Biochimica et Biophysica Acta, 766 (1984) 161–168
Elsevier

BBA 41536

THE USE OF FLUORESCEIN-DIPALMITOYLPHOSPHATIDYLETHANOLAMINE FOR MEASURING pH-CHANGES IN THE INTERNAL COMPARTMENT OF PHOSPHOLIPID VESICLES

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(Received February 17th, 1984)

Key words: Fluorescein-phosphatidylethanolamine; pH change; (Phospholipid vesicle)

The synthesis and characterisation of fluorescein-phosphatidylethanolamine (FPE) is described. The effects of dielectric constant, ionic strength and ambient pH upon the optical absorbance properties of FPE are presented. It is shown that under appropriate conditions, FPE rapidly and quantitatively reports the pH of the aqueous bulk phases when incorporated into phospholipid vesicles. It is also shown that, when the external medium is highly buffered, FPE is capable of specifically reporting only the pH of the intravesicular compartment. The application of FPE for studies of intravesicular pH changes of reconstituted membranous protein systems is discussed.

Introduction

The use of phospholipid vesicles for the study of the vectorial properties of membrane proteins has become a widespread and routinely used technique [1]. In particular, phospholipid vesicles have been employed to demonstrate the ion-transporting properties of the membrane-bound electron transport complexes and of solute-porter systems [2], some of which involve proton movements across the bilayer. Precise measurements of the pH changes on one or both sides of the membrane are thus useful parameters for the elucidation of the stoichiometries and ultimately of the mechanisms of the transport processes. Such pH changes, as a result of transport events, have been successfully and directly measured with pH-electrodes or water-soluble dyes located in the outer medium [3–7]. In principle, inverted pH changes, with respect to those of the external medium, may be measured with water-soluble dyes located in the intravesicular compartment [8,9]. In practice, however, such measurements have rarely been successful due to technical difficulties; for example, it is not possible to ensure that the phospholipid vesicles are completely impermeable to water-soluble dyes.

It has been pointed out that changes of extravesicular pH as a result of processes across the membrane may not only be the consequence of proton translocation, but also of proton release from the membrane outer surface [10]. Measurements of both intra- and extravesicular pH changes, therefore, would provide unequivocal evidence for the existence of proton transport phenomena across membranes.

In this study, it will be shown that by covalently linking fluorescein to phosphatidylethanolamine

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Abbreviations: FPE, dipalmitoyl-DL-phosphatidylethanolaminethionylaminofluorescein; DPPE, DL-α-dipalmitoylphosphatidylethanolamine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; CCCP, carbonyl cyanide *m*-chlorphenylhydrazone.

(PE), a pH indicator can be obtained which may be successfully incorporated into phospholipid vesicles. Under suitable experimental conditions, it provides a reliable quantitative indicator of the intravesicular pH.

Materials and Methods

FPE was synthesised in the absence of light and at room temperature, if not otherwise stated, as follows: 150 mg DPPE, dipalmitoyl (PE) and 85 mg fluoresceinisothiocyanate isomer I were dissolved in 30 ml of 29 : 1 chloroform/triethylamine (water-free solution) and stirred for 48 h until no PE could be detected from an aliquot analyzed by TLC (see below). The solvents were evaporated under reduced pressure at 30°C and the remaining solid residue was dissolved in approx. 3 ml chloroform/methanol/acetone/acetic acid (5 : 1 : 2 : 1, v/v). The resulting orange solution was then loaded onto an 80 cm × 2 cm silica-gel 60 column (mesh 70–230) equilibrated with the same solvent mixture. The flow rate of the column was adjusted to 2 ml/min. Fractions of 4 ml were collected and analyzed for phosphate (see below) and fluorescein (spectroscopically). Fractions containing the product FPE were well separated from free fluorescein and pooled. To this pool (approx. 100 ml), 20 ml water was added to extract most of the acetic acid- and water-soluble compounds. The aqueous phase was adjusted to pH 7.4 initially with solid ammonium carbonate followed by ammonium bicarbonate. The aqueous phase was then discarded and the organic phase was carefully dried by raising the ambient temperature up to 45°C under reduced pressure. The dry compound was then resuspended in water and lyophilised. The last step was repeated twice to remove all water-soluble volatile compounds such as ammonium acetate. 200 mg of a fine red powder, FPE, were obtained and stored at −20°C. TLC analysis was carried out on Kieselgel 60 plates using a chloroform/methanol/acetone/acetic acid (5 : 1 : 2 : 1, v/v) solvent system. This analysis gave two spots, both containing phosphate which corresponded to the two hydration states of the lactone ring in the fluorescein molecule. Two-dimensional TLC analysis on the same plates, according to Awasthi et al. [11], indicated that only one lipid component was present. It was not possible to detect the exact melting point of the FPE due to its decomposition occurring during the measurement.

