Concentration-Dependent Aggregation of CHAPS Investigated by NMR Spectroscopy

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CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) is a zwitterionic surfactant, which has been extensively used in various biological fields. In the present work, the concentration-dependent aggregation of the surfactant in deuterium oxide solution was investigated by NMR spectroscopy. We have found that two break points exist on the basis of the NMR parameters such as chemical shift difference ($\Delta\delta$), self-diffusion coefficient (D), and relaxation rates (R_1, R_2). The first break point corresponds to the widely accepted normal critical micelle concentration (cmc). The second one is unexpected and ascribed to the second cmc, indicating that there is another type of micelle at higher concentrations. Further analysis using 1D selective NOESY with spin-diffusion quenching reveals that at the concentration above the second cmc CHAPS may form a two-layer spherical structure of micelles, with the aliphatic groups of CHAPS molecules in the inner layer interact with the steroid groups in the outer layer. The existence of two types of micelles has also been supported by our TEM experiment. The dependence of CHAPS micelle size on concentration explains why some proteins are soluble and stable only at concentrations above the second cmc. Therefore, our finding provides a basis for optimizing CHAPS concentration in functional and structural studies of membrane proteins.

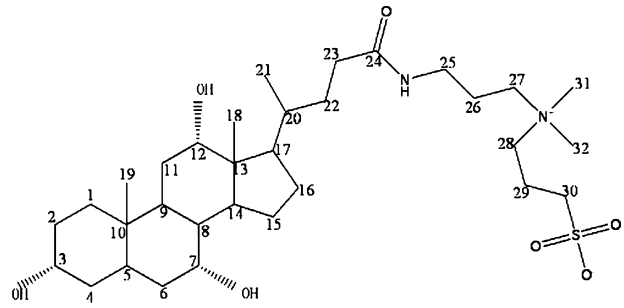
1. Introduction

Detergents are important and indispensable reagents in studies of biological membrane molecules. They are frequently used as lipid bilayer mimetic media to solubilize integral membrane proteins and polypeptides.[1−3] As one of the important detergents designed for membrane biochemistry, CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) is a nondenaturing zwitterionic derivative of cholic acid and combines the useful properties of both sulfobetaine-type detergents and bile salts.[4] It does not have a net charge over a wide pH range, which results in the mildness without altering the charge properties of proteins.

Unlike typical surfactants with polar head and alkyl chain, CHAPS has a characteristic steroid structure with a convex side (β-plane or back) and a concave side (α-plane or face) on which there are a few hydrophilic hydroxyl groups (see Scheme 1). This unique structure may lead CHAPS to atypical aggregation behavior. Because of its special structural property, CHAPS has been predicted to have considerable untapped potentials, which may strengthen its common application in protein structural studies.[5] As an important parameter for a given detergent, the critical micelle concentration (cmc) of CHAPS was reported in a range from 5.4 to 11 mM.[6−8] Therefore, to stabilize a membrane protein, the concentration of CHAPS should be more than its cmc. Nevertheless, in practice, it has been proposed that CHAPS is most effective at preventing protein from self-association at a concentration greater than 20 mM.[9,10] McGuire et al.[11] obtained the optimal solubility and stability of yeast eIF4E with the addition of 25−50 mM CHAPS and predicted that the use of CHAPS could be a promising technique for increasing the solubility and stability of other difficult proteins. Schuck et al.[12] found that some isolated detergent-resistant membranes (DRMs) were insoluble in 20 mM CHAPS, whereas they were fully solubilized by 65 mM CHAPS, and concluded that CHAPS are the most reliable detergents for analyzing possible raft association. Lee et al.[13] suggested that a higher CHAPS concentration could improve protein resolution during 2D-PAGE in the pH range 3−10. Garner et al.[14] revealed that CHAPS did exhibit selective solubilization of the disordered lipid phase at appropriate concentrations and considered that such variation may attribute more to the unique properties of the individual detergents than to the actual raft domains.

As mentioned above, a better understanding of the size, shape, and structure change of CHAPS micelles is essential to understand its interactions with proteins and to determine optimal detergent concentrations for functional and structural

SCHEME 1: Chemical Structure of CHAPS[a]



[a] For convenience, the molecule is divided into two parts: steroid nucleus is thought to be the head of the molecule, and the aliphatic chain is the tail of the molecule.

