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The effects of phospholipid unsaturation and alcohol perturbation at the protein/lipid interface probed using fluorophore lifetime heterogeneity

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Abstract

The influence of phospholipid unsaturation and perturbation by alcohols, on the membrane protein/lipid interface, was probed using the fluorescence decay properties of 1,6-diphenyl-1,3,5-hexatriene (DPH) and DPH attached to the *sn*-2 chain of phosphatidylcholine (DPH-PC), in lipid bilayers and microsomal membranes. With microsomal membranes it was found that it was appropriate to describe the fluorescence decay of DPH-PC as a range of decay rates, accomplished by fitting the data to a bimodal fluorescence lifetime distribution. The major lifetime center had a broad distributional width, indicative of excited state fluorophore heterogeneity. The effect was attributable to protein, and by inference, the protein/lipid interface, since in vesicles made from total microsomal lipids (i.e., without protein) the fluorescence decay was homogeneous. Upon addition of ethanol or hexanol the width of the lifetime distribution of the major lifetime center increased, indicating increased environmental heterogeneity. It was confirmed that the effect was manifest at the protein/lipid interface, and not due to lipid-reorganizational factors, since it could also be obtained using a simple lipid bilayer vesicle system with apocytochrome *c* as a model membrane protein, and DPH instead of DPH-PC. Environmental heterogeneity was also found to increase with increased phosphatidylcholine (*sn*-2) unsaturation. The environmental heterogeneity at the protein/lipid interface could arise from a combination of varying polarities of amino acid side chains and of water that may intercalate in packing defects on the hydrophobic surface of the protein. Therefore the results could be explained on the basis of an increased degree of hydration at the protein/lipid interface. Such an effect offers a route whereby acyl chain perturbation and increased unsaturation might influence protein conformation and hence function.

Key words: Protein–lipid interaction; Phospholipid unsaturation; Membrane perturbation; Alcohol; Fluorescence lifetime; DPH

1. Introduction

The mechanism whereby the altered membrane lipid composition and membrane physical properties may be expressed in terms of altered membrane protein function is still poorly understood. Factors such as the level of phospholipid unsaturation in membrane phospholipids (reviewed in Refs. [1–3]) and perturbation by lipophilic compounds, ethanol being one of the most studied examples, influence many membrane protein mediated processes (reviewed in Refs. [4–6]). While alterations to bulk (i.e., average or overall) membrane 'fluidity' properties may in certain circumstances affect membrane protein function, the current consensus has moved away from this type of explanation and it appears more likely that effects on membranes are expressed in discrete regions. Whether the protein senses a bulk or local physical parameter expressed in a lipid domain the sensing 'event' must be at the protein/lipid interface.

To date the main spectroscopic approach to study of the protein/lipid interfacial region in membranes has

Abbreviations: χ^2_R, chi-squared; DPH-PC, *1*-palmitoyl-*2*-[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]ethyl]carbonyl]-*3*-*sn*-PC; DPH, 1,6-diphenyl-1,3,5-hexatriene; D_w, full-width at half-maximum peak height of the major lifetime center of a Lorentzian lifetime distribution; LUV, large unilamellar vesicles; MLV, multilamellar vesicles; PAPC, *1*-palmitoyl-*2*-arachidonoyl-PC; PC, phosphatidylcholine; PDPC, *1*-palmitoyl-*2*-docosahexaenoyl-PC; POPC, *1*-palmitoyl-*2*-oleoyl-PC; TMA-DPH, 1-(4-trimethyl-ammonium)-6-phenyl-1,3,5-hexatriene; PS, phosphatidylserine.

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been ESR, which spectroscopically identifies 'boundary lipids' (reviewed in Ref. [7]). These lipids have motional properties distinct from the remaining lipids which are remote from the protein and are referred to as 'bulk' lipids. Recent studies in our laboratory [8–13] and elsewhere (e.g., see Refs. [14–16]) have been exploring fluorescence spectroscopic approaches to providing further information on the membrane lipid/protein interface. If the fluorophores are located in a heterogeneous environment then one obtains a *range* of decay rates. This can be modeled as a fluorescence lifetime continuous distribution, where the width of the distribution is proportional to the degree of fluorophore environmental heterogeneity (reviewed in Ref. [17]). In the present work, in terms of DPH attached to PC, for the lipid-fluorophore to be classed as a 'bulk' lipid it should not be in physical contact with the protein surface while in the excited state. From the rate of exchange of lipids at the protein surface and the lifetime of the excited state, it can be estimated that the lipid-fluorophore should be further from the surface than the second 'shell' [9] to be considered to be in the bulk region and in which region environmental heterogeneity would not be experienced. By contrast, lipid-fluorophores within the first two shells would be influenced by the protein surface during the excited state and since it is a heterogeneous environment a range of decay rates would result.

