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Denaturation of Proteins by SDS and by Tetra-alkylammonium Dodecyl Sulfates

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ABSTRACT

This paper describes the use of capillary electrophoresis (CE) to examine the influence of different cations (C^+; $C^+ = Na^+$ and tetra-n-alkylammonium, NR_4^+, where R = Me, Et, Pr, Bu) on the rates of denaturation of bovine carbonic anhydrase II (BCA) in the presence of the anionic surfactant dodecylsulfate (DS^-). Analysis of the denaturation of BCA in solutions of Na^+DS^- and $NR_4^+DS^-$ (in Tris-Gly buffer) indicated that the rates of formation of complexes of denatured BCA with DS^- (BCA^D-$DS^-_{n,sat}$) 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 with rates that depended on the cation; the rates decreased by a factor $> 10^4$, in the order $Na^+ \sim NMe_4^+ > NEt_4^+ > NPr_4^+ > NBu_4^+$ – the same order as the values of cmc (which decrease from 4.0 mM for Na^+DS^- to 0.9 mM for $NBu_4^+DS^-$ in Tris-Gly buffer). The relationship between values of cmc and rates of formation of BCA^D-$DS^-_{n,sat}$ 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 $NR_4^+DS^-$ observed with BCA was not general. Instead, the influence of NR_4^+ on the association of DS^- with these proteins depended on the protein. The selection of cation contributed to the properties (including composition, electrophoretic mobility, and partitioning behavior in aqueous two-phase systems) of aggregates of denatured protein and DS^-. These results suggest

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that variation in the behavior of $NR_4^+DS^-$ with changes in R may be exploited in methods for analyzing and separating mixtures of proteins.

INTRODUCTION

This paper analyzes the interaction between proteins and anionic surfactants (C^+DS^-) that consist of dodecylsulfate (DS^-) paired with the cations Na^+, tetramethylammonium (NMe_4^+), tetraethylammonium (NEt_4^+), tetra-*n*-propylammonium (NPr_4^+), or tetra-*n*-butylammonium (NBu_4^+). 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* equivalents of DS^- to BCA, and results in complexes of denatured protein and DS^-, $BCA^D\text{-}DS^-_{n,sat}$, where *n,sat* is the stoichiometry of DS^- bound to BCA at saturation in solutions containing an excess of DS^- (as observed when BCA is analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis; *n,sat* is approximately 150 for BCA)[1] (Eq. 1).

$$BCA + n\ DS^- \rightarrow BCA^D\text{-}DS^-_{n,sat} \qquad (1)$$

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 $[DS^-]$ below the cmc). At concentrations above the cmc however, rates of formation of $BCA^D\text{-}DS^-_{n,sat}$ were independent of increases in the total concentration of C^+DS^- and depended on the cation, decreasing in the order $Na^+ \sim NMe_4^+ > NEt_4^+ > NPr_4^+ > NBu_4^+$. This order is the same as the order of values of cmc, and suggests that the rates of formation of $BCA^D\text{-}DS^-_{n,sat}$ 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 NR_4^+: -2.7, -2.1, -1.3, 0.4).[2, 3]

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 formation of aggregates between protein and DS^- varied with the protein. For some (e.g., ovalbumin), the rate of formation of protein-DS_n^- was indistinguishable in solutions of Na^+DS^- and $NR_4^+DS^-$. For others (e.g., carboxypeptidase B; CPB), the cation influenced the kinetics of formation and composition of aggregates of protein and DS^-.

Motivation. Treatments of proteins with surfactants are widely used in biotechnology, analytical biochemistry, and medicine.[4-11] 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 adsorption of proteins to surfaces), and the use of surfactants in cleaning, disinfecting, and sterilizing.[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 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 DS$^-$ (which we write as protein^D-DS$^-_{n,sat}$).[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 one equivalent of DS$^-$ for every two amino acid residues).[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.[21-24] Many elements of the interaction of 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 DS$^-$ induces conformational change, are incompletely understood despite extensive investigation.[10, 20, 24-28] Whether or how Na$^+$ (or other cations in buffer solutions of Na$^+$DS$^-$ and protein) participates is not known.

Our primary objective in this study was to compare the influence of cations having a range of hydrophobicity (C$^+$ = Na$^+$; NR$_4^+$, R = Me, Et, Pr, Bu). To identify the effect of NR$_4^+$ on the formation of protein-DS^-_n, we analyzed BCA and several additional proteins using CE, in solutions of C$^+$DS$^-$. Zana et.al. compared the properties (cmc, aggregation number (N), degree of ionization (α^0), microviscosity, micropolarity) of Na$^+$DS$^-$ and NR$_4^+$DS$^-$ in unbuffered aqueous solution; the results suggested that the assembly of DS$^-$ into micelles was more favorable for NEt$_4^+$DS$^-$, NPr$_4^+$DS$^-$, and NBu$_4^+$DS$^-$ than for Na$^+$DS$^-$.[3, 29-33] Trends in the values for cmc and α^0 implicated the binding of NR$_4^+$ to micelles with affinity greater than Na$^+$.[29-32] We anticipated that the

variation of the cation from Na^+ to NR_4^+ would provide a way to manipulate interactions between DS^- and proteins, and to probe the reaction of DS^- with BCA.

Interaction of Proteins with Na^+DS^-: Concentrations Below or Near the Cmc. Isotherms describing the binding of Na^+DS^- to proteins have identified two types of association between protein and DS^-: binding of Na^+DS^- at low stoichiometric equivalencies at concentrations below the cmc, and cooperative binding of Na^+DS^- at high equivalencies at concentrations near or above the cmc. At concentrations $\leq$ 1 mM, Na^+DS^- binds to BSA (~6 equivalents) and cutinase (~14 equivalents) without significant change to the structure of the protein; mushroom tyrosinase, creatine phosphokinase, ubiquitin, and soybean peroxidase are other examples of proteins that show this type of binding.[34-39] 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, cooperative binding of large numbers of Na^+DS^- is accompanied (for most proteins) by the loss of native tertiary structure, and by changes in secondary structure of the native protein.[20, 40]

The concentration of Na^+DS^- that causes denaturation varies with the protein. For myoglobin[37, 41] and ferrocytochrome C[42], binding of monomeric Na^+DS^- at concentrations ($\leq$ 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).[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 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).[43, 44] Factors that may contribute to the resistance of some proteins (e.g., superoxide dismutase, avidin) to denaturation at 22 °C, even at concentrations well above the cmc, include electrostatic repulsion of highly negatively charged proteins (e.g., pepsin) or structural features of the native proteins (e.g., extensive β-sheet structure).[22, 45]

We have studied the association of Na^+DS^- to ubiquitin (UBI), an 8.6-kDa single-chain protein, in detail.[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$_n$) distinguishable by CE. Stoichiometries were approximately defined for several complexes (UBI-SDS$_{~11}$, UBI-SDS$_{~25}$, UBI-SDS$_{~33}$).[47] Identification of these complexes suggested that the denaturation of UBI (ultimately leading to UBI-SDS$_{~42}$) proceeds through several stable intermediates. UBI-SDS$_{~11}$ 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).[10, 34, 48, 495] Investigation of UBI-SDS$_{~11}$ 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.[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 – discussed below), but the concentration of

monomeric Na^+DS^- remains constant.[51-57] 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)[28] increased (from 10^2 s^{-1} to 10^4 s^{-1}) with concentrations of Na^+DS^- in the range 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^-.[28,37,41] At concentrations of $Na^+DS^- \geq 200$ mM (at which rod-like micelles begin to predominate), rates of unfolding increased with a power-law dependence on the concentration of Na^+DS^- (k_{obs} α $[Na^+DS^-]^{3.5}$), suggesting that rod-like micelles are more reactive than spherical micelles are in denaturing S6.[58, 59]

Influence of Cations on the Formation of Micelles of DS^-. The assembly of anionic surfactants into micelles is opposed by the electrostatic repulsion of negatively charged headgroups.[51, 60] Participation of cations, by association with the surface of the micelles and screening of negatively charged headgroups, is essential to the formation of micelles of DS^-.[61] The association of Na^+ to micelles of DS^- has been inferred from the conductivity of solutions of Na^+DS^-.[23,62] Data from this method suggest a value of 0.23 for the degree of ionization (α^0) for micelles of Na^+DS^-, and therefore suggest a value of 0.77 for the degree of association ($1 - \alpha^0$) of Na^+ to 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).[29] Other techniques corroborate the binding of Na^+ to micelles of DS^-.[63-68] Increasing concentrations of Na^+ (e.g., by the addition of NaCl) result in lower values of cmc; the cmc is inversely proportional to ($cmc_0 + [Na^+]_{added}$), where cmc_0 is the cmc in the absence of additional Na^+.[69]

Association of cations also influences the size and shape of micelles. With increasing $[Na^+]$ (from 20 to 140 mM), values of 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 the tighter packing of DS^-, enabled by the screening of SO_4^- headgroups by Na^+.[54, 55, 70] Rod-like micelles require tighter packing of headgroups and greater screening by Na^+ than in spherical micelles; the transition from spherical to rod-like micelles is observed at 140 - 200 mM Na^+DS^-.[56, 57, 61, 71-73]

Properties of $NR_4^+DS^-$. Zana investigated the properties of Na^+DS^- and $NR_4^+DS^-$ prepared by ion-exchange chromatography.[29] Values of cmc in unbuffered aqueous solution decreased from 8.0 to 1.2 mM, in the order $Na^+ \sim NMe_4^+ > NEt_4^+ > NPr_4^+ > NBu_4^+$. This order was also found in the values for the degree of ionization (α^0) for micelles of Na^+DS^- and $NR_4^+DS^-$, decreasing from 0.23 for Na^+DS^- to 0.17 for $NBu_4^+DS^-$. These trends suggested that binding of NR_4^+ to micelles of DS^- is more favorable than binding of Na^+ (presumably due to hydrophobic interactions). Values of the aggregation N for Na^+DS^- and for each $NR_4^+DS^-$ were in the range of 60 - 100, indicating micelles that were spherical and approximately the same size, regardless of the cation.[29] These data indicated that micelles of $NPr_4^+DS^-$ and $NBu_4^+DS^-$ differ from micelles of Na^+DS^- in three ways: (i) the surface density of net negative charge, due to differences in the density of cations bound to the surface of micelles; (ii) hydrophobic interactions between NR_4^+ and DS^- at the surface of micelles; (iii) hydrophobic interactions among NR_4^+.[29-31,74]

EXPERIMENTAL DESIGN.

