Quantitative Study of the Hydrophobic Interaction Mechanism between Urea and Molecular Probes Used in Sensing Some Microheterogeneous Media

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The interactions of 2-phenyl-3,3-dimethyl-3*H*-indole (**1**), 2-(*p*-dimethylaminophenyl)-3,3-dimethyl-5-carboethoxy-3*H*-indole (**3**) and 2-(*p*-aminophenyl)-3,3-dimethyl-5-cyano-3*H*-indole (**4**) with SDS micelles and of **3** and 2-(*p*-aminophenyl)-3,3-dimethyl-5-carboethoxy-3*H*-indole (**2**) with aqueous solutions of β-cyclodextrin (β-CD) in the absence and presence of urea, respectively, were studied by absorption and steady-state fluorescence measurements. It was found that the microviscosity of the interface of sodium dodecyl sulfate (SDS) micelle sensed by **1** increases greatly, while the change in the micropolarity of the interface sensed by **4** is negligible with increasing the urea concentration. The estimated critical micellar concentration (cmc) of the SDS micelle also increases with the urea concentration. It was also found that the binding constant (K_s) of **3** with SDS micelle is reduced by the addition of urea. The steady-state fluorescence measurements of the stoïchiometries of the guest (**2** or **3**):β-CD inclusion complexes indicate that two types of complexes, i.e., 1:1 and 1:2 types, are formed. The association constants, i.e., K_1 for the 1:1 complex and K_2 for the 1:2 complex, were obtained for **2** and **3** in the absence and presence of urea, respectively. The data reveal that the hydrophobic effect plays the major role in the stabilization of 1:1 and 1:2 complexes. The addition of urea gives rise to a remarkable decrease in K_1 and a much greater decrease in K_2. For **2** in the presence of 5 M urea, the formation of the 1:2 complex is inhibited completely. The effects of urea on K_s in SDS micelles and on K_1 and K_2 in aqueous solutions of β-CD are described in terms of its hydrophobic interactions with **2** and **3**. Some more direct evidences about this hydrophobic interaction in SDS micelles and the association constant between urea and **3** are also presented. The above results of the hydrophobic interaction between urea and the 3*H*-indoles and of the urea effect on the micellar interface strongly support the direct mechanism of urea action, through which the mechanism of urea as a protein denaturant can be understood better.

1. Introduction

Urea is often employed as a denaturing agent for proteins, polypeptides, and other biopolymers.[1] However, the mechanism of this denaturing action is not well established since it involves simultaneous operation of several factors, such as the effects of urea on nonpolar, polar, and ionic groups and even the binding of urea by the polypeptide chain in a way that favors unfolded conformations. For these reasons, there has been growing interest in studying of the effect of urea on organized assemblies, such as micelles,[2−8] reversed micelles,[9,10] vesicles,[2] monolayers,[11] polymers,[12] and cyclodextrins,[13,14] which are extensively used as membrane mimetic systems.[15] Two different mechanisms have been proposed to explain the urea action. One is an indirect mechanism,[16] in which urea acts as a "water structure breaker" facilitating the solvation of the hydrocarbon chain of the amphiphile. The other is a direct mechanism,[17] whereby urea participates in the solvation of hydrophobic chain and the polar headgroups of the amphiphile, by replacing some water molecules in the solvation layer. Computer simulations[18] and many experimental investigations,[2−13] at a molecular level, support the direct mechanism by suggesting that urea has a negligible effect on water structure and mainly replaces some water molecules in the solvation layer. It is obvious that studies of the effects of urea on the physical properties of the micellar interface and of the nature of the possible binding of urea with hydrophobic portions of the amphiphile or other compounds are very crucial to confirm the direct mechanism and to understand the mechanism of urea as a protein denaturant.

In the past few years, our research group has been focused on the study of some substituted 3*H*-indoles in various environments.[19−26] It has been observed that the spectroscopy and photophysics of these molecules are largely influenced by the nature of substituents in the para position of the phenyl rings. They are also sensitive to environments,[19−22] thus qualifying them to act as potential probe candidates for microstructures. So far, we have probed successfully the mean structural properties of reversed micelles,[27] aqueous micelles,[25,26,28] and surfactant vesicles.[29] It has been also shown that these molecules are not rigid and that the phenyl ring can librate within the *kT* energy barrier.[19−21,27] This torsional movement is responsible for the geometric changes taking place in the ground and excited states and provides an important deactivation pathway for the S_1 state. For the ester and cyano para-substituted molecules in water, the main nonradiative decay pathway has been ascribed to the formation of a nonemissive twisted intramolecular charge transfer state (TICT) originating in the amino group.[22−24] Very recently, our research group started the research program of studying the complexation between substituted 3*H*-indoles and cyclodextrins.[30,31] It was found that **4** and **5** can form 1:1 and 1:2 (guest:host) complexes with cyclodextrins.[30,31]

The aim of this paper is mainly twofold. We first want to study quantitatively the effect of urea on the microviscosity and micropolarity of the interface of the SDS micelle using **1** and **4** as probes, respectively. Qualitative studies of the urea-induced changes of the microviscosity[8,32] and micropolarity[5,8] of the

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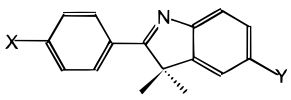

	X	Y
1	H	H
2	NH_2	CO_2Et
3	$N(CH_3)_2$	CO_2Et
4	NH_2	CN
5	$N(CH_3)_2$	CN

Figure 1. Molecular structure of the substituted 3*H*-indoles.

interface of the SDS micelle have been performed. However, to our knowledge, no quantitative data are published in the literature.

The second goal of this paper is to investigate quantitatively the hydrophobic interaction between urea and the aromatic surfaces of **2** and **3** reflected by the effect of urea on the binding (association) constants between these molecules and the SDS micelle and β-CD. Hydrophobic interactions between urea and aromatic hydrocarbons, alcohols, alkanes, and aminoacids are believed to exist.[30−39] Very recently, Monte Carlo simulation[33] clearly showed that denaturants do interact with aromatic hydrocarbons in water. The influence of urea on the interaction between β-CD and different aromatic compounds[13,40] has been also studied to gain a better understanding of the hydrophobic interaction. But more quantitative experimental evidences are very scarce in the literature.

2. Experimental Section

2.1. Materials. The synthesis and purification of the substituted 3*H*-indoles (see Figure 1) were done according to the modified methods of Skrabal *et al*.[41] and was reported by Popowycz.[42] Analytical grade reagent sodium hydroxide, methanol, urea, β-CD (Aldrich), and electrophoresis purity reagent SDS (Bio-Rad Laboratories, CA) were used as received.

