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Thermal Denaturation of Molten Globule State of Cytochrome c Induced by High Concentration of Sodium Dodecyl Sulfate

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The molten globule state can be an intermediate in the protein folding pathway; thus; its detailed description can help understanding protein folding. Sodium dodecyl sulfate (SDS), an anionic surfactant that is commonly used to mimic hydrophobic binding environments such as cell membranes is known to denature some native state proteins, including horse cytochrome c (cyt c). In this article, thermal denaturation of acid denatured cyt c is studied under the influence of SDS to form molten globule like states at above the critical micelle concentration using circular dichroism, ultraviolet and visible absorption as well as fluorescence. Thermal denaturation experiments show that cyt c-SDS complexes with large [SDS]/[cyt] have similar thermal behaviour in both low and neutral pH, suggesting negligible pH effect under these conditions. The protein or SDS absolute concentration is an important factor in addition to the [SDS]/[cyt] ratio in determining the thermal behaviour of the protein-surfactant complex, as monitored by far-UV CD and fluorescence. The results suggest that the protein chain wraps around the micelle at about 20 mM SDS, whereas the micelle nucleates on the protein hydrophobic sites at higher concentration.

Key Words: Cytochrome c, Molten globule state, Sodium dodecyl sulfate, Critical micelle concentration, Thermal denaturation.

INTRODUCTION

Cytochrome c (cyt c) plays an important role in the biological electron transfer system and has been extensively studied[1-3], including its fully resolved three-dimensional structure determined by X-ray and nuclear magnetic resonance[4-6]. For cyt c, several probes (IR, UV-Vis, CD, fluorescence) can be used to monitor the structural changes needed to obtain the variety of states accessible under different solution perturbations (GnHCl, urea, pH, temperature, etc.)[7-15]. When acidified, cyt c is denatured to a primarily random coil structure, destabilized due to the electrostatic repulsion between positively between residues. A molten globule (MG) state of cyt c

can be achieved by adding salt to this acid-denatured state, whereby the electrostatic repulsion is reduced, which is believed to derive the protein to become more compact[16]. This state is characterized by high helical content in its secondary structure, but with little evident tertiary structure[17,18]. It is well known that ionic surfactants can interact very strongly with oppositely charged globular proteins[19]. Studies of such interactions between proteins and surfactants have been carried out for half a century. However, the mechanism by which the surfactants influence protein structure is still not well defined. Among these, bovine serum albumin (BSA) has been most frequently studied with sodium dodecyl sulfate (SDS), a representative anionic surfactant that has been widely used in the purification and characterization of proteins[19-22]. Normally, under saturation binding conditions, 1 g of protein can be expected to bind as much as 1.5-2 g of the surfactant[23,24]. SDS is known to be a strong denaturant for many proteins even at the millimolar level[25,26]. However, its ability to counteract the effect of other denaturants was first reported by Duggan and Luck[27] by addition of SDS to BSA in urea to reduce the viscosity of the solution and addressed more recently by the thermal denaturation experiments of Moriyama[28].

In this article, the interaction between positively charged acid-denatured cyt c and anionic SDS at high concentrations is studied. Molten globule like states can be achieved at very low concentration and above the CMC of SDS[29,30]. Recently, other workers also showed the formation of a molten globule like state of cyt c induced by low concentration *n*-alkyl sulfate[30]. They suggested that hydrophobic interactions play an important role in stabilizing the molten globule state. Here, we have examined the SDS effect at high concentrations on the molten globule state of cytochrome c in thermal denaturation. The thermal denaturation of molten globule states of cytochrome c has a characteristic aspect: the conformational change is reversible below 45°C, but is reversible above this temperature. This present study shows that the molten globule states of cytochrome c induced by high concentrations of SDS, can be almost protected from irreversible conformational change in the thermal denaturation in the present of above CMC concentrations of SDS.

EXPERIMENTAL

Horse cytochrome c (type IV), in the oxidazed form, was purchased from Sigma. Sodium dodecyl sulfate (SDS) was also obtained from Sigma. Other chemicals were of reagent grade. The concentration of SDS used in all experiments was over the critical micelle concentration (CMC). The CMC value of SDS under acidic condition (HCl 20 mM, pH 2) is around 3 mM[31].

