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The polar headgroup of the detergent governs the accessibility to water of tryptophan octyl ester in host micelles

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Abstract

Many attempts have been made to rationalize the use of detergents for membrane protein studies [J. Biol. Chem. 264 (1989) 4907]. The barrier properties of the detergent headgroup may be one parameter critically involved in protein protection. In this paper, we analyzed these properties using a model system, by comparing the accessibility of tryptophan octyl ester (TOE) to water-soluble collisional quenchers (iodide and acrylamide) in three detergent micelles. The detergents used differed only in the chemical nature of their polar headgroups, zwitterionic for dodecylphosphocholine (DPC) and nonionic for octa(ethylene glycol) dodecyl monoether ($C_{12}E_8$) and dodecylmaltoside (DM). In all cases, in phosphate buffer at pH 7.5, the binding of 5 μM TOE was complete in the presence of a slight excess of detergent micelles over TOE molecules, resulting in a significant blue shift and greater intensity of TOE fluorescence emission. The resulting quantum yield of bound TOE was between 0.08 (in DPC) and 0.12 (in DM) with an emission maximum (λ_{max}) of ~335 nm whatever the detergent micelle. Time-resolved fluorescence intensity decays of TOE at λ_{max} were heterogeneous in all micelles (3–4 lifetime populations), with mean lifetimes of 1.7 ns in DPC, and 2 ns in both $C_{12}E_8$ and DM. TOE fluorescence quenching by iodide, in detergent micelles, yielded linear Stern–Volmer plots characteristic of a dynamic quenching process. The accessibility of TOE to this ion was the greatest with $C_{12}E_8$, followed by DPC and finally DM (Stern–Volmer quenching constants K_{sv} of 2 to 5.5 M^{-1}). In contrast, the accessibility of TOE to acrylamide was greatest with DPC, followed by $C_{12}E_8$ and finally DM (K_{sv} = 2.7–7.1 M^{-1}). TOE also presents less rotational mobility in DM than in the other two detergents, as shown from anisotropy decay measurements. These results, together with previous TOE quenching measurements with brominated detergents [Biophys. J. 77 (1999) 3071] provide reference data for analyzing Trp characteristics in peptide (and more indirectly protein)–detergent complexes. The main finding of this study was that TOE was less accessible (to soluble quenchers) in DM than in DPC and $C_{12}E_8$, the cohesion of DM headgroup region being suggested to play a role in the ability of this detergent to protect function and stability of solubilized membrane proteins.

Abbreviations: TOE, tryptophan octyl ester; NATA, *N*-acetyltryptophanamide; DM, dodecylmaltoside; $C_{12}E_8$, octa(ethylene glycol) dodecyl monoether; DPC, dodecylphosphocholine; DOPC, dioleoyl-*sn*-glycero-3-phosphocholine; AOT (Aerosol OT), sodium bis(2-ethylhexyl) sulfosuccinate; MEM, maximum entropy method

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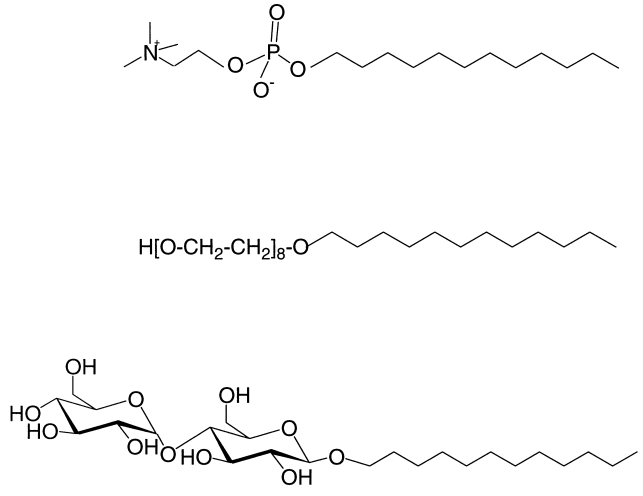
1. Introduction

Various types of detergent micelles have been used as valuable substitutes for membranes for the study of solubilized membrane proteins and amphiphilic or hydrophobic peptides. Detergents are also essential for crystallization studies. Dodecylphosphocholine (DPC), a zwitterionic detergent, is often selected for NMR structural studies because (i) it forms small micelles suitable for this technique and (ii) its polar phosphocholine headgroup mimics natural phospholipid membrane/water interfaces ([1,2] and references therein). The nonionic detergents $C_{12}E_8$ and dodecylmaltoside (DM) are both very efficient at maintaining the functional properties of many membrane proteins [3,4]. They are also suitable for the study of protein fragments [5] or peptides of biological interest [6], by various spectroscopic techniques, including fluorescence spectroscopy and circular dichroism. $C_{12}E_8$ and DM also allowed crystallization of some of the few membrane proteins for which three-dimensional structure could be solved (e.g., the sarcoplasmic reticulum calcium pump, [7]). All three detergents have aliphatic chains of same length and differ only in the nature of their polar headgroups (Scheme 1).



Scheme 1. Chemical formula of the detergents used in this work: DPC, $C_{12}E_8$, DM (from top to bottom).