2.2. Instruments. Absorption spectra were recorded on a Phillips PU 8800 and on a Cary 1 Bio UV-vis spectrophotometer using 1 cm quartz cells. Fluorescence spectra corrected for the emission detection were measured on a Spex Fluorolog-2 spectrofluorimeter with a F2T11 special configuration. The excitation and emission bandpasses used were 2.6 nm and 1.9 nm, respectively. Each solution was excited near the absorption wavelength maximum using 1 cm-path quartz cells. All corrected fluorescence excitation spectra were found to be equivalent to their respective absorption spectra.

2.3. Methods. Fresh sample solutions were used in the absorption and fluorescence measurements. The pH values of all the solutions in this study were adjusted by adding NaOH and no buffers were used. The concentrations of **2**−**4** were 10^{-6} M and that of **1** was 10^{-5} M because of its low fluorescence intensity. Stock solutions of **1**−**4** were prepared in methanol. The fluorescence quantum yields of the various species were measured using the DM3H molecule[19] as a standard in methanol ($\Phi_F = 0.24$). All measurements were carried out at room temperature.

3. Results

3.1. Interactions of 1, 3, and 4 with the SDS Micelle. 3.1.1. Effect of Urea on Microviscosity and Micropolarity. Our recent studies showed that **1** belongs to a unique class of fluorophores in which the deactivation of the excited singlet state is primarily governed by nonradiative internal conversion ascribed to an intramolecular torsional relaxation channel.[21,28,29]

TABLE 1: Microviscosities (η), Binding Constants (K_s), cmc's, and (K_{S0}/K_{Su})[a] Values for 3 in the SDS Micelle (20 mM) at Varying Urea Concentrations

[urea]/M	η/cP	K_s/L·mol^{-1}	cmc/mM	K_{S0}/K_{Su}
0	1.4	11400	7.2	
1	2.0	10340	7.9	1.10
2	2.3	9800	8.3	1.16
3	2.5	8640	8.6	1.32
4	2.6	7250	9.4	1.57
5	3.1	4300	9.8	2.65
6	3.2	2100	10.4	5.43

[a] K_{S0} and K_{Su} are the binding constants in the absence and presence of urea, respectively.

Since the excited state dipole moment of this molecule is much lower than that of its homologs, the polarity and hydrogen-bonding ability of the solvents do not affect the rotational motion of the phenyl moiety. Therefore, any restrictions imposed on the excited state phenyl torsion, e.g., viscosity of solvating medium, are essentially reflected in the quantum yield of fluorescence. The following correlation between Φ_F and viscosity has been obtained:[21]

$$\Phi_F = (9.4 \pm 0.3) \times 10^{-4}(\eta/\text{cP})^{2/3} \quad (1)$$

We have measured Φ_F values of **1** in SDS micelles ([SDS] = 20 mM) at varying urea concentrations. The microviscosities calculated with eq 1 are shown in Table 1.

Owing to the double hydrogen bonding that operates in the ground and excited state of **4** in the presence of water, an empirical equation between the Stokes shift of this molecule and the dielectric constant of various protonated media has been obtained:[25]

$$(\bar{\nu}_A - \bar{\nu}_F)/\text{cm}^{-1} = 21.06D + 5173/\text{cm}^{-1} \quad (2)$$

We have measured the wavenumbers of absorption and fluorescence of **4** in SDS micelles ([SDS] = 20 mM) at [urea] = 0, 1, 2, 3, 4, 5, and 6 M, respectively, and found that the Stokes shift does not change (inside the experimental error) in the range of urea concentration from 0 to 6 M. With eq 2 and the obtained value of Stokes shift, i.e., 6000 ± 100 cm^{-1}, the dielectric constant ($D = 39 \pm 5$) was obtained.

3.1.2. Spectral Characteristics of 3. Absorption and fluorescence spectra of **3** in SDS solutions in the absence and presence of 6 M urea, respectively, are shown in Figure 2 and the spectral characteristics are listed in Table 2.

3.1.3. Binding Constant and Cmc. The association of a substrate or a probe molecule with a micelle is based on the following equilibrium:[43]

$$S_w + D_m \overset{K_s}{\rightleftharpoons} S_m \quad (3)$$

for which the binding constant, K_s, is given by

$$K_s = [S_m]/[S_w][D_m] \quad (4)$$

where $[S_w]$ and $[S_m]$ denote, respectively, the probe molecule concentration in aqueous and micellar phase expressed as molarities in terms of the total volume of the solution, and $[D_m]$ is the molar concentration of surfactant in micellar form. The total probe molecule concentration $[S_t]$ and the total detergent concentration $[D_t]$ will be $[S_w] + [S_m]$ and $[S_m] + [D_m] + \text{cmc}$, respectively. By defining the fraction of the micellar associated probe molecule as $f = [S_m]/[S_t]$, one obtains