Solution preparation: The protein solution was dialyzed against 20 mM HCl, pH 2. The extinction coefficient was used to calculate the concentration of the denatured protein at pH 2. If the initial concentration and volume of the protein solution are $[P]_0$ and V_0, respectively and the stock ligand concentration is $[L]_0$, then the total concentration of protein ($[P]_t$) and ligand ($[L]_t$) can be obtained by accounting for the total volume of the aliquot (V_c) added during the titration experiment[32]:

$$[P]_t = [P]_0V_0 / (V_0 + V_c), \qquad [L]_t = [L]_0V_0 / (V_0 + V_c)$$

Aliquots of SDS were injected into the cytochrome c solution at 5 min intervals to allow for equilibration, each experiment was repeated three times.

Circular dichroism measurements: All measurements were carried out at 20°C with thermostatically controlled cell holders. Far-UV CD spectra were measured with a Jasco spectropolarimeter (Japan), model J-720 equipped with an interface and a personal computer. The instruments were calibrated with ammonium d-10-camphorsulfonic acid[33]. The data were expressed as molar residue ellipticity [θ], which is defined as $[\theta] = 100\ \theta_{obs}$ /cl, where θ_{obs} is the observed ellipticity in degrees, c is the concentration in residue mol cm^{-3} and l is the length of the light path in cm. CD spectra were recorded with a time constant of 4s, d 2 nm band width and scan rate of 5 nm min^{-1}, were signal-average over at least five scans and baseline corrected by subtracting a buffer spectrum. The CD spectra were measured with a 1 mm path length cell from 250 to 190 nm. The rotatory contributions of a protein can be determined by $X = f_HX_H + f_\beta X_\beta + f_RX_R$ where X can be either the ellipticity or the rotation at any wavelength, f is the fractions of the helix (f_H), beta form (f_β) and unordered form (f_R); the sum of f is equal to unity and each f is greater than or equal to zero. With the f values of five proteins obtained by X-ray diffraction studies, the X of the protein at any wavelength is filled by least-squares method, which defines the X_H, X_β, X_R. The CD for the helix, beta and random forms determined thus can be conversely used to estimate the secondary structure of any protein with X at several wavelengths for the same equation. The α-helical content (f_H) was estimated from the ellipticity value at 222 nm ($[\theta]_{222}$) as described as follows[34,35]:

$$f_H = -([\theta]_{222} + 2340/30300) \qquad (1)$$

Absorption measurements: Soret Absorption spectra of cytochrome c were obtained with a spectrophotometer, Model Perkin Elmer at protein concentrations of 5 μM with 1 cm path length cells. The protein concentration was determined spectrophotometrically. Extinction coefficients were used to calculate the concentration of the native protein, $1.06 \times 10^5\ M^{-1}\ cm^{-1}$ at 410 nm at pH 7[36]. The spectrum of ferricytochrome c at pH 7 in 25 mM phosphate buffer was taken as a reference.

Fluorescence measurements: Fluorescence measurements were performed by using a Jasco SP-6200 spectrofluorometer at an excitation wavelength of 285 nm. Trp fluorescence emission was followed at 350 nm[37,38]. SDS significantly affects the fluorescence of free tryptophan under the experimental condition used. The temperature of the cell compartment was kept constant at 20°C by water circulation.

RESULTS AND DISCUSSION

The far-UV CD spectra obtained in the titration of acid denatured cyt c (16 μM) with high concentration of SDS at pH 2 were recorded as shown in Fig. 1. For [SDS] values from 3 mM to 60 mM (Fig. 1), the low pH cyt c far-UV spectra show native-like secondary structure content, suggesting another molten globule like state. These spectra seem independent of SDS concentration once it is above 3 mM. A surface tension test (data not shown) shows that the critical micelle concentration (CMC) for SDS solution at pH 2 is around 3 mM. The fact that the same cyt c spectra were obtained for this range of [SDS] values suggested that micelle formation may affect the stability of this complex but the number (concentration) of micelles is less important.

