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Formation of the Molten Globule-Like State of Cytochrome *c* Induced by *n*-Alkyl Sulfates at Low Concentrations

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The molten globule state of cytochrome *c* is the major intermediate of protein folding. Elucidation of the thermodynamic mechanism of conformational stability of the molten globule state would enhance our understanding of protein folding. The formation of the molten globule state of cytochrome *c* was induced by *n*-alkyl sulfates including sodium octyl sulfate, SOS; sodium decyl sulfate, SDeS; sodium dodecyl sulfate, SDS; and sodium tetradecyl sulfate, STS, at low concentrations. The refolding states of the protein were monitored by spectroscopic techniques including circular dichroism (CD), visible absorbance and fluorescence. The effect of *n*-alkyl sulfates on the structure of acid-unfolded horse cytochrome *c* at pH 2 was utilized to investigate the contribution of hydrophobic interactions to the stability of the molten globule state. The addition of *n*-alkyl sulfates to the unfolded state of cytochrome *c* appears to support the stabilized form of the molten globule. The *m*-values of the refolded state of cytochrome *c* by SOS, SDeS, SDS, and STS showed substantial variation. The enhancement of *m*-values as the stability criterion of the molten globule state corresponded with increasing chain length of the cited *n*-alkyl sulfates. The compaction of the molten globule state induced by SDS, as a prototype for other *n*-alkyl sulfates, relative to the unfolded state of cytochrome *c* was confirmed by Stokes radius and thermal transition point (T_m) measured by microviscometry and differential scanning calorimetry (DSC), respectively. Thus, hydrophobic interactions play an important role in stabilizing the molten globule state.

Key words: anionic surfactants, compactness, cytochrome *c*, hydrophobic salts, molten globule, stabilization.

Abbreviations: CD, Circular Dichroism; %F, percentage of fluorescence intensity; [ε], molar extinction coefficient; [θ], molar ellipticity; SOS, sodium *n*-octyl sulfate; SDeS, sodium *n*-decyl sulfate; SDS, sodium *n*-dodecyt sulfate; STS, sodium *n*-tetradecyl sulfate; U, unfolded state; MG, molten globule state.

The molten globule state is a compact denatured state with a significant native like secondary structure. In fact, it is a large, flexible and disordered tertiary structure (*1–7*). The molten globule-like state and other non-native states of proteins are present in both living cells and in various different physiological processes (*8*). Proteins can be transformed *in vitro* into the molten globule state at low pH. In addition, protein binding to a membrane surface results in "partial denaturation" (*i.e.* being transformed into a non-native state) (*9*).

The effect of polyanions such as poly (vinylsulfate) as a model for a negatively charged surface on the structure of ferric cytochrome *c* at acidic pH has been examined. The polyanion induces small changes in the native structure of the protein at neutral pH, but profoundly shifts the acid induced high spin state of the heme in the active center of cytochrome *c* to a more neutral pH region (*10*).

It is important to elucidate the structure and stabilizing mechanism of the molten globule state, as an intermediate between the native and denatured states, in order to understand the principles for constructing a three-dimensional protein structure. Recently, X-ray small angle scattering studies have shown that the molten globule states of various proteins have a wide range of structures from relatively disordered to highly ordered (*11–13*). This implies that the molten globule state is a largely fluctuating ensemble of various energy minimums. Moreover, the stability of the molten globule state is determined by a delicate balance of interactions, such as electrostatic repulsion between charged residues and opposing forces such as hydrophobic interaction. A significant influence of salts or charges on the stability of the molten globule state reveals that the main driving force of the molten globule state is the reduction of electrostatic repulsion between charged residues, which favors unfolded conformations (*14–16*). However there is a lack of substantial evidence regarding the contribution of hydrophobic interactions to the stability of the molten globule state. However, such interactions have been suggested for the positive heat capacity changes of the ther-

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mal unfolding of the molten globules of apomyoglobin (*17*, *18*) and cytochrome *c* (*19–21*).

The effects of various polyols, such as ethylene glycol, glycerol, erythritol, xylitol, sorbitol, and inositol, on the structure of acid-unfolded horse cytochrome *c* at pH 2 were investigated by several techniques, in order to address the contribution of hydrophobic interactions to the stability of the molten globule state of proteins. The addition of polyols induces the characteristic CD spectra of the molten globule, and this effect is enhanced with increasing concentration and chain length (the number of OH groups) of all polyols except ethylene glycol (*21*).

The importance of charge repulsion to the stability of acidic molten globules has been suggested by Goto and Fink (*15*). They examined the salt- and acid-induced formation of molten globules. They proposed that the conformation of acid-denatured proteins is determined by a balance of charge repulsion between positive groups.

Davis-Searles *et al*. (*22*) have recently reported that sugars induce the molten globule state of cytochrome *c.* This is mainly due to the increased steric repulsion between the protein and the sugar solution. Cosolvent effects of polyhydric compounds are based essentially on their antagonistic properties and non-polar groups. Systematic studies on amino acid solubility and preferential solvent interactions of proteins in polyol-water mixtures clearly demonstrate that the main driving force of protein stabilization by polyols is the enhancement of hydrophobic interactions (*23–26*).

Destabilization of the cytochrome *c* structure has also been observed by the action of lipids/membranes as deduced from NMR (*27*), Fourier-transform infrared (*28*) and Raman (*29*, *30*) studies. Hildebrandt and Stockburger (*30*) proposed a conformational change induced by the electric field around the heme crevice in cytochrome *c,* although Muga *et al*. (*28*) later suggested that such local changes could be the consequence of overall destabilization of the protein structure. The intriguing possibility that the conformationally destabilized state of cytochrome *c* caused by electrostatic and/or hydrophobic interactions might be a folding intermediate on the membrane surface should be addressed. Surfactants can be used as models for studying the effect of such interactions with cytochrome *c*. Moreover, the structure of the unfolded state of the protein needs to be characterized in detail in order to understand role folding mechanism *in vitro* and *in vivo* (*30*).

