J. Membrane Biol. 85, 225–231 (1985)

The Journal of
Membrane Biology

Local Dielectric Properties Around Polar Region of Lipid Bilayer Membranes

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Summary. Local dielectric constant was evaluated from the Stokes shifts of fluorescence spectra of L-α-dansylphosphatidylethanolamine (DPE) incorporated into liposomes made of synthetic phosphatidylcholine (dipalmitoyl or distearoyl) or bovine brain phosphatidylserine. The evaluation was established as follows. First, the Stokes shift of DPE was assured to follow Mataga-Lippert's equation and was a function of the dielectric constant and the refractive index in some standard organic solvents. Second, the change of the refractive index did not contribute much to the change in the Stokes shift. Third, the time resolved fluorescence depolarization of DPE in liposomes showed that the cone wobbling diffusion was rapid relative to the fluorescence lifetime and therefore that the dielectric relaxation did not affect the evaluation of the constant in the polar region of membranes. We then investigated the characteristics of the local dielectric constant in the polar region of the lipid bilayer and found that the dielectric constant varies between 4 and 34 depending upon calcium binding and also gel/liquid-crystal phase transition. Such large changes of the local dielectric constant were further correlated with the dynamic structure of lipid bilayer membranes measured by conventional fluorescence depolarization techniques. The large changes of dielectric constant around the polar region suggest that electrostatic interactions at this region can be altered 10-fold by such ionic or thermotropic factors and therefore that local dielectric properties can play crucial roles in membrane functions.

Key Words dielectric constant · fluorescence study · liposomes · calcium effect · phase transition

Introduction

Biological membranes are composed of proteins and lipid bilayers. Lipid bilayers have two distinct regions: hydrophobic and hydrophilic. The hydrophobic region consists of hydrocarbon chains of lipids and is characterized by its low dielectric constant. The hydrophilic region consists of the polar headgroups of lipids and is characterized by its intermediate polarity between aqueous phase and the hydrophobic region. Some membrane proteins are located at the interface between the aqueous phase and hydrophilic region, while others are integrated within the membrane.

Membrane proteins or domains of membrane proteins function in their own local environments. In some cases, dynamic properties like microviscosity, microelasticity, rigidity, etc., can be essential for their functions (*see* reviews by Chapman, 1982; Vaz et al., 1982; Stubbs & Smith, 1984). In other cases, where electric interactions between proteins (or parts of proteins) are essential for their functions, the local dielectric property is the most important factor because electric forces or potentials are divided by the local dielectric constant between them. Thus, a 10-fold change in the dielectric constant, for instance, could change the interaction between these proteins as much as ten times in magnitude. We will show in this paper that such large change of local dielectric constant can indeed occur in hydrophilic region of lipid bilayer membranes.

A considerable amount of work has been done on dynamics of membrane components (Chapman, 1982; Vaz et al., 1982; Kinosita et al., 1984; Stubbs & Smith, 1984). However, very little has been done on local dielectric properties of membranes in spite of their potential significance.

Here we have investigated the dielectric properties by fluorescence spectroscopic methods. The fluorescence quantum yield and the maximum wavelength of the emission have been shown to be sensitive to the dielectric properties (Stryer, 1965; Turner & Brand, 1968; Waggoner & Stryer, 1970). In fact using the quantum yield of ANS, Träuble and Overath (1973) estimated the local dielectric constant around polar region of DPPC to be 35. However, the response of ANS in membranes depends on membrane potential and on other factors (Azzi et al., 1971; Träuble & Overath, 1973; Träuble & Eibl, 1974), and various nonradiative processes can alter the quantum yield. On the other hand, the Stokes shift of fluorescence, which is defined as dif-

