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Journal

Journal of Molecular Liquids, 45(3-4)

ISSN

0001-8716

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Publication Date

1990-04-01

DOI

10.1016/0167-7322(90)80034-h

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HYDRATION AND PROTEIN SUBSTATES: FLUORESCENCE OF PROTEINS IN REVERSE MICELLES

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(Received 11 October 1989)

SUMMARY

The fluorescence properties of indole derivatives, lysozyme and azurin were investigated in reverse micelles of detergent sodium bis[2-ethylhexyl]sulfosuccinate (Aerosol OT)* in n-hexane. L-tryptophan, l-methyl-tryptophan and n-acetyl-l-tryptophanamide exhibited complex fluorescence decays in reverse micelles. Fluorescence decays were best described using Gaussian bimodal distributions of lifetimes. Increasing hydration levels in the micelles resulted in a decrease in decay heterogeneity, as indicated by a large decrease in lifetime distribution widths. Steady-state polarization and fluorescence emission measurements indicated both an increase in average polarity of the environment around the indole derivatives and an increase in the mobility of the probes with increasing hydration levels. The fluorescence decays of lysozyme and azurin in reverse micelles were also found to be very complex and were described with Gaussian lifetime distributions. Increasing water content in the micelles caused marked decreases in both center and width of the lifetime distributions for these two proteins. Steady-state polarization measurements as a function of the extent of hydration revealed an increase in the average rotational rates of the tryptophan residues in lysozyme upon increasing water content. Thus, static polarization and lifetime measurements indicate that the amount of water present in the micelle may influence the amount of structural flexibility of the polypeptide chains and the rates of interconversion between conformational substates.

*Abbreviations used: Aerosol OT, AOT, sodium bis[2-ethylhexyl]sulfosuccinate; NATA, N-acetyl-l-tryptophanamide; w_0 , $[H_2O]/[AOT]$.

INTRODUCTION

The first comprehensive review on protein motions was by Careri, Fasella and Gratton (ref. 1). The focus was on the characterization of the time scale of different protein time events. The underlying idea of that work was that hydration of the protein matrix is required for most protein motions and that the network of hydration would serve as a common medium for the connection between solvent and protein dynamics. Since that original work, the amount of experimental evidence for protein motions has enormously increased. Protein dynamics is now a well accepted reality of protein systems. Theoretical methods based on molecular dynamics have been used to describe, although for few hundred picoseconds, the motion of each atom of a protein (ref. 2). The result was a complex dynamics with fast, large amplitude motions. Also the effect of water has been included in the calculations using several different approaches (ref. 3). The major results are that (1) motions occur over a very wide time scale (ref. 4); (2) a given kind of motion can also occur over a very wide time scale, i.e., kinetics are non-exponentials; (3) protein structure and dynamics are hierarchically organized (refs. 5,6); (4) proteins of a given type may have the same average structure but each individual molecule may be different in its fine structure (ref. 7); and (5) small differences in the structure can result in large differences in function. Despite the amount of research and the advances on protein dynamics, there are still some basic aspects that need to be understood. Among those aspects is the role of hydration. Essentially, the problem can be explained using the following highly simplified description (ref. 8). (1) At room temperature, in the absence of the hydration shell, the protein is essentially frozen. There is very little motion occurring in the time range of 1 ns and longer. (2) As water is gradually added to the protein, nothing major occurs with respect to the protein dynamics until about 0.2 mg of water per mg of protein. At this value there is a sudden transition and all dynamics reach the so-called "solution value" at about 0.25 mg water per mg protein. The effect of water is minimal on the protein structure, but is paramount on the dynamics (ref. 9). The effect of hydration on protein dynamics has been addressed very little in published literature and its physical origin is virtually unknown. This investigation presents a study of protein hydration using reverse micelles, which can contain a single protein molecule in a microscopic pool. The total amount of water in the pool can be easily controlled. In the following, we present the relevant information on the use of reverse micelles for hydration studies.

Dissolution of amphiphiles in organic solvents may give rise to formation of reverse micelles (for reviews, see refs. 10, 11). These inverted micellar solutions are capable of taking up relatively large amounts of water, forming

"water pools" in the core of the micelles (Fig. 1). The thermodynamics of micelle formation (refs. 12, 13) as well as the characterization of phase-diagrams and

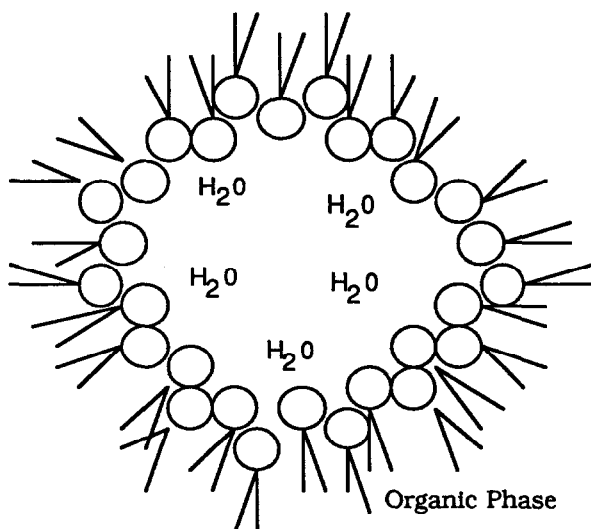


Fig. 1. Schematic representation of a reverse micelle.