Previous studies established that for the free fluorophore DPH, environmental heterogeneity is detected in lipid bilayers of a mixture of phospholipid molecular species, or for gel-phase single species of phospholipids, but not in bilayers of liquid crystalline phase single species phospholipids which were environmentally homogeneous with respect to DPH [8,18]. In lipid bilayers of a mixture of phospholipid molecular species, the looser lipid packing allows 'interstitial' water to penetrate deep into the bilayer, so that DPH molecules locating across the bilayer will be in regions of varying water content. The presence of water adjacent to DPH reduces its excited state lifetime [18,19]. Thus fluorophores closer to the bilayer surface will decay with a faster rate compared to deeper locations, and overall a range of decay rates will be obtained.

When DPH is placed in vesicles where the predominant phospholipid is a single species, analysis of the fluorescence decay indicates a homogeneous environment, however, upon inclusion of a protein, analysis reveals environmental heterogeneity [9,11,12]. In a previous study it was concluded that water may exist at the protein/lipid interface, which, together with other factors such as amino acid side chains protruding into the lipid region, provide regions of varying polarity on the protein surface that would explain this result [12]. When DPH is tethered to PC (DPH-PC), it was shown that the fluorescence decay, while still heterogeneous in cell membranes (as for DPH), by contrast, in vesicles of extracted lipids (i.e., with the protein removed) DPH-PC now reported a homogeneous environment (opposite to DPH) [11]. Thus for DPH in natural membrane systems (i.e., with a complex phospholipid species mixture that allows interstitial water) it is not possible to distinguish the environmental heterogeneity arising due to the fluorophore sampling different amounts of water according to the depth of the fluorophore in the bilayer, from that induced by protein, since both regions are heterogeneous with respect to the probe. However, with DPH-PC, the tethered DPH only experiences environmental heterogeneity at the protein/lipid interface, since it is unable to sample the bilayer normal. Thus, using DPH-PC, the parameter that relates to the degree of heterogeneity induced by protein can be potentially used to 'probe' the protein/lipid interfacial region in intact natural membranes.

Only the basic effects of increasing *cis*-unsaturation of phospholipids in terms of membrane physical properties are understood [1,2], and how this may affect protein functioning is still obscure. While the first double bond greatly reduces the gel-liquid crystalline phase transition temperature, subsequent double bonds have a lesser effect [20], and the same applies to lipid order, as reflected on the range of lipid motion [21]. By contrast, increasing unsaturation increases the rate of motion in proportion to the number of double bonds [21]. Recently, in fluorescence anisotropy studies of DPH-probes, a correlation of increased unsaturation and metarhodopsin function was described [22]. Increasing unsaturation has also been suggested to increase water penetration into lipid bilayers, at least as reflected by a decreased fluorescence lifetime of DPH and TMA-DPH [23]. When the level of phospholipid unsaturation in cell membranes is elevated by dietary means, the effects on physical parameters, as reflected by fluorophore probes that refer to bulk or *average* properties, appear much less responsive [24–26], suggesting the importance of more localized affects possibly located around membrane proteins. In a recent study we found that the rat liver microsomal membrane-protein/lipid interfacial environmental heterogeneity, as probed using DPH-PC, increased if the membranes were isolated from animals that had been subjected to chronic-ethanol ingestion [13]. One of the main effects of ethanol-treatment on these membranes is to modulate the unsaturation in PS, suggesting that it may be responsible for the effect. Thus one aim of the present study was to investigate the effects of unsaturation on the environmental heterogeneity expressed at the protein/lipid interface in model vesicle systems.

Alcohols are representative of the general class of lipophilic compounds that perturb cell membranes and there is also interest in the action of ethanol at the

membrane level, since ethanol intoxication and some effects of chronic-ethanol ingestion are directly manifest at the membrane level (reviewed in Refs. [4–6,27,28]). Also initial events in the action of anesthetics involve a perturbation in the acyl chain region that, if adjacent to a membrane protein, could influence its function. In our previous study [13], ethanol was found to increase environmental heterogeneity, in rat liver microsomal membranes, as probed by DPH-PC, whereas in membranes isolated from animals fed ethanol, chronically, this effect was not found. Whether an increase of environmental heterogeneity expressed at the protein/lipid interface is a general property of alcohol perturbation was therefore a further aim of the present study.

In this study, using DPH-fluorophores to probe both natural (microsomes) and artificial membranes (with apocytochrome *c* as a model protein) it was found that the environmental heterogeneity increased with increased levels of phospholipid *sn*-2 unsaturation or after addition of alcohols. These effects could be assigned to modifications located at the protein/lipid interface, since in protein free systems no environmental heterogeneity was seen.

2. Materials and methods

2.1. Materials

DPH and DPH-PC were obtained from Molecular Probes (Eugene, OR). Phospholipids were from Avanti Polar Lipids (Birmingham, AL). Cytochrome *c* from Sigma (St. Louis, MO). Other chemicals were from Fisher Scientific (Pittsburgh, PA) and were of the highest purity available.

2.2. Preparation of microsomes and lipid extraction

Rat liver microsomes were prepared and enzyme markers determined as reported previously [24]. There were low levels of 5'-nucleotidase and succinate dehydrogenase indicating only small amounts of plasma membrane and mitochondrial contamination, as previously reported. Lipids were extracted from the isolated membranes as described elsewhere [29].