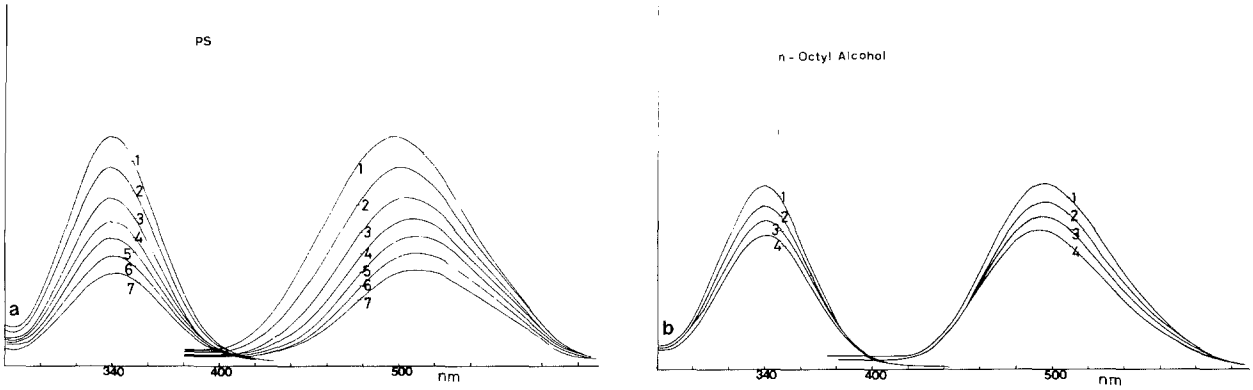

Fig. 1. Excitation and emission spectra of DPE in PS liposomes (*a*) and *n*-octanol (*b*) at various temperatures: 1.7, 8.6, 16.2, 25.2, 34.4, 44.4, 54.4°C (in 1–7, respectively, 1*a*) and 2.5, 20.0, 39.3, 57.4°C (in 1–4, respectively, 1*b*)

ference in energy levels between the ground and excited states of a chromophore, depends only on the dielectric constant and refractive index around the chromophore, if the conformation of the chromophore is left unchanged and no interchromophore interactions occur during the lifetime of the excited state. The dependence on the dielectric constant is immediate when the excitation spectrum remains the same (Mataga & Kubota, 1970; Mataga et al., 1955). It is this property that enables the Stokes shift to be used. Kano, Goto and Ogawa (1981) evaluated local dielectric constants of hydrophobic regions from emission maximum wavelength of pyrene-$(CH_2)_3$-N,N-dimethylaniline incorporated into DMPC- and DPPC-liposomes and found that the constants are between 2 and 4 and also that these constants are changed by the gel/liquid-crystalline phase transitions. However, the dielectric constant reflects reorientation of polar molecules and therefore can be affected by the relaxation time of solvent molecule's reorientation. In other words, if the fluorescence lifetime is of the order of solvent relaxation time, then the evaluated values would be complex and meaningless.

We used DPE as a probe to the hydrophilic region and evaluated the dielectric constant by measuring the Stokes shift. DPE has a dansyl chromophore attached covalently to the amine of the lipid, L-α-phosphatidylethanolamine. Therefore, the chromophore location is clear and the partitioning of the probe in membranes is constant. Furthermore, DPE has been used to detect lipid's dynamic structures by fluorescence depolarization techniques (Waggoner & Stryer, 1970; Faucon & Lussan, 1973; Lussan & Faucon, 1974; Tessie, 1979). By measuring the fluorescence depolarization of DPE we could study the motional and structural state of the chromophore and its environment in the same system in which we evaluated the local dielectric constant. We have also measured time-resolved fluorescence depolarization and the lifetime of DPE and have shown that the reorientation relaxation is sufficiently rapid relative to the fluorescence lifetime. Thus the local dielectric constant evaluated from such data is indeed an accurate value at the location. Furthermore we used DPH as a probe of the hydrophobic region, and could thus compare the change of dielectric constants with the change of the total dynamic structure of the lipid bilayer.

Abbreviations

PC, L-α-phosphatidylcholine; DMPC, L-α-phosphatidylcholine dimyristoyl; DPPC, L-α-phosphatidylcholine dipalmitoyl; DSPC, L-α-phosphatidylcholine distearoyl; PS, L-α-phosphatidylserine; DPE, L-α-dansylphosphatidylethanolamine; DPH, 1,6-diphenyl-1,3,5-hexatriene; ANS, 1-anilinonaphthalene-8-sulfonate.

