

Time-resolved fluorescence and circular dichroism of porphyrin cytochrome *c* and Zn-porphyrin cytochrome *c* incorporated in reversed micelles

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Interactions between fluorescent horse heart cytochrome *c* derivatives (e.g. porphyrin cytochrome *c* and Zn-porphyrin cytochrome *c*) with surfactant interfaces in reversed micellar solutions have been studied, using different spectroscopic techniques. Anionic [sodium bis(2-ethylhexyl)sulfosuccinate, AOT] and cationic (cetyltrimethylammonium bromide, CTAB) surfactant solutions have been used in order to investigate the effects of charge interactions between proteins and interfaces.

Circular dichroism reveals that much of the protein secondary structure is lost in AOT-reversed micelles, especially when the molar water/surfactant ratio, w_o , is high ($w_o = 40$), whereas in CTAB-reversed micelles secondary structure seems to be preserved.

Time-resolved fluorescence measurements of the porphyrin in the cytochrome *c* molecule yields information about the changes in structure and the dynamics of the protein upon interaction with surfactant assemblies both in aqueous and in hydrocarbon solutions. With AOT as surfactant a strong interaction between protein and interface can be observed. The effects found in aqueous AOT solution are of the same kind as in hydrocarbon solution. In the CTAB systems the interactions between protein and surfactant are much less pronounced. The measured effects on the fluorescence properties of the proteins are different in aqueous and hydrocarbon solutions.

In general, the observations can be explained by an electrostatic attraction between the overall positively charged protein molecules and the anionic AOT interface. Electrostatic attraction can also occur between the cytochrome *c* derivatives and CTAB because there is a negatively charged zone on the surface of the proteins. From the fluorescence anisotropy decays it can be concluded that in the CTAB-reversed micellar system these interactions are not important, whereas in an aqueous CTAB solution the proteins interact with surfactant molecules.

Surfactant assemblies in organic media, called reversed micelles or, more general, water-in-oil microemulsions, have been investigated extensively over the past years. Many studies have been carried out towards the elucidation of structure and dynamics of reversed micelles [1–4] but also on the (bio)chemical and (bio)technological applications of these systems [2, 3, 5–9].

In our laboratory an important line of research consists of the incorporation of proteins in reversed micelles in order to perform bioconversions of apolar compounds or to isolate proteins [10–12]. At present, the effects of protein solubilization on both protein and micelle structure and dynamics are not very clear. Because reversed micellar solutions are optically transparent, spectroscopic and ultracentrifugation techniques can be applied to study these effects. Several systems have been the subject of this kind of investigation, some containing cytoplasmatic, others containing membrane proteins [5, 7–9].

Horse heart cytochrome *c* is a protein which *in vivo* is strongly associated via electrostatic interactions with the inner

mitochondrial membrane. The sequence and X-ray structure of this 12.4-kDa heme protein are known [13]. The protein contains nineteen lysine residues, most of which are distributed on the two flanks of the protein molecule, giving it an overall positive charge at neutral pH, but there is also a region where nine of the twelve acidic residues are clustered, thus having a negative charge. The phenomenon of oppositely charged zones on the protein surface is found for cytochrome *c* from most sources and is assumed to be related to the peripheric interactions with the mitochondrial membrane.

Previous studies of cytochrome *c* incorporated in reversed micelles formed by the surfactant sodium bis(2-ethylhexyl)sulfosuccinate (AOT) dealt with the redox activity measured via chemical reductants [14] or hydrated electrons [15]. From these studies it was concluded that the activity is retained when the protein is incorporated. This does not necessarily imply a preserved secondary structure of the protein, because a direct reduction of the heme iron is also possible. Pileni and coworkers [16] conclude from a small-angle X-ray scattering study that cytochrome *c* is strongly associated with the AOT interface. They also find that the protein is still redox active. The steady-state fluorescence properties of the single tryptophan residue of cytochrome *c*, incorporated in AOT-reversed micelles were studied by Erjomin and Metelitzka [17]. An increase of the fluorescence intensity together with a blue shift of the emission maximum was observed. These results

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Abbreviations. AOT, Aerosol-OT [sodium bis(2-ethylhexyl)sulfosuccinate]; CTAB, cetyltrimethylammonium bromide.

were interpreted in terms of an increased viscosity and a decreased polarity in the environment of the tryptophan residue, and therefore a change in protein conformation, because the tryptophan is buried in the protein coil near the heme group.

In the present study we have utilized derivatives of cytochrome *c* from which the heme iron was removed or replaced by a zinc ion, both giving rise to red fluorescence with a rather high quantum yield. The fluorescence and phosphorescence properties of porphyrin cytochrome *c* and Zn-porphyrin cytochrome *c* have been extensively described in a number of publications by Vanderkooi and coworkers [18–21]. The time-resolved and steady-state fluorescence properties of the porphyrin, which is covalently linked to the protein backbone, are very sensitive reporters of changes in the protein structure and environment. We have measured the fluorescence properties of both cytochrome *c* derivatives in aqueous solution and in reversed micellar systems. In addition the circular dichroic spectra in the far-ultraviolet were recorded in order to evaluate the secondary structural characteristics of these protein preparations. Two different surfactants were used for the reversed micellar solutions, an anionic (AOT) and a cationic (CTAB) one. In this way we have tried to elucidate the important role of charge interactions between protein and surfactant.

MATERIALS AND METHODS

Chemicals

AOT [sodium bis(2-ethylhexyl)sulfosuccinate] was obtained from Janssen Chimica and purified according to the method described by Menger and Yamada [22]. CTAB (cetyltrimethylammonium bromide) was from Serva and was used without further purification. Ruthenium tris(bipyridyl) and potassium ferricyanide, which were used for the size measurements of the reversed micelles, were from Strem Chemicals Inc. (Newburyport, MA, USA) and Merck respectively. Rose bengal, which was used as the reference component for the time-resolved fluorescence measurements, was from Eastman Kodak (certified grade) and was purified by thin-layer chromatography as described by Cramer and Spears [23]. Tetraphenyl porphyrin free base was a gift of Ing. R. B. M. Koehorst (Agricultural University, Wageningen). Isooctane (Uvasol, for fluorometry), hexanol (P. A.) and 2-methyl tetrahydrofuran (P. A.) were obtained from Merck. 2-Methyl tetrahydrofuran was distilled prior to use.

