Articles

Properties Influencing Fluorophore Lifetime Distributions in Lipid Bilayers†

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ABSTRACT: The fluorescence lifetime of the membrane fluorophore 1,6-diphenyl-1,3,5-hexatriene has been analyzed according to the distributional approach in a number of lipid bilayer systems. The systems included vesicles of 16:0/18:1-phosphatidylcholine (POPC), egg phosphatidylcholine (EYPC), microsomal phospholipids, and also intact microsomal membranes. With increasing complexity of composition, an increasingly broader width was found in the major component of a bimodal Lorentzian fluorescence lifetime distribution. In order to explain these findings, we propose a model based on environmental heterogeneity and environmental sampling, where the environment is defined as the lipid molecules immediately surrounding the fluorophore. Environmental heterogeneity is thought of as arising from organizational, compositional, and solvent factors. Environmental sampling pertains to the ability of a fluorophore to detect environments in a system and is a function of the fluorophore lifetime and the lipid dynamics. If the fluorescence lifetime is sufficiently short, the fluorophore will only sample a particular environment, and great compositional complexity will mean that each fluorophore in an ensemble will decay to the ground state with a different time. This appears to explain why in our results with DPH a narrow width is obtained for POPC, where vesicles are composed of a single phospholipid molecular species, compared to EYPC and microsomal phospholipid vesicles having complex molecular species composition. This model should serve as a basis for understanding the interrelationships of environmental complexity and lipid dynamics in membranes.

The description of fluorescence decay kinetics in terms of lifetimes is an essential component of fluorescent techniques used to characterize properties of biological materials. In the past it has been analyzed by a multiexponential sum of discrete terms, where often each term is associated with a particular fluorophore state or species. Recently, an alternative approach has been developed in which this analysis is undertaken in terms of a continuous distribution of lifetimes, which models the decay as arising from a multiplicity of fluorophore states (James et al., 1985; James & Ware, 1985, 1986; Alcala et al., 1987a; Lakowicz et al., 1987; Beechem & Gratton, 1988). The result of this approach is a lifetime distribution function, similar to a probability distribution, which determines the proportionate weight attributable to each value of the lifetime. As no theoretical form is generally proposed for such distribution functions, these are at present often represented as sums of Gaussian or Lorentzian distributions. Besides adjustable parameters determining their central values, the widths of these distributions are determined by the standard deviation for the Gaussian or the full width at half-maximum for the Lorentzian. Since broader distributions result from greater multiplicity, these latter parameters characterize the excited state multiplicity.

To date, distributional analysis has been applied to decay data from proteins (Alcala et al., 1987b,c; Eftink & Ghiron, 1987; Lakowicz et al., 1987) and to fluorescent probes incorporated in lipid vesicles and natural membranes (James et al., 1987; Fiorini et al., 1987a,b; Parasassi et al., 1987). Results so far in these latter systems suggest that in certain cases the distributional approach may be comparable or superior to the multiexponential. Fiorini et al. (1987a) found that DPH[1] decay in DMPC and DPPC vesicles below the phase transition temperature was better described by a bimodal Lorentzian distribution function than by a double-exponential analysis, although above the phase transition the two analyses were comparable. James et al. (1987) also investigated the effect of the phase transition on DMPC unilamellar vesicles labeled with parinaric acids and found that the decays were best described by smooth distributions spanning several nanoseconds. In a proerythroblast cell membrane, distributional analysis of DPH decay has been used to obtain information that was not readily apparent from a more conventional lifetime analysis (Parasassi et al., 1987). DPH decay in labeled erythrocyte membranes has also been analyzed in this manner, with the suggestion that changes in the distributional width could be related to membrane structural organization (Fiorini et al., 1987b).