Conformational changes and denaturation of many proteins in surfactant solutions have been studied (*31–33*). Protein denaturation occurs in a number of ways. On the millimolar scale, sodium *n*-dodecyl sulfate (SDS) at higher concentrations is believed to be one of the most effective denaturants (*34*). Moosavi-Movahedi has reviewed the folded and unfolded states of proteins in the presence of both low and high concentrations of surfactants (*34–36*). The mechanism of unfolding and the kinetics of absorbance and fluorescence changes of ferricytochrome *c* induced by sodium dodecyl sulfate have been studied (*37*, *38*). There are no reports on the formation of the molten globule of proteins induced by surfactants except for our previous report on the formation of the molten globule of glutamate dehydrogenase induced by octyl glucoside as a nonionic detergent (*39*). In this paper, we report the formation of a molten globule state of acid-unfolded cytochrome *c* induced by *n*-alkyl sulfates as anionic surfactants.

MATERIALS AND METHODS

Materials—Horse cytochrome *c* (type IV), in the oxidized form, was purchased from Sigma. Sodium octyl sulfate and Sodium dodecyl sulfate were also purchased from Sigma. Sodium decyl sulfate and Sodium tetradecyl sulfate were obtained from Merck and Lancet Co, respectively. Other chemicals were of reagent grade. The concentrations of *n*-alkyl sulfates used in all experiments were under the critical micelle concentrations (cmc). The cmc values of SOS, SDeS, SDS, and STS under acidic condition (HCl 20 mM, pH 2) are to 4.5, 0.67, 0.28, and 0.15 mM, respectively (*40*, *41*).

Solution Preparation—The protein solution was dialyzed against buffers (20 mM HCl, pH 2, and 25 mM phosphate buffer, in pH 7). The extinction coefficients were used to calculate the concentration of the native protein at pH 7 and 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 (*42*):

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

Aliquots of *n*-alkyl sulfate were injected into the cytochrome *c* solution at 5 min intervals to allow for equilibration; each experiment was repeated three times.

Methods—Circular dichroism measurements: All measurements were carried out at 20°C with thermostatically controlled cell holders. CD spectra were measured with a JASCO J-715 spectropolarimeter (Japan) equipped with an interface and a personal computer. The instruments were calibrated with ammonium *d*-10-camphorsulfonic acid (*43*). The results are expressed as the mean residue ellipticity [θ], which is defined as $[\theta] = 100 \times \theta_{obsd}/(lc)$, where θ_{obsd} 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. The CD spectra were measured at protein concentrations of 5 μM with a 1-mm path length cell from 250 to 190 nm.

Absorption measurements: Soret absorption spectra of cytochrome *c* were obtained with a spectrophotometer, Model Shimatzu-3100, 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, 1.06×10^5 M^{-1}·cm^{-1} at 410 nm at pH 7 (*12*). The spectrum of ferricytochrome *c* at pH 7 in 25 mM phosphate buffer was taken as a reference.

Fluorescence measurements: Fluorescence measurements were made on Hitachi MPF-4 spectrofluorometer at an excitation wavelength of 285 nm. Trp fluorescence emission was followed at 350 nm (*44*, *45*). Anionic surfactants significantly affect the fluorescence of free tryptophan under the experimental conditions used. The