$$f/(1 - f) = K_s\{[D_t] - [S_t]f\} - K_s\text{cmc} \quad (5)$$

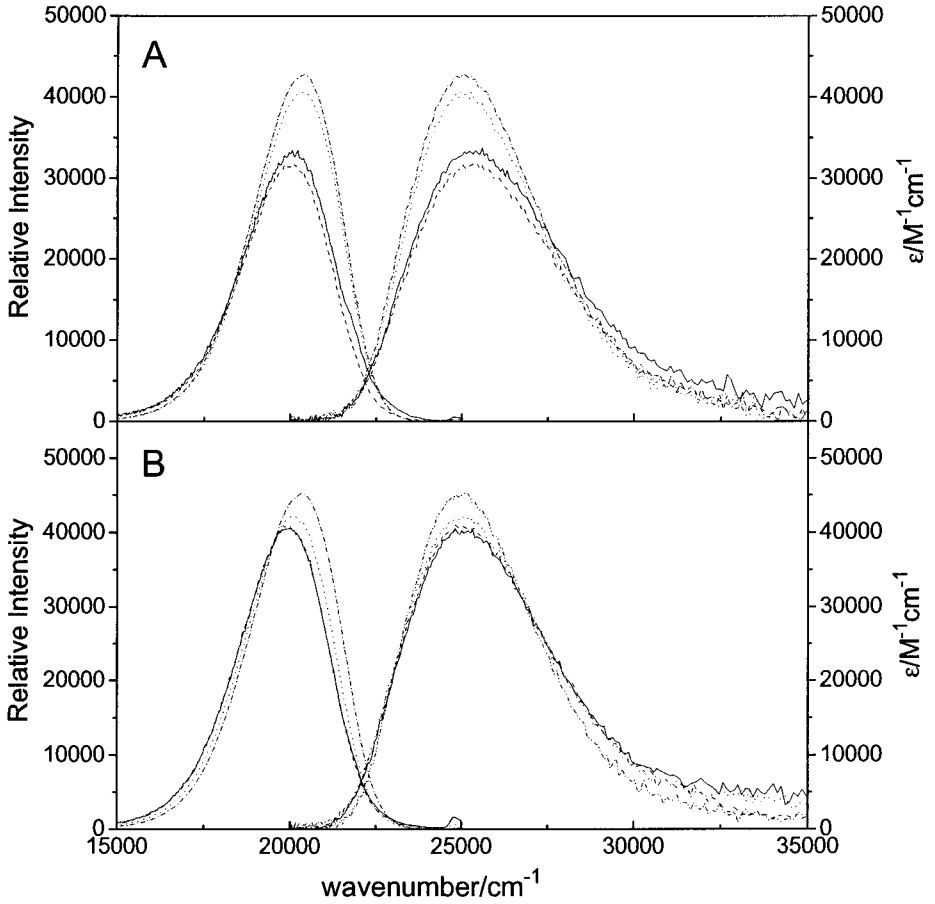

Figure 2. Absorption (right) and fluorescence (left) spectra (normalized according to the respective absorption maximum) of **3** in various SDS environments (pH = 9.5) in the absence (A) and presence (B) of urea, respectively. (A) water (solid); 4 mM SDS (dash); 10 mM SDS (dot); 20 mM SDS (dash dot). (B) 6 M urea (solid); 4 mM SDS + 6 M urea (dash); 10 mM SDS + 6 M urea (dot); 20 mM SDS + 6 M urea (dash dot).

TABLE 2: Spectral Characteristics of the Neutral Molecule 3 in Various Environments

medium	$\bar{\nu}_A$[a] (cm^{-1})	ϵ[b] (M^{-1} cm^{-1})	$\bar{\nu}_F$[c] (cm^{-1})	Stokes shift (cm^{-1})	$fwhm_A$ (cm^{-1})	$fwhm_F$ (cm^{-1})	Φ_F (±0.01)
water (pH = 9.5)	25 600	32 500	19 800	5800	5100	3100	0.012
4 mM SDS (pH = 9.5)	25 500	32 600	19 700	5800	4900	3000	0.017
10 mM SDS (pH = 9.5)	25 200	41 000	20 000	5200	4600	3000	0.30
20 mM SDS (pH = 9.5)	25 200	42 400	20 000	5200	4600	3000	0.31
6 M urea (pH = 9.5)	25 300	39 900	19 700	5600	5300	3100	0.034
4 mM SDS + 6 M urea (pH = 9.5)	25 200	40 100	19 700	5500	4900	3000	0.046
10 mM SDS + 6 M urea (pH = 9.5)	25 200	41 900	19 800	5400	4800	3000	0.091
20 mM SDS + 6 M urea (pH = 9.5)	25 100	44 800	20 000	5100	4500	3000	0.34

[a] Absorption wavenumber taken at the center of mass of the absorption band. [b] Molar absorption coefficient at the peak intensity maximum. [c] Fluorescence wavenumber taken at the center of mass of the fluorescence band.

A plot of $f/(1-f)$ vs $[D_t] - [S_t]f$ gives a straight line (figures not shown), from the slope and the intercept of which the binding constant K_s and cmc can be obtained. Experimentally, f is calculated from the values of fluorescence intensities in surfactant solution (I), in water (I_w), and at complete micellization of the probe molecule (I_m). The value of I can be expressed by the weighted average of I_w and I_m, i.e.,

$$I = I_w(1-f) + I_m f \tag{6}$$

From eq 6, one obtains

$$f = (I - I_w)/(I_m - I_w) \tag{7}$$

The binding constants between **3** and the SDS micelle and cmc's at varying concentrations of urea are compiled in Table 1 and are also shown in Figure 3A.

3.1.4. Changes in the Fluorescence Quantum Yields (Φ_F) of 3. To further understand the effect of urea on the interface of the SDS micelle and the hydrophobic interaction of urea with **3**, we have studied the changes in the Φ_F values of **3** with increasing the concentration of SDS in the absence and presence of urea, respectively, as shown in Figure 4.

3.2. Interactions of 2 and 3 with β-CD. 3.2.1. Spectral Characteristics. The absorption and fluorescence spectra of **3** in the aqueous solutions of β-CD are shown in Figure 5 and the spectral characteristics of both **2** and **3** are listed in Table 3.

3.2.2. Association Constants. First, we consider the formation of a simple 1:1 complex between a fluorescence substrate and β-CD. This process can be described by the following equilibrium:

$$S + CD \overset{K_1}{\rightleftharpoons} SCD \tag{8}$$

where S, CD, and SCD denote the fluorescence substrate, β-CD and 1:1 complex, respectively. The equilibrium constant K_1 is

TABLE 3: Spectral Characteristics of 2 and 3 in Their Neutral Forms Complexed to β-CD

molecule	medium	$\bar{\nu}_A$[a] (cm^{-1})	ϵ[b] (M^{-1} cm^{-1})	$\bar{\nu}_F$[c] (cm^{-1})	Stokes shift (cm^{-1})	$fwhm_A$ (cm^{-1})	$fwhm_F$ (cm^{-1})	Φ_F (±0.01)
2	water (pH = 9.5)	27 700	32 700	20 600	7100	5400	3200	0.024
	0.4 mM β-CD (pH = 9.5)	27 700	32 100	20 800	6900	5500	3500	0.12
	4 mM β-CD (pH = 9.5)	27 700	32 100	20 800	6900	5400	3800	0.37
	5 M urea (pH = 9.5)	27 500	33 800	20 500	7000	5400	3200	0.042
	0.4 mM β-CD + 5 M urea (pH = 9.5)	27 500	33 400	20 600	6900	5400	3200	0.067
	4 mM β-CD + 5 M urea (pH = 9.5)	27 600	33 300	20 800	6800	5300	3500	0.26
3	water (pH = 9.5)	25 600	32 500	19 800	5800	5100	3100	0.012
	0.4 mM β-CD (pH = 9.5)	25 800	39 700	20 500	5300	5200	3400	0.19
	4 mM β-CD (pH = 9.5)	26 000	40 300	20 600	5400	4900	3400	0.39
	5 M urea (pH = 9.5)	25 200	38 100	19 700	5500	4800	3100	0.026
	0.4 mM β-CD + 5 M urea (pH = 9.5)	25 500	41 100	20 300	5200	4800	3400	0.075
	4 mM β-CD + 5 M urea (pH = 9.5)	25 900	39 700	20 500	5400	5000	3400	0.32

[a] Absorption wavenumber taken at the center of mass of the absorption band. [b] Molar absorption coefficient at the peak intensity maximum. [c] Fluorescence wavenumber taken at the center of mass of the fluorescence band.