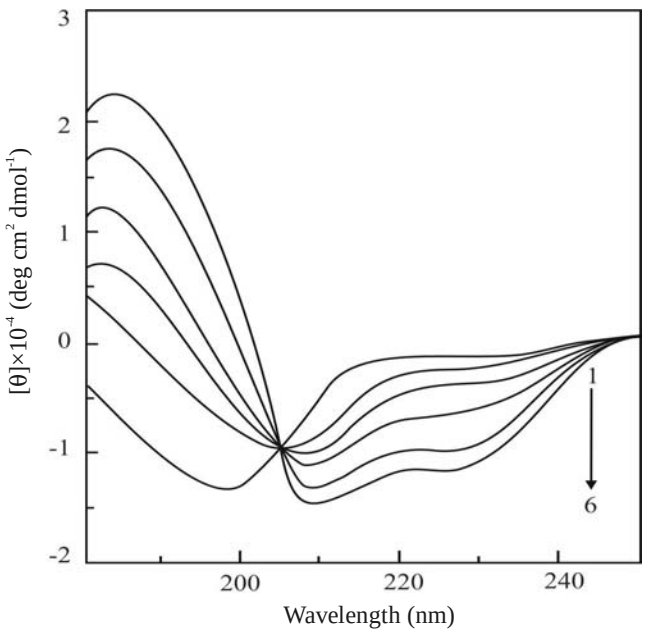

Fig. 1. Far-UV CD spectra of cyt c (ellipticity, [θ]) as a function of SDS concentration at pH 2 and 20°C. (1) 0 mM SDS (denatured state); (2) 0.2 mM SDS; (3) 1.5 mM SDS; (4) 3 mM SDS; (5) 20 mM SDS; (6) 60 mM SDS. Protein concentration was 16 μM. Arrows indicate the direction of the change

The UV-visible absorption in the Soret region of the same protein-surfactant complex as in Fig. 1 is shown in Fig. 2. For [SDS] in the 3-60 mM range, the precipitate redissolves, with only a minor peak intensity decrease; however, especially at high [SDS] (20 mM or above), a band shift is observed, suggesting that a structural change at the heme occurs within this region even though the secondary structure is constant, as shown from far-UV CD.

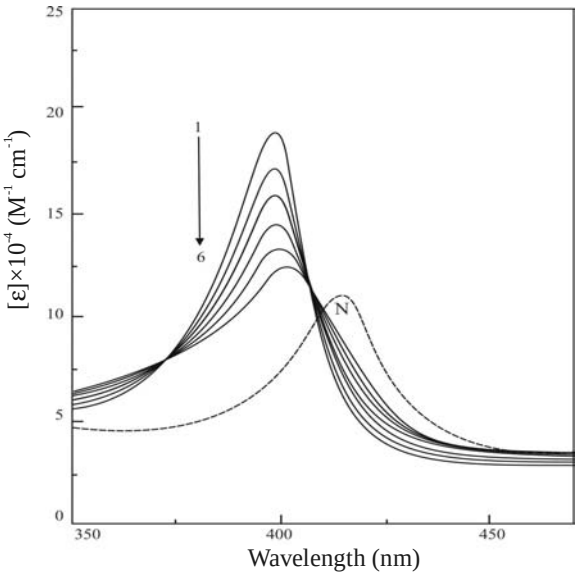

Fig. 2. Soret absorption spectra of cyt c (molar extinction coefficient, [ε]) as a function of SDS concentration at pH 2 and 20°C. (1) 0 mM SDS (denatured state); (2) 0.2 mM SDS; (3) 1.5 mM SDS; (4) 3 mM SDS; (5) 20 mM SDS; (6) 60 mM SDS. Protein concentration was 16 μM. Arrows indicate the direction of the change. Dashed lines show the native state in 25 mM phosphate buffer at pH 7

Fluorescence spectra (Fig. 3) arising from the single Trp in cyt c were also recorded for the same cyt c. SDS samples used in UV CD and Soret absorption experiments. In the native state structure, Trp59 is buried in the hydrophobic core and almost no fluorescence is observed, presumably being quenched by Forster energy transfer to the heme group[39]. However, when the protein is denatured, the Trp residue becomes solvated and intense fluorescence results. As seen in Fig. 3, when [SDS] is above 3 mM, an increase in the fluorescence is observed, and a small red shift occurs, especially at high SDS concentration (> 20 mM), indicating that SDS starts to unfold the protein. Factor analysis of the fluorescence spectra as a function of band shape also shows that there is a distinct maximum in the frequency shift of the band shape at around 10 mM SDS (data not shown), which agrees well UV-visible spectral results.