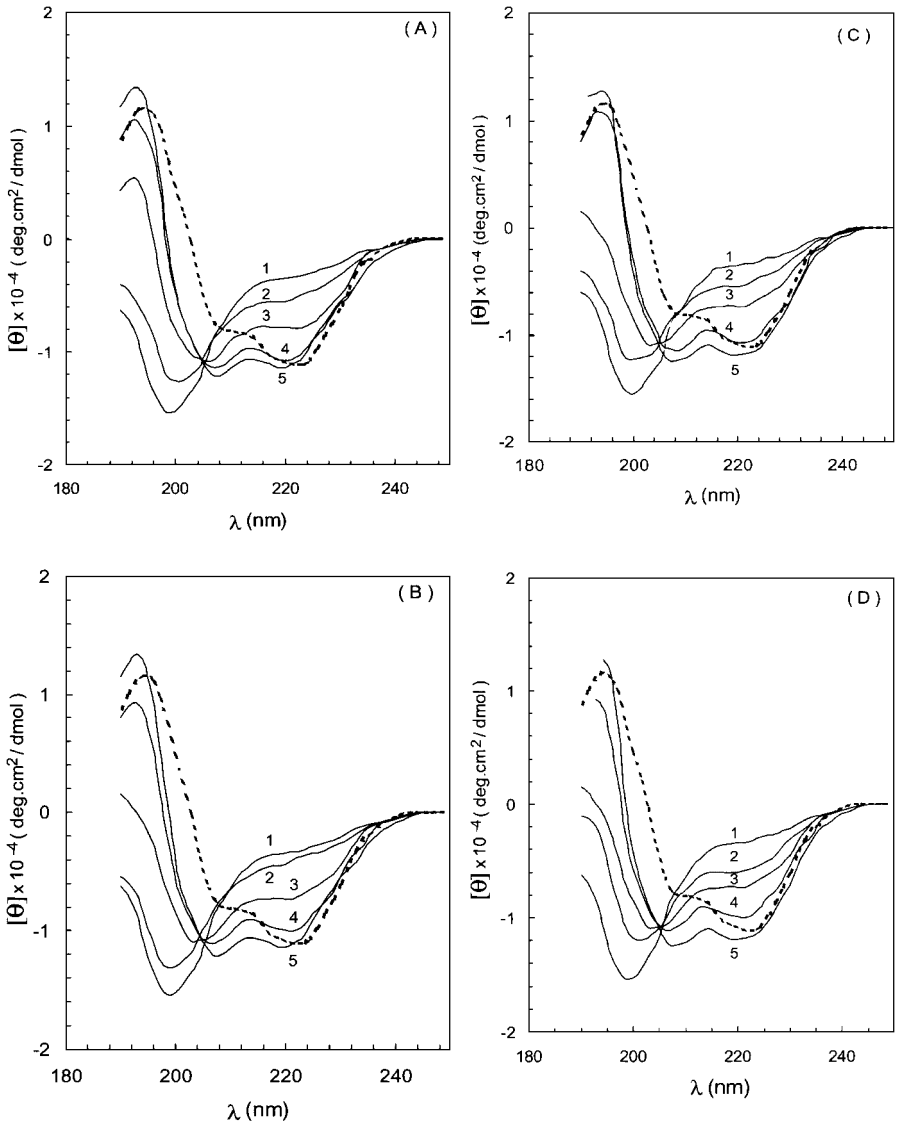

Fig. 1. **Far UV CD spectra of cytochrome *c* (ellipticity, [θ]) as a function of *n*-alkyl sulfates concentration at pH 2 and 20°C.** (A) 1, 0 mM SOS (denatured state at pH 2); 2, 0.26 mM SOS; 3, 0.7 mM SOS; 4, 1.02 mM SOS; 5, 1.2 mM SOS. (B) 1, 0 mM SDeS (denatured state at pH 2); 2, 0.05 mM SDeS; 3, 0.1 mM SDeS; 4, 0.15 mM SDeS; 5, 0.195 mM SDeS. (C) 1, 0 mM SDS (denatured state at pH 2); 2, 0.01 mM SDS; 3, 0.037 mM SDS; 4, 0.06 mM SDS; 5, 0.08 mM SDS. (D) 1, 0 mM STS (denatured state at pH 2); 2, 0.01 mM STS; 3, 0.02 mM STS; 4, 0.03 mM STS; 5, 0.05 mM STS. The spectrum of the native state (dotted line) in 25 mM phosphate buffer at pH 7 is shown for comparison. Protein concentration was 5 μM.

temperature of the cell compartments was kept constant at 20°C by water circulation.

Measurements of viscosity and Stokes radius: The viscosity was measured using a Haake D8 (W. Germany) microviscometer. The intrinsic viscosities, [η], and Stokes radii, *Rs*, of the protein–SDS complexes were determined using the equation (*33*):

$$\eta_{sp}/c \cong [\eta] = \lim_{c\to 0}[(\eta/\eta_0 - 1)/c] = 2.5N_A/M(4/3\pi Rs^3)$$

where η_{sp} is the specific viscosity, c is the protein concentration in grams per milliliter, N_A is Avogadro's number, M is the molecular weight of the protein and π is equal to 3.14

Tm measurements: Thermal transition point (T_m) measurements were carried out with a high sensitivity differential scanning calorimeter (DSC), Scal 1 (Russia, Pushchino), equipped with a personal computer. The scan rate was 1.0 deg/min for all experiments. The protein concentration was 1.0 mg/ml.

RESULTS AND DISCUSSION

Circular Dichroism—Panels A, B, C, and D in Fig. 1 show the effects of various *n*-alkyl sufates (sodium octyl sulfate, SOS, sodium decyl sulfate, SDeS, sodium dodecyl sulfate, SDS, and sodium tetradecyl sulfate, STS) on the far UV-CD spectra of the HCl-unfolded cytochrome *c*. In the absence of n-alkyl sulfate, the protein was substantially unfolded at pH 2 (see Fig. 1, curve 1) and the addition of *n*-alkyl sulfates at low concentrations induced substantial α-helical structure as seen by the lower minima (lower $[\theta]_{208}$, $[\theta]_{222}$] at 208 and 222 nm relative to the native spectrum (see Fig. 1, curves 4 and 5). The CD spectra also show the molten globule state for cytochrome *c* upon the addition of SOS (1.2 mM), SDeS (0.195 mM), SDS (0.08 mM), and STS (0.05 mM). The shapes of the CD spectra are similar to the spectrum of cytochrome *c* in the presence of 0.4 M NaCl (*46*). The CD spectra at different concentrations of various *n*-alkyl sulfates show an isodichronic point at 205.5 nm for cytochrome *c*. This suggests that the refolding process is a two-state transition (*45*). The values of ellipticity at $[\theta]_{222}$ for both native and