While distributional analysis has been used to describe fluorescence decay in membranes, the physical basis of apparent excited-state multiplicity is as yet poorly understood. Fiorini et al. (1987a) propose that their results for disaturated PC vesicles can be understood on the basis of fluorophore environmental heterogeneity with respect to the value of the

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[1] Abbreviations: DMPC, dimyristoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, dipalmitoylphosphatidylcholine; EYPC, egg yolk phosphatidylcholine; HPLC, high-performance liquid chromatography; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; PLPC, 1-palmitoyl-2-linoleoylphosphatidylcholine; PAPC, 1-palmitoyl-2-arachidonylphosphatidylcholine; PDPC, 1-palmitoyl-2-docosahexaenoylphosphatidylcholine; POPS, 1-palmitoyl-2-oleoylphosphatidylserine; TLC, thin-layer chromatography.

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dielectric constant along the membrane normal. Thus the basis for favoring the distributional approach would be that there is a distribution of fluorophores whose lifetimes are determined by their depth in the membrane. In natural membranes, however, additional possible contributions could arise from organizational and compositional factors. To begin to examine the importance of these factors, we have analyzed DPH decay from labeled liver microsomes and lipid vesicles using a bimodal Lorentzian expression for the distribution function in order to determine how these factors affect the half-width parameter of the major component. We find that the value for this parameter increases with increasing expected membrane heterogeneity and propose a model describing some of the physical factors underlying lifetime distributions in membranes.

Experimental Procedures

Materials. DPH was from Molecular Probes (Eugene, OR). POPC and EYPC were from Avanti Polar Lipids (Birmingham, AL) and were used without further purification with routine checking of purity by TLC. Liver microsomal phospholipids were obtained from a total lipid extract (Bligh & Dyer, 1959) in chloroform separated on silicic acid columns. The natural PC subfraction was obtained from the total phospholipids by HPLC (Ellington & Zimmerman, 1987). All phospholipids used were quantified by determination of phospholipid phosphorus.

Microsome and Lipid Vesicle Preparation. Rat liver microsomes were prepared and characterized as previously described (Conroy et al., 1986). DPH labeling was carried out by adding 0.75 nmol of a 0.5 nmol/μL tetrahydrofuran solution to microsomes suspended in a quartz cuvette with 2 mL of Tris-HCl buffer (0.01 M, 0.15 M KCl, pH 7.4) at a concentration of 0.5 mg of microsomal protein/mL. The cuvette was then incubated in the dark at 37 °C for $^1/_2$ h prior to measurement. The molar probe/microsomal phospholipid ratio used was 1/400. Lipid vesicles were prepared by extrusion of freeze-thawed suspensions of phospholipids (Hope et al., 1985) using a Lipex extruder (Vancouver, BC). Preparation entailed placing an aliquot of phospholipid in chloroform along with an aliquot of DPH–tetrahydrofuran solution in a test tube and removing the solvents under a stream of dry nitrogen. Buffer was then added to give a total lipid concentration of 4 mM and the suspension mechanically vortexed for 1 min. Following vortexing, additional buffer was added to give 8 mL of a 1 mM lipid suspension. This was transferred to a 10-mL polycarbonate centrifuge tube and cyclically frozen and thawed five times with liquid nitrogen and a 37 °C water bath. Following the freeze–thaw cycles, the preparation was passed a total of five times through two stacked 0.4-μm polycarbonate filters in the Lipex extruder. The vesicles were used immediately or within 24 h of their preparation, with storage in the dark at 4 °C prior to use. Care was taken during all vesicle preparation steps to avoid undue exposure to light.

Fluorescence Measurements. Fluorescence data were collected with an SLM 48000 multifrequency phase-modulation fluorometer (Urbana, IL) under the control of an IBM PC-XT and associated SLM software. This technique has been previously described in detail (Gratton & Limkemann, 1983; Lakowicz & Maliwal, 1985). A xenon-arc lamp served as source for an excitation wavelength of 360 nm, while emission was monitored through a 420-nm high-pass filter and a Glan-Thompson polarizer set at the magic angle. Ten modulation frequencies between 5 and 90 MHz were used routinely, with an aqueous solution of rabbit liver glycogen serving as the lifetime reference. All experiments were carried out at 37 °C in order to determine effects at physiologically relevant temperatures. The choice of DPH as membrane probe was based on several considerations. DPH is well characterized and has no preference for different membrane phases (Lentz et al., 1976, 1980). Also, since this probe is untethered and uncharged, it should enjoy motional freedom within membranes unconstrained by attachment to other molecules or electrostatic forces.