2.3. Preparation of apocytochrome c

The preparation of apocytochrome *c* was largely as previously described [30]. Briefly, a mixture of 10 mg cytochrome *c* and 50 mg $HgCl_2$ was dissolved in 2 ml 8 M urea, 0.1 M NaCl and left to react in a test tube in the dark for 15 h. The product was then passed through a Sephadex-G25 column with ammonium acetate ($CH_3CO_2NH_4$) in order to separate apocytochrome *c* from the heme and any remaining cytochrome *c*. The fractions containing the protein were monitored by absorption at 285 nm and collected and lyophilized. The sample was then treated to remove protein-bound Hg and dialyzed at 4°C. The removal of heme was confirmed by the lack of absorption at 410 nm. Solutions of apocytochrome *c* were stored in aliquots at −77°C and thawed immediately before use.

2.4. Preparation of vesicles

Aliquots of lipids and DPH (in tetrahydrofuran) or DPH-PC (in chloroform) were placed together in a test tube, the solvent removed by a stream of nitrogen, and the lipids co-dispersed with the fluorophore to form MLV by the addition of 10 mM Tris-HCl, 150 mM NaCl (pH 7.4), followed by vortexing. LUV of ≈ 100 nm diameter were then prepared by extrusion of freeze-thawed suspensions of phospholipids using a Lipex Extruder (Vancouver, BC), as previously described [31].

DPH-PC was introduced into intact membranes by first preparing an MLV suspension of the fluorophore as described above, then sonicating for 3 min at 4°C using a Fisher sonicator with a micro-tip at 50% full power. DPH-PC sonicated vesicles were then incubated at room temperature in the dark for 2 h with the membranes. The membranes were washed by centrifugation (100 000 × *g*, 30 min) and the pellet re-suspended in buffer, followed by re-centrifugation, to remove DPH-PC which had not incorporated into the membrane. The incorporation of DPH-PC into microsomes was verified by following the change in fluorescence anisotropy with time, after addition of DPH-PC vesicles to microsomes, as previously reported [11]. The incorporation was slow, but was complete after ≈ 17 h, since by this time the fluorescence anisotropy had reached a plateau value. The fluorescence anisotropy of the microsomes after 2 h incubation and washing (as used in the lifetime measurements) was close to the 17 h value, this indicating that the unincorporated DPH-PC had been removed.

Apocytochrome *c* was incorporated by addition to preformed PC vesicles, with 10 mol% brain-PS, since it spontaneously incorporates into lipid bilayers with a negative charge [32]. This was confirmed by observing the increase of the fluorescence anisotropy of DPH upon addition of apocytochrome *c*.

2.5. Fluorescence measurements

Fluorescence lifetime data were obtained using an SLM 48000 multifrequency phase-modulation fluorimeter [33,34]. The source of excitation was a Liconix Model 4210NB HeCd laser at 325 nm, modulated by a Pockels cell to obtain a range of frequencies from 5 to

150 MHz. Emission was observed through a 420 nm long (red)-pass filter and a Glan-Thompson polarizer, set at the magic angle. For a lifetime reference an aqueous solution of rabbit liver glycogen was used. All measurements were performed at 37°C.

2.6. Data analysis

Phase and modulation data were subject to analysis using the GLOBALS UNLIMITED software (Laboratory of Fluorescence Dynamics, Univ. of Illinois, Dept. of Physics, Urbana, IL) as described [35,36] and the data fitted to minimize values of the reduced χ^2_R parameter. The experimental error used in the analyses was generally taken as the standard deviation of averaged values for phase and modulation at each frequency (usually ~ 0.002 and 0.2° in the modulation and phase, respectively). This method of choosing errors is common to phase fluorometry and as discussed elsewhere may lead to $\chi^2_R < 1$ [37].

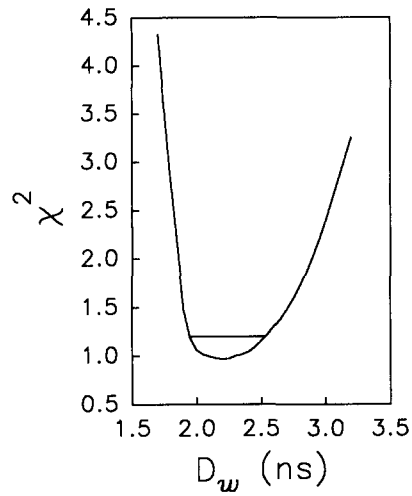

Fig. 1. A χ^2_R-surface plot for D_w for DPH-PC in microsomal membranes. The horizontal line is the 67% confidence level and represents the standard deviation. Experimental details are as described under Materials and methods.