phase-transition behavior (refs. 14-17) of several organic solvent-surfactant-water systems have been extensively investigated. In some cases, it was found that addition of co-surfactants resulted in increased thermodynamic stability of the water-in-oil emulsions. Depending on temperature and on the characteristics of the organic solvent and surfactant employed (here including the presence of impurities, salts for

instance), it is possible to solubilize up to about 50-70 moles of water per mol of surfactant in the inner core of the micelles, which can then be viewed as large (ca. 100Å) droplets of water in the organic phase (ref. 11). One of the best studied reverse micellar systems is that of Aerosol OT (AOT or sodium bis[2-ethyl-hexyl]sulfosuccinate) in hydrocarbon solvents (for review, see ref. 18). An important advantage of the AOT system is that no co-surfactants are needed (ref. 11), thereby reducing considerably the complexity of the equilibria involved in the characterization of this system. Several techniques have been employed in the investigation of the properties of water in the water pool of AOT-hydrocarbon solvent systems. NMR measurements of water proton chemical shifts and coupling constants (ref. 19) as well as ^{13}C chemical shifts of carbons 1 and 3 (ref. 20) and proton chemical shifts of AOT (ref. 21) have indicated the existence of bound water up to a w_0 (ratio of the molar concentration of water to AOT, i.e., $w_0 = [\text{H}_2\text{O}]/[\text{AOT}]$) of about ten. Thus, this is possibly the water that is necessary to hydrate the surfactant molecules on the micellar wall. This was further confirmed by measurements of water activity (ref. 22) and apparent heat capacity (ref. 23) as a function of w_0 . Above $w_0 = 10$ the above cited physico-chemical methods yield results which gradually approach those of pure bulk water. These considerations have led to a general picture in which a host molecule solubilized

in the reverse micelles may experience three types of environments (ref. 11): a) interfacial, in contact with the AOT side-chains in the interface between the organic solvent and water; b) in the bound-water layer; and c) in the water pool. For hydrophilic molecules solubilized in reverse micelles the partition of the molecule in the interfacial region presented by the hydrophobic AOT side chains is probably negligible. Thus, for hydrophilic molecules, the reverse micellar systems essentially reduce to a two-state system: that of the bound water layer and that of the water pool (bulk-like water).

Over the past few years, a large number of small molecules and biopolymers have been solubilized in reverse micelles of AOT in hydrocarbon solvents. The study of proteins in reverse micelles has received a great deal of attention (ref. 11) since it offers a relatively simple way to probe the effects of hydration on protein structure. For enzymes in reverse micelles, it has been suggested that the physico-chemical properties of bound water in the water pool may modulate enzymatic activity (refs. 10,24). In fact, many studies have indicated that some enzymes may exhibit "hyperactivity" at low water content in reverse micelles as compared to their activities in solution (refs. 10, 24).

The purpose of this work was to investigate the structural dynamics of proteins in reverse micelles as a function of water content. Structural fluctuations in proteins typically occur in a time scale from 10^{-11} to 10^{-7} seconds, which can be suitably probed through the use of fluorescence spectroscopy. Protein conformational dynamics has been studied by time-resolved fluorescence measurements. The intrinsic fluorescence decay of proteins is usually complex (for a review see ref. 25) and, in general, sums of exponential components have been used to describe the decay. Alternatively, Alcalá et al. (refs. 26-28) have used continuous distributions of lifetime values to account for the complexity of the fluorescence decay in proteins. Due to the sensitivity of the indole excited state to the physico-chemical properties of the medium, it would be expected that different micro-environments would result in distributions of decay rates. The distribution can be affected by fast interconversion between microenvironments. Therefore, the observed distributions of lifetimes may reflect the dynamics of the protein matrix. In fact, even single-tryptophan proteins have been found to present broad lifetime distributions in solution (ref. 28), probably reflecting multiplicity of conformational substates. Since we anticipate that hydration will affect protein motions, the lifetime distributions should be sensitive to the hydration of the protein.

We present steady-state and lifetime measurements of the single tryptophan protein azurin and of the six-tryptophan protein lysozyme in reverse micelles of AOT in hexane. Our results show that at low water content, the fluorescence decays of these two proteins are very complex and broad

distributions of lifetimes are needed to describe the decay. As the water content is increased the center of the distributions moves to shorter lifetimes toward the value observed for the proteins in solution. The width of the distribution is also sensitive to hydration, and decreases as w_0 is increased. However, even at the highest hydration values attainable in reverse micelles ($w_0 = 70$) the recovered parameters for the lifetime distributions are still far from the solution values. Comparison of steady-state polarization and lifetime data for lysozyme in reverse micelles indicates increased average mobility of the tryptophan residues with increasing hydration levels.

In addition, to gain understanding of the behavior of indole fluorescence as a function of hydration in reverse micelles, we performed measurements on tryptophan, 1-methyl-tryptophan and N-acetyl-L-tryptophanamide (NATA) in reverse micelles. Previous reports on the fluorescence properties of perylene derivatives (ref. 29) and rose bengal (ref. 30) concluded that a simple solubilization process took place as w_0 was increased, with the fluorescent probe moving gradually from the bound-water layer into the "bulk" water pool. We show here that our results with indole derivatives cannot be explained on the basis of this two-state solubilization model. These results suggest that the organization of water in the core of the micelle may modulate the fluorescence decay of indole derivatives.

MATERIALS AND METHODS

Steady-state fluorescence measurements

Excitation and emission spectra were recorded on an ISS, Inc. (Champaign, IL) GREG PC photon counting spectrofluorometer (4 nm slits in both excitation and emission). Spectral center of mass was calculated as

$$\lambda_{av} = \frac{\int \lambda I(\lambda) d\lambda}{\int I(\lambda) d\lambda},$$

and the integrated emission intensity was calculated as

$$I_{total} = \int I(\lambda) d\lambda$$

where λ and $I(\lambda)$ are the emission wavelength and the intensity at a given wavelength, respectively. Static anisotropy measurements were performed in the same instrument using the L format and were corrected by subtracting the background signal from blanks which did not contain the fluorophore.