Data Analysis. The phase and modulation data were subjected to double-exponential analysis and distributional analyses at estimated error values using the SLM software package. For distributions, this software fits the data to a discrete set of exponentials evenly spaced in lifetime with amplitudes determined by the form chosen for the distribution. An optimized version of the nonlinear least-squares algorithm of Brent (1973) is used to vary the center lifetime and width of the distribution so as to obtain the minimum value of the reduced χ^2 parameter. Final parameters for the bimodal Lorentzian distributions presented were obtained in a two-step process. Initially, the fractional intensities and lifetimes from the double-exponential decay were used as starting estimates for the fractional intensity and distribution center parameters, while the half-width parameters were arbitrarily set at 1–2 ns. On the basis of the values of reduced χ^2 achieved, subsequent starting parameters for the nonlinear least-squares analyses were then chosen so as to minimize this value. Originally, both Gaussian and Lorentzian expressions for the distribution were examined by this procedure. A bimodal Lorentzian form was chosen for the distributional analysis for several reasons. Our results suggest that bimodal Lorentzians and Gaussians appear to be about equal in their ability to be distinguished from double-exponential decays on the basis of reduced χ^2 values, and the Lorentzian fits in general required smaller half-width values. Model calculations [Figure 3 of Lakowicz et al. (1987)] show that a double-exponential fit of a bimodal Lorentzian distribution will show larger values of χ^2 than will such a fit of a bimodal Gaussian distribution, suggesting that the Lorentzian might be more easily distinguished. Our results also indicated that the major components of the double-exponential decay and distributional fits would have fractional intensities of some 0.9–0.95, and other model calculations (Alcala et al., 1987a) suggest that single Lorentzians are more easily distinguished from single exponentials than Gaussians or unimodal distributions. In addition, previous data on DPH in lipids and membranes have been analyzed in this manner (Fiorini et al., 1987a,b; Parasassi et al., 1987).

The estimated experimental error used in these analyses was 0.2° in the phase and 0.002 in the modulation, based on the standard deviation of averaged values for phase and modulation at each frequency. Occasionally, these error estimates gave reduced χ^2 values below 0.5, and in these cases error estimates of 0.15° and 0.0015 in phase and modulation, respectively, were substituted.

Results

The results in Table I are the fluorescence decay parameters obtained from double-exponential and bimodal Lorentzian fits to phase and modulation data for DPH in three separate vesicle or microsomal preparations. The reduced χ^2 values represent the median value of these three fits at the error levels noted above. Mean values are not reported because this value would not serve as a better indicator of overall goodness of fit and because error levels for separate replicates are not always the same. Generally, both the double-exponential and bimodal Lorentzian fits showed a component of 0.9–0.95 fractional intensity with lifetimes or central values near 8 ns and a minor

Table I: Double-Exponential and Bimodal Lorentzian Analyses of DPH Fluorescence Decay in Liver Microsomes and Lipid Vesicles[a]

	f_1	τ_1	w_1	f_2	τ_2	w_2	χ^2
POPC							
exponential	0.94 ± 0.02	7.86 ± 0.04		0.06 ± 0.02	1.47 ± 0.08		1.02
Lorentzian	0.93 ± 0.02	7.90 ± 0.06	0.09 ± 0.12	0.07 ± 0.02	1.83 ± 0.09	1.87 ± 0.36	1.03
EYPC							
exponential	0.95 ± 0.01	7.69 ± 0.06		0.05 ± 0.01		1.24 ± 0.15	0.99
Lorentzian	0.94 ± 0.01	7.72 ± 0.06	1.11 ± 0.53	0.06 ± 0.01	1.37 ± 0.25	2.29 ± 0.56	0.96
microsomal PC							
exponential	0.95	7.68		0.05	1.11		1.23
Lorentzian	0.94	7.72	1.67	0.06	1.11	2.23	0.93
microsomal phospholipids							
exponential	0.94 ± 0.01	8.22 ± 0.04		0.06 ± 0.01	1.21 ± 0.14		2.26
Lorentzian	0.95 ± 0.01	8.16 ± 0.03	3.40 ± 0.19	0.05 ± 0.01	0.40 ± 0.12	1.23 ± 0.45	0.89
microsomes							
exponential	0.89 ± 0.01	8.08 ± 0.07		0.11 ± 0.01	0.73 ± 0.08		7.92
Lorentzian	0.91 ± 0.01	8.01 ± 0.10	4.52 ± 0.55	0.09 ± 0.01	0.24 ± 0.13	0.44 ± 0.22	1.01