Selection of C$^+$DS$^-$. We selected Na$^+$DS$^-$ and the series of surfactants NR$_4$$^+DS^-$ for four reasons: (i) The properties of Na$^+$DS$^-$ and NR$_4$$^+DS^-$ (i.e., cmc, α^0) systematically depend on the hydrophobicity of NR$_4$$^+$. (ii) The preparation of NR$_4$$^+DS^-$ from Na$^+$DS$^-$ by ion-exchange chromatography is straightforward. (iii) The optical properties of these systems make analysis straightforward in CE with a UV-absorbance detector: Na$^+$DS$^-$ and NR$_4$$^+DS^-$ are transparent at 214 nm and do not interfere with the detection of proteins, in either native form or in complexes with DS$^-$. (iv) The complete series of surfactants – Na$^+$DS$^-$ and NR$_4$$^+DS^-$ – is soluble in Tris-Gly buffer at 22 °C over a range of concentrations (0 - 100 mM) that is convenient for analyzing the association of DS$^-$ with proteins.

We chose to study Na$^+$DS$^-$ and NR$_4$$^+DS^-$, rather than the series of surfactants consisting of DS$^-$ and alkali metal ions, because differences in the properties of C$^+$DS$^-$ (C$^+$ = Li$^+$, Na$^+$, K$^+$, or Cs$^+$) are relatively small as C$^+$ is varied. For example, as C$^+$ is changed from Li$^+$ to Cs$^+$, values of cmc decrease from 8.9 mM to 6.1 mM (in unbuffered solution at 25 °C);[75] the range of values for cmc is broader for NR$_4$$^+DS^-$ and Na$^+$DS$^-$ (1.2 - 8.0 mM)[29, 76].

Solutions of C$^+$DS$^-$ Tris-Gly Buffer. Values of the cmc for NR$_4$$^+DS^-$ in buffered solutions have not been reported. We determined these values for Na$^+$DS$^-$ and NR$_4$$^+DS^-$ in Tris-Gly buffer with established methods using fluorescent probes (pyrene and 8-anilinonapthalenesulfonate),[77-79] or UV-absorbing probes (e.g., 2-naphthalenecarbinol) in CE experiments.[46, 80] CE was also useful for characterizing the electrophoretic mobility of micelles of Na$^+$DS$^-$ and NR$_4$$^+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 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 protein^D-$DS^-_{n,sat}$ is rapid at concentrations of Na^+DS^- within this range.[25, 38] Concentrations of Na^+DS^- and $NR_4^+DS^-$ were also low enough ($<<$ 200 mM) to justify the assumption that micelles of Na^+DS^- and $NR_4^+DS^-$ were spherical and approximately the same size (values of N in the range 60-100).[29, 54, 81]

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 of tris ($HOCH_2CH_2)_3NH^+$, 8.3 mM), and a mixture of the anion of glycine ($H_2NCH_2CO_2^-$, 8.3 mM) and the zwitterion of glycine ($H_3N^+CH_2CO_2^-$, 184 mM) (concentrations were determined by calculation, using values of pK_a reported for Tris - 8.06, and for glycine - 2.3, 4.3, 7.7, 9.7).[82] 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 NR_4^+) on the formation of protein-DS^-_n, since all of our experiments used Tris-Gly at the same concentration.[83] 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 folding, protein-surfactant interactions, and rational ligand design.[27, 84-86] BCA is monomeric and has no disulfide bonds.[84, 85]

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). Based on stoichiometries of binding that are typical for proteins analyzed in SDS-PAGE, we assume that the formation of BCA^D-$DS^-_{n,sat}$ involves the association of ~150 equivalents of DS^- (i.e., $n \sim 150$, estimated from the binding of ~1.4 g of Na^+DS^- for every 1 g of protein).[19]

Analysis of the Formation of BCA^D-$DS^-_{n,sat}$ 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 complication from DS^- or 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 native protein (μ_N) having net electrostatic charge Z_0 and mass M_0; α is a constant that depends on the shape of the analyte, often estimated as 2/3 for proteins, and C_p is a constant.[87]

$$\mu_N = C_p \frac{Z_0}{(M_0)^\alpha} \qquad (2)$$

The binding of DS^- (and formation of protein-DS^-_n) results in values of electrophoretic mobility higher than μ_N. Equation 3 shows the dependence of the mobility of protein-DS^-_n (μ_{den}) on the electrostatic charge and mass of DS^- (ΔZ_{DS^-} and

M_{DS^-}) and a correction factor ($C_{\psi,n}$) for the non-linear dependence of μ on Z at high surface potentials (> 25 mV).[88, 89]

$$\mu_{den} = C_p C_\psi \frac{Z_0 + n\Delta Z_{DS^-}}{(M_0 + n \cdot M_{DS^-})^\alpha} \qquad (3)$$

Values of μ_{den} are greater than μ_N because the increase in negative charge is significant (upon binding of DS⁻ at a ratio of 1 DS⁻ for every 2 amino acid residues) and is only partially compensated by an increase in hydrodynamic drag (due to an increase in mass by a factor of ~2.4).[90] 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, due to charge regulation.[91] 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 $cm^2\ kV^{-1}\ min^{-1}$.

Generation and Analysis of Protein-DS⁻$_n$ by SurfactantCE (SurfCE).
"SurfCE" is a technique we have developed for surveying the association of ionic surfactants to proteins. SurfCE analyzes reactions of proteins with C⁺DS⁻ carried out within capillaries. Samples of protein, after injection (in their native form) into capillaries filled with solutions of running buffer and C⁺DS⁻, migrate through solutions of C⁺DS⁻ during electrophoresis. Conversion of native protein to protein-DS⁻$_n$ results in peaks reflecting both the composition of protein-DS⁻$_n$ and the time (and therefore rate) of

its formation. SurfCE identifies concentrations of C^+DS^- that cause formation of protein-DS^-_n within the retention time of proteins in the capillary (~3 min. in most of our experiments, which used an applied voltage of 30 kV with 60-cm capillaries having a distance of 50 cm from the inlet to detector). Data from SurfCE can develop qualitative, and in some cases quantitative, information about the kinetics of reaction.

Electropherograms showing peaks only for native protein are the result of either (i) an equilibrium that favors the native form (a thermodynamic outcome), or (ii) slow reactions that require more time than the duration of the experiment (a kinetic outcome). Other methods were necessary to analyze reactions that were too slow for analysis by SurfCE (e.g., Capillary Zone Electrophoresis, or measurement of folded BCA with the fluorescence of dansyl amide).

Other Proteins. We surveyed the interactions of C^+DS^- with proteins other than BCA by SurfCE. These experiments allowed us to examine the possibility that the interactions of BCA with $NR_4^+DS^-$ were exceptional, as well as identify general trends in the influence of NR_4^+ on the formation of complexes of protein and DS^-. We selected seven proteins that met three criteria: (i) values of molecular weight and p*I* that allow for convenient analysis by CE; (ii) absence of disulfide bonds that could complicate the formation of protein^D-$DS^-_{n,sat}$; (iii) commercial availability. The properties of these proteins are summarized in Table 2 of the Results.

RESULTS AND DISCUSSION.

Preparation of $NR_4^+DS^-$, from Na^+DS^-, by cation-exchange. We prepared the surfactants Na^+DS^-, $NMe_4^+DS^-$, $NEt_4^+DS^-$, $NPr_4^+DS^-$, and $NBu_4^+DS^-$ by using a three-step

procedure: (i) re-crystallization of Na^+DS^- from ethanol (twice); (ii) replacement of Na^+ with H_3O^+ by cation-exchange chromatography; (iii) reaction of $H_3O^+DS^-$ with standard solutions of C^+OH^- (NaOH, NMe$_4$OH, NEt$_4$OH, NPr$_4$OH, or NBu$_4$OH).[92]

We used this procedure to prepare samples of Na^+DS^- and $NR_4^+DS^-$ derived from a single batch of re-crystallized Na^+DS^-. Samples of Na^+DS^- prepared by two different methods – re-crystallization (step (i) only) or by ion-exchange (steps (i)-(iii)) – showed agreement in values of cmc, ^{1}H 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 $C_{12}H_{25}$-OSO_3^- 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 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 $NR_4^+DS^-$ in Tris-Gly buffer to determine the cmc of each surfactant. Values of cmc are reported in Table 1 (examples of fluorescence spectra and analysis are in the Supporting Information: Fig. S1A-C).

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

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Table 1. Values of cmc of $n\text{-}C_{12}H_{25}SO_4^-$ as a Function of the Cation

		cmc (mM)		
Cation	Preparation	Tris-Gly[a] (fluorescence[b])	Tris-Gly[a] (CE[c])	H_2O[d] (conductivity)
Na^+	C[e]	4.0	4.3	8.0
Na^+	IE[f]	4.1	4.3	8.0
NMe_4^+	IE	3.8	4.0	5.4
NEt_4^+	IE	2.6	2.9	3.7
NPr_4^+	IE	1.7	2.0	2.2
NBu_4^+	IE	0.9	1.2	1.2

[a] Tris-Gly buffer: 25 mM Tris-192 mM glycine, pH = 8.4. [b] Determined by analyzing the fluorescence spectrum of pyrene in solutions of surfactant (data in the Supporting Information: Figure S1). [c] Determined by analyzing the mobility of 2-naphthalenecarbinol, in solutions of surfactant, by CE (data in Fig. S1D). [d] Values taken from Ref[29]. [e] Re-crystallized twice from ethanol. [f] Prepared by ion-exchange (as described in the text) from a single batch of recrystallized Na^+DS^-.
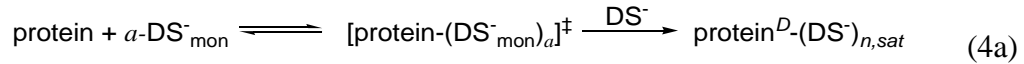
Replacement of Na$^+$ with NR$_4^+$ decreases the cmc of DS$^-$ in Tris-Gly. Values

of cmc in Tris-Gly buffer (Table 1), follow the order Na$^+$ > NMe$_4^+$ > NEt$_4^+$ > NPr$_4^+$ >

NBu$_4^+$. 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 increasingly hydrophobic cations, since the formation of micelles

depends upon the screening of negatively charged R-OSO$_3^-$ headgroups by cations.

Values of 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) on micelle formation.

Zana also identified trends in α^0 and N for NR$_4^+$DS$^-$ in unbuffered solution;[29]

these trends probably hold in solutions of Tris-Gly as well. We assumed that micelles of

Na$^+$DS$^-$ and NR$_4^+$DS$^-$ are spherical and approximately the same size (aggregates of 60 -

90 equivalents of DS$^-$)[29] at concentrations through 10.0 mM, and that the amount of

cation associated to micelles of DS$^-$ increases in the order Na$^+$ < NMe$_4^+$ < NEt$_4^+$ < NPr$_4^+$
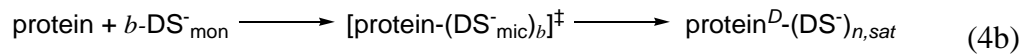
< NBu$_4^+$.