Inorganic phosphate was assayed by development of the TLC-plates with the Zinzadze reagent.

The fluorescence excitation maximum of FPE was found to be 494 nm. The emission maximum was 516 nm, the experimental conditions for the fluorescence measurements were the same as those described in the legend of Fig. 4.

Liposomes for spectral analysis (see Figs. 4 and 5) were prepared as follows: 20 μl from a stock solution of FPE (2 mg/ml) in chloroform/methanol (5 : 1, v/v) were pipetted into a sonication vessel and dried under a gentle stream of nitrogen. 12 mg asolectin and 4 ml 200 mM KCl/2 mM Hepes (pH 7.5) were added. The mixture was then sonicated to clarity at 0°C and under nitrogen with a MSE sonicator. The clear solution was centrifuged for 5 min at 3000 rpm on a bench centrifuge. 2 ml of the supernatant was used to carry out the titrations (Figs. 4 and 5). The total amount of acid or base used was less than 1% of the sample volume. The pH was constantly monitored throughout the course of the titration with a rapidly responding pH electrode.

Proteoliposomes were prepared for the measurement of the buffering capacities and intravesicular pH calibrations, essentially by the so called 'cholate dialysis' procedure [8] as follows: a mixture of 40 : 1 mg/mg asolectin/FPE was well dispersed in a few ml of $CHCl_3$ and dried under nitrogen. To the dry lipid mixture, a solution of 25 mM sucrose/2.5 mM $CaCl_2$/24.5 mM potassium cholate was added to give a final asolectin concentration of 54 mg/ml. The mixture was sonicated as described and adjusted to pH 7.4 after the sample was centrifuged as before. To 1 ml of the clear supernatant, 342 μl of 29.5 μM cytochrome *c* oxidase, 900 mM KCl, 24.5 mM potassium cholate, 25 mM KPO_4 (pH 7.4) were added and rapidly mixed. The sample was dialysed for 4 h against 100 vol. 6.67 $g \cdot l^{-1}$ bovine serum albumin/25 mM sucrose/125 mM KCl/2 mM $CaCl_2$, followed by 4 h against 200 vol. 0.83 $g \cdot l^{-1}$ bovine serum albumin in the same medium, and finally for 14 h with the same volume of a bovine serum albumin-free medium. All dialysis media were adjusted to pH 7.4 prior to use.

The pH changes measured with an electrode were recorded with a Radiometer pH meter PHM64 connected to a custom-built 5-step Butterworth amplifier for noise reduction, the output of which was digitally stored with a Gould 20 MHz oscilloscope and traced with a Bryans BS-271 paper chart recorder. Absorbance measurements were carried out with an Aminco DW2a dual wavelength spectrophotometer equipped with a magnetic stirring device.

Lipid-depleted cytochrome *c* oxidase was prepared according to Yu et al. [12] and contained 9.1 nmol heme *a*/mg protein calculated from the oxidised-reduced spectra using an extinction coefficient of $\varepsilon_{605-630\,nm} = 13.5\ mmol^{-1} \cdot cm^{-1}$ per heme *a*. The composition of the asolectin was as described by Casey et al. [13] and was prepared according to Kagawa et al. [14] and modified as by O'Shea et al. [8].

Bovine serum albumin, essentially fatty acid-free, Hepes, DPPE, fluoresceinisothiocyanate, valinomycin and CCCP were purchased from Sigma Ltd., St. Louis. All other chemicals were of analytical grade.

Results and Discussion

The structural formula of FPE (M_r 1081.35) is shown in Fig. 1.

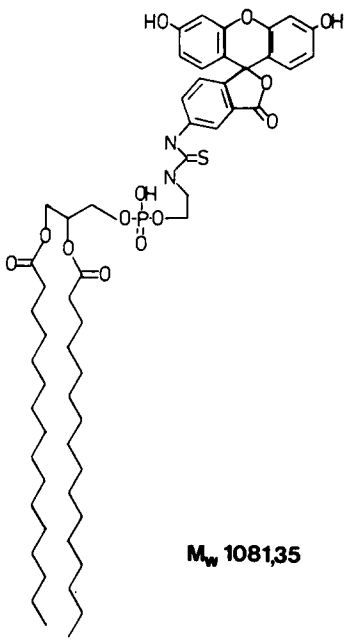

Fig. 1. The fully protonated structure of FPE.