[a] Membranes and vesicles were labeled at a probe/lipid ratio of 1/400 and measured by phase-modulation fluorometry at frequencies between 5 and 90 MHz at 37 °C. Values for fitted parameters represent the mean and standard deviation obtained for three separate vesicle preparations, while the χ^2 values represent the median value obtained from these fits (except in the case of microsomal PC). χ^2 values were calculated by assuming errors of 0.2° and 0.002 in phase and modulation, respectively (see text). $f_{1,2}$ = fraction of exponential term or Lorentzian; $\tau_{1,2}$ = lifetime centers (ns); $w_{1,2}$ = half-widths of Lorentzians (ns).

component with a short lifetime, as commonly found in vesicle systems with DPH. The distribution analyses in general revealed similar lifetime centers to those obtained from the double-exponential analysis. In the following, we use the term w_1 to refer to the half-width parameter of the major component of the bimodal Lorentzian. The w_1 values obtained in these analyses give an indication of the multiplicity of fluorophore excited states.

POPC Vesicles. Previous distributional analysis of DPH decay in single-species phosphatidylcholine vesicles have so far not included species containing unsaturated acyl chains. To examine this case and serve as our starting point, synthetic POPC vesicles were prepared and measured. Decay data and results of the two analyses for a representative replicate are shown in Figure 1. In Figure 1a the vertical lines represent the lifetime centers obtained from the double-exponential fit, while the solid continuous lines represent the bimodal Lorentzian distribution. The fractional proportions and central values of the Lorentzians are equivalent to those obtained for the double exponential. The w_1 parameter indicates very little distribution of decay lifetime in these vesicles, in agreement with earlier results (Fiorini et al., 1987a) for saturated single-species PC above the phase transition. Comparison of reduced χ^2 (Table I) and residual values (Figure 1c,d) suggests the two fits are of comparable quality in describing the data and hence equally valid models. In conjunction with earlier results, this would suggest there is little excited-state multiplicity in vesicles composed of a single PC species.

EYPC Vesicles. To examine the effect of acyl chain compositional diversity on DPH fluorescence decay, EYPC vesicles were measured. EYPC consists of complex mixture of at least nine phospholipid molecular species, of which POPC is in fact the major component (43.3%), followed by PLPC (23%) (Pind et al., 1984). The data in Table I show that the fractional intensities and lifetime centers of the double exponential fit are in agreement with those of the bimodal Lorentzian, as for POPC. The value of w_1 has increased, however, compared to POPC. Despite this increase, the double-exponential and bimodal Lorentzian fits remain comparable on the basis of reduced χ^2 values. The implications of these two models for excited-state diversity are quite different. The increased value of w_1 suggests a greater degree of excited-state multiplicity compared to POPC, while the double-exponential fit suggests little change. This situation may be a case where the limitations imposed by experimental error and the assumption of a bimodal Lorentzian for the distribution preclude any distinction between a double-exponential and a distribution with narrow width on the basis of χ^2.

Microsomal Phospholipid Vesicles. Vesicles made from phospholipids extracted from microsomes represent an intermediate level of compositional complexity between natural membrane and EYPC vesicles in that phospholipid class as well as acyl chain compositional diversity is introduced. Extracted phospholipids include species from phosphatidylethanolamine, -serine, and -inositol classes in addition to phosphatidylcholine species. Results for these vesicles are given in Table I. As with POPC and EYPC vesicles, the fractional intensities and lifetime centers of the major components of the two fits are equivalent. The w_1 parameter, however, has more than doubled in value from that obtained for EYPC. Unlike the earlier vesicle systems, the relative values of χ^2 suggest that the distributional analysis is clearly superior and that a broader distribution of lifetimes is necessary to adequately describe the decay.