Fluorescence lifetime measurements

The fluorescence decays of indole derivatives (tryptophan, 1-methyl-tryptophan, and N-acetyl-L-tryptophanamide), as well as of azurin and lysozyme, were measured in a phase fluorometer using the harmonic content of a Spectra-Physics series 3000 Nd-YAG laser or, in some cases, a Coherent Antares model laser. The light from the mode-locked laser was used to pump a Rhodamine 6G dye laser which was cavity-dumped and externally frequency-doubled, providing 5 to 15 mW light at the excitation wavelength of 295 nm. Fluorescence emission was observed through a WG-335 filter. Color errors due to photomultiplier response were minimized by use of a reference solution of p-terphenyl in cyclohexane (lifetime 1.0 ns) in the measurements. Phase and modulation data were acquired at 8 to 10 frequencies in the 2 to 200 MHz range, with uncertainties of $\pm 0.2^\circ$ and ± 0.004 for phase angles and modulation ratios, respectively. Data were analyzed with a sum of exponential decays or, alternatively, with continuous lifetime distributions. In both cases, the analysis software provided by ISS, Inc. included the above errors in the calculation of χ^2 , which was used to judge the goodness of the fits. The relevant equations concerning the use of lifetime distributions and a discussion of the resolvability of the distributions with respect to exponentials were presented by Alcalá et al. (ref. 26).

Hydration studies

Reverse micelles of Aerosol OT were prepared by dissolution of the detergent in n-hexane to a final concentration of 0.11 M. Concentrated stock solutions of the indole derivatives or proteins were prepared in deionized water at pH 7–8. A small aliquot (2 to 8 μ l) of sample was injected into the fluorescence cuvette containing 3 ml of reverse micellar solution. Water was added to give the desired concentration and the cuvette was gently hand-shaken for 30–60 sec until complete clarification. Hydration levels are expressed as overall concentration of water in the cuvette or as w_0 , the ratio between molar concentrations of water and AOT. Temperature was kept at 30°C using a thermostated sample holder.

Chemicals

The n-hexane used was of spectroscopic grade from Merck (Darmstadt, Germany). Aerosol OT was purchased from Aldrich Chemical Co. (Milwaukee, WI) or Sigma Chemical Co. (St. Louis, MO) and was kindly purified by Dr. M. Polliti at the Universidade de São Paulo (Brasil) to remove acidic or fluorescent impurities. Control measurements showed that background fluorescence and light scatter from the AOT micellar solutions accounted for less than 5% of the fluorescence intensity of the samples under our experimental conditions. Tryptophan, NATA and 1-methyl-tryptophanamide were purchased from Aldrich Chemical Co. (Milwaukee, WI). Azurin was a kind gift of

Dr. A. Finazzi-Agrò (University of Rome). Salt-free lysozyme was kindly provided by Dr. J. Rupley (University of Arizona); alternatively, lysozyme from Sigma (3 × crystallized, dialyzed and lyophilized) was used, yielding similar experimental results. Deionized water (Nanopure II system; Sybron Co., Boston, MA) was used throughout the experiments.

RESULTS

Steady-state measurements on indole derivatives

Figure 2a shows fluorescence emission spectral moments (integrated intensity, closed circles; and spectral center of mass, open circles) for NATA as a function of hydration in

AOT reverse micelles. At low hydration ($w_0 = 0.34$) the emission spectrum was very blue-shifted, with the spectral center of mass at 344.5 nm. As hydration was increased, the center of mass of the emission was progressively shifted to the red, while the emission quantum yield decreased in parallel (Fig. 2a), indicating increased solvent exposure of the fluorescent probes. It should be noted that at the high water regime the center of mass

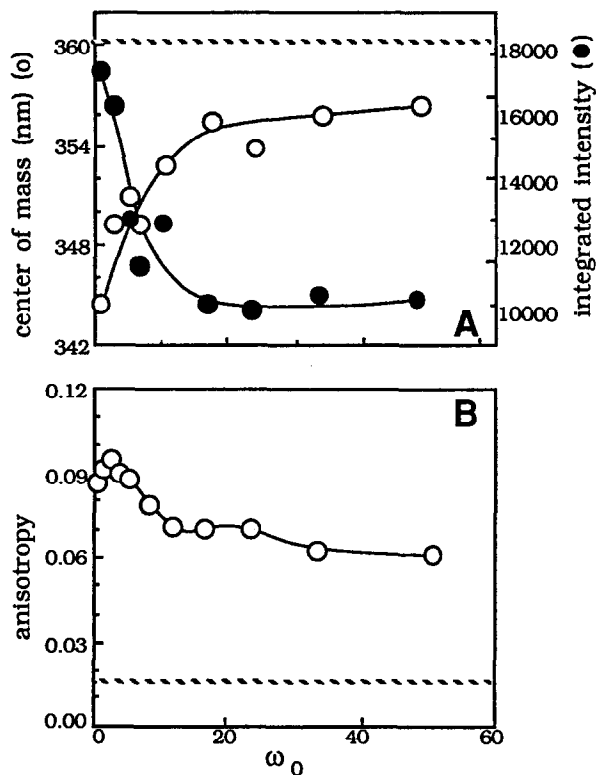


Fig. 2. (A) Emission spectral moments of NATA in AOT reverse micelles as a function of w_0 ; (B) Steady state anisotropy of NATA in reverse micelles as a function of w_0 . The dashed line indicates the solution values.

of the emission spectrum of NATA approached the solution value (dashed line), suggesting that the average polarity in the water pool at high w_0 was similar to

that of aqueous solution. Similar results were found for l-tryptophan in reverse micelles (not shown).

