Effect of Surfactant Hydrophobicity on the Pathway for Unfolding of Ubiquitin

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Supporting Information

ABSTRACT: This paper describes the interaction between ubiquitin (UBI) and three sodium n-alkyl sulfates (SCnS) that have the same charge (Z = −1) but different hydrophobicity (n = 10, 12, or 14). Increasing the hydrophobicity of the n-alkyl sulfate resulted in (i) an increase in the number of distinct intermediates (that is, complexes of UBI and surfactant) that form along the pathway of unfolding, (ii) a decrease in the minimum concentrations of surfactant at which intermediates begin to form (i.e., a more negative ΔGbinding of surfactant for UBI), and (iii) an increase in the number of surfactant molecules bound to UBI in each intermediate or complex. These results demonstrate that small changes in the hydrophobicity of a surfactant can significantly alter the binding interactions with a folded or unfolded cytosolic protein.

INTRODUCTION

Although interactions between cytosolic or non-membrane proteins and surfactants (e.g., lipids, fatty acids, or synthetic detergents) are ubiquitous in biology and biotechnology, no set of chemical principles has emerged that explains these interactions.1–4 For example, interactions between cytosolic proteins and biological surfactants occur in many metabolic pathways,5 in the regulation of cellular function, and in systemic physiological processes such as inflammation.6,7 Not surprisingly, surfactant-binding proteins (e.g., fatty acid-binding proteins) represent many current drug targets for diseases that are characterized by abnormal surfactant sensing, metabolism, and accumulation,8 including targets for obesity,5,9 and obesity-related diseases (e.g., type-II diabetes and non-alcoholic fatty liver disease, NAFLD).10–12 The association of a cytosolic protein and surfactant can also be an early step in pathways of protein misfolding;13–19 examples include the conversion of prion proteins (PrPSc) to toxic conformers (PrPSc).20–24 The association of a cytosolic protein and surfactant can also be an early step in pathways of protein misfolding;13–19 examples include the conversion of prion proteins (PrPSc) to toxic conformers (PrPSc).20–24

In biological systems, a cytosolic protein can interact with surfactants via (i) a transient association with a lipid membrane surface, (ii) the binding to free lipids or fatty acids, or (iii) an interaction with other molecules (drugs or metabolites) with pronounced dipolar (e.g., both hydrophobic and polar) character.5,10,20–24

Our recent studies of protein–surfactant interactions have established that the protein ubiquitin (UBI) and the surfactant sodium dodecyl sulfate (SDS) represent a useful model system25 for studying both specific interactions between proteins and surfactants that do not result in a loss of protein structure and non-specific interactions above the critical micellar concentration (CMC) (which lead to unfolding).25 We study UBI because it is (of the proteins that we have studied26) unique in its ability to bind multiple equivalents of SDS, below the CMC, without unfolding and to form stable, non-exchanging or slowly exchanging structures with defined stoichiometry. We chose SDS as a model surfactant for numerous technical and practical reasons: (i) SDS is highly soluble; (ii) SDS does not interfere with the spectroscopic detection of proteins at 214 nm during capillary electrophoresis (CE); (iii) the carbon chain of SDS is saturated and evenly numbered (n = 12), and near the median range of length of biological lipids and fatty acids (i.e., 4 < n < 24);26 and (iv) the...
sulfate head group of SDS is similar in size to the carboxylic head group of fatty acids—and smaller than a larger lipid sphingosine or ceramide—but its permanent charge makes it electrostatically similar to anionic phospholipids or sulfatides.

We previously used CE and heteronuclear quantum coherence spectroscopy (HSQC) to define the surface properties of folded UBI that result in its simultaneous recognition of multiple molecules of SDS below the CMC. We have also studied the pathway of unfolding of UBI in SDS above the CMC of SDS, by using CE. Capillary electrophoresis is able to detect the binding of a single surfactant molecule to UBI, and to provide qualitative information about rates of association and dissociation. Information from CE has enabled us to identify several discrete complexes of UBI and SDS that form along the pathway of unfolding, including the thermodynamically stable complex that forms (below the CMC) between folded UBI and 11 equiv of SDS (i.e., UBI·(SDS)\(_{11}\)).