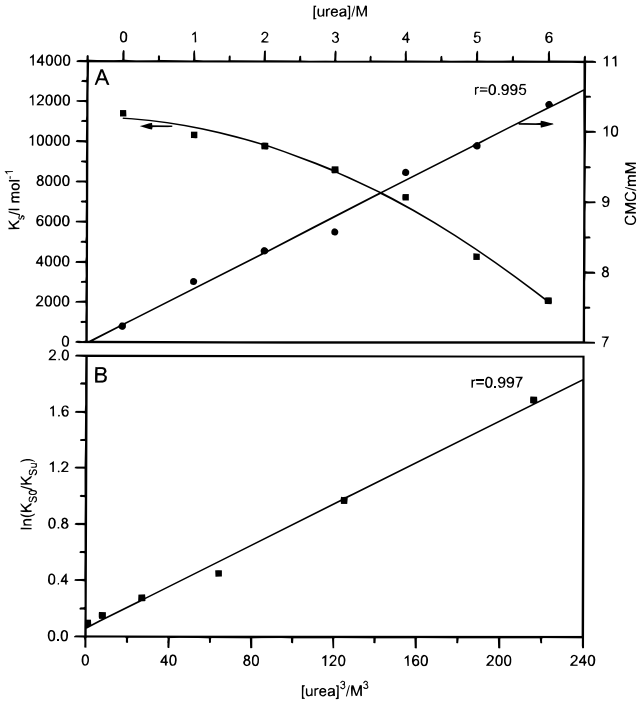

Figure 3. Plots of binding constant and cmc against [urea] (A) and $\ln(K_{S0}/K_{Su})$ against $[urea]^3$ (B) for **3** in SDS micelles.

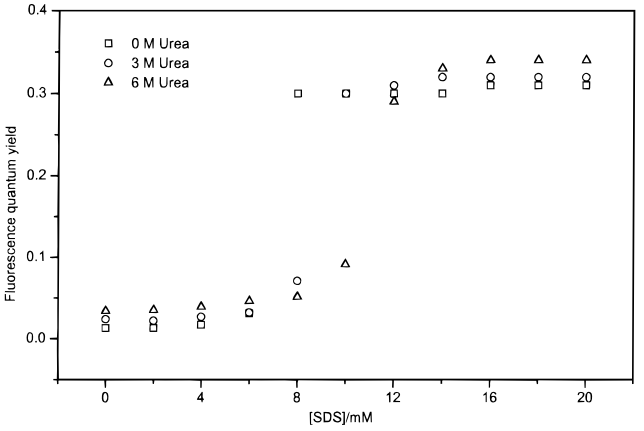

Figure 4. Fluorescence quantum yield of **3** as a function of SDS concentration at [urea] = 0, 3, and 6 M.

then expressed as

$$K_1 = [SCD]/[S][CD] \quad (9)$$

The classical method for the determination of K_1 is the Benesi−Hildebrand double-reciprocal plot.[44] By using the fluorescence intensities in pure water (I_0), in the presence of CD (I), and in the complex (I_1) one obtains the following relationship:[30]

$$1/(I - I_0) = 1/\{K_1(I_1 - I_0)[CD]_0\} + 1/(I_1 - I_0) \quad (10)$$

The plot of $1/(I - I_0)$ against $1/[CD]_0$ should give a straight line, from the slope and intercept of which one can estimate K_1 and I_1.

Figure 6 illustrates the double reciprocal plot for **2** complexed to β-CD. It can be seen that the plot is not well described by a single straight line and should be better described by two segments. This implies that a further complexation is possible followed by that of the 1:1 type. The initial linear portion at higher CD concentrations might be due to the 1:2 complex, while the final linear portion at lower CD concentrations is for the 1:1 complex. Therefore, one has to consider the additional stepwise equilibrium, i.e.,

$$SCD + CD \overset{K_2}{\rightleftharpoons} S(CD)_2 \quad (11)$$

For this equilibrium, one obtains the following equation:[30,45]

$$1/(I - I_0) = 1/\{(I_2 - I_0)K_1K_2[CD]_0^2\} + 1/(I_2 - I_0) \quad (12)$$

where K_2 is the stepwise association constant of $S(CD)_2$ and I_2 is the fluorescence intensity of the substrate in the 1:2 complex. According to the linear correlation described by (12), the values of I_2 and K_1K_2 can be obtained and therefore K_2 might also be obtained.

While the above method of estimating the association constants does work, it does not weight the data properly.[30] Therefore, more reliable values are obtained by the use of a nonlinear regression routine (NLR).[30,46] At lower concentrations, where the 1:1 complex is suggested, the following equation is used by rearranging eq 10:

$$I = \{I_0 + I_1K_1[CD]_0\}/\{1 + K_1[CD]_0\} \quad (13)$$

The initial values of the two parameters (I_1, K_1) are obtained from the experiment and the linear regression method, respectively.