An indication that the pH-sensitive chromophoric group attached to the amino group of DPPE after incorporation in phospholipid vesicles is not located in the lipid phase but in the diffuse double layer, may be inferred from the experiments reported in Fig. 2. The absorbance spectra of FPE when dissolved in solvents of differing dielectric constant are compared. When the probe was dissolved in media of increasing dielectric constant, the absorbance maximum (λ_{max}) was red-shifted from 458 nm in $CHCl_3$ (dielectric constant: 4.8) to 495 nm in the ethanol/chloroform mixture (dielectric constant: 24). The red shift was accompanied by a marked loss in the total absorbance. (Note that the decrease in absorbance in ethanol/chloroform is in effect bigger than it appears from the spectra in Fig. 2 due to the higher concentration of FPE in this solvent than in pure $CHCl_3$). Since the dielectric constant of the water/membrane interface, i.e., the diffuse double layer, is of the order of 20–30 [15], and therefore is comparable with that of the ethanol/chloroform mixture, a similar absorbance maximum of FPE was expected when it was reconstituted into detergent micelles or phospholipid vesicles. This feature is reflected in the experiment shown in Fig. 3, whereupon FPE was incorporated into micelles of the non-ionic detergent, Triton X-100. At neutral pH, the resultant spectrum was very similar to that obtained in ethanol/chloroform (trace B of Fig. 2). An increase of the ambient pH gives rise to a sharp increase of the total absorbance in the λ_{max} region between 490 and 500 nm.

The spectral pattern of FPE taken at a relatively high ionic strength (200 mM KCl) and shown in Fig. 3 is similar to that observed in a medium of low ionic strength (data not shown). The point of maximal increment of the absorption change produced by the addition of base, however, was found to be shifted from pH 7.2 to 7.7, in the latter, which can be interpreted as an equivalent shift of the apparent p*K* of the chromophore.

When FPE was incorporated into phospholipid vesicles (asolectin), a decrease of the net absorbance with respect to that in Triton X-100 was observed. Since the experiments were carried out

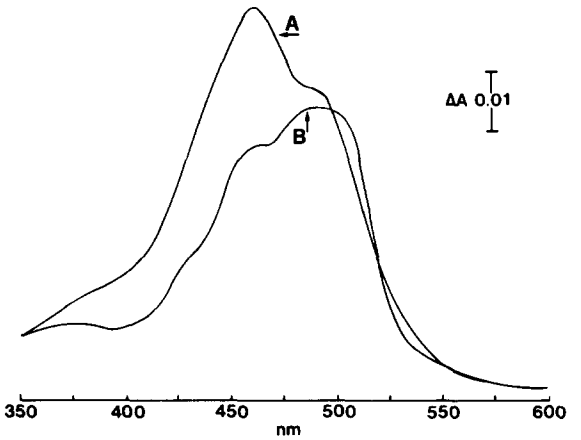

Fig. 2. Spectra of FPE taken in (A) $CHCl_3$, 7.7 μg FPE/ml and (B) 37:3 ethanol/$CHCl_3$, 14.8 μg FPE/ml.

in the presence of optimal concentrations of CCCP and valinomycin, it may be excluded that the spectral difference observed were caused by a different (lower) pH inside the vesicles during the titration as a result of the membrane permeability barrier. This phenomenon may be the consequence of an electrostatic effect upon the probe by the membrane surface potential (see below). Under the conditions described above for FPE in Triton X-100 micelles, a shift of the apparent pK of FPE from 7.5 to 8.5 was also obtained by lowering the ionic strength of the medium from 200 mM KCl to a KCl-free medium (not shown).

