Effect of Proteins on Fluorophore Lifetime Heterogeneity in Lipid Bilayers†

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ABSTRACT: The effect of three different membrane proteins on the fluorescence lifetime heterogeneity of 1,6-diphenyl-1,3,5-hexatriene (DPH) in phospholipid vesicle systems was investigated. For large unilamellar vesicles of dimyristoylphosphatidylcholine (DMPC) and 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) at 37 °C, the fluorescence decay was essentially monoexponential (8.6 and 8.2 ns, respectively) except for a minor component typical of DPH. For gramicidin D reconstituted into DMPC vesicles at a protein/lipid molar ratio of 1/7, the most appropriate analysis of the data was found to be in the form of a bimodal Lorentzian distribution. Centers of the major lifetime components were almost identical with those recovered for vesicles without proteins, while broad distributional widths of some 4.0 ns were recovered. Variation of the protein/lipid molar ratio in sonicated POPC vesicles revealed an abrupt increase in distributional width at ratios approximating 1/15–1/20, which leveled off at about 2.5 ns. For bacteriorhodopsin in DMPC vesicles and cytochrome b_5 in POPC, the most appropriate analysis of the data was again found to be in the form of a bimodal Lorentzian also with broad distributional widths in the major component. Lifetime centers were decreased for these proteins due to fluorescence energy transfer to the retinal of the bacteriorhodopsin and heme of the cytochrome b_5. Fluorescence energy transfer is distance dependent, and since a range of donor–acceptor distances would be expected in a membrane, lifetime distributions should therefore be recovered independently of other effects for proteins possessing acceptor chromophores. Photobleaching of the bacteriorhodopsin and trypsinization of the cytochrome b_5 effectively removed energy transfer, as demonstrated by a return of the lifetime centers to the values obtained for phospholipid vesicles alone. However, the value of the widths remained broad for both proteins (about 3.2 ns). From these results we would conclude that there is a distinct heterogeneity in the environment of the DPH in the protein–lipid interfacial region in lipid bilayers. Although the exact basis for the environmental heterogeneity caused by membrane proteins has yet to be ascertained, since the kinetics of the fluorescence decay process is of general importance in time-resolved fluorescence spectroscopy, these results may have important implications for studies of lipid bilayers containing protein.

The average time a molecule spends in its excited state or its fluorescence lifetime is of central importance in fluorescence spectroscopy. Fluorescence anisotropy, energy transfer, and quenching measurements all depend on a knowledge of this parameter. In addition, the value of the lifetime itself can yield important information due to the dependence of the fluorescence lifetime on the fluorophore environment. Until recently most fluorophore lifetimes were obtained from an analysis of data in the form of multiexponential decays, usually as a biexponential with a single major component. This assumes a homogeneous molecular environment for the fluorophore, if the minor component can be attributed to other processes. This assumption has now been challenged in a number of recent studies on the fluorescence decay of the tryptophan of proteins, lipid bilayer fluorophore probes, and other systems (James et al., 1985, 1987; James & Ware, 1985, 1986; Alcala et al., 1987a–c; Beechem & Gratton, 1988; Eftink & Ghiron, 1987; Eftink & Wasylewski, 1989; Fiorini et al., 1987a,b, 1988; Lakowicz et al., 1987a, 1988; Parasassi et al., 1987; Vincent et al., 1988; Williams & Stubbs, 1988). The alternative methods of analysis used in these investigations allow for multiple or distributed decay lifetimes which can result from heterogeneous fluorophore environments. Biological membranes have an extremely complex composition, so the idea that the hydrophobic environment should be heterogeneous is easily conceivable. Also, this approach would be in keeping with the concepts of lipid domains and microheterogeneity that have been suggested to exist in biological membranes.

At present there are two approaches to obtaining fluorophore lifetime distributions. In the frequency domain technique a Gaussian or Lorentzian form is assumed for the distribution (Alcala et al., 1987a; Lakowicz et al., 1987a), while in the time domain or single photon counting technique an exponential series method has been used (James & Ware, 1986). The width of the recovered distribution gives an indication of the degree of environmental heterogeneity of the fluorophore, where a greater distributional width indicates a greater environmental heterogeneity and hence a more diverse molecular organization in the membrane.

Recently we detailed some of the factors influencing the recovery of information on membrane structural diversity using the distributional approach (Williams & Stubbs, 1988). The major causes of fluorophore environmental heterogeneity were suggested to be organizational features such as bilayer asymmetry and lateral phase separations, penetration of water to different depths into the lipid bilayer constituting the dielectric constant gradient (Fiorini et al., 1987a), and compositional diversity due to the direct interaction of the different membrane components of the membrane with the fluorophore. The

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ability of the fluorophore to detect or sample these factors is due to a combination of the rate of lipid motion and properties of the fluorophore such as shape and charge factors and the fact that different fluorophores may have different "intrinsic" lifetimes in the same systems. Thus, for example, there will be a tendency for broader distributional widths to be recovered for a shorter fluorescence lifetimes and slower rates of lipid motion.