Capillary electrophoresis of UBI and peracetylated UBI in sub-micellar SDS demonstrated that cationic groups on the surface of UBI (i.e., Lys-\(\varepsilon\)-NH\(_3\)) are somehow fundamental in facilitating its binding to SDS below the CMC. For example, the acetylation of all seven positively charged Lys-\(\varepsilon\)-NH\(_3\) groups on the surface of UBI (with acetic anhydride) to yield electrostatically neutral Lys-\(\varepsilon\)-NHCOCH\(_3\) groups inhibited the binding of SDS to native UBI, and prevented the formation of complexes between SDS and native UBI. Peracetylated UBI could only bind SDS at concentrations above the CMC, where the binding coincided with the unfolding of UBI (i.e., SDS only bound peracetylated UBI in the unfolded or non-native state).

Further analysis of complexes of native UBI with multiple equivalents of SDS using HSQC NMR, and a comparison of the results with the known biophysical properties of amino acids in folded UBI (e.g., hydrophobicity, formal charge, electrostatic surface potential, secondary structure, rate of amide H/D exchange, and solvent accessibility), allowed us to identify the properties of amino acids in folded UBI that enable it to bind SDS, and to clarify how cationic groups or regions of electrostatic potential on the protein facilitate binding. This previous study used \(^{13}\)C/\(^{15}\)N-\(^{1}\)H HSQC to demonstrate that the binding of SDS by native UBI does not necessarily involve ionic interactions between R-SO\(_4\)\(^-\) and Lys-\(\varepsilon\)-NH\(_3\) (although energetically important interactions do seem to occur with certain lysines). A comparison of the chemical shift perturbation (\(\Delta\delta\)) for each amino acid residue with its electrostatic surface potential (as calculated from solutions to the non-linear Poisson–Boltzmann equation) suggested that cationic groups in UBI facilitate the binding of SDS by contributing positive electrostatic surface potential that extends beyond the van der Waals radii of the cationic groups, into nearby hydrophobic regions that are formally neutral in charge. Those hydrophobic regions in native UBI that had positive electrostatic surface potential were found to be the preferred sites of binding of SDS to UBI, as evidenced by their large values of \(\Delta\delta\) during \(^{13}\)C-\(^{1}\)H HSQC, or by the attenuation of signals during \(^{13}\)C-\(^{1}\)H HSQC. In particular, and surprisingly (because of its low number of positively charged amino acids), the hydrophobic face of UBI that is centered around Ile44 was specifically unbound peracetylated UBI in the unfolded or non-native state).

The analysis of UBI-S complexes with CE provides information on the number of distinct complexes, as well as the stoichiometry of these complexes. CE provides, for example, detailed information about the number and concentration of distinct complexes, and their exact stoichiometry, at a level of detail that cannot always be obtained using other methods (e.g., isothermal titration calorimetry, circular dichroism, fluorescence spectroscopy, or NMR spectroscopy).

We used CE to analyze the complexes of SC\(_S\) surfactants with UBI after extensive dialysis (170 h), following previously published protocols. The stoichiometry of each complex was determined with CE and protein charge ladders as previously described. We formed charge ladders of UBI by converting positively charged Lys-\(\varepsilon\)-NH\(_3\) and the N-terminal \(\alpha\)-NH\(_3\) into the corresponding amides or thiocarbamoyl derivatives. The products were electrostatically neutral acetates (\(\alpha\)- or \(\alpha\)-NHCOCH\(_3\)) or negatively charged (\(\alpha\)-...
or ε-NH-C$_2$H$_4$SO$_3^-$ thiocarbamoyl sulfonate groups there-of (the phenyl group being electrostatically neutral). Each protein derivative with the same number of acetylated or thiocarbamoylated amino groups had a different net charge (Z), regardless of the location of the chemical modifications, and appeared as a distinct peak in a capillary electropherogram. The mobility of each peak of the charge ladders provided a self-calibrating tool to estimate the stoichiometry p of complexes UBI-(SC$_n$)$_p$. With the mobilities of the rungs of the UBI charge ladder (μ)$_n$ and the neutral marker (μ)$_0$ that is caused by the binding of surfactant to UBI. This correction is described in the Materials and Methods section (Supporting Information).

**RESULTS AND DISCUSSION**

**Dialysis of UBI with Free SC$_n$S and Analysis of UBI-(SC$_n$)$_p$ Complexes Using Capillary Electrophoresis.**

In order to determine the specific number of complexes of UBI and surfactant that form along the unfolding pathway of UBI in SC$_n$S, and to determine the stoichiometry of each complex, we used CE to study the formation of the complexes UBI-(SC$_n$)$_p$ formed after extensive dialysis against solutions of surfactant, at different concentrations of SC$_n$S.

