Biochimica et Biophysica Acta 820 (1985) 107–114
Elsevier

BBA 72778

Incorporation of a novel photochromic phospholipid molecule into vesicles of dipalmitoylphosphatidylcholine

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(Received May 29th, 1985)

Key words: Photochromism; Phospholipid; Phase transition; Azobenzene photoisomerisation; Light scattering; Fluorescence

A phospholipid molecule has been synthesised, bearing an azobenzene linkage within one acyl chain. The lipid has been incorporated into vesicles of dipalmitoylphosphatidylcholine, and the effects of photoisomerisation on the vesicle permeability and phase behaviour studied by light scattering and fluorescence spectroscopy. The phase transition temperature of DPPC is reduced, and the transition is broadened by the *trans* Azo-lipid. After photolysis the transition temperature is further reduced, and non-equilibrium effects are evident. Vesicles containing the Azo-lipid can sustain a pH gradient before photolysis, but pH slowly equilibrates following irradiation. Results indicate that photoisomerisation causes no loss of bilayer integrity. Electron microscopy shows that the Azo-lipid alone forms vesicles on dispersion using an ethanol injection technique.

Introduction

Photochromism has recently received considerable attention in biochemistry. The role of photoisomerisation in vision is well known [1]. There are however many other areas where photochromism can be exploited to provide control and synchronisation of biochemical systems. Particular examples of photochromic reaction control include the modulation of acetyl choline binding to receptor using photoisomerisable blocking agents [2] and the photoregulation of the concentration of metal ions using photoisomerisable chelating agents [3]. Other examples are the use of photochromic crown ethers and cryptands to modulate ion permeability through membranes [4] and the direct- and indirect modification of enzyme activity via bound photochromic effectors [5] or control of the phase equilibrium in micellar systems [6]. Photochemical control of biochemical processes has advantages which include the potential for reversing reaction by change of excitation wavelength, the possibility of fast kinetic studies on systems otherwise difficult to synchronise and the ability to modulate levels of biologically-active effectors in a rapid non-invasive manner.

To date little work has been done using photochromic lipid molecules, despite the possibility of amplification of effect of light by non-linear processes involving phase behaviour or ion permeability of bilayer membranes. Some interesting work has been reported using a cationic detergent containing an azobenzene chromophore [7], though this has been confined to studies of osmotic shrinkage and release of a lipophilic membrane-

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bound indicator, bromophenol blue. The origin of changes seen in the latter paper are somewhat uncertain, and stem in part from the effect of fairly high concentrations of the photosensitive cationic detergent on the bilayer membrane properties.

In order to minimise perturbation of bilayer membranes used and to avoid exchange of photoactive species through solution, it is desirable to use a phospholipid molecule containing the isomerisable grouping. This paper describes the synthesis and preliminary characterisation of such a phospholipid containing a photoisomerisable acyl chain, and reports some studies of the effect of this molecule on bilayer vesicles of dipalmitoylphosphatidylcholine.

Materials and Methods

Dipalmitoyl-L-α-phosphatidylcholine (DPPC) and lysophosphatidylcholine from egg yolk were purchased from Sigma Chemical Company, and were used without further purification. Before acylation, lysophosphatidylcholine was dried overnight over phosphorus pentoxide.

The azobenzene-containing acid, 4-(*n*-butyl)-4′-(3-carboxypropyl)azobenzene, was prepared by condensation of 4-(*n*-butyl)nitrosobenzene with 4-(*p*-aminophenyl)butyric acid. The latter acid was prepared by reduction of 4-(nitrophenyl)butyric acid (Aldrich) [8], or by catalytic hydrogenation using 10% Pd on charcoal.

