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Orientation and Dynamics of a Novel Fluorescent Cholesterol Analogue in Membranes of Varying Phase

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Fluorescent analogues of cholesterol offer a powerful approach for monitoring cholesterol behavior in membranes because of their high sensitivity, suitable time resolution, and multiplicity of measurable parameters. In this work, we have monitored the orientation and dynamics of a novel fluorescent cholesterol probe, 6-dansylcholestanol (DChol), in membranes of different phase type utilizing sensitive fluorescence techniques including the red-edge excitation shift (REES) approach. Our results show that fluorescence emission maximum, anisotropy, and lifetime of DChol are dependent on the phase of the membrane. Interestingly, DChol exhibits significant red-edge excitation shift (REES) that appear to depend on the phase of the membrane. Analysis of membrane penetration depth by the parallax method shows that the dansyl group of DChol is localized at the interfacial region of the membrane (~15.6 Å from the center of the bilayer). This is in excellent agreement with the previously reported location of cholesterol in fluid-phase membranes. We propose that DChol could be a potentially useful cholesterol analogue in future studies of model and biological membranes.

Introduction

Cholesterol is an essential constituent and the single most abundant molecule in the plasma membrane of eukaryotic cells. It plays a crucial role in membrane organization, dynamics, function, and sorting,[1,2] and it is often found distributed nonrandomly in domains or pools in biological and model membranes.[1−3] Many of these domains (sometimes called lipid rafts) are believed to be important for the maintenance of membrane structure and function. Such membrane domains have been implicated in important cellular functions such as membrane sorting and trafficking,[4] signal transduction processes,[5] and the entry of pathogens into the cell.[6,7]

Lipid probes have proved to be very useful in membrane biology because of their ability to monitor lipid molecules by a variety of physicochemical approaches at increasing spatiotemporal resolution.[8] Fluorescent sterols offer a powerful approach for studying cholesterol behavior in membranes because of their high sensitivity, suitable time resolution, and multiplicity of measurable parameters.[9,10] One class of probes commonly used for such studies is sterols that are covalently linked with extrinsic fluorophores. The advantage of this class of probes is the choice of the fluorescent label to be used, and therefore, specific probes with appropriate photophysical properties can be designed for specific applications.

Wiegand et al. recently introduced a novel fluorescent cholesterol probe [6-dansylcholestanol (DChol); see Figure 1] in which the dansyl group is covalently linked to the sixth carbon atom of the steroid backbone of cholesterol.[11] The dansyl group is widely used to fluorescently label lipids, proteins, and peptides because of the environmental sensitivity of its fluorescence and favorable lifetime.[12] The dansyl group exhibits a relatively large Stokes shift, and its emission spectrum is highly sensitive to environmental polarity. It has the additional advantage of being one of the smallest available fluorophores. Interestingly, DChol was shown to faithfully mimic cholesterol in cellular assays.[11] For example, the rates of esterification exhibited by DChol and [^{3}H]cholesterol by ACAT (acyl CoA cholesterol acyltransferase), the enzyme that esterifies native cholesterol, were similar in CHO cells. In addition, the efflux kinetics and subcellular distribution profiles of DChol and [^{3}H]cholesterol were found to be similar.[11] Upon inhibition of ACAT, the unesterified DChol was shown to accumulate in the endoplasmic reticulum, as also occurs for cholesterol. In addition, DChol can be readily incorporated into methyl-β-cyclodextrin, which helps in cellular cholesterol modulation.[11] Taking all of these properties together, DChol appears to be a promising fluorescent cholesterol analogue in cellular systems. Importantly, it was previously

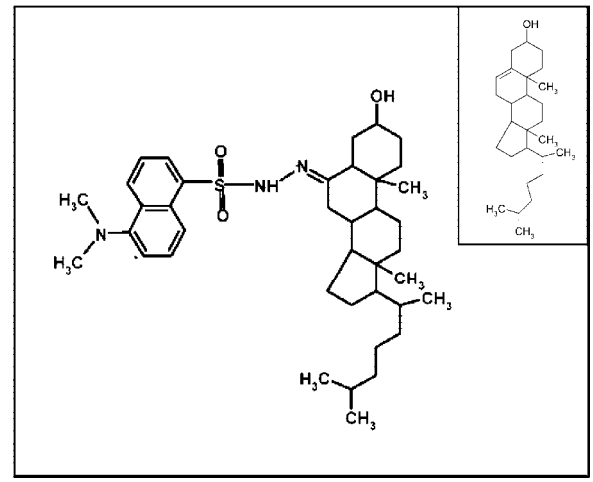

Figure 1. Chemical structure of DChol. The structure of cholesterol is shown in the inset for comparison.

