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Biophysical Chemistry 61 (1996) 151–160

Biophysical Chemistry

Binding and relaxation behaviour of prodan and patman in phospholipid vesicles: a fluorescence and ^{1}H NMR study

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Received 9 February 1996; accepted 22 April 1996

Abstract

The relative location, binding behaviour and the solvent relaxation behaviour of the polarity sensitive membrane probes 6-propionyl-2-(dimethylamino)naphthalene and 6-palmitoyl-2-[[trimethylammoniumethyl]methylamino]naphthalene chloride in vesicles composed of 1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine or egg yolk lecithin have been compared using steady-state and time-resolved fluorescence as well as high resolution NMR measurements. The reconstructed time-resolved emission spectra show unambiguously that the observed spectral shifts in vesicle systems have to be assigned to time-dependent solvent relaxation processes rather than to a probe relocation mechanism. All fluorescence as well as the NMR relaxation data suggest a deeper localization of Patman in the membrane, sensing a less polar and/or more restricted probe environment.

Keywords: Time-dependent Stokes shift; Dielectric solvent relaxation; Head group dynamics; High resolution ^{1}H NMR; Prodan; Patman

1. Introduction

The function of peripheral membrane proteins is regulated in many cases by the chemical composition and the physical properties of the headgroup domain of the phospholipid bilayer. The proteolysis of vitamin K dependent proteins in blood coagulation may serve as an example for the catalytic role of phospholipid surfaces in protein activations [1]. Although such aspects focus the interest on structural parameters of the interfacial region, fluorescence spectroscopy of membrane labels has been mostly employed to characterize the hydrophobic bilayer interior [2–4]. The low number of fluorescence spectroscopic characterizations of the phospholipid headgroup region may be the result of a relatively small amount of structural information accessible by the dominating fluorescence method for membrane characterization, the determination of the fluorescence anisotropy [5].

The fluorescence probe Prodan (6-propionyl-2-(dimethylamino)-naphthaline) is thought to be localized in the phospholipid headgroup region and exhibits extreme large Stokes shifts with increasing polarity of the medium (e.g. from 401 nm in cyclohexane to 531 nm in water) [6]. In the phospholipid

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PII S0301-4622(96)02185-0

gel-state its emission maximum is around 437 nm, while in fluid crystalline phase it is shifted to about 490 nm. Thus, Prodan has found widespread use in membrane studies because of its large Stokes shift associated with phospholipid phase transitions [7–12]. The fluorescence probe Patman (6-palmitoyl-2-[[trimethylammoniumethyl]methylamino]naphthalene chloride) has been synthesized in order to localize the chromophore — which is essentially identical to that of Prodan — more defined in the membrane by attaching a palmitoyl instead of the propionyl chain. Moreover, a Me_3N^+-group was introduced to orient the alkylamino-end of the fluorophore toward the lipid-water interface [13].

In this work we use steady-state and time-resolved spectroscopy as well as ^1H NMR-measurements to define the relaxation behaviour and the relative localization of the two very polarity sensitive probes Prodan und Patman in model membranes. It is known [14,15] that the trimethylammonium group plays an important role in monitoring the structure and dynamics of the lipid headgroups. Thus, the influence of the fluorescence probe on the motional dynamics of the choline headgroup region was determined by high resolution ^1H NMR, measuring the relaxation times. The reconstruction of time-resolved emission spectra (TRES) allows a direct measurement of the relaxation process. We show that Patman yields blue-shifted steady-state spectra and considerably slower solvent relaxation, as observed by the TRES. All results from fluorescence spectroscopy and NMR support our conclusion that Patman senses a less polar and/or more restricted environment than Prodan.

2. Materials and methods

Egg yolk phosphatidylcholine (PC) was from Avanti Polar Lipids, 1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC) was from Fluka. Prodan and Patman were purchased from Molecular Probes. Except for some of the binding studies the dyes were already present when preparing the lipid systems. The fluorophores were added to the lipid in chloroform from an ethanolic stock solution (4 mM) to a final lipid/dye ratio of 100:1 for time-resolved and 200:1 for steady-state measurements. The solvent was removed under a stream of N_2 and the remaining lipid-dye mixture was kept under vakuum overnight. Tris buffer (20 mM Tris, 100 mM NaCl, pH 7.5) was added and the lipid film was allowed to swell for 1 h above the phase transition temperature with occasional vortexing to yield multilamellar vesicles (MLV). 10% sucrose were added to prevent the sedimentation of the liposomes. Small unilamellar vesicles (SUV) were prepared by sonication above the phase transition. The vesicles in the obtained clear solution were allowed to anneal for 30 min and centrifuged in order to remove MLV and titanium particles. Lipid concentration was approximately 1 mM for the time-resolved and 0.1 mM for steady-state fluorescence studies. For the NMR-experiments the SUV were prepared from egg yolk phosphatidylcholine in heavy water (33 mM phospholipid, 100 mM NaCl, pH 6.5) at 5°C under a stream of argon in the presence of Prodan and Patman (3.3 mM), respectively. DSC-measurements were performed as described previously [16]. An average mass-weighted radius of 15 nm has been obtained with a distribution width (standard deviation) of 5.5 nm.

