Hydrodynamic Radii of Protein-Free and Protein-Containing Reverse Micelles As Studied by Fluorescence Recovery after Fringe Photobleaching. Perturbations Introduced by Myelin Basic Protein Uptake

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The determination of hydrodynamic radii of reverse micelles composed of sodium bis(2-ethylhexyl) sulfosuccinate (AOT)/isooctane/water has been carried out using a nonperturbative technique: fluorescence recovery after fringe pattern photobleaching. The technique allows the precise measurement of hydrodynamic radii of protein-containing as well as protein-free micelles. This is achieved by the covalent labeling of a protein (myelin basic protein) and of a small dipeptide (*N*-acetylglycyl-L-lysine methyl ester) with a fluorescent dye (fluorescein isothiocyanate). The measurements were performed at low and high water content of the system as defined by the water-to-surfactant molar ratio $w_0 = [H_2O]/[AOT]$. At $w_0 = 5.6$ and 50 mM AOT, the hydrodynamic radii were $R_h = 29$ Å and $R_h = 44.5$ Å for protein-free and protein-containing micelles, respectively. The radii do not seem measurably affected by the surfactant concentration or the amount of protein present. In contrast, at $w_0 = 22.4$, the effect of AOT concentration was prominent. Measurements carried out at various surfactant concentrations, extrapolated to [AOT] = 0, gave a single R_h value of 51 Å for both protein-filled and protein-free micelles. All the results compared with previous measurements carried out by quasi-elastic light scattering were found to be in excellent agreement, provided the concentration effect of the surfactant was taken into account at high w_0 values. Moreover, under the latter experimental conditions, intermicellar interactions are significantly perturbed by the presence of the protein in the aqueous core of the micelles. The possible biological relevance of such attractive interactions involving myelin basic protein and the lipid monolayers of the myelin sheath is suggested.

Introduction

Mixtures of surfactant, water, and hydrocarbons in wide areas of phase diagrams can form fluid, transparent, isotropic solutions called microemulsions or reverse micelles. These consist of a dispersion of water droplets stabilized by an interfacial monolayer of surfactant in organic solvents.[1] The finding that the aqueous core of reverse micelles can solubilize various macromolecules[2-5] has opened new avenues of investigation into various aspects of membrane mimetic chemistry[6] and also protein–enzyme biotechnology.[7] However, the fundamental dynamics of this system, which involve processes of rapid collision and exchange, remain relatively unexplored. Furthermore, the mechanism of incorporation of biomolecules is hardly understood, as is the extent to which the solubilized proteins can perturb the structural properties and dynamics of these organized assemblies, although it is clear they depend on the water content of the microemulsion.[8] Elucidation of these questions requires a detailed characterization of both protein-free and protein-containing droplets. In this regard, a prerequisite is the precise determination of the hydrodynamic radius (R_h).

Previous experiments carried out by nonperturbative physical methods such as quasi-elastic light scattering (QELS) have been able to document changes in R_h upon protein incorporation, and a model has been proposed to explain these observations.[9] The latter measurements however suffer from a shortcoming since the resulting R_h values are an average of protein-containing and protein-free radii. We have overcome this problem in the present work by the use of fluorescence recovery after fringe pattern photobleaching (FRAPP) to determine the R_h of sodium bis(2-ethylhexyl) sulfosuccinate/water/isooctane reverse micelles containing myelin basic protein (MBP), a major protein from bovine brain[10] labeled with fluorescein isothiocyanate (FITC). Because the fluorescent probe is covalently bound to MBP and not to the surfactant, it is expected to provide minimal perturbation to the structure and the equilibrium properties of the system, as will be established. We have also shown that FRAPP experiments allow the measurements of protein-free micelles, provided the fluorescent probe could be inserted in the water core of the micelles without any measurable modification of their R_h. This was accomplished by the binding of FITC to the dipeptide *N*-acetylglycyl-L-lysine methyl ester.

One of the most interesting observations reported here concerns the alteration of the interaction potential of micelles after protein incorporation. This effect is more prominent at high water-to-surfactant molar ratios ($[H_2O]/[AOT] = w_0 = 22.4$) than at low w_0 values (5.6) where the main effect observed is an increase of the micellar size due to optimum protein incorporation.

Material and Methods

Chemicals. Sodium bis(2-ethylhexyl) sulfosuccinate (AOT), a gift from Cyanamid Co. (France), was purified according to Wong[11] and carefully dried in vacuo. The purity of the surfactant

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was checked according to the recommendations of Luisi et al.[12] FITC isomer I was from Sigma. Isooctane (Uvasol grade) was purchased from Merck (Darmstadt, FRG). *N*-Acetylglycyl-L-lysine methyl ester acetate was from Serva (Heidelberg, FRG). All materials used were of the best grades commercially available. Myelin basic protein was extracted from bovine brain, purified by the method of Deibler et al.,[13] and lyophilized.