For single species of phospholipids in the gel phase, such as DPPC[1] and DMPC, the slow rate of lipid motion leads to a low sampling rate and the presence of the dielectric constant gradient leads to the recovery of a distributional width for DPH, due to its solvent sensitivity and its ability to free sample at different distances from the bilayer center (Fiorini et al., 1987a). In the liquid-crystalline phase, for DPH, bilayers of single species of phospholipids are environmentally homogeneous with little or no distributional width (James et al., 1987; Fiorini et al., 1987a). However, for complex mixtures of phospholipid molecular species and classes again a broad distributional width is recovered (Williams & Stubbs, 1988). Cholesterol has been shown to narrow the distributional width of DPH in such systems, probably due to the decreased penetration of water into the bilayer (Fiorini et al., 1987b, 1988). Recently the effect of cholesterol on the distributional width of dehydroergosterol has been used to study its exchange between vesicles (Nemecz & Schroeder, 1988).

In comparisons of the width of the major component of a bimodal Lorentzian distribution for DPH in liver microsomal membranes with that obtained for vesicles of the extracted phospholipids, we noted a greater value for the intact membranes (Williams & Stubbs, 1988). This indicated to us that the protein component of membranes may also be affecting the environmental heterogeneity of the fluorophore. To explore this possibility further, we have investigated a small selection of membrane proteins reconstituted into lipid vesicles. Our strategy was to use lipids that alone in vesicles were environmentally homogeneous with respect to DPH (i.e., had virtually no distributional width) and then to examine the effect of the presence of these proteins. Earlier studies show that DPH partitions more or less equally into different lateral domains of membranes with and without proteins (Lentz et al., 1976, 1980; Moore et al., 1978); therefore, it should be an appropriate probe for the detection of heterogeneity induced by proteins. In our current results, we found a broad distributional width for DPH in each instance despite differences in the types of protein used. From this we would conclude that membrane proteins can cause significant fluorophore environmental heterogeneity in membranes.

Experimental Procedures

Materials. DPH was obtained from Molecular Probes (Eugene, OR). Phospholipids were from Avanti Polar Lipids (Birmingham, AL) and quantified through determination of lipid phosphorus (Bartlett, 1959). Gramicidin D (*Bacillus brevis*) was purchased from Sigma and used without further purification. Bacteriorhodopsin was prepared as previously described (Scotto & Zakim, 1988). Cytochrome b_5, a gift of Dr. P. W. Holloway (Department of Biochemistry, University of Virginia Medical School), was prepared according to the method of Ozols (1974), except for an additional gel filtration step in deoxycholate, and stored frozen prior to use. All other chemicals used were of reagent grade or better.

Preparation of LUV. Aliquots of phospholipid in chloroform and DPH in THF were placed in a test tube (probe/lipid molar ratio 1/400) and the solvents removed under a stream of dry nitrogen. Buffer (10 mM Tris, 150 mM NaCl, pH 7.4) was then added to give a total lipid concentration of 800 μM and the suspension mechanically vortexed for 1 min. Following vortexing, the suspension was diluted to give 8 mL of a 200 μM lipid suspension (as multilamellar vesicles). LUV (diameter $\sim$100 nm) were then prepared by extrusion of freeze–thawed suspensions of phospholipids using a Lipex extruder (Vancouver, BC) as previously described (Hope et al., 1985). These 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.

Gramicidin D. DMPC LUV with gramicidin D were prepared according to a procedure based on the method of LoGrasso et al. (1988) with the additional steps required to make LUV. Gramicidin D was added from stock solutions in chloroform/methanol (97/3 v/v) to DMPC and DPH (probe/lipid molar ratio 1/400) previously dried under dry nitrogen in a test tube. LUV were then prepared as described above for phospholipids with both the extruder and suspension maintained at a temperature $\sim$10 °C above the phase transition of the lipid.

Using a simple sonication procedure, we found that POPC vesicles could be prepared in which DPH fluorescence decay lacked distributional width. This procedure was used to examine the effect of varying the protein/lipid ratio. By use of this method, a large number of samples could be prepared and measured in a single experiment under conditions minimizing sample and instrumental variations. Such a single experiment would have been more difficult for LUV. Vesicles were prepared from the 800 μM phospholipid suspensions described for LUV by using a Model 300 Fisher probe sonicator at half power, followed by dilution to 200 μM lipid concentration and centrifugation at 600g for 2.5 min to remove dust, titanium particles, and large aggregates. Sonication consisted of three 1-min bursts followed by 30-s pauses at 25 °C. We note that this procedure would not result in a strictly small unilamellar vesicle preparation, but this was not of primary concern in this experiment. Recovery of gramicidin D in the final preparation was determined by using the ratio of gramicidin absorbance at 280 nm after centrifugation to the absorbance of the known quantity of protein added before centrifugation, while POPC was measured through determination of lipid phosphorus. It should be noted that the organic solvent history of the gramicidin may influence the final conformation adopted by this polypeptide (Killian et al., 1988). In all experiments with gramicidin, the chloroform–methanol solvent used results in a nonchannel conformation (LoGrasso et al., 1988).

