0273-2289/01/95/0163/$13.00

Fluorescence Investigation of Affinity Interaction Between Bovine Serum Albumin and Triazine Dye in Reversed Micelles

TIAN-XI ZHANG,* HUI-ZHOU LIU, AND JIA-YONG CHEN

Laboratory of Separation Science and Engineering,
Institute of Chemical Metallurgy, Chinese Academy of Sciences,
PO Box 353, Beijing 100080, P.R. China,
E-mail: zhangtianxi@hotmail.com

Received December 1, 2000; Revised June 21, 2001;
Accepted June 29, 2001

Abstract

The effect of Cibacron Blue 3GA (CB) on the fluorescence emission spectra of bovine serum albumin (BSA) was investigated in cationic reversed micelles formed with cetyltrimethylammonium bromide (CTAB) compared with that in aqueous phase. The anionic CB had electrostatic interactions with cationic CTAB and affinity interactions with BSA in the reversed micelles. The addition of CB in the reversed micellar phase led to a great decrease in the fluorescence intensity of BSA and a remarkable red shift of the wavelength of emission maximum (λ_{max}). The fluorescence intensity of BSA decreased and the λ_{max} decreased 5 nm (blue shift) without the addition of CB in the reversed micellar phase. The fluorescence intensity of BSA with the addition of CB had the strongest value in the aqueous phase with the presence of CTAB, a less strong value in the reversed micellar phase, and a weak value in the aqueous phase without the presence of CTAB. The increase in λ_{max} of BSA with the addition of CB in the reversed micellar phase might indicate the decrease in the hydrophobic microenvironment of the Trp residue of BSA, contrary to those microenvironments in the absence of CB.

Index Entries: Affinity interaction; reversed micelles; bovine serum albumin; Cibacron Blue 3GA; fluorescence emission spectra.

Introduction

Reversed micelles are optically transparent nanometric droplets of water in apolar organic solvents stabilized by the aggregated surfactants

*Author to whom all correspondence and reprint requests should be addressed.

(1,2). They have attracted much interest from biotechnologists in recent years because enzymes, protein, and nucleic acids can be dissolved in them with retention of activity and stability *(3–7)*. Several research groups have investigated protein-protein interactions, such as protease-inhibitor complexes and antibody-antigen reactions, in the reversed micelles. Walde et al. *(8)* reported that exchange and recognition of macromolecules (trypsin and its inhibitor) could take place in micellar solutions at the same rate as with low molecular weight molecules. Nicot et al. *(9)* investigated the digestion of myelin basic protein in the reversed micelles by three proteolytic enzymes: trypsin, cathepsin D, and *Staphylococcus aureus* V8 protease. It has been found that the number of cleavage sites is considerably decreased with micellar digestion generating long hydrophobic peptides compared with proteolysis in aqueous solution. Specific protein-protein interactions in reversed micelles were carried out by measuring antigen-antibody binding using fluorescence polarization spectroscopy *(10)*.

Reversed micellar systems have been studied to separate proteins in liquid-liquid extraction processes *(11–13)*. Significant improvement in the process selectivity can be achieved by introducing affinity ligands to the reversed micellar phase *(14–18)*. Anionic surfactants such as bis-(2-ethylhexyl)-sulfosuccinate (AOT) have often been used with alkyl glucoside as a ligand in affinity-reversed micellar systems. Selective separation of trypsin from a mixture involving many kinds of contaminating proteins has been achieved using trypsin inhibitor immobilized in the reversed micelles *(19,20)*. One group of ligands, triazine dyes, has been used to extract lysozyme and bovine serum albumin (BSA), with the result of a significant increase in the solubilization of lysozyme with high selectivity *(21–23)*.

The affinity interaction between triazine and protein in aqueous solutions has been widely studied *(24,25)*. It seems that little attention has been paid to the affinity interaction between triazine and protein in reversed micelles. In our previous work, the effect of Cibacron Blue 3GA (CB) and BSA as guest molecules on the microstructure of reversed micelles was investigated with electrical conductivity measurement *(26)*. The conductivity of reversed micellar systems decreases with the addition of CB and decreases even further with the addition of both CB and BSA. The conductivity of reversed micellar phase is three orders of magnitude lower than that of aqueous phase under the same CB concentration *(26)*.

In the present study, CB is used as an affinity ligand and is directly introduced into the reversed micelles formed with cetyltrimethylammonium bromide (CTAB). The anionic CB has electrostatic interactions with cationic CTAB and shows affinity interactions with BSA. The fluorescence emission spectra of the reversed micellar system were investigated since BSA has intrinsic fluorescence. The effect of CB on fluorescence emission spectra of BSA in the reversed micelles was studied compared with that in an aqueous phase.