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,

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 a

equivalents of monomeric DS$^-$ (DS$^-_{mon}$) associate with the protein in the initial step,

causing the protein to partially unfold, leading to cooperative association of DS^- and denaturation. Rates of denaturation should not increase with the concentration of C^+DS^- above the cmc, since additional DS^- above the cmc is present only in micelles. Since values of cmc indicate the maximum possible concentration of monomeric DS^- in a solution of C^+DS^-, the hypothesis of Eq. 4a, and the values of cmc in Table 1, predict *rates of denaturation in the order $Na^+ > NMe_4^+ > NEt_4^+ > NPr_4^+ > NBu_4^+$ in solutions above the cmc.*

$$\text{protein} + a\text{-}DS^-_{mon} \rightleftharpoons [\text{protein-}(DS^-_{mon})_a]^{\ddagger} \xrightarrow{DS^-} \text{protein}^D\text{-}(DS^-)_{n,sat} \qquad (4a)$$

(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 – 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 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, $[\text{protein-}(DS^-_{mic})_b]$ is the structure of protein as it nucleates the condensation of b-equivalents of DS^-_{mon} and the formation of a micelle of DS^-. This hypothesis predicts rapid denaturation at concentrations of C^+DS^- that follow the trend in value of cmc and *therefore at concentrations that increase in the order $NBu_4^+ < NPr_4^+ < NEt_4^+ < NMe_4^+ < Na^+$.*

$$\text{protein} + b\text{-}DS^-_{mon} \longrightarrow [\text{protein-}(DS^-_{mic})_b]^{\ddagger} \longrightarrow \text{protein}^D\text{-}(DS^-)_{n,sat} \qquad (4b)$$

(iii) If denaturation is induced by interactions with pre-formed micelles of C^+DS^- (Eq. 4c), the kinetics of denaturation should characterize the influence of C^+ bound to micelles of DS^-. In Eq. 4c, [protein-$(DS^-_{mic})_{\sim60\text{-}100}$] is a structure involving the direct association of protein with a pre-formed micelle. We could not, however, predict whether NR_4^+ bound to the surface of micelles would increase rates of reaction (through favorable hydrophobic interactions of proteins with both DS^- and NR_4^+) or decrease rates (by blocking the interaction of micelles of DS^- with proteins).

$$\text{protein} + (DS^-_{mic})_{\sim60\text{-}100} \longrightarrow [\text{protein-}(DS^-_{mic})_{\sim60\text{-}100}]^{\ddagger} \longrightarrow \text{protein}^D\text{-}(DS^-)_{n,sat} \quad (4c)$$

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 factors of $\sim10^4$ 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).[25] Rates also increase with the concentration of Na^+DS^- beyond the cmc, but only by a factor < 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

20

Na$^+$ with NR$_4^+$, and testing the hypotheses of Eq. 4a and 4b, we hoped to shed light on the mechanism of the reaction.
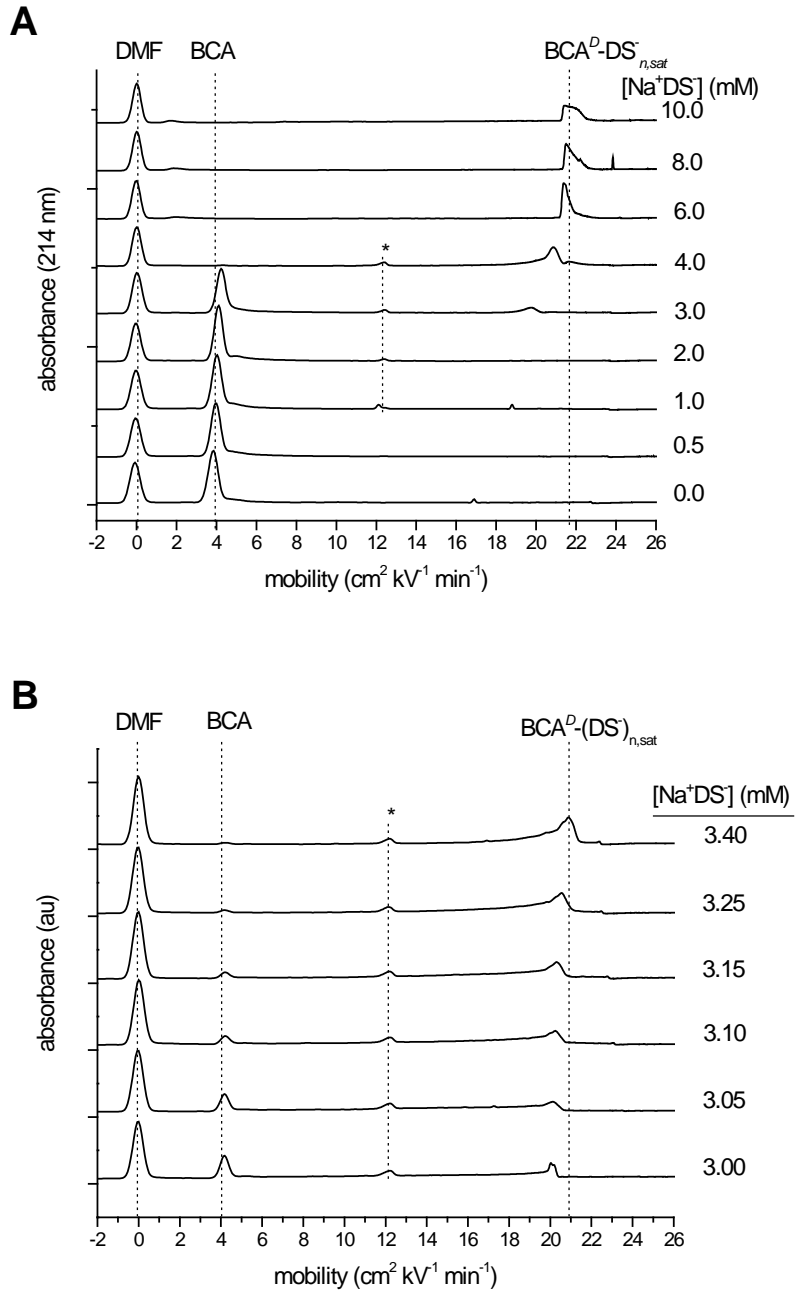
Analysis by SurfCE: Formation of BCA^D-DS$^-_{n,sat}$ in Solutions of Na$^+$DS$^-$. We used SurfCE to carry out and analyze reactions of BCA with Na$^+$DS$^-$. Traces obtained by injecting and analyzing samples of native BCA (8 μM) in capillaries with 0 - 10.0 mM Na$^+$DS$^-$ showed peaks for either native BCA or BCA^D-DS$^-_{n,sat}$ (Fig. 1A). SurfCE showed that the generation of BCA^D-DS$^-_{n,sat}$ is rapid at concentrations ≥ 6.0 mM; the reaction is complete within the interval of time of native BCA takes to reach the detector (~2.6 min.), under the conditions used to collect the data in Fig. 1A. The broadness of the peak in 4.0 mM Na$^+$DS$^-$ indicated that the time required for conversion to BCA^D-DS$^-_{n,sat}$ is 2 - 3 minutes at the cmc.

In the range of 3.00 - 3.40 mM Na$^+$DS$^-$, SurfCE data showed shoulders of peaks for BCA^D-DS$^-_{n,sat}$ (Fig. 1B); conversion of BCA to BCA^D-DS$^-_{n,sat}$ 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 the complete conversion to BCA^D-DS$^-_{n,sat}$ in 3.00 mM Na$^+$DS$^-$ (data available in Fig. S3). Data obtained by SurfCE was 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).

Further discussion of the electropherograms in Fig. 1B is provided in the Supporting Information. Quantitative analysis of the absorbance over the broad range in

Figure 1. Electropherograms obtained by SurfCE show the formation of BCA^D-DS$^-_{n,sat}$ in solutions of Na$^+$DS$^-$. Samples of native BCA (8 μM) were injected into capillaries (60 cm in total length, 50 cm from the inlet to detector) filled with Na$^+$DS$^-$ in Tris-Gly buffer (25 mM Tris-192 mM glycine, pH = 8.4), and were analyzed by electrophoresis at 30 kV. Traces stacked in **(A)** cover the range 0.0 - 10.0 mM, and traces stacked in **(B)** focus on the range 3.00 - 3.40 mM. The small peak at ~12 cm^2 kV^{-1} min^{-1} (*) is not related to BCA. Fig. S4 in the Supporting Information identifies the peak as an artifact of stacking, caused by the injecting samples without Na$^+$DS$^-$ into capillaries containing running buffer with Na$^+$DS$^-$.
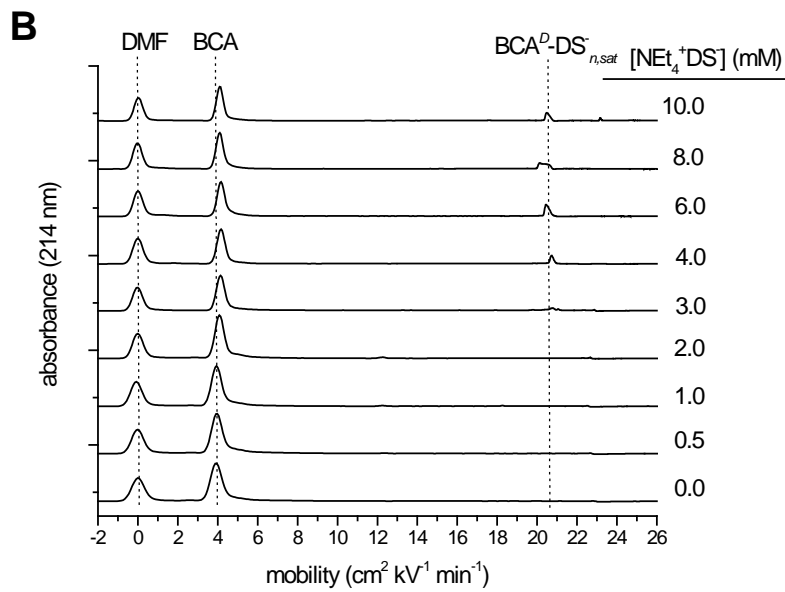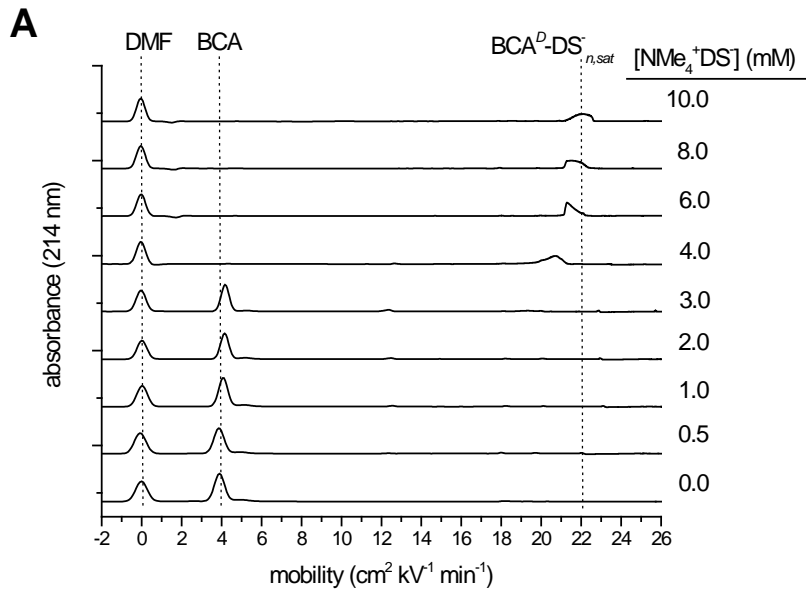
Fig. 1

mobility ($4 < \mu < 22$ cm^2 kV^{-1} min^{-1}) shows that the shapes of peaks are consistent with pseudo-first-order kinetics for the conversion of native BCA to BCA^D-DS$^-_{n,sat}$, 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; Fig. S4).