Bacteriorhodopsin. Bacteriorhodopsin (1.5 mg) was mixed with 15 mg of DMPC LUV in a final volume of 3 mL. The vesicles were mixed at 5 °C for 1 h to allow complete incorporation (Scotto, 1988). Lipid vesicles, lipid–protein complexes (proteoliposomes), and unincorporated protein were separated from each other by centrifugation on 10–75% linear glycerol gradients containing 10 mM Hepes, 100 mM KCl, 0.1 mM EDTA, and 0.02 mM azide (pH 7.5) in a SW-28 rotor for 18 h at 27 000 rpm and 30 °C. Purified proteoliposomes that banded in the lower portion of the gradient ($R_f \sim 0.75$) were removed by side puncture of the gradient and separated from the glycerol by group separation chromatography on unsub-

[1] Abbreviations: DMPC, dimyristoylphosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, dipalmitoylphosphatidylcholine; LUV, large unilamellar vesicles; NBD, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl); POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPOP, *p*-bis[2-(5-phenyloxazolyl)]benzene; THF, tetrahydrofuran; w_1, major component distributional full width at half-maximum.

stituted Sepharose MacroBeads (Pharmacia). Bacteriorhodopsin was measured by coprecipitation with soluble RNA (Polachek & Cabib, 1981) followed by protein determination with bicinchoninic acid (Smith et al., 1985). The protein/lipid molar ratio was determined from quantitation of inorganic phosphorus (Scotto & Zakim, 1985), with a ratio of 1/59 determined for the preparation used. Vesicles were labeled with DPH (probe/lipid molar ratio 1/400) by addition of 0.5–1.0 μL of a THF solution followed by a 60-min incubation at 37 °C in the dark. Photobleaching of the retinal was achieved by exposure to a 450-W UV lamp with the amount of chromophore remaining assessed by determination of the absorbance at 550 nm. Photobleached samples were then labeled with DPH.

Cytochrome b_5. Incorporation at protein/lipid molar ratios of 1/50 was achieved by addition of the protein to 2 mL of 200 μM POPC LUV in a cuvette followed by a 30–45-min incubation in the dark at 37 °C. For removal of the chromophoric heme, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone treated bovine pancreatic trypsin was added as powder (1 mg) to 2 mL of vesicles incubated with protein, and the total suspension was allowed to further incubate for 4 h at room temperature in the dark (Fleming & Strittmatter, 1978; Kleinfeld & Lukacovic, 1985; P. W. Holloway, personal communication). Vesicles without protein were similarly incubated as controls.

Fluorescence Measurements. Fluorescence lifetime data were obtained by using an SLM 48000 multifrequency phase modulation fluorometer controlled by an IBM PC-XT computer. Excitation was from a Liconix Model 4210NB HeCd laser at 325 nm which was then modulated by a Pockels cell to obtain a range of frequencies from 5 to 150 MHz. Emission was observed through a 420-nm red-pass filter and a Glan-Thompson polarizer, set at the magic angle. For a reference either POPOP in ethanol [with a lifetime of 1.35 ns (Lakowicz et al., 1981)] or an aqueous solution of rabbit liver glycogen as a scatterer was used. This multifrequency technique has been previously described in detail (Gratton & Limkemann, 1983; Lakowicz & Maliwal, 1985). The estimated experimental error used in the analyses was generally taken as 0.2° in the phase and 0.002 in the modulation, on the basis of the standard deviation of averaged values for phase and modulation at each frequency. We note that in some instances individual experiments may have errors outside this range but have adopted this practice since it is in common usage and facilitates comparison between experiments.

Fluorescence Anisotropy. Steady-state fluorescence anisotropy was measured with Glan-Thompson polarizers and 420-nm red-pass filters by using the relationship $(V - HG)/(V + 2HG)$, where V is the emission intensity observed through a vertically aligned polarizer for vertically polarized excitation, H is that for a horizontally aligned polarizer and vertically polarized excitation, and G is the ratio of vertically to horizontally polarized emission for horizontal excitation. The steady-state anisotropy gives a measure of a combination of the rate and order of lipid motion [see, e.g., Stubbs (1983)], with the emphasis being on the order component in the case of DPH. Therefore, although the fluorescence anisotropy measurement gives a reasonable assessment of lipid order, there is some slight uncertainty in that the contributions of the rate and order components are not known. In the present work, the anisotropy value also serves as an indicator of the incorporation of protein into vesicles, in that this parameter for DPH is substantially increased by the presence of protein [see, e.g., Stubbs et al. (1984)].

Data Analysis. Phase and modulation data were subject to double-exponential analysis and distributional analyses using the SLM software package. This software uses an optimized version of a nonlinear least-squares algorithm (Brent, 1973) to fit the data to minimal values of the reduced χ^2 parameter for the particular analysis and errors chosen. Bimodal Lorentzian forms for the distribution were used for several reasons. First, model calculations (Lakowicz et al., 1987a) suggest Lorentzian distributions are more easily distinguished from double exponentials than Gaussians on the basis of reduced χ^2 values. Also, Lorentzians generally required smaller half-width parameters than Gaussians, and finally, previous data on DPH in lipids and membranes have also been analyzed in this manner. For the Lorentzian distribution the analysis was taken to two half-widths, which is the fixed default value for the current version of the SLM software (version 1.4). Although this value truncates the tails of the Lorentzians, the long tails may in fact be considered to be less important, especially when it is considered that the Lorentzian distribution is only being used as an approximation for the "real" distribution. Nevertheless, we explored the effect of varying this parameter using the GLOBAL analysis software (Beecham & Gratton, 1988; Beecham, 1987). Increase in the number of half-widths to 7–10 increased the χ^2 slightly; above this value the χ^2 increased considerably. The full width at half-maximum of the major components decreased. A similar situation appears to be found with the SLM software revision 1.5 (to be released; G. Mitchell, personal communication). While with GLOBAL we found systems lacking distributional width were more difficult to handle (in that the distributional width recovered was extremely variable, perhaps reflecting the shallow χ^2 surface), importantly, all the general trends were found to be the same using the SLM or GLOBAL software.