Preparation of porphyrin cytochrome *c* and Zn-porphyrin cytochrome *c*

Porphyrin cytochrome *c* was prepared according to a method which is a slight modification of the methods described by Flatmark and Robinson [24] and by Fisher et al. [25]. Anhydrous hydrogen fluoride (HF, 2–3 ml), obtained from Matheson (Oevel, Belgium), was distilled in a Teflon beaker, which was placed in a Dewar flask filled with liquid nitrogen. 100 mg of lyophilized horse heart cytochrome *c* (Sigma, type III) was added to the liquid HF and the solution was stirred with a Teflon rod. After 2–3 min, the reaction vessel was taken out of the liquid nitrogen and the HF was removed by applying a vigorous stream of nitrogen. When all the HF was removed a fluorescent, purple-colored, paste was left. This was dissolved in a small volume (3–4 ml) 0.1 M ammonium acetate pH 5.0.

The solution was dialysed against the same buffer for 24 h. To check that no alterations other than quantitative iron removal had occurred during this procedure, absorption, emission and circular dichroic spectra were measured of this preparation.

Zinc was incorporated by adding a tenfold molar excess of zinc acetate to a porphyrin cytochrome *c* solution in 0.1 M ammonium acetate pH 5.0. This solution was stirred for about an hour at room temperature in the dark (Zn porphyrin cytochrome *c* is reported to be light-sensitive [19]). The reaction was followed by the change of the visible absorption spectrum. After the reaction was completed the excess zinc acetate was removed by dialysis at 4 °C in the dark.

Preparation of reversed micellar solutions

50 μ l of a concentrated protein solution (0.3 mM) in 20 mM potassium phosphate pH 7.0 was injected into 2.5 ml of a 0.24 M solution of AOT in isooctane or CTAB in a 12% (v/v) hexanol/isooctane mixture and the solution was extensively Vortex-stirred until it became clear. Buffer was added until the desired w_o ($= [H_2O]/[surfactant]$) value was obtained and after that the total volume of sample was adjusted to 3.0 ml with isooctane. In this way the samples had a final surfactant concentration of 0.2 M. The CTAB solutions contained 10% hexanol with respect to the total volume.

Fast protein liquid chromatography

Chromatographic comparison of porphyrin cytochrome *c* and Zn-porphyrin cytochrome *c* with the native protein was performed on a Pharmacia fast protein liquid chromatography (FPLC) system. This system consisted of a GP 250 gradient programmer, a V-7 injection valve and two P500 dual piston pumps. Detection occurred with a UV-1 monitor equipped with an HR flow cell. Detection wavelength was 280 nm.

Samples and buffers were filtered through 0.22- μ m Millipore filters. Buffers were degassed under vacuum afterwards.

Gel permeation chromatography was performed on a Superose 12, HR 10/30 column; 50- μ l samples of the protein in a 20 mM potassium phosphate, 150 mM potassium chloride pH 7.0 buffer were injected and eluted with the same buffer at a flow rate of 0.5 ml/min.

The cation-exchange experiments were done on a Mono S 5/5 column. Elution occurred with a 20 mM potassium phosphate buffer pH 7.0 with a gradient of 0–1 M potassium chloride. The flow rate was 1 ml/min. After each experiment the column was reequilibrated with 5 vol. starting buffer.

Absorption, steady-state fluorescence and circular dichroism measurements

Absorption spectra were recorded on a Cary-16 spectrophotometer. Fluorescence emission spectra were measured with an Aminco SPF-500 spectrofluorimeter. Slit widths were 4 nm for both the excitation and the emission monochromators. Circular dichroism was measured with a Jobin Yvon mark V Auto-dichrograph equipped with a Silex microcomputer for data acquisition. Concentrations for the CD measurements were 25 μ M for the aqueous protein samples and 5 μ M for the reversed micellar solutions. Cuvets with a path length of 0.5 mm were used throughout.

Time-resolved fluorescence measurements

Time-resolved fluorescence of ruthenium tris(bipyridyl) was measured with the mode-locked continuous-wave argon-ion laser and single-photon-counting set-up as described previously [26]. Excitation was performed with the 457.9-nm laser line. Pulses had nanojoule energies and widths of about 100 ps FWHM (full width at half maximum). The pulse repetition rate of 76 MHz was decreased to 300 kHz using an extra cavity electro-optic modulator set-up [27]. The cuvet holder was thermostatted and during the experiments temperature was kept at 25°C.

The fluorescence emission was detected perpendicular with respect to the direction of the exciting beam via tandemized 0.25-m (Jarrell Ash, 82-410) monochromators. Detection wavelength was 613 nm, bandwidth 3 nm. A sheet of Polaroid HN38 linear polarizer was placed at magic angle (54.74° to the vertical) in front of the first monochromator slit. In order to prevent pile-up effects the excitation energy was reduced with neutral density filters so as to obtain a frequency of detected photons of 15 kHz [28], which is 5% of the excitation frequency. Data were collected in 512 channels of the multichannel analyzer and transferred afterwards to a MicroVax II computer for analysis.

For excitation of the protein samples a continuous wave dye-laser was used, synchronously pumped by the argon-ion laser, providing pulses of about 5 ps FWHM. The dye used was rhodamine 6G, giving tunable output from 580 nm to 630 nm. Excitation wavelengths used were 610 nm for the porphyrin cytochrome *c* and 590 nm for the Zn-porphyrin cytochrome *c* experiments. Emission was selected using a combination of an interference and a cut-off filter (Balzers B40, 625 nm plus Schott RG 630 for porphyrin cytochrome *c* and Balzers B40, 647 nm plus Schott RG 645 in the case of Zn-porphyrin cytochrome *c*). A sheet of linear polarizer (Polaroid HN22), mounted on a ball bearing holder, driven by two computer-controlled rotation magnets, was used to select emission, polarized parallel or perpendicular to the polarization direction of the exciting beam. Long-term fluctuations in the excitation power were averaged out by rotating the polarizer every 10 s.

Data of parallel and perpendicular polarized emission were collected in two subgroups (1024 channels each) of the multichannel analyzer. Analysis was performed with the reference convolution method described in a previous publication [28]. This means that the impulse response profile of the instrument was measured via the single-exponential fluorescence decay of a reference compound under exactly the same conditions as the other experiments. The reference compound used in this study was rose bengal, dissolved in water. To obtain a good value for the reference lifetime, τ_r , we have also measured the fluorescence decay of tetraphenyl porphyrin free base, dissolved in 2-methyl tetrahydrofuran. Simultaneous analysis of the two single-exponential decays [28] yielded lifetimes of 70 ± 2 ps for rose bengal and 11.00 ± 0.01 ns for tetraphenyl porphyrin at both excitation wavelengths used. In the rest of the experiments the τ_r value of 70 ps was fixed. All experiments were in duplicate.

