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Use of a fluorescein derivative of phosphatidylethanolamine as a pH probe at water/lipid interfaces

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A fluorescent indicator for the determination of pH in the vicinity of water/lipid interfaces was produced by the covalent linkage of fluoresceinisothiocyanate to *Escherichia coli* phosphatidylethanolamine. When embedded in monolayers spread on an air/water interface, its apparent p*K* was shown to be only slightly affected by the nature of the polar headgroups or the packing density of the host phospholipids. Its properties were not affected by the ion content of the subphase. For small unilamellar vesicles, its properties were only affected when localized in the inner layer. This probe could therefore be of value in the study of proton fluxes along biological membranes.

Introduction

Many biological processes related to membrane bioenergetics are thought to be driven by localized pH gradients in membranes [1]. Such mechanisms can only be verified by direct measurement of proton concentrations in the vicinity of the membrane. Fluorescence spectroscopy would seem well suited for this application, since many chromophores are pH sensitive. In order to measure the pH in the neighbourhood of a lipid membrane, Frommherz employed a pH-sensitive fluorescent chromophore covalently bound to a fatty acid chain [2]. The value of this technique has been confirmed by other workers [3,4]. In order to match the probe more accurately to the relevant membrane phospholipids, Thelen and co-workers bound the chromophore directly to the polar group of a constituent phospholipid [5].

In practice, two characteristic features of a lipid/water interface must be taken into account: (i) the marked fall in dielectric constant of the medium going from the aqueous phase ($\epsilon = 80$) to the fatty acid chains ($\epsilon = 2$); (ii) electric fields are generated by surface charges on the polar headgroups which can make the interfacial ionic concentrations differ from those in the bulk phase. On the basis of both theoretical considerations and careful experimentation, Fernandez and Frommherz demonstrated that the p*K* of an ionizable group such as a pH sensitive dye is highly sensitive to both these characteristic features [6]. It is therefore important to check that the properties of a fluorescent probe are not affected by the organization of the polar heads of the lipid matrix.

We describe here the synthesis and use of a fluorescein derivative of phosphatidylethanolamine. Its fluorescence properties were determined

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Abbreviations: FITC, fluorescein isothiocyanate; FPE, fluorescein phosphatidylethanolthiocarbamide; SUV, small unilamellar vesicle.

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for different polar headgroups and ionic content of the subphase under various conditions of packing density in monolayers.

Materials and Methods

Chemicals

All phospholipids were obtained from Sigma (U.S.A.) or were purified from *Escherichia coli* as described in Ref. 7. Fluorescein isothiocyanate (FITC) was purchased either from Sigma (U.S.A.) or from Serva (F.R.G.) All solvents were of analytical grade. Ultrapure water for the monolayer experiments was prepared using a Milli. Q system (Millipore, France).

Synthesis of FPE (fluorescein phosphatidylethanolthiocarbamide)

The synthetic scheme was adapted from methods described in the literature [5,8]. 5 mg of *E. coli* PE, 10 mg of FITC and 25 μl of triethylamine (stored in contact with anhydrous KOH) were dissolved in 2 ml of peroxide-free tetrahydrofuran (THF). This solution was kept overnight in the dark at room temperature. Solvents were evaporated in vacuo. Since FITC is present in large excess, it must be eliminated before purification of FPE. The crude reaction mixture was poured onto a G-25 Sephadex (coarse) column (Pharmacia, Sweden) (50 ml) packed in $CHCl_3$/CH_3OH (95 : 5, v/v) saturated with water, and rapidly eluted with the same solvent system (70 ml). Prior to use, the column was packed with methanol/water (1 : 1, v/v). Two runs were required to eliminate most of the residual FITC. FPE was further purified by preparative thin-layer chromatography on silica gel G with chloroform/methanol/water (65 : 25 : 4, by vol.) as eluting solvent. FPE was obtained at over 98% purity on the basis of the observed fluorescence signal, and from phosphorus determination by the Dittmer reaction. After drying under a stream of nitrogen, the FPE was kept at $-20\,^{\circ}C$.