3. Results

In previous studies we have established that for DPH and for DPH-PC in cell membranes a bimodal Lorentzian analysis is clearly superior in terms of χ^2_R and residuals [11–13], as illustrated in Table 1 for DPH and DPH-PC in liver microsomes. In general, only one of two forms of analysis were found to be appropriate, on the basis of χ^2_R and residuals, either a biexponential or a bimodal Lorentzian distribution (both forms allowing for a minor component – see below). The distributional analysis assumes excited state environmental heterogeneity, whereas the exponential assumes homogeneity, for the particular fluorophore location. For the (bimodal) distributional form of analysis to be favored over the biexponential, three criteria had to be met: (i) the χ^2_R for the (bimodal) distributional analysis had to be improved over that for a biexponential analysis (i.e., the ratio of the χ^2_R for a biexponential to that for a bimodal Lorentzian ($\chi^2_{Rexp/Lor}$) should be > 1); (ii) the residuals had to show improvement over the frequency range used, and (iii) the width at half-peak-maximum of the major lifetime center of the bimodal Lorentzian distribution (D_w) had to be significantly greater than zero, as indicated by a χ^2_R-surface plot (e.g., see Fig. 1). Thus, for instance, analysis of vesicles of extracted lipids, i.e., without protein, failed the criteria for a bimodal distribution, an exponential decay being favored due to the lack of χ^2_R improvement of the distributional analysis over the exponential. Also D_w was essentially zero, which alone would make a distri-

Table 1
Fluorescence decay analysis of DPH-PC and DPH in natural membrane and vesicles of extracted lipids

	χ^2_R	τ_1	w_1	f_1	τ_2	w_2	f_2
DPH-PC							
Microsomes							
2-exp	3.29	6.84 ± 0.06		0.87 ± 0.02	1.55 ± 0.30		0.06 ± 0.01
2-Lor	0.90	6.10 ± 0.07	2.18 ± 0.02	0.97 ± 0.01	0.64 ± 0.13	0.07 ± 0.09	0.03 ± 0.01
Vesicles of extracted microsomal lipids							
2-exp	0.79	7.91 ± 0.16		0.81 ± 0.05	4.22 ± 0.39		0.19 ± 0.05
2-Lor	0.91	7.92 ± 0.17	0.04 ± 0.03	0.81 ± 0.05	4.20 ± 0.36	0.01 ± 0.00	0.19 ± 0.05
DPH							
Microsomes							
2-exp	3.11	8.81 ± 0.15		0.87 ± 0.01	2.31 ± 0.23		0.13 ± 0.01
2-Lor	1.18	8.42 ± 0.18	1.85 ± 0.06	0.92 ± 0.01	2.57 ± 0.52	0.78 ± 0.42	0.08 ± 0.01
Vesicles of extracted microsomal lipids							
2-exp	0.60	8.58 ± 0.04		0.94 ± 0.01	2.14 ± 0.14		0.06 ± 0.01
2-Lor	0.56	8.53 ± 0.08	0.70 ± 0.15	0.96 ± 0.01	1.89 ± 0.22	0.08 ± 0.08	0.04 ± 0.01

2-exp, biexponential; 2-Lor, bimodal Lorentzian; $\tau_{1,2}$, lifetime (centers, ns); $w_{1,2}$, widths at half height of distribution (ns); $f_{1,2}$, fractional intensities; χ^2_R, chi-squared.

butional analysis pointless, since the analysis is showing that there is no environmental heterogeneity. Regarding the value of D_w, the smaller the value, the less was the improvement of χ^2_R for a fluorescence lifetime distribution over an exponential fit, as previously discussed [38]. At $0 < D_w < 0.8 \cdot 10^{-9}$ s the $\chi^2_{Rexp/Lor}$ is close to unity and the two fits become indistinguishable and the more complex distributional fit strictly should be rejected.

In the present study we have chosen the bimodal Lorentzian (rather than Gaussian or other forms) to conform with common usage in phase fluorometry (e.g., see [14–16,18,39–44]). It should be noted that the *choice* of the form of the lifetime distribution (Lorentzian, Gaussian etc.,) had no effect on the conclusions of the study which used the *degree* of heterogeneity for comparative purposes. The only other plausible analysis returning a comparable χ^2_R to the distributional analysis, was a triple exponential decay, however, this solution yielded physically unrealistic parameters.

Regarding the choice of probe for detection of environmental heterogeneity induced by protein as discussed above, water penetration into the bilayer can also be sensed by DPH, in cell membranes or vesicles of complex lipid mixtures. The exception is one or two component phospholipid species mixtures, as used in this study (below), otherwise for intact membranes DPH-PC had to be used since it is unable to sample heterogeneity along the bilayer normal and is therefore insensitive to water penetration, due to the tethering of the DPH to the PC *sn*-2 chain.

The fluorescence decay of DPH fluorophores is widely known to yield a 'minor lifetime component' typically of $\sim (1–3) \cdot 10^{-9}$ s (< 10%) and this was also observed here. The basis of this is considered to be a combination of the photophysical properties of DPH type fluorophores and/or photodegradation [45–47]. We note that the properties of the minor component (lifetime center, distributional width, fraction etc.) did not show any systematic variation as a function of added protein, alcohols or with varied phospholipid unsaturation.