Since the last step before lyophilization consists of a dialysis against 0.1 M acetic acid, the lyophilized protein was extensively dialyzed against pure (MilliQ) water and lyophilized again. This prevents the presence of any electrolyte in reverse micelles as well as low pH values, which dramatically decrease the intensity of FITC fluorescence.[14]

Labeling with FITC. This was carried out with MBP and the dipeptide under the following conditions: 1 μmol of FITC in 20 μL of acetone was added by increments of 5 μL to 0.55 μmol of MBP solubilized in 1 mL of 50 mM borate (pH 9.0). The mixture was vigorously stirred at 0 °C for 20 min and left overnight at 4 °C with slow mixing. After centrifugation, the solution was filtered through a Sephadex G-15 (Pharmacia) 10- × 2-cm diameter column, equilibrated with a 0.13 M (1%) ammonium bicarbonate buffer, and adjusted to pH 8.5 with 1 M NH_4OH. MBP–FTC fractions were separated from unbound dye and lyophilized. The reaction with *N*-acetylglycyl-L-lysine methyl ester acetate was carried out by a slightly different procedure: 40 μmol of dipeptide dissolved in 4 mL of 0.13 M ammonium bicarbonate (pH 8.5) was added to 5.5 μmol of FITC as a dry powder. The mixture was then vigorously stirred for 2 h at 0 °C and left overnight with slow mixing at 4 °C. The labeled dipeptide was separated from unbound dye on a Bio-Rad P-2 12.5- × 0.9-cm diameter column, equilibrated in bicarbonate buffer (pH 8.5), collected, and kept frozen.

Preparation of Microemulsions. The micellar solutions were prepared by addition of measured volumes of isooctane to dry preweighed amounts of AOT. The required volume of MilliQ (Millipore) purified water was delivered with Hamilton syringes, and the samples were shaken until optically clear. Dried, preweighed quantities of MBP were added and left at room temperature. Usually, solubilization was achieved in less than 1 h; when necessary, mixtures were sonicated for 2 min. For the dipeptide, measured volumes of FTC–dipeptide aqueous solution were directly injected into the AOT–isooctane solution with a Hamilton syringe in order to obtain the required w_0 values, and gently shaken.

Instruments

The absorption spectra were measured on a Cary Model 118 spectrophotometer. A millimolar extinction coefficient of 10.7 cm^{-1} was used for MBP at 278 nm. The optical density of micellar solutions of MBP–FTC was kept between 0.1 and 0.2 at 495 nm (ϵ = 72 100 M^{-1} cm^{-1} at pH >8.1) (ref 14). Fluorescence spectra were measured on a SLM 8000 spectrofluorometer coupled to a Hewlett-Packard 9815 A minicomputer. Steady-state anisotropy measurements were performed on the same instrument in the T format configuration, as already described.[5]

FRAPP Measurements. The experimental setup is shown in Figure 1. The principle of the experiment is as follows. The fluorescence of the dye molecules (FITC) bound to MBP can be destroyed by intense laser (Spectra Physics) illumination (λ_{exc} = 488 nm). In a first step, the sample is illuminated with a high-intensity fringe pattern produced by two crossed laser beams; this creates a nonuniform dye concentration distribution, since some of the dye molecules located in the bright fringes are irreversibly destroyed. Then, the relaxation of this concentration profile is monitored with the same fringe pattern, but of much weaker

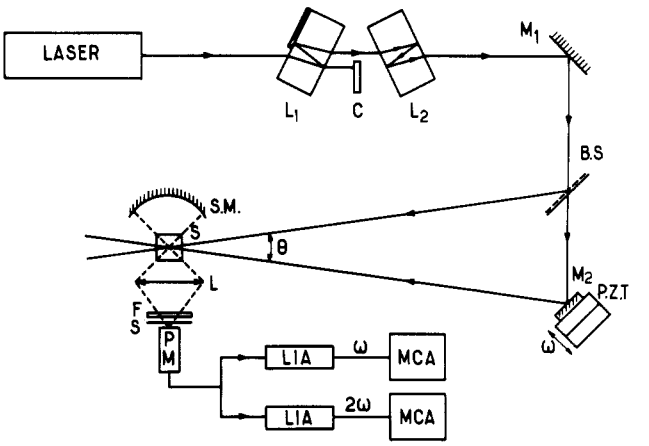

Figure 1. Scheme of the experimental setup for FRAPP measurements: The laser is an argon ion laser (Spectra Physics); L_1 and L_2, glass plates producing two beams of unequal intensities; C, chopper; M_1 and M_2, mirrors; PZT, piezoelectric stack; SM, spherical mirror; L, high aperture lens; S, sample; F, filter centered at the fluorescence wavelength; S, shutter; LIA, lock-in amplifier; MCA, multichannel analyzer.