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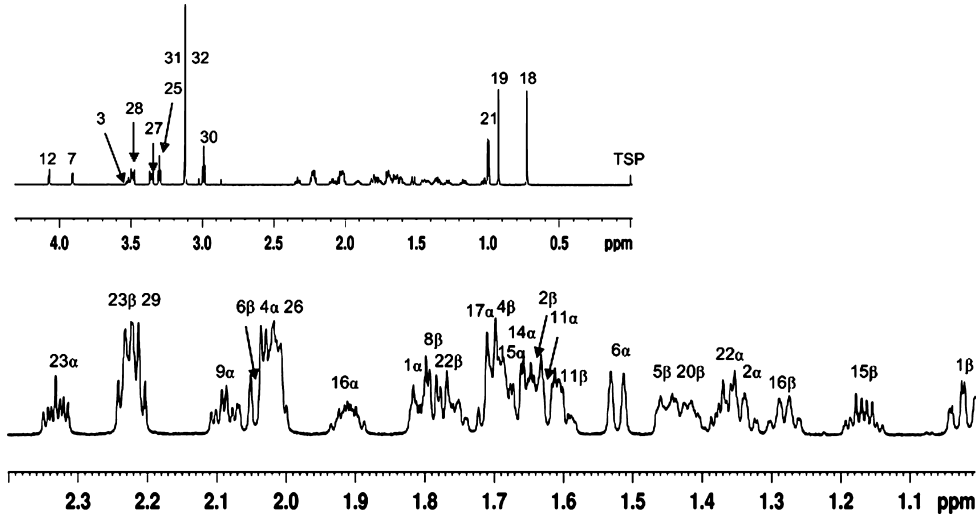

Figure 1. ^{1}H NMR spectrum labeled with assignments of CHAPS protons (3.8 mM) in D_2O. The full spectrum is shown in the upper left-hand corner, the region between 1.0 and 2.4 ppm is enlarged for detail.

studies of membrane proteins. Funasaki et al.[15,16] estimated that CHAPS forms dimers mainly in the conformation of parallel back-to-back association before micelles. Lipfert et al.[17] found that CHAPS micelles indeed grow with the increase of detergent concentrations. However, the whole structure of the micelle could not be predicted, and the dependence of the apparent micelle size on concentration is unknown. Consequently, the mechanism of the formation of CHAPS micelles still needs further investigation.

Here we use NMR to probe the micelle formation of CHAPS. We used a number of NMR parameters, such as chemical shifts, self-diffusion coefficients, and relaxation rates, to probe the aggregation behavior and conformational change of the detergent systems. On the basis of our NMR data, we found that there are two different aggregation states for CHAPS, which are concentration-dependent. Further analysis using 1D selective NOESY with spin-diffusion suppression revealed the aggregate models of the two types of micelles.

2. Experimental Section

Materials. The purities of both CHAPS (Sigma-Aldrich) and deuterium water (D_2O, Arcos) were 98%. $Me_3Si-CD_2CD-CO_2Na$ (TSP) obtained from ISOTEC (a member of Sigma-Aldrich family) was used as an external reference. The above-mentioned reagents were used without any further purification.

Sample Preparation. CHAPS samples were prepared by dissolving it in D_2O directly without any additives. High concentration CHAPS was prepared first and then diluted to desired low concentrations (range from 0.8 to 78.4 mM, total 23 samples, pH 6.5). To remove the dissolved paramagnetic oxygen and make the solution homogeneous, sonication was done for 10 min for every sample. The solution was transferred to 5 mm NMR tubes and sealed with paraffin film immediately, and then stored at ambient temperature and normal pressure overnight before use.

NMR Experiments. All NMR experiments were performed at 298 K on a Bruker AVANCE 500 MHz spectrometer, except two-dimensional ^{1}H−^{1}H correlation spectroscopy (COSY), ^{1}H−^{13}C heteronuclear multiple-quantum coherence spectroscopy (HMQC), heteronuclear multiple-bond correlation spectroscopy (HMBC) and selective one-dimensional NOESY experiments. The 1D ^{1}H NMR experiment was accomplished by a small pulse flip-angle of $\pi/6$. The relaxation times T_1 and T_2 were measured by using the inversion recovery and the Carr−Percell−Meiboom−Gill (CPMG) experiment, respectively.[18] A bipolar gradient pulse pair longitudinal eddy-current delay experiment was used to determine the self-diffusion coefficients, D.[19] The selective 1D NOESY with spin-diffusion quenching was performed on a Bruker AVANCE III 800 MHz spectrometer equipped with a cryo-probe and pulsed field gradients. The NOESY spectra were recorded at six surfactant concentrations: 2.5, 7.1, 13.8, 31.8, 61.9, and 110.1 mM, with a spectral width of 9600 Hz, 32 K data points in time domain, 32 scans, and a relaxation delay of 4 s. A 80 ms Gauss-shaped π pulse was used for selective refocusing in the 1D NOESY.[20−22] To differentiate NOEs caused by direct cross-relaxation from those caused by spin-diffusion, the spectra were recorded with six different mixing times: 3, 20, 40, 60, 80, and 100 ms. To obtain resonance assignments of CHAPS, 2D COSY, HMQC, and HMBC spectra were also performed on a Bruker AVANCE III 800 MHz spectrometer.