Steady-state anisotropy measurements of NATA as a function of w_0 are shown in Figure 2b. At low hydration, the anisotropy of the probe was high (about 0.09 in the w_0 range 0.34 to 5.0). This high anisotropy indicated that the rotational motion of the probe was severely restricted in this situation, possibly suggesting that the probe was attached to the micelle wall. As w_0 was increased above 5.0, the anisotropy decreased to a value of about 0.06 at the highest water content investigated. This could mean that a solubilization process occurred, with the probe going from the micelle wall to the inner water pool. However, it should be noted that even at a w_0 of 60 (close to the maximum amount of water that can be taken up by the micelles) the anisotropy value was still far above the value of 0.015 found for NATA in solution (Fig. 2b, dashed-line).

Fluorescence lifetime measurements on indole derivatives in reverse micelles

The fluorescence decays of tryptophan, 1-methyl-tryptophan and NATA were measured as a function of hydration level. For the three compounds, attempts to describe the decay with a single exponential lifetime yielded high χ^2 values (not shown), indicating that the decay was heterogeneous in all the hydration range investigated. Two-exponential fits of the data provided better descriptions of the decay, but use of bimodal Gaussian distributions of lifetimes resulted in marked decreases in the χ^2 values (Tables I, II, and III). Analysis of the F statistics (ref. 31) associated with the decreases in χ^2 on-going from the biexponential to the bimodal distribution models, indicated that the improvements in fits were statistically significant at confidence levels above 99% for most of the data sets. Figure 3

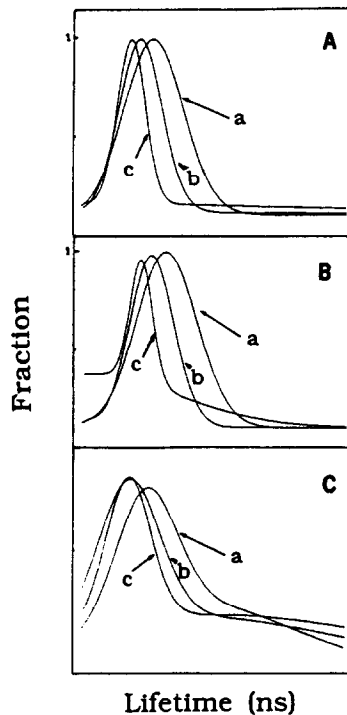


Fig. 3. Bimodal Gaussian lifetime distributions for (A) NATA; (B) 1-methyl-tryptophan, (C) l-tryptophan. a, b, and c indicate three different increasing hydration levels. Data are shown in Tables I, II, and III.

shows the recovered bimodal Gaussian lifetime distributions for the three indole derivatives investigated. Figure 3 shows that for the three compounds the fluorescence decay could be almost entirely described by a major lifetime distribution component centered at 1.7–2.0 ns, depending on hydration level. In the case of NATA (Fig. 3a), this major component accounted for 86–100% of the decay under all hydration conditions tested, whereas the relative contribution of the minor distribution component appeared to be higher in the decays of 1-methyl-tryptophan and L-tryptophan (Figs. 3b and 3c, respectively). For the three compounds, however, increasing hydration levels appeared to have similar qualitative results. In all cases, the center and the width of the major distribution component decreased upon increasing hydration (Fig. 3), causing the decay to appear less heterogeneous at high hydration levels.

TABLE I.
Lifetime analysis of NATA in reverse micelles.

Global bi-exponential analysis				Bimodal Gaussian analysis					
w_0	f_1	f_2	χ^2	c_1	w_1	c_2	w_2	f_1	χ^2
$\frac{H_2O}{AOT}$	$(\tau_1=3.11 \text{ ns})$ $(\tau_2=1.31 \text{ ns})$								
0.7	0.69	0.31	5.97	2.65	1.06	--	--	1.00	0.62
1.3	0.60	0.40	3.66	2.39	0.93	--	--	1.00	0.87
2.3	0.51	0.49	2.72	2.14	0.78	0.01	99.85	0.99	0.40
3.3	0.43	0.57	2.67	1.98	0.69	0.01	93.26	0.99	0.60
5.0	0.36	0.64	2.11	1.88	0.60	0.01	44.52	0.99	0.24
8.3	0.32	0.68	1.56	1.75	0.52	0.01	9.36	0.96	0.46
11.7	0.31	0.69	0.89	1.66	0.38	0.01	4.63	0.88	0.10
16.7	0.30	0.70	0.71	1.63	0.34	0.01	4.30	0.86	0.36
28.3	0.30	0.70	0.53	1.64	0.37	0.01	4.86	0.88	0.08
41.7	0.31	0.69	0.38	1.66	0.38	0.01	4.63	0.87	0.16
58.3	0.32	0.68	0.36	1.70	0.42	0.01	4.50	0.88	0.69
75.0	0.34	0.66	1.14	1.75	0.51	0.01	8.96	0.94	0.20
83.3	0.39	0.61	3.16	1.76	0.43	0.01	12.70	0.92	1.51

TABLE II.
Lifetime analysis of 1-methyl-tryptophan in reverse micelles.