The nitroso compound was prepared from 4-(*n*-butyl)aniline both by oxidation with stabilised peroxymonosulphate (as 'Oxone' from Aldrich) [9], and by oxidation using peroxybenzoic acid [10]. The latter procedure gave a superior yield (approx. 40%) of 4-(*n*-butyl)nitrosobenzene, and proved to be the more reproducible. The nitroso compound was purified by column chromatography on silica gel (Merck Kieselgel 60) eluting with chloroform/hexane (10 : 90, v/v). This procedure was preferred to steam distillation in that it minimised decomposition of the product. 1.3 g of 4-(*n*-butyl)nitrosobenzene and 1 g of 4-aminophenylbutyric acid were dissolved in 6 ml of glacial acetic acid at room temperature, and the mixture was allowed to stand for several hours. Crystalline 4-(*n*-butyl)-4′-(3-carboxypropyl) azobenzene separated out and was filtered off. The product was dissolved in chloroform, washed with 10% aqueous sodium bicarbonate and dried over anhydrous sodium sulphate. Purification was by column chromatography on Kieselgel 60 eluting with chloroform/methanol (95 : 5, v/v). The acid was characterised by mass spectrometry and showed major peaks at masses of 91, 133, 163 and 324 (molecular ion). Thin-layer chromatography on silica gel showed the material to be pure.

Conversion of the azo-acid to phospholipid was by mixed anhydride acylation in the presence of 4-(dimethylamino)pyridine, as previously described for similar acylations [11].

Lipid bilayer vesicles having pH gradients between the inner trapped volume and the bulk medium were prepared by a method similar to that of Deamer et al. [12]. Vesicles were prepared by sonication of a dispersion of DPPC mixed with the photochromic phospholipid (Azo-PC) in 200 mM unbuffered potassium chloride solution (3 mg total lipid per ml of solution). Before sonication the lipids were mixed in ethanolic solution and evaporated to a thin film under nitrogen. During the sonication the solution was allowed to heat up to approx. 50°C, so that the DPPC was above its characteristic phase transition temperature. Sonication was conducted in short bursts over a total time of 2 min using an MSE sonicator. Vesicles were allowed to cool and were applied to a short column of Sephadex G-25 medium porosity gel (Pharmacia Ltd.) equilibrated with 400 mM sucrose, 30 mM sodium phosphate (pH 7.5), and material eluted close to the void volume was collected. Vesicle samples eluted from the column were diluted 5-fold with the eluting buffer and 9-aminoacridine hydrochloride was added to a total concentration of approx. 1 μM. The pH gradient was established by the addition to the vesicle suspension of a trace of valinomycin and of pentachlorophenol as a proton carrier. The establishment of the pH gradient was monitored by the decrease in fluorescence of the 9-aminoacridine with time as a consequence of uptake into the vesicles. Addition of Triton X-100 reversed the quenching of fluorescence as expected, since the vesicles are solubilised by the detergent.

Deamer et al. [12] discuss an alternative method to establish a pH gradient, based on entrapment of

potassium ferricyanide within the vesicles and addition of ascorbate externally in the presence of a redox carrier such as dichlorophenolindophenol. This method was found to be unsuitable for our purposes. Illumination of the system in the absence of Azo-PC caused a rapid reestablishment of fluorescence of 9-aminoacridine, indicating collapse of the proton gradient, and optical measurements indicated a marked increase in turbidity following illumination. We ascribe these effects to osmotic changes consequent on the photodecomposition of the entrapped ferricyanide.

The effect of Azo-PC on osmotic shrinkage of liposomes was measured by 90° light scattering at 537 nm using a Perkin-Elmer 2000 spectrofluorimeter. An aliquot of lipid in ethanol was evaporated under nitrogen and dispersed in distilled water at 50°C by vortex mixing for several minutes. The total lipid concentration used was typically 0.14 mg/ml. After cooling to room temperature, the osmotic changes were initiated by rapid addition and mixing of 100 μl of molar potassium chloride solution to 3 ml of lipid dispersion, and changes in scattering were monitored over several minutes. Lipid dispersions prepared by this method are multilamellar. Kano et al. [7] used sonicated vesicles and monitored osmotic changes by measurement of absorbance at 220 nm. However, both isomers of Azo-PC absorb at this wavelength while 537 nm light is not absorbed, but is efficiently scattered by the larger multilamellar vesicles.

Vesicles containing Azo-PC were photolysed for varying periods of time using a high pressure mercury arc (250 W) fitted with a heat filter and a 366 nm bandpass filter.