Capillary electrophoresis confirmed the existence of multiple, distinct, thermodynamically stable (over the time for a CE experiment) complexes of UBI and SC$_n$S (Figure 1). These complexes have electrophoretic mobilities (μ) that range between that of native UBI (N) in the absence of surfactants (μ = 0.3 cm$^2$ kV$^{-1}$ min$^{-1}$; the peak for N overlaps with that of the neutral marker DMF with μ = 0 cm$^2$ kV$^{-1}$ min$^{-1}$) and that of denatured UBI (D) that is saturated with surfactant. Denatured UBI formed at concentrations of 50 mM for SC$_{10}$S (μ = 20 cm$^2$ kV$^{-1}$ min$^{-1}$), 10 mM for SC$_{12}$S (μ = 22 cm$^2$ kV$^{-1}$ min$^{-1}$), and 0.8 mM for SC$_{14}$S (μ = 22 cm$^2$ kV$^{-1}$ min$^{-1}$, Figure 1).

Complexes of UBI and SC$_n$S surfactants could be resolved by CE into as many as seven distinct complexes, which we call N, G$_1$#, G$_2$#, G$_3$#, G$_6$#, G$_9$ and D, using a nomenclature previously described for UBI–SDS complexes. We were able to estimate the number of surfactant molecules bound to UBI in each complex by comparing the electrophoretic mobility of the complex with the mobility of charge ladders of UBI. As expected, the number of molecules of SC$_n$S incorporated into the UBI-(SC$_n$)$_p$ complexes increased with the concentration of each surfactant.

The first thermodynamically stable intermediate (G$_2$) that formed in each surfactant had a different number of SC$_n$S surfactants (ranging between 9 and 13) associated with the surface of the protein. The stoichiometry of the first stable complex (G$_2$) was higher in solutions of SC$_{14}$S than in solutions of SC$_{12}$S or SC$_{10}$S (discussed further below). Apparently, the stoichiometry of UBI–SC$_{14}$S complexes was not reduced by the steric constraints of packing longer surfactants onto the limited surface of UBI. This observation suggests two possible characteristics for the formation of the first stable complexes of anionic surfactants with UBI: (i) that hydrophobic and electrostatic interactions operate cooperatively in causing this association between UBI and SC$_n$S, and/or (ii) that a surfactant that is already bound to UBI can itself interact (hydrophobically) with another surfactant in solution;

![Figure 1. Electropherograms of UBI-(SC$_n$)$_p$ complexes obtained after dialyzing native UBI against tris--glycine (25 mM tris, 192 mM glycine, pH 7.5, 25 °C) containing the indicated concentrations of SC$_n$S (from 0 to 40 mM) for 170 h. The total concentration of free UBI in the injected aliquot is [UBI] ≈ 50 μM. Each of the electropherograms is labeled with the concentration of surfactant: (a) UBI-(SC$_{10}$)$_p$; (b) UBI-(SC$_{12}$)$_p$; and (c) UBI-(SC$_{14}$)$_p$. Colored numbers under each electropherogram designate surfactant stoichiometry (p) of surfactant molecules in UBI-(SC$_n$)$_p$ complexes estimated from protein charge ladders. Each inset on the right-hand side of panels a–c represents an expanded electropherogram collected at a specific concentration of surfactant.](image-url)
regulation in UBI-(SC₈S)ₚ complexes that probably occurs upon the binding of SC₈S to UBI is equal to that resulting from the acetylation of lysines in a charge ladder of UBI. We also corrected for the increased molecular weight and hydrodynamic drag resulting from the binding of surfactant molecules. For analytical details see the Experimental Design section.

Protein charge ladders can only be used to directly approximate the stoichiometry (i.e., mobility) of UBI-(SC₈S)ₚ complexes that have low stoichiometry (i.e., p < 16 SC₈S molecules). In order to estimate the stoichiometry of UBI-(SC₈S)ₚ complexes with higher stoichiometry (i.e., p > 16 SC₈S molecules), the plot of mobility versus the number of acylated residues for the charge ladder (Figure 2a) must be mathematically extrapolated. For example, there are only seven Lys-ε-NH₄⁺ groups in UBI (and one N-terminal α-NH₄⁺); the thio carbamoylation of each R-NH₂ to R-NHC=SNH-C,H₄SO₄⁻ is electrostatically equivalent (approximately) to the binding of two molecules of SC₈S. The R-NHC=SNH-C,H₄SO₄⁻ charge ladder can, therefore, estimate the stoichiometry of UBI-(SC₈S)ₚ complexes with p < 16 molecules of surfactant.

