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Abscisic acid-induced microheterogeneity in phospholipid vesicles

A fluorescence study

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Changes in the thermal behavior of DMPC (dimyristoyl-L-phosphatidylcholine) and an equimolar mixture of DMPC and DMPE (dimyristoyl-L-phosphatidylethanolamine) induced by the plant hormone abscisic acid (ABA) have been investigated using fluorescent probes. The fluorescence decay of the hydrophobic probe 1,6-diphenyl-1,3,5-hexatriene (DPH) in these vesicles has been measured using frequency-domain fluorometry, and has been analyzed using both models of discrete exponential components and continuous lifetime distributions. In the DMPC vesicles, using the distributional approach, higher center and width values were observed in the presence of abscisic acid (ABA), indicating a decrease in the dielectric constant of the lipid phase that we attribute to a decrease in the water concentration within the bilayer. Moreover, the presence of ABA in the liposomes increased the phospholipid phase transition temperature. The addition of ABA to the DMPC/DMPE mixture strongly increased the microheterogeneity of the system as reported by the FWHM (full-width at half-maximum) of the distributional approach.

1. Introduction

The plant hormone ABA exerts a number of physiological effects [1,2]. However, the mechanism of action by which ABA can regulate plant apical dominance, seed dormancy, stomatal aperture, transport of assimilates and roots' water permeability is still obscure [3–5]. Additional in-

terest in the function and mechanism of action of ABA has arisen, due to the identification of ABA in animal tissues, in particular, in pig and rat brains [6]. The interaction between ABA and cells is believed to occur via the plasma membrane and recent studies [7–10] report variations in membrane water permeability and ion transport induced by ABA. These effects are enhanced when phosphatidylethanolamine is present in the membrane [8,9], leading to the hypothesis of a direct interaction between ABA and phosphatidylethanolamine. Since these structure-function relationships have been introduced, we have initiated spectroscopic studies to characterize the effect of ABA on phospholipid bilayer architecture.

In the present paper, we report a fluorescence study on the modifications induced by ABA on

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Abbreviations: ABA abscisic acid; DMPC dimyristoyl-L-phosphatidylcholine; DMPE dimyristoyl-L-phosphatidylethanolamine; DPH 1,6-diphenyl-1,3,5-hexatriene; Laurdan 2-dimethylamino(6-lauroyl)naphthalene; POPOP 1,4-bis(5-phenyl-2-oxazolyl)benzene; FWHM full-width at half-maximum.

synthetic phospholipid vesicles. The study used DPH to monitor the behavior of the hydrophobic core and 2-dimethylamino(6-lauroyl)naphthalene (Laurdan) to report on the polar head group region of the bilayer. DPH is generally considered to be localized in the hydrophobic core of the bilayer [11]. The decay behavior of DPH has been shown to be sensitive to the physical state of the surrounding phospholipid acyl chains [12–14]. When inserted into phospholipid membranes, DPH shows a multi-exponential decay with different lifetime values depending on the physical state (gel or liquid crystalline) of the bilayer.

Laurdan is a fluorescent probe most probably located at the hydrophobic-hydrophilic interface of the bilayer [15,16]. It shows a marked steady-state emission red-shift in the phospholipid liquid-crystalline phase, with respect to the gel phase. The Laurdan emission red-shift is due to dipolar relaxation phenomena, that are related to the physical state and the dynamics of the surrounding phospholipid polar head groups [16].

Most of the methods to approach the problem of membrane microheterogeneity are based on measurement of fluorescence lifetimes. Klausner et al. [17] initiated work in this field with an attempt to determine phase coexistence and membrane microheterogeneity using the different values of the fluorescence lifetime of DPH in the gel and fluid phases in liposomes. More sophisticated methods were introduced recently which provide a better characterization of membrane heterogeneity. These methods are based on the analysis of the fluorescence decay of DPH and other membrane probes using continuous lifetime distributions. The width of the lifetime distribution has been correlated with the microheterogeneity of the membrane [18].