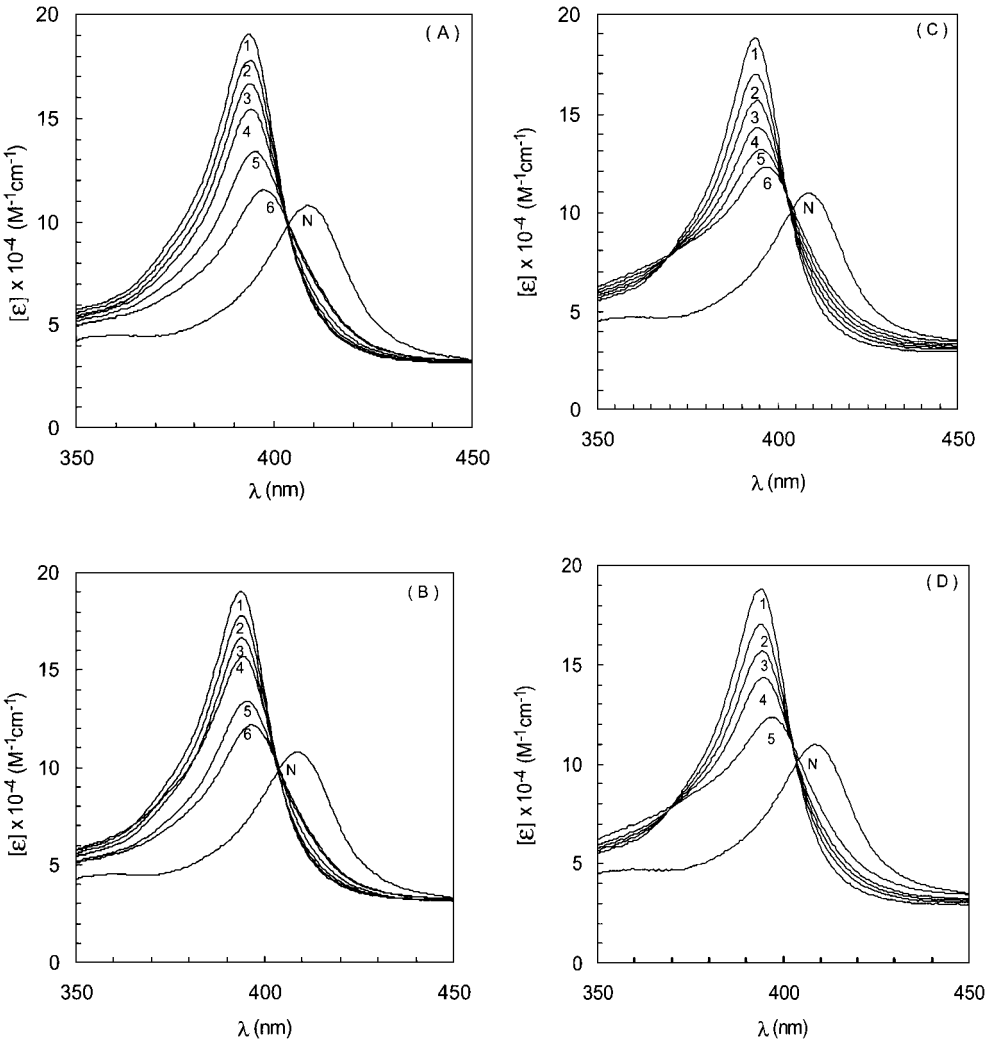

Fig. 2. **Soret absorption spectra of cytochrome *c* (molar extinction coefficient, [ε]) as a function of *n*-alkyl sulfate concentrations at pH 2 and 20°C.** (A) 1, 0 mM SOS (denatured state at pH 2); 2, 0.26 mM SOS; 3, 0.43 mM SOS; 4, 0.6 mM SOS; 5, 0.82 mM SOS; 6, 1.2 mM SOS. (B) 1, 0 mM SDeS (denatured state at pH 2); 2, 0.1 mM SDeS; 3, 0.12 mM SDeS; 4, 0.15 mM SDeS; 5, 0.17 mM SDeS; 6, 0.195 mM SDeS. (C) 1, 0 mM SDS (denatured state at pH 2); 2, 0.02 mM SDS; 3, 0.04 mM SDS; 4, 0.06 mM SDS; 5, 0.07 mM SDS; 6, 0.08 mM SDS. (D) 1, 0 mM STS (denatured state at pH 2); 2, 0.02 mM STS; 3, 0.03 mM STS; 4, 0.04 mM STS; 5, 0.05 mM STS. The spectrum of the native state (N) in 25 mM phosphate buffer at pH 2 is shown for comparison. Protein concentration was 5 μM.

n-alkyl sulfates complexed were similar. However the values of ellipticity at $[\theta]_{208}$ changed upon the addition of *n*-alkyl sulfates. The negative increment in $[\theta]_{208}$ is believed to have occurred because the protein α-helix conformation is kept in a fixed position for molten globule state at low concentrations of *n*-alkyl sulfates, as indicated by the far UV-CD spectrum. Considering these findings, the *n*-alkyl sulfate–induced conformations are not an indication of the native state. In fact, they are regarded as molten globule states of the protein with similar secondary structures. The helical contents in the unfolded state and the native state of cytochrome *c* are 4 and 30% respectively, on the basis of the ellipticity values at 222 nm as calculated by the method of Chen *et al*. (*47*). The helical content of the molten globule-state of cytochrome *c* induced by low concentrations of sodium octyl sulfate (SOS), sodium decyl sulfate (SDeS), sodium dodecyl sulfate (SDS), and sodium tetradecyl sulfate (STS) are 31, 31, 33.6, and 33.6%, respectively, according to this method.

Soret Absorption Spectra—The heme absorption of cytochrome *c* in the vicinity of 400 nm reflects the spin state of the iron, which is dependent on the conformational state of the protein (Fig. 2). Whereas the native protein (low spin state) shows a maximum at 410 nm with an extinction coefficient of 1.06×10^5 $M^{-1}\cdot cm^{-1}$, the unfolded state (high spin state) has a maximum at 394 nm and an extinction coefficient of 1.90×10^5 $M^{-1}\cdot cm^{-1}$. The absorption wavelength maxima and the molar absorptivities obtained coincide with the corresponding spin state of cytochrome *c* (*46*, *48*). The absorption coefficients of *n*-alkyl sulfates are also negligible at 400 nm, thus we can clearly study the effects of *n*-alkyl sulfates on cytochrome *c*. Figure 2 (panels A, B, C, and D) shows the changes in the Soret absorption spectra from the unfolded state to the molten globule state in the presence of various *n*-alkyl sulfates. It is apparent that the maximal wavelength increases as the intensity decreases. The Soret spectra at different concentrations of SOS, SDeS, SDS, and STS show an isosbestic point at 405 nm for each. The interaction of cytochrome *c* with *n*-alkyl sulfates at different concentrations causes different absorptivity at pH 2. The addition of low concentrations of *n*-alkyl sulfates to the acid-unfolded state of cytochrome *c* decreases the absorption intensities. It is important to note that cytochrome *c* in the presence of high concentrations of *n*-alkyl sulfates starts to unfold (data not shown).

