

Surfactant Interactions with Zein Protein

Namita Deo,[†] Steffen Jockusch,[‡] Nicholas J. Turro,^{*,‡} and P. Somasundaran^{*,†}

NSF IUCR Center for Advanced Studies in Novel Surfactants, Langmuir Center for Colloid and Interfaces, Columbia University, New York, New York 10027, and Department of Chemistry, Columbia University, New York, New York 10027

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Interactions of a model surfactant, sodium dodecyl sulfate (SDS), with a water-insoluble model protein, zein, were investigated to gain an understanding of the effects, such as skin irritation and protein denaturation, of surfactants that are common in personal-care products. To elucidate the mechanisms of such effects, the zein protein interaction with SDS in aqueous solutions was investigated using a multipronged approach involving a range of techniques, such as UV–visible and fluorescence spectroscopy, TOC (total organic carbon analysis), light scattering, and viscosimetry. The zein protein solubilization increases with an increase in the SDS concentration. Solubilization of zein occurs in two distinct stages followed by a complete unfolding of the protein. In the first stage ([SDS] \sim 4 mM; critical complexation concentration), SDS is incorporated into the globular zein structure, forming small hydrophobic microdomains. From the pyrene fluorescence lifetime decay measurements, the aggregation number of SDS in such hydrophobic microdomains was found to be markedly lower than the aggregation number of pure SDS micelles in the bulk solution. The vibrational fine structure of pyrene fluorescence, however, showed the core of SDS–zein complex micelles to be more hydrophobic than that of the SDS micelles. In the second stage ([SDS] \sim 200 mM; unfolding concentration), the protein unfolds, as is evidenced by viscosity and dynamic light scattering measurements.

Introduction

A full knowledge of the mechanisms of the interactions of cell membrane components with surfactants is required to understand the effects of personal-care products containing surfactants on skin. It is believed that skin irritation by surfactants is caused by the binding of the surfactant to proteins present in the skin.^{1–3} Keratin proteins, located in the stratum corneum, are dominantly present on human skin and hair. The matrix in the stratum corneum of the skin offers a defense against environmental insult.⁴

Ionic surfactants are well-known for their strong binding property to globular proteins. The charged headgroup of an ionic surfactant molecule binds electrostatically to the oppositely charged amino acid group of the protein. In addition to the above, the alkyl chain of the surfactant molecules interact through hydrophobic bonding to the nonpolar parts on the surface as well as in the interior of the globular proteins. The unfolding of the protein is believed to occur beyond the saturation of the protein by the surfactants.

In this study, zein protein has been used as a model protein in place of keratin because of its similarity. Zein protein, a water insoluble protein,⁵ has often been employed to estimate the protein denaturation potential of surfactants and is referred to as the “zein solubilization

test”.^{1,3,6–8} Zein storage proteins are frequently found in maize endosperm, where they are deposited as protein bodies within the rough endoplasmic reticulum.^{9,10} They are practically insoluble in water even at low concentrations of salt and require a high percentage of ethanol in aqueous systems to maintain a folded conformation.^{11,12} The surface charge of zein protein varies according to the pH of the environment. As a result of the structural reformation of proteins in the presence of surfactants, it can be expected that proteins play a major role in biomembrane destabilization processes. To elucidate the mechanisms of such an interaction, the zein protein interaction with sodium dodecyl sulfate (SDS) was investigated in this study by using a multipronged approach involving techniques such as UV–visible and fluorescence spectroscopy, TOC (total organic carbon analysis), light scattering, and viscosimetry. The interaction of SDS with zein has been studied before using methods such as the zein solubilization test^{1,7} and the Langmuir balance technique.⁷ In contrast to the previous studies, our multipronged approach allows a more detailed understanding of the solubilization and unfolding process of the zein protein. In a separate work,¹³ zein protein has been described to be integrated into liposomes to form a membrane model system for skin. We investigated in that work the disintegration of such surfaces by SDS. We found that the primary step of membrane disintegration

* Authors to whom correspondence should be addressed.

[†] NSF IUCR Center for Advanced Studies in Novel Surfactants, Columbia University.

[‡] Department of Chemistry, Columbia University.