Direct comparison of results for microsomal phospholipids with EYPC is limited in that changes in both acyl chains and phospholipid class composition have taken place. Microsomal phospholipids in general might reasonably be expected to possess a greater degree of unsaturation in their acyl chains than would EYPC, and the increase in w_1 might very well be related to this difference alone. To test this possibility, the PC fraction of the total phospholipids was isolated by HPLC and formed into vesicles. Fractional intensities, lifetime centers, and the w_1 parameter obtained for these vesicles (Table I) are comparable to those obtained for EYPC, which suggests that the increase in distributional width in this system can mainly be attributed to the effect of increased phospholipid class diversity. That phospholipid class can affect the decay lifetime is supported by preliminary experiments with synthetic POPS vesicles: here the lifetime center of the major component was found to be about 1 ns larger than for POPC.

Liver Microsomes. Decay data and residual values for a representative replicate are presented in Figure 2. The trend toward increasing values of w_1 is continued, with an increase of some 1 ns observed. Increased discrepancy in reduced χ^2 values between the double exponential and the bimodal Lorentzians is also observed, with the residual plot for the double exponential (Figure 2c) showing larger and more systematic deviations. In this natural membrane, while all of the effects of acyl chain and phospholipid class compositional diversity

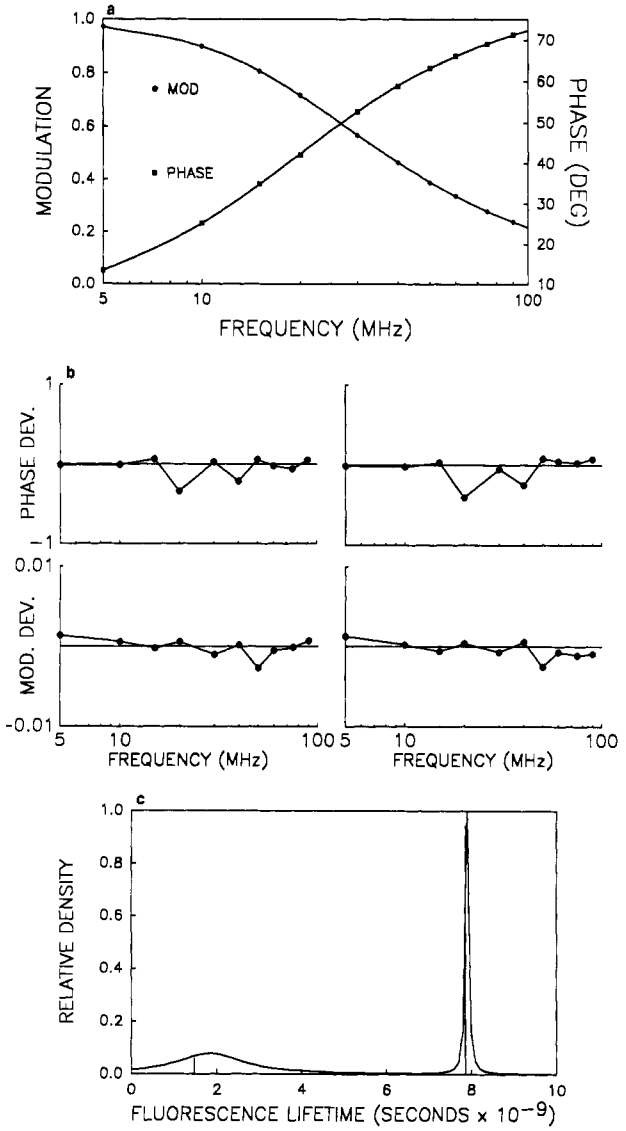

FIGURE 1: (a) Bimodal Lorentzian fits (solid lines) to phase and modulation data for POPC labeled with DPH. (b) Deviations for both the Lorentzian (left panels) and double-exponential fits. (c) Bimodal Lorentzian distribution of decay times. Also shown as vertical lines are the positions from the double-exponential analysis. Values of the parameters are given in Table I and experimental details are as described under Experimental Procedures.