2. Results

2.1. DPH studies

2.1.1. Anisotropy

From 5 to 20 mol% ABA in DMPC vesicles caused no appreciable variation in plots of the values of DPH anisotropy vs. temperature with

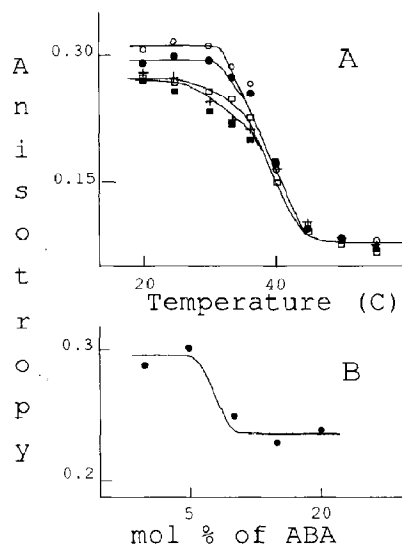


Fig. 1. (A) Steady-state DPH anisotropy values vs. temperature in an equimolar DMPC-DMPE mixture (●) in the presence of 5 (○), 10 (□), 15 (+) and 20 (■) mol% ABA, with respect to the phospholipids. (B) Effect of ABA concentration on the steady-state DPH anisotropy at 20 °C.

respect to the phospholipids. Higher ABA concentrations were not tested in order to avoid severe perturbation of the phospholipid bilayer structure. In equimolar DMPC/DMPE vesicles, the values of DPH anisotropy vs. temperature decreased as a function of ABA concentration (fig. 1). The effect was only observable at temperatures below the phospholipid phase transition. Also, the phase transition interval was broader and smoother. The largest variations in anisotropy values were observed between 10 and 15 mol% ABA (see fig. 1B).

2.1.2. Lifetime

The fluorescence decay of DPH in multilamellar DMPC vesicles was measured between 2 and 40 °C with and without 10 mol% ABA. Data were analyzed using models of discrete exponential components [19] and continuous lifetime distributions [20,21]. The discrete exponential analysis was performed for a double-exponential decay, resulting in a main component with a lifetime value dependent upon temperature varying from 9.6 to 8.3 ns (fig. 2A). The second component was about 1 ns with a very low relative intensity of less

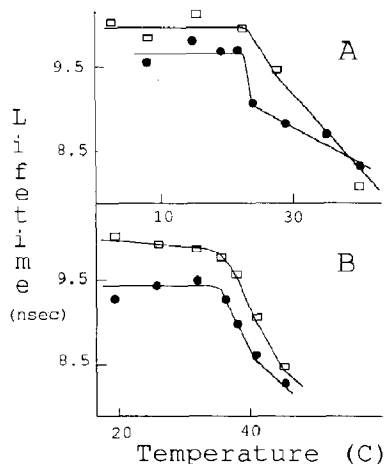


Fig. 2. DPH average lifetime value vs. temperature (A) in DMPC and (B) in the equimolar DMPC-DMPE mixture, in the absence (●) and presence (□) of 10 mol% ABA.

than 1%. This minor component, previously attributed to photochemical degradation products [12], was found in all samples using both analysis models and was affected by neither the temperature nor the presence of ABA. In the presence of 10 mol% ABA, the main lifetime component varied with temperature from 10.0 to 8.2 ns (fig. 2A). Moreover, in the presence of ABA, the phase transition temperature of DMPC was raised by about 3°C.