Fluorescence decays (λ_{ex} = 337 nm) were recorded with commercial single photon counting equipment (Edinburgh Instruments 199 S) and analyzed using an iterative reconvolution technique, as described [17]. Steady-state spectra and -anisotropies were recorded with an Aminco Bowman II spectrometer. Temperature was controlled within 0.2°C. Time-resolved emission spectra (TRES) were calculated from the fit parameters of the multiexponential decays detected from 390 to 530 nm and the corresponding steady-state intensities [18]. The TRES were fitted by log-normal functions [19]. Correlation functions $C(t)$ are calculated from the emission maxima $\nu(t)$ of the TRES at defined time t after excitation [18]:

$$C(t) = \frac{\nu(t) - \nu(\infty)}{\nu(o) - \nu(\infty)} \quad (1)$$

where $\nu(0)$ and $\nu(\infty)$ are the emission maximum (in cm^{-1}) at times zero and ∞, respectively. In all cases the solvent response cannot be satisfactorily described by a monoexponential relaxation model. In order to characterize the overall time scale of the

solvation response, we use an (integral) average relaxation time:

$$\langle \tau \rangle \equiv \int_0^\infty C(t)\,dt \qquad (2)$$

The NMR spectra were obtained at 250 MHz (AM/250 Bruker at the IOM Leipzig) at 22°C. The relaxation time T_1 was measured by the $\pi - \pi/2 -$ pulse sequency and T_2 by the Carr, Purcell/Meiboom, Gill-method ($\pi/2$-π-π-π-....). Overlapping signals were analyzed by the computer programme PEAKFIT. In comparison to other chemical groups the choline headgroup $C\text{–}N(CH_3)_3^+$ is characterized by a very high freedom of mobility even in lecithin multilayers because of the fast rotational motion of the $-CH_3$ and the $-N(CH_3)_3$ group, respectively, with a threefold symmetry [14,20]. After sonication the multilayers break up, forming small bilayer vesicles and show headgroup ^{1}H-NMR signals of only a few hertz in width. Particularly the $C\text{–}N(CH_3)_3^+$ headgroup in small vesicles can adopt numerous different orientations with respect to the lipid surface. For a given methyl rotor the rotor axis can wander [21] over a large angular range ($\geq \pm 60°$). Aside from a very fast decay of the magnetization by 11% during 10 ms after the pulse, which is attributed to remaining multilayers, the relaxation processes for T_1 and T_2 show an exponential behaviour for times greater than 20 ms after the first pulse [15]. An exponential decay of the magnetization is expected if the angular range of fluctuation is very large [21]. Therefore, we will interprete the results under the assumption that the angular range of a methyl rotor axis lies between $\pm 90°$. Considering these arguments it is justified to describe the proton relaxation of the choline methyl group without the introduction of an order parameter, describing the restricted angular reorientation of the methyl rotor axis. Using this simplification the well-known theory of Woessner [22] can be applied for the relaxation of protons of methyl groups undergoing fast anisotropic motion (τ_c) of the methyl rotor and the second independent correlation time τ_{c1} characterizing the reorientation of the $N(CH_3)_3$ axis. Introducing the correlation time

$$1/\tau_{c3} = 1/\tau_{c1} + 4/\tau_c \qquad (3)$$

and considering the results of Feigenson and Chan [21] and the scale of reorientation times, compiled by Higinbotham et al. [23], we can propose the validity of the approximations

$$(2\pi f \tau_{c3})^2 \ll 1 \wedge (2\pi f \tau_{c1})^2 \gg 1,$$

where f is the resonance frequency of the spectrometer ($f = 250$ MHz). This assumption simplifies the interpretation, because τ_{c3} only depends on τ_c.

Therefore, the following equations derived from the formulas of Woessner can be used only in a small range of frequencies for monitoring the effects of additives on the dynamics in the head group region:

$$1/T1 = 9/4\,\gamma^4 \hbar^2 r^{-6} \tau_{c3} \qquad (4)$$

$$1/T2 = 9/40\,\gamma^4 \hbar^2 r^{-6} \tau_{c1} \qquad (5)$$

where γ is the magnetogyric ratio for protons, $\hbar$ is the Planck constant devided by 2π and r is the proton-proton distance for a methyl group.