TEM Measurements. Transmission electron microscopy (TEM) measurements were performed using a JEM-2010 200 kV transmission electron microscope. The aqueous sample solutions were dropped into copper EM grids (precoated with the thin film of carbon) and dried at room temperature.

3. Results and Discussion

Resonance assignments of CHAPS were carried out on the basis of the 1D ^{1}H NMR spectrum, 2D homonuclear COSY spectrum, 2D heteronuclear HMQC, HMBC spectrum, and the reference data available.[23] A ^{1}H NMR spectrum of CHAPS in D_2O is shown in Figure 1.

^{1}H NMR chemical shifts are well-known to be sensitive to the local electronic environment of protons and are an excellent parameter to probe micelle aggregation.[24−28] The observed proton chemical shifts as a function of surfactant concentration can be used not only to provide direct and strong evidence of micelles aggregation but also to determine the cmc. As shown in Figure 2, an interesting phenomenon is that the changes of proton chemical shifts with CHAPS concentration do not follow a single transition profile, but there are two distinct break points for all protons. Generally, the variations of the chemical shifts reflect the micelle formation, and the break point corresponds to the cmc value. Thus the CHAPS concentrations at the two break points were tentatively considered as cmc_1 and cmc_2, respectively. The chemical shift changes in Figure 2a are of

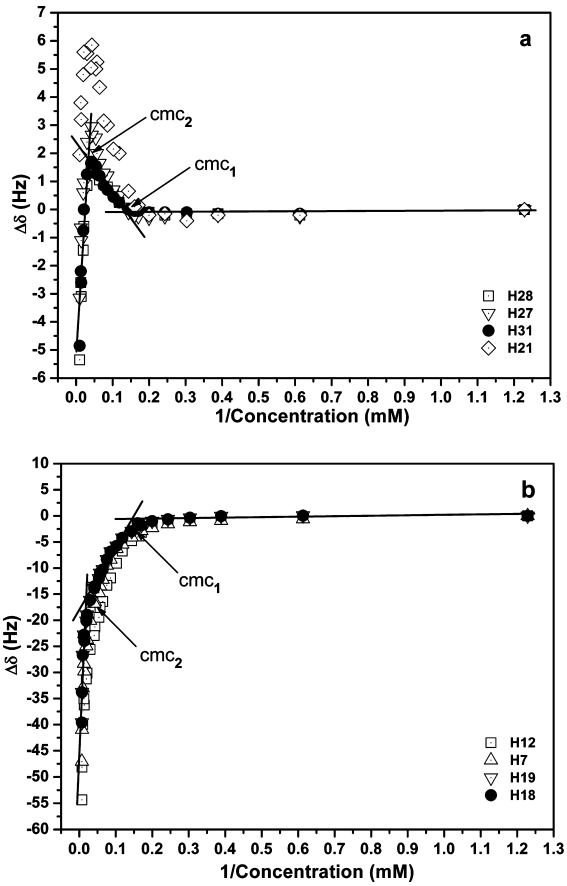

Figure 2. Relative ^{1}H chemical shifts versus the reciprocal concentration of CHAPS in D_2O at 298 K: (a) chemical shift changes of the tail group protons; (b) chemical shift changes of the head group protons.