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by SDS is the structural reformation of zein followed by the extraction of zein from the membrane by SDS.¹³

Experimental Section

SDS (Fluka), pyrene (Sigma), and phosphate buffer at pH 7.4 (GibcoBRL) were used as received. Zein protein (Fisher Scientific, used as received) possessed the following properties: molecular weight 19–22 kDa (determined by SDS gel), 23–26 kDa (determined by amino acid sequencing); isoelectric point pH = 7.2 (10^{-3} M KNO_3); amino acid composition¹⁴ Ala = 13.7, Arg = 1.2, Asx = 4.7, Cys = trace, Glx = 21.2, Gly = 2.1, His = 0.9, Ile = 4.1, Leu = 20.0, Lys = 0.2, Met = trace, Phe = 6.3, Pro = 9.7, Ser = 6.6, Thr = 2.4, Trp not determined, Tyr = 3.1, Val = 3.6.

Solubilization of Zein Protein by SDS. Two different sets of solubilization experiments were conducted, one in triple-distilled water (TDW) and another in phosphate buffer saline, to establish the salt effect in the zein solubilization processes. Each 1.0% zein sample was interacted with SDS at different concentrations for 48 h. The undissolved protein was separated through centrifugation followed by membrane filtration (0.2 μm). The protein concentration in solution was analyzed by TOC. The amount of SDS in the solution after filtration was determined by a two-phase titration method.¹⁵

Solubilization of Pyrene. Each 0.1 g of zein protein and 0.01 g of pyrene in TDW/phosphate buffer (10 mL) was interacted with SDS at different concentrations for 48 h. The undissolved protein and pyrene were separated by centrifugation and membrane filtration (0.2 μm). The supernatants were diluted using 100 mM SDS solutions to assay the total solubilized pyrene concentration. The pyrene concentration in the supernatant was measured by the absorbance change at $\lambda = 335$ nm. Pyrene solubilization by SDS in phosphate buffer and TDW without buffer was also conducted following the same procedure. The absorbance measurements were made at 25 °C using a Shimadzu UV-240 spectrophotometer.

Fluorescence Measurements. Fluorescence spectra were recorded on a SPEX FluoroMax 2 spectrofluorometer (Jobin Yvon) using pyrene as the fluorescence probe. The excitation wavelength was 335 nm. For the micropolarity measurements, fluorescence intensities at 373 and 383 nm were recorded using sample cells of 10-mm path length. The fluorescence lifetime measurements were performed by single-photon counting on an OB900 fluorometer (Edinburgh Analytical Instruments) using a nitrogen flash lamp as the excitation source.

Viscosity Measurements. A calibrated capillary viscometer (Canon Instruments) was used for measuring the efflux time to calculate the relative viscosity based on the efflux time of TDW at 25 °C. The viscometer was cleaned with chromic acid and TDW and thoroughly dried with acetone before the measurements. The efflux time for TDW was checked before every measurement for reproducibility of the results.

Determination of the Hydrodynamic Diameter of Zein as a Function of the SDS Concentration. The mean size hydrodynamic diameter of zein as a function of the SDS concentration was determined by using a photon correlator spectrophotometer (BI-9000AT, Brookhaven Instrument Corp.) at 25 °C at an angle of 90°. The polydispersity index was higher than 0.4, indicating that the particle size distribution is heterogeneous. The particle size distribution of the protein after equilibration with 400 mM SDS showed a marked change, suggesting that the protein undergoes a significant structural reformation.

Results and Discussions

Solubilization of zein protein by SDS was investigated by determining the total amount of carbon dissolved. Figure 1 illustrates solubilization of zein protein as a function of the SDS concentration. Zein is a hydrophobic protein and is practically insoluble in water in the absence of the surfactant. However, the solubility increases markedly above SDS concentrations of 2 mM. To inves-

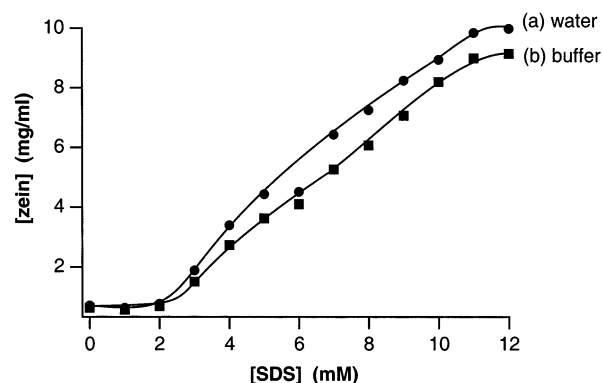


Figure 1. Solubilization of zein protein by dodecyl sulfate in different environments determined by TOC analysis: water (a) and phosphate buffer saline (pH = 7.4; b). The error limit is 10%.