3. Results

3.1. Steady-state spectroscopy

Steady-state emission spectra ($\lambda_{ex} = 337$ nm) as a function of temperature have been recorded for Prodan and Patman in small unilamellar vesicles (SUV) and multilamellar liposomes (MLV) composed of DMPC. A large Stokes shift is observed in all systems when crossing the phase transition temperature T_c from the gel to the liquid crystalline phase. The spectra of Prodan are broader and red-shifted compared to Patman. While the spectra of Patman in SUV and MLV are similar, the spectra of Prodan in SUV are much broader than in MLV for $T \leq T_c$ (Fig. 1). These differences can be expressed quantitatively in terms of the general polarisation (GP) [24] or simply by the ratio I_{blue}/I_{red}. Fig. 2 shows that the broad spectra in SUV lead to small GP-values in the gel phase which decrease more or less continuously near T_c. In contrast the sharpest decrease of the I_{blue}/I_{red}-ratio around T_c is obtained for Prodan in MLV, while the behaviour of Patman in both vesicle types is quite similar. In accordance with the results of Parasassi [24] a defined value for I_{blue}/I_{red} is found for all samples at high temperature in the liquid crystalline phase. The behaviour of Patman in

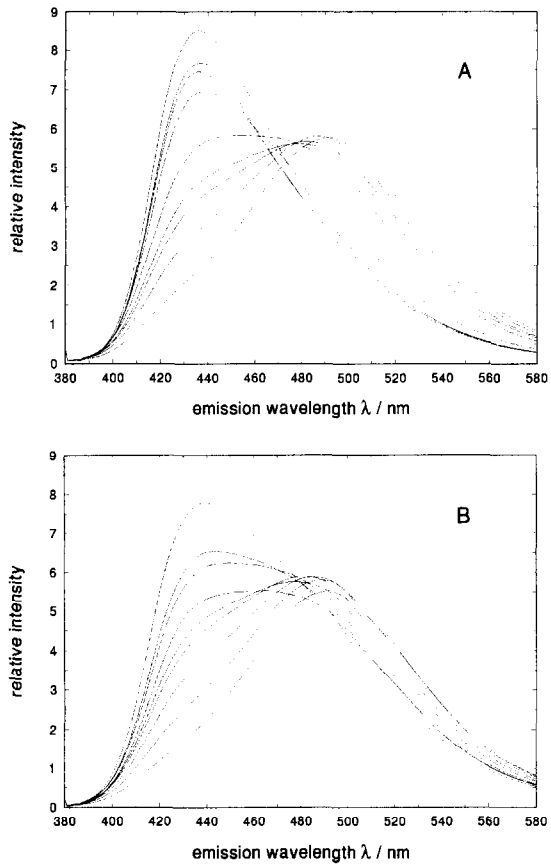

Fig. 1. Fluorescence emission spectra for Prodan in DMPC vesicles at different temperatures, measured at excitation wavelength 360 nm. With decreasing intensity at 430 nm, temperatures are; 15°C, 20°C, 22°C, 23°C, 25°C, 27°C, 30°C, 35°C, 45°C; (A) in multilamellar vesicles (MLV); (B) in small unilamellar vesicles (SUV).

MLV is quite similar to that observed for Laurdan in MLV as might be expected since both dyes possess a long acyl chain. While the use of SUV leads only to a minor decrease of the cooperativity of the transition for Patman (the decrease of I_{blue}/I_{red} around T_c is somewhat less sharp) Prodan is clearly able to differentiate between both types of vesicles.

Steady-state spectra with different excitation wavelengths were recorded for both dyes below (15°C), near (23°C) and above the phase transition (30°C, 45°C) (Fig. 3). While Prodan shows a red-shifted spectrum when excited at the extrem red edge of the absorption spectrum even in the gel phase, no such shifts are observed for Patman. The shifts for