The formation of BCA^D-DS$^-_{n,sat}$ at concentrations of Na$^+$DS$^-$ below the cmc (4.0 mM) showed that interactions with monomeric DS$^-$ can lead to the formation of BCA^D-DS$^-_{n,sat}$; pre-formed micelles of Na$^+$DS$^-$ are not required for the denaturation of BCA. Analysis of BCA in solutions of Na$^+$DS$^-$ by SurfCE did not detect intermediates along the pathway to BCA^D-DS$^-_{n,sat}$.

NR$_4^+$ decreases the rate of formation of BCA^D-DS$^-_{n,sat}$ in SurfCE. SurfCE experiments analyzing BCA in solutions of NR$_4^+$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 NMe$_4^+$DS$^-$ and NEt$_4^+$DS$^-$ (0 - 10 mM); data for NPr$_4^+$DS$^-$ and Nu$_4^+$DS$^-$ are in the Supporting Information. The amount of BCA^D-DS$^-_{n,sat}$ generated in 10.0 mM NR$_4^+$DS$^-$ decreased across the series, in the order NMe$_4^+$ ($\sim$ Na$^+$) > NEt$_4^+$ > NPr$_4^+$ $\sim$ NBu$_4^+$. Solutions of NEt$_4^+$DS$^-$, even at concentrations well above the cmc, generated only small amounts of BCA^D-DS$^-_{n,sat}$ (< 20% conversion within the ~3 min. period of time allowed by SurfCE); only native BCA was observed in solutions 0 - 10 mM NPr$_4^+$DS$^-$ and NBu$_4^+$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 BCA^D-DS$^-_{n,sat}$). We

Figure 2. Samples of native BCA (8 μM) were analyzed by SurfCE in solutions of **(A)**

NMe$_4^+$DS$^-$ and **(B)** NEt$_4^+$DS$^-$, at concentrations of 0 - 10 mM. SurfCE experiments used

an applied voltage of 30 kV and capillaries that were 60 cm in length, with 50 cm

between inlet and detector. *N,N*-dimethylformamide (DMF, 2 mM) was added to

samples as an internal standard for the purpose of indicating electro-osmotic flow. In

(B), the small peaks near $\mu = 21$ cm^2 kV^{-1} min^{-1} are probably BCA^D-DS$^-_{n,sat}$; other

experiments allowing longer intervals of time (i.e., CZE) show that the peak for native

BCA disappears as the peak near 21 cm^2 kV^{-1} min^{-1} increases in area.
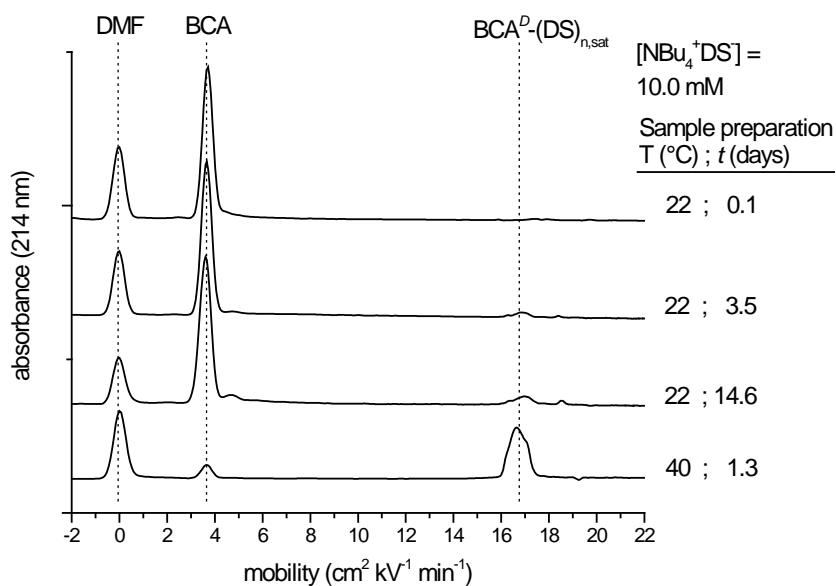
Fig. 2 (A)

concluded that the formation of $BCA^D\text{-}DS^-_{n,sat}$ in solutions of $NR_4^+DS^-$ is not related to the formation of micelles of $NR_4^+DS^-$. The data however remain compatible with the prediction of Eq. 4a: substitution of NR_4^+ for Na^+ results in decreasing amounts of $BCA^D\text{-}DS^-_{n,sat}$ because the denaturation of BCA is induced by monomeric DS^-. We hypothesized that the formation of lower amounts of $BCA^D\text{-}DS^-_{n,sat}$ in $NR_4^+DS^-$ with increasingly hydrophobic NR_4^+ was the result of decreasing concentrations of monomeric DS^-, due to micelle formation at lower values of cmc.

SurfCE data in Fig. 2 however provided a window of observation limited to reactions of BCA with DS^- that take place within 2 - 3 minutes. From these experiments, the underlying reasons for the absence of $BCA^D\text{-}DS^-_{n,sat}$ in solutions of $NPr_4^+DS^-$ and $NBu_4^+DS^-$ are unclear. Other experiments – observation over longer periods of time, or acceleration of reactions by heating – were necessary to determine whether the SurfCE results were outcomes of thermodynamics favoring native BCA, or slow kinetics of denaturation.

Generation of $BCA^D\text{-}DS^-_{n,sat}$ in $NBu_4^+DS^-$. BCA remained folded in solutions of 10 mM $NBu_4^+DS^-$, 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 (Fig. 3) (analysis of samples after 100 days showed a decrease in total absorbance; we did not determine the reason for the apparent loss in total protein). We identified the small peak that developed at $\mu \sim 17$ cm^2 kV^{-1} min^{-1} as $BCA^D\text{-}DS^-_{n,sat}$, by showing that it has the same mobility as the major peak for samples heated to 40 °C (for 32 hours). These results showed that the formation of $BCA^D\text{-}DS^-_{n,sat}$ in $NBu_4^+DS^-$ is slow, and that CZE data showing native BCA in 10 mM $NBu_4^+DS^-$ are early snapshots of

Figure 3. Denaturation of BCA in 10.0 mM NBu$_4^+$DS$^-$ at 22 °C. BCA in 10.0 mM NBu$_4^+$DS$^-$, kept at 22 °C, was analyzed at various time points by CZE (times given to the right of the trace). CZE experiments used an applied voltage of 30 kV and a capillary that was 60 cm in length, with 50 cm between the inlet and detector. The bottom trace is for reference; a sample of BCA^D-DS$^-_{n,sat}$ was generated by heating a solution of BCA in 10.0 mM NBu$_4^+$DS$^-$ to 40 °C.
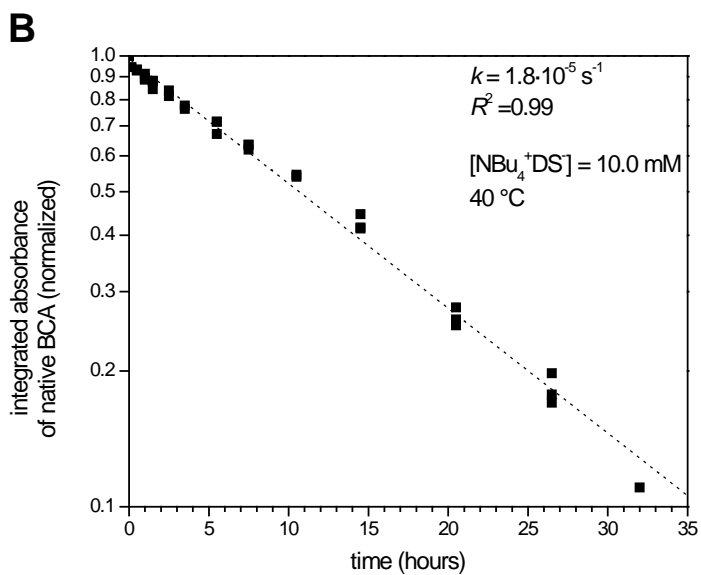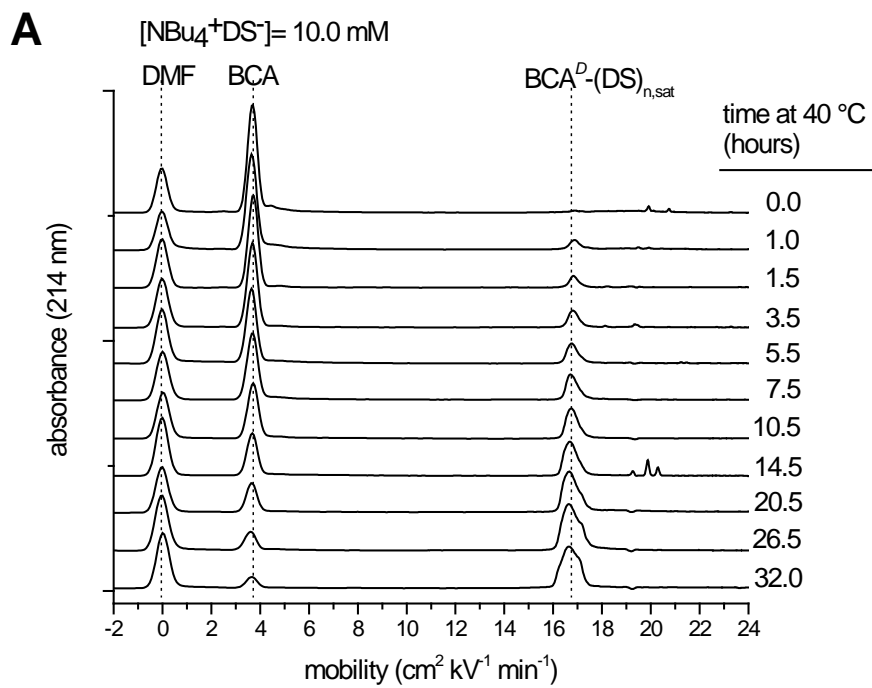
a slow reaction at 22 °C (half-life greater than 10 days). The formation of $BCA^D\text{-}DS^-_{n,sat}$

at 22 °C is slower in 10.0 mM $NBu_4^+DS^-$ than in 10.0 mM Na^+DS^- by a factor $> 10^4$ (the

approximation is based on reactions that require an amount of time < 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

Fig. 2 were due to the kinetics of the system: decreasing amounts of $BCA^D\text{-}DS^-_{n,sat}$ 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 $BCA^D\text{-}DS^-_{n,sat}$ at 40 °C. To circumvent the sluggish

kinetics in solutions of $NBu_4^+DS^-$ at 22 °C, we analyzed the rate of formation of $BCA^D\text{-}$

$DS^-_{n,sat}$ at 40 °C (a temperature still substantially below the melting temperature of BCA,

which is 64 °C)[84]. Traces collected by CZE, at times up to 32 hours, showed the

conversion of BCA to $BCA^D\text{-}DS^-_{n,sat}$ in solutions of 10 mM $NBu_4^+DS^-$ (Fig. 4A).