Phase behaviour of phospholipid vesicles was monitored by 90° light scattering in the fluorimeter using 537 nm illumination. Vesicles were prepared by brief sonication of a vortexed lipid dispersion prepared in 20 mM phosphate buffer (pH 7.5), containing 16% sucrose by weight to match the refractive index of the lipid. Samples were heated at a rate of 1–2 Cdeg $\cdot$ min^{-1} and data were recorded on the second heating cycle: a slow heating and cooling always preceded data collection, so that vesicles were equilibrated.

Absorption spectra and second derivative spectra were recorded using a Perkin-Elmer Lambda 5 spectrophotometer. Spectra of vesicles were taken using as reference a similar phospholipid dispersion containing no Azo-PC, so that scatter was compensated.

Electron microscopy was on a Corinth 500 transmission instrument. Samples were negatively stained with 1% w/v aqueous uranyl acetate on Formvar/carbon coated grids from Agar Aids Ltd. Vesicles of Azo-PC were prepared by injection of an ethanolic solution into vortexing 20 mM phosphate buffer, pH 7.5 heated to 50°C. The final ethanol concentration was 1% v/v. The dispersion was passed through a 0.22 μm Millipore filter and applied to grids.

Results

Ultraviolet light photolysis of a sonicated dispersion of Azo-PC in vesicles of DPPC is accompanied by the change in absorption spectrum shown in Fig. 1. This change is reversed when the

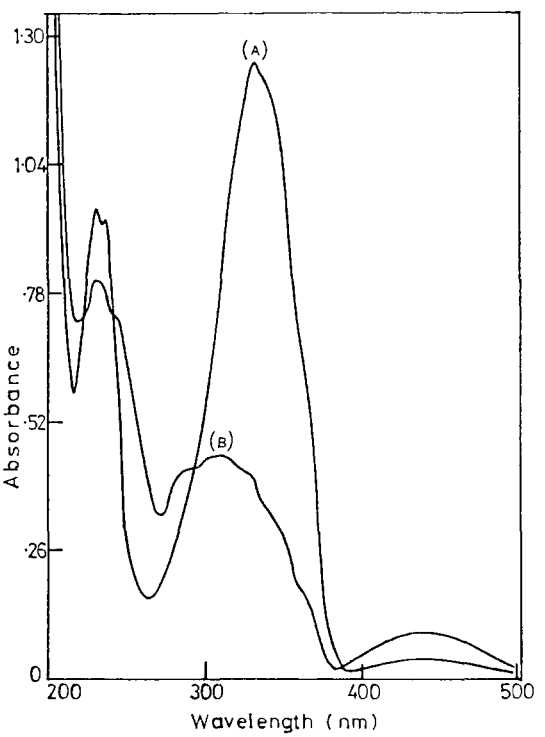

Fig. 1. Ultraviolet photolysis of Azo-PC (20% (w/w)) in DPPC injection vesicles. Total lipid concentration is 0.42 mg/ml in distilled water. (A) Absorption spectrum before photolysis. (B) After 6 min photolysis using a high pressure mercury arc lamp with an 80 nm passband filter centred at 366 nm.

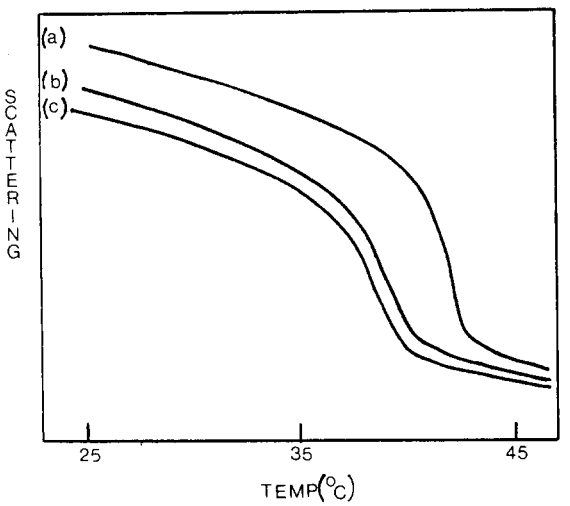

Fig. 2. Light scattering vs. temperature profiles for (a) DPPC vesicles, (b) DPPC vesicles containing 10% Azo-PC by weight (before photolysis) and (c) sample (b) after photolysis for 6 min at 366 nm. Lipid concentration and conditions are given in the text.