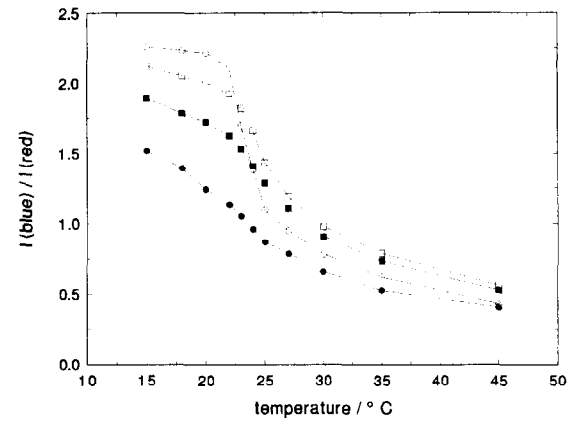

Fig. 2. Ratios of fluorescence intensities of the blue and red emission maxima (I_{blue}/I_{red}); intensity values are taken at 440 and 490 nm for Prodan and 428 and 478 nm for Patman, respectively; excitation wavelength is 360 nm:
DMPC-SUV, Prodan (·); DMPC-MLV, Prodan (○); DMPC-SUV, Patman (■); DMPC-MLV, Patman (□).

Prodan are largest near the phase transition and still detectable far above T_c.

Steady-state anisotropy measurements of both dyes as a function of temperature are able to monitor the phase transition, yielding a much steeper decrease of the anisotropy values r_{ss} near T_c (not shown). The steady-state anisotropy depends on both λ_{ex} and λ_{em}. The r_{ss}-values increase with increasing λ_{ex} and decrease with increasing λ_{em}. The values

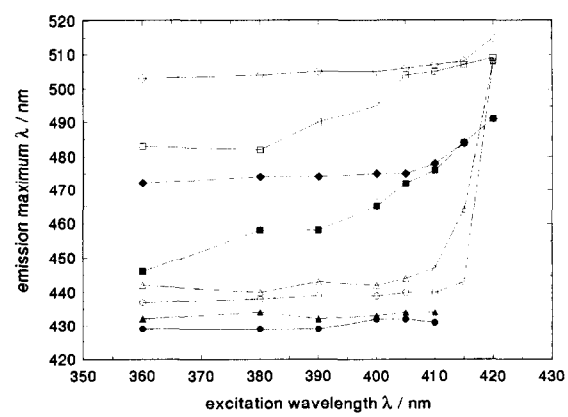

Fig. 3. Fluorescence maxima as a function of the excitation wavelength (red-edge excitation) in DMPC-SUV at different temperatures: filled symbols represent Patman, open symbols Prodan; the temperatures are 15°C (circles); 23°C (triangles); 30°C (boxes); 43°C (diamonds).

are larger for Patman compared with Prodan at both 15°C and 30°C at all excitation and emission wavelengths, suggesting a more restricted environment (not shown).

3.2. Binding behaviour

Both Prodan and Patman show only weak fluorescence when dissolved in buffer, but yield strong fluorescence when bound to membranes. Thus, information about the time course of the membrane binding can be obtained by measuring the fluorescence intensity as function of time after addition of dye (from 4 mM EtOH solution) to the preformed vesicles. Prodan shows constant intensity after addition to SUV, while for Patman an exponential-like increase in the fluorescence intensity (in total 70%) is observed reaching a constant intensity value about 5 min after addition (Fig. 4). This result suggests that the uptake of Prodan is faster than mixing, while at least part of the uptake is considerable slower for Patman. An investigation of steady-state and time-resolved fluorescence of Patman in pure buffer gave no evidence for aggregate formation like a concentration dependent spectral shift or excimer fluorescence.

Spectra obtained when the dyes had been added before vesicle formation are essentially independent of time after preparation when the spectrum is recorded and significantly blue-shifted compared to

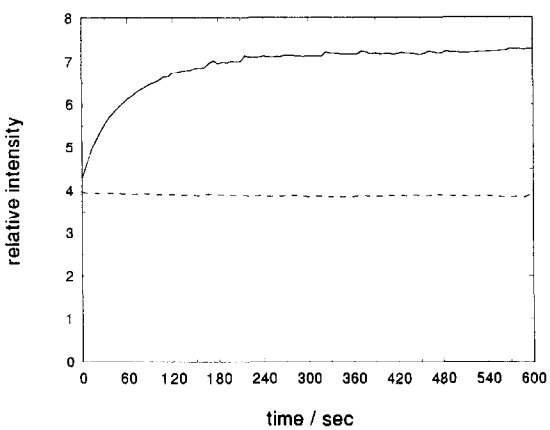

Fig. 4. Time dependence of fluorescence intensity after addition of dye to preformed PC-SUV; lipid concentration is 10^{-4} M, temperature is 25°C. Patman (—— ——); Prodan (----).