Global bi-exponential analysis				Bimodal Gaussian analysis					
w_0	f_1	f_2	χ^2	c_1	w_1	c_2	w_2	f_1	χ^2
$\frac{H_2O}{AOT}$	$(\tau_1=3.27 \text{ ns})$ $(\tau_2=1.33 \text{ ns})$								
0.7	0.80	0.20	2.72	3.10	1.09	0.13	0.25	0.99	1.63
1.3	0.73	0.27	0.53	2.81	1.07	0.19	0.30	0.99	0.39
2.5	0.62	0.38	0.61	2.54	0.81	0.29	0.28	0.97	0.76
5.0	0.51	0.49	1.43	2.23	0.84	0.22	0.35	0.99	0.60
10.0	0.46	0.54	1.96	2.25	0.56	0.43	0.84	0.90	0.58
16.7	0.46	0.54	1.58	2.25	0.64	0.42	0.78	0.91	0.45
26.7	0.47	0.53	1.45	2.16	0.42	0.30	2.72	0.73	0.15
36.7	0.48	0.52	0.97	2.14	0.46	0.20	3.05	0.74	0.24
50.0	0.47	0.53	1.09	2.12	0.42	0.30	3.11	0.70	2.23
66.7	0.47	0.53	1.50	2.10	0.38	0.24	3.29	0.67	0.14

TABLE III.
Lifetime analysis of L-tryptophan in reverse micelles.

Global bi-exponential analysis				Bimodal Gaussian analysis					
w_0	f_1	f_2	χ^2	c_1	w_1	c_2	w_2	f_1	χ^2
$\frac{H_2O}{AOT}$	$(\tau_1=3.41 \text{ ns})$ $(\tau_2=0.91 \text{ ns})$								
0.3	0.76	0.24	4.08	2.34	1.10	5.08	2.74	0.71	0.30
1.8	0.64	0.36	3.12	1.81	1.02	4.70	2.74	0.65	1.11
4.7	0.55	0.45	6.84	1.72	1.06	4.37	4.55	0.76	0.44
11.3	0.53	0.47	5.24	1.67	0.85	4.04	6.21	0.78	0.37
23.0	0.54	0.46	5.63	1.69	0.82	5.00	6.04	0.79	0.31

Increasing hydration could solely increase the solubility of the indole derivatives in the water pool inside the micelles. To test this simple solubilization model, we analyzed our lifetime data in terms of an internally consistent set of lifetimes and associated fractional intensities, using the Globals Unlimited software developed by Drs. Beechem, Gratton and Mantulin at the University of Illinois. Data were analyzed for each compound by linking the two exponential lifetimes (corresponding to bound and free-probe in the micelle) throughout hydration levels and allowing the fractional amplitudes to vary to provide the best fit of the data. Tables I, II, and III show the results obtained with the global analysis for NATA, l-methyl-tryptophan and L-tryptophan, respectively. For the three compounds, the decay presented a major component of 3.1–3.4 ns and a smaller component of 0.9–1.3 ns. In all cases, the fractional intensity of the major long-lived component appeared to decrease up to a w_0 of 10–15, and then displayed a tendency to increase again at higher hydration levels (which was more pronounced in the measurements with NATA, Table I). Thus, our results do not support a simple exchange of the fluorescent probes between two different environments (namely, bound and free probes) with increasing hydration levels. In order to more easily assess the effects of hydration on the fluorescence decay of NATA in reverse micelles, we analyzed our lifetime data with a unimodal Gaussian distribution of lifetimes.

The results thus obtained are shown in Figure 4. Increasing hydration produced interesting effects on the center of the lifetime distributions of NATA in reverse micelles (Fig. 4a). Up to $w_0 = 28$ (ca. 2 M overall water concentration) the center of the distribution was gradually quenched. Above this hydration level, the center of the lifetime distribution started to increase slightly again up to $w_0 = 83$. It is interesting to note that the solution lifetime found for NATA (Fig. 4a (open circle) at $[H_2O]=55.55 \text{ M}$) is similar to that observed in reverse micelles in the driest possible state ($w_0 = 0.7$). In fact, extrapolation of the observed increase in lifetime above $w_0 = 28$ (dashed line) suggests that the lifetime values at the

high water regime in reverse micelles were moving toward the pure aqueous solution value. This finding is incompatible with a simple two-state model for solubilization of NATA in the water pool with increasing w_0 . If a simple solubilization phenomenon occurred, one would expect the measured parameter (in this case the center of the lifetime distribution) to move monotonically toward the solution value. The observed bell-shaped curve suggests that at an intermediate w_0 value around 28 the structure of water inside the micelle may be such as to maximize some quenching mechanism that results in decreased lifetime. Above $w_0 = 28$, the structure of water might be looser, leading to diminished quenching efficiency and, thus, an increase in lifetime toward the solution value.

Figure 4b shows the width of the lifetime distribution of NATA as a function of overall water concentration in the cuvette. Also shown is the width obtained in the analysis of NATA lifetime in aqueous solution (dashed line), which is of 0.05 ns, indicating a single exponential decay. It should be noted that although the width of the lifetime distribution is substantially decreased upon increasing hydration, even at the highest w_0 attainable in reverse micelles ($w_0 = 83$) the decay is still described by a broad distribution as compared to the single exponential decay kinetics observed in water solution.

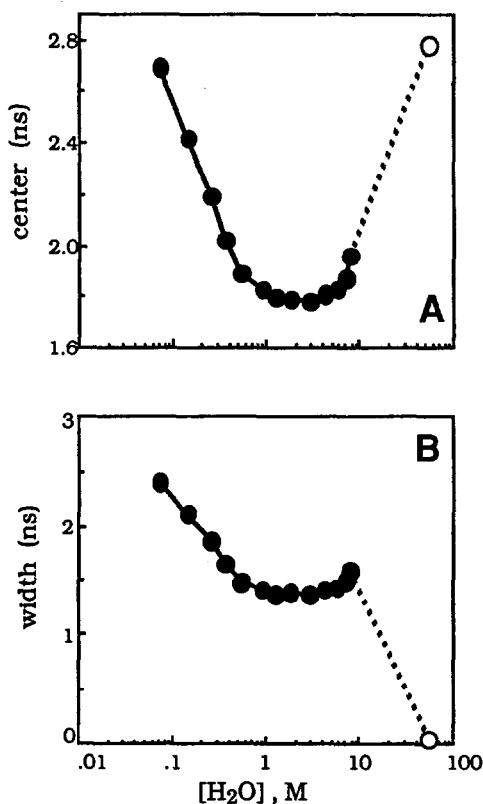


Fig. 4. (A) Center and (B) width of the lifetime distribution of NATA in AOT reverse micelles. Data were analyzed using a uni-model Gaussian distribution. Open symbols correspond to the solution value.