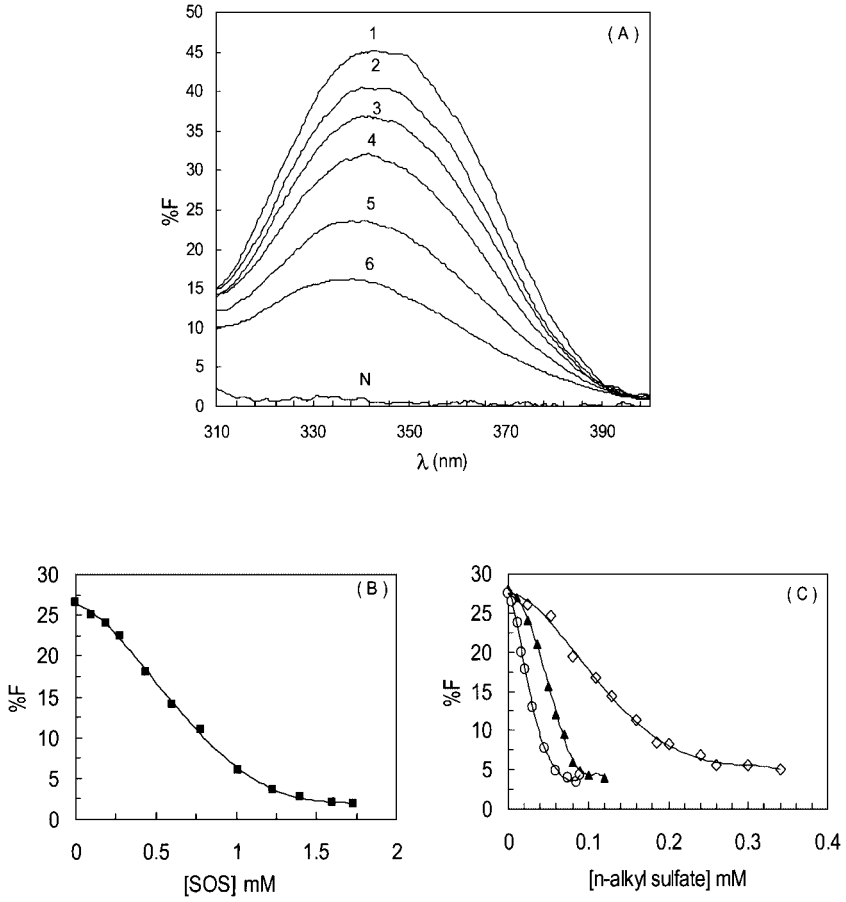

Fig. 3. **(A) Flourescence spectra of cytochrome *c* (percentage of fluorecence intensity, *%F*) as a function of SDS concentration at pH 2 and 20°C ($\lambda_{Excitation}$ = 285 nm and $\lambda_{Emission}$ = 350 nm).** 1, 0 mM SDS (denatured state at pH 2); 2, 0.01 mM SDS; 3, 0.037 mM SDS; 4, 0.05 mM SDS; 5, 0.07 mM SDS; 6, 0.08 mM SDS. The spectrum of the native state (N) in 25 mM phosphate buffer at pH 7 is shown for comparison. **(B) Plot of fluorescence intensity versus SOS (filled squares) concentration. (C) The plots of fluorescence intensity *versus* the concentrations of SDeS (open diamonds), SDS (filled triangles), and STS (open circles).**

The molten globule state induced by SOS, SDeS, SDS, and STS occurred at λ_{max} of 397 nm with extinction coefficients of 10.9×10^4, 11.4×10^4, $9.2 \times 10_4$, and 11×10^4 $M^{-1}\cdot cm^{-1}$, respectively.

Fluorescence Spectra—Figure 3A shows the effect of SDS on the fluorescence spectra of the acid-unfolded state of cytochrome *c*. According to Fig. 3A, the addition of low concentrations of SDS to the acid-unfolded state of cytochrome *c* (<0.12 mM) causes a decrease in the fluorescence intensity. However, under same conditions, the addition of higher concentrations of SDS (>0.12 mM) dequenched the fluorescence spectra (data not shown). Here, the interaction of cytochrome *c* with SDS (0.08 mM), (see curve 6, Fig. 3A) is consistent with the results obtained for cytochrome *c* in the presence of 0.4 M NaCl as previously reported by Goto *et al*. (*46*). The correspond-

Table 1. **Intrinsic viscosity, Stokes radius and thermal transition point of native, acid-unfolded and various structural states of cytochrome *c* induced by various concentrations of SDS[a].**

Protein states	[η] (M^{-1})	*Rs* (Å)[b]	*Rg* (Å)[c]	T_m (°C)
Native	17.7 ± 0.2	14.1 ± 0.2	13.5 ± 0.1	77*
Acid-unfolded	82.8 ± 0.2	23.6 ± 0.2	24.2 ± 0.3	51*
SDS (0.06 mM)	31.5 ± 0.3	17.1 ± 0.2	NM	NM
SDS (0.07 mM)	28.3 ± 0.3	16.5 ± 0.2	NM	NM
SDS (0.08 mM)	23.0 ± 0.3	15.4 ± 0.2	NM	69.5*
SDS (0.12 mM)	39.3 ± 0.3	18.4 ± 0.2	NM	NM
SDS (0.16 mM)	53.5 ± 0.3	20.4 ± 0.3	NM	NM
SDS (0.2 mM)	102.0 ± 0.3	25.3 ± 0.3	NM	NM
SDS (0.3 mM)	197.0 ± 0.3	31.5 ± 0.3	NM	NM

[a]SDS was added to the acid-unfolded state of cytochrome *c*. [b]The values were calculated from equation:

$$\eta_{sp}/c \cong [\eta] = \lim_{c\to 0}[(\eta/\eta_0 - 1)/c] = 2.5N_A/M(4/3\pi Rs^3)$$

[c]The data are taken from Ref. *11*. The molten globule state induced by SDS at 0.08 mM concentration was obtained from the acid-unfolded state of cytochrome *c*. NM, not measured. *T_m is dependent on the instrument scan rate; these data use a DSC scan rate of 1.0 deg/min.