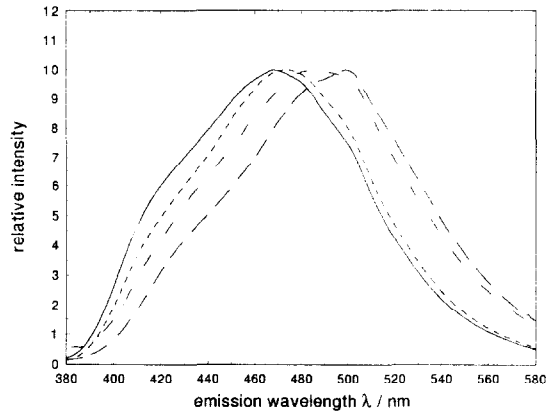

Fig. 5. Fluorescence emission spectra of Prodan and Patman in PC-SUV; excitation wavelength is 360 nm; lipid concentration is 10^{-4} M, temperature is 25°C. Patman added before vesicle formation (—— ——); Patman added after vesicle formation (---); Prodan added before vesicle formation (--- —— ---); Prodan added after vesicle formation (— — —).

those where the dyes have been added after vesicle formation (Fig. 5). The latter effect which is more pronounced for Prodan than for Patman does not disappear within 12 h and is also reflected in the mean fluorescence lifetimes. While the lifetime for Patman added before vesicle formation ($\tau_v = 2.30$ ns) is only slightly longer than that for Patman added after vesicle formation ($\tau_n = 2.10$ ns), this difference is much more pronounced for Prodan labeled vesicles ($\tau_v = 2.30$ and $\tau_n = 1.85$ ns, respectively). These results show that location and fluorescence properties are more dependent on time of addition for Prodan than for Patman. For both preparation methods the form of the spectra is nearly independent of preparation.

3.3. Decay behaviour and time-resolved emission spectra

The fluorescence decays can be well described using a biexponential model for both dyes at all emission wavelengths. For $\lambda_{em} \geq 470$ nm one decay component is always obtained with a negative pre-exponential factor. An increase in the mean decay times is observed with increasing λ_{em}, as expected for increasing contributions of relaxed states.

TRES and correlation functions were calculated for both Patman and Prodan in SUV composed of

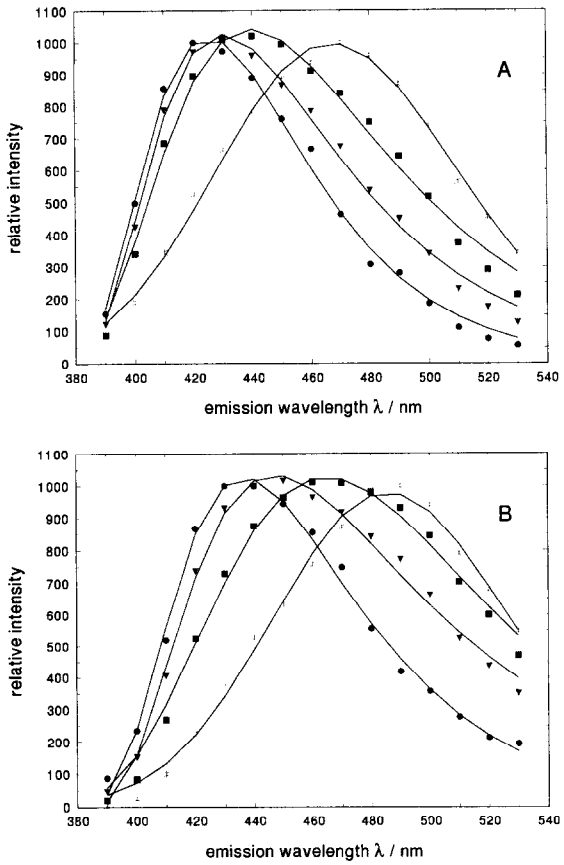

Fig. 6. Reconstructed time-resolved emission spectra of Patman and Prodan in DMPC-SUV at 30°C for four different times after excitation; times are 0.2 ns (circles); 1.0 ns (triangles); 2.0 ns (boxes); 5.0 ns (asterix). (A) Patman, (B) Prodan.

DMPC at 30 and 43°C. The TRES of Patman are blue-shifted (~ 15 nm) compared with those of Prodan (Fig. 6). For both temperatures the solvent response of Patman is about two fold slower than for Prodan (Fig. 7). The average relaxation times are 2.2 and 3.5 ns (30°C) and at 0.7 and 1.8 ns (43°C) for Patman and Prodan, respectively.