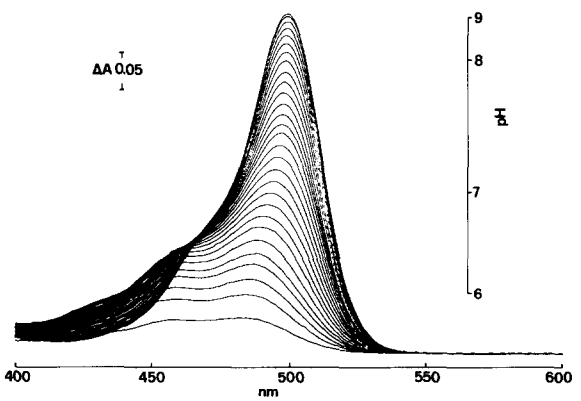

Fig. 3. Titration of FPE in Triton X-100. 20 μl 2 mg/ml FPE in 5:1 $CHCl_3$/methanol were dried under a gentle stream of nitrogen. 4 ml 0.1% Triton X-100, 200 mM KCl, 2 mM Hepes were added and sonicated in a bath sonicator to clarity. Final FPE concentration was 10 μg/ml, apparent pK_{FPE} 7.2. The titration was carried out with 0.5 M KOH.

The foregoing observations indicate that the chromophoric group of the FPE may be located in the diffuse double layer associated with the membrane/water interface and as such will be in rapid electrochemical equilibrium with the bulk medium. The latter conclusion is further supported by the observation that, when incorporated into phospholipid vesicles, FPE responds to a pH jump within the mixing time of the system, as shown in Fig. 6. Since the probe was observed to respond to both pH and the surface potential of the membrane, conditions were required for the measurement of the bulk medium pH, without possible interferences of the surface potential changes.

Criteria for the measurement of bulk phase pH

(a) Composition of the medium. Upon addition of monovalent salts to FPE-containing phospholipid vesicles (supplemented with CCCP and valinomycin), an absorbance increase was observed, which was larger if an equal concentration of divalent or trivalent cations was added. Thus, it appeared that the ionic strength and, particularly, the valency of the cations present in the medium affected the response of the probe. Such a phenomenon is explained by the Gouy-Chapman theory [16] which describes the relationship between the membrane surface potential and the membrane surface charge density which is related to the ionic composition of the bulk medium. Thus, the effect of a change of medium ionic strength can induce a change of the membrane surface potential which then may electrostatically affect the absorbance characteristics of FPE. From this theoretical approach, it can be concluded that to limit surface potential problems, the bulk media should be adjusted to a fairly high ionic strength and have a divalent cation concentration of about 1–3 mM [17], depending upon the cation chosen. In fact, the presence of monovalent cations alone in the bulk medium of a phospholipid vesicle suspension with incorporated FPE did not prevent absorbance changes upon salt addition, due to insufficient shielding of the surface charge.

In order to exclusively monitor small pH changes of the weakly buffered inner compartment of the phospholipid vesicles, the external medium must be highly buffered to exclude any pH response of the probe oriented in the extra vesicular

space. This was achieved by initially preparing the phospholipid vesicles in 125 mM KCl/25 mM sucrose/2 mM $CaCl_2$ (last dialysis medium) and resuspending them in 100 mM KCl/2 mM $CaCl_2$/50 mM potassium-Hepes (pH 7.3). Such conditions also fulfilled the requirements discussed above with respect to limit the absorbance changes due to the addition of cations. Since Hepes and sucrose are known not to interact with the membrane surface [18], it was possible to use both indiscriminately, depending upon the buffering properties required. However, in order to maintain an equal ionic strength in both compartments, sucrose and KCl were used to equate both the osmotic and ionic status of Hepes.

The medium composition discussed represents a compromise between the requirements for monitoring intravesicular pH changes whilst excluding surface phenomena and the conditions needed to measure the vectorial activity of the protein reconstituted in the phospholipid vesicles. In addition, the medium employed here is very similar in its composition to those often used in reconstitution studies [2–8].

(b) Selection of suitable measuring wavelengths. A linear increase of the FPE optical absorbance over physiological pH ranges was obtained over a fairly broad spectral bandwidth. Fig. 5 shows that at 488 nm, the absorbance was linear when the ambient pH was changed by almost 2 pH units, whereas at 504.5 nm, the probe response exhibited two linear sections over the same pH range. Since the expected intraliposomal pH change which would occur during activity measurements of reconstituted transport proteins are rather small, the use of FPE even at 504.5 nm will generate reliable results. It is relevant to mention that measurements of redox-linked proton translocation at this wavelength have often been carried out [4], since the oxidised/reduced spectrum of cytochrome *c* exhibits an isosbestic point at 504.5 nm at which no interference of its redox changes on the absorbance measurements occur. Artefactual absorbance changes caused by light scattering of the vesicular suspension may be avoided by application of dual-wavelength spectroscopy. For this technique, any wavelength greater than 550 nm may be used without precaution of changing linearity of the probe response (as indicated in Fig. 4).