Materials and Methods

Chemicals and Reagents

BSA (67 kDa, p*I* 4.7) was purchased from Beijing Hongxing Huaxue Factory, China. CB was obtained from Sigma. Beijing Chemical Reagents supplied CTAB, *n*-hexanol, and *n*-hexane (analytical grade). Other chemicals were all commercially available reagents of analytical grade. Deionized and distilled water was used throughout the experiments.

Solutions

The original reversed micellar solutions used contained 50 m*M* CTAB and 15% (v/v) *n*-hexanol in *n*-hexane, denoted as CTAB (50 m*M*)/15% (v/v) hexanol/hexane/0.8% (v/v) H_2O. The aqueous phase was 10 m*M* phosphate buffer solution. Protein solutions were made in buffer stocks in concentrations of BSA equal to 100 mg/mL. CB (typically 23.68 μ*M*) with a molar ratio of CB to CTAB (50 m*M*) of 4.74×10^{-4} was dissolved in buffer solution. The reversed micellar solutions for fluorescence measurements were made by injection method. The protein solution was injected into the organic phase and shaken vigorously to form a clear solution.

Fluorescence Spectra

Fluorescence emission spectra of all samples were recorded on a Hitachi (F-4500) fluorescence spectrophotometer with excitation wavelength at 295 nm. Excitation and emission slit widths were set at 5.0/5.0 nm. The fluorescence spectra of reversed micellar phase were compared with those of aqueous phase.

Results and Discussion

The fluorescence probe for the characterization of state of water in reversed micelles has been investigated using sensitive dyes such as hemicyanine dye and aromatic fluorophore Prodan *(27,28)*.

Lundgren et al. *(29)* investigated the effects of hydration on acrylodan-labeled BSA and human serum albumin (HSA) (BSA-Ac and HSA-Ac) in AOT reversed micelles in the absence of ligand. Their results demonstrate the importance of hydration on protein behavior in environments in which water is limited and the complexity of the interplay between hydration and protein function and dynamics *(29)*. Lysozyme, HSA, and liver alcohol dehydrogenase were studied in reversed micelles by frequency domain fluorescence spectroscopy *(30)*. The emission of the tryptophanyl residues of the proteins was monitored. The rotational correlation times for the internal motions and the overall protein rotation in reversed micelles decreased with increasing water concentration. Lysozymes showed peculiar rotational dynamics that reflect denaturation occurring as the protein increases its water content in the reverse micelle. This influence was not observed for the other proteins *(30)*.

A BSA molecule has 2 Trp and 19 Tyr residues that mainly produce the intrinsic fluorescence. Both Trp and Tyr residues contribute to fluorescence of BSA when the excitation wavelength is <280 nm. The fluorescence of Tyr residues is so small that the contribution to BSA fluorescence could be assumed negligible when the excitation wavelength is >290 nm. Thus, Trp residues mainly contribute to the fluorescence emission spectra of BSA *(31)*. In the present study, the fluorescence of Trp residues of BSA can only be considered since excitation wavelength was set 295 nm. The microenvironment of Trp residues could be changed since the affinity interaction occurred between BSA and CB in reversed micelles, which results in the change in fluorescence emission spectra of the system.

Fluorescence Emission Spectra of CB

CB is one triazine dye that could have fluorescence at a certain excitation wavelength. Figure 1 shows the fluorescence emission spectra of CB of reversed micelles compared with that of aqueous phase. CB had low fluorescence intensity (the maximum value of fluorescence intensity is ~190 in reversed micelles) with an excitation wavelength at 295 nm. The fluorescence intensity of CB of the reversed micelles was higher than that of aqueous phase. The wavelength of emission maximum (λ_{max}) had a blue shift for 3 nm, which might indicate that the polarity of the CB microenvironment decreases owing to the lower polarity of reversed micellar core than that of aqueous phase. CB had low concentration (23.68 μM) with the molar ratio of CB to CTAB (50 mM) of 4.74×10^{-4}. Thus, the perturbation of the system in the presence of CB could be assumed negligible.

Figure 2 shows the effect of water content in reversed micellar phase on fluorescence emission spectra of CB. The fluorescence intensity of CB decreased with increasing water content in the reversed micelles. The λ_{max} had a red shift for 2 nm when water concentration was >0.63 M. The free water in the core of reversed micelles increased with an increase in water content, indicating an increase in polarity of the CB microenvironment. This indicates that the fluorescence emission spectra of CB could reflect the microenvironmental change in the reversed micelles.