RESULTS

The first requirement was to find phospholipid systems that would be environmentally homogeneous with respect to DPH. On the basis of earlier results (James et al., 1987; Fiorini et al., 1987a; Williams & Stubbs, 1988) POPC and DMPC above the gel–liquid-crystalline phase transition were chosen. To investigate the effect of proteins, two complex membrane proteins capable of quenching DPH fluorescence in addition to the simple transmembrane polypeptide gramicidin D were reconstituted into vesicles. The fluorescence decay parameters obtained from double-exponential and bimodal Lorentzian fits to DPH phase and modulation data from these various vesicle preparations are shown in Tables I and II.

Phospholipid Vesicles. Double-exponential and bimodal Lorentzian fits for DMPC and POPC LUV showed major lifetime components near 8 ns, with insignificant w_1 (major component distributional width at half-maximum) and equivalent χ^2 parameters (Table I). Lifetimes and centers recovered for DMPC were in general slightly higher than those recovered for POPC, which might be expected on the basis of differing unsaturation. Phase and modulation data from a representative replicate of DMPC LUV are shown in Figure 1, along with residual plots for the exponential and Lorentzian fits. The residual plots show little difference between a double exponential and a bimodal Lorentzian with narrow w_1. In addition, the phospholipid data also suggest that the change in excitation source from the arc lamp at 360 nm we used previously (Williams & Stubbs, 1988) to a HeCd laser at 325 nm has had little effect on the recovered decay parameters.

Gramicidin D Vesicles. Phase and modulation data for DMPC LUV containing gramicidin D are shown in Figure 2a. Bimodal Lorentzian analysis of these data resulted in

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

		α_1	τ_1 (ns)	w_1 (ns)	α_2	τ_2 (ns)	w_2 (ns)	χ^2
DMPC (n = 11)	exp	0.93 ± 0.02	8.55 ± 0.16		0.07 ± 0.02	2.58 ± 0.48		0.55
	lor	0.92 ± 0.01	8.58 ± 0.15	0.09 ± 0.15	0.08 ± 0.01	2.81 ± 0.34	0.82 ± 0.95	0.54
gramicidin D/DMPC	exp	0.91 ± 0.01	8.81 ± 0.16		0.09 ± 0.01	2.47 ± 0.28		2.32
(1/7 mole fraction) (n = 3)	lor	0.95 ± 0.02	8.66 ± 0.18	3.98 ± 0.22	0.05 ± 0.02	2.13 ± 0.35	1.31 ± 1.28	0.31
bacteriorhodopsin/DMPC	exp	0.68	7.02		0.32	0.87		22.21
(1/59 mole fraction)	lor	0.75	6.62	6.76	0.25	0.80	1.19	1.66
with 90-min photobleach	exp	0.89	8.84		0.11	2.38		1.43
	lor	0.89	8.87	3.30	0.11	2.91	2.30	0.35
POPC (n = 3)	exp	0.90 ± 0.01	8.24 ± 0.11		0.10 ± 0.01	2.40 ± 0.22		0.69
	lor	0.90 ± 0.01	8.24 ± 0.11	0.04 ± 0.07	0.10 ± 0.01	2.40 ± 0.22	0.00	0.73
with trypsin (n = 3)	exp	0.93 ± 0.02	8.19 ± 0.01		0.07 ± 0.02	2.16 ± 0.38		0.69
	lor	0.94 ± 0.01	8.15 ± 0.06	0.56 ± 0.59	0.06 ± 0.01	2.38 ± 0.51	0.92 ± 1.01	0.58
cytochrome b_5/POPC	exp	0.81 ± 0.02	6.99 ± 0.13		0.19 ± 0.02	2.31 ± 0.06		1.30
(1/50 mole fraction) (n = 3)	lor	0.94 ± 0.03	6.41 ± 0.33	4.21 ± 0.62	0.05 ± 0.03	1.57 ± 0.17	0.42 ± 0.75	0.40
with trypsin (n = 3)	exp	0.88 ± 0.01	7.84 ± 0.10		0.12 ± 0.01	2.10 ± 0.01		1.49
	lor	0.89 ± 0.02	7.77 ± 0.07	3.23 ± 0.48	0.11 ± 0.02	2.40 ± 0.53	2.07 ± 0.99	0.73

[a] Vesicles (LUV), labeled at a DPH/lipid ratio of 1/400, were measured by phase-modulation fluorometry at frequencies between 5 and 120 MHz at 37 °C. Errors for fitted parameters (where noted) represent the standard deviation obtained from the number of experiments indicated, while the χ^2 values represent the median value obtained. χ^2 values were calculated by using errors of 0.2° and 0.002 in phase and modulation, respectively (see text). $\alpha_{1,2}$, fraction of exponential or Lorentzian; $\tau_{1,2}$, lifetime centers (ns); $w_{1,2}$, full widths at half-maximum of Lorentzians (ns).