Areas of peaks for native BCA and $BCA^D\text{-}DS^-_{n,sat}$ allowed us to quantify the amount of

native BCA and to estimate a value of $1.8 \cdot 10^{-5}$ s^{-1} for the pseudo-first-order rate constant

for the disappearance of native BCA (Fig. 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 equivalents of DS^- by BCA.[94] The rate of formation of $BCA^D\text{-}DS^-_{n,sat}$

in 10.0 mM $NPr_4^+DS^-$ at 40 °C ($2.1 \cdot 10^{-4}$ s^{-1}) was greater than that in 10.0 mM $NBu_4^+DS^-$

by a factor of ~10 (Figure S6).

The formation of $BCA^D\text{-}DS^-_{n,sat}$ at 40 °C in solutions of 10.0 mM Na^+DS^-,

$NMe_4^+DS^-$, 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 at these

conditions. We analyzed the rates of denaturation of BCA by measuring the fluorescence

Figure 4. Denaturation of BCA in 10.0 mM NBu$_4^+$DS$^-$ at 40 °C. (A) CZE traces

analyzing aliquots taken from mixtures of BCA (10 μM) and 10.0 mM NBu$_4^+$DS$^-$ heated

to 40 °C. At times noted, aliquots were injected into capillaries (60 cm in length, 50 cm

between the inlet and detector) filled with 10.0 mM NBu$_4^+$DS$^-$ in Tris-Gly buffer (22 °C),

and analyzed by electrophoresis at 30 kV. **(B)** Points represent the fraction of BCA that

was in native form, determined from areas of peaks for native BCA and BCA^D-DS$^-_{n,sat}$,

(corrected for differences in the residence time at the UV-detector). All points from three

trials are included. Analysis by single-exponential decay (dotted line) provided an

estimate for the first-order rate constant.

Fig. 4

A

[NBu$_4$$^+DS^-$] = 10.0 mM

DMF   BCA   BCA^D-(DS)$_{n,sat}$

time at 40 °C (hours)

0.0
1.0
1.5
3.5
5.5
7.5
10.5
14.5
20.5
26.5
32.0

absorbance (214 nm)

mobility (cm^2 kV^{-1} min^{-1})

B

integrated absorbance of native BCA (normalized)

$k = 1.8 \cdot 10^{-5}$ s^{-1}
$R^2 = 0.99$

[NBu$_4$$^+$DS] = 10.0 mM
40 °C

time (hours)

31

intensity of dansyl amide (DNSA) bound to the active site of folded BCA; the fluorescence of DNSA at 460 nm increases by a factor >100 upon binding the active site of BCA.[25, 95, 96] By adding DNSA to aliquots taken from reaction mixtures of BCA and C^+DS^- 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 Fig. S7).

Rates of denaturation spanned factors of $\sim 10^4$ in solutions of 10.0 mM C^+DS^- at 40 °C, and decreased in the order $Na^+ \sim NMe_4^+ > NEt_4^+ > NPr_4^+ > NBu_4^+$. Denaturations in solutions of either Na^+DS^-, $NMe_4^+DS^-$, 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 Fig. 5 overlap).
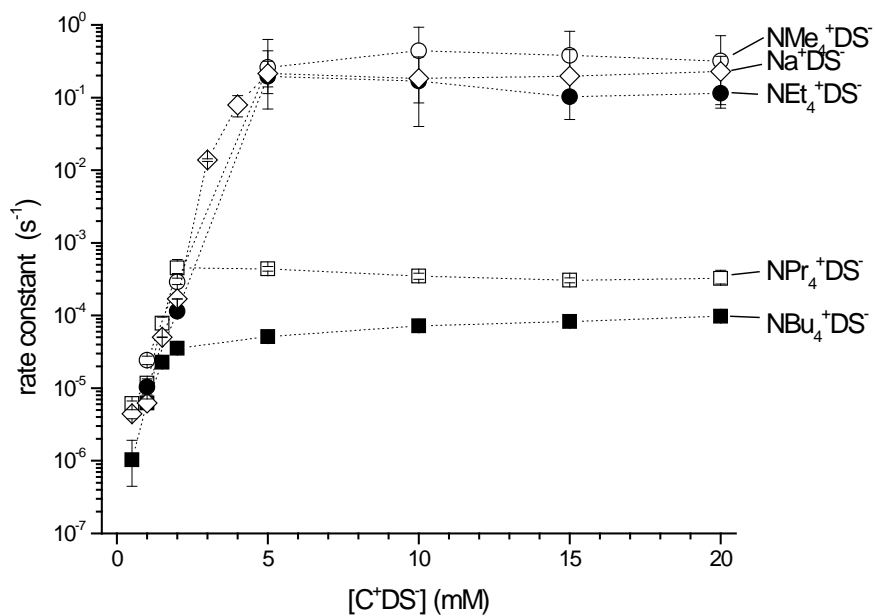
The data in Fig. 5 reveal three key features in the kinetics of denaturation of BCA: (i) Rates are similar for different C^+DS^- when the concentration of C^+DS^- is below the cmc. (ii) The increase in rate essentially stops at the cmc (rates increase by factors ≤ 10 as $[C^+DS^-]$ rises just beyond the cmc, and are indistinguishable at $[C^+DS^-]$ well above the cmc).[97] (iii) the increase in rate constant is not a simple function of the concentration of C^+DS^-. Below the cmc, the relationship between the rate of denaturation and the concentration of DS^- is approximately given by Eq. 5a and 5b.
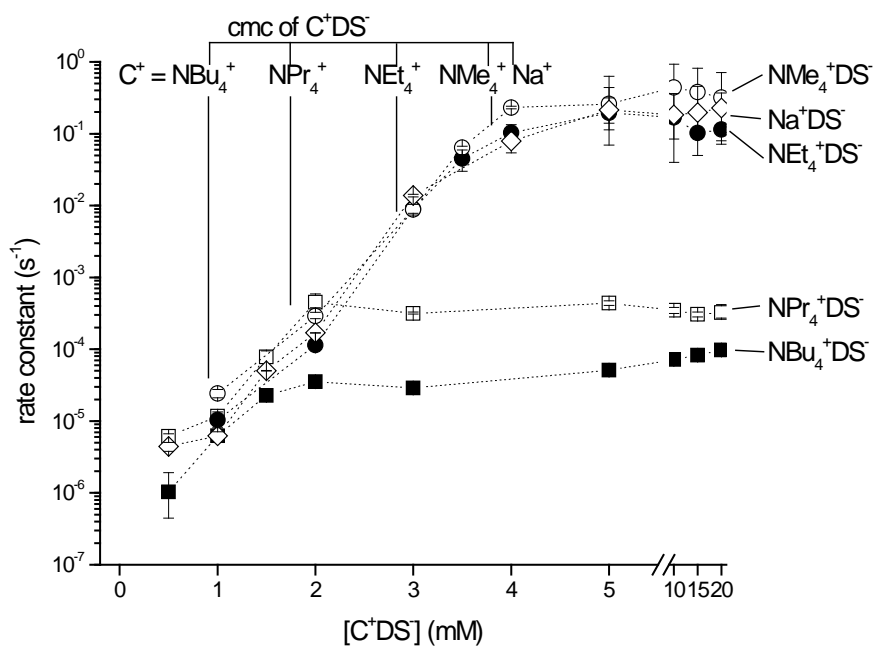
$$k_{\text{den}} \; \alpha \;\; 10^{[DS^-]} \quad\quad\quad\quad (5a)$$

$$\log k_{\text{den}} \; \alpha \;\; \Delta G^{\ddagger} \, \alpha \, [DS^-] \quad\quad\quad\quad (5b)$$

Figure 5. Rate Constants for the Denaturation of BCA in Solutions of $NR_4^+DS^-$ at 40 °C. Points show rate constants that were measured by analyzing aliquots taken from mixtures of BCA (0.2 μM) and C^+DS^- in Tris-Gly buffer heated to 40 °C. Aliquots were analyzed by adding DNSA, and measuring the fluorescence intensity at 460 nm (λ_{ex} =280 nm; concentrations of DNSA and BCA were 2 μM and 0.1 μM respectively). Rate constants were estimated by fitting a single-exponential decay to the decrease in fluorescence intensity over time. For rapid reactions (rate constants of 10^{-1} - 10^0 s^{-1}), samples were analyzed ~4 - 8 s after mixing C^+DS^- and BCA. For "slow" reactions (rate constants < 10^{-3} s^{-1}), intervals of time were 0.5 - 4.0 hrs. Points are averages of three or four repetitions, and error bars span minimum and maximum values. Straight, dotted lines that connect the points are to guide the eye. Inset: Magnification of data at 0 - 5.0 mM $NR_4^+DS^-$. We did not observe any change in the amount of native BCA in solutions of any surfactant at concentrations of 0.25 mM after 48 hours; we did not analyze these samples at longer periods of time.
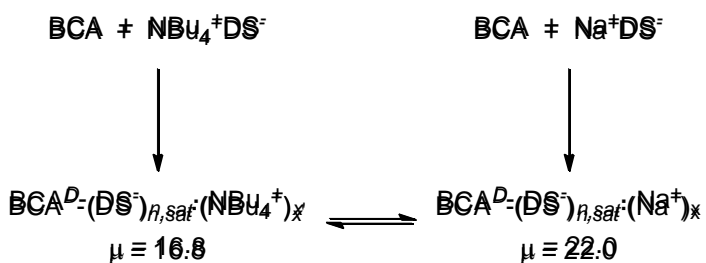
Figure 5.

Inset:

In Eq. 5, k_{den} is the rate constant for denaturation (with units s^{-1}), and [DS$^-$] (mM) is the concentration of C$^+$DS$^-$ below the cmc.