Table 2. **ΔG (H_2O), m values and inflection transition points for the molten globule state of cytochrome c at 20°C upon interaction with n-alkyl sulfates.**

n-Alkyl sulfates	ΔG (H_2O) kJ·mol^{-1} [a*]	m (kJ·mol^{-1}·M^{-1})[b*]	C_m (mM)[c*]
SOS	7.41 ± 0.01	10.7	0.70
SDeS	10.10 ± 0.01	71.4	0.14
SDS	11.63 ± 0.01	409.2	0.03
STS	11.90 ± 0.01	490.0	0.02
Na_2SO_4[d]	9.92	7.69	1.30

[a]ΔG (H_2O) was calculated by the least-square method from Eq. 2. [b]A parameter reflecting the hydrophobicity of the transition state. [c]The midpoint concentration of transition. [d]The raw data taken from the sigmoidal curve for the unfolded to molten globule state of cytochrome c upon the addition of Na_2SO_4 (*46*). [*]The data were determined from the fluorescence intensity, molar extinction coefficient and molar ellipticity sigmoidal curves (Figs. 3, B and C, and 4, A and B).

ing experiments were also carried out for SOS, SDeS, and STS.

Sigmoidal Curves—Figures 3 (B and C) and 4 (A and B) show the profile transitions of the unfolded to the molten globule state of cytochrome c induced by various concentrations of n-alkyl sulfates as measured by the change in emission at 350 nm, absorption at 394 nm, and ellipticity at 222 nm. These figures also show the effects of the number of hydrophobic chains on the inflection point of the cited profiles. When the hydrophobic chain increases, the inflection points of the profiles. The results show that the refolding ability of cytochrome c increases according to the number of hydrophobic chains.

Intrinsic Viscosity and Stokes Radius—Table 1 shows intrinsic viscosity and Stokes radius of the acid-unfolded state, different structural states induced by various concentrations of SDS, and the native state of cytochrome c at 20°C. The Stokes radii indicate different values for the native, molten globule (protein in the presence of 0.08 mM SDS) and acid-unfolded states. These results show the compaction of the molten globule state (Rs = 15.4 ± 0.2 Å) relative to acid-unfolded state (Rs = 23.6 ± 0.2 Å) for cytochrome c. The Rs values are nearly consistent with the gyration radius (Rg) values (Table 1). It is important to note that SDS at low concentrations (<0.12 mM) induces the formation of the molten globule state of cytochrome c whereas at higher concentrations (>0.12 mM) it promotes unfolding of the protein.

Thermal Unfolding as Measured by Differential Scanning Calorimetry—The thermal unfolding transition of the native, molten globule induced by SDS and the acid-unfolded states of cytochrome c at 20 mM HCl, pH 2, were measured by differential scanning calorimetry, and the values of the thermal transition points (T_m) are tabulated in Table 1. Table 1 shows that the T_m of the molten globule state is higher than that of the acid-unfolded state but lower than that of the native state of cytochrome c.

Thermodynamic Analysis of Molten Globule Formation—Figures 3 (B and C) and 4 (A and B) show the sigmoidal curves (drawn by a numerical analysis method, called qubic-spline in the MATLAB program, version 6.1) for the unfolded (U) to the molten globule state (MG) of cytochrome c upon the addition of n-alkyl sulfates. Cyto-

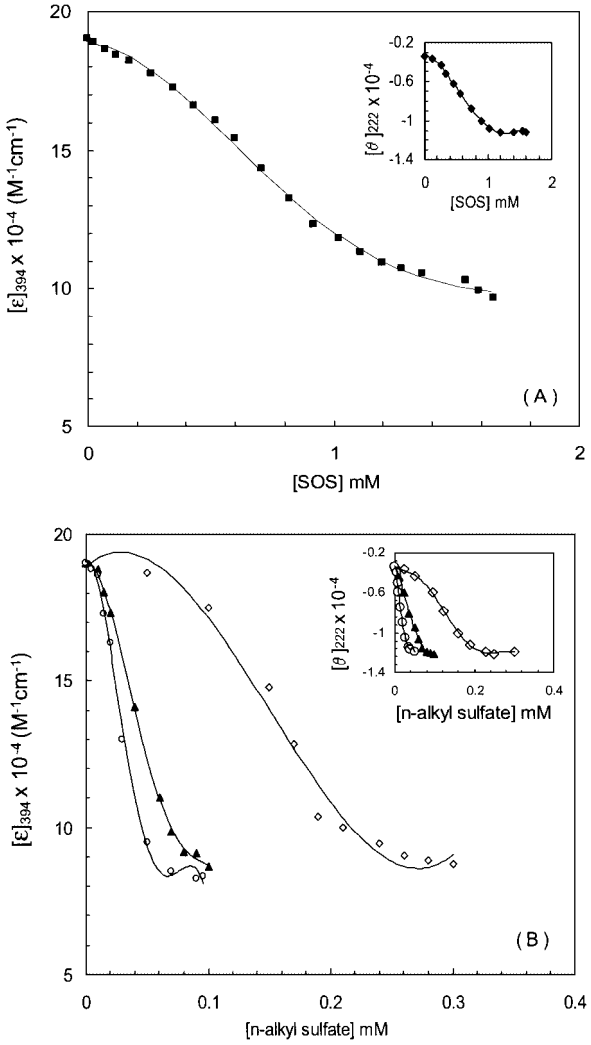

Fig. 4. **(A, B) Plots of molar extinction coefficient ($[\varepsilon]_{394}$) *versus* total concentrations of n-alkyl sulfates at 20 mM HCl and 20°C.** (A, B) insets: Plots of molar ellipticity ($[\theta]_{222}$) *versus* concentrations of n-alkyl sulfates at 20 mM HCl and 20°C. SOS (filled squares); SDeS (open diamonds); SDS (filled triangles); and STS (open circles).

chrome c is a small protein with a single subunit. Therefore, a two-state analysis based on the Pace theory was performed (*49*). It is now possible to obtain equilibrium constants (K) for the U-MG states and to calculate the corresponding Gibb's free energy changes, ΔG, as follows:

$$\Delta G = -RT \ln K = -RT \ln (A_{obs} - A_U)/(A_{MG} - A_{obs}) \quad (1)$$

where R is the gas constant, T is the absolute temperature, A_U, A_{MG}, and A_{obs} are the physical parameters of extinction coefficient, molar ellipticity and percentage of fluorescence of U, MG, and any observed states, respectively.