protons in the tail group of CHAPS, such as H21, H27, H28, and H31. It is evident that the chemical shifts almost do not change at concentrations lower than cmc_1. This implies that the surfactant molecules in solution remain in the monomeric state. Subsequently, the chemical shifts move from upfield to downfield when the concentration increases over the cmc_1. However, when the total surfactant concentration is higher than cmc_2, the chemical shifts drops dramatically with the increase of the concentration. On the other hand, the chemical shifts of protons in the head group of CHAPS always decrease with the increase of the detergent concentration after each break point (Figure 2b). Moreover, the changes of chemical shifts with CHAPS concentration for the head group protons are significantly larger than those protons from the tail groups, indicating that CHAPS may micellize by the self-association of the steroid nucleus (head group).[29] Although the observed chemical shift for a given proton is a weighted average of those in monomeric and micellar states, the existence of two break points reveals that there are two possible aggregation states. The chemical shift difference in different sized micelles may arise from the change of packing density of the molecules in the micelles. The distinct changes of the chemical shifts of the protons in the tail group in different ranges of CHAPS concentration (Figure 2a) suggest that the tail group protons are in two extremely different environments at the two aggregation states. In contrast, the chemical shifts of the head group protons change in a unidirectional manner with the increase of CHAPS concentration (Figure 2b), indicating that the environments around the protons from head groups are not extremely different in the two aggregation states.

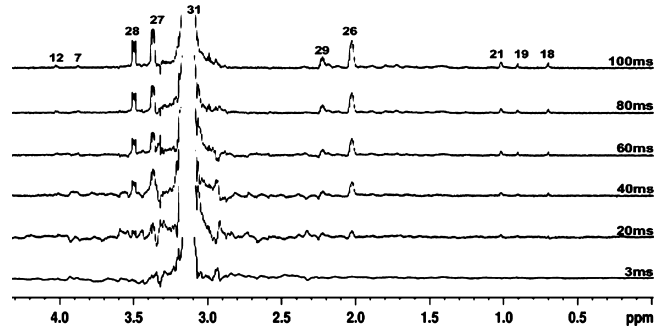

Figure 3. 1D selective NOESY spectra of CHAPS at different NOE mixing times. Here, proton H31 is selectively inverted. The concentration of CHAPS is 61.9 mM.

The existence of two possible aggregation states has also been confirmed by the measurement of relaxation rates of CHAPS, shown in the Supporting Information. There are also two break points in the plots of relaxation rates (the spin−spin relaxation rate R_1 and spin−lattice relaxation rate R_2) versus the reciprocal CHAPS concentration.

In general, one cmc exists for one detergent, which appears as a break point in the chemical shift curve. Sometimes, the second cmc is observed for some detergents at a concentration of 3−10 times of the first cmc, such as SDS, C12BCl, and some mixed micelles.[25,26,30−32] From Figure 2, the two apparent cmc values obtained are 7.2 ± 0.1 and 32.3 ± 0.1 mM, respectively. The cmc_1 value determined here is in good agreement with the data obtained by other methods.[6−8] Therefore, it is unlikely that the presence of the two break points is fortuitous. By comparison, the second cmc observed here may be due to the use of a larger range of CHAPS concentration.

In the study of SDS and some mixed micelles, it has been suggested that the occurrence of the second cmc is due to a new rearrangement of the micellar structures, such as the size or shape changes of the micelles.[25,26,30,31] Therefore, it is interest to check whether the micelles of CHAPS also undergo some similar structural transitions.

The nuclear Overhauser effect (NOE) plays an important role in NMR based molecular structure determination; however, the application of 2D NOESY spectra to structure determination of large molecules is usually hindered by the spin-diffusion effect. Besides, for the CHAPS system, the resonances of the protons at the head group are close to each other, making it difficult to find their cross-peaks in 2D NOESY spectra.[16] Therefore, to obtain the details about the arrangement of CHAPS in the micelles, selective 1D NOESY was employed here. In this experiment, the spin-diffusion effect was quenched by the application of a selective π pulse in the middle of the NOE mixing period, and cross-relaxation rates could be measured more accurately. Consequently, more reliable internuclear distances could be obtained.

The reliability of the selective NOESY was checked by measuring the cross-peak as a function of mixing time. Elimination of the spin-diffusion effects is demonstrated in Figure 3, in which the 1D NOESY spectrum (methyl proton H31/H32 was selective inverted) of CHAPS at a concentration of 61.9 mM is plotted. As the mixing time increases, peaks for protons H18, H19, and H21, which result from cross relaxation, increase linearly in intensities. In addition, other protons near H18, H19, and H21 did not show any detectable NOE signals at all. This indicates the spin-diffusion effect was efficiently suppressed in the experiment.