Microvesicles

Lipids and probe were first mixed in an organic solvent which was evaporated. The lipidic film was resuspended in phosphate buffer (pH 7.5). The suspension was sonicated for more than 1 h in a bath sonicator (Ultrasons 75 T801, France). Residual multilamellar liposomes were centrifuged out at $100\,000 \times g$ during 10 min.

The fluorescence emission of the small unilamellar vesicles (SUVs) was observed on a spectrofluorimeter (JY3, Jobin Yvon, France) (λ_{exc} = 480 nm, λ_{em} = 520 nm, slit width = 10 nm).

Monolayer and fluorescence experiments

A front-face fluorimeter designed for the study of monolayers was used [9,10]. The trough was constructed of plexiglass in order to reduce the level of scattered light. The film was compressed using a movable Teflon barrier. The surface pressure was measured by the platinum plate method [11].

Monolayers were obtained by spreading a mixture of phospholipids and FPE (molar ratio 98 : 2) in solution in $CHCl_3$/CH_3OH (5 : 1, v/v) onto an aqueous subphase (10 mM phosphate buffer at a well-defined pH). The ionic strength of the subphase was increased in some experiments by adding NaCl. The size of the trough ($60 \times 240 \times 10$ mm) was such that edge effects did not affect the behavior of the film. After spreading the film, the organic solvents were allowed to evaporate for a period of 3 min. Compression was always carried out slowly (less than 0.1 nm^2/min).

For the fluorescence experiments, the exciting beam (wavelength centered on 462 nm, bandwidth larger than 10 nm) was focused on the monolayer in a 4 mm diameter spot. The incident beam was linearly polarized, either parallel to the monolayer (X) or to the incidence plane (YZ), by means of a Glan prism. The emitted light was measured perpendicular to the plane of the monolayer through an interference filter (wavelength centered on 520 nm, bandwith approx. 10 nm) with a photomultipler. Under our experimental conditions, the signal from light scattered by the aqueous subphase was of the same order of magnitude as the fluorescence signal.

Orientation of the absorption moment of the fluorescent chromophore was obtained by using linearly polarized incident light and by computing the ratio X/YZ of the fluorescent light emitted when the exciting beam was polarized either parallel to the monolayer (X) or to the incidence plane (YZ) [9]. This ratio is a function of both the mean

orientation of the chromophore and of the fluctuation angle, taking oscillations of the probe around its equilibrium position into account.

Determination of the apparent pK (pK_{app})

pK_{app} was taken as the subphase pH at which the fluorescence F emitted by the film for a given surface pressure π obeyed the relationship.

$$\frac{F(pK_{app}, \pi) - F(\text{pH } 4, \pi)}{F(\text{pH } 7.5, \pi) - F(pK_{app}, \pi)} = 1$$

the indicated pHs being those of the subphase. This operational definition was based on the observation that the fluorescence was not related in any obvious way to the subphase pH for values of pH outside the range 4.5–7.2. It was obtained by compressing films on subphases with different values of pH, and recording the fluorescence intensity and the surface pressure as functions of the molecular area (see Fig. 1).

Results

Apparent pK changes on film compression

When compressing a film containing FPE, the fluorescence was observed to increase. This was primarily due to the increase in the probe density in the illuminated area. This relative change was observed to be dependent on the pH of the subphase for values of pH between 4.5 and 7. As shown in Fig. 1, for a given surface pressure, the plot of the fluorescence of the film against pH of the subphase was sigmoidal. From these curves, it was possible to compute an apparent pK (pK_{app}) as defined in Methods. From Fig. 1, it was concluded that this pK_{app} depended on the molecular packing. This is shown clearly in Fig. 2. The pK_{app} increased on compression.

Dependence of pK_{app} on the nature of the host lipid

Figs. 2 and 3 show the changes in pK_{app} on compression when the probe was embedded in films formed from two different lipids: one uncharged (phosphatidylethanolamine) and the other negatively charged (lipid extract from *E. coli*). By comparing the results for each system when the film was spread on the same subphase, it was found that the two pK_{app} values were almost identical. Nevertheless, the changes of pK_{app} on compression of the film were larger for the charged host film than for the neutral one.