Using a model of a continuous distribution of lifetime values, the decay of DPH in DMPC vesicles was described by a main Lorentzian distribution, with lifetime values centered from 9.5 to 8.3 ns as a function of temperature (fig. 3A). The temperature dependence of the FWHM values associated with the main distributional component is reported in fig. 3B. An FWHM value of about 0.3 ns was obtained at temperatures below that of the phospholipid phase transition, decreasing to a minimum value of 0.05 ns above the phase transition temperature of DMPC. In the presence of 10 mol% ABA the main distributional component of DPH was centered at lifetime values varying with increasing temperature from 10.0 to 8.3 ns. With respect to the values obtained in the absence of ABA, the temperature dependence of FWHM values associated with the main distributional component of DPH was different. The minimum

FWHM value of 0.05 ns was observed below or above the phase transition temperature of DMPC (fig. 3B); however, a larger FWHM value (0.22 ns) was noted at the phase transition temperature (24°C). Using the reduced χ^2 value to judge the goodness-of-fit, both the discrete and distributional models gave satisfactory results. However, the distributional approach yielded an average reduction in the χ^2 value of about 20% with respect to the two-component fit. In the distributional approach, the Lorentzian shape produced better fits than the uniform or Gaussian shape. An evaluation of the uncertainties of the parameters is shown in table 1, where the maximum parameter variation that caused an increase of 1.2 in the reduced χ^2 value is reported as the error associated with the parameter.

When analyzed in terms of discrete exponential components, the decay of DPH in an equimolar DMPC-DMPE mixture showed a main component with a temperature-dependent lifetime value (fig. 2B). In the presence of 10 mol% ABA, the lifetime value varied from 9.9 to 8.1 ns. Moreover, the DMPC-DMPE transition temperature (T_m) was observed at 38°C when ABA was added to the sample (fig. 2B). By using the distributional approach and in the absence of ABA, the main decay component was centered at values depen-

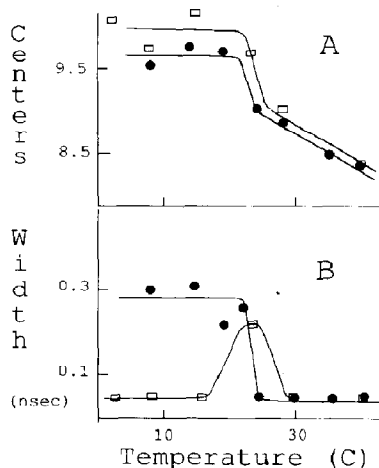


Fig. 3. (A) Center and (B) FWHM (width) values vs. temperature of the DPH continuous lifetime distribution in DMPC vesicles, in the absence (●) and presence (□) of 10 mol% ABA.

Table 1

Results of the discrete exponential and continuous distribution analysis for the DPH decay data in DMPC-DMPE at various temperatures

The errors reported represent the variation in the parameter value that gives a 1.2 increase in the reduced χ^2 value. Main discrete component, τ , and associated fractional intensity, f . Main distributional component center, c , full width at half maximum, w , and fractional intensity f . A Lorentzian distribution was used for the distribution analysis.

T ($^{\circ}\text{C}$)	Exponential		Distribution		
	τ	f	c	w	f
20	9.29 ± 0.08	0.985 ± 0.002	9.32 ± 0.09	0.40 ± 0.10	0.987 ± 0.001
26	9.39 ± 0.08	$0.988^{+0.002}_{-0.001}$	9.43 ± 0.08	0.40 ± 0.10	0.990 ± 0.010
32	9.40 ± 0.10	$0.990^{+0.010}_{-0.002}$	9.40 ± 0.10	0.40 ± 0.10	$0.993^{+0.040}_{-0.001}$
36	9.40 ± 0.10	$0.990^{+0.010}_{-0.002}$	9.35 ± 0.10	0.40 ± 0.10	$0.994^{+0.040}_{-0.002}$
38	9.05 ± 0.06	$0.993^{+0.007}_{-0.001}$	9.08 ± 0.05	0.36 ± 0.09	$0.995^{+0.040}_{-0.001}$
41	8.50 ± 0.10	$0.999^{+0.001}_{-0.001}$	8.60 ± 0.10	0.33 ± 0.20	$1.002^{+0.040}_{-0.002}$
45	8.30 ± 0.09	$0.997^{+0.003}_{-0.001}$	8.30 ± 0.09	$0.05^{+0.08}_{-0.00}$	$0.997^{+0.040}_{-0.001}$
50	8.02 ± 0.06	$0.999^{+0.010}_{-0.001}$	8.03 ± 0.05	$0.05^{+0.08}_{-0.00}$	$0.999^{+0.050}_{-0.001}$

dent upon the temperature from 9.4 to 8 ns (fig. 4A). Below the phase transition temperature of the phospholipid mixture ($T_m = 38^{\circ}\text{C}$ [22]), the asso-