This study is aimed to characterize the accessibility to soluble quenchers of a small hydrophobic analog of Trp, TOE, embedded in micelles of these detergents. The quenchers used were iodide ions and acrylamide, and accessibility was determined from their efficiency to quench TOE fluorescence. It has been suggested that the indole ring of TOE is embedded in the hydrophobic core of DM micelles, because it is highly accessible to DM with brominated alkyl chain, but close to the headgroup region [8]. This location is likely due to the probable hairpin conformation of TOE in DM micelles, suggested from molecular modeling [8]. Before performing quenching experiments, we assessed the binding of TOE to the various detergent micelles and the pH dependence of the steady-state fluorescence of bound TOE. As a complement, time-resolved fluorescence intensity and anisotropy decays of bound TOE were also analyzed. We found that the various detergent headgroups formed different barriers to the diffusion of the two water-soluble compounds used, with DM micelles being the less permeable and the more rigid system. These 'barrier' properties, among various other parameters (e.g., [4]) may partly account for the ability of various detergents to protect the functional state of a membrane protein.

2. Materials and methods

2.1. Solutions and chemicals

TOE, *N*-acetyltryptophanamide (NATA), KI and acrylamide were purchased from Sigma–Aldrich. DM was from Calbiochem, $C_{12}E_8$ from Nikko Chemicals Co., NTD (Tokyo) and DPC from Avanti Polar Lipids (AL, USA). Stock solutions of TOE (usually 2.5 mM) were prepared in ethanol (from Merck, quality Uvasol). Stock solutions of the three detergents were prepared in water at concentrations of 20 and 200 mM. A 5-M stock solution of KI was prepared in water and 0.1 mM $Na_2S_2O_3$ was added to prevent oxidation [9]. Concentrated acrylamide solution (5 M) was prepared in water.

Water was distilled and then purified by a Milli-Q system. All buffers were filtered through Millex HA filters (0.45 μm, Millipore).

2.2. Absorption measurements

Absorption spectra were recorded on an HP 8452A or HP8453 diode array spectrophotometer, with a thermostatically controlled sample holder (20°C). The sample was continuously stirred. The pathlength through the cuvette was 1 cm.

2.3. Steady-state fluorescence measurements

Fluorescence intensities were measured on a Spex Fluorolog spectrofluorometer. The temperature in the cuvette was controlled with a thermostat and the sample was continuously stirred. We used a standard quartz cuvette (1×1 cm). Excitation spectra were corrected for the spectrum of the lamp and both excitation and emission spectra were corrected for fluctuations in lamp intensity (usually very small, $<1\%$).

2.4. Data analysis

Quenching in the presence of iodide was analyzed using the classical Stern–Volmer equation (see, for reviews, [10,11]):

$$F_0/F = 1 + K_{sv}[Q]$$

where K_{sv} is the Stern–Volmer quenching constant and [Q] is the quencher concentration. K_{sv} is related to the bimolecular quenching constant k_q according to the following formula:

$$K_{sv} = k_q \tau_0$$

where τ_0 is the lifetime, in the absence of quencher, of the fluorophore.

In the presence of acrylamide, nonlinear Stern–Volmer plots were analyzed using the modified equation:

$$F_0/F = (1 + K_{sv}[Q])\exp V[Q]$$

where V can be considered as a sphere of action around the fluorophore in which the presence of a quencher molecule results in instantaneous (static) quenching. The radius r of this sphere is obtained from:

$$V/N = 4\pi r^3/3$$

N being the Avogadro number [9].

2.5. Time-resolved fluorescence measurements

Fluorescence intensity and anisotropy decays were determined by the time-correlated single photon counting technique, from the polarized components, $I_{vv}(t)$ and $I_{vh}(t)$, using the experimental setup of the SB1 window of the Super-ACO synchrotron radiation machine (Anneau de Collision d'Orsay), as previously described ([12] and references therein). The excitation wavelength was selected using a double monochromator (Jobin Yvon UV-DH10). A Hamamatsu MCP-PMT detector (model R3809U-02) was used. Time resolution was about 20 ps and data were accumulated in 2048 channels. Automatic sampling cycles were carried out, including 30 s accumulation time for the instrument response function and 90 s acquisition time for each polarized component, so that a total of $(2–4)\times10^6$ counts was reached for each measure of fluorescence intensity decay. Fluorescence intensity and anisotropy decays, $I(t)$ and $r(t)$, respectively, were analyzed as sums of exponential terms by the maximum entropy method (MEM) [13] according to the following formula:

$$I(t) = \Sigma \alpha_i \exp(-t/\tau_i)$$

where α_i is the normalized amplitude and τ_i the lifetime of the intensity decay, and

$$r(t) = \Sigma \beta_i \exp(-t/\theta_i)$$

where β_i is the anisotropy and θ_i the rotational correlation time of the anisotropy decay.

3. Results

3.1. Binding of TOE to the various detergent micelles

We first compared the binding of TOE (5 μM) to $C_{12}E_8$, DPC and DM micelles on the basis of the resulting fluorescence intensity changes (Fig. 1A). In all cases, binding resulted in a blue-shift of the