3.4. NMR-Spectroscopy

In Fig. 8 the ^{1}H-NMR spectra of the $(CH_3)_3N$-groups of the lecithin, in presence of Patman and Prodan, respectively, are given. The ^{13}C-H satellites from the choline methyl peak are also visible, each representing 0.5% of the intensity of the central peak. The aliphatic proton signals of the chromophores were not separable because of overlap with lipid signals. The aromatic protons of the fluorescence probes were not detectable, even after 512 scans. This latter fact is caused by extensive line broadening caused by the strong fixation of the chromophores in the bilayer. The data obtained for the relaxation times are compiled in Table 1.

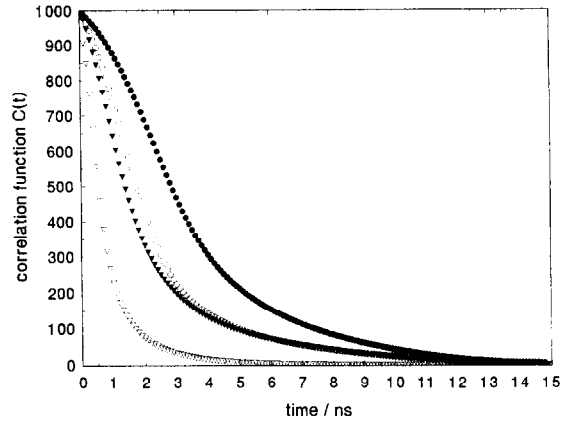

Fig. 7. Correlation functions $C(t)$ calculated according to Eq. (1) for Patman (circles) and Prodan (triangles) in DMPC-SUV at 30°C (filled symbols) and 43°C (open symbols).

Using a proton-proton distance of $r = 1.8$ Å [25] for the methyl group, a correlation time $\tau_{c3} = 86$ ps

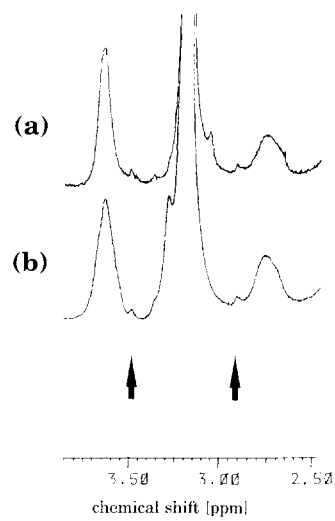

Fig. 8. ^{1}H NMR spectrum of the $N(CH_3)_3^+$-group of sonicated vesicles. Egg yolk lecithin (33 mM) in 100 mM NaCl in D_2O at pD 6.5, (a) with Prodan (3.3 mM), (b) with Patman (3.3 mM). The arrows denote the ^{13}C–H satellites of the choline methyl peak.

Table 1
Proton relaxation in small bilayer liposomes containing fluorescent probes (egg yolk lecithin: 33 mM; probe 3.3 mM; NMR measurement at 22°C)

Sample	Relaxation	Probe	Lecithin			
		$N(CH_3)_x^{+a}$	$N(CH_3)_3^+$	$CH_2-C=C-$	$HC=CH$	CH_3
Chem. shift		[a]	3.21 ppm	2.03 ppm	5.27 ppm	0.90 ppm
Lipid	T_1 [s]	–	0.31 ± 0.01	0.35 ± 0.03	0.39 ± 0.03	0.59 ± 0.05
	T_2 [ms]	–	95 ± 5			
Lipid + Patman	T_1 [s]	0.21 ± 0.03	0.28 ± 0.01	0.31 ± 0.02	0.36 ± 0.03	0.50 ± 0.04
	T_2 [ms]	30 ± 4	61 ± 3			
Lipid + Prodan	T_1 [s]	0.42 ± 0.05	0.30 ± 0.02	0.34 ± 0.03	0.36 ± 0.03	0.49 ± 0.05
	T_2 [ms]	63 ± 10	83 ± 3			

[a] Peak assignement: Patman, $(CH_3)_3N^+$ at 3.30 ppm; Prodan, $(CH_3)_2N$ at 3.08 ppm

can be calculated from Eq. (4) for the choline headgroup protons. This value is not enhanced significantly by the binding of the fluorescent probes. However, the reorientation time $\tau_{c1} = 2.8$ ns, determined from Eq. (5), changes to 4.3 ns in the presence of Patman and to 3.2 ns in the presence of Prodan.

4. Discussion

The fluorescent probe Patman has not found wide application as a membrane probe compared to its parent fluorophore Prodan, although its molecular structure should lead to a more defined localization in membranes, which is favourable for interpretation of membrane properties. It has been discussed whether the fluorescence behaviour of a polarity sensitive probe near a phase transition is better described by solvent relaxation processes or a probe relocation mechanism [7,8,13,24]. It was the aim of this study to compare the time-resolved spectral behaviour of Prodan and Patman in order to clarify their relaxation behaviour and relative localization in the membrane.