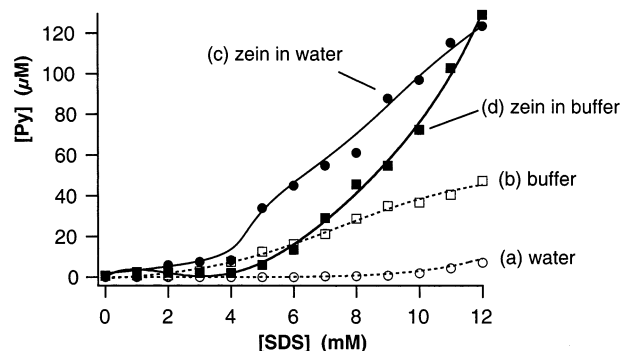


Figure 2. Pyrene dissolution (determined by UV spectroscopy) by different amounts of SDS in water (a), phosphate buffer saline (pH = 7.4; b), zein in water (c), and zein in phosphate buffer saline (pH = 7.4; d). The error limit is 10%.

tigate the protein solubilization in a system close to natural conditions, experiments were performed in a phosphate buffer solution (pH = 7.4; ionic strength = 0.1 M). Only a minor increase in the protein solubilization was observed in the presence of buffer (Figure 1); therefore, it was concluded that ionic strength does not play a major role in the zein protein solubilization.

Pyrene, a commonly used probe molecule for microheterogeneous aqueous solutions,^{16–18} was employed to investigate the nature of the complex between SDS and zein protein. The nonpolar pyrene possesses a stronger affinity to nonpolar environments, such as SDS micelles or zein–SDS complexes, than to polar environments, such as water. The dissolved amount of pyrene in zein–SDS complexes and SDS micelles was determined by UV spectroscopy (Figure 2). In the absence of zein protein in pure water, almost no pyrene (water solubility $\sim 10^{-7}$ M) was detected at SDS concentrations below 8 mM (Figure 2a). Above 8 mM of SDS, measurable amounts of pyrene were dissolved. The change in the pyrene solubility at 8 mM is in accordance with the critical micelle concentration (cmc) of SDS (8 mM in water).¹⁹ The increase in the pyrene concentration above the cmc is attributed to the pyrene solubilization in the SDS micelles. As is expected, in phosphate buffer (0.1 M salt concentration)

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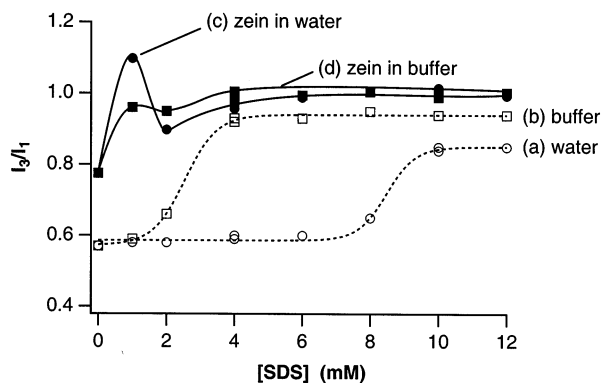


Figure 3. Vibrational fine structure (I_3/I_1) of the pyrene fluorescence as a function of the SDS concentration in the presence and absence of zein: water (a), phosphate buffer saline (pH = 7.4; b), zein in water (c), and zein in phosphate buffer saline (pH 7.4; d). The error limit is 5%.

the cmc of SDS shifted to a lower value (1.5 mM), and the pyrene dissolution increased linearly above the cmc due to the availability of SDS micelles (Figure 2b). Parts c and d of Figure 2 show solubilization of pyrene in the presence of zein protein as a function of the SDS concentration in aqueous solutions with and without phosphate buffer. A distinct change in the pyrene solubility was found at 4 mM. This critical SDS concentration (4 mM) suggests the existence of a critical complexation concentration (cc). Interestingly, the pyrene solubilization increased significantly above 4 mM (cc), much more than that in the case of the pure micelle systems (Figure 2a,b). This marked increase in the pyrene dissolution in the presence of zein is attributed to the formation of a large number of hydrophobic domains in the zein–SDS complexes, which accelerated the pyrene solubilization.