RESULTS AND DISCUSSION

Characterization of the cytochrome *c* derivatives

The purity of the protein preparations was checked by gel permeation and ion-exchange chromatography using an

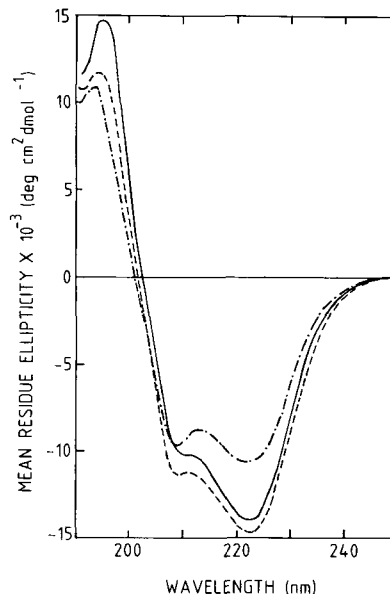


Fig. 1. Circular dichroism of ferri (—), porphyrin (---) and Zn-porphyrin (-·-·-) cytochrome *c* at 25°C. Protein concentration was 25 μ M, cuvet path length 0.5 mm

FPLC apparatus. Samples of native Fe(III) cytochrome *c*, porphyrin cytochrome *c* and Zn-porphyrin cytochrome *c* were compared with these methods. On the Superose-12 molecular sieve column the three protein samples had exactly the same elution volume, indicating that the molecular mass of the protein does not change upon iron removal and zinc incorporation. At the detection wavelength of 280 nm some minor impurities in the protein solutions could be observed but the contribution of these never exceeded 1% and were no more important in the modified cytochrome preparations than in the native sample.

When using the Mono S cation-exchange column a difference was observed between native Fe(III) cytochrome *c* and porphyrin cytochrome *c* on the one hand and Zn-porphyrin cytochrome *c* on the other. In a 0–1 M KCl gradient the first two proteins elute at 0.5 M KCl whereas the Zn derivative elutes at 0.4 M KCl. This means that charge interactions between Zn-porphyrin cytochrome *c* and column material are weaker than in the case of the other two proteins.

Porphyrin cytochrome *c* has the four-banded visible absorption spectrum characteristic for free base porphyrins. The two bands with maxima at 505 nm and 540 nm are due to the Q_y transition, the other ones (with maxima at 567 nm and 621 nm) to the Q_x transition [18]. The Soret peak has its maximum at 404 nm. Upon incorporation of Zn in the porphyrin, Q_y and Q_x become degenerate and the four bands collapse into two, with maxima at 550 nm and 586 nm. The Soret band shifts to 422 nm and sharpens.

The fluorescence emission spectra of the cytochrome *c* derivatives have been presented before [18, 19]. Upon 500-nm excitation porphyrin cytochrome *c* has emission maxima at 621 nm and 684 nm. Zn-porphyrin cytochrome *c*, excited at 550 nm, has emission maxima at 589 nm and 642 nm.

A good (qualitative) measure of the protein secondary structure is the circular dichroism (CD) in the far-ultraviolet region (190–250 nm). CD spectra of native Fe(III) cytochrome *c*, porphyrin cytochrome *c* and Zn-porphyrin cytochrome *c* are given in Fig. 1. The spectra of native Fe(III)

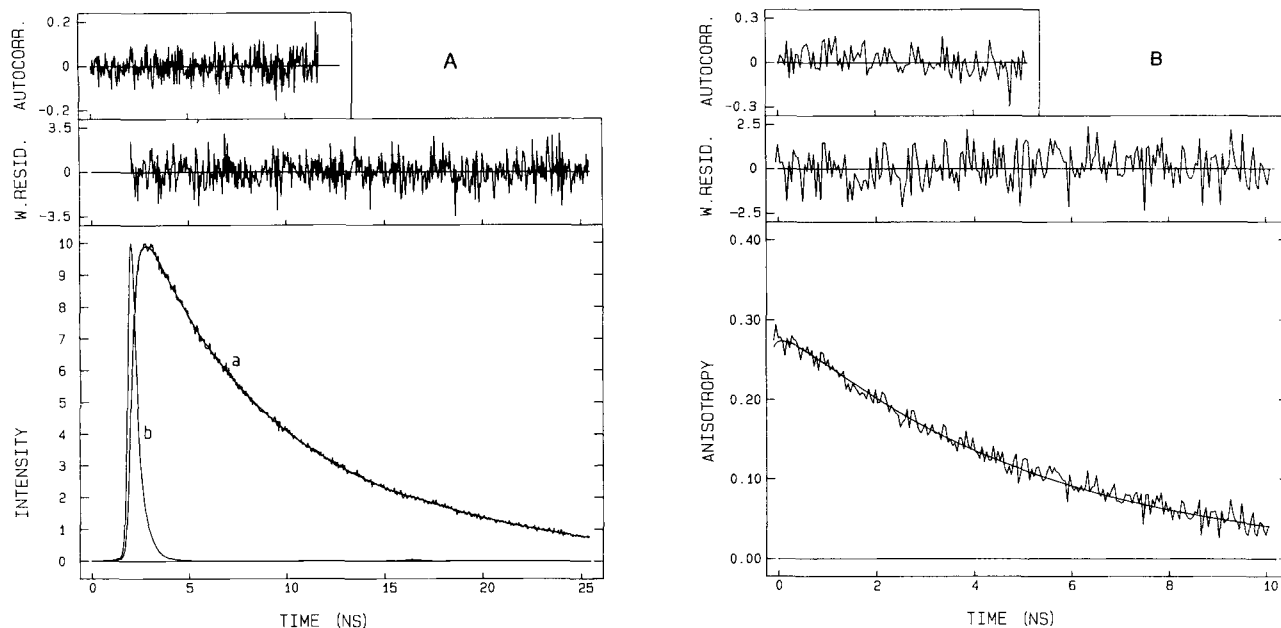


Fig. 2. Fluorescence (A) and anisotropy (B) decay of porphyrin cytochrome *c* in aqueous solution at 25°C. (A) Excitation and emission wavelengths were 610 nm and 625 nm respectively. Time equivalence per channel was 0.046296 ns. The first 25 ns of the fluorescence is shown (a), together with the response of rose bengal (b), which was used as the reference compound. The decay was fitted with a triple-exponential function having the decay parameters $\alpha_1 = 0.16 \pm 0.01$, $\tau_1 = 0.54 \pm 0.01$ ns, $\alpha_2 = 0.22 \pm 0.01$, $\tau_2 = 4.1 \pm 0.1$ ns, $\alpha_3 = 0.62 \pm 0.02$, $\tau_3 = 9.4 \pm 0.1$ ns. The quality of the fit is indicated by the weighted residuals and the autocorrelation function, shown in the upper panels. Intensity is in arbitrary units. (B) The first 10 ns of the experimental anisotropy is shown together with the single-exponential fit. Anisotropy parameters are $\beta = 0.290 \pm 0.001$, $\phi = 5.00 \pm 0.05$ ns