According to this interpretation of Fig. 5, NR$_4^+$ 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$^-$ that can be reached in the presence of C$^+$. We conclude that the rate of denaturation is determined by the concentration of monomeric DS$^-$, and not by micelles of C$^+$DS$^-$. The kinetics indicate a rate-limiting step that involves monomeric DS$^-$. Values for the rate constant k_{den} do not have a linear dependence on [DS$^-$]; the rate-limiting step therefore involves the interaction of BCA with many equivalents of DS$^-$ (we do not know how many equivalents of DS$^-$ from these data). Although interactions of monomeric DS$^-$ with BCA may be cooperative, the data of Fig. 5 suggest that these interactions are not the same as those involved in the self-association of monomeric DS$^-$ to form micelles of DS$^-$. Instead, the initial step for denaturation may be the association of monomeric DS$^-$ (possibly several equivalents) with native BCA that induces local unfolding and exposure of hydrophobic surface area; subsequent association of more monomeric DS$^-$ to partially unfolded protein may cause the denaturation of BCA and formation of BCA^D-DS$^-_{n,sat}$. Since we do not observe shifts in μ in SurfCE to suggest stable complexes of BCA with a small number of equivalents of DS$^-$, 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-DS$^-_{n,sat}$ suggest that NBu$_4^+$ and Na$^+$ associate with BCA^D-DS$^-_{n,sat}$. Values of μ for BCA^D-DS$^-_{n,sat}$ were lower in 10.0 mM NBu$_4^+$DS$^-$

$(16.8 \text{ cm}^2 \text{ kV}^{-1} \text{ min}^{-1})$ than in 10.0 mM Na^+DS^- $(22.0 \text{ cm}^2 \text{ kV}^{-1} \text{ min}^{-1})$ (Figure 6). The shift in μ suggested the influence of the cation on the composition of $\text{BCA}^D\text{-DS}^-_{n,sat}$. To demonstrate that the mobility of $\text{BCA}^D\text{-DS}^-_{n,sat}$ depends on the cation (Na^+ or NBu_4^+) in solution, but does not depend on how $\text{BCA}^D\text{-DS}^-_{n,sat}$ is prepared, we carried the reactions in Scheme 1.

Scheme 1:

$$\text{BCA} + \text{NBu}_4^+\text{DS}^- \qquad\qquad \text{BCA} + \text{Na}^+\text{DS}^-$$

$$\downarrow \qquad\qquad\qquad\qquad\qquad \downarrow$$

$$\text{BCA}^D\text{-(DS}^-)_{n,sat}\cdot(\text{NBu}_4^+)_x \;\;\rightleftharpoons\;\; \text{BCA}^D\text{-(DS}^-)_{n,sat}\cdot(\text{Na}^+)_x$$
$$\mu \equiv 16.8 \qquad\qquad\qquad\qquad \mu \equiv 22.0$$

We generated two samples of $\text{BCA}^D\text{-DS}^-_{n,sat}$ – one in $\text{NBu}_4^+\text{DS}^-$ and another in Na^+DS^- – by heating BCA (8 µM) in solutions of 10.0 mM $\text{NBu}_4^+\text{DS}^-$ or 10.0 mM Na^+DS^- (40 °C, 56 hours). We analyzed $\text{BCA}^D\text{-DS}^-_{n,sat}$ from both preparations, after treating 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) ten-fold dilution into 10.0 mM $\text{NBu}_4^+\text{DS}^-$ (and in parallel, ten-fold dilution into 10.0 mM Na^+DS^-); (iii) incubation for 20 min. at 22 °C. Both preparations of $\text{BCA}^D\text{-DS}^-_{n,sat}$, after equilibration in 10.0 mM $\text{NBu}_4^+\text{DS}^-$, gave CZE traces showing agreement in μ (~ 16.8 $\text{cm}^2 \text{ kV}^{-1} \text{ min}^{-1}$, top set of traces in Fig. 6; running buffer contained 10.0 mM $\text{NBu}_4^+\text{DS}^-$). CZE traces also agreed for $\text{BCA}^D\text{-DS}^-_{n,sat}$ that had been equilibrated in 10.0 mM Na^+DS^- (μ ~ 22.0 $\text{cm}^2 \text{ kV}^{-1}$ min^{-1}, bottom set of traces in Fig. 6).

Figure 6. Analysis of Equilibrated Samples of BCA^D-DS$^-_{n,sat}$ by CZE. Samples of

BCA^D-DS$^-_{n,sat}$, prepared by heating BCA in either 10.0 mM NBu$_4^+$DS$^-$ or 10.0 mM

Na$^+$DS$^-$ for 56 hours, were diluted ten-fold into solutions of 10.0 mM DS$^-$ and mixtures

of NBu$_4^+$ and Na$^+$. After equilibration for twenty minutes at 22 °C, samples were

analyzed by CZE. Traces are shown for the analysis of BCA^D-DS$^-_{n,sat}$ prepared initially

in NBu$_4^+$DS$^-$ (peaks up) or in Na$^+$DS$^-$ (peaks down). Samples analyzed in [NBu$_4^+$] =

[Na$^+$] = 5.0 mM (marked by *) were equilibrated at 40 °C for 6 hrs.
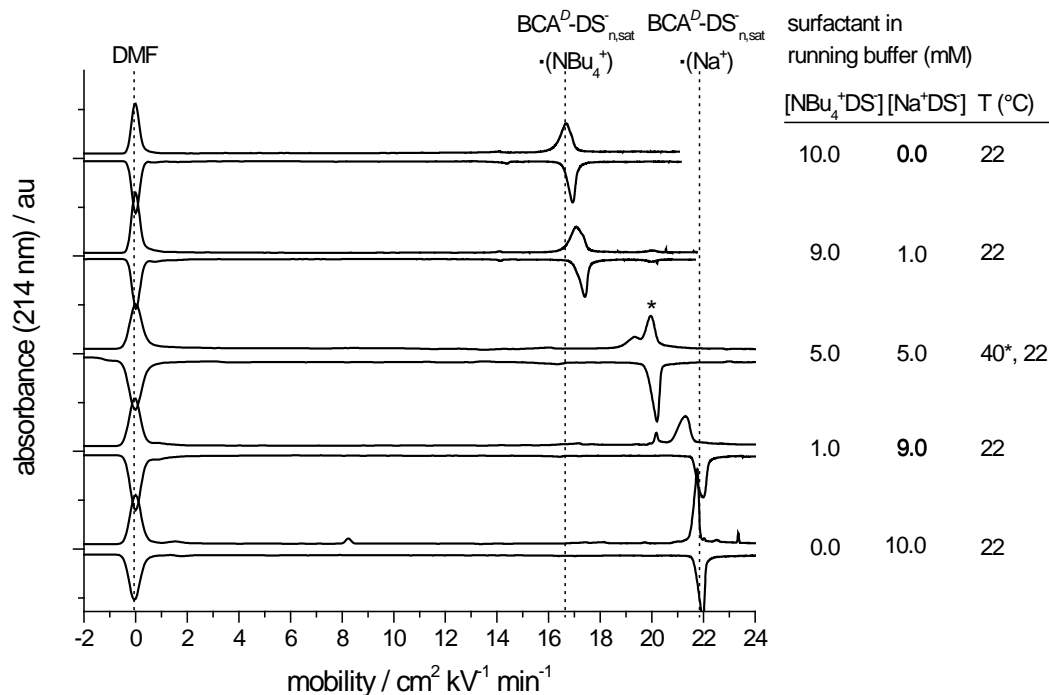
We drew three conclusions from the results: (i) Conversion between

BCA^D-$DS^-_{n,sat}$ with $\mu = 16.8$ and BCA^D-$DS^-_{n,sat}$ 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 μ for BCA^D-$DS^-_{n,sat}$ depends on the cation, suggesting the Na^+

and NBu_4^+ associate with BCA^D-$DS^-_{n,sat}$, and that the complexes should be viewed as

BCA^D-$DS^-_{n,sat}\cdot(C^+)$; iii) The influence of Na^+ and NBu_4^+ on the mobility of BCA^D-$DS^-_{n,sat}$

is determined by association to BCA^D-$DS^-_{n,sat}$ and is independent of how BCA^D-$DS^-_{n,sat}$ is

formed.

Analysis of the data in Figure 6 to estimate the stoichiometry and affinity of

NBu_4^+ and Na^+ for BCA^D-$DS^-_{n,sat}$ is included in the Supporting Information (Fig. S8).

Other Proteins. We wished to determine whether the influence of NR_4^+ on the

formation of aggregates of protein and DS^-, protein-DS^-_n, 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^+DS^- and $NR_4^+DS^-$. Figure 7 shows SurfCE traces for these

proteins in solutions of Na^+DS^- and of $NBu_4^+DS^-$; data for analysis in $NMe_4^+DS^-$,

$NEt_4^+DS^-$, and $NPr_4^+DS^-$ are available in Fig. S9.

The results of SurfCE revealed a range of behaviors among the proteins tested. For

several proteins (β-LacB, α-Lac, MYO, UBI), SurfCE data collected in $NR_4^+DS^-$ and

Na^+DS^- was similar, suggesting that the influence of the cation on the formation of

protein-DS^-_n was relatively minor. For others (BCA, CPK,[98] and β-LacA), replacing Na^+

with NBu_4^+ decreased the amount of protein-DS^-_n generated in SurfCE. For CPB,

the effect of the cation was distinctive; replacing Na^+ with NPr_4^+ or NBu_4^+ enabled the

formation of complexes of CPB with DS^- that are not observed in solutions of Na^+DS^-.