The core of the domain of protein–SDS complexes is expected to be more hydrophobic than the core of the SDS micelles as a result of the participation of the hydrophobic chains of zein protein in the complex formation. Pyrene was employed as the fluorescence probe to determine the micropolarity of the protein–SDS complexes. The vibrational fine structure of the pyrene fluorescence depends strongly on the polarity of the environment. The ratio of the intensities between the the third (I_3) and the first (I_1) fluorescence peaks of pyrene is commonly used as the polarity probe.^{16,17} In the absence of zein protein, in pure SDS systems (Figure 3a,b), below the cmc a low I_3/I_1 value was observed ($I_3/I_1 = 0.57$), which is in agreement with the location of the pyrene molecules in the polar water or buffer solution. A sharp increase of the I_3/I_1 value was observed at the cmc of SDS [8 mM for pure water (a) and ~1.5 mM for the buffer solutions (b)]. Above the cmc, the I_3/I_1 value remained constant within the investigated SDS concentrations, which is characteristic of the SDS micelles.²⁰ Pyrene- and zein protein-saturated solutions (without SDS) in the absence and presence of phosphate buffer showed a I_3/I_1 value of 0.78, which is higher than that without zein protein ($I_3/I_1 = 0.57$). This indicates that some of the pyrene is located in a more hydrophobic environment, such as the hydrophobic regions in zein protein. But the amount of solubilized pyrene in zein without SDS is very low, as is indicated in Figure 2. The addition of SDS caused an increase in the I_3/I_1 value (Figure 3c,d). Because the $I_3/I_1 = 1.0$ is higher for the protein–SDS complexes than that for the pure SDS micelles ($I_3/I_1 = 0.85$), it is concluded that the hydrophobic

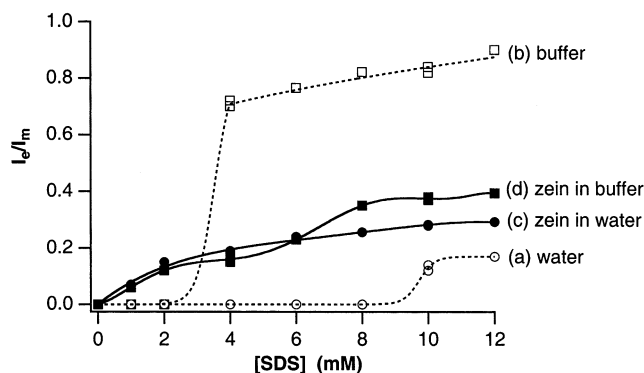


Figure 4. Ratio of excimer–monomer fluorescence (I_e/I_m) of pyrene as a function of the SDS concentration in the presence and absence of zein: water (a), phosphate buffer saline (pH = 7.4; b), zein in water (c), and zein in phosphate buffer saline (pH 7.4; d). The error limit is 10%.

pockets in the protein–SDS complexes are less polar than the core of the SDS micelles.

If two or more pyrene molecules are located in the same micelle or hydrophobic domain, then the fluorescence of pyrene is rapidly quenched to form an excimer (excited-state dimer), which has a typical fluorescence, bathochromically shifted to the monomer fluorescence.²¹ The ratio of the fluorescence intensity between excimer and monomer (I_e/I_m) is a parameter that can be utilized to determine the relative amounts of excimer to monomer, which in turn provides information on the distribution of the probe molecules in the hydrophobic domains.²² In the absence of zein protein, in pure SDS micelles above the cmc of SDS a strong excimer emission at 480 nm was observed for pyrene-saturated solutions. As was expected, below the cmc of SDS no excimers were detected. Figure 4 shows the I_e/I_m ratios of the pyrene fluorescence at different SDS concentrations. In the absence of zein protein, in pure SDS micelles distinct breaks were observed (Figure 4a,b) at concentrations corresponding to the cmc of SDS in the presence and absence of phosphate buffer. In contrast to the above, in the presence of zein, significant amounts of excimers were observed at 1 mM of SDS and above. This indicates that hydrophobic microdomains were formed involving SDS molecules on zein. The microdomains must be large enough to accommodate in some cases more than one pyrene molecule. Because the microdomains of zein–SDS complexes are expected to remain far away from each other, the possibility of formation of excimers between two pyrene molecules originating from two different microdomains is rare.