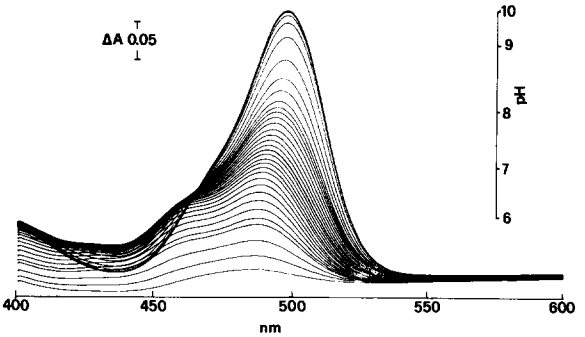

Fig. 4. Titration of FPE incorporated in (uncoupled) asolectin vesicles (see Materials and Methods). 10 μg/ml FPE, 3 mg/ml asolectin, 250 nM valinomycin, 2.5 μM CCCP; apparent pK_{FPE} 7.5. The titration was carried out with 0.5 M KOH.

For the calibration procedure of the intravesicular pH changes described in the next section, the wavelength couple 504.5–556.2 nm was used.

Measurement of intraliposomal buffering capacity and calibration of absorbance changes of FPE-proteoliposomes

Liposomes with incorporated electrogenic membrane proteins have often been applied to determine the stoichiometric ratios of ion-translocation processes. When these systems involve proton movements, they are usually assayed by the exclu-

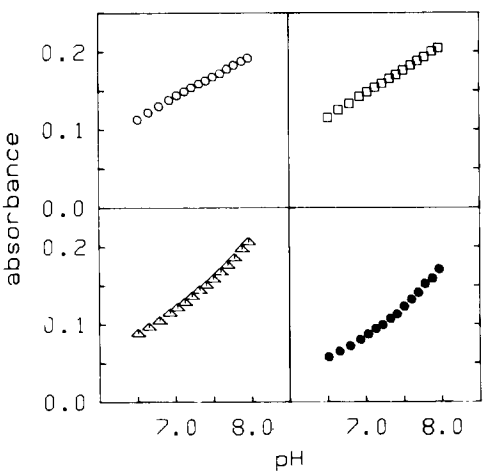

Fig. 5. Absorbance as a function of pH. Experimental conditions as in Fig. 4. ○, 485 nm; □, 488 nm; △, 500 nm; ●, 504.5 nm.

sive measurement of extravesicular pH changes. Calibration of these pH changes are accomplished by the addition of measured amounts of acid or base to the external bulk phase [6,7,19]. The outer and inner vesicular buffering capacities may then be calculated according to the procedure, extensively discussed for whole mitochondria by Mitchell and Moyle [19].

To measure the intravesicular pH of the FPE-proteoliposomes by optical absorbance, the vesicles must be suspended in a medium with a buffering capacity sufficiently large such that no extravesicular pH change occurs as a result of any vectorial enzymatic process. The absorbance contribution from the probe sensitive to the extravesicular medium is thus excluded.

In order to determine the relationship between a change of absorbance and a pH change in the intravesicular compartment, two measurements must be taken. The intravesicular buffering capacity is determined in terms of $\Delta H^+/\Delta pH$ [19], i.e., a given pH change is related to the number of equivalents of acid or base added. The second measurement relates a change of absorbance with a change of pH. Equation of the two relationships will then provide a calibration of a change of absorbance with respect to equivalents of added acid or base in the intravesicular compartment.