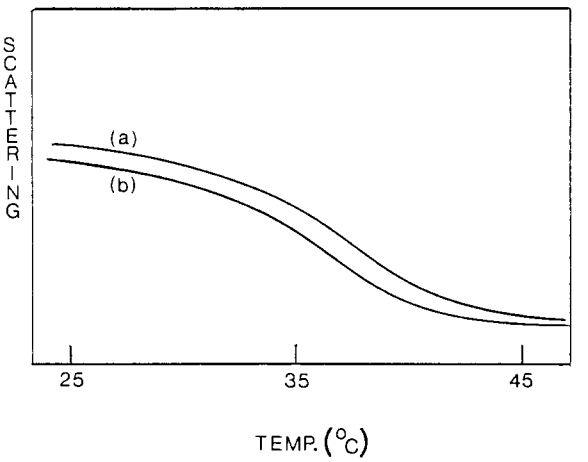

Fig. 3. Light scattering vs. temperature for Azo-PC in DPPC vesicles (a) before photolysis, (b) after photolysis for 6 min at 366 nm. Azo-PC was 20% (w/w). Other conditions are given in the text.

sample is subsequently illuminated with unfiltered visible light from a tungsten lamp. The absorption spectrum of the mixture corresponding to the photostationary state can be conveniently resolved into contributions from the *trans* isomer and the ultraviolet-generated *cis* isomer using the second derivative spectrum, which minimises the effects of spectral overlap.

Phase behaviour of DPPC / Azo-PC mixtures

The temperature profile for light scattering from a lightly sonicated dispersion of DPPC is shown in Fig. 2. The phase transition is clearly seen, with the mid-transition temperature at approx. 41°C. The effect on the phase behaviour of 10% (w/w) of the Azo-PC is also shown. The phase transition is still present, but is broadened somewhat and is

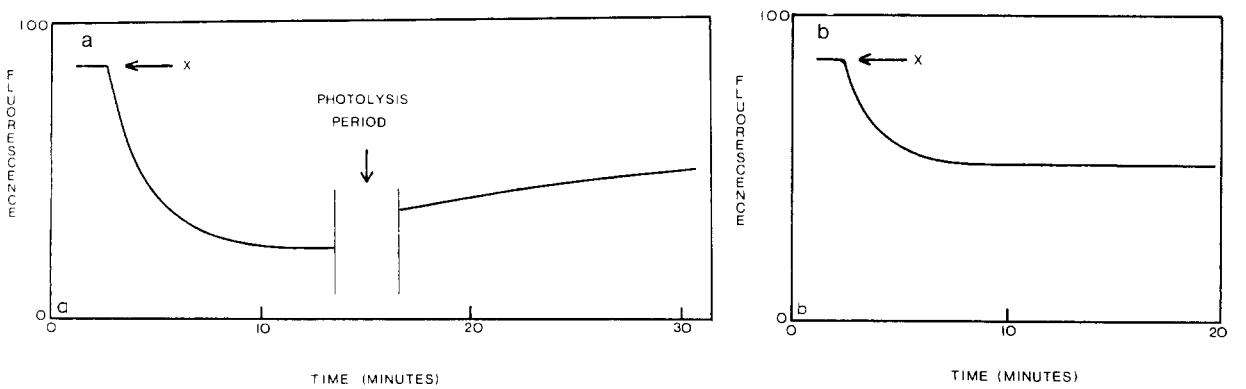

Fig. 4. (a) Fluorescence quenching and recovery profiles for 9-aminoacridine uptake into, and release from DPPC vesicles containing Azo-PC (11% (w/w)). A total of 3 mg lipid was sonicated into 1 ml of 200 mM KCl unbuffered. Samples were passed through a Sephadex G-25 column equilibrated in 400 mM sucrose, 30 mM sodium phosphate buffer (pH 7.5), containing 10 mM EDTA, and diluted to 15 ml. 9-Aminoacridine was added to 2 μM total concentration. X indicates the time of addition of pentachlorophenol and valinomycin to establish the proton gradient, as discussed in the text. (b) Effect of prephotolysis of K^+-loaded vesicles prepared as in (a) above. Vesicles were irradiated before the addition of 9-aminoacridine and valinomycin/pentachlorophenol.