The steady-state emission spectra of Prodan are broader and red-shifted compared to Patman, suggesting a more polar and heterogeneous environment of the Prodan chromophore [7]. While the spectra of Patman are quite similar in SUV and MLV, Prodan is able to distinguish between both types of vesicles for $T \leq T_c$, showing different widths of the emission spectra. Since the spectra in the gel phase of Prodan in SUV are much broader than in MLV, we infer a more heterogeneous environment of Prodan in SUV. This might be the result of the high intrinsic curvature of the SUV, facilitating the penetration of water in the headgroup region and, thus, adding a significant contribution of more polar and faster relaxing binding sites [16]. These results are illustrated by the ratio I_{blue}/I_{red}. It shows an abrupt change at the phospholipid phase transition temperature for Prodan and Patman in MLV, while for Prodan in SUV the transition is much broader (Fig. 2). Such a less cooperative behaviour of lipids in vesicles prepared by sonication is known from DSC-experiments [26]. The results of Parasassi et al. [24] were confined to MLV-studies. For this type of vesicles our results with a common value for both probes at high temperature and similar values in the gel phase are comparable to those obtained by Parasassi. The ability to differentiate between SUV and MLV clearly seems to be associated with the length of the acyl chain: While the behaviour of Prodan is strikingly different in both types of vesicle systems, only minor differences were observed with Patman. This hypotheses will be tested with Laurdan for which a behaviour similar to Patman can be assumed as a lauroyl chain is much more similar to a palmitoyl than a propionyl chain.

The observation of red-shifted emission spectra with increasing excitation wavelength λ_{ex} as well as the decrease of steady-state anisotropies (r_{ss}) with increasing emission wavelength λ_{em} is a first and simple indication for the occurence of solvent relaxation. The decrease in r_{ss} with increasing λ_{em} is caused by the selection of longer living, relaxed fluorophores, which have more time to reorient.

It should be stressed that there has been consider-

able debate on the origin of the above described steady-state fluorescence emission behaviour of Prodan and similar probes [7,8,24]. While it is difficult on the basis of steady-state measurements alone — as used in these publications — to decide between solvent relaxation and a probe relocation to a more polar environment this can be achieved by TRES. The detection of blue-shifted TRES for short times after excitation and the observation of a large time-dependent Stokes shift during the first 5 ns after excitation (Figs. 6 and 7) unambigiously favour dipolar solvent relaxation as the mechanism leading to the observed flourescence characteristics. In this context the term 'solvent' is used for the intimate surrounding of the fluorophore, e.g. the hydrated phospholipid headgroups and the hydrated carbonyl region. Since the solvent relaxation of polar solvents occurs in the picosecond regime [27], dye molecules in the bulk solvent will not contribute to a relaxation process on the experimentally given nanosecond time scale. As can be seen from the TRES (Fig. 6) and more clearly from the correlation functions (Fig. 7) the relaxation is considerably faster for Prodan than for Patman. This seems to be a general result, as we observed the same behaviour both in different asymmetric phosphatidylcholines (1-stearoyl-2-lauroyl-sn-glycero-3-phosphatidylcholine; 1-O-stearyl-2-O-lauryl-sn-glycero-3-phosphatidylcholine) [28] and in mixtures of PC and phosphatidylserine (PS) in absence or presence of Ca^{2+} [29]. The slower relaxation and the blue-shifted TRES of Patman suggest that this dye is localized in a less polar and/or more restricted environment compared to Prodan. As the polarity along the membrane normal decreases from the headgroup region to the bilayer interior [30], it seems reasonable to conclude that the chromophore of Patman is embedded deeper in the membrane than the chromophore of Prodan, most probably because of the long palmitoyl chain.

An important aspect for the practical use of these probes in membrane studies is the binding behaviour to phospholipid vesicles. If Prodan is added to PC-SUV, the steady-state intensity reaches its final value within the time of mixing, suggesting a very rapid uptake of Prodan into the membrane.

However, the resulting steady-state spectrum is significantly red-shifted compared to the spectrum, where Prodan had been added prior to vesicle formation. Concomitantly, the mean lifetime is considerably shorter, if Prodan is added after vesicle formation. We assume that the percentage of dye bound in the outer leaflet is higher, when the fluorophore was added after vesicle formation. The high curvature in SUV should facilitate the penetration of water molecules into the outer leaflet of the membrane [16]. Thus, on the average, the Prodan molecules sense a somewhat more polar environment, yielding a red-shifted emission spectrum and shorter lifetimes. This behaviour is independent of the time after addition of dye, suggesting that the ratio of Prodan in the outer to Prodan in the inner bilayer leaflet does not change substantially with time, i.e. any flip-flop of probes seems to be slow.