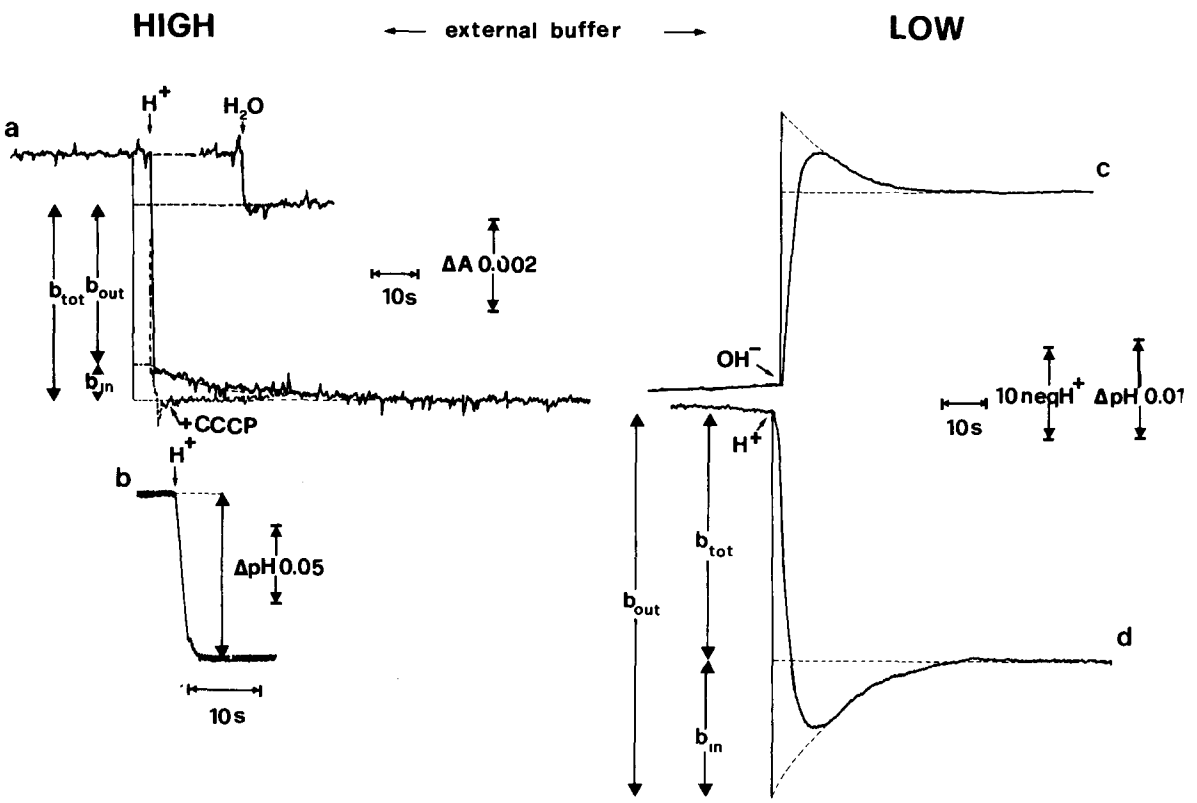

Fig. 6. The measurement of the intravesicular pH buffering capacity of FPE-proteoliposomes and the pH calibration of the absorbance of FPE. FPE-proteoliposomes were prepared as described in Materials and Methods. The reaction conditions during the experiments shown by traces a and b ('HIGH' external buffer): 100 mM KCl, 2 mM $CaCl_2$, 50 mM Hepes (pH 7.3), 2.1 mg/ml asolectin, 1.56 μM valinomycin; Temp. 15°C. Trace a shows the effect of a pH jump on the optical absorbance of FPE at the wavelength couple 504.5–556.2 nm in the absence and presence of the proton ionophore, CCCP. Trace b shows the response of a pH electrode suspended in the same reaction vessel (effect of CCCP not shown). The experiments shown in traces c and d were recorded solely with a combined pH electrode. The reaction conditions employed ('low' external buffer) were as follows: 125 mM KCl 25 mM sucrose 2 mM $CaCl_2$, 0.1 mM potassium-Hepes, 0.27 mg/ml asolectin, 0.83 μM valinomycin. The pH was adjusted to 7.3 and the ambient temperature was 20°C. Acid/base pulses were carried out using aliquots of a calibrated 10 mM HCl solution or 10 mM freshly prepared NaOH (traces c and d); 5 μl 100 mM HCl or 5 μl H_2O were added at the arrow marks (traces a and b).