Effect of CB on Fluorescence Emission Spectra of BSA

Figure 3 shows the effect of CB on fluorescence emission spectra of BSA in aqueous phase. BSA had high fluorescence intensity (the maximum relative intensity was ~6700). The fluorescence of BSA was almost quenched with the addition of CB. The maximum value of relative intensity of the system with the addition of CB was ~137, which is 2.04% that of BSA. The fluorescence emission spectra of BSA were strongly influenced by the affinity interaction between BSA and CB, which could lead to a significant change in the microenvironment of Trp residues. The CB-BSA complex had a lower ability of fluorescence emission than that of BSA.

The fluorescence emission spectra of BSA in the absence of CB (Fig. 3) was compared with that of CB (Fig. 1). The maximum value of relative

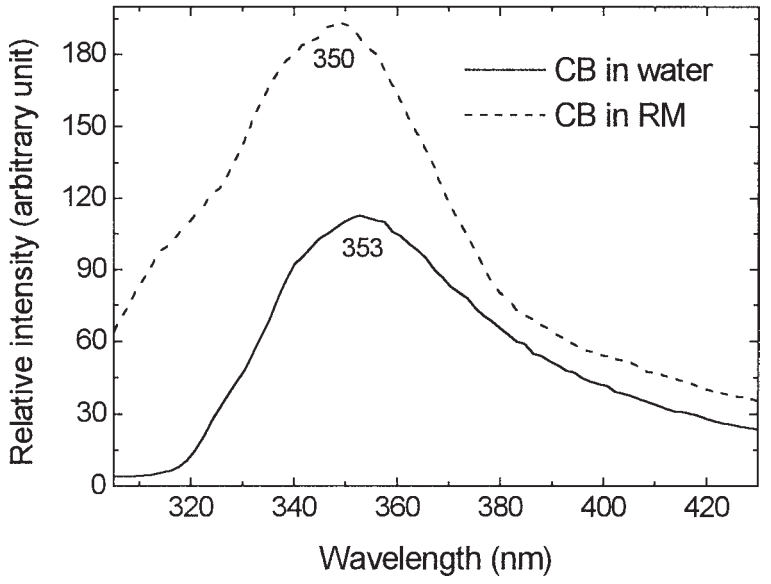

Fig. 1. Fluorescence emission spectra of CB in reversed micelles (RM) and in water. CB = 23.68 μ*M*, H_2O = 1.0 *M* (in reversed micelles); excitation at 295 nm, slit at 5.0/5.0 nm.

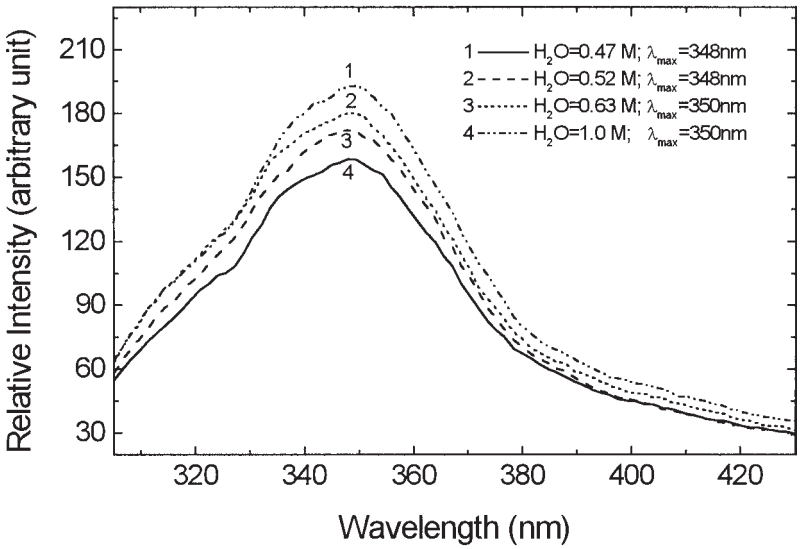

Fig. 2. Effect of water concentration in reversed micellar phase on fluorescence emission spectra of CB. CB = 23.68 μ*M*; excitation at 295 nm, slit at 5.0/5.0 nm.

intensity of CB was ~113, which is 1.69% that of pure BSA. There was a large difference in fluorescence intensity between CB and BSA. The fluorescence of CB could be assumed negligible in comparison with that of BSA if CB and BSA are considered respectively.