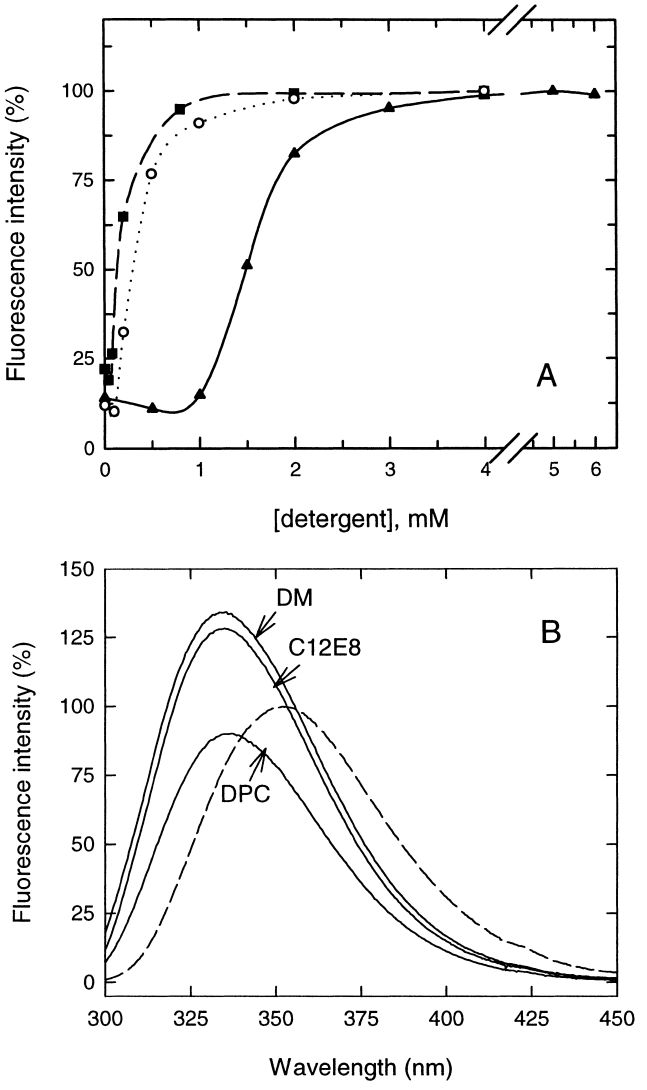


Fig. 1. Binding of TOE to $C_{12}E_8$ (■), DPC (▲), and DM (○) micelles (A) and fluorescence emission spectra of bound TOE (B). (A) TOE (5 μM) was added to 25 mM phosphate buffer (pH 7.5) at 20°C, and left to equilibrate for 4 min to allow its fluorescence to stabilize. Aliquots of $C_{12}E_8$ (■) or DPC (▲) were then added sequentially, with continuous stirring, at 4-min intervals. Fluorescence intensity was continuously recorded with λ_{ex} and λ_{em} set at 280 and 335 nm, respectively, and slitwidths of 1.25 mm (bandwidths ~5 nm) at both wavelengths. The fluorescence intensities obtained after each detergent addition, corrected for the slight blank values (detergent alone in buffer), were plotted as a percentage of the maximal value, as a function of final detergent concentration. ○, Binding of TOE to DM, as previously obtained [8]. (B) TOE (5 μM) was directly added to the above phosphate buffer, already supplemented with 4 mM DM, $C_{12}E_8$ or DPC, as indicated. λ_{ex} and slitwidths were as above. Fluorescence emission spectra were recorded after ~2 min of equilibration and were corrected for blank spectra. Dashed line: emission spectrum of NATA (5 μM) in pure water, obtained as described above, for reference.

TOE fluorescence emission spectrum (λ_{max} from ~350 nm in buffer to 335 nm in micelles) and a significant increase in the fluorescence intensity, consistent with the transfer of the TOE indole moiety from a polar environment (the bulk water phase) to a less polar (and/or more viscous) environment within the detergent micelle. Increases in fluorescence showed that with $C_{12}E_8$ and DPC, binding began at a detergent concentration close to the cmc value (respectively 0.09 mM and 1.1 mM, see Table 1) and was complete at respectively ~1 mM and ~3 mM, i.e., in the presence of a slight excess of detergent micelles over TOE molecules (taking into account detergent aggregation number, Table 1). The binding curve with $C_{12}E_8$ is similar to that obtained with DM [8] except for a slight shift in detergent concentration consistent with differences in the cmc of the two detergents (0.18 mM for DM; Table 1).

The fluorescence emission spectra of TOE (5 μM)

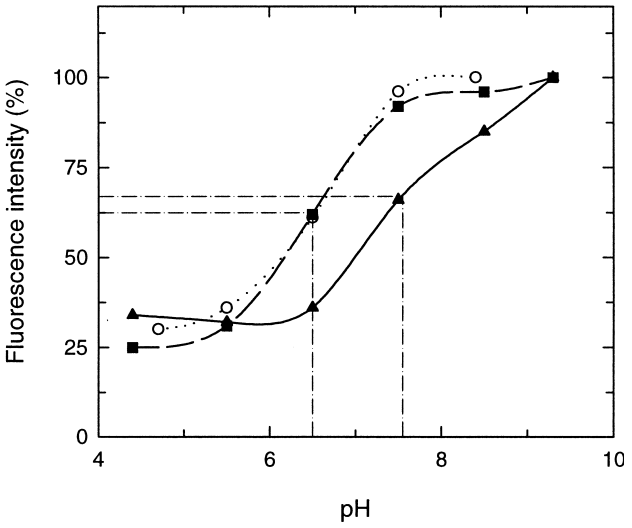


Fig. 2. pH dependence of TOE fluorescence intensity in $C_{12}E_8$ (■), DPC (▲) and DM (○) micelles. TOE (5 μM) was added to 25 mM phosphate buffer containing 4 mM $C_{12}E_8$ (■) or DPC (▲), at various pH values (4.4, 5.5, 6.5, 7.5, 8.5, and 9.3) and at 20°C. Emission spectra were recorded after ~3 min of equilibration, with λ_{ex} set at 280 nm, and slitwidths of 1.25 mm (bandwidths ~5 nm) for both excitation and emission. They were corrected by subtracting blank spectra (detergent alone in buffer). The fluorescence intensity at the final λ_{max} value (335 and 340 nm in $C_{12}E_8$ and DPC, respectively) was plotted as a function of pH. The curve previously obtained for DM (at 336 nm) [8] is plotted with open circles. For DPC, the fluorescence intensities are the means of duplicate measurements. The dash-dot lines indicate the half-effect in $C_{12}E_8$ and DPC.