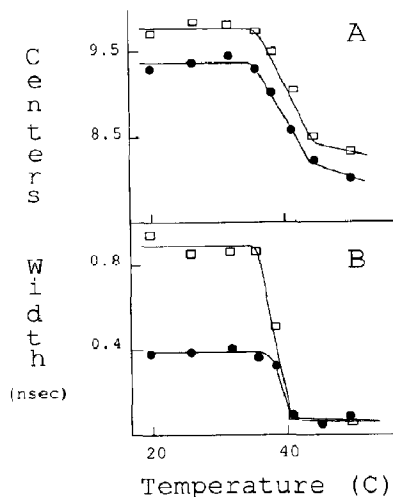


Fig. 4. (A) Center and (B) FWHM (width) values vs. temperature of the DPH continuous lifetime distribution in vesicles composed of an equimolar DMPC-DMPE mixture, in the absence (●) and presence (□) of 10 mol% ABA.

ciated FWHM value was about 0.4 ns, decreasing to a value of about 0.1 ns at higher temperatures (fig. 4B). In the presence of 10 mol% ABA, the

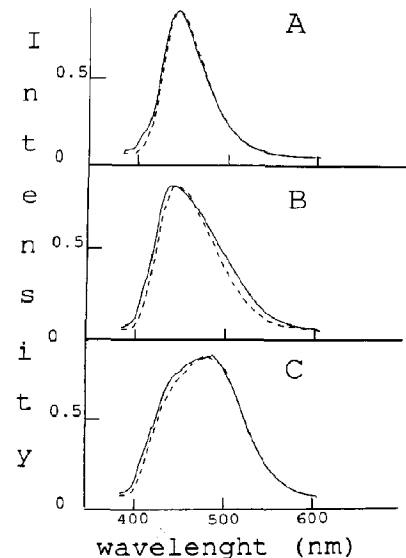


Fig. 5. Laurdan steady-state emission in DMPC vesicles at 6.2°C (A), 23.5°C (B), and 37°C (C) in the absence (dashed line) and presence (continuous line) of 10 mol% ABA.

center of the main DPH distributional component varied with temperature from 9.8 to 8.2 ns (fig. 4A). Below the DMPC-DMPE transition temperature, the associated FWHM values were approx. 0.85 ns, decreasing to 0.1 ns at higher temperatures (fig. 4B). Using the distributional analysis, the reduced χ^2 value decreased about 20% with respect to the discrete exponential model, in both the absence and presence of ABA.

2.2. Laurdan studies

The steady-state emission spectra of Laurdan in DMPC vesicles at three different temperatures, in the absence and presence of 10 mol% ABA are reported in fig. 5. A 2 nm blue-shift was observed at 6°C in the presence of ABA (fig. 5A). The spectrum obtained at 23.5°C in the presence of 10 mol% ABA showed a small broadening in both the blue and red parts of the spectrum (fig. 5B). Above the DMPC transition temperature (measurements at 37°C), the emission of Laurdan was broader in the blue region when ABA was present in the vesicles (fig. 5C). In the equimolar DMPC-

DMPE mixture, the effects induced by the presence of ABA were enhanced. The Laurdan emission was blue-shifted by 4 nm in the presence of 10 mol% ABA when the vesicles were in the gel phase (measurements at 14°C, fig. 6A). A 36 nm blue-shift was observed in the Laurdan emission in the presence of ABA at the transition of the DMPC-DMPE mixture (measurements at 38°C, fig. 6B). Above the phospholipid phase transition temperature, the presence of ABA induced an even larger effect on the Laurdan emission: the spectrum became quite broad, showing the coexistence of both the blue and red Laurdan emission (fig. 6C).