The behaviour of Patman has been shown to be different. Two reasons for the observed rise of the fluorescence intensity after addition to vesicles may be cited. Either, the adsorption of Patman slows down with time because of the increasing surface charge, provided by the Me_3N^+-groups of Patman or the dye undergoes a slow reorientation within the membrane (after an initial fast uptake) to a less polar environment yielding higher quantum yields.

The spectrum of Patman in PC-SUV in case of addition after vesicle formation is also blue-shifted compared to the spectrum obtained when Patman is present during vesicle formation; however, the blue-shift is smaller than for Prodan. Correspondingly, the lifetime difference is smaller, too. A cone-like fluorophore with one long acyl chain resembles a lysophosphatidylcholine which should prefer the outer monolayer in highly curved vesicles. Therefore, while Patman should be restricted to the outer leaflet because of its charge in case of addition to preformed vesicles, a somewhat higher concentration of this dye in the outer leaflet may be also assumed for addition of the dye during vesicle formation. This difference in distribution of Patman between outer and inner monolayer may account for the small differences in the steady-state spectra and lifetimes. Moreover, the smaller effect compared to Prodan might be attributed to the deeper incorporation of Patman in a membrane region where polarity differences between inner and outer leaflet in highly curved SUV are not very important, in agreement with our results from the TRES. Because of the low stock EtOH-concentration involved (0.025%, ~ 4 mM) it seems un-

likely to us that the observed differences in the fluorescence behaviour are caused by the presence of EtOH in the case of dye addition after vesicle formation.

In view of the highly restricted mobility of the aromatic residue Eq. (5) can be used only for an estimation of the reorientation time of the $N(CH3)_x$ group of a fluorophore. Nevertheless, from the comparison of the NMR relaxation times T_2 (Table 1), one can conclude that the chromophore of Prodan has a higher freedom of mobility than Patman concerning the reorientation time τ_{c1} of the rotation axis of the $N(CH_3)_x$ groups. This statement is in accordance with the TRES data. A comparison of results from NMR and fluorescence is justified for egg PC in spite of large differences in the dye concentrations, because the sensitivity of the membrane structure against perturbations by additives is relatively low at temperatures substantially higher than at the phase transition [20]. Using lanthanide shift reagents we have proofed by NMR that the size of the SUVs (25–30 nm diameter) was not changed by addition of the dyes (3.3 mM).

According to Table 1 it is obvious that the incorporation of the fluorescent molecules have no significant influence on the T_1 values of any of the considered lecithin signals. From Eq. (3) a value of $\tau_c = 0.35$ ns can be calculated for the intrinsic local anisotropic rotation of the choline headgroup, which is not influenced by the additives. This lack of sensitivity of T_1 against 'perturbations' of the lipid structure was also observed during other modifications, e.g. a moderate oxidation of the fatty acid chains by ionizing radiation [15]. However, the incorporation of the fluorophores leads to a remarkable decrease of T_2, in spite of the relatively small molar fraction of probe molecules. The calculated enhancement of the mean reorientation time τ_{c1} of the choline headgroup by Prodan is equal to 15%. The stronger increase (55%) of τ_{c1} induced by Patman can be interpreted by a stronger fixation of the chromophore at the lipid surface attributed to its positive charge or to a shift of the chromophore towards the glycerol backbone, supposed as the most rigid part of the lipid molecule [20]. As a result of the packing constraints in SUV one can expect that the cone-like probe shows an enrichment in the external lipid layer linked to the hydrophobic core by its single alkyl chain. In this connection it is of interest that the NMR signal in SUV was dominated by contributions from the external layer [15].

5. Conclusion

Although Prodan and Patman possess essentially the same chromophore, their binding and fluorescence behaviour as well as the NMR data show marked differences suggesting their complementary use in studies of the membrane headgroup region. Patman exhibits blue-shifted emission spectra, slower uptake and considerably slower solvent relaxation, in accordance with a deeper localization in the membrane compared to Prodan. The large time-dependent Stokes shifts seen with both dyes demonstrate that solvent relaxation rather than probe relocation mainly contributes to the red-shift in the emission spectra observed during the phase transition of the dye-labelled vesicles.

Acknowledgements

We thank Dr. S. Vajda for data analysis programs and Dr. H. Ruf for performing the DLS measurements. M.H. is a recipient of the Liebig-Stipendium of the Fond der Chemischen Industrie.

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