Table 1
Properties of the detergents used in this work

Detergent	Monomer M_r (Da)	cmc (mM)	Aggregation no. (micelle size, M_r)	ν (cm^3/g)	Ref.
DM	511	0.18[a]	110–140 (56200–71500)	0.81[b]	[30,31]
$C_{12}E_8$	538	0.09	90–120 (48400–64500)	0.973	[30]
DPC	352	1.1	50–60 (17600–21100)	0.937	[1,32]

[a]Slightly lower values have also been reported, from 0.125 mM [33] to 0.150–0.17 mM [8,34].
[b][35].

in the various detergents (4 mM) are shown in Fig. 1B. The quantum yield ϕ of TOE in the three detergents was estimated from the ratio of total TOE peak area to the total peak area for NATA ($\phi = 0.10$, from [12]). Similar TOE quantum yield values were obtained with DM and $C_{12}E_8$ ($\phi = 0.12$ and 0.11, respectively) whereas ϕ was slightly lower with DPC (0.08).

3.2. pH dependence of TOE fluorescence in detergent micelles

As the interface properties of the micelle should modify the pK_a values of ionizable groups, we analyzed the pH dependence of TOE (5 μM) fluorescence in an excess of $C_{12}E_8$ and DPC (4 mM) micelles (Fig. 2). In $C_{12}E_8$, the effect of pH on TOE fluorescence was very similar to that previously ob-

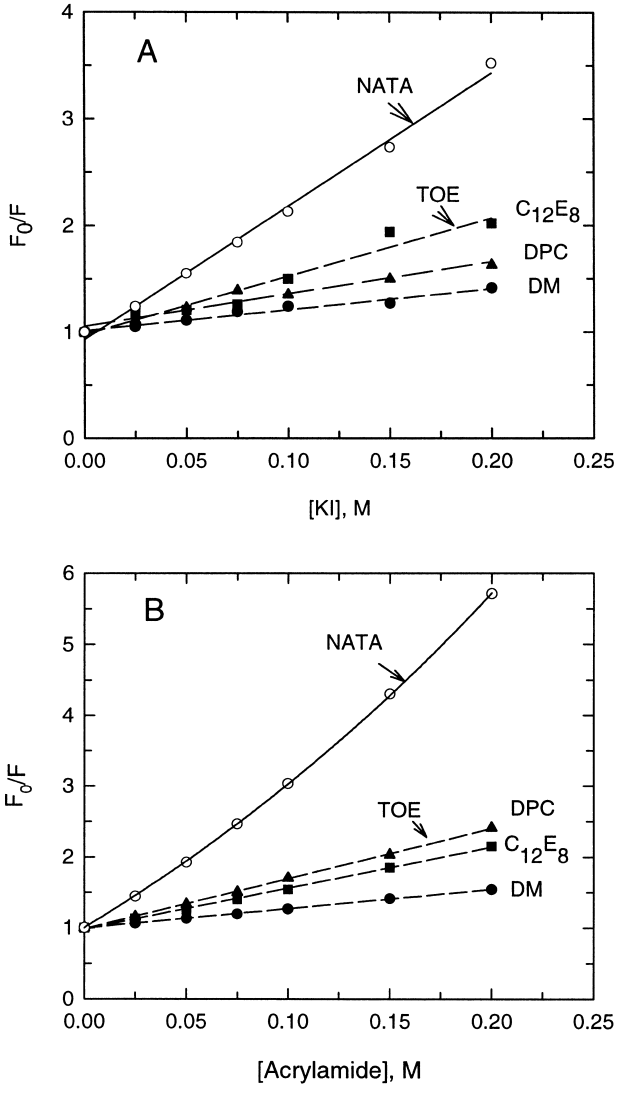


Fig. 3. Stern–Volmer plots of iodide (A) and acrylamide (B) quenching of TOE in DM (●), $C_{12}E_8$ (■) and DPC (▲) micelles, and of NATA in buffer (○). (A) Each data point was obtained from a separate experiment in which TOE (5 μM) was added to 10 mM phosphate buffer (pH 7.5) at 20°C, supplemented with 4 mM DM, $C_{12}E_8$ or DPC. Aliquots of KCl and KI were then sequentially added, at intervals of 50 s, so that [KCl]+[KI] = 0.2 M. Fluorescence intensity was continuously recorded with λ_{ex} and λ_{em} set at 290 nm (slitwidths 1.25 mm) and 335 nm (slitwidths 2.5 mm), respectively. The final value (F) obtained after KI addition was corrected for slight blank values. The reference value, F_0, was that obtained in the presence of 0.2 M KCl. A similar experiment (except that λ_{em} was set at 354 nm) was performed for NATA (5 μM) in buffer alone. A straight line was fitted to the data. (B) TOE (5 μM) was added to 10 mM phosphate buffer (pH 7.5) at 20°C, supplemented with 4 mM DM, $C_{12}E_8$ or DPC. Aliquots of acrylamide were then added sequentially, at 100-s intervals. Fluorescence intensity was continuously recorded with λ_{ex} and λ_{em} set at 295 nm (slitwidths 1.25 mm) and 335 nm (slitwidths 2.5 mm), respectively. The fluorescence intensities obtained at each acrylamide concentration were corrected for slight blank values. For comparison, a similar experiment (except that λ_{em} was set at 354 nm) was performed for NATA (5 μM) in buffer alone. A straight line was fitted to the data for TOE in the presence of detergent, whereas the modified Stern–Volmer equation was used for NATA.

served in DM (Fig. 2). Emission spectra in $C_{12}E_8$ were slightly red-shifted (from 328 to 335 nm) with increasing pH (not shown) and fluorescence intensity increased by a factor of four, such that the curve could almost be superimposed on that for DM. The apparent pK_a value (pH of half-effect) of TOE excited state(s) was 6.5 in $C_{12}E_8$, as compared to 6.7 in DM.