shifted to lower temperature. These data were both obtained after samples had been slowly heated to 50°C and allowed to cool to 20°C over a period of 30 min. Data were obtained on subsequent reheating. Vesicles containing Azo-PC were subsequently photolysed (at 20°C) to the photostationary state using light of between 320 and 400 nm. On heating these vesicles the phase behaviour was very similar to that seen before isomerisation until the region of the phase transition was reached, when deviations were observed. The light scattering efficiency at the higher temperatures was somewhat lower after photolysis. However, this heating curve was not reproducible once the vesicles had been allowed to cool back to 20°C. After cooling the light scattering efficiency of the photolysed vesicles had decreased relative to a control which had not been photolysed. Reheating of these vesicles showed that the phase transition was still evident, but at a slightly lower temperature than before photolysis. Vesicles were subsequently illuminated with unfiltered visible light for 5 min at room temperature. The light scattering efficiency was found to be once again increased, and was now slightly greater than that of vesicles before exposure to ultraviolet light. However, on thermal cycling the scattering efficiency reached that of the unphotolysed control.

Incorporation of larger amounts of the Azo-PC into DPPC vesicles caused further broadening of the phase transition and also moved the mid-transition to lower temperature. Fig. 3 shows the phase transition observed by light scattering for a lightly sonicated dispersion of DPPC containing 20% Azo-PC (w/w).

Bilayer permeability

Vesicles formed from a mixture of DPPC and Azo-PC were able to sustain a pH gradient, generated as described in Materials and Methods. Fig. 4a shows the build up of the proton gradient as monitored by the fluorescence quenching of 9-aminoacridine added to the buffer. The fluorescence quenching can be reversed by ultraviolet photolysis, but the effect is not instantaneous. It is not clear whether the rate of recovery of fluorescence is limited by the rate of collapse of the proton gradient, or whether the leakage of accumulated fluorophore is limiting. In either event, the rate of recovery is consistent with an increased vesicle permeability rather than a gross perturbation of bilayer integrity (e.g. detergents such as Triton X-100 cause an immediate recovery of fluorescence, even at low detergent concentration). Photolysis of vesicles loaded with K^+ (before addition of valinomycin and pentachlorophenol), followed by reversal of isomerisation by illumination with visible light, gave a reduced fluorescence effect on subsequent addition of the ionophores and 9-aminoacridine. It is likely that during photolysis (either with visible or ultraviolet light) some leakage of entrapped K^+ occurred, so that a smaller pH gradient resulted (Fig. 4b). Vesicles prepared in the presence of the fluorescent marker calcein [13] showed no evidence of leakage on photolysis.