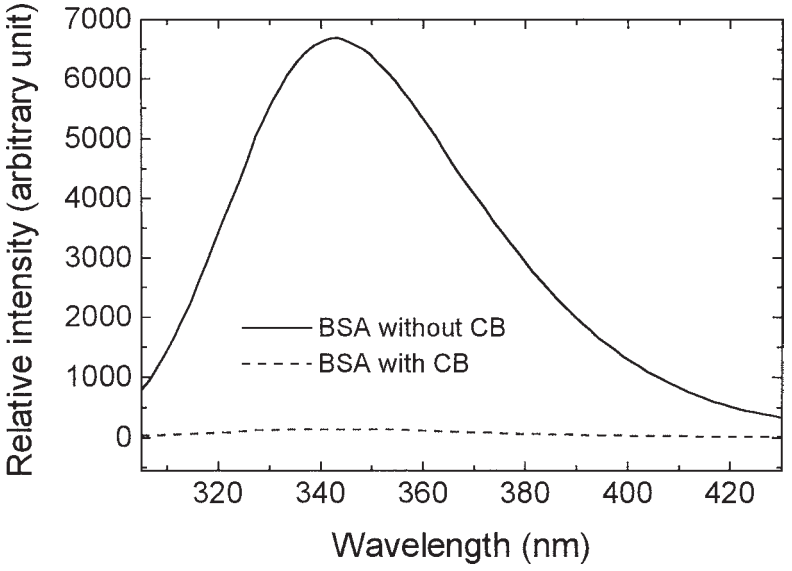

Fig. 3. Effect of CB on fluorescence emission spectra of BSA in aqueous phase. CB = 23.68 μ*M*, BSA = 0.5 mg/mL, pH = 6.96; excitation at 295 nm, slit at 5.0/5.0 nm.

Figure 4 shows the effect of CB on fluorescence emission spectra of BSA in aqueous phase with the addition of CTAB. The addition of CTAB led to a decrease in fluorescence intensity of the system and a blue shift of λ_{max} of 11 nm (from 343 to 332 nm) in the absence of CB. This might indicate that the hydrophobic microenvironment of Trp residues of BSA increases. The conformation of BSA could change since there was an electrostatic attraction between BSA and CTAB owing to the solution pH being greater than the p*I* of BSA (pH 6.96), which influences the microenvironment of Trp residues.

The addition of CB caused the fluorescence intensity of the system to decrease further in the presence of CTAB in aqueous phase. The λ_{max} (343 nm) did not change in the presence of CB and CTAB compared with pure BSA. The microenvironment of Trp residues of BSA could not be significantly changed. The CB-BSA complex may favor the maintenance of BSA conformation owing to the affinity interaction between BSA and CB.

Figure 5 shows the effect of CB on fluorescence emission spectra of BSA in reversed micellar phase. The fluorescence intensity of BSA decreased sharply with the addition of CB. The maximum value of fluorescence intensity of BSA was 4425 in the absence of CB in the reversed micelles. The maximum intensity was 865 with the addition of CB, which is 19.5% that of BSA without the addition of CB. The λ_{max} had a red shift of 10 nm (from 338 to 348 nm), which indicates that the hydrophobic microenvironment of Trp residues of BSA decreases. There was an electrostatic attraction between BSA and CTAB owing to the solution pH being greater than the p*I* of BSA (pH 6.96). The affinity interaction between CB and BSA could lead Trp

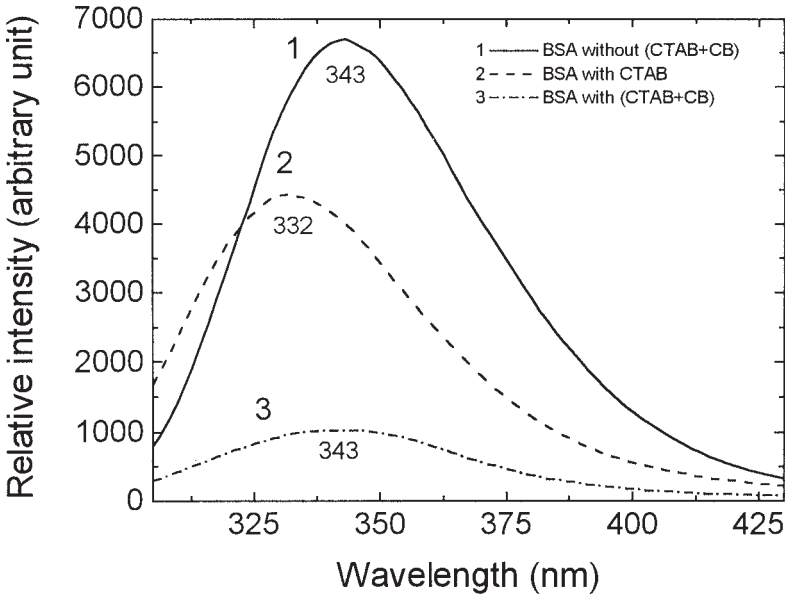

Fig. 4. Effect of CB on fluorescence emission spectra of BSA in aqueous phase with addition of CTAB. CB = 23.68 μ*M*, BSA = 0.5 mg/mL, pH = 6.96, CTAB = 2.0% (w/v); excitation at 295 nm, slit at 5.0/5.0 nm.