In contrast, in DPC, the fluorescence intensity curve was significantly shifted towards higher pH, with a half-effect obtained at pH 7.5. This was also the apparent pK_a of TOE in membranes of DOPC [14]. In addition, the fluorescence changes occurred over a wider range of pH ($\sim$3 pH units versus 2, from 6.5 to at least 9.3) and the red shift in the emission spectrum was also larger (from 331 to 340 nm). These effects may reflect both the ionization of TOE and, indirectly, an effect of pH on the zwitterionic polar heads of DPC.

Subsequent quenching experiments were compared at pH 7.5, in conditions in which TOE fluorescence is due mainly to its neutral form in $C_{12}E_8$ and DM, and probably to both its ionized and neutral form in DPC.

3.3. I^- and acrylamide quenching of TOE in the various detergent micelles

Fig. 3A shows the Stern–Volmer plots of iodide quenching of TOE in the various detergents and of NATA in buffer alone, for reference. The data were well fitted by a straight line, typical of a simple collisional (dynamic) mechanism. TOE seemed to be less accessible to KI when embedded in detergent micelles than NATA, considered as fully accessible. However, significant differences were observed between the three detergents, as shown from the values of the Stern–Volmer and bimolecular quenching constants, K_{sv} and k_q, given in Table 2, with accessibility increasing in the order DM < DPC < $C_{12}E_8$. DM headgroups efficiently protected TOE from contacts with I^- (the ratio of k_q for TOE in DM to k_q for NATA in buffer was only 24%), whereas DPC and $C_{12}E_8$ headgroups were more permeable to this ion and/or larger transverse motions of TOE occurred in micelles of these detergents. For DM, these data are consistent with previous results in which the dynamic mechanism of quenching by I^- was also checked by both steady-state and time-resolved measurements [8].

To determine the role of the charge on the iodide in the limited accessibility of TOE in detergent, similar experiments were performed with acrylamide as a neutral quenching probe (Fig. 3B). In this case, the fitting of the reference curve (with NATA) required the use of the modified Stern–Volmer equation, which takes into account the presence of a static quenching process, in addition to the dynamic one. Linear fits were adequate for TOE in detergents indicating that static quenching was negligible for the

Table 2
Parameters of TOE fluorescence quenching in various detergents

Fluorophore	Medium	Quencher	$\langle\tau\rangle^g$ (ns)	K_{sv}^d (M^{-1})	k_q^e ($M^{-1}\,s^{-1}$)	V^f (M^{-1})
NATA	Buffer	Iodide	3[a]	12.5	4.2×10^9 (100%)	
TOE	DM	Iodide	2.0[b,c]	2.0	1.0×10^9 (24%)	
TOE	$C_{12}E_8$	Iodide	2.0[b]	5.5	2.7×10^9 (64%)	
TOE	DPC	Iodide	1.7[b]	3.0	1.8×10^9 (43%)	
NATA	Buffer	Acrylamide		17.4	5.8×10^9 (100%)	1.52
TOE	DM	Acrylamide		2.7	1.3×10^9 (22%)	
TOE	$C_{12}E_8$	Acrylamide		5.8	2.9×10^9 (50%)	
TOE	DPC	Acrylamide		7.1	4.2×10^9 (72%)	

[a]Determined in water [12].
[b]Determined in 4 mM detergent, 25 mM phosphate buffer (pH 7.5, 20°C) (see legend to Fig. 4).
[c]Mean value of three experiments in similar conditions.
[d]Obtained from Fig. 3A,B.
[e]Calculated as $k_q = K_{sv}/\langle\tau\rangle$.
[f]From Fig. 3.
[g]$\langle\tau\rangle = \Sigma\alpha_i\tau_i$.

acrylamide concentration range tested (0–0.2 M). The parameters of the fits are given in Table 2. Again, limited accessibility of TOE (to acrylamide) was observed in DM (~22% from the k_q ratio) whereas $C_{12}E_8$ was more permeable. In DPC, TOE was significantly more accessible to acrylamide (72%) than to iodide (43%). As the mean lifetimes of TOE in the three detergents were similar (see Table 2 and following paragraph) K_{sv} and k_q gave the same order of accessibility.

For NATA, the static component, described by a sphere of action with a radius of ~0.8 nm, was consistent with published data [9].

Finally, DM appeared to limit drastically the accessibility of TOE to both KI and acrylamide, whereas $C_{12}E_8$ and DPC were more permeable to both these compounds. Charge effects are not important for either of the two non-ionic detergents, in contrast to DPC.