Materials and Methods

L-α-phosphatidylcholine (dimyristoyl, dipalmitoyl and distearoyl) were purchased from Sigma. Phosphatidylserine was purified from bovine brain according to Sanders (1967). All lipids were checked on thin-layer chromatography (Merck, developing solvent: chloroform/methanol/water 65:25:4 and chloroform/methanol/acetic acid/water 25:15:4:2) and appeared as single spots. L-α-dansylphosphatidylethanolamine (DPE) was synthesized according to the method of Waggoner and Stryer (1970) and purified on 2-mm-thin layer plates. Concentrations of lipids and DPE were determined by measuring the amount of phosphate (Gerlach & Deuticke, 1963). All manipulations of lipids were done under a stream of nitrogen. All other reagents and organic solvents were analytical grades purchased from Wako Pure Chemicals Industry.

Labeling and preparations of liposomes were done as follows. Lipids and DPE were dissolved in chloroform/methanol (2:1, vol/vol) and aliquots were dried under reduced pressure

and then suspended in buffer (10 mM Tris-HCl, pH 7.3). The suspension was sonicated for about 10 min with a tip-sonicator (Chouonpa Industry, Model USV-150V, Tachikawa, Japan). DPH labeling was done by adding an aliquot of 1 mM DPH solution in tetrahydrofuran to liposomes in buffer before sonication (THF < 0.2%). The final concentrations of lipids, DPE and DPH were 0.3 to 1.0 mg/ml, 1/100–500 (molar ratio) and 1/800 of lipids, respectively.

Measurements of polarized fluorescence and fluorescence spectra were done with Hitachi fluorescent spectrophotometer MPF-3 equipped with a temperature-controlling system. The temperature was measured with a calibrated thermistor immersed in the measuring cuvette. For fluorescence depolarization measurements DPE or DPH were excited with vertically polarized light at 340 or 360 nm ($\Delta\lambda$ = 8 nm), respectively. The vertically and horizontally polarized fluorescence was measured at 90 degree configuration at either 500 or 450 nm ($\Delta\lambda$ = 8 nm). To minimize stray light, optical filters were used in the light paths of either incident side (U-360, 340, Hoya) or detection side (L42, C-500).

The total fluorescence intensity I_T, the difference intensity I_D, and the fluorescence anisotropy r were calculated from the observed intensities of polarized fluorescence I_V (vertically) and I_H (horizontally) as follows:

$$I_T = I_V + 2SI_H \quad (1)$$

$$I_D = I_V - SI_H \quad (2)$$

$$r = I_D/I_T \quad (3)$$

where S is the ratio of the sensitivities of the detection system for vertically and horizontally polarized light. S was taken as equal to I'_V/I'_H obtained with horizontally polarized excitation. Time-resolved fluorescence was measured and analyzed as in Kawato et al. (1977).

Dielectric constants of organic solvents were measured in a custom-cell by using 4260A universal bridge (Hewlett Packard) and their refractive indexes were measured with Abbe's refractometer (Elmer, Tokyo).

Results

Fluorescence Emission Spectra of DPE

The fluorescence emission spectra of DPE in various organic solvents showed a marked red-shift as the polarity of the solvent increased whereas the excitation spectra showed no shift. Although a strong temperature dependence of the fluorescence emission spectra was observed in lipid bilayers (Fig. 1*a*), no temperature-dependent spectral shifts were observed in most of the organic solvents tested (Fig. 1*b*): e.g. methanol, ethanol, propanol, butanol, octanol, butylamine and octylamine. In most cases the peaks of the excitation spectra are at 340 nm (only slightly shifted to blue in butylamine and octylamine) and the peaks of the emission spectra are between 480 and 510 nm which vary with the polarity of the solvent. It should be noted that in some organic solvents with low dielectric constants, e.g. hexane and chloroform, the emission spectra showed temperature-dependent spectral shifts probably due to reversed-micelle formation.