cytochrome *c* and porphyrin cytochrome *c* are almost identical in shape and intensity except for the 190–200-nm region but in this region the experimental error is enhanced because of the increasing light absorption of protein and buffer and the decreasing output of the xenon arc. The CD spectrum of Zn-cytochrome *c* is significantly different. The peak at 222 nm has lost about 25% of the intensity in comparison with the other two spectra, while the peak at 208 nm is unchanged. Therefore, incorporation of the Zn ion in the porphyrin causes some changes in the secondary structure of the protein. This can be due to the fact that the Zn ion lies somewhat out of the plane of the porphyrin ring, which is usually the case with Zn-porphyrins [29], whereas it is known from Fe(III) cytochrome *c* that Fe lies almost exactly in the plane of the porphyrin [30]. Another difference is that Zn in porphyrins usually has five coordination bonds where Fe in Fe(III) cytochrome *c* has six (although the sixth coordination with methionine-80 is a weak one). The CD observation of a changing protein structure upon Zn incorporation is in good agreement with the FPLC results.

Time-resolved fluorescence of the proteins in aqueous solution

The fluorescence and anisotropy decays of porphyrin cytochrome *c* are shown in Fig. 2. Excitation was at 610 nm because of the high initial anisotropy at this wavelength [18, 31, 32]. The fluorescence decay must be described by a sum of at least three exponentials. Because the only emitting group in the protein is a free base porphyrin, which in a noninteracting environment is reported to have a single-exponential decay [33], the multi-exponential fluorescence decay must be explained by microheterogeneity in the porphyrin environment. A good parameter to characterize multiple ex-

ponential decays is the average lifetime $\langle \tau \rangle$ [34], defined as:

$$\langle \tau \rangle = \frac{\sum_i \alpha_i \tau_i^2}{\sum_i \alpha_i \tau_i} \quad (1)$$

where α_i are the relative contributions of the exponential components to the decay and τ_i the fluorescence lifetimes. For porphyrin cytochrome *c* we find a $\langle \tau \rangle$ value of 8.6 ns, which is substantially higher than the single lifetime of 6.5 ns reported by Vanderkooi et al. [19]. This difference is probably due to the fact that Vanderkooi et al. used a phase-modulation instrument, yielding modulation-frequency-dependent average fluorescence lifetimes [34].

The anisotropy decay is described by a single exponential. The high initial anisotropy, β , of 0.290 ± 0.001 allows an accurate determination of the rotational correlation time, ϕ . For porphyrin cytochrome *c*, ϕ is 5.00 ± 0.05 ns. From the rotational correlation time the hydrated radius, r_h , of the protein can be calculated according to the relationship:

$$\phi = \frac{4\pi r_h^3 \eta}{3kT} \quad (2)$$

where η is the solvent viscosity (kg/m·s), k the Boltzmann constant and T the absolute temperature. For $\phi = 5.00$ ns at 25°C the calculated $r_h = 1.77$ nm, which is in excellent agreement with X-ray data [13, 35].

For Zn-porphyrin cytochrome *c* the fluorescence and anisotropy decays upon 590-nm excitation are shown in Fig. 3. As can be seen the initial anisotropy is low. The use of a higher excitation wavelength to obtain higher anisotropies did not work because the excitation efficiency of the Zn-

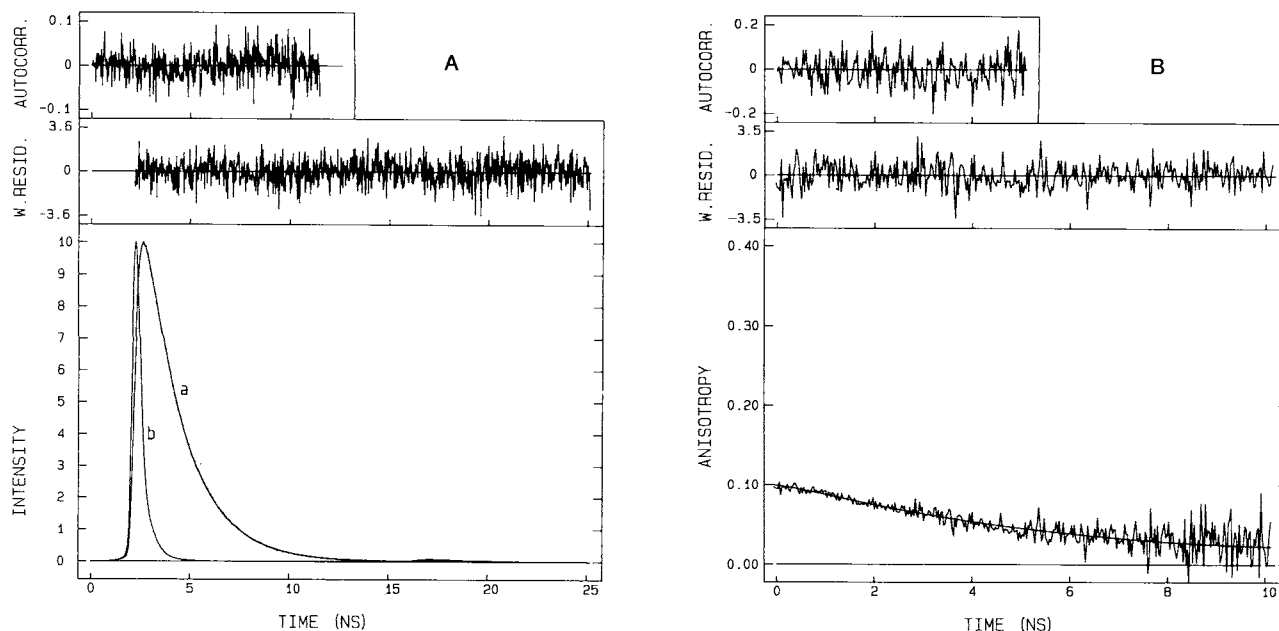


Fig. 3. Fluorescence (A) and anisotropy (B) decay of Zn-porphyrin cytochrome *c* at 25°C. (A) Excitation was at 590 nm; emission wavelength was 645 nm, time equivalence per channel 0.027932 ns. The fit is a double exponential having parameters $\alpha_1 = 0.18 \pm 0.01$, $\tau_1 = 0.49 \pm 0.02$ ns, $\alpha_2 = 0.82 \pm 0.02$, $\tau_2 = 1.92 \pm 0.03$ ns. (B) The anisotropy decay was fitted with a single-exponential function having $\beta = 0.100 \pm 0.002$, $\phi = 5.1 \pm 0.4$ ns. Other details as in Fig. 2

porphyrin becomes so low (low absorption coefficient) that impurities in the preparation (probably traces of free base porphyrin cytochrome *c*) disturb the measurements.