Fluorescence emission spectra of proteins in reverse micelles

Fluorescence emission spectra were acquired for azurin and lysozyme in reverse micelles as a function of w_0 . Figure 5 illustrates the result obtained for the center of gravity of the emission for lysozyme as a function of w_0 . Increasing w_0 from 1 to 60 produced a red shift in the spectral center of mass from 342 nm to 351 nm, thus approaching the solution value of 352 nm (dashed line). This effect is qualitatively similar to that obtained for NATA or tryptophan (Fig. 2) and probably reflects increased polarity of the tryptophan environment upon hydration of the protein.

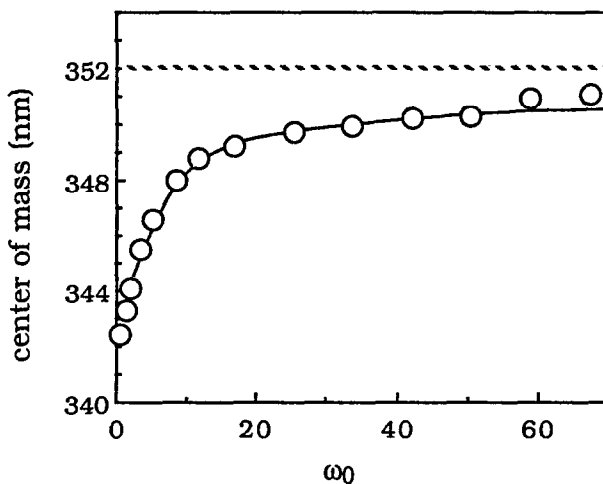


Fig. 5. Center of mass of the emission spectrum of Lysozyme in AOT reverse micelles as a function of hydration.

Lifetime measurements on proteins in reverse micelles

Lifetime measurements were performed on both azurin and lysozyme at various degrees of hydration. The purpose of using these two proteins was to compare the effects of hydration on a single tryptophan and on a multiple-tryptophan containing protein. For both proteins in reverse micelles, the decays were found to be complex and were analyzed with Gaussian unimodal lifetime distributions. The results obtained are shown in Figure 6.

Figure 6a shows that the average lifetime (as indicated by the center of the lifetime distribution) was progressively quenched with increasing overall water concentration in the cuvette. This feature was more marked for azurin (closed circles) but was still clear with lysozyme (open circles). The fluorescence lifetimes of both proteins approached the solution value with increasing water concentration. For azurin a monotonic profile of lifetime quenching was observed as a function of water concentration, whereas, for lysozyme the lifetime appeared to

decrease from $w_0 = 0.7$ (0.07 M overall water concentration) to $w_0 = 8.5$ and then stayed approximately constant up to $w_0 = 33$ (3.7 M overall water concentration). Above $w_0 = 33$, the lifetime again decreased up to $w_0 = 76$.

Figure 6b shows the width of the Gaussian distribution of lifetimes of azurin and lysozyme in reverse micelles. For azurin (closed circles), the width decreased markedly as a function of water concentration in the cuvette. It should be noted, however, that extremely broad distributions were found even at the high water regime in reverse micelles, suggesting large heterogeneity in the environments sensed by the single tryptophan in azurin. Interestingly, the width of the lifetime distribution in lysozyme (open circles) appeared to be less sensitive to hydration, which may indicate that, as an average, the environmental heterogeneity felt by the tryptophan residues in lysozyme is less affected by hydration than in the case of the single tryptophan in azurin.

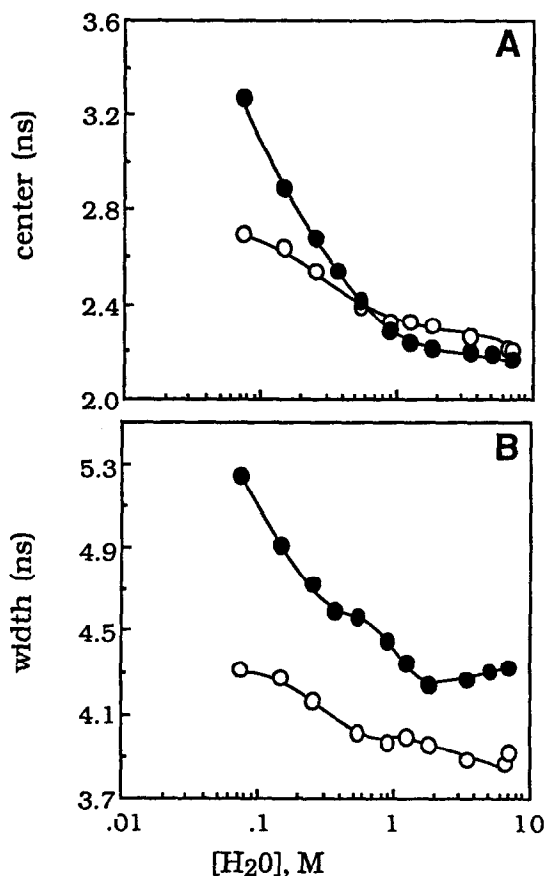


Fig. 6 (A) Center and (B) width of a Gaussian lifetime distribution for lysozyme (open symbols) and azurin (closed symbols).