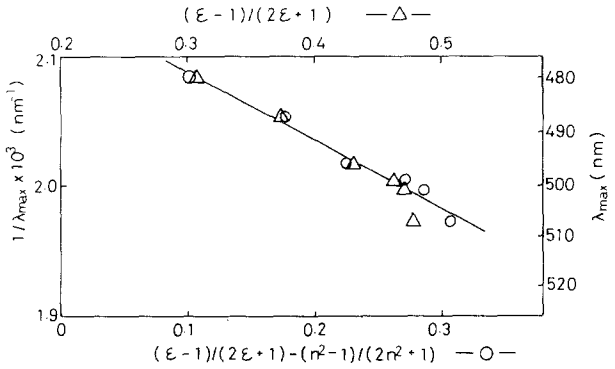

Fig. 2. A relation of dielectric constant and λ_{max} of DPE fluorescence determined in various organic solvents, in which the spectra showed no temperature dependence: methanol (ε = 32.6, n = 1.3323), ethanol (24.3, 1.3666), propanol (20.1, 1.3918), octanol (10.3, 1.4305), butylamine (5.4, 1.4019) and octylamine (3.4, 1.4298), where ε denotes dielectric constant and n denotes refractive index. To show the contribution of refractive index, $1/\lambda_{max}$ was plotted *vs.* $(\varepsilon - 1)/(2\varepsilon + 1) - (n^2 - 1)/(2n^2 + 1)$ (○) and was replotted *vs.* $(\varepsilon - 1)/(2\varepsilon + 1)$ only (△)

Relation between Dielectric Constant and Peak Wavelength of Fluorescence Emission Spectrum of DPE

Peak wavelength of DPE fluorescence in organic solvents are related to dielectric constants and refractive indexes of the solvents as shown in Fig. 2 (○). When the refractive index was ignored, the relation shifted along the abscissa (△). This indicates that the change of dielectric constant dominantly determined the change of the Stokes shift. The linear relation between $1/\lambda_{max}$ of the fluorescence and $(\varepsilon - 1)/(2\varepsilon + 1)$ is well predicted by theories (Mataga et al., 1955; Mataga & Kubota, 1970). In Fig. 2, the peak wavelength of the excitation spectrum was not used because it was constant. We used this relation to determine dielectric constant around the chromophore after clarifying the reorientation process of the polar headgroups about the DPE chromophore.

Fluorescence Anisotropy Decay

Time-resolved fluorescence anisotropy decay of DPE in PS liposomes showed double phases, an initial decay followed by a constant phase (Fig. 3), typical for wobbling-in-cone diffusion as is the case

of DPH in liposomes and biological membranes (Kawato et al., 1977; Kinosita et al., 1977, 1981, 1982). Various kinetic parameters, e.g. diffusion constants in cone, cone angles, fluorescence lifetimes, etc., are summarized in the Table. The data indicate that the reorientation of the lipid's polar headgroups (*see* ϕ in Table) is sufficiently rapid relative to the lifetimes of the fluorescence.

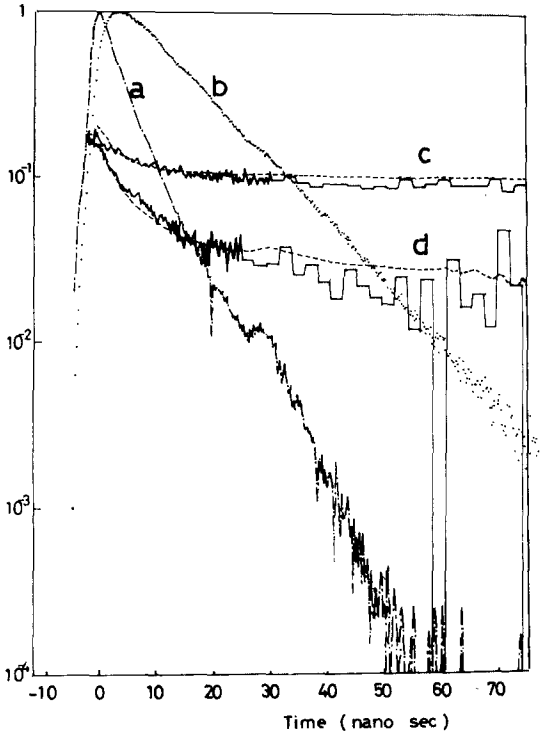

Fig. 3. Time-resolved fluorescence intensity and anisotropy decay of DPE in PS liposomes: *a*, flash pulse for the excitation filtered with U-340 (Hoya, 340 nm); *b*, total fluorescence intensity decay curve $I_T(t)$; *c* and *d*, anisotropy decay curves with (1.88 mM) and without calcium. PS concentration was 3 mg/ml. All other parameters are in the Table