Table II: Double-Exponential and Bimodal Lorentzian Analyses of DPH Fluorescence Decay in Gramicidin D/POPC Vesicles[a]

mole fraction of gramicidin D		α_1	τ_1 (ns)	w_1 (ns)	α_2	τ_2 (ns)	w_2 (ns)	χ^2
0.199	exp	0.95	7.70		0.05	1.85		4.70
	lor	0.95	7.69	2.52	0.05	2.09	2.61	2.93
0.154	exp	0.94	7.73		0.06	2.30		0.85
	lor	0.97	7.61	2.44	0.03	1.69	1.02	0.62
0.077	exp	0.95	7.84		0.05	3.22		0.79
	lor	0.99	7.70	2.15	0.01	1.93	0.00	0.62
0.051	exp	0.98	7.77		0.02	1.37		1.65
	lor	0.97	7.82	0.32	0.03	2.31	2.31	1.86
0.042	exp	0.98	7.80		0.02	2.26		1.05
	lor	0.98	7.80	0.03	0.02	2.26	0.13	1.13
0	exp	0.97	7.76		0.03	2.44		0.31
	lor	0.97	7.76	0.00	0.03	2.44	0.00	0.34

[a] Sonicated vesicles, labeled at a DPH/lipid ratio of 1/400, were measured by phase-modulation fluorometry at frequencies between 5 and 90 MHz at 37 °C. χ^2 values were calculated by using errors of 0.2° and 0.002 in the phase and modulation, respectively. $\alpha_{1,2}$, fraction of exponential or Lorentzian; $\tau_{1,2}$, lifetime centers; $w_{1,2}$, full width at half-maximum of Lorentzians.

markedly improved fits in comparison to the double-exponential analysis, as indicated by the reduced χ^2 parameters (Table I) and the residual error plots in Figure 2b. The averaged value for w_1 from three separate vesicle preparations was about 4.0 ns, with representative bimodal Lorentzian distributions for gramicidin D/DMPC LUV and LUV without the protein compared in Figure 2c. To further characterize the effect of gramicidin D on distributional width and vesicle properties, the effects of varying protein/lipid molar ratio on the steady-state anisotropy and distributional width were measured in sonicated POPC vesicles (Table II and Figures 3 and 4). The steady-state anisotropy showed a gradual, continuous increase in the value of the anisotropy with increasing amounts of protein, with discernible changes noted at the lowest ratio of about 1/40. The distributional width, however, remained small until a protein/lipid molar ratio of about 1/15–1/20 was reached, whereupon it increased abruptly to a value of about 2.5 ns and remained roughly constant down to a ratio of about 1/5. Due to scattering problems, greater error in the experimental data was present at the highest protein/lipid ratio, causing fitting difficulties and increased values for χ^2. The χ^2 parameters recovered for Lorentzians showing width were lower than those for the double-exponential fits, as for the DMPC LUV, although differences were in general not as great. Simulation of phase

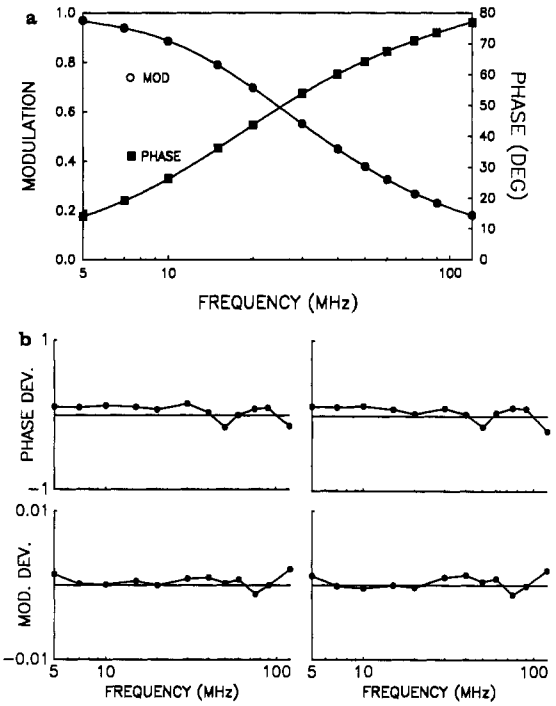

FIGURE 1: (a) Bimodal Lorentzian fits (solid lines) to phase and modulation data for DMPC LUV labeled with DPH. (b) Deviations for both the Lorentzian (left panels) and double-exponential fits. Values of the parameters are given in Table I with the experimental details described under Experimental Procedures.

and modulation values using parameters derived from the two experimental fits did, however, show reasonably similar values, indicating the appropriateness of the close χ^2 values. Analyses using lower error values resulted in χ^2 values differing by a slightly greater degree (not shown).