In the case of higher concentrations, where both 1:1 and 1:2 complexes are suggested, the following equation is used:[45]

$$I = \{I_0 + I_1K_1[CD]_0 + I_2K_1K_2[CD]_0^2\}/\{1 + K_1[CD]_0 + K_1K_2[CD]_0^2\} \quad (14)$$

The initial values of I_1 and K_1 are obtained from the results of

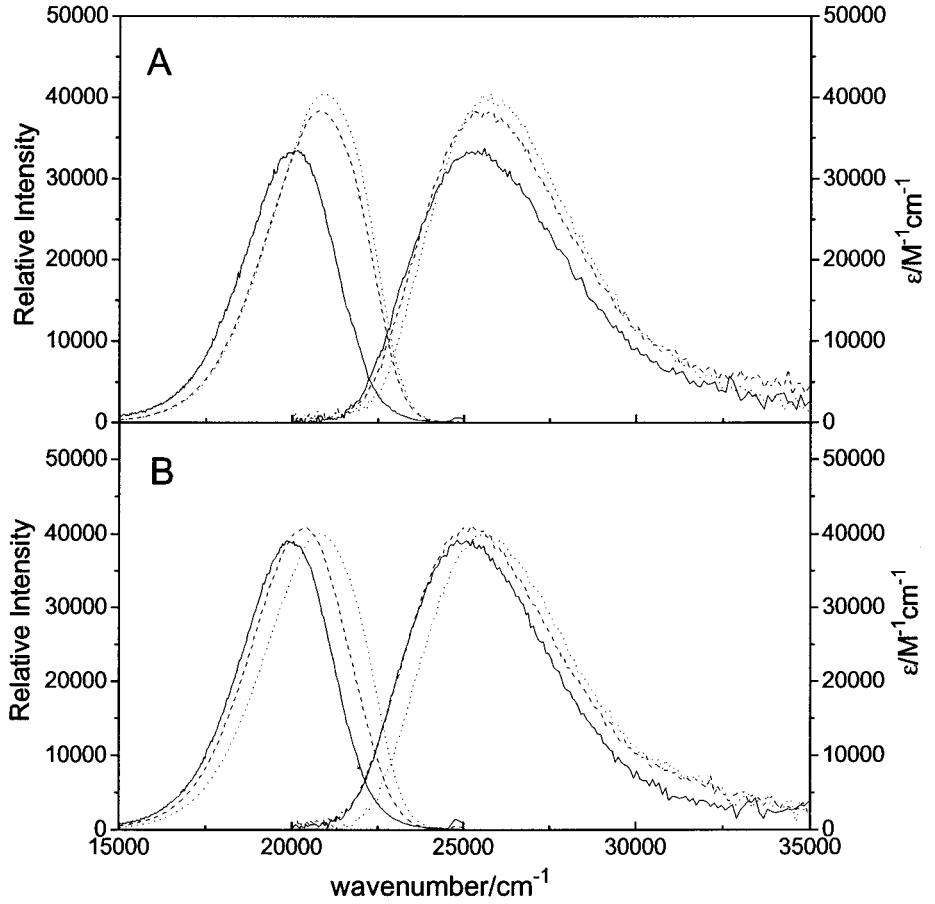

Figure 5. Absorption (right) and fluorescence (left) spectra (normalized according to the respective absorption maximum) of **3** in various β-CD environments (pH = 9.5) in the absence (A) and presence (B) of urea, respectively. (A) water (solid); 0.4 mM β-CD (dash); 4 mM β-CD (dot). (B) 5 M urea (solid); 0.4 mM β-CD + 5 M urea (dash); 4 mM β-CD + 5 M urea (dot).

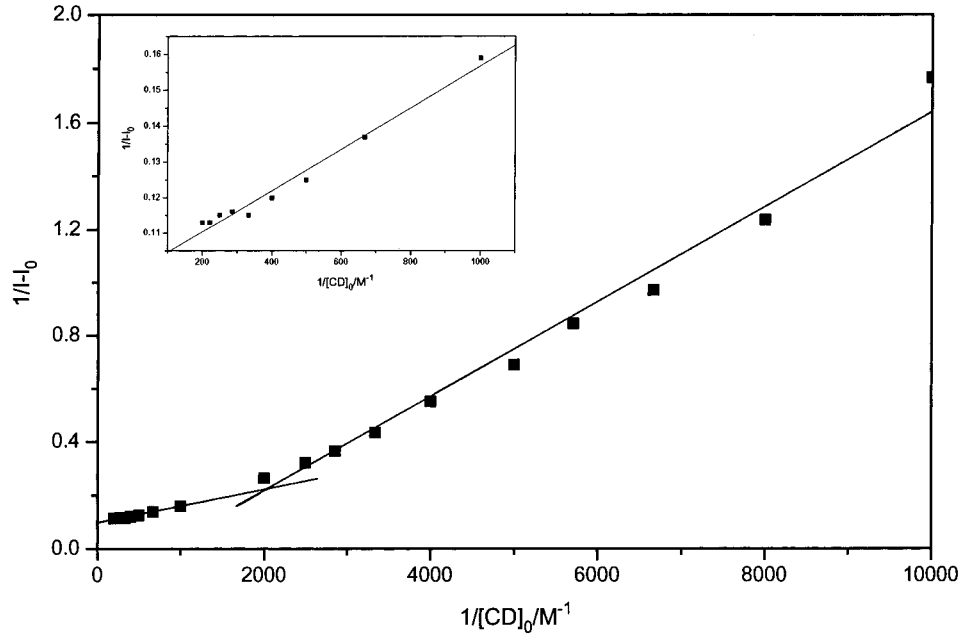

Figure 6. Double reciprocal plot for **2** complexed to β-CD in the absence of urea following eq 10. The insert shows the initial linear portion.

the nonlinear regression analysis using eq 13 and those of I_2 and K_2 are from the results of the linear regression on the basis of eq 12. In all cases, the fit converged well with a correlation coefficient $r^2 > 0.99$ (see Figure 7). Results of the nonlinear regression analysis are listed in Table 4.

The association constants K_1 and K_2 of **4** and **5** as well as the molecular volumes of **2**−**5** are also included in Table 4. To study the factors influencing the stabilization of the complexes, the ratios of the molecular volume as well as ln K_1 and ln K_2 for the six pairs among **2**−**5** are listed in Table 5.

3.2.3. Effect of Urea on K_1 and K_2. In order to study the effect of urea on the interactions of **2** and **3** with β-CD, we have estimated the values of K_1 and K_2 at [urea] = 3 and 5 M, respectively. Again, the nonlinear regression method was used

TABLE 4: Molecular Volumes (V_m)[a] and Lengths[b] of 2−5 and the Association Constants (K_1, K_2) for Their Neutral Forms Complexed to β-CD

					K_1/M^{-1}, K_2/M^{-1}		
molecule	V_m/Å^3	l^c/Å	$l_1{}^d$/Å	$l_2{}^e$/Å	[urea] = 0 M	[urea] = 3 M	[urea] = 5 M
2	388	16.6	6.9	9.7	840 ± 130, 2660 ± 210	810 ± 370, 660 ± 80	170 ± 20
3	441	17.7	8.0	9.7	1770 ± 350, 5070 ± 460	780 ± 130, 1260 ± 60	540 ± 160, 720 ± 50
4	332	13.3	6.9	6.4	750 ± 120,[f] 760 ± 60[f]		
5	382	14.5	8.1	6.4	1430 ± 220,[f] 1880 ± 150[f]		

[a] Taken from ref 26. [b] Calculated from the molecular structure optimized using the AM1 semiempirical method.[24] [c] Total molecular length. [d] Length from the indolic nitrogen atom to the left end (see Figure 1). [e] Length from the indolic nitrogen atom to the right end. [f] Taken from ref 30.