At least two exponentials are needed to describe the fluorescence decay, indicating that in the Zn-derivative also there is some microheterogeneity of the protein in the neighbourhood of the porphyrin. The average lifetime $\langle \tau \rangle = 1.85$ ns. This is much shorter than the 3.2 ns reported by Vanderkooi et al. [19]. On the other hand Thomas et al. [36] have reported a lifetime of 2.0 ns of the Zn-substituted derivative of *Hansenula anomala* cytochrome *c* at 10°C, which is close to our value.

The analysis of the anisotropy decay is hampered by the combination of the short fluorescence lifetime and the low initial anisotropy of 0.100 ± 0.002 (Fig. 3). A rotational correlation time of 5.1 ± 0.4 ns is found. This value is in good agreement with the one found for porphyrin cytochrome *c* (note that the error in ϕ is much higher in the case of Zn-cytochrome *c*). From the measured rotational correlation time we can deduce that only monomers are present in our Zn-porphyrin cytochrome *c* preparation and no dimers as reported by Thomas et al. for the *H. anomala* Zn-porphyrin cytochrome *c* [37]. The same conclusion was derived from the FPLC results.

Size measurements of reversed micelles

To be able to interpret the anisotropy decay results of protein-filled reversed micelles, which will be discussed below, we need to obtain estimates of sizes and rotational correlation times of the empty micelles. For the AOT system literature values are available [38–40] but no reports have appeared yet about the CTAB system, to our knowledge. Therefore we have performed size measurements using the time-resolved fluorescence quenching technique described by Atik and Thomas [39]. As fluorophor we used ruthenium tris(bipyridyl) and as quencher potassium ferricyanide $[\text{K}_3\text{Fe}(\text{CN})_6]$.

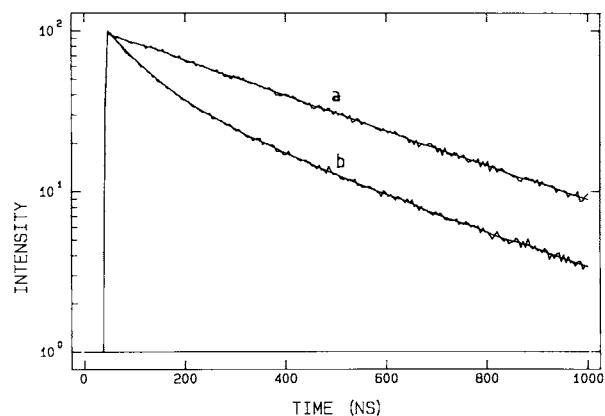


Fig. 4. Fluorescence decays of ruthenium tris(bipyridyl) in CTAB-reversed micelles, $w_o = 20$, at 25°C. Excitation wavelength 457.9 nm, emission wavelength 613 nm and time equivalence per channel 4.711 ns. The first 1000 ns of the decays is shown. The upper curve is the unquenched decay, fitted with a single-exponential function having $\tau = 374$ ns. Because of the long lifetime no deconvolution is needed. The lower curve shows the fluorescence decay in the presence of 1.0 mM potassium ferricyanide. This decay is fitted according to Eqn (3). Parameters are $\tau = 374$ ns, $k_q = 5.95 \times 10^6$ and $[\text{WP}] = 0.92$ mM

Assuming that exchange of probe and quencher molecules between the micelles is negligible during the fluorescence lifetime of the probe and that the quencher molecules have a Poisson distribution among the micelles, the quenching of the fluorescence can be described by (cf. [39]):

$$I(t) = I(0) \exp\{-t/\tau - n[1 - \exp(-k_q t)]\} \quad (3)$$

with τ , the unquenched fluorescent lifetime of the probe in the reversed micelles, k_q , the intrawater pool quenching rate constant and $n = [\text{quencher}]/[\text{WP}]$, the average number of quencher molecules per water pool. A typical example of an experiment is shown in Fig. 4.

From the water pool concentration the core radius of the micelles, r_c , can be calculated. If we want to obtain the rotational correlation times according to Eqn (2) we need to know the hydrodynamic radius, r_h , and the solvent viscosity. r_h is simply obtained by adding the average surfactant length to r_c . As lengths we have taken 1.2 nm for AOT [38] and 2.4 nm for CTAB [41]. The solvent viscosity is that of isooctane at 25°C in the case of AOT ($\eta = 4.74 \times 10^{-4}$ kg/m·s). In the CTAB system the continuous phase consists of a mixture of 1-hexanol and isooctane in a ratio of about 1:20 (v/v) according to Hilhorst et al. [11]. The measured viscosity of this mixture amounts to 5.00×10^{-4} kg/m·s. The results of the measurements and calculations are listed in Table 1. It must be noted that the r_h values and the rotational correlation times especially are only estimates because the surfactant lengths and, in case of the CTAB system, the composition of the continuous phase are not known exactly. Besides that, the micelles may interact mutually, which will also affect the rotational correlation times.

Circular dichroism of reversed micellar solutions

The effect of incorporation in two different reversed micellar systems, one containing an anionic (AOT) the other one a cationic (CTAB) surfactant on the two cytochrome *c* derivatives was measured by circular dichroism. For comparison also denaturing solutions of 5 M guanidine·HCl were measured. The results are summarized in Table 2. It should be noted that it is very difficult to measure circular dichroism of these samples because the protein concentrations are low (5 μ M) and, in addition, there is much background signal from the solution due to the presence of the surfactants or the guanidine·HCl. Therefore we estimate the error in the reported values for the reversed micellar solutions to be about 20%. In the case of cytochrome *c* the CD intensity at 222 nm, θ_{222} , is a measure of the amount of helix because this protein has no sheet structures [13, 30, 35]. Despite the high error in the CD data it is clear that the protein structure is more affected in AOT- than in CTAB-reversed micelles, whereas secondary structure is totally lost in the guanidine·HCl solution. An unexpected observation is that in the AOT micelles θ_{222} decreases with increasing w_o . This is the case for porphyrin and Zn-porphyrin cytochrome *c*. One would expect that in large micelles the protein structure resembles that in bulk water more than in small micelles, which is not the case.

Time-resolved fluorescence of the proteins in micellar solutions

The time-resolved fluorescence properties of the two proteins in aqueous solution have been discussed. Here we will concentrate on results obtained with micellar solutions. In this case the fluorescence decays of both proteins can be described with a sum of three exponentials. The average fluorescence lifetimes in the micellar systems as a function of w_o are displayed in Figs 5 and 6 for porphyrin and Zn-porphyrin cytochrome *c* respectively.