Dielectric Constants

Dielectric constants of polar regions in liposomes were calculated and were plotted against the temperature (Fig. 4*a,b*). In PC and PS liposomes, the dielectric constants were 34 and stayed constant above their phase transition temperatures: 41°C for DPPC, 52°C for DSPC, and about 10 to 20°C for bovine brain PS. It should be noted here that the dielectric constants were the same between PC and PS liposomes despite the charge difference. The dielectric constants were as small as about 4, well below the phase transition temperatures. Furthermore, addition of calcium to PS liposomes lowered the dielectric constant considerably (Fig. 4*b*). Details of these observations will be discussed later in connection with dynamic structures of lipid bilayer membranes.

Dynamic Structures of PC Liposomes

The dynamic structures of PC liposomes and their phase transitions are clearly shown in Fig. 5*a* and *b*. All fluorescence data appeared in sigmoidal curves. In each curve, the state with higher anisotropy corresponds to the gel state of the lipid bilayer and the one with low anisotropy corresponds to the liquid-crystalline state. DPH data showed the clear phase transitions at 23°C for DMPC and 41°C for DPPC in accordance with previous works (Andrich & Vanderkooi, 1976), which are the same as those measured by other techniques (Chapman et al., 1967; Chapman, 1975). DPE data showed the transitions at lower temperatures than these temperatures again in accordance with previous works (Faucon & Lussan, 1973; Tessie, 1979). Such differences between DPH and DPE data were clear in sonicated liposomes (Faucon & Lussan, 1973). However, an effect due to impurities (Tessie, 1979) is absent in

Table. Fluorescence parameters of DPE in PS liposomes

	Temp. (°C)	r^s	ϕ (ns)	r_∞	D_w (nsec^{-1})	θ_c (deg)	α_1	τ_1 (nsec)	α_2	τ_2 (nsec)
PS	30	0.081	1.3	0.028	0.176	64.0	0.65	2.5	0.35	11.4
PS + 1.88 mM $CaCl_2$	30	0.118	0.9	0.099	0.167	45.8	0.67	3.2	0.33	15.3

[a] r^s, steady-state fluorescence anisotropy in Fig. 2; ϕ and r, apparent relaxation time and residual anisotropy in time-resolved anisotropy decay; D_w, wobbling diffusion constant; θ_c, cone angle; α_1, τ_1, α_2, and τ_2, intensity decay parameters for double exponential approximation. *See* Kawato et al. (1977) or Kinosita et al. (1981) for details of the analysis. We used 0.280 as the fundamental anisotropy, r_0, of DPE in the analysis.

our experiments because measurements for Fig. 5*a* and *b* were performed in the same specimen. The observed differences in transition temperatures may reflect the differences of dynamic structures of the two regions (hydrophobic and hydrophilic) of lipid bilayers.

Dynamic Structures of PS Liposomes

Differences of dynamic structures between the two regions are more clearly shown in Fig. 5*c* and *d*, especially in the calcium-effects experiments. PS liposomes showed a phase transition at 10 to 20°C,