Figure 5 (A and B) shows the plot of ΔG against total n-alkyl sulfate concentration ($[n\text{-alkyl sulfate}]_{total} = [n\text{-alkyl sulfate}]_{free} + [n\text{-alkyl sulfate}]_{bound}$). The free energies of molten globule formation in the absence of n-alkyl sul-

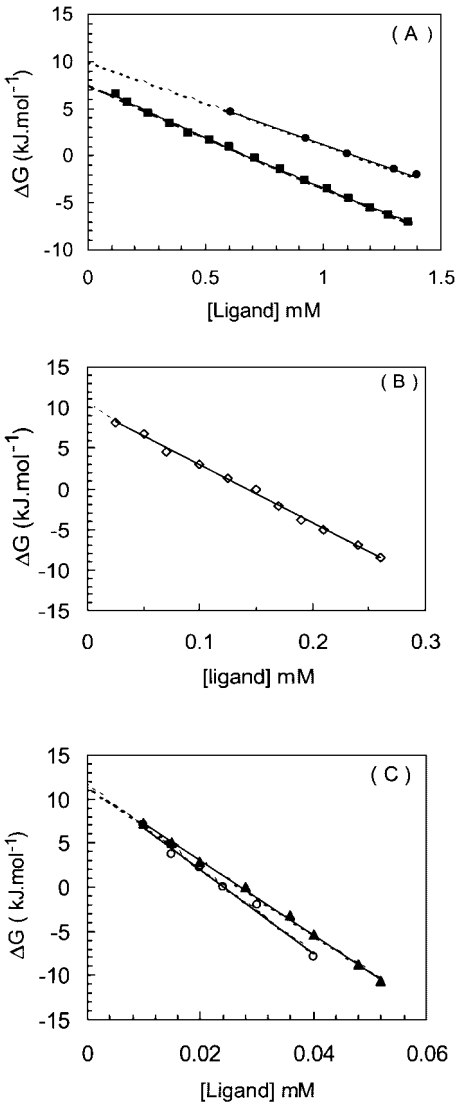

Fig. 5. **(A–C) Free energies values (Δ*G*) *versus* concentrations of ligands (*n*-alkyl sulfates and Na_2SO_4).** (A) Na_2SO_4 (filled circles) [the raw data were taken from the sigmoidal curve in reference (*46*)]; SOS (filled squares); (B) SDeS (open diamonds); (C) SDS (filled triangles); STS (open circles).

fates, ΔG (H_2O), were calculated by the least-squares method from the following equation (*49*):

$$\Delta G = \Delta G\,(H_2O) - m\,[n\text{-alkyl sulfate}] \qquad (2)$$

where m is the slope of linear curve reflecting the cooperativity and also hydrophobicity of the transition state. The m-value correlates very strongly with the amount of protein surface exposed to the solvent upon unfolding (*50*). A similar dependence on accessible surface area has been found for the heat capacity change (ΔC_p), which was confirmed for a set of proteins (*50*, *51*). The m-values and heat capacity changes correlate well with each other, and also ΔC_p of the protein is linearly related to the fraction of hydrophobic residues (*52*). Thus, for proteins that undergo a simple two-state unfolding mechanism, the amount of surface exposed to solvent upon unfolding is a main structure determinant for m-values, ΔC_p and hydrophobicity. The $\Delta G(H_2O)$ and m values are tabulated in Table 2. The free energy values shown as the dotted linear line in Fig. 5A belong to Na_2SO_4, which was calculated by the authors [raw data were taken by sigmoidal curve from reference (*46*)].

It is apparent from Table 2 that the values of the midpoint concentration (C_m) and m-values of the conformational transitions induced by SOS, SDeS, SDS, and STS are not identical; as the chain lengths of n-alkyl sulfates increases, the values of C_m and "m" decrease and increase, respectively.

We have reported that anionic surfactants are the most commonly used denaturants for proteins at high concentration; and they are responsible of the folded state of some proteins at low concentration (*33*, *34*). The structure and stabilizing mechanism of molten globule states of proteins have been widely investigated. It is believed that the main driving force of the salt-induced molten globule state is the reduction of the electrostatic repulsion between the charged groups in the protein molecule (*16*, *46*, *53*).

Kamiyama *et al*. reported that the addition of polyols induces the characteristic CD spectra of a molten globule, and this effect is enhanced with increasing concentration and chain length (the number of OH groups) of polyols (*21*).

The addition of salts reduces the electrostatic repulsion, while it has only a small effect on hydrogen-bonding and hydrophobic interactions at low concentrations. This produces a favorable net force for the formation of the molten globule. This is in contrast to the effects of polyols, which have only small effects on the electrostatic interactions. The dielectric constant of water is not so markedly reduced as to bring about the formation of ion-pairs at high polyol concentrations. Kamiyama *et al*. demonstrated that polyols can stabilize the molten globule state of cytochrome *c*. The stabilizing mechanism and structure of the molten globule state induced by polyols have been discussed in terms of the preferential solvent interactions and osmotic pressure of the medium in comparison with the salt-induced molten globule state (*21*, *53*). In this paper we provide evidence for the stabilization of the molten globule state of cytochrome *c* by n-alkyl sulfates as salts with hydrophobic chains. Accordingly, the authors label n-alkyl sulfates as hydrophobic salts. This is new evidence that hydrophobic interactions contribute substantially to the stabilization of the molten globule state, as expected from the positive heat capacity change of thermal unfolding (*17*, *25*).

In this paper, n-alkyl sulfates were selected because of their dual electrostatic and hydrophobic interactions. These are amphipatic compounds with a polar head and a nonpolar tail group. Studies of n-alkyl sulfates with identical polar heads but different nonpolar tails will allow us to determine the contribution of electrostatic and hydrophobic forces to the induction of protein refolding, such as to molten globule conformation. The effects of various n-alkyl sulfates on the acid-unfolded states of cytochrome *c* show that the longer chain length increases the ability of the molten globule state to be formed. Therefore, hydrophobic forces play a dominant role in inducing the molten globule state.