For porphyrin cytochrome *c* in AOT micelles a decrease of the average lifetime, $\langle \tau \rangle$, is observed in comparison to the value in bulk water. This difference increases at higher w_o . This is the same unexpected phenomenon as has been noticed with the CD results. Above a w_o value of 30, the average lifetime becomes constant.

Table 1. Sizes and correlation times of AOT- and CTAB-reversed micelles as a function of the water content at 25°C

w_o	AOT-reversed			CTAB-reversed		
	r_c	r_h	ϕ	r_c	r_h	ϕ
	nm		ns	nm		ns
5	1.2	2.4	7	—	—	—
10	1.7	2.9	12	1.9	4.3	41
20	3.4	4.6	47	3.1	5.5	85
30	4.3	5.5	80	4.1	6.5	140
40	5.5	6.7	145	5.2	7.6	223
50	6.7	7.9	238	6.3	8.7	335

Table 2. Circular dichroism of porphyrin cytochrome *c* and Zn-porphyrin cytochrome *c* in different solutions at 25°C

KP_i = potassium phosphate; GuHCl = guanidium·HCl

Solution	w_o	θ_{222} for	
		porphyrin cytochrome <i>c</i>	Zn-porphyrin cytochrome <i>c</i>
deg cm ² dmol ⁻¹			
20 mM KP _i pH 7.0	—	— 14100	— 10400
0.2 M AOT/isooctane	5	— 12800	— 8500
	10	— 10700	— 6500
	40	— 6700	— 3800
0.2 M CTAB/isooctane/hexanol	10	— 12100	— 10200
	40	— 11900	— 10100
20 mM KP _i , 5 M GuHCl pH 7.0	—	— 2200	— 2500

In the CTAB micelles the lifetimes of porphyrin cytochrome *c* are even shorter than in AOT. In this case there is almost no effect of w_o on $\langle \tau \rangle$. Only at $w_o = 10$ is the average lifetime somewhat shorter. This observation agrees with the CD results where we also observe less dependence on the water content of the micelles in CTAB than in AOT. Apparently the interactions between protein and surfactant are stronger with AOT than with CTAB. This is in good agreement with the fact that *in vivo*, cytochrome *c* appears to be bound to negatively charged phospholipid cardiolipin [42].

We have also measured the fluorescence decays in aqueous surfactant solutions. For porphyrin cytochrome *c* in a 20 mM potassium phosphate 5 mM AOT pH 7.0 solution a $\langle \tau \rangle$ value of 7.50 ns is observed. As in the reversed micellar solutions, this value is shorter than in normal buffer solution. Probably the interactions between the protein and the surfactant in the aqueous AOT solution are of the same nature as in the reversed micellar solutions.

In a 20 mM potassium phosphate 5 mM CTAB pH 7.0 solution the observed $\langle \tau \rangle$ value is 9.20 ns, which is longer than the value found in buffer solution. In reversed micellar CTAB solutions a shortening of the average fluorescence lifetime was observed. So in case of CTAB the interactions between protein and surfactant in aqueous micellar solutions are different from those in reversed micellar solutions.

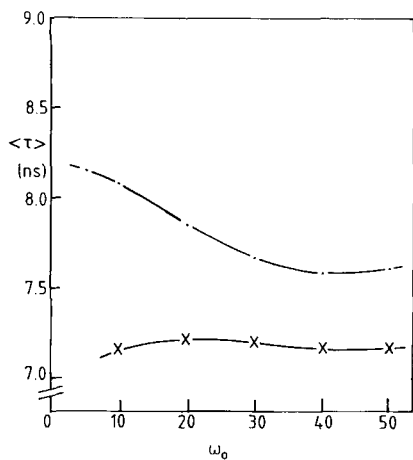


Fig. 5. Average fluorescence lifetimes of porphyrin cytochrome *c* in AOT-reversed (---) and CTAB-reversed (-x-x-) micelles as a function of w_0 at 25°C. Experimental conditions were the same as described in the legend of Fig. 2. Fluorescence decays were fitted with a triple-exponential function

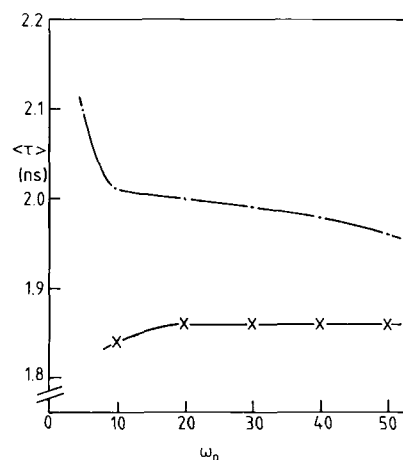


Fig. 6. Average fluorescence lifetimes of Zn-porphyrin cytochrome *c* in AOT-reversed (---) and CTAB-reversed (-x-x-) micelles as a function of w_0 at 25°C. Experimental conditions were as in Fig. 3. Fluorescence decays were fitted with sum of three exponentials instead of two, which were sufficient in the case of Zn-cytochrome *c* in aqueous solution

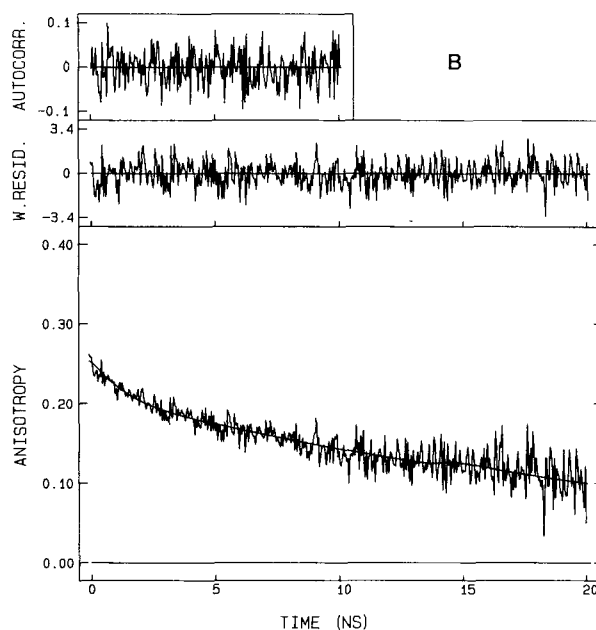
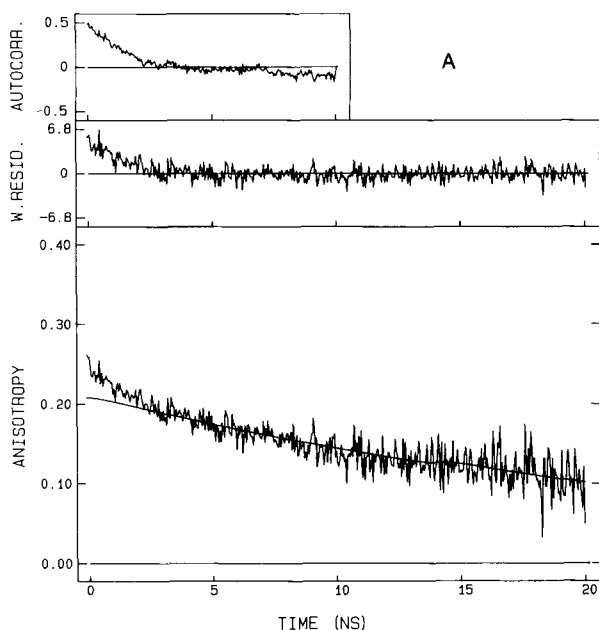