An important parameter in aggregates involving surfactants is the aggregation number (N_{agg} , number of SDS molecules involved in one micelle or microdomain). To estimate N_{agg} , fluorescence lifetime measurements were performed using pyrene as the fluorescence probe. The measurement involves the determination of the decay of the pyrene monomer fluorescence under conditions of simultaneous monomer decay and excimer formation. The overall decay behavior was then analyzed, using the assumption of an intramicellar excimer formation model and Poisson statistics for the probe distribution, which leads to the equation for the time dependence of the pyrene

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Table 1. Properties of SDS Micelles and Zein–SDS Complexes^a

SDS		zein–SDS	
below cmc		below ccc	
water (cmc = 8 mM) $\tau_0^m = 133$ ns	phosphate buffer (cmc = 1.5 mM) $\tau_0^m = 119$ ns	water (2 < ccc < 4 mM) $\tau_0^m = 321$ ns	phosphate buffer (2 < ccc < 4 mM) $\tau_0^m = 120$ ns
above cmc		above ccc	
water (cmc = 8 mM) $\tau_0^m = 168$ ns $\bar{n}_{\max} = 0.66$ $N_{\text{agg}} = 69$	phosphate buffer (cmc = 1.5 mM) $\tau_0^m = 160$ ns $\bar{n}_{\max} = 0.66$ $N_{\text{agg}} = 110$	water (2 < ccc < 4 mM) $\tau_0^m = 263$ ns $0.25 < \bar{n}_{\max} < 0.6$ $15 < N_{\text{agg}} < 30$	phosphate buffer (2 < ccc < 4 mM) $\tau_0^m = 246$ ns $0.3 < \bar{n}_{\max} < 0.7$ $15 < N_{\text{agg}} < 40$

^a cmc and ccc of the SDS molecules, pyrene fluorescence lifetime of pyrene monomers (τ_0^m), maximum average number of pyrene molecules per microdomain (\bar{n}_{\max}), and aggregation number of the SDS molecules (N_{agg}).

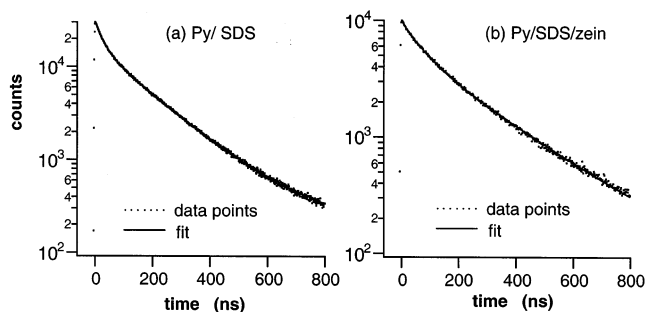


Figure 5. Decay of the pyrene monomer fluorescence recorded at 371 nm after excitation at 337 nm of the pyrene-saturated (0.115 mM) SDS solutions (20 mM; a) and pyrene- (0.12 mM) and zein-saturated SDS solutions (12 mM; b). The decays were fitted to eq 1.

monomer fluorescence (eq 1):²³

$$I_m(t) = I_m(0) \exp[-k_0 t + \bar{n}(\exp(-k_e t) - 1)] \quad (1)$$

where $I_m(0)$ and $I_m(t)$ are the monomer fluorescence intensities at time 0 and at time t , k_0 is the decay rate constant for the monomer fluorescence, and k_e is the excimer formation rate constant. The aggregation number, N_{agg} , can be estimated from the value of \bar{n} (average number of pyrene molecules per micelle) and the concentration of pyrene.

$$\bar{n} = \frac{[\text{pyrene}]}{[\text{micelle}]} = \frac{[\text{pyrene}]N_{\text{agg}}}{[\text{SDS}] - \text{cmc}} \quad (2)$$

Figure 5a shows a typical decay trace of the fluorescence of the pyrene monomers (monitored at 371 nm) after the excitation of pyrene ($\lambda_{\text{ex}} = 337$ nm) in the SDS micelles. The decay traces were fitted to eq 1, and the monomer fluorescence lifetimes ($1/k_0$), the excimer formation rate constant (k_e), and the aggregation numbers obtained for the micellar system are in good agreement with the published values ($1/k_0 = \tau_0^m = 167$ ns;²³ $N_{\text{agg}} = 69$).²⁴ In the presence of phosphate buffer, the aggregation number increased to $N_{\text{agg}} = 110$, which was expected due to the increase in the ionic strength.²²