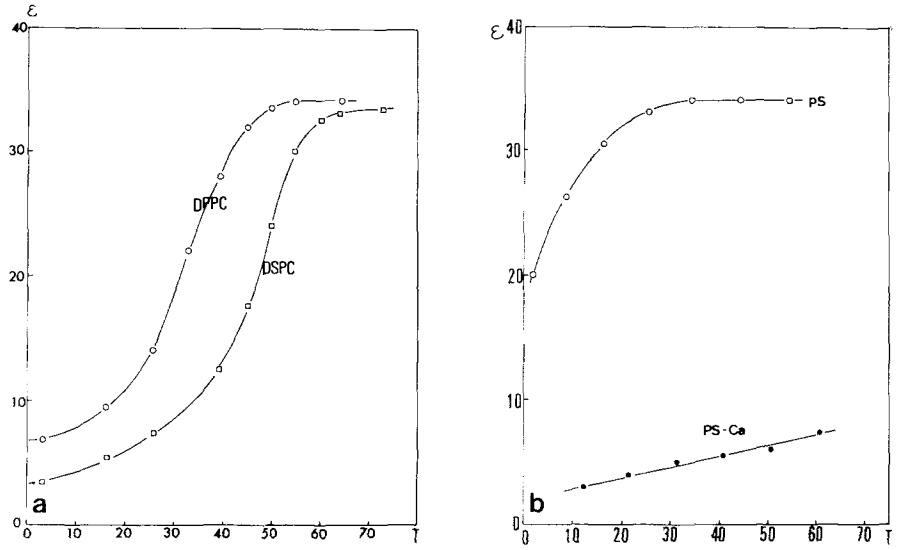

Fig. 4. Temperature dependence of local dielectric constant around polar region of lipid bilayer: *a*, in DPPC and DSPC liposomes, and *b*, in PS liposomes with and without 0.23 mM $CaCl_2$. The suspensions were buffered with 10 mM Tris-HCl at pH 7.3

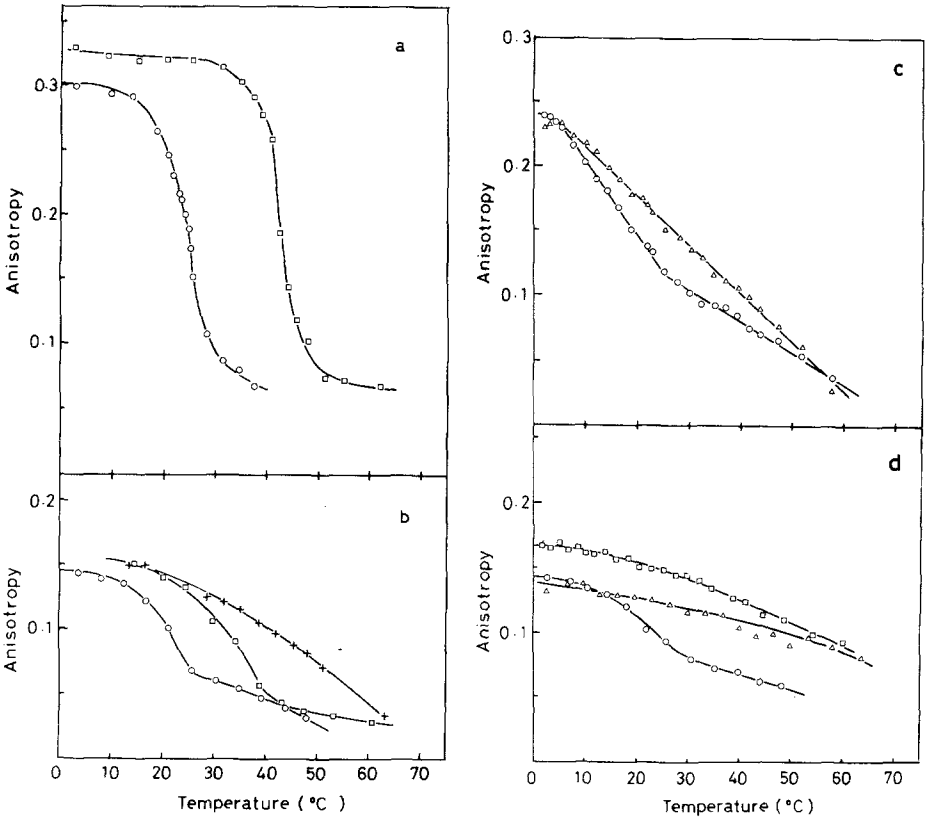

Fig. 5. Temperature dependence of fluorescence anisotropy of DPH (*a*,*c*) and DPE (*b*,*d*): liposomes of DMPC (○), DPPC (□) and DSPC (×) in (*a*,*b*) and liposomes of PS from bovine brain in (*c*,*d*). PS concentration was 0.375 mg/ml and calcium chloride concentrations were 0 (○), 0.23 (△) and 0.47 mM (□)