In order to assess the influence of hydration on the dynamics of the protein matrix, steady-state anisotropy measurements were performed as a function of water content for lysozyme in reverse micelles. Figure 7a shows that the static anisotropy of lysozyme was mostly constant up to $w_0 = 25$ and then increased slightly up to $w_0 = 50$. Since the behavior of the average lifetime of lysozyme as a function of hydration was known (Fig. 6a), it was possible to calculate an average rotational correlation time for lysozyme in reverse micelles. The average rotational correlation time was calculated from the Perrin equation as

$$\phi = \frac{\tau}{\frac{r_0}{r} - 1},$$

where τ is the average lifetime, r_0 is the limiting anisotropy for indole at $\lambda_{\text{exc}} = 300 \text{ nm}$ ($r_0 = 0.3$ according to Valeur and Weber, ref. 32), and r is the measured anisotropy. Figure 7b shows that the average rotational correlation time thus calculated for lysozyme in reverse micelles decreased as a function of water content. This suggests that hydration resulted in increased mobility of the polypeptide chain. However, it should be noted that even at the highest w_0 value investigated the average rotational correlation time calculated for lysozyme was still much higher than the solution value (about 1ns) (ref. 33), suggesting that the mobility of lysozyme was still quite restricted as compared to the mobility in aqueous solution.

DISCUSSION

The fluorescence properties of model indole compounds in reverse micelles were found to be markedly dependent on the amount of water present. Steady-state measurements indicated that the polarity of the environment around the indole moiety increased with increasing hydration (Fig. 2a). On the other hand, polarization measurements suggested that at low hydration the indole derivatives were partially attached to the micelle wall, and increasing hydration resulted in increased probe mobility (Fig. 2b). This feature could, in principle, be

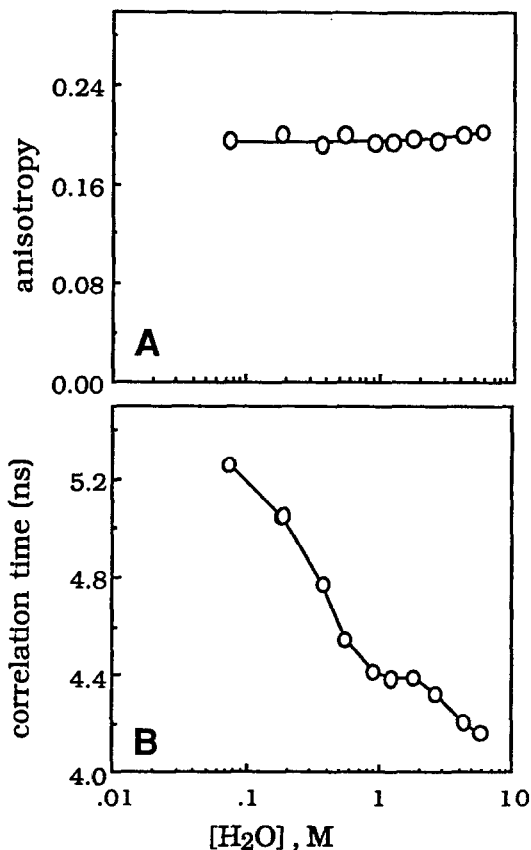


Fig. 7. (A) Steady-state anisotropy of lysozyme in AOT reverse micelles as a function of hydration. (B) Average rotational correlation time for lysozyme in AOT reverse micelles as a function of hydration. (Excitation wavelength was 300 nm).

attributed to a simple solubilization of the probe in the water pool, but this interpretation, as discussed below, is not compatible with our time-resolved measurements.

The fluorescence decay of indole derivatives in reverse micelles was complex. Global analysis of the datasets obtained under various hydration conditions in terms of a two-state model gave incompatible results with a single solubilization process (Tables I, II, and III). For the three probes tested, the fits yielded two lifetime components, a major component at 3.1–3.4 ns and a smaller component at 0.9–1.3 ns. The lifetimes of tryptophan and NATA in aqueous solution at pH 7 are close to 3 ns (ref. 34), and, hence, it would seem that our long lifetime component could be associated with probe in the water pool, whereas the short lifetime component could arise from probe which was attached to the micelle wall. Inspection of the fractional intensities of each component as a function of w_0 (Tables I, II, and III) shows that the fraction of the short component increased significantly up to $w_0 = 10$ –15, which would imply that the amount of solubilized probe decreased as hydration increased. Even if the long lifetime component were associated with bound probe and the short-lived component with the solubilized probe, it should be noted that the fraction of the short component increased up to $w_0 = 10$ –15 and then started decreasing again (this was especially clear for the measurements on NATA, Table III, which cover a larger w_0 range). This implies that above $w_0 = 15$ the solubility of the probe in the water pool would be decreased. Given the inadequacy of the exponential analysis to describe the system, we have analyzed the data with continuous lifetime distributions. For the three probes tested, the decay could be well described by bimodal Gaussian lifetime distributions (Tables I, II, and III). Figure 3 shows that increasing hydration resulted in an apparent decrease in heterogeneity of the fluorescence decay, as indicated by the decrease observed in the width of the major lifetime component with increasing hydration. The distribution analysis also did not support a simple solubilization mechanism: in Fig. 4, the decay of NATA as a function of water concentration was analyzed with a single Gaussian distribution. Although this analysis yielded worse fits than those obtained with bimodal Gaussian distributions, it enabled a direct evaluation of the effect of hydration on the average lifetime. Figure 4a shows that the average lifetime was quenched up to an overall water concentration of about 2 M. Above this value, the lifetime started increasing again up to an overall water concentration of about 9 M, the highest hydration value experimentally accessible in the micelles. Furthermore, the dependence of the lifetimes on the concentration of water was bell-shaped and extrapolation of the experimental curve to higher water concentrations (Fig. 4a, dashed line) pointed toward the lifetime of NATA in aqueous solution (shown as an open circle at 55 M water). It