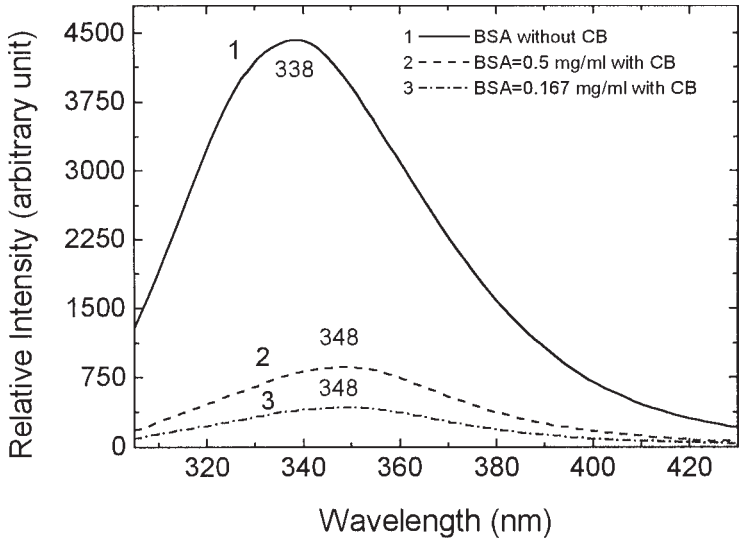

Fig. 5. Effect of CB on fluorescence emission spectra of BSA in reversed micellar phase. CB = 23.68 μ*M*, H_2O = 1.0 *M*, pH = 6.96; excitation at 295 nm, slit at 5.0/5.0 nm.

residues of BSA close to the water core of reversed micelles, which resulted in a decrease in the hydrophobic microenvironment of Trp residues. The fluorescence emission spectra of BSA did not significantly change with a decrease in BSA concentration, only a decrease in fluorescence intensity.

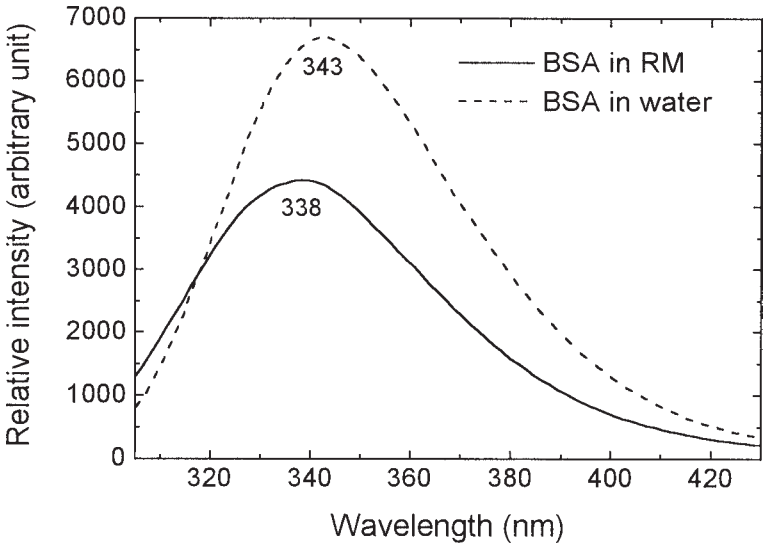

Fig. 6. Fluorescence emission spectra of BSA in reversed micelles (RM) and water. BSA = 0.5 mg/mL, pH = 6.96, H_2O = 1.0 *M* (in reversed micelles); excitation at 295 nm, slit at 5.0/5.0 nm.

Comparison of Fluorescence Emission Spectra of BSA of Reversed Micellar Phase with That of Aqueous Phase

To reflect clearly the effect of the microenvironment of reversed micelles on fluorescence emission spectra of BSA, Fig. 6 shows BSA fluorescence spectra of the reversed micellar phase compared with that of aqueous phase in the absence of CB. The fluorescence intensity of BSA in the reversed micellar phase decreased with a blue shift of λ_{max} of 5 nm. There was a different water property between reversed micellar phase and aqueous phase. The water of the reversed micellar phase was confined in the core of the water pool, which could result in more of a decrease in water polarity of reversed micelles than that of bulk water. The BSA of the reversed micelles was in a more hydrophobic microenvironment than that of aqueous phase. The hydrophobicity of the Trp microenvironment in the reversed micelles increased, which led to a blue shift of fluorescence spectra of BSA.