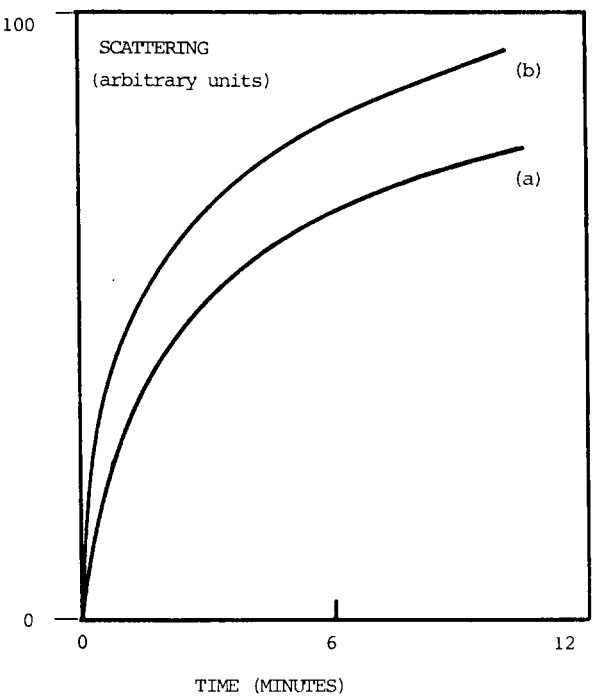

Fig. 5. Effect of photolysis on the osmotically-induced shrinkage of liposomes of DPPC containing 20% Azo-PC (w/w). Trace (a) shows the time-course of scattering changes in vesicles before photolysis, and trace (b) shows that for a similar sample photolysed using a 366 nm bandpass filter with a 250 W high-pressure mercury arc lamp. Vesicles had reached the photostationary state after 10 min photolysis. Osmotic shrinkage was induced by the rapid addition of potassium chloride to a final concentration of 32.25 mM, and light scattering monitored at 537 nm. Lipid concentration was 0.14 mg/ml. Other conditions are given in the text.

The enhanced permeability to water of ultraviolet-photolysed vesicles is demonstrated by the rate of change of light scattering induced in vesicle dispersions by osmotic shock (Fig. 5). On the time scale of the experiments these effects were not reversible, suggesting that bilayers are not freely permeable to potassium ions under the conditions used. This is not necessarily inconsistent with the data in Fig. 4b, in that ion permeability after photolysis is distinct from permeability during isomerisation when induced dynamic fluctuations in structure will occur. In addition, light scattering measurements were made using multilamellar vesicles, while the experiments on pH gradients used unilamellar vesicles. In the former case the rate of leakage of small ions relative to water is likely to be influenced by multiple diffusion barriers. Azo-PC is itself able to form vesicles. Electron microscopy showed that vesicles prepared by the ethanol injection technique were typically 400–600 Å in diameter.

Discussion

Azo-PC behaves as a typical phospholipid and in admixture with DPPC forms closed bilayer vesicles at the concentrations used in this study. In addition, electron microscopic studies showed typical vesicular structures for dispersions of Azo-PC alone, though these structures have not yet been fully characterised. Mixed phospholipid vesicles containing Azo-PC are able to trap solutes and, before photoisomerisation, can sustain a gradient of pH across the bilayer. The host phospholipid matrix does not markedly affect photoisomerisation and ultraviolet derivative spectra show that the photostationary state composition is similar whether photolysis is in vesicles or in ethanolic solution. Illumination with visible light rapidly reverses photoisomerisation, but the thermal back-isomerisation is slower with a half-time of the order of thirty minutes in DPPC vesicles at 70°C. It is therefore possible to measure thermal phase behaviour of vesicles between room temperature and 55°C without thermal reversal of isomerisation on the time scale of the measurements. However, for light scattering experiments it is necessary to illuminate with long wavelength light which is not absorbed.

Light scattering measurements are not ideal for measurements of phase behaviour, since the scattering efficiency is an extensive property. Moreover, it is difficult to directly compare results between samples of different composition because the refractive index of the sample influences scattering efficiency. However, other methods also suffer disadvantages, and light scattering is a continuous and non-perturbing process. Fluorescence probes have frequently been used to study lipid phase behaviour, but in this case the presence of Azo-PC limits the choice of fluorophore because of possible fluorescence quenching by resonance energy transfer and the need to excite fluorescence outside the absorption bands of the Azo-PC.

The *trans* Azo-PC isomer broadens the main gel to liquid-crystalline phase transition of DPPC and shifts the midpoint to lower temperature. After isomerisation in gel phase lipid the light scattering profile shows the transition shifted to a slightly lower temperature but the temperature profile is not greatly affected. However, there is clear evidence of non-equilibrium effects, since on initial heating the light scattering profile of photolysed lipid follows that of the non-photolysed sample up to the phase transition temperature. If the photolysed sample, after heating and cooling to allow equilibration, is exposed to visible light the *trans* isomer reforms rapidly. The light scattering profile up to the phase transition differs from that before ultraviolet photolysis unless the sample is thermally cycled through the phase transition.