is interesting that very similar lifetimes were found both at the lowest possible hydration regime in reverse micelles and in aqueous solution. One possible explanation for this is that the structure of water in the hydration shell around the indole may limit the efficiency of quenching of indole fluorescence. Thus, at very low hydration there is simply not enough water to quench the fluorescence; as hydration increases in reverse micelles, a structured water layer is formed covering the polar heads of the detergents and also the fluorescent probe, which at low water levels remains attached to the micelle wall (Fig. 2b). Several methods have been previously employed to characterize the structure of water in reverse micelles (refs.19-23), revealing that indeed a highly structured water is present up to an overall concentration of about 1 M (i.e., $w_0 \cong 10$). This structured water layer could be optimally organized to quench the fluorescence of the indole derivatives. Further additions of water result in a progressive disorganization of the structure of water, which could lead to less effective quenching. The limit situation would be the aqueous solution, where the structure of water could be too loose to effectively quench the indole derivatives.

Previous studies on the fluorescence of perylene derivatives and of rose bengal in AOT reverse micelles (refs. 29,30) indicated that a simple solubilization process occurred upon increasing water levels. As discussed above, this conclusion is not supported by our results on indole derivatives, which may indicate that the structure, rather than just the average polarity of the surrounding water, may affect the fluorescence decay of the indole moiety.

An alternate explanation for our findings could arise from the compartmentalized nature of the reverse micellar system. Due to the small size of the water pool in the micelles, an impurity (even if in minimal amounts) which presented a significant overlap integral between its absorption and indole fluorescence emission could act as an acceptor of resonant energy transfer from the indole. This could result in the apparent quenching of indole fluorescence, since increasing hydration might permit a closer proximity or better positioning of the impurity with respect to the indole moiety. The extinction coefficient of the AOT used in the work was as low as $< 1 \text{ M}^{-1}\text{cm}^{-1}$ in the range 330 to 380 nm, which corresponds to the emission of the indole derivatives. Even though this extinction coefficient was very low, indicating that the AOT was in fact very pure, we cannot rule out the presence of trace impurities in our measurements. In addition, since at present we do not have an explanation for the water-mediated quenching of indole fluorescence in reverse micelles, our results should be viewed as a qualitative description of the effect of hydration on the fluorescence of indole derivatives.

We extended our studies to investigate the fluorescence decay of proteins in reverse micelles. The decays were found to be very complex and were described

by unimodal Gaussian lifetime distributions. Alcalá et al. (refs. 26-28) used lifetime distributions to describe complex fluorescence decays in proteins. Broad lifetime distributions were found for even single-tryptophan proteins, and the width and center of the distribution appeared to be related to the interconversion rates between different conformational substates in proteins (refs. 28, 35).

Our results for azurin and lysozyme in reverse micelles showed that increasing water resulted in a decrease in both the center and the width of the lifetime distributions (Fig. 6). These results likely indicate faster interconversion rates between conformational substates (ref. 35) as hydration increased in the micelles. It should be noted that the lifetime parameters for the two proteins tested decreased continuously with increasing hydration (Fig. 6), and did not display the bell-shaped water-dependence of the indole derivatives (Fig. 4a). Both the average lifetime and distribution width for the two proteins decreased toward their solution values with increasing hydration but even at the highest attainable hydration level in the micelles the lifetime parameters were still far from reaching their solution values.

Further evidence of increased protein dynamics as hydration proceeded in reverse micelles was obtained from anisotropy measurements which enabled us to calculate average rotational correlation times for the tryptophan residues in lysozyme at various hydration levels (Fig. 7). Our results are, thus, in qualitative agreement with previous reports on the effects of hydration on several dynamic properties of lysozyme (ref. 9), which showed that, at low hydration, the protein presented a rigid structure and that increasing hydration sharply increased the dynamics of the protein matrix.

The activities of several enzymes have been studied in reverse micelles as a function of w_0 (for reviews, see refs. 11 and 24). At very low w_0 , the measured activities were low; increasing w_0 results in an overshoot of activity at w_0 around 10-15 and the activity decreases again towards its aqueous solution value with further additions of water. This feature was interpreted as resulting from hyperactivity of the enzymes entrapped in reverse micelles, and several models have been suggested to account for this behavior (ref. 36). These models take into account, for example, the partition of substate molecules between the organic solvent and the water pool inside the micelles, and it has also been suggested that the shape of the micelles, as well as the presence of electric fields arising from the negatively charged detergent polar heads may influence activity (ref. 11). The overshoot of activity at intermediate w_0 is apparently not compatible with previous studies (ref. 9) which showed that all measured physico-chemical properties of lysozyme, including enzyme activity, gradually approached the aqueous solution value as hydration was increased. In addition, our fluorescence measurements also indicate a progressive and monotonic increase in the flexibility of the

protein matrix with increasing hydration. The correlation between enzyme activity and flexibility of the polypeptide chain in reverse micelles should provide information on the role of hydration in catalysis. We are currently developing a model which takes into account the compartmental nature of the micellar solutions, and which may help explain some of the apparent incompatibilities between our results on the dynamics of proteins and their activities in reverse micelles

ACKNOWLEDGEMENTS

This work was supported by NIH grant RR03155 (EG). The experiments and data analysis described in this paper were performed in the Laboratory for Fluorescence Dynamics (LFD) in the Department of Physics at the University of Illinois at Urbana-Champaign (UIUC). The LFD is supported jointly by the Division of Research Resources of the National Institutes of Health (RR03155) and the UIUC. The authors gratefully acknowledge the assistance of J. Butzow in the preparation of this manuscript.

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