We note that in this system the lipid/protein ratio was decreased up to, but not beyond, the point where nonbilayer structures would be expected [somewhere between a ratio of 1/4 and 1/5 (Chapman et al., 1977; Rice & Oldfield, 1979)]. What possible additional effect these types of structures might have had on the distributional width remains an open question.

Bacteriorhodopsin Vesicles. The results of bimodal Lorentzian analysis for bacteriorhodopsin/DMPC LUV before and after photobleaching along with that for representative DMPC/LUV are shown in Table I and Figure 5. The Lorentzian fit to unbleached vesicles showed a substantial w_1

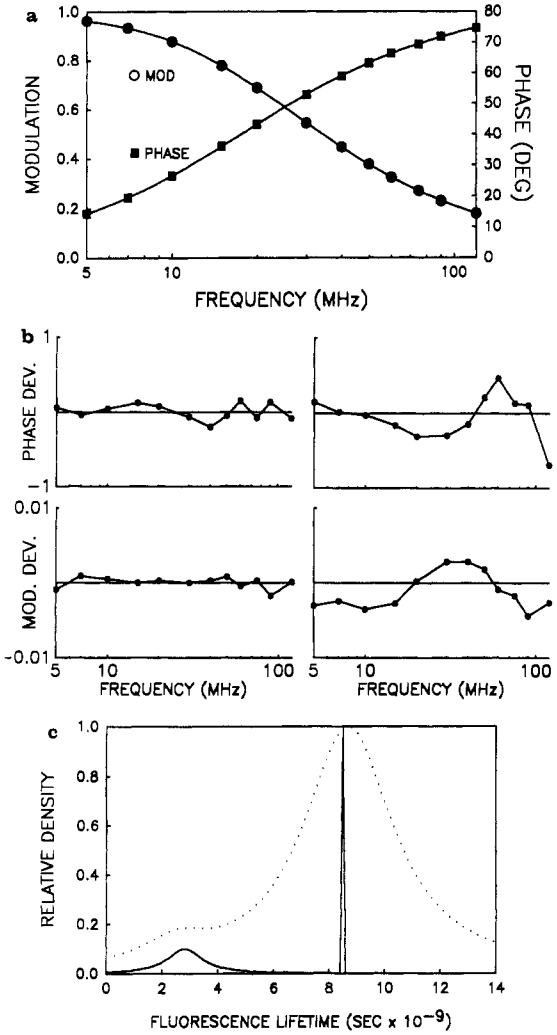

FIGURE 2: (a) Bimodal Lorentzian fits (solid lines) to phase and modulation data for DMPC/gramicidin (1:7 molar ratio) labeled with DPH. (b) As for Figure 1. (c) Bimodal Lorentzian distribution of decay times for DMPC (solid lines) and gramicidin D/DMPC (dotted lines). Values of the parameters are given in Table I with the experimental details described under Experimental Procedures.

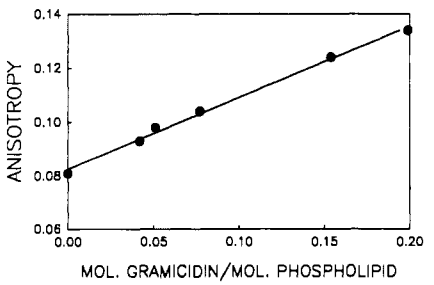

FIGURE 3: Steady-state anisotropy versus protein/lipid molar ratio for sonicated gramicidin D/POPC vesicles. Details are as described under Experimental Procedures.

of 6.76 ns and a 10-fold improvement in the χ^2 parameter in comparison to the double exponential. Unlike gramicidin D, the exponential lifetimes and distributional centers as well as the fractional intensities of the major component underwent significant decreases in comparison to the phospholipid. This reduction in DPH lifetime is indicative of the energy-transfer processes as previously reported for this protein (Rehorek et al., 1983). This also will affect the lifetime heterogeneity independently of the presence of the protein itself. Therefore, bacteriorhodopsin/DMPC LUV photobleached (before DPH addition) to minimize energy transfer were also examined.

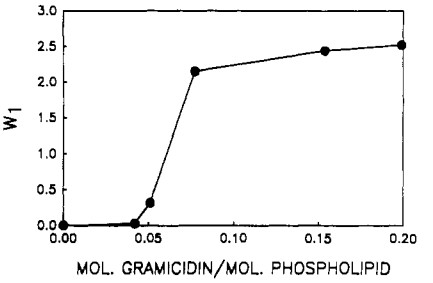

FIGURE 4: W_1 (major component distributional width) versus protein/lipid molar ratio for sonicated gramicidin D/POPC vesicles. Details are as described under Experimental Procedures.

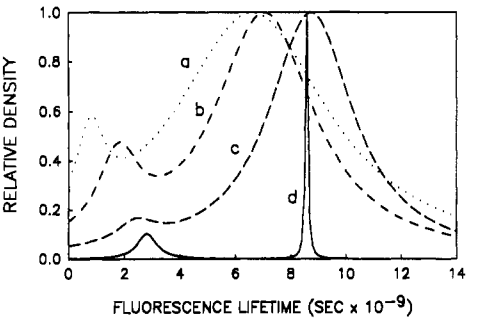

FIGURE 5: Bimodal Lorentzian distribution for bacteriorhodopsin/DMPC LUV after (a) 0 min of photobleaching (dotted line), (b) 40 min of photobleaching (short dashes), and (c) 90 min of photobleaching (long dashes), and (d) DMPC LUV alone (solid line).