Dependence of pK_{app} on the ionic content of the subphase

Electrical properties of lipid/water interfaces are known to be strongly dependent on the ionic strength of the aqueous phase. The thickness of

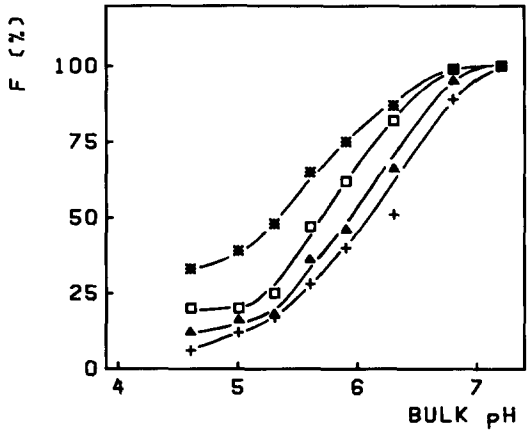

Fig. 1. Fluorescence intensity of FPE in a PE monolayer versus the bulk subphase pH for different packing density of the monolayer. The intensity at pH larger than 7.5 was taken as 100% in each case. The subphase was a phosphate buffer 10 mM with 0.1 M NaCl. The surface pressure of the film was: lift-off (•), 10 mN/m (□), 20 mN/m (△), 30 mN/m (+).

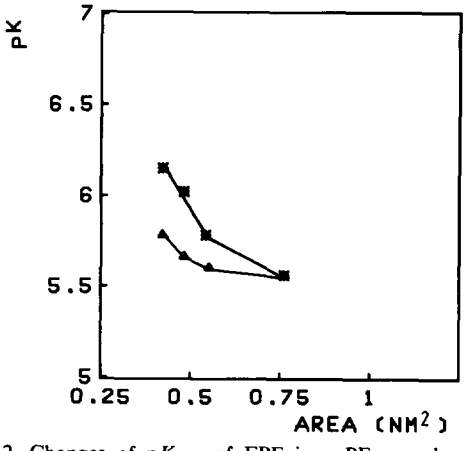

Fig. 2. Changes of pK_{app} of FPE in a PE monolayer as a function of the molecular area. The subphase was a phosphate buffer 10 mM in the presence (•) or absence (△) of a 0.1 M NaCl concentration.

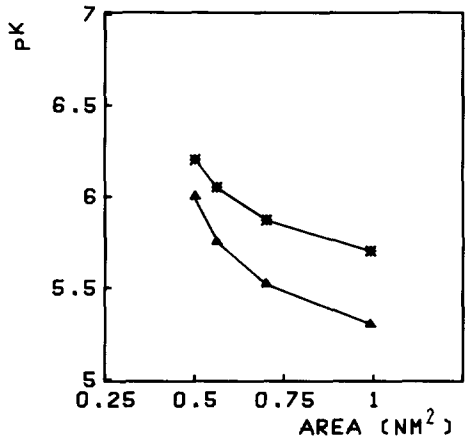

Fig. 3. Changes of FPE pK_{app} in a *E. coli* lipid extracts monolayer as a function of the molecular area. The subphase was a phosphate buffer 10 mM in the presence (*) or absence (△) of a 0.1 M NaCl concentration.

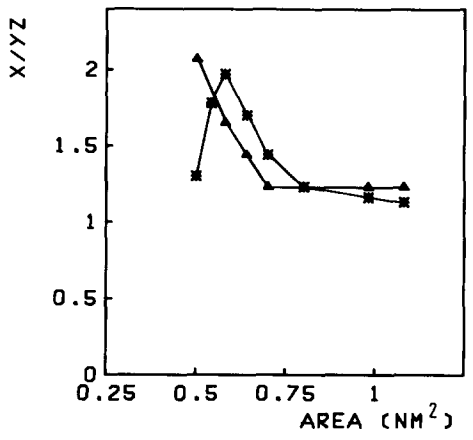

Fig. 4. Changes in the orientation ratio *X/YZ* of FPE in a *E. coli* lipid extract monolayer versus the packing density. The subphase pH was set at 4.5; 0.1 M NaCl was present ((△) or not (*).