1D selective NOESY spectra of CHAPS at different concentrations are shown in Figure 4. As the concentration increases,

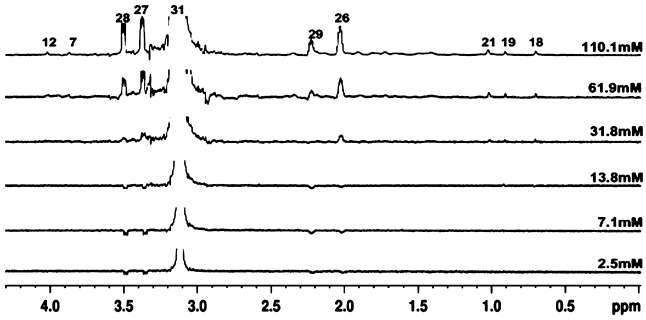

Figure 4. 1D selective NOESY spectra of CHAPS at six different concentrations. Here, the NOE mixing time is 60 ms: H31 was selective inverted.

the signal intensities of NOEs between CH_3 groups (H31 and H32) and their proximal intramolecular protons (H28, H27, H29, and H26) increase too. These NOEs might originate from intramolecular interactions since they are also present in the 1D NOESY spectra below the cmc_1. However, the sign of the NOEs changed from positive to negative after the second break point, indicating that the overall tumbling time of the molecule or the effective correlation time exceeds a certain number. Apparently, this should result from the change of the micellar size and the restriction of the local motions. Interestingly, NOEs between H31 (H32) and H18, H19, H21, H7, and H12 were not observed at low CHAPS concentrations ($\leq cmc_1$), indicating that the molecules most probably exist in an extended form as shown in Scheme 1 where H31 (H32) is far away from H18, H19, H21, H7, and H12. However, those NOEs appeared and became significant at the concentration above cmc_2. The observed NOEs can be explained by three possible models as shown in Figure 5. If CHAPS molecules adopt the conformation shown in model a in micelles, some protons in the head group would have similar chemical shift dependences on CHAPS concentration to some protons in the tail group. Actually, the protons in the head and tail groups have very different chemical shift dependences (Figure 2). Thus model a is incorrect. In model b, the charged groups of the two molecules are aligned in opposite directions. In this case, the molecules cannot form micelles. Thus, model b is incorrect also. In model c, we assume that CHAPS molecules form normal micelles at cmc_1 (first type of micelles, Figure 5d) in which the noncharged head groups are located inside the micelle while the charged tail groups are outside. At CHAPS concentrations larger than cmc_1, the extra CHAPS molecules can be inserted in among tail groups through hydrophobic interactions as shown in model c and Figure 5d. At cmc_2, all the tail groups in the inner layer of the micelle are in close contacts with the head groups in the outer layer. As a result, the second type of micelles with two layers is formed. The interactions for the tail groups of the inner layer CHAPS are quite different from those of the outer layer in the second type of micelles and also very different from those in the first type of micelles. In principle, two distinct sets of resonances should be observed for the second type micelles at cmc_2. In practice, only one set of resonances was observed. This should be caused by the fast exchange of the CHAPS molecules from the inner layer to the outer layer on the NMR chemical shift time scale (i.e., the exchange rate is much larger than the chemical shift differences between the two environments (a few hertz)). The observed resonance shifts are the weighted averages of the chemical shifts of the protons in the two different environments. Nevertheless, two distinct patterns of chemical shift changes were observed for the protons in the tail groups, reflecting the two distinct environments (Figure 2a). Though the head groups of CHAPS in the outer and inner layers are different in interactions, the difference does not cause the reverse of the direction of the chemical shift change, resulting in a unidirectional change of chemical shifts for the protons in the