intensity in order to prevent a further bleaching of the dye. The system is monitored by simply measuring the fluorescence intensity as a function of time. The diffusion coefficient D is obtained from the characteristic time τ of the fluorescence recovery curve

$$D = i^2/4\pi^2\tau$$

where i is the fringe spacing. The apparent hydrodynamic radius is calculated from

$$R_h^a = kT/6\pi\eta D$$

where k is the Boltzmann constant, T the absolute temperature, and η the isooctane viscosity. In order to increase the signal-to-noise ratio, we have used a phase-modulated interferometer, which produces a modulated fluorescence signal detected by a lock-in amplifier. For each sample, three experiments were performed at different values of the interfringe spacing (in the range of 10–100 μm) in order to check the i^2 dependence of the fluorescence recovery characteristic time. Each recovery curve was then fitted with a single exponential.

QELS measurements were done as described by Chatenay et al.[9] Both experimental techniques were carried out at 20 °C.

Results

Upon incorporation in reverse micelles, FITC covalently bound to an amino group has absorption and fluorescence spectra very similar to those obtained in aqueous solutions and already published by different investigators.[14,15] Indeed, for MBP–FTC both absorption and fluorescence excitation spectra yielded, as expected at pH >8, a maximum at λ = 495 nm and a shoulder at 460 nm. The binding of the fluorescent dye to the protein or to the dipeptide was further evidenced by steady-state anisotropy measurements (λ_{exc} = 488 nm). In aqueous solutions, FITC displayed a value of 0.02 (in arbitrary units), in reverse micelles at low w_0 values, the anisotropy increased to 0.127, while after binding to MBP the value rose again to 0.262, indicating a sharp decrease in the mobility of the fluorescent dye.

Protein-Free Droplets. The measurements were carried out at two w_0 values, 5.6 and 22.4, i.e., at low and high water content of the micellar system, using a FITC labeled dipeptide (see methods section). At w_0 = 5.6 and at 50 mM AOT, the R_h of protein-free droplets was measured by FRAPP. The value obtained, R_h = 29 ± 1 Å, is identical with that of protein or peptide-free droplets measured by QELS and reported previously.[9] At high water content (w_0 = 22.4), the radius R_h = 60 ± 1 a, measured in 50 mM AOT, differs from that measured by QELS experiments, R_h = 50 ± 1 Å. Since it appears very unlikely that

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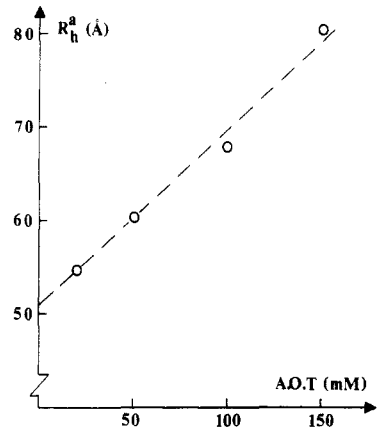

Figure 2. Variation of the apparent hydrodynamic radius (R_h^a) of protein-free reverse micelles, measured by FRAPP at $w_0 = 22.4$ and using a fluorescent dipeptide (see methods section).

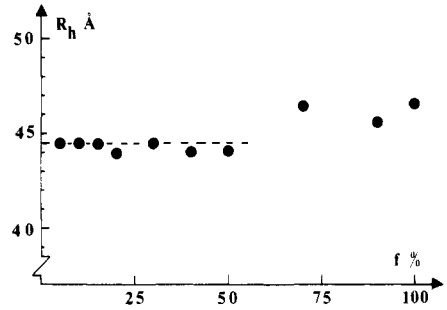

Figure 3. Hydrodynamic radius (R_h) of reverse micelles measured by FRAPP at $w_0 = 5.6$ and 50 mM AOT, as a function of protein occupancy factor f. (f is defined as the percent of protein molecules per number of droplets initially present in the system.)