3.4. Time-resolved fluorescence intensity decays of TOE in the various detergents

A knowledge of fluorescence lifetimes is required for the interpretation of quenching experiments, in particular to convert the Stern–Volmer quenching constant into bimolecular quenching constant, allowing comparison of accessibility of various fluorophores. Therefore, we recorded fluorescence intensity decays for TOE in the three detergent micelles, and in methanol for comparison. In no case was emission decay monoexponential, and three to four (in DM) lifetime populations were detected at the emission maximum (Fig. 4). Mean lifetimes were from 1.63 ns (in methanol) to 2 ns (in $C_{12}E_8$ and DM, see Table 2). The similarity in mean lifetimes was also consistent with the similarity in emission maxima of TOE in the same conditions (335 nm in detergent to 338 nm in methanol). The slightly lower mean lifetime value for TOE in DPC than in $C_{12}E_8$ or DM, is also consistent with its lower quantum yield value (see Fig. 1B). Looking in more detail at the lifetime distributions shows a subnanosecond lifetime population (0.1–0.3 ns, see legend to Fig. 4), a second population close to the ns (0.7–1 ns) and a third one of a few ns (2.2–3.4 ns) except in DM where this longest lifetime seems split into two populations. The main contribution (calculated as $\alpha_i\tau_i/\Sigma\alpha_i\tau_i$) to

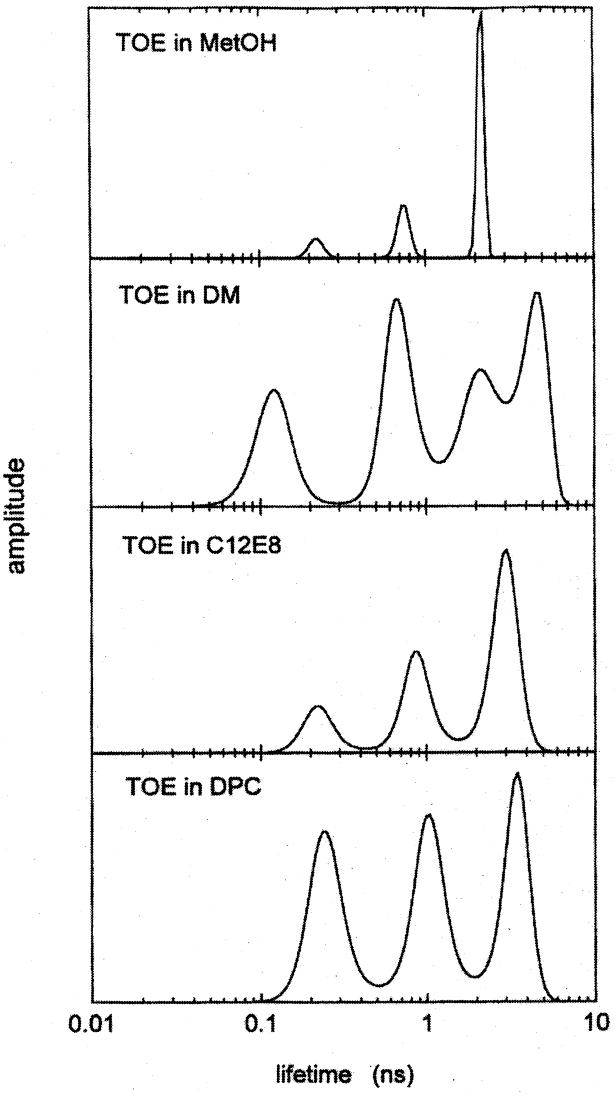


Fig. 4. MEM recovered lifetime distributions of TOE in DM, $C_{12}E_8$ and DPC micelles versus that for TOE in methanol. TOE concentration was 5 μM in 25 mM phosphate buffer (pH 7.5, 20°C), containing 4 mM DM, $C_{12}E_8$ or DPC. λ_{ex} = 280 nm, λ_{em} = 335 nm, excitation and emission bandwidths: 6 and 8 nm, respectively. The normalized area α_i and barycenters τ_i of each peak of the lifetime distribution were as follows. In DM: $\alpha_1 = 0.20$; $\alpha_2 = 0.30$; $\alpha_3 = 0.24$; $\alpha_4 = 0.27$; $\tau_1 = 0.12$ ns; $\tau_2 = 0.71$ ns; $\tau_3 = 2.09$ ns; $\tau_4 = 4.40$ ns; $\chi^2 = 0.992$. In $C_{12}E_8$: $\alpha_1 = 0.15$; $\alpha_2 = 0.30$; $\alpha_3 = 0.54$; $\tau_1 = 0.23$ ns; $\tau_2 = 0.90$ ns; $\tau_3 = 2.96$ ns; $\chi^2 = 1.04$. In DPC: $\alpha_1 = 0.33$; $\alpha_2 = 0.34$; $\alpha_3 = 0.32$; $\tau_1 = 0.26$ ns; $\tau_2 = 1.05$ ns; $\tau_3 = 3.40$ ns; $\chi^2 = 0.96$. In both $C_{12}E_8$ and DPC, a minor contribution ($\alpha < 1\%$), with a lifetime of 20 ns, was also detected. For the reference in methanol, TOE concentration was 20 μM, λ_{em} was set at 338 nm and bandwidths were 8 and 12 nm for excitation and emission, respectively: $\alpha_1 = 0.10$; $\alpha_2 = 0.24$; $\alpha_3 = 0.66$; $\tau_1 = 0.22$ ns; $\tau_2 = 0.74$ ns; $\tau_3 = 2.17$ ns yielding $\langle\tau\rangle = 1.63$ ns; $\chi^2 = 1.06$.

the steady-state intensity comes from the longest (or the two longer, in DM) lifetime population. The complexity of decay in DM has already been demonstrated and correlated with the existence of Trp rotamers and dipolar relaxation processes around the excited state [8]. Dipolar relaxation in methanol is too fast to be detected, so the three populations observed in methanol probably correspond to three rotamers of TOE (in slow exchange with the other rotamers on the fluorescence decay time scale). In $C_{12}E_8$ and DPC, dipolar relaxation is also suggested from emission wavelength dependence of fluorescence intensity decays (unpublished results).