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shown that derivatization of the sixth position carbon atom of the steroid ring of cholesterol does not alter the biophysical properties of cholesterol in model membranes.[13] In this work, we have monitored the organization and dynamics of DChol in membranes of different phase types utilizing sensitive fluorescence techniques including the red-edge excitation shift (REES) approach. In addition, we analyzed the membrane penetration depth of the dansyl group in DChol using the parallax method.[14]

Experimental Section

Materials. 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) and cholesterol were obtained from Sigma Chemical Co. (St. Louis, MO). 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-*sn*-glycero-3-phosphotempocholine (Tempo-PC), and 1-palmitoyl-2-(5-doxyl)stearoyl-*sn*-glycero-3-phosphocholine (5-PC) were obtained from Avanti Polar Lipids (Alabaster, AL). Anthroyloxy-labeled fatty acids such as 2-(9-anthroyloxy)stearic acid (2-AS) and 12-(9-anthroyloxy)stearic acid (12-AS) were obtained from Molecular Probes (Eugene, OR). Lipids were checked for purity by thin-layer chromatography on silica-gel-precoated plates (Sigma) in chloroform/methanol/water (65:35:5, v/v/v) and were found to give only one spot in all cases with a phosphate-sensitive spray and on subsequent charring.[15] Concentrations of phospholipids were determined by phosphate assay subsequent to total oxidation by perchloric acid.[16] DMPC was used as an internal standard to assess lipid oxidation. 6-Dansylcholestanol (DChol) was synthesized as decribed previously.[11] The concentration of a stock solution of DChol in methanol was estimated using its molar extinction coefficient (ε) 4500 M^{-1} cm^{-1} at 335 nm. All other chemicals used were of the highest purity available. Solvents used were of spectroscopic grade. Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout.

Sample Preparation. All experiments were performed using large unilamellar vesicles (LUVs) of 100-nm diameter of either POPC, DPPC, or DPPC with 40 mol % cholesterol. All samples contained 1 mol % DChol. In general, 640 nmol of total lipid and 6.4 nmol of DChol were mixed well and dried under a stream of nitrogen while being warmed gently (~35 °C). After further drying under a high vacuum for at least 3 h, the lipid mixture was hydrated (swelled) by addition of 1.5 mL of 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4 buffer, and each sample was vortexed for 3 min to uniformly disperse the lipids and form homogeneous multilamellar vesicles. The buffer was always maintained at a temperature well above the phase transition temperature of the phospholipid used as the vesicles were made. The lipids were therefore swelled at a temperature of 40 °C for POPC samples and 60 °C for DPPC samples. LUVs of 100-nm diameter were prepared by the extrusion technique using an Avestin Liposofast Extruder (Ottawa, Ontario, Canada) as previously described.[17]Briefly, the multilamellar vesicles were freeze−thawed five times using liquid nitrogen to ensure solute equilibration between trapped and bulk solutions and then extruded through polycarbonate filters (pore diameter of 100 nm) mounted in an extruder fitted with Hamilton syringes (Hamilton Company, Reno, NV). The samples were subjected to 11 passes through the polycarbonate filters to give the final LUV suspension. Background samples were prepared in the same way except that DChol was not added to them. The optical density of the samples measured at 335 nm was ~0.15 in all cases, which rules out any possibility of scattering artifacts in the anisotropy measurements. Samples were incubated in the dark for 12 h at room temperature (~23 °C) for equilibration before fluorescence measurements. All experiments were performed with at least three sets of samples at room temperature (~23 °C).