3. Discussion

The only fluorescence parameter of DPH that varies in the presence of ABA in DMPC vesicles is the lifetime. Indeed, no appreciable variation of anisotropy values is observed in the presence of variable ABA concentrations. Since the polarization value represents the weighted average of the fluorophore molecules to the total emission, small variations are difficult to detect or to assign. However, in the DMPC-DMPE mixture, DPH polarization values vary in the presence of ABA. Low ABA concentrations (5 mol%) do not produce significant variations in the polarization values. Instead, in the presence of 10 and 15 mol% ABA an appreciable variation is observed in the gel phase and in the phase transition regions. A further increase in ABA concentration to 20 mol% did not increase the anisotropy further. This variation in anisotropy can be due to several factors, including a disordering effect of ABA on the bilayer or to a lifetime change in DPH.

With the aim of gaining a better understanding of the physical origin of the observed modifications of the anisotropy values, lifetime experiments were performed. The multifrequency phase and modulation technique allows the resolution of heterogeneous emission into component lifetimes and their relative fractional weights. DPH shows a multi-exponential decay when inserted into phospholipid membranes, with a significant dependence on the vesicles' physical state [12–14].

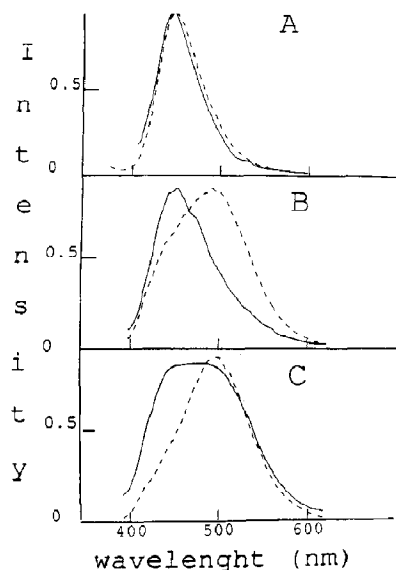


Fig. 6. Laurdan steady-state emission spectra in vesicles composed of an equimolar DMPC-DMPE mixture at 14.4°C (A), 37.4°C (B), and 54.2°C (C) in the absence (dashed line) and presence (continuous line) of 10 mol% ABA.

Lifetime studies performed with DPH in isotropic solvents show that the lifetime value of DPH is strongly dependent upon the dielectric constant of the medium [12,22]. In model membranes, the heterogeneity of physical properties can originate from differences in phospholipid composition, structure, and water penetration. A gradient of water concentration perpendicular to the membrane surface normally exists [23] and could give rise to multiple DPH lifetime values. Since DPH can be surrounded by different phospholipid phases and be distributed at various depths within the membrane normal [24] a continuous distribution of lifetime values appears more suitable for a physical description of the system. In the distributional approach, sample heterogeneity is reflected by the FWHM value. Indeed, in the study of natural membranes, meaningful results were obtained only by using the distributional approach [14]. The analysis of the phase and modulation data following the model of continuous lifetime distribution results in a significant decrease in the reduced χ^2 value with respect to the discrete exponential model [13,14]. The discrete exponential analysis corresponds to the analysis of the center of the distribution. In fact, the two analysis methods give identical results with respect to the average lifetime value.