3.5. *Time-resolved fluorescence anisotropy decays of TOE in the various detergents*

Anisotropy decays of TOE in micelles at emission maximum were also heterogeneous, with two to three measurable components, reflecting different modes of depolarizing rotation of the indole moiety of TOE. The parameters fitting the anisotropy decays are given in Table 3, along with the static anisotropy, r_s. Depolarization is partly due to very fast rotation of the indole moiety within the micelle: this is shown by the shortest rotational correlation time (θ_1) values in $C_{12}E_8$ and DPC (<100 ps), with significant relative preexponential factor β_1. In DM, the occurrence of such very fast rotation is indicated by the sum of the preexponential factors β ($\beta_1+\beta_2=0.125$) being lower than the theoretical limiting anisotropy value for indole in these conditions ($\sim$0.17–0.18 at $\lambda_{ex}=280$ nm) [15]. Components in the nanosecond range (1–2 ns) were observed in all three detergents. Only in DM, was a longer component that may correspond to the Brownian rotation of the whole micelle detected ($\theta_2=17.6$ ns). These data show that the TOE molecule is more mobile in $C_{12}E_8$ and DPC than in DM. This is also reflected by the values for static anisotropy, r_s, which increase in the order $C_{12}E_8 < DPC < DM$.

4. Discussion

TOE has been shown to be a suitable hydrophobic model of Trp in proteins both in model membranes [14,16] and membrane mimetic systems, such as DM micelles [8] or AOT reverse micelles [17]. In DM micelles, the indole moiety of TOE is embedded within the micelle and is highly accessible to bromine atoms on the alkyl chain of brominated DM analogs [8]. Molecular modeling in water and in vacuum suggested a hairpin conformation of this probe, in which the indole ring mainly occupies the *trans* position. This conformation was in agreement with the lifetime distribution data with a (nearly) major long lifetime, indicating that, for the corresponding conformation, the indole ring was apart from quenching contacts either with the carbonyl or the ammonium groups. In this conformation, the indole ring is partially folded on the octyl chain.

In this study, we assessed the permeability to water-soluble quenchers and the subnanosecond/nanosecond dynamics of the micelle headgroup region with three widely used detergents, DM, $C_{12}E_8$ and DPC. In all three cases, TOE readily bound to the detergent micelles and the spectroscopic properties of bound TOE (quantum yield: 0.08–0.12, emission maximum $\lambda_{max}\sim335$ nm, fluorescence lifetimes: 1.7–2 ns) suggested that the indole moiety was located in an environment of intermediate polarity. At λ_{max}, lifetime distribution showed similar patterns in $C_{12}E_8$, DPC and methanol as a reference. Three lifetime populations are detected, the longest one being the major one (or of similar amplitude than the others, in DPC). There may be a direct correla-

Table 3
Fluorescence anisotropy decay parameters of TOE in various detergents

Detergent	β_1	β_2	β_3	θ_1	θ_2 (ns)	θ_3	r_s
DM	0.072	0.053		2.09	17.6		0.091
$C_{12}E_8$	0.043	0.067	0.063	0.07	0.26	1.28	0.034
DPC	0.207	0.093		0.05	2.12		0.049

The β_i and θ_i values given here are the area and barycenter of each peak of the rotational correlation time distribution, respectively. r_s is the static anisotropy. Experimental conditions are given in the legend to Fig. 4.

tion between these three defined lifetime populations and Trp rotamers as proposed for proteins, peptides and model systems (see [18,19]; and for recent references [20–22] for instance). In DM, the occurrence of dipolar relaxation was suggested (due to slowly relaxing water molecules in the hydrated headgroup regions of the micelles), and may explain the more complex lifetime distribution pattern at the emission maximum in these micelles. Such slow solvent relaxation was also reported from observation of red edge excitation shift (REES) for TOE embedded in DOPC [14]. In $C_{12}E_8$ and DPC, such a relaxation process may exist but it is likely fast as in MeOH, and unobservable at the emission maximum.

Increasing the pH led to enhancement of the fluorescence intensity in all micellar systems, probably due to the suppression of the quenching effect of the ammonium group of the TOE moiety as proposed for tryptophan in buffer solutions [23]. This could, however, be an indirect effect of the amino group ionization state on the electrophilic properties of the carbonyl moiety, which is likely the most general quencher group in proteins [22,24]. A specific interplay between the ionization (and steady-state fluorescence) properties of TOE and the zwitterionic headgroup of DPC, was shown from the pH dependence curve of TOE fluorescence in DPC micelles. This curve was broader in DPC than in DM or $C_{12}E_8$. This suggested that the TOE ammonium group displayed a higher apparent pK in the excited states, possibly because electrostatic interactions between this group with the negatively charged phosphate moiety of the detergent took place. However, a possible charge heterogeneity of TOE in DPC at pH 7.5 does not result in any additional complexity of the lifetime distribution in this detergent nor in a significant broadening of the lifetime populations. Only the amplitude of the shortest lifetime is significantly higher in DPC than in the other micelles at pH 7.5. This is explained by the assumed pK shift. The rather similar lifetime distributions in the detergents ensure that the accessibility of similar TOE excited states are observed in the quenching experiments.

Quenching of TOE in micelles was performed with iodide which is thought to be a selective quencher of surface residues in proteins, and acrylamide, which may quench both external and embedded residues [10]. Acrylamide is a highly efficient quencher (i.e., the probability of extinction after a collision between quencher and fluorophore is close to 1), whereas iodide is slightly less efficient. The mechanisms of quenching, as analyzed by time-resolved fluorescence quenching of NATA, are thought to be electron transfer for acrylamide and an exchange reaction for iodide (see [25] and references therein).

