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Relationships between membrane water molecules and Patman equilibration kinetics at temperatures far above the phosphatidylcholine melting point

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ABSTRACT

The naphthalene-based fluorescent probes Patman and Laurdan detect bilayer polarity at the level of the phospholipid glycerol backbone. This polarity increases with temperature in the liquid–crystalline phase of phosphatidylcholines and was observed even 90 °C above the melting temperature. This study explores mechanisms associated with this phenomenon. Measurements of probe anisotropy and experiments conducted at 1 M NaCl or KCl (to reduce water permittivity) revealed that this effect represents interactions of water molecules with the probes without proportional increases in probe mobility. Furthermore, comparison of emission spectra to Monte Carlo simulations indicated that the increased polarity represents elevation in probe access to water molecules rather than increased mobility of relevant bilayer waters. Equilibration of these probes with the membrane involves at least two steps which were distinguished by the membrane microenvironment reported by the probe. The difference in those microenvironments also changed with temperature in the liquid–crystalline phase in that the equilibrium state was less polar than the initial environment detected by Patman at temperatures near the melting point, more polar at higher temperatures, and again less polar as temperature was raised further. Laurdan also displayed this level of complexity during equilibration, although the relationship to temperature differed quantitatively from that experienced by Patman. This kinetic approach provides a novel way to study in molecular detail basic principles of what happens to the membrane environment around an individual amphipathic molecule as it penetrates the bilayer. Moreover, it provides evidence of unexpected and interesting membrane behaviors far from the phase transition.

1. Introduction

Naphthalene derivatives (e.g. Prodan, Laurdan, and Patman) have been available for over thirty years as probes of membrane structure and dynamics [1–3]. Their utility stems from their strong sensitivity to general solvent effects. Specifically, their emission spectra shift by about 60 nm toward longer wavelength when water molecules present in the head-group region of the bilayer are able to align with the excited-state dipole of the photo-excited probe in the charge-transfer state [4–7]. Based on comparisons between effects of protic and aprotic solvents, specific solvent interactions (presumably involving hydrogen bonds), also contribute to the solvatochromatism of these probes [8–10]. These strong solvent effects have been used to provide indirect estimations of membrane order and fluidity, which appear to correlate with water invasion into the bilayer [11,12]. Typically, these solvent effects are quantified by calculating a parameter termed "Generalized Polarization" (GP) [4,11] or by deconvolution of spectral components by non-linear regression [13,14]. With these methods, physical phenomena such as lipid phases have been readily observed [4,11,15–18].

Saturated phospholipids display a strong phase transition from a solid ordered phase to a liquid disordered phase (liquid–crystalline or L_α) at temperatures that depend on the length of the chains and the chemical structure of the heads. Although many methods for studying these phases suggest that the liquid disordered phase displays stable properties as temperature is raised beyond the main phase transition, the fluorescence of these naphthalene derivatives suggests otherwise. Studies of either saturated phosphatidylcholine or phosphatidylglycerol consistently show a steady increase of apparent membrane polarity (i.e. decreased GP value or stronger solvent relaxation) as a function of temperature well above the lipid melting point [4,13,19].

The molecular basis for this sensitivity of membrane polarity to high temperatures is not known. Early observations with Laurdan attributed this phenomenon simply to the responsiveness of the probe to dipolar relaxations without further detail [4]. A more specific explanation was

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offered by investigators studying Laurdan spectra and anisotropy with dilaurylphosphatidylcholine and dipalmitoylphosphatidylglycerol in the liquid disordered state [19]. This explanation argued that changes in the emission spectrum as a function of temperature reflect concomitant increases in the kinetic energy of membrane water molecules facilitating their ability to rotate and therefore respond to the fluorophore's excited state dipole.

We report here a study designed to delineate the mechanism behind the changes in membrane polarity observed in the liquid crystalline state. We have considered the hypothesis proposed above (faster water rotation) as well as an alternative that these effects result instead from increases in the number and/or penetration depth of bilayer water molecules. We take advantage of recent observations from kinetic analyses of probe equilibration suggesting that these naphthalene derivatives may reside in at least two configurations in the membrane and that one can learn additional detail about membrane dynamics by considering the properties of those configurations [20,21]. For such studies, Patman appears ideally suited [20], and we have therefore focused our attention on the behavior of that probe with unilamellar vesicles of various saturated and unsaturated phosphatidylcholines. The results unexpectedly provided evidence that phospholipid behavior is more diverse at temperatures far about the phase transition than previously reported.