Fig. 7. Fluorescence anisotropy decay of porphyrin cytochrome *c* in AOT-reversed micelles, $w_0 = 20$, at 25°C. Experimental conditions were the same as in Fig. 2. (A) Decay fitted with a single-exponential function having parameters $\beta = 0.21 \pm 0.003$, $\phi = 25.1 \pm 0.6$ ns. (B) Decay fitted with a double-exponential function having parameters $\beta_1 = 0.05 \pm 0.002$, $\phi_1 = 1.0 \pm 0.2$ ns, $\beta_2 = 0.21 \pm 0.002$, $\phi_2 = 25.4 \pm 0.7$ ns

In the case of Zn-porphyrin cytochrome *c* different effects are found. First, three fluorescence lifetimes are needed to describe the decays satisfactorily while in aqueous solution only two were needed. Second, the average lifetimes increase upon incorporation in the AOT-reversed micelles and are not significantly different from the value in aqueous solution and in CTAB-reversed micelles. The increase of $\langle \tau \rangle$ is most strongly found in AOT micelles at $w_0 = 5$. From $w_0 = 10$ up to $w_0 = 50$ the $\langle \tau \rangle$ value hardly changes in AOT. In the aqueous surfactant solutions the changes are also small. $\langle \tau \rangle = 2.02$ ns is found in 5 mM AOT and $\langle \tau \rangle = 2.04$ ns in 5 mM CTAB. The only similarity between the results for porphyrin cytochrome *c* and those for Zn-porphyrin cytochrome *c* is that the lifetimes in aqueous and in

hydrocarbon CTAB solutions differ while in AOT the effects of aqueous and hydrocarbon surfactant solutions are comparable. The different fluorescence behaviour of the two proteins is difficult to explain. Firstly, there is the effect of the Zn ion on the fluorescence properties of the porphyrin and, secondly, the alterations in the protein structure which are evident from CD spectra and FPLC experiments, both of which may play a role.

From the analysis of the anisotropy decays the first striking observation is that all decays of the proteins in (aqueous and hydrocarbon) surfactant solutions must be described by a sum of two exponentials, one with a rotational correlation time of about 1 ns, the other one longer. A typical experiment is shown in Fig. 7. The origin of the fast

Table 3. Fluorescence anisotropy parameters of porphyrin cytochrome *c* and Zn-porphyrin cytochrome *c* in different solutions at 25 °C

Solution	w_o	Porphyrin cytochrome <i>c</i>				Zn-porphyrin cytochrome <i>c</i>				
		β_1	ϕ_1	β_2	ϕ_2	β_1	ϕ_1	β_2	ϕ_2	
			ns		ns		ns		ns	
0.2 M	5	0.04 ± 0.003	1.0 ± 0.4	0.23 ± 0.003	30 ± 0.9	—	—	—	—	
AOT/isooctane	10	0.04 ± 0.004	1.3 ± 0.4	0.22 ± 0.004	28 ± 1.2	—	—	—	—	
	20	0.05 ± 0.002	1.0 ± 0.2	0.21 ± 0.002	25 ± 0.7	—	—	—	—	
	30	0.05 ± 0.002	1.8 ± 0.2	0.20 ± 0.003	26 ± 1.0	—	—	—	—	
	40	0.06 ± 0.002	1.1 ± 0.2	0.20 ± 0.003	28 ± 1.0	—	—	—	—	
	50	0.06 ± 0.002	2.1 ± 0.2	0.19 ± 0.003	30 ± 1.1	—	—	—	—	
20 mM KP _i , 5 mM AOT pH 7.0	—	0.04 ± 0.002	1.9 ± 0.2	0.23 ± 0.002	60 ± 12	—	—	—	—	
0.2 M	10	0.02 ± 0.002	0.9 ± 0.2	0.25 ± 0.002	15.3 ± 0.3	0.009 ± 0.004	2.0 ± 1.0	0.089 ± 0.004	20 ± 4	
CTAB/isooctane/ hexanol	20	0.03 ± 0.003	1.3 ± 0.2	0.25 ± 0.003	12.4 ± 0.2	0.009 ± 0.003	1.2 ± 0.4	0.092 ± 0.003	12 ± 1	
	30	0.04 ± 0.004	0.6 ± 0.3	0.25 ± 0.006	9.7 ± 0.4	0.012 ± 0.009	2.0 ± 1.0	0.087 ± 0.009	10 ± 1	
	40	0.04 ± 0.004	0.7 ± 0.2	0.26 ± 0.005	8.7 ± 0.4	0.008 ± 0.004	1.2 ± 0.5	0.093 ± 0.004	8 ± 1	
	50	0.04 ± 0.004	0.7 ± 0.2	0.27 ± 0.005	7.8 ± 0.3	0.003 ± 0.005	1.6 ± 0.6	0.094 ± 0.004	7 ± 1	
20 mM KP _i , 5 mM CTAB pH 7.0	—	0.04 ± 0.003	0.9 ± 0.1	0.26 ± 0.002	13.7 ± 0.2	0.009 ± 0.003	1.3 ± 0.5	0.080 ± 0.002	15 ± 2	

component in the decay is not completely clear. Keh and Valeur [43] have proposed a model for the depolarization of the fluorescence in which the rotation of probe molecules within the micelles, characterized by ϕ_{int} , is coupled to the overall rotation of the micelles, represented by ϕ_{mic} . From this model the following expression for the anisotropy decay was derived:

$$r(t) = \frac{r_o}{\phi_{mic} - \phi_{int}} [\phi_{mic} \exp(-t/\phi_{int}) - \phi_{int} \exp(-t/\phi_{mic})]. \quad (4)$$

In this equation there is a combination of two exponentials, one of them having a negative preexponential factor. Attempts to fit the anisotropy decays of porphyrin cytochrome *c* and Zn-porphyrin cytochrome *c* in reversed micelles according to this model failed. Therefore we assume that the fast component in the anisotropy decay is caused by an enhanced mobility of the porphyrin group. Such a view is supported by the fact that this rapid component is also present in the aqueous surfactant solutions while its contribution is more important in AOT than in CTAB. The results of the analysis of the anisotropy decays are listed in Table 3.