which is a bit higher than the reported values (Jacobson & Papahadjopoulos, 1975; Browning & Seelig, 1980), probably due to a difference of chain composition of the lipids. Calcium addition abolished the phase transition as reported (Jacobson & Papahadjopoulos, 1975). DPE data showed that the hydrophobic region stayed in the gel state even at 60°C, whereas DPH data showed that the hydrophobic region was as fluid as calcium-free liposomes in the liquid-crystalline state at that temperature. The calcium effect is described in more detail by the time-resolved fluorescence study. As shown in the Table, the cone-diffusion-constants were not much affected by the addition of calcium, whereas the cone angle changed considerably from 64 to 45.8 degrees. We conclude that the viscosity in the cones was the same but only cone angle changed.

Discussion

We have estimated the local dielectric constants around polar regions of lipid bilayer membranes from the Stokes shift of DPE fluorescence. Detailed analysis of the fluorescence clearly justified such estimation. The major points are that the solvent-solute interaction is the major occurrence during the fluorescence lifetime and that the solvent's reorientation relaxation is sufficiently rapid relative to the lifetime. We checked these and established the evaluation. Strictly speaking, what we measured is the chromophore's reorientation. Since the chromophore is attached to the headgroup of a lipid, the actual reorientation of the lipid's headgroup itself should be much faster. Therefore, the fluorescence emission occurs after most reorientations of polar molecules around the probing chromophore finished. The conclusion is that the dielectric dispersion of the system does not affect the evaluation of the local dielectric constant. The fact that the time-integrated spectra almost corresponds to late-gated fluorescence spectra of DPE in egg-lecithin has already been shown by Ghiggino et al. (1981).

Thus estimated, the local dielectric constant around polar region of lipid bilayer membranes showed the following characteristics: (1) The temperature dependence of dielectric constant in liposomes closely follows the change of dynamic structures of the polar region; (2) Both PS and PC liposomes have similar local dielectric constants in spite of difference of surface charges and dynamic structures above their phase transition temperatures; (3) Calcium addition changed the local dielectric constant considerably in PS liposomes; (4) The local dielectric constants of PC and PS liposomes are different around 0 to 10°C in spite of the same dynamic structures of the polar regions. However, this difference disappears on the addition of calcium without any changes in the dynamic structures of the polar region of PS liposomes. Therefore, the difference without calcium probably originates from charge difference. The apparent discrepancy between (2) and (4) can be explained as a charge effect was compensated by the difference of dynamic structures, particularly of the cone angles. The compensation may be more interesting if it has more general aspects. However, the subject is left unsolved.

The local dielectric constant reflects dynamic structures and bulk water around the location. We could demonstrate its large change (almost ten times) due to calcium addition and gel/liquid-crystalline phase transitions.

We reported about the local dielectric properties which DPE chromophore actually experienced in its location. However, the dielectric properties thus evaluated could only offer averaged properties sensed by the chromophore. The size of the probing chromophore will cast questions about perturbing effect and significance of averaged values. What we want to know is actual local dielectric constant which some functional units of membrane components will sense at their particular location in biological membranes. Such units, of course, have their own sizes. Therefore, a notion of infinitesimally small probe is not appropriate to understand the idea of local dielectric constant in biological systems; or rather a size of the order of amino acid residues may be a preferable probing size. Although the particular values may vary to some extent according to their sizes, the outlooks may be similar to the present systems.

Lack of knowledge about the local dielectric properties has limited progress in developing a concept about interactions among membrane proteins. The large changes reported in this system will open up new insights into interactions among membrane components in biological membranes.

We thank Dr. Suguru Kawato for his generous help in measuring time-resolved fluorescence measurements and also Dr. Haruki Nakamura for the measurement of dielectric constants of organic solvents. We appreciate helpful discussions with Drs. Susumu Terakawa and Kimon Angelides. We thank Miss Ritsuko Sakurai for her help in preparing this manuscript.

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Received 10 October 1984, revised 21 December 1984