Figure 7 shows the effect of CB on the fluorescence emission spectra of BSA of the reversed micellar phase compared with that of aqueous phase. The fluorescence intensity of BSA with the addition of CB had the strongest value (1027) in the aqueous phase in the presence of CTAB, a less strong value (865) in the reversed micellar phase, and a weak value (137) in the aqueous phase in the absence of CTAB. The fluorescence intensity of the system with the presence of CTAB in both the reversed micellar and aqueous phases was higher than that in the absence of CTAB in the aqueous phase. The maximum value of fluorescence intensity in the absence of CTAB

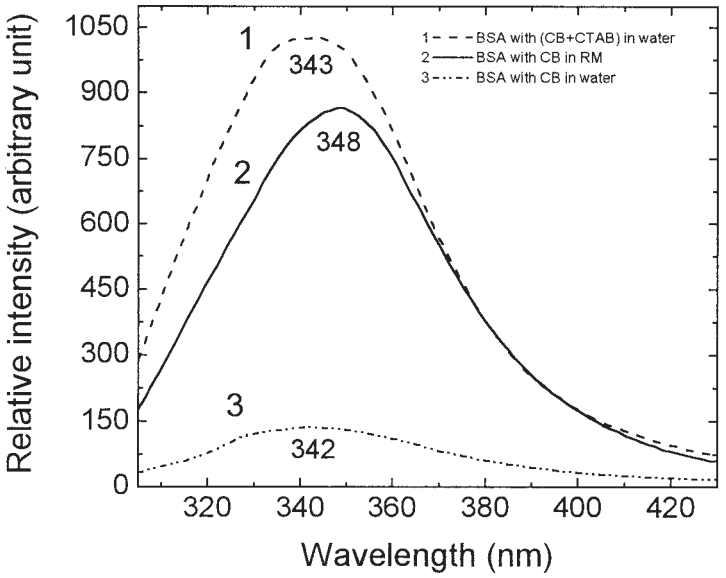

Fig. 7. Effect of CB on fluorescence emission spectra of BSA in reversed micelles (RM) and in aqueous phase. BSA = 0.5 mg/mL, pH = 6.96, CTAB = 2.0% (w/v), CB = 23.68 μ*M*, H_2O = 1.0 *M* (in reversed micelles); excitation at 295 nm, slit at 5.0/5.0 nm.

in the aqueous phase was 15.8% that in the reversed micellar phase. The presence of CTAB could hinder the quenching of fluorescence of BSA. The electrostatic interaction between CTAB and CB could decrease the affinity interaction between BSA and CB.

The λ_{max} of BSA with the addition of CB in the aqueous phase was not changed in the presence of CTAB and decreased 1 nm without the addition of CTAB in comparison with that of pure BSA. This indicates that the microenvironment of Trp residues in the presence of CB was not significantly influenced by the addition of CTAB in the aqueous phase. However, the λ_{max} of BSA with the addition of CB in the reversed micellar phase increased 5 nm (red shift), from 343 to 348 nm. This might indicate that the hydrophobic microenvironment of the Trp residue of BSA decreases. The electrostatic and hydrophobic interaction between BSA and CTAB could also take phase in the reversed micelles. This is contrary to those microenvironments without the addition of CB. The affinity interaction between BSA and CB of reversed micellar phase was somewhat different from that of aqueous phase.

Effect of CB Concentration on Fluorescence Emission Spectra of BSA in Reversed Micelles

To gain insight into the formation of complexes between CB and BSA, the effect of CB concentration of the fluorescence emission spectra of BSA in reversed micelles was investigated. During the static quenching, the relationship between intensity of fluorescence, *F*, and concentration of

quenching agent CB, [*Q*], can be obtained by means of the binding constant, *K*, and the number of bindings, *n*. If there are *n* binding sites on BSA molecules (*P*), we can write relational expressions as follows:

$$nQ + P \rightleftharpoons Q_nP \tag{1}$$

$$K = \frac{[Q_nP]}{[Q]^n\,[P]} \tag{2}$$

in which [*Q*], [*P*], and [Q_nP] are the concentration of quencher CB, free BSA, and complexes between CB and BSA, respectively. If [P_0] is the total concentration of BSA, and [P_0] = [Q_nP] + [*P*], then

$$[Q_nP] = [P_0] - [P] \tag{3}$$

and

$$\frac{[P]}{[P_0]} = \frac{[F]}{[F_0]} \tag{4}$$

in which F_0 and *F* are the fluorescence intensities at a 348-nm emission wavelength in the absence and presence of quencher CB. In association with Eqs. 1–4, one can finally describe the relationship between the intensity of fluorescence, *F*, and the concentration of quenching agent, [*Q*], as follows:

$$\log \frac{(F_0 - F)}{F} = \log K + n \log[Q] \tag{5}$$

Figure 8 shows the relationship between the fluorescence emission spectra of BSA and CB concentration in reversed micellar phase under different water concentrations. There are two linear relationships, whose slopes and intercepts are taken to correspond to the number of binding molecules, *n*, and binding constant, *K*, respectively. The binding constant, *K*, increases sharply from 2.0×10^6 to $6.61 \times 10^7\ M^{-1}$ when the water concentration increased from 0.81 to 1.0 *M* in the reversed micelles. This could be the stronger interaction between cationic CTAB and anionic CB in lower water concentration in the reversed micelles, which then decreases the affinity interaction between CB and BSA. The binding constant, *K*, between CB and BSA is $1.18 \times 10^5\ M^{-1}$ in aqueous phase *(21,23)*. The binding constant, *K*, of the reversed micellar phase is one or two orders of magnitude higher than that of aqueous phase. This indicates the stronger interaction between CB and BSA in the reversed micelles than that in aqueous phase owing to the nanosize of reversed micelles. The number of binding molecules, *n*, in the reversed micelles increased slightly from 1.2 to 1.5 with increasing water concentration from 0.81 to 1.0 *M*. This result suggests that not many

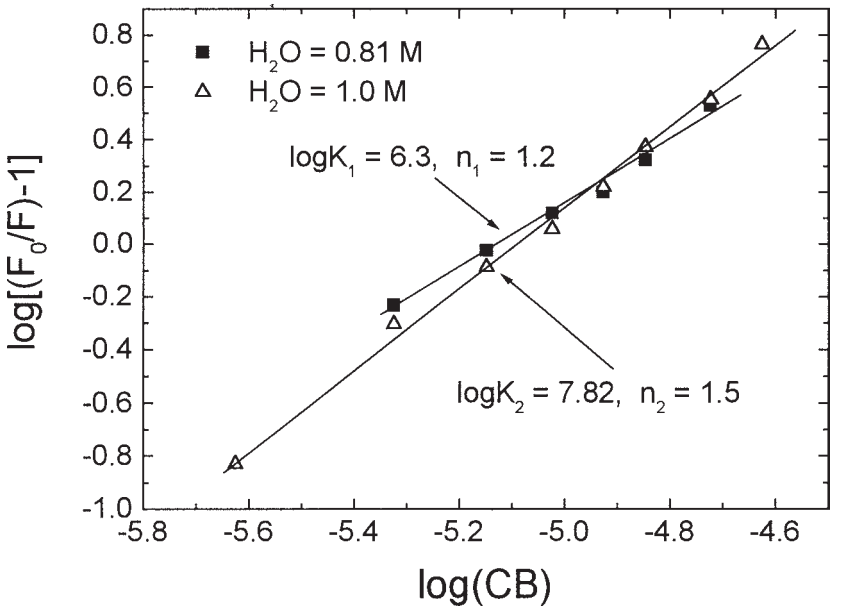

Fig. 8. Effect of CB concentration on fluorescence emission spectra of BSA in reversed micelles. BSA = 0.5 mg/mL, pH = 6.96; excitation at 295 nm, slit at 5.0/5.0 nm. F_0 and F are fluorescence intensities at a 348-nm emission wavelength in the absence and presence of CB, n is the number of binding molecules; and K is the binding constant.

CB molecules are required to bind a BSA molecule in the reversed micelles although the molecular weight between BSA and CB (large and small molecules) is much different.

Conclusion

CB had a significant influence on the fluorescence emission spectra of BSA. The fluorescence intensity of BSA in the aqueous phase was almost quenched with the addition of CB. The influence of CTAB on BSA fluorescence spectra was decreased by the affinity interaction between CB and BSA. The fluorescence intensity of BSA in the reversed micellar phase was greatly decreased with the addition of CB.

The λ_{max} in the reversed micelles formed with CTAB had a blue shift of 5 nm compared with pure BSA in the absence of CB. The λ_{max} in the aqueous phase with the addition of CTAB had a blue shift of 11 nm in the absence of CB. The λ_{max} of BSA with the addition of CB in the aqueous phase was not changed in the presence of CTAB and decreased 1 nm without the addition of CTAB in comparison with pure BSA. However, the λ_{max} of BSA with the addition of CB in the reversed micellar phase increased 5 nm (red shift), which might indicate that the hydrophobic microenvironment of the Trp residue of BSA decreases.

Acknowledgment

We wish to thank Xiao-Fang Wei for the use of the fluorescence spectrophotometer to acquire excitation spectra and for further discussions on the revised manuscript. We acknowledge the financial support of the National Natural Science Foundation of China (no. 29836130).