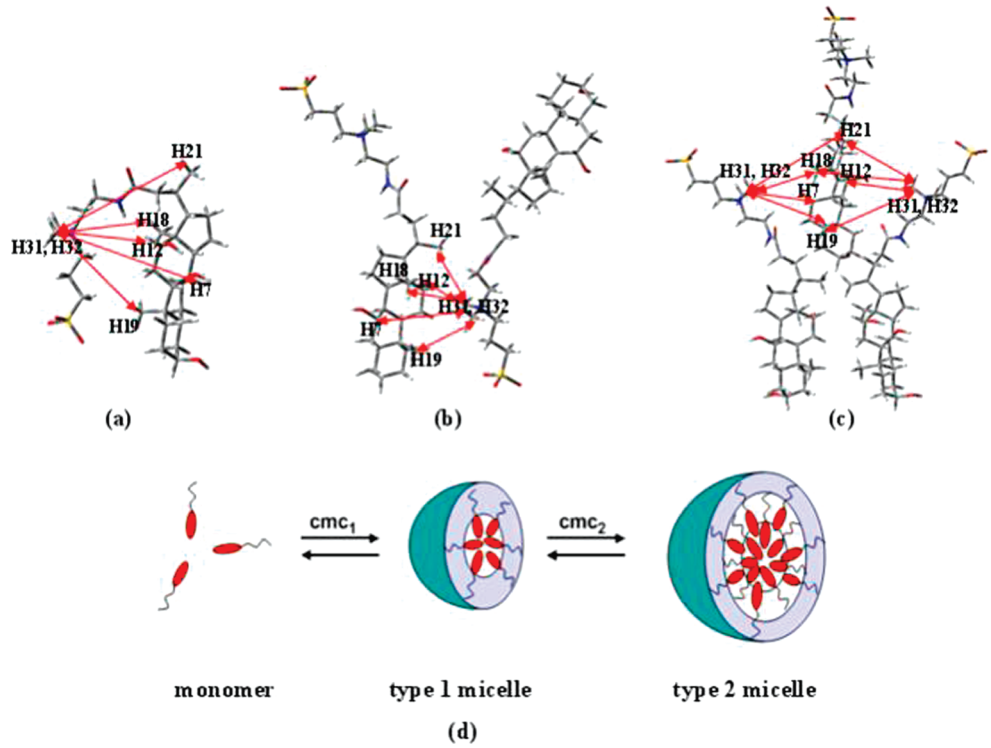

Figure 5. (a) Molecules folding back; therefore, tails turning to the hydrophobic core accordingly. (b) Molecular rearrangement antiparallel. (c) Heads of outer molecules interacting with the tails of inner molecules. (d) Concentration-dependent models of CHAPS micelles, which transform from monolayer to bilayer. The key protons have been linked with red arrows, which coincide with the 1D selective NOESY results.

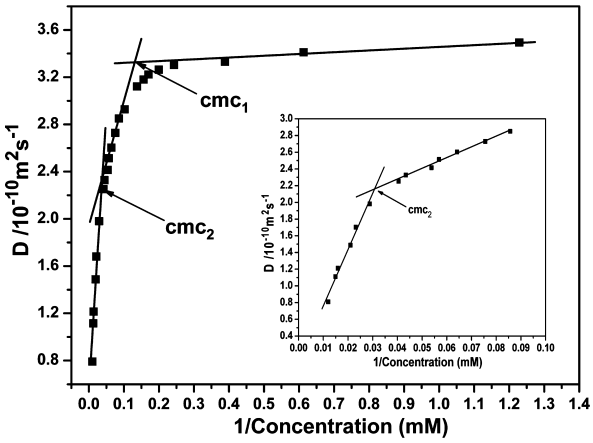

Figure 6. Self-diffusion coefficients of CHAPS molecules versus the reciprocal concentration in D_2O at 298 K.

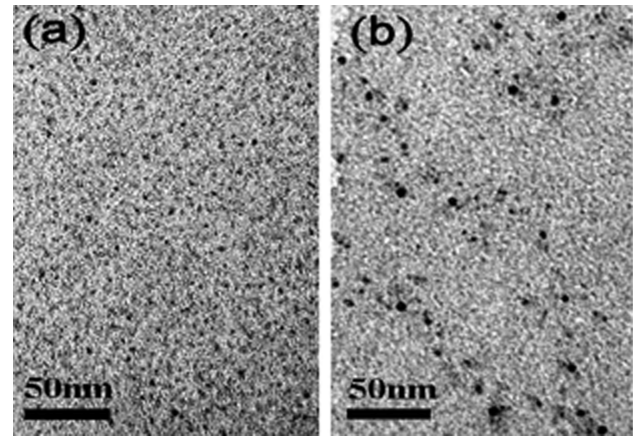

Figure 7. TEM images of the micelles formed by CHAPS in D_2O: (a) CHAPS concentration 20 mM; (b) CHAPS concentration 80 mM.

head group with CHAPS concentration. Therefore, only model c is supported by both chemical shift and NOE data.