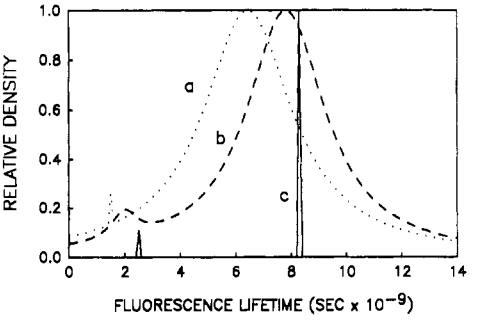

FIGURE 6: Representative bimodal Lorentzian distributions for cytochrome b_5/POPC LUV before (a, dotted line) and after (b, dashed line) treatment with trypsin as described under Experimental Procedures and for POPC LUV with trypsin (c, solid line).

Bimodal Lorentzian analyses of vesicles bleached for 40 and 90 min and subsequently labeled with DPH (Figure 5 and Table I) continued to show improvement over the double exponential. The Lorentzians showed the value for the lifetime center of the major component gradually restored to a value similar to that observed for DMPC alone, while the w_1 value, although decreased, remained large.

Cytochrome b_5 Vesicles. Like bacteriorhodopsin, cytochrome b_5 possesses a chromophore that can undergo energy transfer with donor fluorophores [see, e.g., Kleinfeld and Lukacovic (1985)]. The hydrophilic segment containing the heme acting as the acceptor, however, can be enzymatically removed with trypsin (Fleming & Strittmatter, 1978; Kleinfeld & Lukacovic, 1985). The results of bimodal Lorentzian analysis of DPH fluorescence decay for vesicles before and after trypsinization along with representative POPC LUV are shown in Table I and Figure 6. Bimodal Lorentzian analysis of the vesicles before treatment with trypsin gave a 3-fold improvement in the χ^2 parameter in comparison to the double exponential, with an average value of about 4.2 ns for w_1. The average lifetime center of the major component, about 6.4 ns,

was decreased from that found for the phospholipid vesicles without protein and showed an even greater reduction than that observed for unbleached bacteriorhodopsin. Following trypsinization, bimodal Lorentzian analysis also gave improved χ^2 parameters in comparison to the double-exponential analysis. The lifetime center returned to a value similar to that of the POPC vesicles, while the w_1 remained large at about 3.2 ns. Trypsin alone had no significant effect on the DPH fluorescence lifetime parameters, indicating that the width observed in trypsinized vesicles arises from the effect of the protein.

Discussion

The main obervation arising from this study was that the fluorescence decay of DPH in lipid bilayers containing protein is better described by bimodal Lorentzian distributions of lifetimes with substantial distributional width in the major component rather than by simple exponential decays. This effect was observed in three structurally distinct proteins. Gramicidin D, which inserts completely into the bilayer in the form of a dimeric ion channel [for recent review see Cornell (1987)], contrasts with bacteriorhodopsin with seven transmembrane helical segments (Henderson & Unwin, 1975) and cytochrome b_5, which incompletely inserts into bilayers (Dailey & Strittmatter, 1981; Kleinfeld & Lukacovic, 1985). Thus, although we recognize, along with others (Alcala et al., 1987a; Lakowicz et al., 1987a), that further refinement of distributional analysis is needed, it would appear for DPH that environmental heterogeneity induced by the presence of proteins in membranes leads to fluorescence lifetime distributions. This supports our earlier suggestion that proteins alone can have this effect, on the basis of the observation of a broader distributional width for microsomal membranes as compared to vesicles of extracted microsomal phospholipids (Williams & Stubbs, 1988).

Previously we have outlined factors governing fluorescence lifetime distributions within the framework of environmental sampling and environmental heterogeneity (Williams & Stubbs, 1988). These concepts may also be applied in the present situation with proteins. The "boundary layer" of lipids extending to several "shells" beyond the protein surface might be expected to be environmentally heterogeneous, causing fluorophore distributional width, if this diversity can be sampled. Previously, there was some discussion in the literature as to whether or not a boundary layer did indeed exist as distinct from the bulk lipid. Fluorescence and ESR investigations tended to support the notion of distinct regions, while NMR experiments tended to discount it (Kang et al., 1979). The solution to this problem is directly related to the time scale of the experimental technique [Jahnig, 1979; Jahnig et al., 1982; see also the reviews of Devaux and Siegneuret (1985) and Kinosita and Ikegami (1988)]. Thus, ESR and fluorescence experiments measure events on a time scale of approximately 10^{-7}–10^{-8} s, while NMR measures events on a time scale of 10^{-6}–10^{-5} s. Since the rate of exchange of lipids surrounding proteins has been determined to be on the order of 10^{-7} s [e.g., Paddy et al. (1981), Marsh et al. (1982), and East et al. (1985)], it would become difficult to explore this region by using NMR, since the lipids adjacent to the protein would become averaged with those in the bulk of the bilayer; i.e., the environmental heterogeneity is not "sampled". An approximation of the sampling potential of DPH as a fluorophore can be gained by estimating the distance an average DPH probe travels during its excited state. For lateral diffusion, this can be taken as $(4Dt)^{1/2}$, where D is the lateral diffusion constant and t is the lifetime of the excited state. Assuming a value of about 1×10^{-7} cm^2/s for D, which is the value obtained for several phospholipid probes in DMPC at 38 °C (Derzko & Jacobson, 1980), and a lifetime of 8×10^{-9} s results in a value of 5–6 $\times 10^{-8}$ cm. This would suggest that on average DPH can be thought of as essentially sampling only changes in the environment of the single lipid shell surround it during its excited state. Other differences may relate to the size and mobility of the probes used in the respective experiments, but the time scale of the experiment appears to be a key factor.