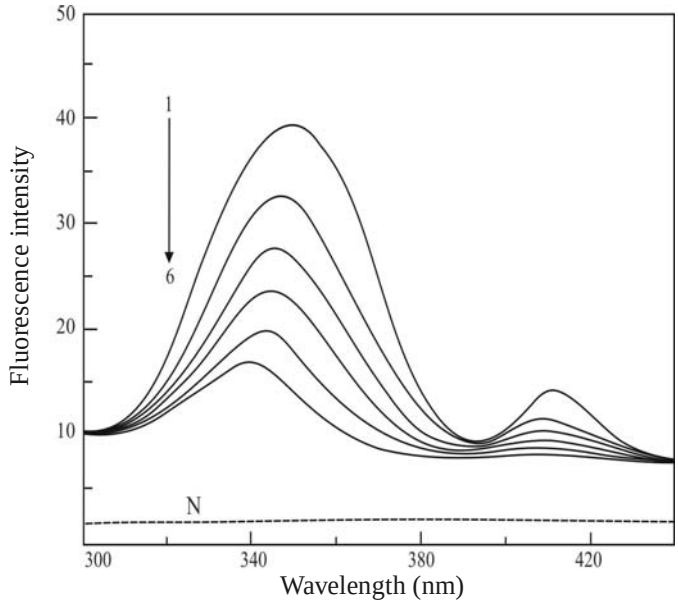

Fig. 3. Fluorescence spectra of titration of 16 μM acid-denatured cyt c with SDS at pH 2 and 20°C. (1) 0 mM SDS (denatured state); (2) 0.2 mM SDS; (3) 1.5 mM SDS; (4) 3 mM SDS; (5) 20 mM SDS; (6) 60 mM SDS. Protein concentration was 16 μM. Arrows indicate the direction of the change. Dashed lines show the native state in 25 mM phosphate buffer at pH 7

In the above titration experiments, molten globule like state was observed at above the CMC by use of far-UV CD, UV-visible and fluorescence. Thermal denaturation experiments monitored with far-UV CD were carried out to the molten globule like state found above. Fig. 4 shows the thermal denaturation behaviours of molten globule like state of cyt c induced by SDS at CMC concentration. At low temperature, molten globule like state shows a typical α-helical pattern, similar to that of the sodium *n*-alkyl sulfates-induced molten globule state at low pH[35]. For the molten globule like state, which has a high concentration of SDS (25 μM) and a high [SDS]/[cyt c] ratio of *ca.* 1500, a linear loss of ellipticity at 222 nm is observed from low temperature (5°C) without any thermally stable region. No band-shape disruption is observed for molten globule like even up to 80°C and when the sample is cooled down to 20°C, 82 % of the CD signal change at 222 nm is recovered. Meanwhile maintenance of the $[\theta]_{222}/[\theta]_{207}$ ratio before and after heating indicates that this process is mostly reversible. In addition, isodichronic point at 202 nm are observed in the thermal denaturation experiments for molten globule like state of cyt c induced by SDS at above CMC concentration, suggesting at two-state process, probably from α-helical to random coil conformation. For [SDS]/[Cyt] ratio of *ca.* 1500, molten globule like CD spectra typical of helical structures was

observed at high temperatures and no aggregation was observed for the whole temperature range. An isodichronic point at *ca*. 204 nm is found this sample, which could be consistent with a two-state transition with an increase of temperature, but only a broad transition with no sigmoidal behaviour. Upon cooling, CD intensity at 222 nm was fully recovered for sample and a similar $[\theta]_{222}/[\theta]_{207}$ ratio is obtained, indicating that high [SDS] unfolding process is fully reversible.