In the case of porphyrin cytochrome *c* in AOT micelles we see a slight increase of the contribution of the fast component in larger micelles (Table 3). This implies that the mobility of the porphyrin within the protein becomes more important in larger micelles. This coincides with the CD results where we found that the protein structure is affected most strongly in AOT micelles with a high water content. The slowly decaying component in the fluorescence anisotropy, characterized by ϕ_2 , reports on the overall motion of the protein, which is composed of the mobility of the protein within the micelles, represented by ϕ_{int} , and the mobility of the micelle as a whole, described by ϕ_{mic} . When these two components of the protein motion are independent ϕ_2 , ϕ_{int} and ϕ_{mic} are related as follows (cf. [43]):

$$\frac{1}{\phi_2} = \frac{1}{\phi_{int}} + \frac{1}{\phi_{mic}}. \quad (5)$$

The observed ϕ_2 of porphyrin cytochrome *c* in AOT micelles are not very dependent on the water content. From $w_o = 5$ up to $w_o = 30$ a slight decrease of the rotational correlation time is observed and from $w_o = 30$ to $w_o = 50$ the values increase again. We have seen before that the other spectroscopic properties of this protein change with a variation of w_o (especially at w_o values below 20). Therefore we believe that there are two effects which have a compensating influence on the rotational correlation times. The first one is a decrease of ϕ_{int} with increasing w_o . When the size of the micellar water pools increases it can be expected that the mobility of the protein within the micelles also increases. The second effect is that ϕ_{mic} increases when the micelles grow. Comparing the rotational correlation times of the filled and the unfilled micelles we can conclude that at $w_o < 20$ the micelles have to expand upon incorporation of a protein molecule. This can be expected because the core radii of the unfilled micelles at $w_o = 5$ and at $w_o = 10$ are smaller than the radius of the protein molecule. For $w_o \geq 20$ it is not possible to make a statement about the size of the filled micelles. The measured ϕ_2 values are smaller in this case than the rotational correlation times of the empty micelles but this is probably due to the increasing mobility of the protein within the micelles.

Solubilization of cytochrome *c* in AOT-reversed micelles was also studied by Pileni and coworkers [16] with the small-angle X-ray scattering technique. A decrease of the micellar size at $w_o = 20$ upon protein incorporation was found, from which the authors argue that the protein is mainly located at the interface of the micelles and that addition of cytochrome *c* has the same effect on the system as addition of surfactant, lowering the apparent w_o and thus causing a shrinking of the micelles. From our measurements we can only conclude that there are indeed important interactions between porphyrin cytochrome *c* and the AOT-reversed micelles, even at the highest w_o value studied, but the statement of Pileni et al., that addition of the protein gives an increase of the interfacial area, cannot be positively affirmed.

In the aqueous AOT solution there are comparable interactions between protein and surfactant as was also concluded

from the fluorescence lifetimes. The anisotropy decay in this case also has two components. The contribution of the fast component is of the same order of magnitude as in the reversed micellar systems and the values found for ϕ_2 is, although high in standard error, much larger than the 5 ns found in normal aqueous solution. Apparently in an aqueous AOT solution also the protein interacts with surfactant aggregates.

In CTAB-reversed micelles the contribution of the fast component to the anisotropy decay is smaller than in the case of AOT (see Table 3). The measured ϕ_2 values are significantly shorter than in the case of AOT and decrease monotonically with increasing w_o . From this observation we conclude that porphyrin cytochrome *c* gains more internal mobility when the size of the reversed CTAB micelles increases. It is not expected that the size of the filled micelles decreases with increasing water content, thus causing the shortening of the rotational correlation time of the whole micelle. At $w_o = 50$ the value of ϕ_2 is quite close to that measured in normal aqueous solution. Because the empty CTAB micelle at $w_o = 50$ is very large, we do not expect that the overall rotation of the micelle has any contribution to the measured anisotropy decay. This indicates that the protein mobility within the micelle is almost the same as in bulk water.

In the aqueous CTAB solution a significant effect of the surfactant on the protein anisotropy decay is found. Again a fast component is found in the decay. The ϕ_2 , which reflects the protein overall rotation, is nearly three times longer than in normal aqueous solution. Apparently, the interactions between protein and CTAB are more important in aqueous than in hydrocarbon surfactant solutions. Probably the changes in the fluorescence properties of the protein in the reversed micellar solutions are caused either by the high effective bromide concentration in the water pools or by interactions with surfactant-bound water molecules. Direct interactions between the protein and the surfactant interface are unlikely, because in that case the protein rotation would be more hindered, resulting in a longer rotational correlation time. The effects, which are observed in the aqueous CTAB solution, can only be explained in terms of direct interactions between the protein and the surfactant. The increase of the fluorescence lifetime can be explained if surfactant molecules interact with the protein, thus causing a shielding of the exposed part of the porphyrin ring. In the neighbourhood of this part of the porphyrin ring the protein has a zone where most of the acidic amino acid residues are clustered, which might lead to electrostatic attraction between the protein and CTAB molecules. In the reversed micellar system these interactions may be prevented by the high effective bromide concentrations in the water pools.

For Zn-porphyrin cytochrome *c* the dynamic properties are more difficult to obtain from the fluorescence anisotropy decay. The reason for this is, as we stated before, that the fluorescence lifetime is short and the initial anisotropy low (see Fig. 3). In the case of Zn-porphyrin cytochrome *c* in AOT micelles (aqueous and reverse) no meaningful values for the rotational correlation times can be obtained from the polarized fluorescence decay data. A good fit of the anisotropy decay can be obtained with every ϕ_2 value higher than 25 ns. Data are therefore omitted in Table 3.

In the case of CTAB-reversed micelles reliable values for the rotational correlation times can be obtained for Zn-porphyrin cytochrome *c*, although the standard error in the fitted parameters is much higher than in the case of porphyrin cytochrome *c*. The results are listed in Table 3 and they are comparable to those for porphyrin cytochrome *c*. Although

the two proteins do not have exactly identical structures, the dynamic properties in CTAB aqueous and reversed micelles are almost the same.

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