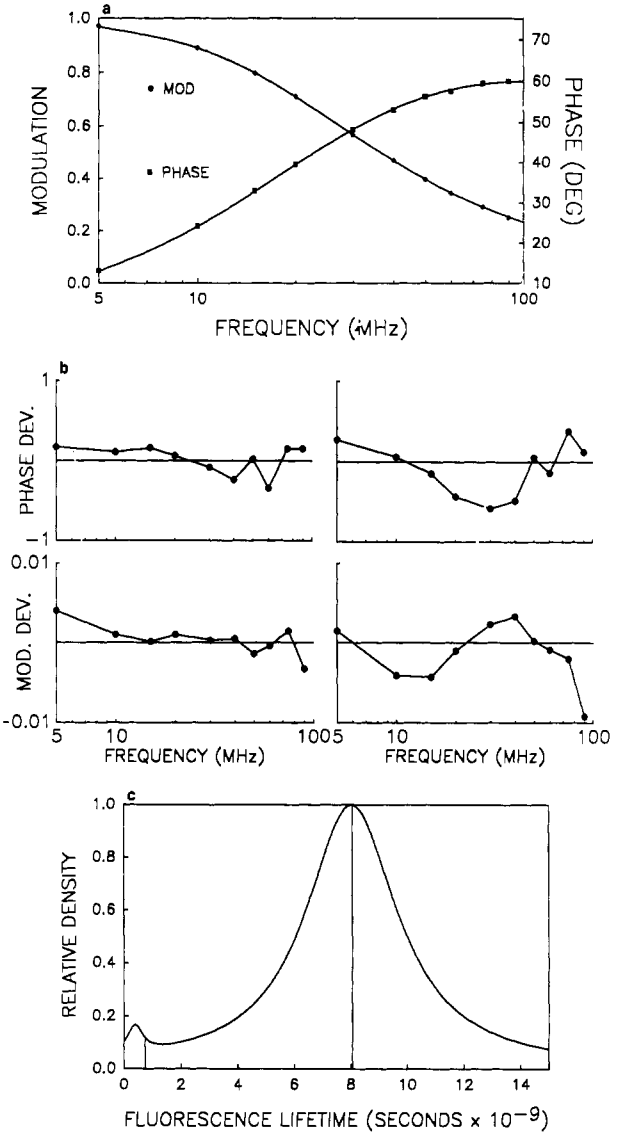

FIGURE 2: (a) Bimodal Lorentzian fits (solid lines) to phase and modulation data for microsomes labeled with DPH. (b) and (c) are the same as for Figure 1. Values of the parameters are given in Table I and experimental details are as described under Experimental Procedures.

observed above would be expected, further effects related to the presence of protein components and the overall organization of the membrane might be expected. These factors would appear to further increase the excited-state multiplicity of DPH.

Discussion

Our objective in examining DPH decay in the different membrane systems was to determine whether or not any correlation could be made between the parameter describing distributional width and membrane organizational and compositional features. The reasoning behind this approach can be seen on examination of previous results with the single phosphatidylcholine molecular species DOPC, POPC, PLPC, and PAPC (Stubbs et al., 1981). Here, the lifetime of the major component of a double-exponential analysis of DPH decay was found to vary between 7.3 and 8.4 ns, decreasing with increasing unsaturation. If a measurement were to be made on vesicles consisting of a mixture of these lipids, it would be reasonable to expect a range of lifetime centers arising from a superposition of these individual lifetimes. This illustrates why the DPH fluorescence decay might be expected to be distributed in systems with a complex composition.