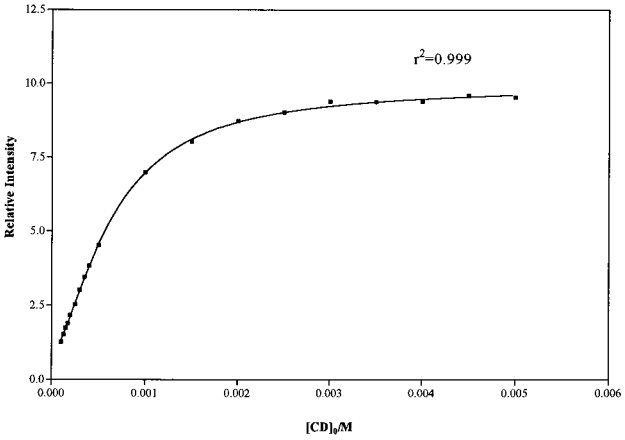

Figure 7. Plot of the relative fluorescence intensity versus $[CD]_0$ for **2** complexed to β-CD in the absence of urea. The full line is the nonlinear regression fit to the experimental data points following eq 14.

TABLE 5: Ratios of Molecular Volumes, ln K_1 and ln K_2, for Six Molecular Pairs

pair	ratio of molecular volumes	ratio of ln K_1	ratio of ln K_2
3−**2**	1.14	1.11 ± 0.06	1.08 ± 0.02
3−**4**	1.33	1.13 ± 0.06	1.29 ± 0.03
3−**5**	1.15	1.03 ± 0.05	1.13 ± 0.03
2−**4**	1.17	1.02 ± 0.05	1.19 ± 0.03
2−**5**	1.02	0.93 ± 0.04	1.05 ± 0.02
5−**4**	1.15	1.10 ± 0.05	1.14 ± 0.02

(eq 14). In all cases in the presence of urea, the fits converged well with correlation coefficients $r^2 > 0.99$ and reasonable standard errors at 95% confidence intervals (see Table 4) except for **2** at [urea] = 5 M. In the latter case, the estimated results of the four parameters I_1, I_2, K_1, and K_2 as well as the standard errors were not reasonable. In contrast, the fit following eq 13 gave satisfactory results indicating that only a 1:1 complex is probably formed. Double reciprocal plot for **2** at [urea] = 5 M only exhibits one linear segment, while the plot of $1/(I - I_0)$ vs $1/[CD]_0{}^2$ does not exhibit a straight line as shown in Figure 8, which again suggests that the 1:2 complex is not formed. Thus, only K_1 for the 1:1 complex was obtained in this situation. The evaluated association constants K_1 and K_2 in the presence of urea can be found in Table 4.

4. Discussion

4.1. Quantitative Effect of Urea on the Interface of SDS Micelles. If the direct mechanism of the urea action is true, the physical properties of the interface of the SDS micelle will be altered by the presence of urea. One can see from Table 1 that the microviscosity of the interface of SDS micelles increases substantially with increasing the concentration of urea. Similar results have been obtained in the literature.[8,32] It was reported that the microviscosity of the interface of SDS micelle, sensed

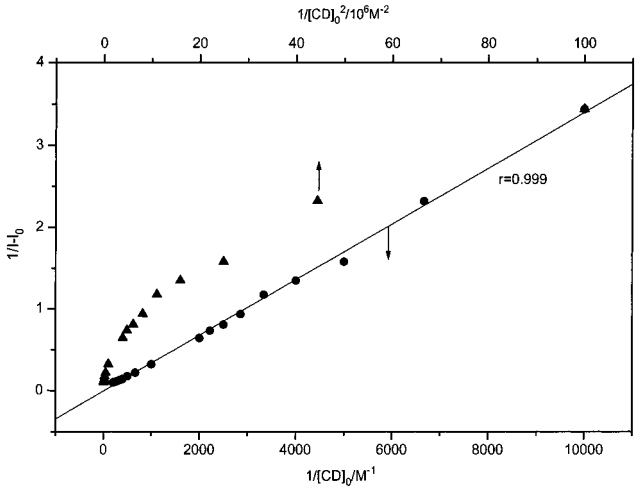

Figure 8. $1/(I - I_0)$ as a function of $1/[CD]_0$ and $1/[CD]_0{}^2$, respectively for **2** complexed to β-CD in the presence of 5 M urea.

by *N*,*N*,*N*′,*N*′-tetramethyldiaminodiphenyl ketoimine, is 60% greater in 5 M urea than that without urea.[32] Using 4-octanoyl-2,2,6,6-tetramethylpiperidinyl-1-oxy as a probe, Baglioni *et al.*[8] reported data showing that urea produces an increase of the microviscosity at the SDS micellar interface of about 100% in the presence of 6 M urea. Our quantitative data indicate that the microviscosity of the micellar interface increases about 130% due to the presence of 6 M urea. The effect of urea on the micropolarity of the micellar interface has also been studied qualitatively.[5,8] The investigation of Baglioni and co-workers[8] shows that urea slightly decreases the polarity of the micelle interface, while that of Iglesias and Montenegro[5] indicates that the solubilization process of urea and its derivatives at the micelle interface does not change its local polarity. Our quantitative result reveals that the effect of urea on the polarity of the micelle interface is very weak.