References

1. Luisi, P. L. and Magid, L. J. (1986), *CRC Crit. Rev. Biochem.* **20,** 409–421.
2. Leodidis, E. B. and Hatton, T. A. (1990), *J. Phys. Chem.* **94,** 6412–6420.
3. Martinek, K., Levashov, A. V., Klyachko, N. L., Khmelnitsky, Y. I., and Berezin, I. V. (1986), *Eur. J. Biochem.* **155,** 453–459.
4. Luisi, P. L., Giomini, M., Pileni, M. P., and Robinson, B. H. (1988), *Biochim. Biophys. Acta* **947,** 209–249.
5. Skagerlind, P. and Holmberg, K. J. (1994), *Dispers. Sci. Technol.* **15,** 317–323.
6. Bommarius, A. S., Hatton, T. A., and Wang, D. I. C. (1995), *J. Am. Chem. Soc.* **117,** 4515–4523.
7. Nicot, C. and Waks, M. (1995), in *Biotechnology and Genetic Engineering Reviews*, vol. 13, Tombs, M. P., ed., Intercept, England, pp. 266–302.
8. Walde, P., Peng, Q., Fadnavis, N. W., Battistel, E., and Luisi, P. L. (1988), *Eur. J. Biochem.* **173,** 401–409.
9. Nicot, C., Vacher, M., Denoroy, L., Kahn, P. C., and Waks, M. (1993), *J. Neurochem.* **60,** 1283–1289.
10. Groome, N. P., Vacher, M., Nicot, C., and Waks, M. (1990), *Biochem. Int.* **21,** 1–7.
11. Dekker, M., Hilhorst, R., and Laane, C. (1989), *Anal. Biochem.* **178,** 217–226.
12. Hatton, T. A. (1989), in *Surfactant-Based Separation Processes*, Scamehorn, J. F. and Harwell, J. H., eds., Marcel Dekker, New York, pp. 55–90.
13. Luisi, P. L., Giomini, M., Pileni, M. P., and Robinson, B. H. (1988), *Biochim. Biophys. Acta* **947,** 209–246.
14. Woll, J. M., Hatton, T. A., and Yamush, M. L. (1989), *Biotechnol. Prog.* **5,** 57–62.
15. Coughlin, R. W. and Baclaski, J. B. (1990), *Biotechnol. Prog.* **6,** 307–309.
16. Paradkar, V. M. and Dordick, J. S. (1991), *Biotechnol. Prog.* **7,** 330–334.
17. Kelley, B. D., Wang, D. I. C., and Hatton, T. A. (1993), *Biotechnol. Bioeng.* **42,** 1199–1208.
18. Kelley, B. D., Wang, D. I. C., and Hatton, T. A. (1993), *Biotechnol. Bioeng.* **42,** 1209–1217.
19. Adachi, M., Yamazaki, M., Harada, M., Shioi, A., and Katch, S. (1997), *Biotechnol. Bioeng.* **53,** 406–408.
20. Adachi, M., Shibata, K., Shioi, A., Harada, M., and Katoh, S. (1998), *Biotechnol. Bioeng.* **58,** 649–653.
21. Sun, Y., Ichikawa, S., Sugiura, S., and Furusaki, S. (1998), *Biotechnol. Bioeng.* **58,** 58–64.
22. Sun, Y., Gu, L., Tong, X. D., Bai, S., Ichikawa, S., and Furusaki, S. (1999), *Biotechnol. Prog.* **15,** 506–512.
23. Zhang, T. X., Liu, H. Z., and Chen, J. Y. (1999), *Biotechnol. Prog.* **15,** 1078–1082.
24. Vijayalakshmi, M. A. and Bertrand, O. (1989), *Protein-Dye Interactions: Developments and Applications*, Elsevier Applied Science, London.
25. Ramírez-Vick, J. E. and García, A. A. (1996–97), *Sep. Puri. Methods* **25,** 85–129.
26. Zhang, T. X., Liu, H. Z., and Chen, J. Y. (1999), *Colloids Surf. A* **162,** 259–264.
27. Karukstis, K. K., Frazier, A. A., Loftus, C. T., and Tuan, A. S. (1998), *J. Phys. Chem. B* **103,** 8163–8170.
28. Hof, M., Lianos, P., and Laschewsky, A. (1997), *Langmuir* **13,** 2181–2183.
29. Lundgren, J. S., Heitz, M. P., and Bright, F. V. (1995), *Anal. Chem.* **67,** 3775–3781.
30. Marzola, P. and Gratton, E. (1991), *J. Phys. Chem.* **95,** 9488–9495.
31. Lakowicz, J. R. (1983), *Principles of Fluorescence Spectroscopy*, Plenum, New York.