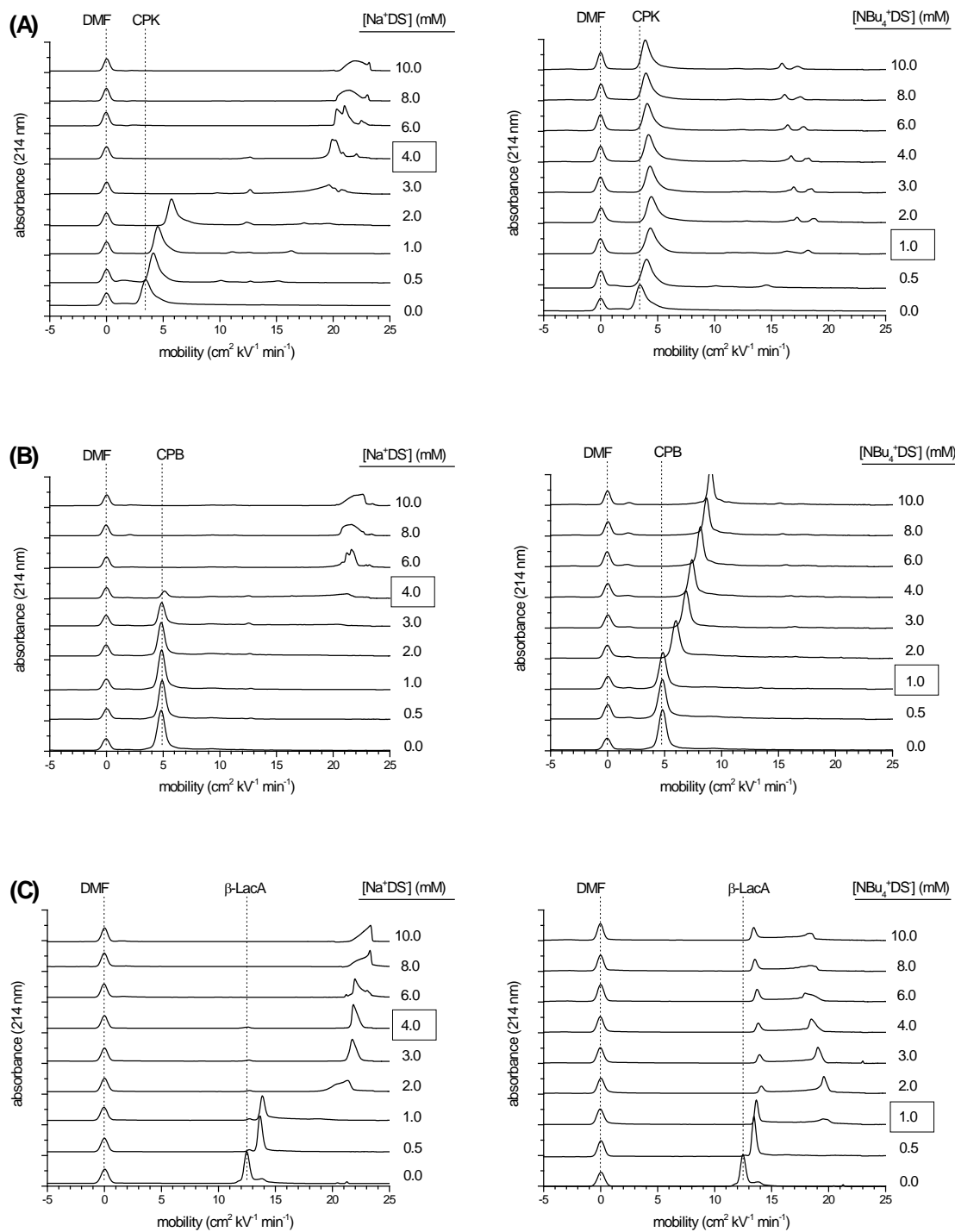**Table 2.** Proteins Analyzed by SurfCE in Solutions of Na^+DS^- and $NR_4^+DS^-$

protein	enzyme accession no.[a]	molecular weight[a]	pI[a]	$[Na^+DS^-]$ for $\Delta\mu$[b]	$\Delta\mu_{Na^+,NBu_4^+}$[c]
bovine carbonic anhdrase (BCA)	P00921	30.0	5.9	3.0	18.0
creatine phosphokinase (CPK)[d]	P005663	86.2	6.7	3.0	18.5
carboxypeptidase B (CPB)	P09955	34.7	5.7	4.0	12.8
β-lactoglobulin A (β-LacA)	P02754	18.4	4.8	2.0	5.0
β-lactoglobulin B (β-LacB)	P02754	18.3	4.8	2.0	4.0
α-lactalbumin (α-Lac)	P00711	14.2	4.8	1.0	4.5
myoglobin (MYO)	P68082	17.0	7.4	0.5	4.5
ubiquitin (UBI)	P62990	8.6	6.6	1.0	2.0

[a] Data for the molecular weight (kDa) and pI were obtained from the Swiss-Prot database. [b] Concentrations of Na^+DS^- that cause shifts in mobility > 5 cm^2 kV^{-1} min^{-1} in SurfCE experiments, determined from the data in Fig. 9 (SurfCE experiments observed reactions taking place within 2 - 3 min.). [c] Difference between the mobility of the major peaks observed in SurfCE (reactions taking place within 2 - 3 min.) using 10 mM Na^+DS^- and 10 mM $NBu_4^+DS^-$. [d] CPK is a homo-dimer and contains no disulfide bonds.

Figure 7. SurfCE data for the analysis of **(A)** CPK, **(B)** CPB, **(C)** β-LacA, **(D)** β-LacB, **(E)** α-Lac, **(F)** MYO, **(G)** UBI in solutions of Na^+DS^- or $NBu_4^+DS^-$. Samples of protein (~0.3 mg/mL) in Tris-Gly were injected into capillaries filled with solutions of surfactant, and analyzed by electrophoresis (30 kV using capillaries that were 60 cm in length and 50 cm between inlet and detector). In the data for CPK in $NBu_4^+DS^-$, small peaks at $\mu \sim$ 17 and 19 $cm^2 \, kV^{-1} \, min^{-1}$ have not been identified. Data for analysis in solutions of $NMe_4^+DS^-$, $NEt_4^+DS^-$, and $NPr_4^+DS^-$ are available in Fig. S9.

Fig. 7

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(G)

Left panel — absorbance (214 nm) vs mobility (cm² kV⁻¹ min⁻¹), [Na⁺DS] (mM): 10.0, 8.0, 6.0, 4.0, 3.0, 2.0, 1.0, 0.5, 0.0. DMF UBI markers.

Right panel — absorbance (214 nm) vs mobility (cm² kV⁻¹ min⁻¹), [NBu₄⁺DS] (mM): 10.0, 8.0, 6.0, 4.0, 3.0, 2.0, 1.0, 0.5, 0.0. DMF UBI markers.
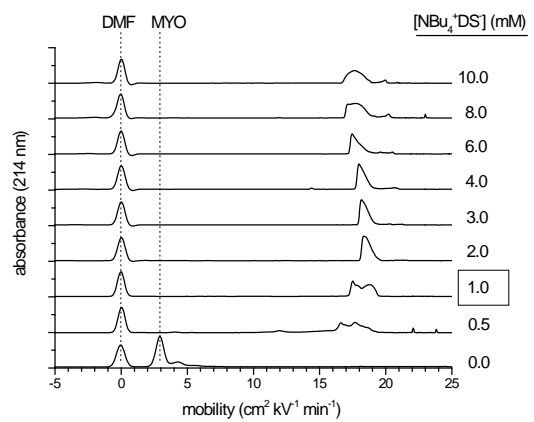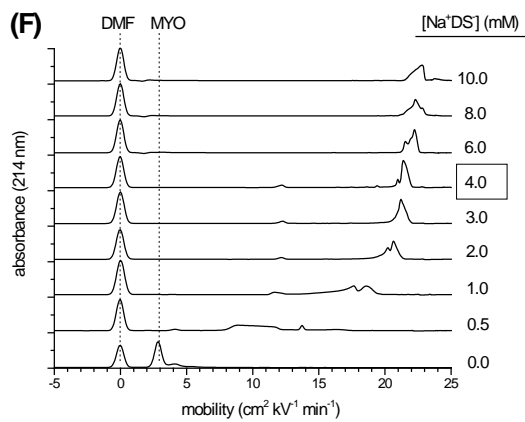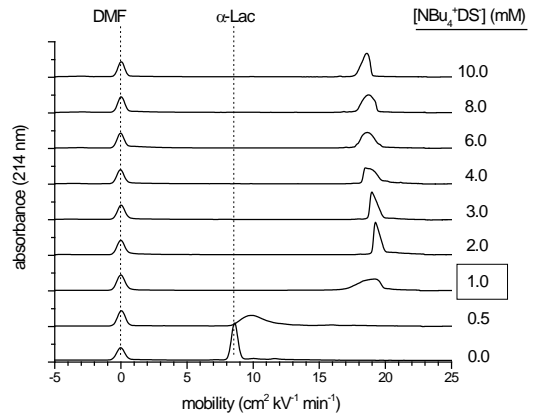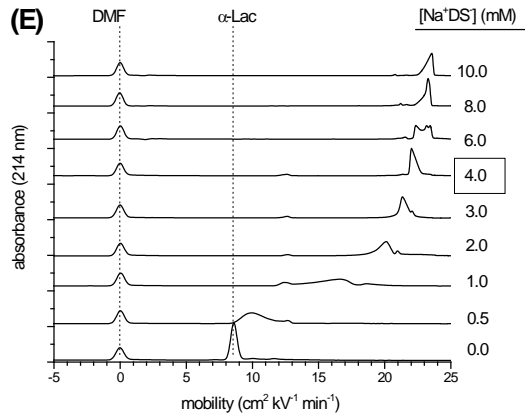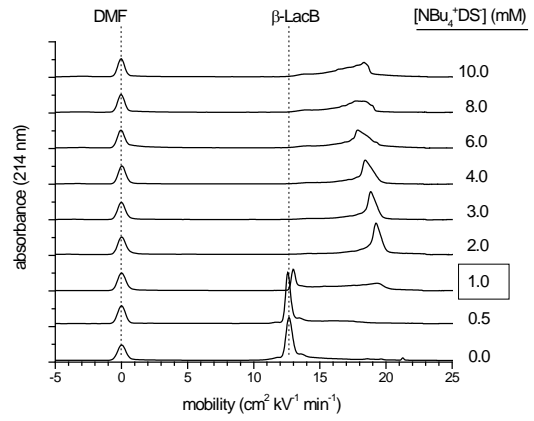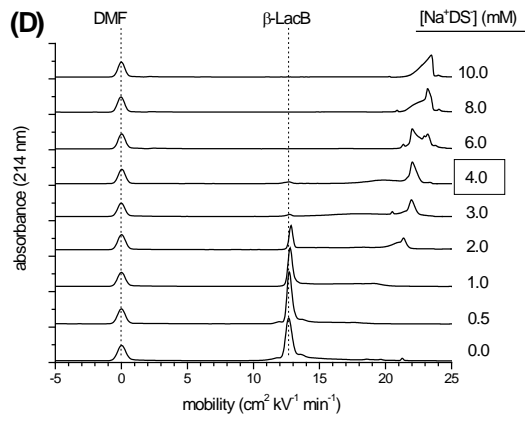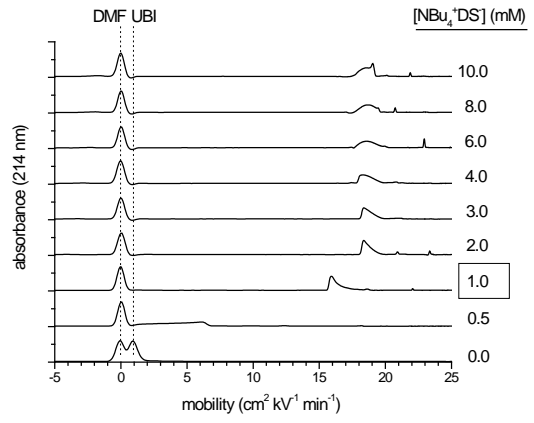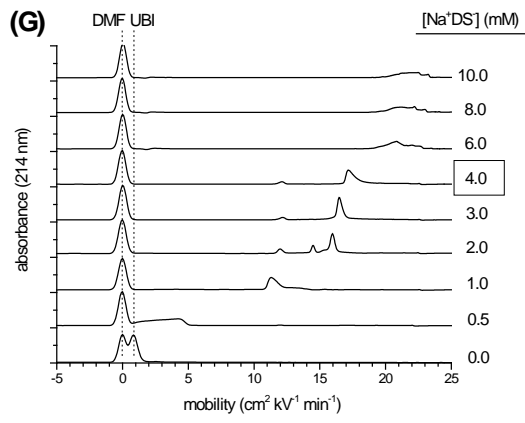
The influence of NR_4^+ appeared to distinguish these proteins by their mechanism of denaturation. Proteins that showed only minor changes upon replacement of Na^+ with NR_4^+ in SurfCE were also those that denatured in solutions of Na^+DS^- at concentrations (1 - 2 mM) well below the cmc (i.e., β-LacB, α-Lac, MYO, UBI). Denaturation of these proteins may be induced by interactions with monomeric DS^- at low concentration; we would not expect the influence of NR_4^+ on the cmc or on the properties of micelles of $NR_4^+DS^-$ to affect the denaturation of these proteins.