The effect of urea on cmc can also help understanding the mechanism of urea action. A linear correlation ($r = 0.995$, see Figure 3A) between cmc and urea concentration is obtained, which can be described by the following equation:

$$\text{cmc/mM} = 7.25 + 0.52([\text{urea}]/\text{M}) \qquad (15)$$

This kind of dependence of cmc on urea concentration has been observed before.[47] The dependence of cmc on urea concentration can be interpreted in terms of the interaction of urea with the hydrophobic chain of the SDS molecule.[48]

The discussion on the spectral characteristics of **3** is helpful. It can be seen from Figure 2 and Table 2 that in the absence of urea the formation of SDS micelles leads to a red shift, a decrease in the bandwidth, and a large increase in the molar absorption coefficient (ϵ) in the absorption spectra of **3**. Similar phenomena were observed for **3** in the micelle of CTAB.[26] Water acts as a hydrogen bond donor to the lone pair of the

terminal nitrogen atoms of the amino- or dimethylamino-substituted 3*H*-indole, causing reduced conjugation of the phenyl ring with the indolic moiety causing a blue shift, an increase in the bandwidth, and a decrease in the ϵ values.[23,25,26] These results thus indicate that the end of the anilino moiety of **3** approaches the interface of the SDS micelle and that this molecule is shifted from a hydrogen-bonding environment where the terminal nitrogen atom is protonated in water to an environment inside the micelles where it is protected to a certain extent from water interactions. Since the electron lone pair on the terminal nitrogen in the dimethylamino substituent is less available for hydrogen-bonding complexation in the electronic ground state as compared to the amino substituent in **2**, the observed red shift for **3** is not large as compared to that of **2**.[26]

It is interesting to note that in the presence of 6 M urea, the formation of SDS micelles gives rise to a negligible red shift and a smaller increase in the molar absorption coefficient compared to that in the absence of urea. Two possibilities responsible for this phenomenon may be postulated: one is that the environment for **3** in the bulk phase, i.e., the mixture of urea, SDS monomers, and water does not differ greatly from that at the interface of SDS micelle where urea molecule has been suggested to replace some water molecules; the other is that **3** might transfer from the interface to the bulk phase on addition of urea. Since concentrated aqueous solution of urea induces a red shift of the absorption of **3** relative to water as shown in Table 1, this transfer should make the red shift less obvious when SDS micelles are formed.

The decrease of the binding constant with increasing urea concentration (see Figure 3A) suggests that a part of **3** transfers from the interface to the bulk phase. This confirms the above hypothesis made on the basis of spectral characteristics. Here, we suggest that hydrophobic interactions between urea and **3** in the bulk phase are playing the main role on the transfer of **3**. Because of the interaction of urea with **3** and SDS monomers, one may imagine that the local environment composed of the three components will be more or less similar to that at the interface of SDS micelle although the former structure is looser.

It is quite interesting to note that a plot of $\ln(K_{S0}/K_{Su})$ vs $[\text{urea}]^3$ (K_{S0} and K_{Su} denote binding constants in the absence and presence of urea, respectively; see Table 2) exhibits a straight line with a correlation coefficient $r = 0.997$ (see Figure 3B).

Association constant has been employed to reflect the hydrophobic interaction between urea and aromatic hydrocarbons.[33] It seems then plausible to propose the following equilibrium:

$$S_w + U \overset{K_a}{\rightleftharpoons} S_u \qquad (16)$$

where K_a is the association constant and U represents the urea molecules directly interacting with **3** and S_u can be regarded as the "aggregate" of a single **3** with some urea molecules. Note that the concentration of this part of urea ([U]) should be in the same order of the concentration of **3** and is much smaller than the total concentration of urea, i.e., [urea]. The water solvation layer of **3** is changed by the interaction of urea, but the interaction between **3** and water still exists to some extent. This situation is similar to that of **3** interacting with the SDS micelle as described by eq 3, where the interaction of **3** with water is not avoided completely by the association of **3** with the SDS micelle since only the anilino moiety of **3** approaches the interface of the SDS micelle as mentioned above. Combining eqs 3 and 16, i.e., (3) − (16), one obtains:

$$S_u + D_m \rightleftharpoons S_m + U \qquad (17)$$

This equation stands for the equilibrium of **3** between urea "aggregate" and SDS micelle. Thus, the binding constant in eq 17 should be K_{Su}. Now, it is clear that K_{S0}/K_{Su} is actually the association constant between urea and **3**, i.e., K_a.

Monte Carlo simulation[33] showed that there is a short optimum distance (e.g., 4.40 Å and 4.00 Å for urea−benzene and urea−naphthalene, respectively[33]) between urea and the surface of aromatic hydrocarbons when hydrophobic interaction between them takes place. With this in mind, one can imagine that urea molecules interacting with a single **3** tend to aggregate on a plane, a very small area parallel to the molecular plane of **3**, and at a short optimum distance. If this is true, [U] will be directly proportional to the cube root of total [urea] and the straight line shown in Figure 3B actually indicates a linear correlation between ln K_a and [U]. When the urea molecules in the "aggregate" is viewed as a whole, this kind of dependence is similar to that on ln K (or standard free energy) and molecular volume.[26] Therefore, the linear correlation between ln K_a and $[\text{urea}]^3$ is taken as an evidence of the hydrophobic nature of the interaction.

Now, we will discuss the effect of urea on the change of Φ_F of **3** as shown in Figure 4. The low value of Φ_F of this molecule in water has been interpreted in terms of a formation of a nonemissive TICT state.[22,23] It is well-known that an increase of viscosity leads to a decrease of the TICT rate formation and consequently to an increase of Φ_F,[49] while an increase of polarity results in the opposite effect.[50] Since both the viscosity[51] and the dielectric constant[52] of water is increased by the addition of urea, the urea effect on Φ_F is small (but significant compared to the experimental error) in water and, similarly, in SDS solutions at [SDS] less than 6 mM. The direct comparison of the effect of urea is impossible from [SDS] = 6 to 12 mM since the cmc's of SDS differ at varying concentrations of urea. When [SDS] exceeds 12 mM, Φ_F of **3** slightly increases again with increasing the concentration of urea. However, the reason of this Φ_F increase at [SDS] exceeding 12 mM is different from that at [SDS] less than 6 mM. The substantial increase in the microviscosity of the micellar interface should lead to an obvious increase of Φ_F, but this effect is actually compensated by the opposite effect owing to the transfer of **3** from the micellar interface to the bulk phase. Similar results have been observed for *p*-toluidinonaphthalenesulfonate (TNS) in SDS and CTAB micelles.[6] It can be seen that on the basis of the effect of urea on Φ_F, the urea-induced changes of the physical properties of the micellar interface and the transfer of **3** from the micellar interface to the bulk phase can be confirmed further.