For iodide, the reference value obtained with NATA in buffer is consistent with published data ($K_{sv} = 12.5\ M^{-1}$ versus $12.0\ M^{-1}$, [26]). DM headgroups provide an efficient barrier to I^- (relative accessibility, estimated from the ratio of k_q, was only 24%) whereas DPC and $C_{12}E_8$ were more permeable to this ion. For DM, this was not due to an ionic effect (as observed for quenching of an indole derivative by I^- in negatively charged SDS micelles, [9]). The low level of permeability with DPC was partly due to an ionic effect, as shown by the results obtained with acrylamide. In contrast, $C_{12}E_8$ was highly permeable to I^-. The calculated accessibility was 64%, and actual value must be even higher because corrections should be made for the difference in translational diffusion of NATA in water and the whole detergent micelle in which TOE is embedded. The quenching rate constant k_q is indeed proportional to the sum of the translational diffusion coefficients of quencher and fluorophore (D_Q+D_F) (the translational diffusion coefficient being given by the Stokes–Einstein equation: $D = kT/6\pi\eta R$). The reported diffusion coefficient of iodide (D_Q) is $2.0\times10^{-5}\ cm^2\ s^{-1}$ whereas that of NATA is thought to be similar to that of L-Trp (i.e., $0.55\times10^{-5}\ cm^2\ s^{-1}$; see for instance [26] and references therein; neglecting differences in hydration, geometry or density, $D_1/D_2 = R_2/R_1 = (M_{r2}/M_{r1})^{1/3}$, with M_r equal to 245 and 204 for NATA and L-Trp, respectively). For TOE in detergent, the relevant diffusion coefficient is that of the micelle. The diffusion coefficient of dodecylphosphocholine has been experimentally determined and is in the range of $8–9\times10^{-7}\ cm^2\ s^{-1}$ [1] whereas those of $C_{12}E_8$ and DM micelles, of larger micelle size (2–3-fold, see Table 1) are therefore slightly lower: all are negligible in comparison to that of iodide. The correction to be applied is therefore $(D_Q+D_F)/D_Q$, here equal to $(D_{I^-} + D_{NATA})/D_{I^-}$, i.e., 1.3. For $C_{12}E_8$, for instance, accessibility is 83% (versus 64% before correction).

In acrylamide, our reference K_{sv} value for NATA was again consistent with previously reported values (17.4 versus 17.5 M^{-1} in [9]). The accessibility of TOE increased in the order DM < $C_{12}E_8$ < DPC. In these conditions in which ionic repulsion (or attraction) is not involved, the high accessibility in DPC may be correlated with the small size of headgroup (see the M_r of the three detergents in Table 1). TOE may also show a broader location in the micelle, being on average slightly less deeply anchored in the DPC micelle, due to the presence of neutral and ionized forms (see the pH dependence of fluorescence). Changes in orientation and location of membrane-bound probes with ionization has already been described (see for instance, [27]). It may also have a higher level of transverse mobility with respect to the micelle. Consistent with this, the anisotropy decay results indicate that the rotational mobility of TOE is higher in both $C_{12}E_8$ and DPC than in DM. Reasoning similar to that used above applies to diffusion coefficient correction. A rough estimate of the correction factor $(D_Q+D_F)/D_Q$ of 1.7 was obtained (with $D_Q = D_{(acrylamide)} = 1.4$ $D_{L-Trp} \sim 0.8\times10^{-5}$ cm^2 s^{-1}, the molecular mass of acrylamide being 71). Application of this correction suggests that TOE is completely accessible to acrylamide in DPC (72% corrected to 122%). Values of over 100% may result from imprecision in the correction factor or slight favoring of the partitioning of acrylamide in the DPC micelle.

Even if it is located at the surface of a macromolecular object, the fluorophore only occupies (and is therefore only accessible over) a small area of this object; this may also lower the apparent k_q value. Such an effect has only previously been discussed in the case of a fluorophore attached to a macromolecule (a protein) impermeable to the quencher (iodide), and is dependent on the rotational diffusion coefficient of the macromolecule [28]. This case is different because the micelles (playing here the role of the protein) are at least partly permeable to the quenchers. This effect should not affect significantly comparison between the various detergents.

Note also that using mean lifetimes in the calculation of the quenching constant from the steady-state measurements implicitly assumes that all rotamers have an equal chance of being quenched. Since the longest (or the two longer in DM) lifetime populations mainly contributes to the fluorescence intensity, the calculated accessibility at least closely reflects that of the corresponding conformers.

5. Conclusion

Many attempts have been made to rationalize the use of detergents for membrane protein studies. The present results provide clear evidence that the headgroup region is significantly less permeable to soluble quenchers in DM micelles than in $C_{12}E_8$ and DPC micelles. The micelle-bound probe (TOE) is also significantly less mobile in DM than in the other detergents. The headgroup cohesion of DM may be one parameter favoring the stabilizing effect of this type of detergent on various membrane proteins, by limiting the water penetration and thus protecting hydrophobic parts of the proteins from denaturation. A similar conclusion was reached in recent studies of Laurdan in octylglucoside [29]. These results also show that the zwitterionic headgroup of DPC discriminates between ionic and neutral quenchers. Overall, they provide initial reference data for analyzing Trp insertion in peptide (or protein)–detergent complexes. To progress along this line, the study of a more complex reference system where Trp is inserted in hydrophobic model peptides in under way using the same approach.

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