The values of electrophoretic mobilities that define a specific group of complexes (e.g., G₂) increased as the hydrophobicity of the surfactant increased (mobilities are listed over each electropherogram in Figure 1). The higher mobility of complexes with surfactants with longer n-alkyl tails (e.g., SC₁₀S, SC₁₂S) suggests that these complexes contain greater numbers of surfactants than analogous complexes of UBI-(SC₄S)ₚ.

### Hydrophobicity of SC₈S Affects the Stoichiometry of Thermodynamically Stable Complexes G₂, G₄, and D.

Capillary electrophoresis demonstrated that UBI forms a specific G₂ complex (i.e., UBI-(SC₈S)ₚ) with each surfactant. The concentration of surfactant at which each G₂ complex forms (which we refer to as the “critical complex concentration”) decreases as the surfactant hydrophobicity increases. The stoichiometry of each G₂ complex (p) increased with the length of the chain of the SC₈S species. For example, for SC₁₀S, p = 9; for SC₁₂S, p = 11; for SC₁₄S, p = 13. The complexes between UBI and each of the three surfactants that formed near or above the CMC probably include UBI in a non-native structure; these complexes (i.e., G₄ and D) had greater differences in stoichiometry than the complexes such as G₂, that formed far below the CMC (and probably consist of natively folded UBI). This point is demonstrated by the increasing slopes of the three regression lines of p vs n: ~1.0 for G₂, ~2.5 for G₄, and ~9.2 for D (Figure 3b). The difference between these slopes suggests that hydrophobic interactions between UBI and n-alkyl sulfates become stronger at higher concentrations of surfactant, and greater hydrophobicity of surfactant. The stable intermediates G₄ and D, which formed at higher concentration of surfactants than G₂, are, therefore, capable of binding a greater number of longer surfactant (i.e., C₁₄S) than shorter surfactant (i.e., C₁₀S). This trend toward larger aggregation numbers for longer surfactants is not surprising (in this particular system) when considering that hydrophobic interactions seem to be the major driving force in binding of surfactants to UBI at high concentrations of surfactant (>CMC). The positive correlation between aggregation number and hydrophobicity is not necessarily a general effect: in systems other than UBI and alkyl sulfates—for example, in systems where surfactants of different hydrophobicity might have similar binding constants—one might expect that higher aggregation numbers would be observed for smaller alkyl chains, because a greater number of molecules is required to cover the surface of the protein.

Our previous HSQC study demonstrated that the binding of 11 SDS to native UBI caused substantial changes in the Δδ's of residues throughout this protein. The greatest perturbations were, however, seen in residues at the hydrophobic patch that centers around Ile44. This patch—which is on the positively charged face of the electrostatically Janus-faced UBI—is the...
binding site for many different polypeptides that possess UBDs.29-31 The results of this study and our previous study suggest that multiple surfactants bind to the hydrophobic Ile44 region of UBI. We do not assume, however, that all 13 SCnS molecules in the G2 complex, for example, bind at the Ile44 hydrophobic face. The surface area of this hydrophobic patch of UBI is simply not large enough to accommodate 13 molecules of SCnS (unless the surfactants were undergoing hydrophobic interactions with one another that facilitated their stacking upon the Ile44 patch, outward to solvent—which we cannot exclude).

In summary, we estimated that each pair of additional methylene units in the series of n-alkyl sulfates (i.e., n = 10, 12, or 14) resulted in the additional binding of approximately two surfactant molecules in the transition from N to G2; about five surfactant molecules in the transition from N to G4; and approximately 18 surfactant molecules in the transition from N to D (Figure 3a). The effect of increasing the hydrophobicity of the surfactant on the stoichiometry of the UBI-(SCnS)p complexes is therefore cumulative, and larger for later (G4 and D) than earlier intermediates (G2) along the pathway to complete denaturation and saturating interaction with surfactant.

Hydrophobicity of SCnS Affects the Number of Observable Complexes with Distinct Electrophoretic Mobilities. An increase in the length of the n-alkyl sulfate increases the number of different protein–surfactant complexes that form after G2. For example, the transition from G2 to G4 that occurs during the binding of SC10S is characterized by a single broad peak (see group labeled G4* in the three right insets in Figure 1); the G4-to-G6 transition that occurs during the binding of SC12S is characterized by two resolvable peaks; the G5-to-G6 transition in SC14S yielded three resolvable peaks (that is, one or two G5* complexes could be resolved in the presence of SC12S or SC14S, but not SC10S). The observed reduction in the width for peaks of G4* in the presence of longer surfactants is consistent with a greater stability of complexes with intermediate stoichiometry for longer n-alkyl sulfates, such as UBI-(SC14S)14 and UBI-(SC14S)20.