Although the distributional approach has some attraction for describing fluorophore decay in membranes, definitive criteria allowing a clear judgement as to whether a distribution function or a multiexponential series better describes a particular observation are lacking. At least two investigations to date have examined the use of the reduced χ^2 values in this regard. Alcala et al. (1987a) place the comparison on a quantitative basis by considering the ratio of width to center values at various levels of experimental error and note that a single exponential should be distinguishable from a Lorentzian when the width/center ratio exceeds 0.07 at a 1% error level. However, on the basis of calculations comparing multiexponential and distributional approaches, Lakowicz et al. (1987) conclude that reduced χ^2 values in general provide very little distinction between these two approaches. In particular, they show that a double exponential may be indistinguishable from a single Lorentzian or Gaussian distribution and that a triple exponential may be indistinguishable from a bimodal Lorentzian and Gaussian. The latter authors resolve this dilemma by noting that the choice is dictated by the

properties of the systems undergoing measurement and their correspondence to the models underlying the method of analysis, an approach we take in this work.

Considered from the viewpoint of multiexponential versus distribution, the double exponential could be favored in the case of single-species PC and EYPC while the distribution is favored in more compositionally complex systems. However, since the two methods are hard to distinguish when the distributional width is small, this viewpoint may obscure an underlying trend, suggesting a connection between compositional complexity and distributional width that may be attributable to specific factors. The overall trend observed in the w_1 parameter might then be interpretable as showing the transition from a case of little excited-state diversity (POPC or single-species vesicles) to moderate excited-state diversity (EYPC) and finally to greater diversity (natural membranes). The w_1 parameter in itself has no direct physical meaning; however, this shift in thinking may provide better insight into the actual behavior of fluorophores in membranes and the future development of quantitative models that relate distributional width specifically to fluorophore and membrane physical properties.

As a step toward better understanding of the physical basis of distributional width, we wish to propose a model that envisions environmental heterogeneity as the cause of lifetime distributions and considers factors governing the manner in which these environments are sampled. For the purpose of this discussion, we define the "fluorophore environment" to be the lipid molecules and solvent immediately surrounding the fluorophore, in essence the "solvent cage" provided by the membrane. The time for the decay of a excited-state fluorophore to the ground state is modified by environmentally dependent excited-state interactions competing for the fluorescence emission process. These include a number of factors such as the vibrational relaxation of the excited state, internal conversion, conformational change, hydrogen bonding, and orientational relaxation (Badea & Brand, 1979).

Environmental heterogeneity can then be thought of as arising from three factors: the overall *organization* of a membrane, the *compositional* diversity of the lipids, and the *solvent* properties of the membrane. The primary factor is the overall organization of the membrane, by which we refer to such features of membrane structure as lateral phases, bilayer asymmetry, head-group interactions, and lipid–nonlipid interactions. Fluorophores in different organizational domains will differ in that their environments differ. Within organizational domains, compositional factors arise due to the large variety of lipid classes and species. A fluorophore in a particular organizational domain may experience little environmental heterogeneity if the domain is composed mainly of one lipid class or species, for example, or greater heterogeneity if the domain is composed of many lipids. Among solvent effects would be included the dielectric gradient as previously suggested by Fiorini et al. (1987a). All of these factors are of course interrelated, and although the divisions are arbitrary, they help to describe particular aspects of membranes.

In the vesicle systems examined in the present study all three factors contribute to the recovered w_1 values. First, there is the contribution to heterogeneity arising from the diversity of phospholipid molecular species and phospholipid classes as discussed above. In addition, an increase in acyl chain unsaturation might be expected to increase the dielectric constant gradient and thereby increase w_1. Finally, an organizational factor would be expected in stepping from vesicles made from natural phospholipids to intact natural membranes, and here again an increase in w_1 is observed.

The broader width obtained on increasing the number of lipid components implies greater environmental heterogeneity. This could be caused directly by the greater diversity of lipid molecules constituting the heterogeneous environment or by an increased solvent penetration. At the present time it is difficult to experimentally distinguish between these possibilities. However, in a situation where there is a decrease in lipid order (i.e., looser lipid packing) one would expect a greater degree of solvent (water) penetration into the bilayer. This could occur without changing the number of different lipid components, for example, by addition of perturbants, or the degree of unsaturation. For the systems in the present study, the fluorescence anisotropy values of POPC, microsomal phospholipids, and intact microsomes are 0.071 (Stubbs et al., 1981), 0.081, and 0.118 (Y. Nie, C. D. Stubbs, and E. Rubin, submitted for publication), respectively. These show a trend of *increasing* lipid order with increasing distributional width, suggesting that here compositional diversity may be the major contributing factor. The relative contributions of compositional diversity and solvent effects to the distributional width provide an important area for future study.

