0.9 for ΔZ is inferred for the change in charge caused by the removal of a unit of positive charge in the conversion of Lys ε-NH₃⁺ groups to Lys ε-NHCOCH₃ groups (which is comparable, electrostatically, to the binding of DS⁻/C₀). One plausible reason for a value of ΔZ that is less than a full unit of charge is a small change in the local pH, or a shift in the pKₐ for some ionizable residues of the protein.
(92) Details of the experimental procedures are available in the Supporting Information.
(93) One reason for why values of cmc determined with pyrene are lower than those determined with 2-naphthalenecarbinol is that pyrene is more hydrophobic than 2-naphthalenecarbinol. Pyrene may associate with DS⁻/C₀ or induce micelle formation (resulting in changes in its fluorescence) at concentrations of DS⁻ that are slightly lower than the cmc determined by 2-naphthalenecarbinol.
(94) Although this contribution leads to a decrease of ~15% in the concentration of NBu₄⁺/DS⁻ by the end of the reaction, there was good agreement between the data and the line obtained by fitting to a first-order exponential decay (R² = 0.99).
(97) We assumed that the cmc values are not significantly different at 22 and 40 °C. In unbuffered aqueous solution, the cmc values increase by less than 0.5 mM with an increase in temperature from 25 to 40 °C.
(98) The peaks for CPK shifted by less than 5 cm² kV⁻¹ min⁻¹ when analyzed in Na⁺/DS⁻ and NR₄⁺/DS⁻ and suggested the association of a small number of equivalents of DS⁻. The mobilities of derivatives of CPK acetylated at Lys ε-NH₃⁺ residues suggested that the formation of CPK-DS⁻ in SurfCE takes place with an increase of less than two units of negative charge.
(99) The secondary structure of CPB, characterized by circular dichroism, was similar in solutions with and without NBu₄⁺/DS⁻ (concentrations of 0.5–10 mM).