the incorporation of a small dipeptide into the aqueous core of a rather large reverse micelle might increase the R_h to such an extent, the possibility of concentration effects due to micellar interactions was investigated. We have therefore carried out a series of FRAPP experiments at several AOT concentrations ranging from 25 to 100 mM. As shown in Figure 2, the value of R_h decreases with decreasing surfactant concentrations and the curve extrapolates to a value of 50 ± 1 Å for zero AOT concentration. The extrapolated value is therefore consistent with the values obtained by QELS.[9]

Protein-Containing Droplets. The R_h of MBP–FTC-containing droplets was measured under similar experimental conditions. As shown in Figure 3, at $w_0 = 5.6$ and at 50 mM AOT, the R_h of droplets remains constant over a wide range of protein occupancy. The protein occupancy factor f is defined here as the percentage of protein molecules, determined from absorption at 280 nm, per number of droplets initially present in the system and calculated from the surfactant concentration and the aggregation number (i.e., the number of surfactant molecules per micelle). The value of R_h determined here is 44.5 ± 1 Å, again in excellent agreement with the plateau value obtained as a function of f (for $f > 3.3$) in earlier QELS experiments, where $R_h = 43 \pm 1$ Å.[9]

Since at high water content FRAPP experiments with protein-free droplets have shown a marked dependence of R_h on AOT concentration, the R_h of MBP–FTC-containing micelles was measured at several surfactant concentrations. As illustrated in Figure 4, the experimental curves extrapolate to $R_h = 51 \pm 1$ Å, for zero AOT concentration. Furthermore, the extrapolation is identical for all the values of protein occupancy measured.

At first, this result seems to be inconsistent with the increase of R_h values as a function of protein micellar occupancy observed by earlier QELS experiments, at a single AOT concentration (50 mM) and yielding to a value of 57 Å.[9] Therefore, we have again performed a series of QELS experiments for $f = 55\%$, at several AOT concentrations. Figure 5 illustrates the result of such experiments. Here again, when the curve is extrapolated for zero

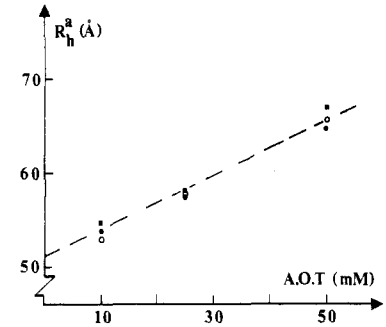

Figure 4. Apparent hydrodynamic radius (R_h^a) of reverse micelles, measured by FRAPP as a function of AOT concentration, at $w_0 = 22.4$, for values of $f = 80\%$ (■), 55% (●), and 30% (○), respectively.

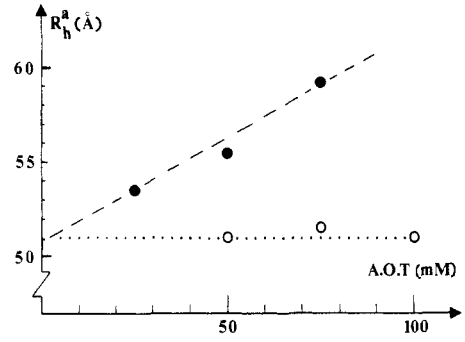

Figure 5. Variation of the apparent hydrodynamic radius (R_h^a) of protein-containing and protein-free reverse micelles with AOT concentration, measured by QELS at $w_0 = 22.4$. The protein occupancy factor is $f = 55\%$ (●) and $f = 0$ (○). Experiments were carried out as previously described.[9]

AOT concentration, the same value of $R_h = 51 \pm 1$ Å is obtained. However, for $f = 0$ the value of $R_h = 51 \pm 1$ Å is independent of AOT concentration. It can be concluded that, at $w_0 = 22.4$, the R_h values of protein-free and protein-containing droplets are identical for zero AOT concentration and for all the values of f measured. These results underline the fact that, at high water content, care must be exercised in the estimation of R_h at finite surfactant concentrations, since intermicellar interactions can significantly affect the diffusion measurements.

Discussion

In an earlier publication[9] we have discussed in detail a model to account for the structural modifications observed in micellar systems by QELS after MBP incorporation. The model, derived from the water–shell model described by Bonner et al.,[16] is purely geometrical, each protein molecule being enclosed in a large micelle built up, when required, from several initially empty droplets. One of the goals of this investigation was to refine previous measurements carried out by another nonperturbative technique.[9] A fundamental difference between the two techniques lies in the fact that while QELS measurements lead to an average R_h value of protein-filled and protein-free micelles, FRAPP allows the size determination of exclusively the protein-filled micelles. Incidentally, we have also demonstrated that the presence of a fluorescent dipeptide does not measurably affect the size of the aqueous core of reverse micellar droplets. The method which thus provides a direct estimation of their radius can be safely used for further studies of "empty" (protein-free) micelles, at any amount of water present. In this respect, it is interesting to note that the free fluorescent label entrapped in the micelles does not bleach.