2. Materials and methods

All of the experimental methods used in this study have been described in [20]. Lipid stocks (all purchased from Avanti Polar Lipids, Alabaster, AL) were dissolved in chloroform then dried under a nitrogen stream followed by high vacuum. Unilamellar vesicles were prepared by extrusion (100 nm pore size). When high salt concentrations were used (1 or 5 M NaCl, KCl, or $CaCl_2$), vesicles were equilibrated with aqueous solutions of the salt for at least an hour with repeated raising and lowering of temperature through the relevant lipid main phase transition. Steady-state fluorescence with Patman and Laurdan (Molecular Probes; now Life Technologies, Grand Island, NY) was acquired at dual emission wavelengths optimized for separation of nonpolar (short wavelength, 435 nm) and polar (long wavelength, 500 nm) spectral components or as spectra with excitation of 350 nm and a 4 nm excitation and emission bandpass. Suspensions of vesicles in citrate buffer (20 mM Na citrate, 150 mM KCl, pH 7; 50 μM phospholipid) were temperature-equilibrated in the spectrofluorometer for at least 6 min prior to initiating data acquisition. After 100 s, Patman or Laurdan was added (250 nM). When applicable, the value of the spectrum GP was calculated from data at 435 and 500 nm (difference of intensity at 435 and 500 nm divided by the sum of the two as explained) [11,17]. Steady-state anisotropy values were obtained using Glan–Thompson polarizers with corrections for wavelength-dependent differences in polarizer transmission and calculated as described previously for our instrument [17].

3. Calculations

3.1. Monte Carlo simulations

Monte Carlo simulations of spectra involved the following three assumptions:

1. The emission spectrum was assumed to be described approximately by a single normal probability distribution.
2. Water molecules were assumed to relax around the electric field of the excited state as a first-order exponential decay.
3. The emission wavelength for individual probe molecules was assumed to shift toward longer wavelengths directly proportional to the degree to which water molecules have relaxed around the excited state.

Four constants were applied based on observed values: the average excited state lifetime (τ, 3 ns for Patman, [5]), the observed emission maximum for unrelaxed probe (λ_0, 433 nm), the observed emission maximum for fully relaxed probe (λ_R, 480 nm), and the standard deviation for the unrelaxed emission spectrum (σ_λ, 34 nm). The independent variables in the simulations were the probability of water molecules in the vicinity of the probe (P_w) and the rate constant for water relaxation (k_w, ~0.6 ns^{-1}, [5,22]). A total of five random numbers were applied to each probe (R_1... R_5). R_1 was used to determine the energy of fluorescence emission ($1/\lambda$) for each probe molecule by setting it equal to the cumulative probability given by integrating the normal distribution

$$R_1 = \frac{1}{2}\left[1 + \operatorname{erf}\left(\frac{(1/\lambda - 1/\lambda_0)}{\sigma_\lambda\sqrt{2}}\right)\right] \tag{1}$$

where the integration range in the error function was 0 to $1/\lambda$. The unknown, λ, was solved numerically from Eq. (1) by interpolation.

R_2 was used to determine whether a water molecule was present for each probe. Hence, if $R_2 < P_w$, a water molecule was assigned to the probe and allowed to alter the energy of emission so that the final emission wavelength, λ_{em} was greater than λ from Eq. (1). Otherwise, the value of λ from Eq. (1) was retained. For simplicity, each probe with $R_2 < P_w$ was paired with a single water molecule, and changes in emission energy were attributed to the rotational positioning of that single water. This decision to pair the probe with a single water molecule is undoubtedly an oversimplification [23], but it does not interfere with the nature of the simulation results.

For those probes influenced by water in the simulation, R_3 was used to determine t, the time elapsed between probe excitation and emission:

$$t = -\tau \ln(R_3) \tag{2}$$

The starting angle of each water molecule relative to the orientation of its probe partner was determined using R_4. Only the angle between a vector parallel to the excited-state dipole of the probe and one parallel to the permanent dipole of water was considered for determining emission energy. However, since the angle between these dipoles (θ_Z) may be oriented in any direction along the X-Y plane in three-dimensional Euclidean space, the probability of water's orientation relative to the Z axis was weighted by $\sin^2(\theta_Z)$, which generates the following cumulative probability function:

$$R_4 = \frac{1}{\pi}\left[\theta_Z - \frac{1}{2}\sin(2\theta_Z)\right] \tag{3}$$

The value of θ_Z was obtained from R_4 numerically by interpolation using Eq. (3). Lastly, the speed of rotation of each water molecule (r_w) was determined with a fifth random number, R_5, which was set equal to the cumulative distribution function of the classic Maxwell–Boltzmann distribution.

$$R_5 = \operatorname{erf}\left(\frac{r_w}{\sqrt{2}a}\right) - \sqrt{\frac{2}{\pi}\left(\frac{r_w e^{-\left(r_w^2/2a^2\right)}}{a}\right)} \tag{4}$$

where $a = k_w/\sqrt{2}$ and the integration range in the error function was 0 to r_w. The value of r_w was determined from R_5 by interpolation using Eq. (4).

The position of each water molecule at the time of emission was then calculated by combining results from Eqs. 2–4.

$$\theta_{Z,t} = \theta_Z e^{-r_w t} \tag{5}$$