The effect of protein is to induce heterogeneity into the fluorescence decay of DPH fluorophores. This has been previously demonstrated in this laboratory for natural and model systems and it has been concluded that the protein/lipid interface provides a heterogeneous fluorophore environment, as discussed above. However, if the bulk lipid region and protein/lipid interface had each contributed separate fluorescence lifetime centers, would a distributional analysis of the combination erroneously produce a broad D_w indicative of fluorophore heterogeneity? To investigate this, the experimentally recovered lifetime centers for DPH-PC in POPC/brain-PS ($7.46 \cdot 10^{-9}$ s) and POPC/brain-PS/apocytochrome *c* ($7.27 \cdot 10^{-9}$ s) were used to back-simulate two phase and modulation data sets (assuming no distributions and including minor components in the simulation). The two data sets were then combined and re-analyzed as a bimodal Lorentzian distribution. The resultant D_w was $0.1 \cdot 10^{-9}$ s, a value much narrower than the real D_w recovered from analysis of the actual decay from POPC/brain-PS/apocytochrome *c*, which was $0.46 \cdot 10^{-9}$ s. Thus we conclude that the D_w value reflects a real fluorophore environmental heterogeneity and not an artifactual D_w generated from two lifetimes analyzed as a single centered distribution.

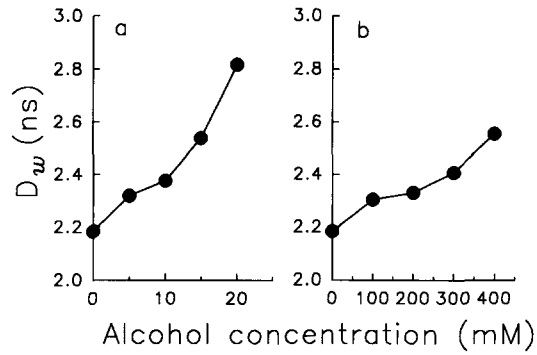

Fig. 2. Effect of hexanol (left panel) and ethanol (right panel) on D_w for DPH-PC in microsomal membranes. The error margin for D_w was $\pm 0.02 \cdot 10^{-9}$ s (from a triplicate determination with microsomes). Experimental details are as described under Materials and methods.

3.1. Effects of perturbation by alcohols

When ethanol or hexanol was titrated into liver cell microsomal membranes, labeled with DPH-PC, the D_w recovered from a bimodal Lorentzian analysis increased (Fig. 2). Also the major lifetime center decreased by a small degree (from $6.1 \cdot 10^{-9}$ s to $5.9 \cdot 10^{-9}$ s, for 400 mM ethanol). Hexanol has a higher partition coefficient into the lipid bilayer, due to its longer chain length and this was reflected in the greater potency of the effect compared to ethanol. The lifetime center after 20 mM hexanol addition decreased to $5.14 \cdot 10^{-9}$ s.

To test whether this effect could also be seen in a simpler model system, the same experiment was performed with apocytochrome *c*, added to pre-formed brain-PS/POPC vesicles (1:9 molar) with apocytochrome *c* (10 mol% of total phospholipid). With apocytochrome *c*, it was necessary to add a negatively charged phospholipid, in this case PS, so that it could interact with, and insert into the bilayer. Insertion (into POPC/brain-PS vesicles) was verified by monitoring the increase in the fluorescence anisotropy of the DPH as apocytochrome *c* was added, an increase being characteristic of the effect of membrane protein on

DPH anisotropy (e.g., see [48]) (results not shown). Although brain-PS consists of a number of molecular species, without protein, biexponential analyses of the fluorescence decay data for DPH was found to be appropriate, since analysis using a Lorentzian distribution did not improve the χ^2_R, and the recovered D_w was zero.

Experiments were designed to verify that the effect of alcohols on D_w was not due to a heterogeneous location of the DPH-PC, possibly reflecting any heterogeneity in the natural PC distribution in the membrane. It was thus reasoned that if the alcohol effect on D_w could be obtained in a model vesicle system, with a single protein and a different fluorophore (free DPH instead of DPH-PC), then this would validate the conclusion that alcohol increased the environmental heterogeneity at the protein/lipid interface in the natural membrane system. Further, the possibility that the result for intact membranes might have been explainable on the basis of complex rearrangements of lipid domains or asymmetry, such as are known to exist in cell membranes, could also be effectively eliminated. Also, PS/PC vesicles, with apocytochrome *c* (1:9, apocytochrome *c*:total phospholipid (molar ratio)), have previously been characterized using ^{31}P- and ^{2}H-NMR and the lipids were found to be in a bilayer configuration [49]. In the presence of apocytochrome *c*, calculated to be sufficient to minimize the proportion