To estimate \bar{n} and N_{agg} in the SDS–zein complexes, pyrene fluorescence lifetime experiments were also performed in the presence of zein protein at different SDS concentrations (Figure 5b, Table 1). Above the ccc, the aggregation number of SDS molecules in each microdomain in the zein–SDS complexes was decreased compared to that in the pure SDS micelles. The aggregation

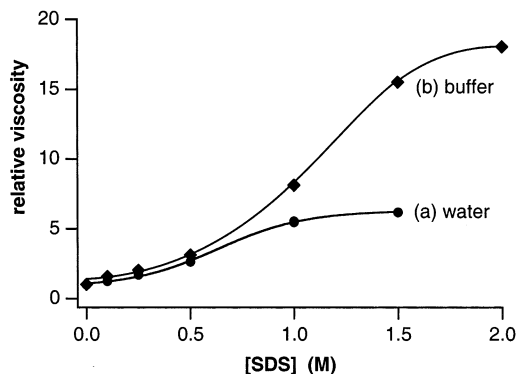


Figure 6. Viscosity of aqueous SDS solutions measured using a capillary viscometer without (a) and with (b) zein. The error limit is 5%.

numbers in zein–SDS complexes range from about 15 to 40 and are relatively independent of the ionic strength (Table 1).

By steady-state fluorescence spectroscopy of zein–SDS complexes at concentrations of SDS below the ccc, a significant amount of pyrene excimer emission was observed (above 1 mM SDS). The fluorescence lifetime results to determine the aggregation number at these SDS concentrations ($1 < [\text{SDS}] < 4$ mM) were inconclusive, and an aggregation number could not be obtained. On the basis of the fast initial fluorescence decay of the pyrene monomer fluorescence and the fast excimer formation kinetics, it can be concluded that in the solubilized zein protein at SDS concentrations below the ccc, the pyrene molecules are located close to each other in the small hydrophobic pockets. Because the pyrene molecules are located close to each other, the fluorescence of the monomer would decay fast, followed by fast excimer formation kinetics.

Viscosity measurements were performed using a capillary viscometer to determine the relative viscosity of zein protein as a function of the SDS concentration (Figure 6b). Only a minor viscosity increase was observed below a SDS concentration of 0.2 M. A significant increase in the viscosity of the zein–SDS solutions was observed at 0.5 M, and the further addition of SDS resulted in an additional viscosity increase, suggesting a gradual transformation from globular to elongated structures, until a plateau is reached at approximately 1.5 M. Upon further addition of SDS, the viscosity remained almost constant, implying the complete unfolding of the protein. Control experiments involving a viscosity measurement on pure SDS solutions (without zein protein) showed only a small viscosity increase with the increasing SDS concentrations (Figure 6a), which is much smaller than that in the presence of zein protein (Figure 6b). These results are compatible with an increase in the effective volume

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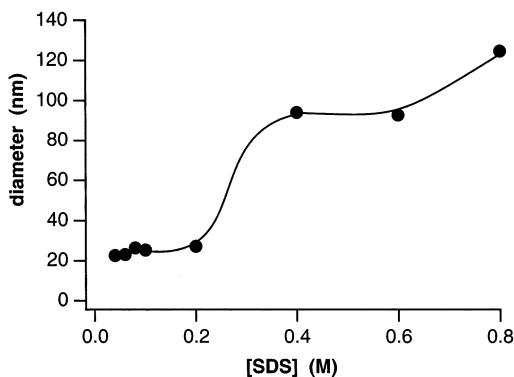


Figure 7. Change in the hydrodynamic diameter of zein as a function of the SDS concentration determined by dynamic light scattering. The error limit is 10%.

occupied by the zein–SDS complexes (unfolded structure) compared to that of the pure zein protein (coiled structure).

The hydrodynamic measurements were performed using dynamic light scattering to determine the size of the zein–SDS complexes as a function of the SDS concentrations (Figure 7). With an initial increase in the SDS concentration, the computed diameter of the SDS–protein complexes remained constant up to 0.2 M. A significant increase in the hydrodynamic diameter of the zein–SDS complexes was observed at 0.4 M, and the further addition of SDS caused only a small increase in the diameter.