The relatively small effect of photoisomerisation of Azo-PC on the phase behaviour of DPPC bilayers is surprising. In the *trans*-form the Azo-PC has a linear chain, while the *cis*-isomer formed on ultraviolet photolysis has a non-linear chain like that of an unsaturated lipid. It might therefore be expected that the *cis*-isomer would interfere strongly with packing of acyl chains, and perhaps exert a 'fluidising' effect. In view of the non-equilibrium effects mentioned for the observed phase behaviour it seems likely that the material is phase-separated, at least in part. The equilibrium distribution of *cis* and *trans* isomers within the bilayer need not be the same, and since photolysis was conducted in the gel phase, heating through the phase transition might be needed to reequilibrate the lipid distribution. Phase separation

would tend to minimise the effects of Azo-PC on bilayer phase behaviour, but is difficult to detect with certainty. An X-ray study of oriented bilayers might be useful in this respect.

Osmotic shrinkage experiments show that vesicles containing Azo-PC are more permeable to water after ultraviolet photolysis. However, leakage of charged solutes is not markedly enhanced and calcein-loaded vesicles showed no leakage after photolysis. The proton gradient generated using valinomycin is collapsed by photolysis but the effect is not instantaneous, and suggests simply that proton permeability is higher after isomerisation. The pH gradient generated after photolysis was less than that in an unphotolysed sample, suggesting that some potassium ion leakage might have occurred during photolysis. These effects are consistent with an increase in bilayer disorder, possibly at the interface with phase-separated lipid, but with no loss of bilayer integrity.

Leakage studies were performed at room temperature where the lipid mixtures were in the gel phase. It seems likely that the largest permeability changes would be near the phase transition temperature, since in this region the photolysis effect would be amplified by changes in phase behaviour. This aspect is currently under investigation.

The Azo-PC used in the experiments described is prepared from lysolecithin obtained from egg yolk lecithin, and as such is heterogeneous in fatty acid composition in the 1-position. The predominant fatty acids esterified are palmitic and stearic acid. It would be desirable to prepare an Azo-PC with a single known fatty acid chain in the 1-position, so that the lipid might be characterised as a pure compound and its phase behaviour measured unmixed with host lipid. This has been accomplished using palmitoyllysophosphatidylcholine as starting material in the lipid synthesis. However, the palmitoyllysophosphatidylcholine proved to be rather insoluble in the solvent system used for acylation, and the yield of this lipid was much lower than that for the heterogeneous Azo-PC. Detailed characterisation of the chemically pure and homogeneous Azo-PC will be described elsewhere, as sufficient material becomes available.

There are several interesting avenues of research which can be explored using the Azo-PC described, and which do not require chemically homogeneous samples. The absorption spectrum of Azo-PC has extensive overlap with the fluorescence spectrum of tryptophan, and for this reason the lipid is an excellent acceptor for resonance energy transfer from tryptophan. For this reason Azo-PC is very well suited to studies of peptide- and protein-lipid interactions where protein binding to vesicles is monitored by quenching of tryptophan fluorescence. This method is presently being used to study the binding of the lytic toxins melittin and delta-haemolysin to phospholipid vesicles, and will be published separately. We have also shown that the Azo-PC can be incorporated into biological materials using a phosphatidylcholine-specific transfer protein with a dispersion of Azo-PC alone. This will allow studies of membrane bound enzymes, to explore the possibility of photoregulation of enzyme activity through changes in membrane phase behaviour.

Finally, in collaboration with colleagues at the University of North Wales, Bangor, it has been shown that Azo-PC will form a surface monolayer on water, and that this monolayer can be transferred onto a surface using Langmuir-Blodgett techniques. This observation opens the way for studies of electrical properties of such films, and investigation of photochromism in oriented samples, as well as allowing surface pressure-area measurements to determine the effect of isomers on lipid packing.

Acknowledgements

The authors wish to thank Professor Karel Wirtz of the University of Utrecht for his kind gift of phosphatidylcholine-specific exchange protein, and Dr. C.C. Chadwick who provided sarcoplasmic reticulum vesicles. Thanks are also due to Drs. D.M. Taylor, B. Zaba and D. Laidmann of the University of North Wales, Bangor for their hospitality and assistance in preliminary Langmuir-Blodgett studies. Y.P.Y. wishes to thank EMBO for the award of a Short Term Fellowship.

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