The absorbance measurements are, however, complicated by the fact that there is a larger number of lipid molecules facing the extravesicular phase than the intravesicular phase [20]. There will, therefore, be a larger number of probe molecules also facing the outer phase, the contribution to the observed absorbance change for a given pH change will consequently be much larger from the extravesicularly facing probe molecules than from the intravesicularly located molecules. Furthermore, as it is advisable to optimise the response of the intravesicularly facing probe, the buffering capacity of this compartment will be made relatively much smaller than that of the extravesicular compartment. The two latter conditions introduce a technical difficulty which adds some complication to the procedure applied by Mitchell and Moyle [19]. The problem arises with respect to the $\Delta H^+/\Delta pH$ calibration of the vesicles. If FPE-proteoliposomes were suspended in a medium of relatively high buffering capacity, i.e., a medium appropriate for the measurement of intravesicular absorbance changes, a large error would be introduced due to the very large difference in buffering capacities of the two compartments. The assumption was made, therefore, that the composition of the extravesicular medium would not affect, to any significant extent, the intravesicular buffering capacity. Thus, the $\Delta H^+/\Delta pH$ calibrations could be carried out in a medium of relatively low buffering capacity (see Fig. 6), according to the technique of Mitchell and Moyle [19].

Inspection of the pH-electrode traces (traces c and d) illustrated in Fig. 6 indicates the protocol applied: a suspension of FPE-proteoliposomes in a reaction vessel equipped with a pH electrode was challenged with a known quantity of acid (d) or base (c). This caused a step change and an excursion of the pH, the kinetics of which reflected two separate processes. The initial rapid change represents the response solely of the extravesicular compartment to the pH jump (i.e., b_{out}). The latter, slower phase represents the rate of equilibration of the imposed pH gradient across the vesicular membrane to the final equilibrium value of the system (b_{tot}). By back extrapolation (see Fig. 6) of the presumed exponential relaxation process it was possible to discriminate the two events (see Ref. 19). It was thus possible to calculate the intravesicular buffering capacity (b_{in}) to be 5.0 ± 0.7 nmol $H^+/0.1$ pH unit per μmol lipid. It is noteworthy that this value was found to vary significantly, depending upon the lipid preparation and the enzyme system used. However, under circumstances whereby the buffering capacities of the two compartments (i.e., low buffer concentrations) were kept low, it was consistently observed that the buffering capacity of the system was not dependent upon the ambient pH.

Traces a and b of Fig. 6 indicate analogous experiments to those described above; however, in addition to the measurement of pH with an electrode (trace b) the optical absorbance (trace a) of FPE was also monitored. Furthermore, the extravesicular buffering capacity was maintained at a relatively much larger value than that of the intravesicular compartment. With the addition of a quantity of acid sufficient to cause a measurable change of the pH of the system, two phases were distinguishable from the absorbance change. The first, larger and more rapid phase represents the response of the extravesicularly facing probe (i.e., b_{out}); the second phase represents the response of the intravesicularly facing probe (i.e., b_{in}), and as in the experiments illustrated by traces c and d, the final equilibrium value represents the buffering capacity of the whole system as measured by FPE. In the presence of a proton ionophore (trace a + CCCP), no biphasic pH relaxation kinetics were observed; the absorbance responded in a step-wise manner equivalent to the total absorbance change as a consequence of the pH jump. The actual pH changes were recorded with the aid of an electrode (see trace b). It was thus possible to relate the absorbance changes of the intravesicularly facing dye to known pH changes and, therefore, as the intravesicular buffering capacity is known, to equivalents of acid or base. From such measurements it was possible to calculate an absorbance change of: $0.002 \pm 0.0002/0.1$ pH unit per μmol lipid ($n = 6$) for the intravesicularly facing FPE.

It is apparent from Fig. 6 that a dilution artifact occurred with an addition to the reaction medium. Fig. 6 also illustrates that the total response of the externally facing probe is about 5-times larger than that of the internally facing probe for the same pH change. It should be stressed, therefore, that the extravesicular medium must be suffi-

ciently well buffered such that no extravesicular pH changes occur as a consequence of any enzymatically induced vectorial pH changes. Under the experimental conditions employed for membrane protein studies, the buffer systems described above adequately fulfil this latter criterion.

The appropriate conditions have been described for the application of FPE as a pH indicator of the inner compartment of phospholipid vesicles. The probe may, however, also find use for the measurement of surface phenomena. It was suggested that FPE is located in the diffuse double layer at the membrane/water interface and, as such, it is sensitive to the membrane surface potential. Since at low ionic strength the probe responds to changes of the ionic composition of the bulk phases, it is feasible, that under these conditions, if the buffering capacity of the system is kept high, the probe may exclusively report surface potential changes. We are presently investigating this novel application of FPE.

Acknowledgements

P.S. O'Shea is grateful to EMBO for the award of a postdoctoral fellowship. This work was supported by the Swiss National Foundation 3.739-0.80, the Emil-Barell Stiftung and the Sandoz Stiftung.

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