Several significant points deserve comment. At low water content, the R_h values obtained by both techniques are in excellent agreement and confirm the validity of the above-mentioned model. At $w_0 = 5.6$, experiments have been carried out at a finite AOT concentration (50 mM), since no concentration effect has been reported whatsoever by investigators using various physical methods: ultracentrifugation, fluorescence, photon correlation spectroscopy, nuclear magnetic resonance.[16-19] All these results

are in excellent agreement with the data presented here.

At high water content, the geometrical model does not hold; we have demonstrated that, at $w_0 = 22.4$, the radii of protein-containing and protein-free micelles are identical (51 ± 1 Å). In fact, such a result should be expected: a rough calculation taking into account the increase of the volume of the aqueous core after addition of the volume of the solubilized protein leads to a final increase in R_h of about 3 Å. On the other hand, QELS measurements have shown, at a finite concentration of AOT (50 mM), an apparent increase of the R_h with protein occupancy from 50 to 57 Å.[9] We conclude that this increase reflects, as clearly shown in Figure 5, a modification of the intermicellar interactions, due to the incorporation of a protein of 18.500 daltons into the micelles.

It should be emphasized that such a modification does not significantly affect self-diffusion experimental results; indeed, self-diffusion coefficients are mostly insensitive to direct interactions between micelles, as recently demonstrated.[20] Although this modification might depend on the occupancy factor (f), it is not evidenced by self-diffusion experiments.

Finally, two distinctive types of perturbation are observed by FRAPP experiments after MBP solubilization in the micellar system, depending on the initial size of protein-free droplets. At low w_0 values (5.6), the radius of the micelles increases from 29 to 43 Å, indicating a consistent change in the structural properties of the system at the molecular level. Conversely, at high w_0 values (22.4), the radius of the droplets does not measurably vary after protein incorporation ($R_h = 51 \pm 1$ Å). However, a more subtle alteration affecting the dynamic properties of the system appears at $w_0 = 22.4$; i.e., the variation of the apparent R_h as a function of AOT concentration and the change of the slope of the curve after protein solubilization (Figures 4 and 5). This type of effect originates from intermicellar interactions which are obviously modified by the presence of MBP in the water core. Incidentally, these modifications probably also take place at $w_0 = 5.6$, but they are masked by the much more important effect of structural changes at the molecular level.

In this respect, we can suggest two possible explanations: (i) by interacting with the polar heads, the protein may also modify the overlapping of the aliphatic tails of the surfactant monolayers from different host micelles; (ii) alternatively, the protein may alter the magnitude of the time constant during which the rapid process of collision, exchange, and formation of the "transcient dimer"[21] by two micelles occurs. One can therefore question the role of such attractive interactions[22] in maintaining the compaction of the intact myelin sheath, since it has been shown that reverse micelles of AOT/isooctane/water constitute an excellent biomimetic system for myelin proteins.[4,5]

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Nonisothermal Calorimetric Studies of Pyridine Intercalation in $CdPS_3$

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Measurements of intercalation of pyridine into $CdPS_3$ from the vapor-saturated liquid and of deintercalation of pyridine under vacuum have been performed in a heat-flux scanning calorimeter with a heating rate of 1.4 mK s^{-1}. The absolute values of the enthalpies of intercalation and of deintercalation are similiar with a mean value of 76.5 ± 0.7 kJ mol^{-1} pyridine. The kinetic mechanism is a complicated one in which diffusion or nucleation–growth processes play an important role in the control of the overall rate of the reaction. A table of formulas for kinetic analysis derived in terms of the parameters measured in a dynamic calorimetric batch experiment is given for different reaction mechanisms.

1. Introduction

Metal phosphorus sulfides with the formula MPS_3 ($M = M^{2+}$) have been the subject of numerous investigations since the first reports of their synthesis.[1-3] These compounds are structurally related to the dichalcogenides (MX_2), having sandwich-type repeat units consisting of metal ions and P_2 subunits situated in octahedral holes between pairs of sulfide layers.[4,5] Adjacent sulfide layers are held together only by weak van der Waals interaction. Properties of the pure materials such as their magnetic[6-8] and electronic structures[9,10] have been of interest because of the two-dimensional nature of the MPS_3 lattice. The differing abilities of the pure compounds to intercalate a variety of inorganic, organic, and organometallic species into the van der Waals gap[7,11-16] and the effect of intercalation on host properties have also been characterized.[7,8,17-19]

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