4.2. Quantitative Effect of Urea on the Interaction of Fluorescent Probes with β-CD. It is quite interesting to note from Table 3 that a small blue shift appears for **3** going from water to aqueous solutions of β-CD, which is contrary to the small red shift observed for **5**.[30] For **2**, no shift is observed, while for **4**, a considerable red shift was observed.[30] The red shifts of **4** and **5** can only result from the terminal nitrogen partly protected from water interactions into the β-CD cavity. Thus, the 1:1 complexes of **4** and **5** fitted by the anilino moiety are believed to exist. Because of the structural similarity between **2** and **4** as well as between **3** and **5**, the 1:1 complexes of **2** and **3** should be also formed. Actually, the different spectral characteristics between **2** and **3** show that the amino substituent in **2** and the dimethylamino substituent in **3** shift the wavenumber of absorption to different extents. This should be the evidence that the 1:1 complexes of **2** and **3** fitted by the anilino moiety exist. On the other hand, since the hydrogen bonding between water and the indolic nitrogen increases the conjugation

of the indolic moiety with the phenyl moiety,[23] a blue shift can be expected when the indolic nitrogen is partly protected from water interactions. There is no evidence showing the ester substituent can shift the wavenumber.[23] Therefore, the blue shift of **2** and **3** can only result from the effect of the indolic nitrogen. On the basis of the above discussion, we suggest that **2** and **3** are more deeply entrapped into the β-CD cavity than **4** and **5**, respectively, such that the effect of the indolic nitrogen is predominant over that of the terminal nitrogen. Clearly, the situations of **4** and **5** are opposite. Considering the molecular lengths of these molecules (Table 4) calculated from an AM1 geometry optimization,[24] and the internal diameter (6.0−6.5 Å) and the length (7.9 Å) of the β-CD cavity,[53] one cannot exclude completely the possibility of the formation of 1:1 complexes fitted by the indolic moiety of **2**−**5**. Nevertheless, the spectral characteristics of **2**−**5** cannot provide any definite answer.

In the presence of urea, a blue shift for **3** can still be seen when 1:1 and 1:2 complexes are formed. For **2**, the change of the absorption wavenumber is negligible in all environments. As the spectral characteristics of **2** and **3** in the presence of urea are compared with those in the absence of urea, one observes small red shifts. This is not unexpected since **2** and **3** probably escape from the cavity of β-CD due to the hydrophobic interactions of urea. The decrease of Φ_F on addition of urea for both **2** and **3** in the aqueous solutions of β-CD (see Table 3) further shows that **2** and **3** transfer from the β-CD cavity to the bulk phase.

Table 5 shows that within experimental accuracy the ratio of ln K_2 is similar to the ratio of molecular volume for any pair. However, the similarity between the ratio of ln K_1 and the ratio of molecular volume appears only between the two ester-substituted 3*H*-indoles or between the two cyano substituted 3*H*-indoles, i.e., for the **3**−**2** and **5**−**4** pairs. For other pairs, the difference between the ratio of ln K_1 and the ratio of molecular volumes seems not negligible, although it is not very large. The similarity between the ratio of ln K and the ratio of molecular volume indicates the hydrophobic effect is playing the key role in the stabilization of these complexes,[30] especially for the 1:2 complexes, in which most of the 3*H*-indole molecule is entrapped into two β-CD cavities. In CTAB micelles, a very good linear correlation was obtained between the standard transfer free energy (or ln K) and the molecular volume for seven substituted 3*H*-indoles including **2**−**5**.[26] In the present study, the linear correlation approximately exists between ln K_2 and the molecular volume but not between ln K_1 and the molecular volume (figures not shown). This is because in the 1:1 complexes, the solvent effect (interactions with water) on the ester substituent is different from that on the cyano substituent, indicating that the solvent effect on the 1:1 complexes should not be ignored completely in this study.

Having ascertained the nature of the interaction of **2** and **3** with β-CD, we can now analyze the urea effect. It can be seen from Table 4 that both K_1 and K_2 values decrease due to the addition of urea. This phenomenon can be rationalized by the destabilization effect of urea on both complexes. The fact that urea induced increase in ΔG value accompanying the formation of the complex between trimethyl(ferrocenylmethyl)ammonium cation and β-CD supports our results.[13] Since the solvation of β-CD receptor is essentially unaffected by the presence of urea,[40] the destabilization of the complexes can only be ascribed to the hydrophobic interaction of urea with **2** and **3**. In our recent work,[54] it was found that the interaction pattern between 3*H*-indoles and β-CD is not altered by the presence of urea. In other words, the nature of the interaction is hydrophobic both in the presence and in the absence of urea. This further supports the hydrophobic interaction mechanism of urea. So far, the competitive effect of urea in the inclusion process between guests and β-CD has been reported by some authors.[13,40,55−57] However, to our knowledge, the reported results are mainly on the 1:1 complex but not on the 1:2 complex.

It is also noted from Table 4 that the effect of urea on K_2 is more remarkable than on K_1. Double reciprocal plots for **2** and **3** (figures not shown) indicate that the crossing point of the two segments corresponds to a smaller $1/[CD]_0$ value or larger $[CD]_0$ value on the addition of urea. This clearly indicates that urea makes the formation of 1:2 complex more difficult. The different urea-induced thermodynamic functions[13] in the 1:1 complexation process from those in the successive 1:2 complexation process are probably the important factor responsible for this. Nevertheless, the detailed mechanism is not clear. For **2** with 5 M urea, the competitive effect of urea is large enough to totally annihilate the formation of 1:2 complexes.

Using a method similar to that in SDS micelles, one can also estimate the association constant between urea and **3** from the values of K_1 in the absence and presense of urea, respectively. The obtained values are 2.27 and 3.27 at [urea] = 3 and 5 M, respectively. The values are slightly higher than the corresponding values listed in Table 2, i.e., 1.32 and 2.65, respectively.

5. Concluding Remarks

The quantitative studies through fluorescence measurements indicate that the microviscosity of the interface and cmc value of the SDS micelle increase on increasing the urea concentration. This result reflects that urea does participate in the solvation of the polar headgroup and the hydrophobic chain of the SDS molecule by replacing some water molecules in the solvation layer.

The reduction in the binding constant between **3** and the SDS micelle and in the association constants of **2** and **3** with β-CD results from the hydrophobic interactions of urea with the aromatic surfaces of these 3*H*-indoles. The hydrophobic interaction is also believed to exist between urea and the hydrophobic chain of the SDS micelle, which should be responsible for the increase of cmc. The linear correlation between $\ln(K_{S0}/K_{Su})$ and $[\text{urea}]^3$ in SDS micelles for **3** provides a more direct evidence of this hydrophobic interaction. Quantitative data about the hydrophobic interaction of urea, e.g., the association constant, are lacking in the literature. Thus, the new method suggested in this paper is worth studying further.

The results presented in this paper strongly support the direct mechanism of urea action and the methods used provide a way of understanding the denaturing effect of urea on biopolymers.

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