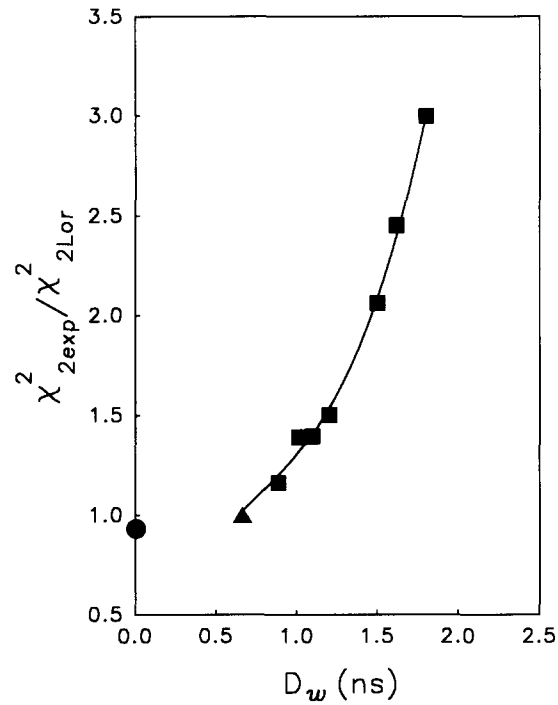

Fig. 4. The effect of increased hexanol on the values of the χ^2_R for a biexponential analysis divided by that for a bimodal Lorentzian ($\chi^2_{Rexp/Lor}$) for DPH in POPC/brain-PS LUV (PC/PS, 9:1 molar). (a) Without apocytochrome *c*, circle ($D_w = 0$); (b) with apocytochrome *c* ($D_w = 0.66$), triangle; (c) with apocytochrome *c* and 5–30 mM hexanol, squares. Experimental details are as described under Materials and methods.

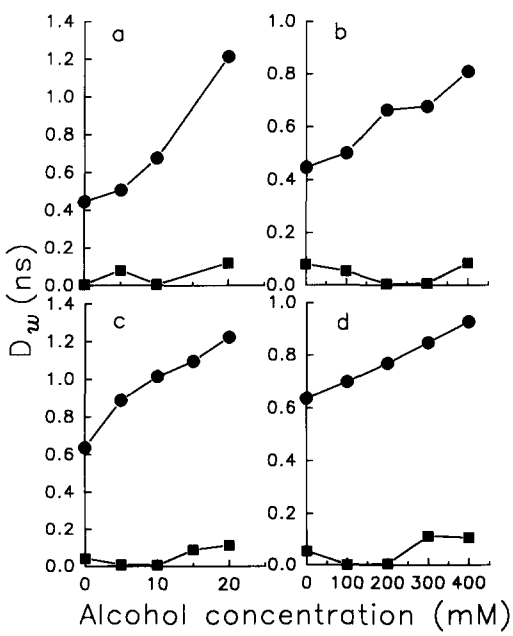

Fig. 3. Effect of hexanol (a,c) and ethanol (b,d) on D_w of DPH-PC (a,b) and DPH (c,d) in POPC/brain-PS (10%, molar) LUV in the presence (solid circles) and absence (solid squares) of apocytochrome *c* (apocytochrome *c*/total phospholipid, 1:9, molar). Error margins were $0.045 \cdot 10^{-9}$ s for DPH-PC and $0.041 \cdot 10^{-9}$ s for DPH (determined from triplicate measurements on POPC/brain-PS). Experimental details are as described under Materials and methods.

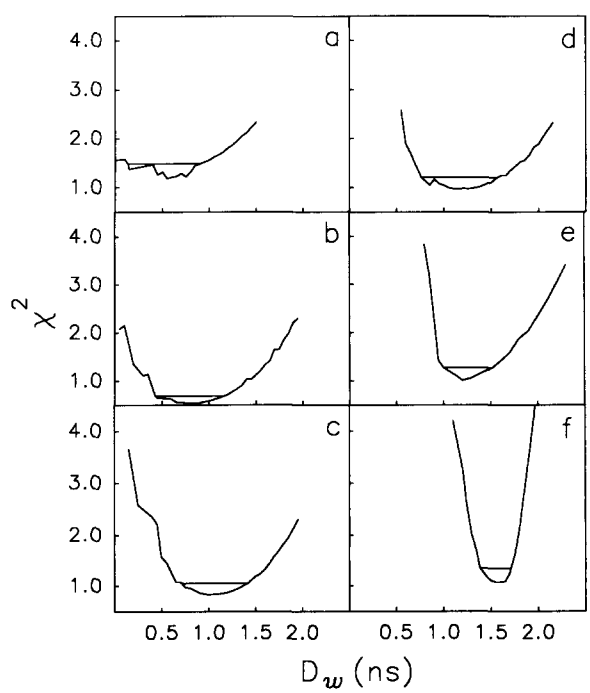

Fig. 5. χ^2_R-surface plots for D_w for DPH in POPC/brain-PS LUV (9:1, molar) in the presence of apocytochrome *c* (10 mol% of total phospholipids) in the presence of (a) 0; (b) 5; (c) 10; (d) 15; (e) 20 and (f) 25 mM hexanol. Experimental details are as described under Materials and methods.

of bulk lipids, upon addition of alcohols the value increased significantly (see Fig. 3). First it was confirmed that D_w increased upon alcohol addition with DPH-PC labeled PS/PC membranes, in the presence of apocytochrome *c* (see Fig. 3 (a,b)). Then the effect was examined with DPH, and as shown in Fig. 3 (c,d), D_w again increased with alcohol addition. Note, that without apocytochrome *c*, a biexponential analysis produced a lower χ^2_R as expected, since the heterogeneity is introduced by the protein. This means that to ask what happens to D_w without protein has strictly speaking no physical meaning since the analysis rejects the heterogeneous solution. Nevertheless, as a control Fig. 3 also shows that without apocytochrome *c*, neither addition of alcohols to DPH-PC or DPH labeled PS/PC, induced a significant increase of D_w, showing the increase to be dependent on the presence of the protein.