Alkyl sulfates are salts with hydrophobic chains. The conformation of acid-unfolded proteins is determined by the balance of charge repulsion between positive groups. When *n*-alkyl sulfates are adding to the acid-unfolded state of cytochrome *c*, the shielding of intramolecular electrostatic repulsive forces in the unfolded state by the negative polar head of *n*-alkyl sulfate binding reflects the intrinsic forces that favor the formation of the molten globule state. It was previously reported that two binding sets exist in protein-surfactant complexes, the first involving electrostatic interactions and the second having a hydrophobic nature (*54–58*). Once all the ionic sites are saturated, the hydrophobic contribution predominates. Alkyl sulfates play an important role in electrostatic interactions at low concentrations and they have a hydrophobic nature at high concentrations. Due to the nature of the acid-unfolded state of cytochrome *c* with a positively charged surface, it was also suggested that the first interaction of a monovalent anionic surfactant is electrostatic. It is important to note that the presence of a hydrophobic moiety together with the electrostatic contribution of *n*-alkyl sulfates upon interaction with cytochrome *c* produces the MG state, whereas the interaction of protein with *n*-alkyl sulfates at high concentration produces the predominant hydrophobic force that induces protein denaturation. In this manner, short-range interactions between nonpolar groups on a protein and the nonpolar tail of *n*-alkyl sulfates at low concentrations induce the compaction state, but at high concentrations of *n*-alkyl sulfates, the hydrophobic interactions predominate relative to the electrostatic contribution and protein unfolds.

On the other hand, the dielectric constant plays an important role in the protein dynamics of charged side chains. With enhancement of the dielectric constant, the protein dynamics of the charged side chains increase (*59–61*). Alkyl sulfates at low concentration decrease the dielectric constant of MG transition relative to the acid-unfolded state of cytochrome *c*. This demonstrates that *n*-alkyl sulfates at low concentration as salts with hydrophobic chains decrease the dynamics and increase protein compaction (data not shown).

The spectroscopic properties of the molten globule state strongly support the view that *n*-alkyl sulfates stabilize the molten globule state of cytochrome *c.* The molten globule state, in fact, shows a native-like amounts of α-helix. The spectroscopic properties of the molten globule state closely resemble the ones, as reported by Goto *et al*. (*46*), thus implying a close structural similarity.

A key parameter for characterizing the molten globule state is compactness or globularity, but only limited data have been reported on the direct measurement of this quantity (*62–65*). The intrinsic viscosity and Stokes radius, *Rs*, (*26*, *33*, *66–69*) are crucial measurements of the compactness of molten globule states. The Rs values in Table 1 show the most compact state is the native form. The molten globule state induced by SDS at low concentration (0.08 mM, SDS) is more compact than the acid-unfolded state of cytochrome *c* (pH 2). The results show that the (*Rs*) value of the molten globule state is closer to the native form than to the unfolded state in the case of cytochrome *c* (Table 1). Others have also reported that the molten globule state is the closer intermediate to the native state (*70*, *71*). It is also noteworthy that the *Rs* values indicate the folding of the acid-unfolded state of cytochrome *c* during the addition of low concentrations of SDS (less than 0.12 mM). These states are expanded slightly compared to the native state, and are much more compact than the acid-unfolded state. Results at high (>0.2 mM) concentrations of SDS show the *Rs* value relative to the native state increases and that the acid-unfolded state at pH 2 still retains some residual structure. Another good diagnosis of the stability and globularity of the molten globule state is the thermal transition point (T_m) (*21*, *53*, *72*, *73*). The values of the thermal transition point (T_m) in Table 1 show that the most stable form is the native state. The molten globule state induced by SDS (0.08 mM) is more stable and also more compact than the acid-unfolded state of cytochrome *c* (pH 2). These results confirm that the molten globule state induced by SDS (0.08 mM) is more compact than the acid-unfolded state of cytochrome *c*. Our results show the thermal profile of the acid-unfolded state of cytochrome *c* at pH 2, which is thought to be partially unfolded. Others have reported that the acid-unfolded state at pH 2 still retains some residual structure (*11*). No tendency to aggregate was observed for any of the states.

One of the best criteria for determining protein stability is free energy in the absence of ligand, $\Delta G(H_2O)$ or *m*-values. Table 2 shows the *m*-values and $\Delta G(H_2O)$ for the molten globule state of cytochrome *c* upon the addition of *n*-alkyl sulfates such as SOS, SDeS, SDS, and STS. Table 2 shows the increase in $\Delta G(H_2O)$ and *m*-values that corresponds to the length of the hydrophobic chains. Table 2 also indicates the appearance of a higher stabilized molten globule state, which corresponds to the interaction of cytochrome *c* with *n*-alkyl sulfates. Therefore, our results show a direct role of hydrophobicity to the stability of the molten globule state.

The *m*-values were calculated for the molten globule formation induced by Na_2SO_4 (7.69 kJ·mol^{-1}·M^{-1} at 20°C), which is the lowest, and C_m, which is the highest, compared to *n*-alkyl sulfates. Therefore, the formation of molten globule states of cytochrome *c* induced by *n*-alkyl sulfates is stabilized by the presence of hydrophobic tails of different lengths.

Concluding Remarks—The molten globule state of cytochrome *c* induced by *n*-alkyl sulfates at low concentrations is a stabilized form with higher free energy in the absence of ligand, $\Delta G(H_2O)$ and *m*-values. The greater free energy and *m*-values correspond to the hydrophobic chain length of *n*-alkyl sulfates, which play a salt-like role exposing the molten globule state. The hydrophobic salts show a greater affinity for the molten globule state, nearly 50 times of the salts without hydrophobic chains.

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