Given that the properties of DPH allow sampling of different regions of the bilayer both distant from and adjacent to a protein, an important remaining question is the underlying location and cause of the environmentally diverse region sampled by the DPH. Regarding the location of the environmental diversity underlying the fluorescence lifetime distributions, there are two possibilities. A distribution could arise from an environmentally diverse boundary lipid region extending several lipid shells beyond the protein or could be due to diversity at the hydrophobic protein surface (Lentz, 1988). If it arises from the boundary lipid region, then one would expect a distributional width to be recovered for a lesser protein/lipid ratio than if the protein surface were responsible. Protein perturbation of acyl chain ordering is known to persist over several lipid shells and is revealed by increased DPH steady-state fluorescence anisotropies (Moore et al., 1978; Jahnig, 1979; Jahnig et al., 1982; Lentz et al., 1983). If this perturbed region were also to give rise to a distributional width, then one would expect such a width to be obtainable over the same range of protein/lipid ratios. In fact, our results for gramicidin D/POPC indicate that while the anisotropy indicates perturbation at quite low protein/lipid ratios, a distributional width is only obtainable at higher ratios and may therefore require a shorter range protein–lipid or protein–probe interaction.

Environmental heterogeneity in protein–lipid systems could stem from at least three causes: differing external solvent penetration, rotational heterogeneity in the boundary region leading to lifetime heterogeneity, or contact with different regions on the hydrophobic surface of a protein. First, Fiorini et al. (1987a) have suggested that differing degrees of solvent penetration along the bilayer normal can result in a broad distributional width for DPH in lipid bilayers, especially below the gel–liquid-crystal phase transition temperature. Such differing degrees of solvent penetration could occur as a gradient over several lipid shells in the boundary region or perhaps only immediately adjacent to the protein due to special interactions with the protein surface or perturbed orientations of initial lipid shell acyl chains. So far this idea lacks experimental evidence; our data would not seem to favor the idea of a gradient extending over several lipid shells. A second possible cause relates to probe rotational heterogeneity. Rotational heterogeneity has previously been detected in lipid systems with DPH; whether or not this is accompanied by lifetime heterogeneity is unclear (Davenport et al., 1986; Wang et al., 1986). From our data, at least for gramicidin D, this would not seem an important causative factor, since the steady-state anisotropy data indicate effects on DPH rotational motion at protein levels where no corresponding effect is seen on distributional width. A third cause relates to the effects direct protein–lipid or protein–probe contact might have on the decay lifetime. A structure for gramicidin recently proposed, for example, shows spatially organized tryptophan side chains and suggests lipid chains "keyed tightly into the vertical hydrophobic contours" (Langs, 1988). Probes located in the

immediate lipid shell surrounding a protein clearly differ in their surroundings from those located in bulk lipid or further distant in the boundary layer, and such differences might influence their relaxation processes prior to emission.

In addition to those effects related directly to the presence of proteins in membranes, our results suggest that energy-transfer processes induced by the presence of protein chromophores can also result in distributional width. In the Forster analysis of energy transfer, the lifetime of the donor is related to its distance from the acceptor. Due to the variability in the distance between DPH donors and protein chromophore acceptors, a distribution of lifetimes would also be expected for any energy-transfer process. This has been demonstrated for an acceptor and donor attached to the ends of a flexible alkyl chain (Lakowicz et al., 1987b) and for proteins [e.g., Amir and Haas (1987) and Lakowicz et al. (1988)]. Preliminary results with model donor–acceptor vesicle systems containing DPH and NBD-labeled phospholipids also show qualitatively similar behavior (Stubbs et al., 1989). Hence, for proteins with acceptors, the w_1 value would be expected to reflect both an effect due to energy transfer and an effect caused by environmental heterogeneity at the protein–lipid interfacial region. At present, we are unable to separate the relative contributions of these two effects, except by physically removing the energy-transfer contribution. That this would then lead to a generalized decrease in the distributional width does not, of course, necessarily follow.

The finding that increased and quantitatively similar fluorophore lifetime distributional widths are observed for dissimilar proteins might suggest factors exist independent of specific protein structure, although this question is by no means resolved in this study. The interesting question of the effect of protein conformation also remains to be explored. By offering a new approach for the investigation of protein–lipid interactions, distributional analysis would at least appear to offer a means of insight into these and other questions. Finally, if these results for DPH in protein-containing membranes are representative of the common behavior of fluorescent probes, they may have some significance for fluorescence spectroscopy in general due to the central importance of the fluorescence lifetime parameter.

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