Discussion

Zein protein, a water insoluble hydrophobic protein with a globular structure, has been used as a model protein in this study. Because of the tight packing of the native structure, entropy tends to unfold the original conformation of globular proteins to compensate for the interplay of intraprotein and protein–solvent noncovalent interactions. The thermodynamic stability of the native conformation of the protein arises from the minimization of the total free energy of interaction, which originates in a unique balance between large stabilizing and destabilizing forces. The thermodynamic stable state of the unfolded protein is probably the complete exposure of all the amino acid residues and peptide bonds to the solvent, which means that the unfolded state is one single population of a highly expanded, fully solvated, and disordered con-

formation. The addition of negatively charged surfactants, such as SDS, increases the protein solubility.

On the basis of TOC, UV absorption, fluorescence, viscosity, and light scattering measurements, we propose a model for zein–SDS interactions, which is illustrated in Figure 8. Zein protein is a hydrophobic protein with a globular structure and is relatively insoluble in water. The optical absorption and fluorescence measurements employing pyrene as a probe suggest that the globular structure of zein protein does not change measurably if small amounts of SDS are added ($[\text{SDS}] < 4 \text{ mM}$). The TOC, UV absorbance, and fluorescence measurements show a significant change in property at the SDS concentrations between 2 and 4 mM (ccc). As the SDS concentration is increased ($4 < [\text{SDS}] < 200 \text{ mM}$), zein swells and more SDS is incorporated in the zein–SDS complexes. Hydrophobic domains are formed in the zein–SDS complexes involving approximately 15–40 SDS molecules, as is shown by the pyrene fluorescence lifetime measurements. The viscosity and dynamic light scattering measurements show a second significant change in property at a SDS concentration of approximately 0.4 M. At the SDS concentrations higher than 0.4 M, the viscosity and hydrodynamic diameter increase significantly, suggesting the unfolding of the zein–SDS complexes (Figure 8).

The unfolding process could be described as follows: The initial electrostatic attraction between the positively charged headgroups of the protein and the negatively charged dodecyl sulfate molecules increases the solubility of the protein. After all the positive charges are neutralized by the negative charges of the surfactants, repulsion between the negatively charged headgroups of SDS comes into play as a structure-determining feature. Such an electrostatic repulsion between the headgroups could initiate the unfolding, and the hydrophobic SDS chains can then penetrate the globular structure of the protein to interact with the hydrophobic backbone of the protein. Upon further addition of the surfactant, small charged microdomains are formed on the hydrophobic backbone of the protein, which repel each other, causing the complete unfolding of the protein.

Conclusions

The water-insoluble zein protein dissolves in the presence of SDS. With increasing SDS concentration, solubilization of zein occurs in two distinguishable stages

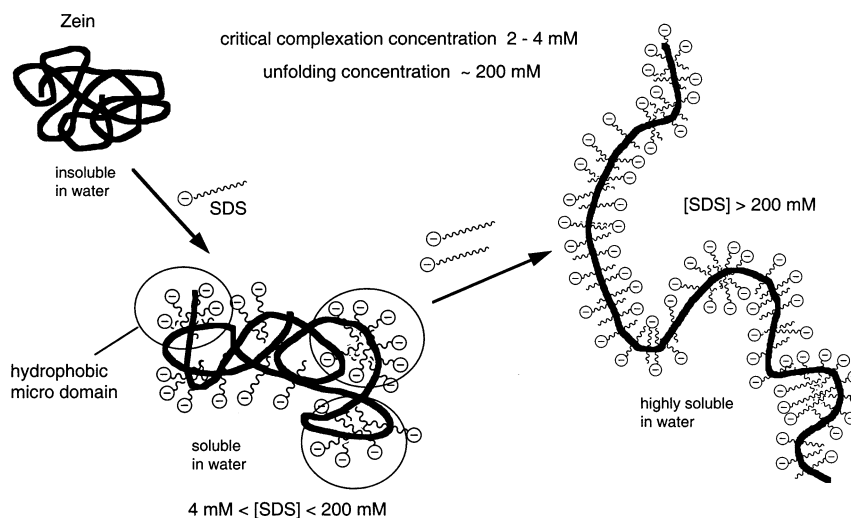


Figure 8. Effects of SDS on the structural reformation of zein leading to its unfolding.

followed by complete unfolding of the protein. In the first stage ($2 < [\text{SDS}] < 4$ mM; ccc), SDS is incorporated into the globular zein structure, forming small hydrophobic microdomains. In the second stage ($[\text{SDS}] \sim 200$ mM), the protein becomes unfolded.

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