Proteins that did not denature in $NPr_4^+DS^-$ and $NBu_4^+DS^-$ were also those that require concentrations of Na^+DS^- (3 - 4 mM) near the cmc for denaturation (BCA and CPK). These concentrations of Na^+DS^- may be important for denaturation because the affinity of these proteins for DS^- may be low. As was the case for BCA, the influence of NR_4^+ on the denaturation of CPK may be due to concentrations of monomeric DS^- that are limited by the formation of micelles of $NR_4^+DS^-$ at decreasing values of cmc. The formation of $CPB\text{-}DS^-_n$ in $NBu_4^+DS^-$ with mobilities of 6 - 10 $cm^2\ kV^{-1}\ min^{-1}$ suggested a different type of involvement of NBu_4^+; we do not understand the reason for the gradual shift in μ for this protein, other than it seems to involve association of DS^- without complete denaturation.[99]

Application of $NBu_4^+DS^-$ in Methods for Separating Proteins. In principle, the selective and differential formation of protein-DS^-_n from proteins in a mixture should be useful in the analysis or purification of proteins. Gudiksen demonstrated the resolution of proteins in CE in solutions of Na^+DS^-, below the cmc, by exploiting differences in the rates of denaturation.[38] The combination of Aqueous Two-Phase Partitioning (ATPP) and the selective formation of protein^D-$DS^-_{n,sat}$ in $NBu_4^+DS^-$ should also be useful for

separating proteins. Most applications of ATPP in protein purification rely on differences in partition coefficient in mixtures of aqueous dextran and poly(ethylene glycol) (PEG); the dextran-rich phase is considered more hydrophilic than the PEG-rich phase.[100-102,100, 102-104] We hypothesized that ATPP would be able to separate native proteins from protein^D-DS$^-_{n,sat}$.

To show how the treatment of proteins with NBu$_4^+$DS$^-$ can enhance separations by ATPP, we analyzed the partitioning of MYO (a protein that denatures in NBu$_4^+$DS$^-$ at 22 °C in SurfCE experiments) and BCA (a protein that is kinetically stable towards denaturation in NBu$_4^+$DS$^-$ 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$_4^+$Cl$^-$; (iii) 16.7 mM Na$^+$DS$^-$; (iv) 16.7 mM NBu$_4^+$DS$^-$; (v) 20.0 mM Na$^+$DS$^-$ for five minutes, followed by the addition of NBu$_4^+$Cl$^-$, resulting in a concentration of 16.7 mM for DS$^-$, Na$^+$, NBu$_4^+$, and Cl$^-$. The procedure in (v) was designed to obtain BCA^D-DS$^-_{n,sat}$ and MYO^D-DS$^-_{n,sat}$ in solutions containing DS$^-$ and NBu$_4^+$, without heating. We added 360 μLs of the samples in (i) - (v) to 300 μLs of a stock mixture consisting of 8% PEG and 12% dextran (w/vol) 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$^-$, for partitioning samples (iii) - (v), was 9.1 mM (at this concentration of surfactant, neither denatured BCA or denatured MYO refold). After vortexing 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 Fig. 8).

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/vol; average molecular weight 20 kDa) and dextran (5.5% w/vol; average

molecular weight 500 kDa). Partition coefficients were determined by measuring the

absorbance of protein (280 nm) in each phase. **(B)** CZE traces analyzing mixtures of

BCA and MYO prior to ATPP (bottom) and protein recovered from the dextran-rich

phase after ATPP (top). After partitioning, the dextran-rich phase was dialyzed against

Tris-Gly buffer to remove $NBu_4^+DS^-$. 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 CZE

consisted of Tris-Gly buffer containing 100 μM 4-carboxybenzenesulfonamide (**1**);

association of **1** to BCA results in a shift in mobility by ~1 $cm^2\,kV^{-1}\,min^{-1}$ and improves

the resolution of BCA from MYO.

Fig. 8

A

B

47
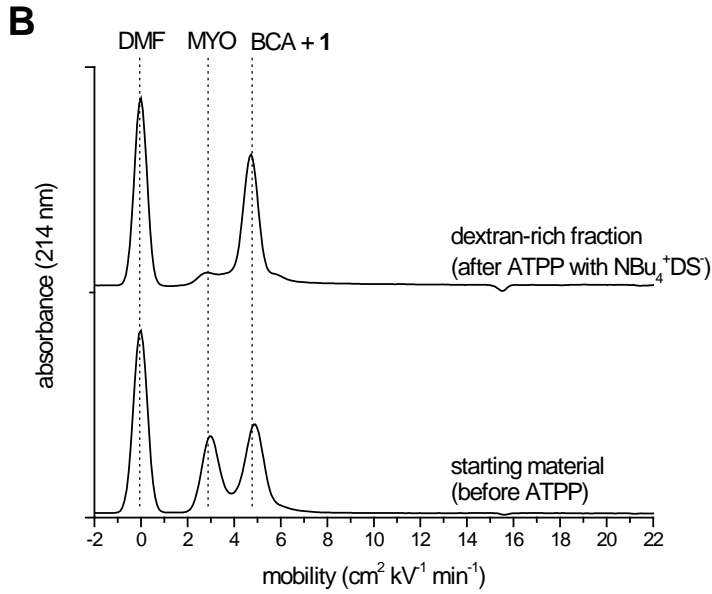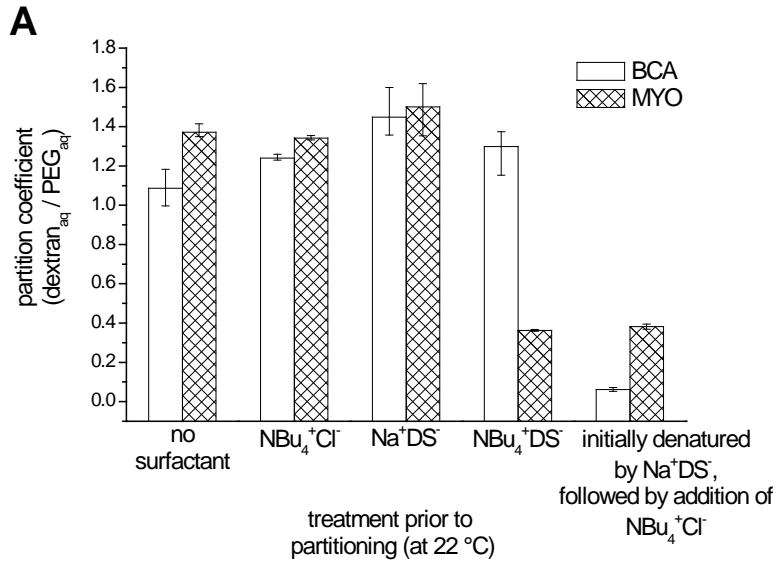
While partition coefficients for BCA and MYO were similar in solutions without surfactant, or in solutions of Na^+DS^-, the partition coefficients were different by a factor > 3 when mixtures contained $NBu_4^+DS^-$. MYO was converted to $MYO\text{-}DS^-_n$ in solutions of $NBu_4^+DS^-$, and partitioned more favorably into the PEG-rich phase, while BCA remained and partitioned in its native form. These results suggested that these proteins can be separated by ATPP with the use of $NBu_4^+DS^-$.

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 $NBu_4^+DS^-$. After partitioning the proteins in a mixture of 3.6% PEG and 5.5% dextran, we analyzed protein recovered from the dextran-rich phase. The CZE traces in Fig. 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 $protein^D\text{-}DS^-_{n,sat}$ in $NBu_4^+DS^-$ 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 $NBu_4^+DS^-$), 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 NR_4^+ on the formation of $BCA^D\text{-}DS^-_{n,sat}$ from DS^- and native BCA. SurfCE showed that variation of the cation, from Na^+ through increasingly hydrophobic NR_4^+, results in decreasing amounts of $BCA^D\text{-}DS^-_{n,sat}$ formed in reactions performed in

capillaries (windows of observation of 2 - 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, rates of formation of $BCA^D\text{-}DS^-_{n,sat}$ increased sharply with the concentration of DS^- (the rates of denaturation were proportional to $10^{[DS^-]}$) but were independent of C^+; these data indicate that the cation is a spectator to the interaction of DS^- with BCA below the cmc. Rates of formation of $BCA^D\text{-}DS^-_{n,sat}$ did not increase with the concentration of C^+DS^- above the cmc, but showed a wide range (a factor $> 10^4$) 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 is denatured by DS^-).

The rate-limiting step in the denaturation of BCA by DS^- involves interaction with monomeric DS^-. Analysis of BCA in solutions of Na^+DS^- and $NR_4^+DS^-$ allowed us to characterize the importance of the cmc of C^+DS^- on the kinetics of denaturation. At concentrations of C^+DS^- up to the cmc, BCA denatured with 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^-, and 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. NBu_4^+ facilitates the assembly of DS^- into micelles, but does not accelerate 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 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 NR$_4^+$ on the formation of protein-DS$_n^-$ in SurfCE varied among the eight proteins tested. The results in solutions of NBu$_4^+$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 NBu$_4^+$DS$^-$ in ATPP to separate MYO and BCA. Variation among proteins in the rates of denaturation by NBu$_4^+$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).

Supporting Information Available: Chemicals used; experimental protocols; additional figures (Figures S1 - S20).

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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-NH$_3^+$ groups to Lys ε-NHCOCH$_3$ groups (which is comparable, electrostatically, to the binding of DS$^-$). 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 pK_a for some ionizable residues of the protein (Ref. 82).

(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$^-$ or induce micelle formation (resulting in changes in its fluorescence) at concentrations of DS$^-$ slightly lower than the cmc determined by 2-naphthalenecarbinol.

(94)   Although this contribution leads to a decrease of ~15% in the concentration of NBu$_4^+$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 ($R^2 = 0.99$).

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(98)   Peaks for CPK shifted by less than 5 cm^2 kV^{-1} min^{-1} when analyzed in Na$^+$DS$^-$ and NR$_4^+$DS$^-$, and suggested the association of a small number of equivalents of DS$^-$. The mobilities of derivatives of CPK acetylated at Lys ε-NH$_3^+$ residues

suggested that the formation of CPK-DS^-_n in SurfCE takes place with an increase of less than two units of negative charge.

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