The second aspect of a model describing the basis for obtaining different distributional widths of fluorophore decays is the factors affecting the detection of environmental heterogeneity, which we term *sampling*. The idea of environmental sampling was invoked by Fiorini et al. (1987a) to explain their results showing a decreased width at higher temperatures in disaturated PC systems. Structural factors such as charge, size, and shape obviously influence which organizational domains or environments a fluorophore can probe or detect. Thus, the sampling relates to how many different environments an individual fluorophore samples during its excited-state lifetime. The fluorophore lifetime is thus an important property affecting sampling; shorter lifetimes tend to decrease, and longer lifetimes increase, the number of sampled environments. The fluorophore lifetime will be dictated primarily by its molecular composition and secondarily by its environment, so that the choice of fluorophore will be critical in the sampling process. If the time taken for an excited fluorophore to decay is rapid compared to the time taken for the surrounding environment to change, then only that particular environment will be sampled. In this case each member of an ensemble of fluorophores in a membrane of heterogeneous composition will contribute a different time for the return to the ground state, and a range or distribution of fluorescence lifetimes results. In contrast, if the time taken for a individual fluorophore to decay is long compared to the time taken for the environment to change, then that fluorophore may experience many distinct environments during its decay. It then becomes possible for each member of an ensemble to experience the same averaged effects. A narrowed distribution of lifetimes would then be expected. It is also apparent that the lipid dynamics of the membrane are significant, since the rate of lipid motion affects how rapidly environments change. Hence, both factors combine to influence the environmental heterogeneity experienced by an ensemble of fluorophores.

The photophysics of the fluorophore are of obvious interest regarding the sampling process. DPH photophysics have been previously reviewed by Zannoni et al. (1983) and Dale (1984). The fluorescence heterogeneity analyses of a range of different types of DPH probes, including charged species and those conjugated to lipids, reveal biexponential decay kinetics as surveyed by Cranney et al. (1983). This has been shown repeatedly for DPH itself, as in the present study. However,

evidence has been presented showing the minor (shorter lifetime) component to be due to a photoproduct of DPH (Barrow & Lentz, 1985; Parasassi et al., 1984), although it has yet to be characterized in any detail. While its presence can introduce some uncertainty into the analysis, we do not feel it is sufficient to have any major effect on the interpretation of the results. So far the only other probe that has been subjected to distributional analysis is parinaric acid (James et al., 1987), though again this probe displays nonmonoexponential decay kinetics in otherwise homogeneous systems, a feature common to most membrane probes. At the present time it is difficult to suggest which probes would be most suitable for investigating environmental heterogeneity in membranes. Indeed we would suggest that different probes would be better suited to studying the different aspects. Currently DPH may offer the most promise for detecting those factors underlying environmental heterogeneity.

Although our experimental data may deal more with the heterogeneity resulting from compositional changes rather than with the issue of environmental sampling, this concept points the way for future investigations. In particular, it should help to predict the effects compositional changes or the introduction of membrane perturbants affecting lipid dynamics have on distributional width.

Conclusions

We have demonstrated that increasing the complexity of membranes through compositional changes results in increased distributional width for DPH decay analyzed with a bimodal Lorentzian distribution function. It is proposed that this increase in width can be rationalized as reflecting increased environmental heterogeneity of the fluorophore due to changes causing greater complexity. The general causes of environmental heterogeneity are broadened to include compositional and organizational factors as well as the effects of the dielectric gradient. In addition to environmental heterogeneity, it is proposed that the sampling of this process is a function of both the fluorophore lifetime and the lipid dynamics. Future studies in this area should delineate in more detail how these factors contribute to this process.

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