the Gouy-Chapman layer falls and the electrical potential decreases as the ionic content increases. pK_{app} was observed to be affected by changes in the salt content of the subphase as shown in Figs. 2 and 3. Lower pK_{app} values were observed when monolayers were spread on subphases to which 0.1 M NaCl had been added, the host lipids being either neutral (PE) or negatively charged (extracts from *E. coli*). Interestingly the change in pK_{app} on film compression was close to zero when a PE film was spread on a subphase with a high ionic content (Fig. 2).

Effect of the phase transition of the host phospholipid on FPE pK_{app}

The phospholipid phase transition in a monolayer can be observed by changing its molecular area. At 21°C, dipalmitoylphosphatidylcholine (DPPC) is in the liquid expanded state when its surface pressure is equal to 5 mN/m, but is found in the solid-condensed state at 36 mN/m. Determination of the pK_{app} of FPE in DPPC monolayers showed it was not influenced by the phase states of the host.

Change in orientation of FPE on film compression

By use of a linearly polarized incident light (see Methods), changes in the orientation of the probe were evaluated from the characteristic ratio *X/YZ*.

For a neutral film (PE) this ratio was observed to be independent (1.13 ± 0.05) of film compression, subphase pH (either 4.5 or 7) or subphase ionic content (presence or absence of 0.1 M NaCl) (data not shown).

When the film was negatively charged (extracts from *E. coli*), *X/YZ* remained constant on film compression on a subphase at pH 7, irrespective of the presence of 0.1 M NaCl (data not shown). In contrast, on an acidic subphase (pH 4.6), *X/YZ* was observed to rise with increasing molecular packing (Fig. 4). This increase was monotonic when the ionic content was high (0.1 M NaCl), although a sudden fall was observed at high surface pressure in the absence of salts in the subphase.

pK values in small unilamellar vesicles (SUVs)

SUVs are often used as model systems for biological membranes. Due to their high curvature (external diameter less than 30 nm), it has been shown that the molecular organization of the internal and external layers is not identical [12,13]. On the basis of the results on monolayers, the pK_{app} of FPE were not expected to be the same on the two leaflets of a SUV. In order to determine the pK_{app} values, we took advantage of the relatively slow diffusion of H^+ and OH^- across a bilayer [14,16]. The SUV were first equilibrated at a given pH (7.5, or 4.0), then the pH was shifted slightly by adding a small aliquot of HCl or NaOH. The fluorescence change associated with

TABLE I

PK_{app} OF FPE IN EGG PHOSPHATIDYLCHOLINE SUV

Ionic content	Outer layer	Inner layer
No salt added	6.2	7.2
+0.1 M NaCl	6.4	7.4

The pH buffer was a 10 mM phosphate.

this shift in pH was observed to be biphasic. The external layer was affected immediately (during mixing), but the internal layer took several seconds to equilibrate to the new pH. In this way the pH could be titrated for both layers. The results are shown on Table I for egg phosphatidylcholine (PC). They confirm the difference in organization between the two layers in a SUV. For the outer layer, the pK_{app} values were similar to those found in the monolayer experiments. For the inner layer, there was a marked shift to higher values. Such high values of pK_{app} were not observed in the monolayers at any degree of lipid molecular packing (Fig. 2). However, as for the PE monolayers, the values of pK_{app} were not affected by the ionic content of the subphase.