The measurements of self-diffusion coefficients of CHAPS also support the hypothesis of the existence of two types of micelle structures. The self-diffusion coefficient of CHAPS versus its reciprocal concentration is displayed in Figure 6. The self-diffusion coefficients of CHAPS decrease with concentration, indicating the population of the molecules in aggregation states increased. There are also two break points in the plot of the diffusion coefficient against concentration. To confirm the existence of bilayer micelle structure, the hydrodynamic radius of the micelles has been estimated using the Stokes−Einstein equation: $R_h = K_BT/6\pi\eta D$, where T is the temperature, K_B is the Boltzmann constant, and η is the viscosity of the solution (the initial value is considered as that of water, $\eta_0 = 0.892$ cP).[33] To properly analyze the concentration-dependent D, the relative viscosity was carefully calibrated by measuring the self-diffusion coefficients of HOD over the entire surfactant concentration range. The relative diffusion coefficients D_{HOD}/D_0 are given in the Supporting Information, where D_0 is the value of pure D_2O, which is 1.97×10^{-9} m^2/s at 298 K. Therefore, the hydrodynamic radius of micelles can be calculated using the equation below: $R_v = K_BT/6\pi D_v(D_{HOD}/D_0\eta_0)$, D_v and D_{HOD} are the self-diffusion coefficient of CHAPS and HOD at the same concentration, respectively. Assuming that the micelle is perfectly spherical and interactions are absent. The calibrated self-diffusion coefficients of CHAPS, at concentrations of 20 and 80 mM, were $(2.1 \pm 0.2) \times 10^{-10}$ and $(7.8 \pm 0.1) \times 10^{-11}$ m^2/s, the corresponding hydrodynamics radii were 1.5 ± 0.2 and 2.7 ± 0.1 nm, respectively. The hydrodynamic radius of the micelle at 80 mM is about 1.8 times of that at 20 mM. This result coincides with the formation of the second micelle by adding another layer of CHAPS, which is partially inserted in the first layer through head−tail interactions.

The morphology of the CHAPS micelles deduced from NMR measurements was further characterized by direct observation with transmission electron microscopy (TEM). The spherical shape of the CHAPS micelles is clearly evident in Figure 7. The dark spots shown in Figure 7a have an approximate uniform radius of 1.4 ± 0.1 nm. The micellar size becomes larger at concentrations above cmc_2, as shown in Figure 7b. The micelles become polydisperse with a mean radius of 2.3 ± 0.2 nm, which is approximately 1.6 times as large as that shown in Figure 7a. This result is in good agreement with the shape and the dimension of CHAPS micelles deduced from NMR spectroscopy. However, the size from TEM is slightly smaller than that measured by NMR. This may be due to the fact that the micelles are fully hydrated in the solution during the NMR measurements but dried under TEM conditions. Actually, the radius measured by NMR is also underestimated, because the diffusion coefficient measured is a weighted average of those in the aggregation and free states. In vacuum and without any interactions the energy minimized length of a single molecule CHAPS is 1.7 nm by GassView 3.0 modeling. All these results are in a good agreement with the model proposed here.

It has been reported that protein conformations in micelles are strongly influenced by the size and thickness of the micelle,[34] and the sizes of protein−detergent complexes are sensitive to the surfactant concentration.[35] Because the micelle dimension of CHAPS is related to the surfactant concentration, it becomes reasonable that CHAPS is more effective for increasing the solubility and stability of large proteins at higher concentration.[9−14] The finding mentioned above may be helpful in the selection of the concentration of CHAPS in protein studies.

4. Conclusions

Our data show that two cmc values exist for the concentration-dependent CHAPS micellar system. Further analysis reveals that the structure of CHAPS micelles can be transformed from the spherical monolayer (type 1 micelle) to the approximate bilayer (type 2 micelle) as the concentration increases above the second cmc. The increase of the micelle size can be directly observed with TEM. This explains why some proteins are soluble and stable only at concentrations above the second cmc. Our findings provide a basis for optimizing CHAPS concentrations in functional and structural studies of membrane proteins.

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Supporting Information Available: Spin−spin relaxation rate (R_1) and the spin−lattice relaxation rate (R_2) versus the reciprocal CHAPS concentration are given in Figure S1 and S2. Two break points appear in each curve, which correspond to cmc_1 and cmc_2, respectively. The diffusion coefficients of HOD versus the concentrations of CHAPS are also provided in Figure S3. This material is available free of charge via the Internet at http://pubs.acs.org.

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