Variations in the averaged DPH lifetime value, as well as the center of the main distributional component (both as a function of temperature) show an increase in the phospholipid phase transition temperature of about 3°C in the presence of ABA. The higher phase transition temperature can be due to closer packing of the phospholipids, induced by the presence of ABA. From the results of the distributional analysis, further information about the influence of ABA on the heterogeneity of the system can be obtained. In DMPC vesicles, the width of the main distributional component of DPH is narrower in the gel phase when ABA is present. The narrow lifetime distribution in the gel phase may indicate a decreased heterogeneity of the dielectric constant, due, perhaps, to a decrease in water penetration in the bilayer. By increasing the cholesterol concentration in extracted erythrocyte membranes, a similar effect was observed, and was attributed to a water concentration gradi-

ent in the bilayer core [18]. In the gel phase of the equimolar DMPC-DMPE mixture, the presence of ABA induces an increase in the width of the distribution by a factor of about two. In this case, the specific interaction between ABA and phosphatidylethanolamine can explain the creation of phospholipid domains, possessing differing permeability to water, and the microheterogeneity detected by the lifetime distribution analysis. From conformational calculations [25,26], the phosphatidylethanolamine polar head group has been shown to be more accessible than the choline moiety to interaction with ions or charged groups, since it possesses a large degree of freedom in terms of both internal flexibility and rotation. By contrast, the choline group shows maximum stability when oriented parallel to the plane of the bilayer, strongly interacting with the neighboring phosphate. The presence of ethanolamine can alter the membrane surface charge distribution and renders the charges available for interaction with ABA. An analogous observation has been reported on the induction of phase segregation in vesicles composed of DMPC and DMPE due to the presence of Ca^{2+} in the medium [27]. The anisotropy experiments can also be interpreted on this basis. The lifetime increase due to the addition of ABA for the DMPC-PE mixtures is too small to account for the observed decrease in polarization. Instead, the distributional analysis shows an increase in the microheterogeneity due to ABA additions. Most likely, the increase in microheterogeneity also reflects an increase in disorder, which in turn is revealed by the decrease in the anisotropy value.

Interactions between ABA and the polar head groups of the phospholipids are also detected by the fluorescence response of Laurdan. The fluorescent part of the molecule is likely to be located at the hydrophobic-hydrophilic interface of the bilayer, with the lauric acid tail in the hydrophobic core. In the phospholipid's liquid-crystalline phase and within the phase transition, Laurdan displays a red-shifted spectrum [16]. The red-shift has been attributed to relaxation processes that involve motions of the phospholipid polar head groups [15]. If solvent molecules can move during the fluorescence lifetime, the Laurdan ex-

cited-state molecular dipole will orient the neighboring solvent dipoles, and the steady-state Laurdan emission spectrum will be red-shifted [15].

At temperatures below the phase transition of DMPC and the DMPC-DMPE mixture, no relaxation phenomena are observed from the Laurdan steady-state emission spectra, even when ABA is added to the phospholipids. Within the DMPC phase transition and at higher temperatures, a slight broadening of the spectra is observed when ABA is added to the vesicles. More relevantly, ABA induced a dramatic effect on the Laurdan emission in the DMPC-DMPE mixture, within the transition and at higher temperatures. At the phase transition of the DMPC-DMPE mixture and in the presence of ABA, the red part of the Laurdan spectrum still showed a low intensity, indicating hindered motion of the polar head groups. Above the DMPC-DMPE phase transition and in the presence of ABA, the Laurdan emission spectrum is extremely broad, showing the coexistence of unrelaxed blue-emitting and relaxed red-emitting fluorophore molecules. The blue portion of the spectrum should then originate from Laurdan molecules inserted into an ABA-PE-rich domain, while the red portion of the spectrum should originate from Laurdan molecules inserted into a relatively PE-poor domain or, conversely, in a domain where the ABA-PE interactions are reduced. These observations support the previous hypothesis of an interaction between ABA and the bilayer polar head group charges, favored by the presence of DMPE. The interaction dramatically decreases the motional freedom of the bilayer polar residues, as demonstrated by the presence of an unrelaxed blue component of the spectrum above the DMPC-DMPE phase transition, but increases the heterogeneity of the system.

The conclusion we draw from this preliminary study is that ABA can induce closer packing of the phospholipids in the bilayer, probably causing reduction of water penetration in the hydrophobic core. Moreover, in vesicles composed of different phospholipids, the presence of ABA favored an increase in heterogeneity within the bilayer. ABA decreased dynamic motion in the region of the phospholipid head group and this effect was

dramatically enhanced by the presence of ethanolamine within the hydrophilic residues, suggesting a direct interaction between ABA and the ethanolamine charges.