Discussion

The use of interfacial pH probes is frought with problems of artefacts. Firstly, the indicator must be localized close to the interface, and must remain there during the experimental measurements. Use of a pH-sensitive chromophore covalently linked to a fatty acid chain has been found to overcome this problem [2]. This approach was chosen here using FPE produced by covalent linkage of fluorescein to a phospholipid. Secondly, lipid/water interfaces are affected by the steep change in the dielectric constant of the environment, and by the very strong electric fields produced by the ionized polar head groups. These factors are known to affect the pK_{app} of pH indicators [6]. Fortunately, they are only short range effects. If the chromophore is sufficiently separated from the polar heads it will not be affected either by their high electric fields or low dielectric constants. For FPE, the values of pK_{app} were only slightly affected by compression of a PE monolayer. The change was less than 0.2 pH units for an ionic content of the subphase of 0.1 M, i.e. when the electrical terms were attenuated. The small alteration observed was attributed to changes in the dielectric constant in the environment of the probe [6]. Absence of a disturbance of the probe environment is also supported by the observation that the average orientation of the probe was not altered by film compression. For this zwitterionic lipid, when the ionic content of the subphase was reduced, a slight influence of the polar heads was detected. At low surface pressure (expanded film), it was negligible, although after compression it attained 0.4 pH units as compared with a high ionic content subphase at high surface pressure (Fig. 2). This is only a small change, indicating the weakness of the contribution of the electric field (dipolar origin). This conclusion is also supported by the lack of change in the average orientation of the probe.

On compression of the *E. coli* lipid extracts spread on a low ionic content subphase, changes in pK_{app} were observed (Fig. 3). They were somewhat larger than those found for the PE monolayers, but were nonetheless quite small (less than 0.7 pH unit), indicating a small contribution from the electrical term. In the presence of 0.1 M NaCl, changes in pK_{app} comparable to those for PE were observed. Thus, it would appear that FPE is far enough away from the polar head region to be largely unaffected by their associated electric field. The values for the orientation parameter X/YZ also support this conclusion, since the average orientation was the same on compression at pH 7 whatever the ionic content of the subphase. For subphases at low pH (4.6), orientation was strongly affected by compression. This is in agreement with results using dansylcephalin where the position of the chromophore has been shown to be dependent on lipid packing [3]. It is known that the state of ionization of polar phospholipids is strongly dependent on the pH of the subphase [17]. At low pH, protonation of the polar heads will reduce the interfacial surface charge, and perhaps also affect the organization of the headgroup region. In this case, FPE acts as a structural probe in the interfacial region. But it should be stressed that this effect on the orientation of the probe was only observed at low bulk pH. This is outside the physiological range.

A general conclusion is that the values of pK_{app} for FPE are only marginally affected either by the packing and nature of the host monolayer, or by the ionic content of the subphase (less than 1 pH unit between extreme conditions). This probe does not, therefore, appear to be influenced significantly by the dielectric or electrostatic environment in the interface between the hydrophobic and aqueous phases. Using PE on a high ionic content subphase, the pK_{app} value was in fact close to that of the parent chromophore (fluorescein) dissolved in water. This result indicated that the chromophore projected away from the interface, and that its environment was essentially that of the bulk water phase.

A puzzling observation was the marked difference in pK_{app} values for the internal layer of the SUVs. This suggested that there are distortions in the packing of the polar head regions in this highly curved lipid matrix. This is in line with the results of NMR studies of SUVs [12,13]. Care should, therefore, be taken when using this probe to study proton fluxes across membranes of very small particles. In this case, the values of pK_{app} for both sides of the vesicles need to be determined.

Our values of pK_{app} differ from published values for similar compounds [6,8]. However, Thelen and co-workers [5] used a phospholipid whose fatty acid chains were fully saturated. In the present study, in order to avoid possible phase separation due to the nature of the fatty acid chains when the host matrix is composed of unsaturated chains, we chose to use phosphatidylethanolamine purified from *E. coli*. From the pK_{app} values, it appears that the chromophore is more sensitive to interfacial phenomena (larger shift in pK_{app} relative to the parent fluorescein) when bound to a fully saturated phospholipid. In this case, the chromophore may lie nearer the lipid polar head groups of the host.

The FPE derivative described here represents a useful tool for the study of lateral proton conduction along lipid polar headgroups [18–20] since its fluorescence characteristics are particularly sensitive to the presence of protons. In addition, it is localized in the layer adjacent to the monolayer away from the influence of the polar headgroups. Thus in combination with surface potential measurements, this probe could be employed to demonstrate the existence of steep pH gradients from the monolayer surface towards the bulk phase [21].

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