The increase in the number of G4* complexes with distinct mobilities between G2 and G4 as the surfactant hydrophobicity increases suggests that (i) different complexes are forming (with unique stoichiometry) as a result of different modes of binding of UBI, or (ii) the binding of additional molecules of surfactant is energetically unfavorable because, for example, a re-arrangement of previously bound molecules must occur before the binding of additional surfactant. Despite the differences in stoichiometry of these intermediate G4* complexes, each must be thermodynamically stable; otherwise, it would revert to G2 via desorption of bound surfactants, or would produce a peak with intermediate mobility (as appears to be the case with SC10S, see inset in Figure 1a).

The complexes of G5 and D that formed upon the binding of SC10S or SC12S were separable by CE. The G5 and D complexes that formed in SC14S, however, were not resolved by CE. The peak corresponding to the G5 intermediate is broader in SC14S than in shorter surfactants (Figure 1). We suggest that the low resolution of G5 and D in SC14S is caused by the increased heterogeneity of complexes (e.g., “mixed protein–surfactant micelles”38,41).

Using CE To Estimate the Free Energies of Binding of Anionic Surfactants to UBI in Saturated (Dialyzed) Complexes. We can use CE to estimate the average affinity of binding of surfactants to UBI on the basis of the concentration of surfactant at which each different complex formed (i.e., the “CCC”). This concentration of surfactant influences the distribution of protein–surfactant complex species within each group (N, G1*, G2, G3*, G4, G5, G6*, and D), as would be anticipated if hydrophobic interaction were important in determination of pathways of interaction of surfactants with proteins. Increasing the length of the alkyl chain of the surfactant decreases the equilibrium concentration of surfactant required to form complexes within a specific group. For example, dialyzed samples of UBI (50 μM) formed G2 intermediate at concentrations [SC10S] = 12.5 mM, at [SC12S] = 1.2 mM, and at [SC14S] = 0.04 mM, Figure 1. The equilibrium concentration of surfactant required to form G2 is reduced by a factor of 10−30 (corresponding to a difference in free energy of ΔΔG° ≈ 1.3–1.6 kcal/mol) for each pair of additional methylene units added to the hydrophobic tail of the surfactant in the series. This value of ΔΔG° corresponds to −21.4 cal mol−1 Å−2 energy of burying a hydrophobic surface (assuming the entire surface is buried). This value is somewhat higher than the reported value of −15 cal mol−1 Å−2 for burying hydrophobic surface due to the ordering of water molecules.42 The calculated ΔΔG° of interactions of excess of
SC₅S surfactants with UBI thus lies within the expected range of energies for burying hydrophobic surface of proteins.

**CONCLUSIONS**

We have studied interactions of ubiquitin with n-alkyl sulfates (n = 10, 12, or 14). An increase in the hydrophobicity of an n-alkyl sulfate affected several properties of UBI-(SC₅S)ₙₕ complexes. In particular, increasing the hydrophobicity of an n-alkyl sulfate resulted in (i) an increase in the affinity of surfactants for UBI, (ii) an increase in the number of distinct complexes of UBI-(SC₅S)ₚ, and (iii) an increase in the stoichiometric ratio of surfactant to UBI in each native or denatured complex. These increases were semi-quantitatively in line with expectations for increasing areas of interacting hydrophobic surfaces.

The interactions between proteins and surfactants have been historically difficult to study because the surfaces of most proteins are chemically heterogeneous and structurally dynamic (i.e., each conformation within a native ensemble contains multiple types of functional groups positioned in different configurations). We believe that it will be possible to infer greater detail concerning the interactions of proteins and surfactants than we have achieved with UBI in this study and our two previous studies by limiting further the complexity of the problem via the study of smaller proteins. The Trp-cage protein (PDB: 1L2Y), for example, is one of the smallest folded proteins found in living systems. This 20-residue-long protein (derived from the saliva of Gila monsters) consists of three turns of an α-helix and a single flexible loop; its structure and pathway of folding have been studied extensively. Identifying (or engineering) a small folded protein, similar to Trp-cage protein, that could bind SDS below the CMC, and then systematically substituting amino acids via mutagenesis, would further clarify how details of the surface chemistry of a folded protein (e.g., aromaticity—a property that we have not studied) might facilitate the self-assembly of multiple surfactants onto that surface.

**ASSOCIATED CONTENT**

® Supporting Information
Additional experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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**Notes**

The authors declare no competing financial interest.

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