Further studies are needed to extend the reported results to the physiological effects of ABA and its interactions with natural membranes, mainly considering the complex composition of phospholipids and proteins in cell membranes. Nevertheless, the above results provide evidence that fluorescence spectroscopy can detect changes due to the addition of ABA to multilamellar vesicles. The better packing for the DMPE vesicles and the increase in the microheterogeneity in DMPC-DMPE vesicles can suggest a physical mechanism by which ABA effects the passive transport of water and small solutes across the membrane bilayer [7–10].

4. Experimental

DMPC and DMPE were obtained from Avanti Polar, (Birmingham, AL) and were used without further purification. DPH was obtained from Molecular Probes (Junction City, OR), Laurdan was a generous gift from Professor Gregorio Weber, synthesized following reported methods [28] and purified by TLC, using a mixture of $\text{CHCl}_3/\text{CH}_3\text{OH}$ (2:1, v/v) as eluent. ABA ((±)-*cis/trans*-abscisic acid) was from Sigma (St. Louis, MO). All solvents were spectroscopic grade. Phospholipid vesicles were prepared according to reported methods [12,16]. The final concentration ratio between the probe (DPH or Laurdan) and the phospholipids was 1:600 for lifetime determinations and 1:800 for steady-state measurements. ABA-containing samples were prepared by adding the appropriate amount of an ABA solution in methanol to the phospholipid-probe mixture in chloroform. For polarization experiments, the final ABA concentration was varied from 5 to 20 mol%, with respect to phospholipids and for lifetime measurements 10 mol%. Solvents were evaporated under an N_2 stream, then the dried samples were resuspended in Dulbecco's phosphate-buffered saline, warmed above the phospholipid transition temperature and vortex-mixed

[12,16]. All sample manipulations were conducted under a red safety light to avoid the formation of photochemical products.

DPH steady-state polarization measurements were performed using an SLM 4800 fluorometer (SLM/Aminco Instrument, Urbana, IL) in the L format. Excitation was at 360 nm with 8 nm bandwidth. The emission was observed at 430 nm with 16 nm bandwidth. Scattering contributions to the mean fluorescence polarization were always negligible. The polarization values were corrected for the intrinsic polarization of the instrument. Samples were continuously stirred by a magnetic device under the cell holder. Temperature was controlled by a thermostated water bath and measured in the sample cuvette. Steady-state fluorescence spectra of Laurdan and DPH emission spectra were obtained using the SLM 4800 fluorometer. The excitation was at 360 nm and excitation and emission bandpasses were set at 4 nm. Emission spectra were not corrected for the instrumental response. Fluorescence lifetime measurements were obtained using the multi-frequency phase fluorometer [29] available at the PULS laboratory at the ADONE storage ring (Frascati, Italy). The light source was derived from the synchrotron radiation at the low-energy beam line. The 360 nm excitation wavelength was obtained via a Jobin-Yvon (10 UV) monochromator with a 16 nm bandpass. The fluorescence was viewed through a Schott cut-off filter (KV 418). Phase and modulation data were collected for 8–10 modulation frequencies in the range 8–100 MHz. A solution of POPOP in ethanol was used in the reference cuvette (lifetime value = 1.35 ns). Under our experimental conditions the fluorescence of unlabeled samples was undetectable.

Phase and modulation data were acquired using ISS electronics (ISS s.r.l., La Spezia, Italy) interfaced to an Apple II computer, and were analyzed using software from ISS (Champaign, IL) written for an IBM-PC computer. The data were analyzed using a model of both discrete exponential components [19] and of continuous lifetime distribution [20,21] to describe the fluorescence decay kinetics. The reduced χ^2 value was used to judge the goodness of fit [30]. The software for this analysis

(Globals Unlimited) was provided by the UIUC Laboratory for Fluorescence Dynamics (Urbana, IL).

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