In the model system while the initial χ^2_R ratio ($\chi^2_{Rexp/Lor}$), before alcohol addition, was too low for the bimodal Lorentzian to be clearly favored over the biexponential analysis. This contrasted with the natural membrane, with DPH-PC (or DPH), where the greater diversity of heterogeneous fluorophore locations led to a much greater $\chi^2_{Rexp/Lor}$. However, upon addition of alcohol the recovered D_w of DPH (and DPH-PC), in the apocytochrome *c*-vesicles, gradually increased, and along with it the $\chi^2_{Rexp/Lor}$ increased, to values well exceeding unity (shown in Fig. 4 for DPH). Thus, while without alcohol the distributional analysis had to be rejected, after alcohol addition, at the higher concentrations, the bimodal Lorentzian distributional fit to the data became preferable. This conclusion is reinforced from the χ^2_R–surface plots, shown for the full hexanol concentration series in Fig. 5 (for DPH), which become progressively steeper, while the confidence in D_w increased with hexanol concentration. The result shows that alcohol addition increases protein induced environmental heterogeneity of DPH at the protein/lipid interface, since in the absence of protein the bimodal analyses were not superior in terms of χ^2_R to the biexponential and the recovered D_w was effectively zero. In a study with acetylcholine receptor enriched membranes from *Torpedo nobiliana* [50], it was shown that hexanol decreased the fraction of motionally restricted spin-labeled PC, i.e., in the fraction of probes located at the protein/lipid interface. The conclusion was made that the ESR probe was displaced by the hexanol into the bulk lipid region, however, if such an effect with the DPH-PC had occurred here, a decrease rather than increase in D_w would have accrued.

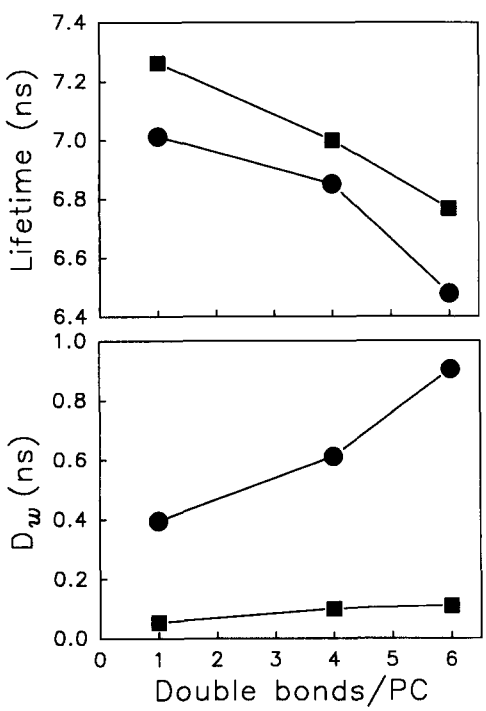

Fig. 6. Effect of increased PC-unsaturation for one, four and six-*sn*-2 *cis*-double bonds (POPC, PAPC and PDPC, respectively) in the presence of apocytochrome *c*, showing the fluorescence lifetime center of DPH-PC (TOP) and D_w (BOTTOM), with (solid circles) and without apocytochrome *c* (solid squares). Vesicles consisted of POPC/brain-PS (9:1, molar) with apocytochrome *c* (10 mol% of total phospholipids). Error margins were $0.19 \cdot 10^{-9}$ s for DPH-PC lifetimes and $0.045 \cdot 10^{-9}$ s for D_w (determined from triplicate measurements on POPC/brain-PS). Experimental details are as described under Materials and methods.

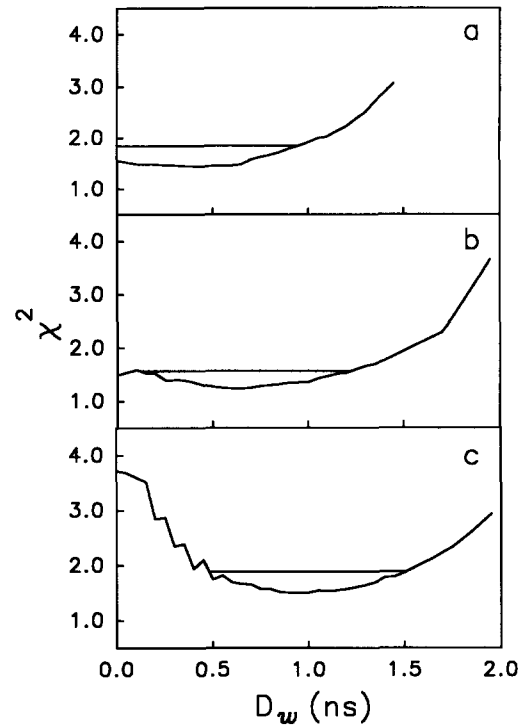

Fig. 7. χ^2_R–surface plots for D_w for DPH-PC in vesicles with varied PC-unsaturation in the presence of 10 mol% apocytochrome *c* with the same compositions as described in the legend to Fig. 6. (a) POPC, (b) PAPC and (c) PDPC.