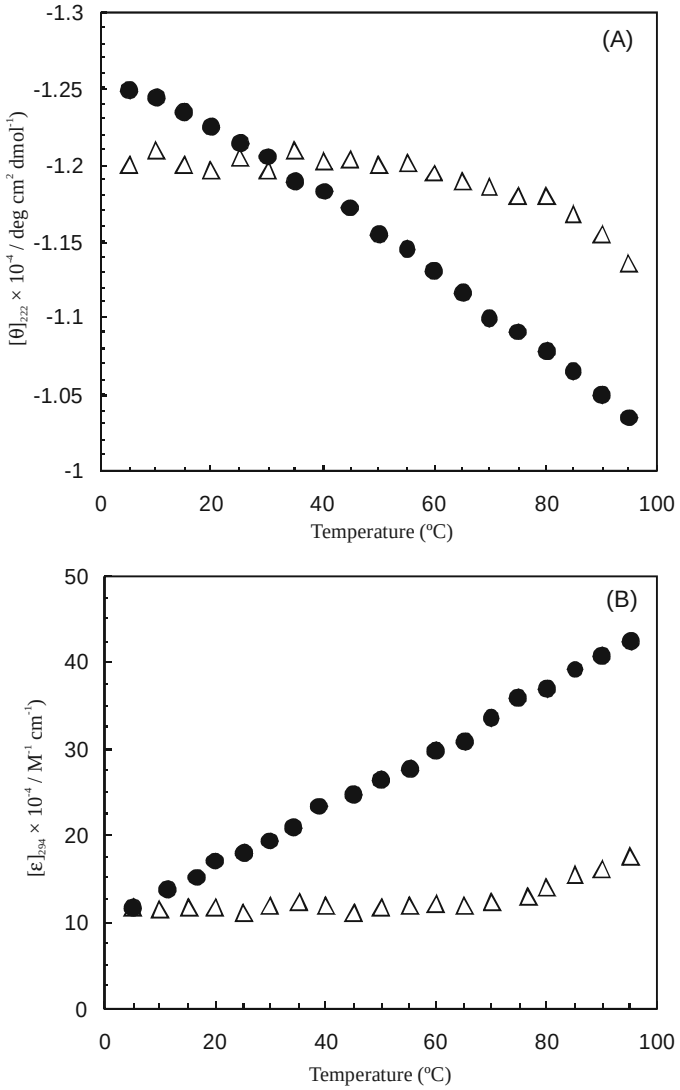

Fig. 4. Thermal denaturation of cyt c-SDS complex (MG-state) monitored by (A) Far-UV CD, (B) UV-Visible spectroscopy. Shown is 16 μM cyt c with a [SDS]/[cyt] ratio of 1500 at low pH (open triangle). Thermal denaturation of native cyt c has been shown with closed circle symbol at neutral pH

SDS, as well as other ionic surfactants, is known for its ability to denature some native proteins, including cyt c, by unordering its tertiary structure but leaving much secondary structure intact[40-47]. It has been reported that SDS above its CMC stabilizes peptides in helical conformations[48,49], whereas below its CMC, SDS has also been found to stabilize β-strands[50,51]. Addition of a small amount of SDS (negative) to aqueous lysozyme (net positive) solution has been reported to cause precipitation[52,53], but complete redissolution of the precipitate occurs when [SDS]/[protein] increases to > 19. Duggan and Luck[27] very early observed that the addition of SDS prevents the rise in viscosity of urea-denatured serum albumin. Recent research on cyt c denaturation by urea with SDS at physiological pH suggests that the existence of SDS will prevent the complete denaturation of cyt c by instead forming some stable partially folded states[54]. An other recent study reported the formation of a molten globule like state of acid-denatured cyt c with *n*-alkyl sulfates at low concentrations[30]; however, they focused on the effect of different chain lengths and they concluded that hydrophobic interactions play an important role in stabilizing the molten globule state.

In this work, SDS is shown to refold acid-denatured cyt c into molten globule like state that is obtained at high (above its CMC) SDS concentrations. Refolding experiments with the positively charged DTA surfactant also showed an ellipticity increase (molten globule like helix formation) for 6 mM at pH *ca.* 2 (its CMC). The full MG-like state is formed at *ca.* 16 mM, a concentration that is much smaller than the *ca.* 0.3 M required for NaCl[16-18], indicating that the hydrophobic effect, enhanced by the lipid, is an important factor for this protein refolding. The huge difference between SDS and NaCl induced refolding is probably also affected by the higher SDS binding efficiency. A revisit of the model for protein-surfactant interaction may give us some helpful information to understand the mechanism of protein folding. The "*necklace and bead structure*" of protein surfactant complexes has two possibilities for a dominant mode of interaction: (1) The protein wraps around the micelle and (2) the micelles nucleate on the protein hydrophobic sites[25,55-57]. Although we can not prove or disprove this model, we can evaluate the consistency of either model with our observed data. In structure (2), the more extended protein conformation could further expose the Trp residue to water, resulting in a red shift compared with structure (1). More importantly, it certainly would be less compact, resulting in less quenching due to the increased average separation between Trp and heme. Thus the characteristic of structure (2) are consistent with the experimental results at high [SDS]. Thermal denaturation experiments show that cyt c-SDS complexes with large [SDS]/[cyt] (and/or high [SDS]) have similar thermal behaviour in both low and

neutral pH, suggesting negligible pH effect under these conditions. However, the protein or SDS absolute concentration is an important factor in addition to the [SDS]/[cyt] ratio in determining the thermal behaviour of the protein-surfactant complex, as monitored by far-UV CD and fluorescence. Separate *necklace and bead* structures could explain the cyt-SDS complex behaviour under different conditions. For *ca.* 1.6 mM cyt c, the results suggest that the protein chain wraps around the micelle at about 20 mM SDS, whereas the micelle nucleates on the protein hydrophobic sites at higher [SDS] concentration.

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