3.2. Effects of increased phospholipid unsaturation

The effect of increasing *sn*-2 *cis*-PC unsaturation on environmental heterogeneity at the protein/lipid interface was investigated using the apocytochrome *c* model system and vesicles of three PC molecular species representing common constituents of cell membranes, with and without apocytochrome *c*. These were POPC, PAPC and PDPC, with one, four and six *sn*-2 *cis*-double bonds, respectively, with 10 mol% brain-PS. For these vesicles (in the absence of protein) the decay of DPH-PC was found to be homogeneous and a biexponential fit appropriate (i.e., $D_w \approx 0$, $\chi^2_{Rexp/Lor} \approx 1$) and analysis as a distribution failed to show a significant D_w (see Fig. 6). By contrast, in the presence of apocytochrome *c* the bimodal Lorentzian analysis recovered D_w values that increased for increased unsaturation (Fig. 6). Although, the value of D_w was still rather low (i.e., $< 0.8 \cdot 10^{-9}$ s), for PDPC (i.e., the most unsaturated-PC) the $\chi^2_{Rexp/Lor}$ increased into the acceptable range, as re-enforced by the χ^2_R–surface plots (see Fig. 7). The important point here was that the experiment revealed a trend for a greater protein induced D_w (i.e., environmental heterogeneity) with increased PC-unsaturation.

4. Discussion

In this work effects of PC-unsaturation and alcohol perturbation on protein induced fluorescence lifetime heterogeneity of DPH fluorophores were demonstrated. While the basic effects of phospholipid *sn*-2 *cis*-unsaturation and alcohol perturbation on lipid bilayer-acyl chain order and dynamics are well known there have been few studies addressing the effects at the level of the protein/lipid interface.

In lipid bilayers fluorophore environmental heterogeneity may be induced by a fluorophore existing in a range of diverse environments while in its excited state, as previously discussed [8]. Cell membranes, apart from a diverse range of proteins, have a complex acyl chain and phospholipid headgroup composition, therefore a range of different fluorophore environments, broad enough to result in a wide range of decay rates, may be reasonably anticipated. The main condition for this to occur is that the fluorophore should 'sample' the different environments available to it at a rate that is *slower* than the rate of excited state decay. While the value of the fluorescence lifetime yields information on the average polarity of the entire fluorophore environment (bulk lipid *and* protein/lipid interfacial environments), D_w as defined in the present study, reveals the extent of structural diversity of the protein/lipid interface itself. This is because the rate of exchange of a lipid-fluorophore (e.g., DPH-PC) at the protein/lipid interface is slower, by at least an order of magnitude, as compared to the excited state lifetime [9].

Increased unsaturation, increases hydration in a lipid bilayer [23,51]. This can be shown by a decrease in the fluorescence lifetime of a membrane associated fluorophore such as DPH or TMA-DPH [23] and in the present work we have extended this observation to the protein/lipid interface, the results indicating that increased unsaturation leads to greater 'hydration' in this region. This is probably due to the effect that the inflexible *cis*-double bonds have on the acyl chain arrangement around the protein, leaving packing 'defects' which could accommodate water molecules. Recently we showed that addition of cholesterol, known to have the opposite effect of *reducing* interstitial water in lipid bilayers due to its acyl chain condensing effect, using a similar approach to that of the present study, also reduced the level of water molecules residing at the protein/lipid interface [12].

Both ethanol and hexanol were found to increase D_w with a potency reflected by the greater partition coefficient of the latter into a membrane. The increased environmental heterogeneity would also appear to be due to increased protein/lipid interfacial hydration, although it is difficult to distinguish between increased interstitial water and increased alcohol, since both would have the effect of reducing the excited state lifetime of the fluorophore. However, previously it was concluded that addition of ethanol led to an increase of water and also of ethanol itself in the lipid bilayer [51] so that it may be reasonable to conclude that the same applies to the protein/lipid interface. Whether the effect of ethanol on D_w at the protein/lipid interface at pharmacologically relevant concentrations (100 mM), could also lead to altered membrane protein function cannot be ascertained from the present type of study. Here the primary goal was to examine the effects of general membrane disordering at the level of the protein/lipid interface. The results would, however, indicate that experiments to assess correlation of membrane protein function, with the effects shown here would be a worthwhile subject for future study.

The mechanistic consequences of altered levels of hydration at the protein/lipid interface have yet to be explored. It is probably now no longer appropriate to consider the protein and lipid regions in membrane as a separate entities. It seems likely that amino acid side chains hydrogen bond either directly to lipids or to water molecules that form part of the hydration layer in the lipid headgroup region so that, in essence, water may form a structural part of the protein. Thus from the results of this study we postulate that perturbation of this interaction is liable to modify the conformation of the protein. To test this hypothesis, systems in which hydration is systematically varied, as in the present

study, could be investigated in terms of protein conformation and function. This type of approach is the aim of current experimentation in this laboratory.

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