Depth Measurements Using the Parallax Method. The actual spin (nitroxide) contents of the spin-labeled phospholipids (Tempo- and 5-PC) were assayed using fluorescence quenching of anthroyloxy-labeled fatty acids (2- and 12-AS) as described previously.[18] For depth measurements, liposomes were made by the ethanol injection method.[19] These samples were made by codrying 160 nmol of DOPC containing 10 mol % spin-labeled phospholipid (Tempo- or 5-PC) and 1 mol % DChol under a steady stream of nitrogen with gentle warming (~35 °C), followed by further drying under a high vacuum for at least 3 h. The dried lipid film was dissolved in ethanol to give a final concentration of 40 mM. The ethanolic lipid solution was then injected into 1.5 mL of 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4 buffer, while vortexing, to give a final concentration of 0.11 mM DOPC in buffer. The lipid composition of these samples was 90% DOPC and 10% Tempo- (or 5-PC). Duplicate samples were prepared in each case except for samples lacking the quencher (Tempo- or 5-PC), for which triplicates were prepared. Background samples lacking DChol were prepared in all cases, and their fluorescence intensity was subtracted from the respective sample fluorescence intensity. Samples were kept in the dark for 12 h before fluorescence measurements.

Steady-State Fluorescence Measurements. Steady-state fluorescence measurements were performed with a Hitachi F-4010 spectrofluorometer using 1-cm-path-length quartz cuvettes. Excitation and emission slits with a nominal bandpass of 5 nm were used for all measurements. Background intensities of samples in which DChol was omitted were subtracted from each sample spectrum to cancel out any contribution due to the solvent Raman peak and other scattering artifacts. The spectral shifts obtained with different sets of samples were identical in most cases. In other cases, the values were within ±1 nm of those reported. Fluorescence anisotropy measurements were performed at room temperature (~23 °C) using a Hitachi Glan-Thompson polarization accessory. Anisotropy values were calculated from the equation[12]

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (1)$$

where I_{VV} and I_{VH} are the fluorescence intensities (after appropriate background correction) measured with the excitation polarizer oriented vertically and the emission polarizer vertically and horizontally oriented, respectively. G is the grating factor and is the ratio of the efficiencies of the detection system for vertically and horizontally polarized light; it is equal to I_{HV}/I_{HH}. All experiments were done with at least three sets of samples and average values of anisotropy are shown in Figure 4.

For depth measurements, samples were excited at 335 nm, and emission was collected at 498 nm. Excitation and emission slits with a nominal bandpass of 5 nm were used. Fluorescence was measured at room temperature (~23 °C) and averaged over two 5-s readings. Intensities were found to be stable over time. In all cases, the intensity from background samples without DChol was subtracted. Membrane penetration depths were calculated using eq 5 (see Results).

Time-Resolved Fluorescence Measurements. Fluorescence lifetimes were calculated from time-resolved fluorescence

intensity decays using IBH 5000F coaxial nanosecond flashlamp equipment (Horiba Jobin Yvon, Edison, NJ) with DataStation software in the time-correlated single-photon-counting mode. This machine uses a thyratron-gated nanosecond flash lamp filled with nitrogen as the plasma gas (~1 bar) and is run at 40 kHz. Lamp profiles were measured at the excitation wavelength using Ludox (colloidal silica) as the scatterer. To optimize the signal-to-noise ratio, 5000 photon counts were collected in the peak channel. All experiments were performed using excitation and emission slits with a bandpass of 8 nm. The sample and the scatterer were alternated after every 10% acquisition to ensure compensation for shape and timing drifts occurring during the period of data collection. This arrangement also prevents any prolonged exposure of the sample to the excitation beam, thereby avoiding any possible photodamage of the fluorophore. Data were stored and analyzed using DAS 6.2 software (Horiba Jobin Yvon, Edison, NJ). Fluorescence intensity decay curves so obtained were deconvoluted with the instrument response function and analyzed as a sum of exponential terms

$$F(t) = \Sigma_i \alpha_i \exp(-t/\tau_i) \quad (2)$$

where $F(t)$ is the fluorescence intensity at time t and α_i is a pre-exponential factor representing the fractional contribution to the time-resolved decay of the component with a lifetime of τ_i such that $\Sigma_i \alpha_i = 1$. The program also includes statistical and plotting subroutine packages.[20] The goodness of the fit of a given set of observed data and the chosen function was evaluated by the χ^2 ratio, the weighted residuals,[21] and the autocorrelation function of the weighted residuals.[22] A fit was considered acceptable when plots of the weighted residuals and the autocorrelation function showed random deviation about zero with a minimum χ^2 value not more than 1.4. Intensity-averaged mean lifetimes $\langle\tau\rangle$ for triexponential decays of fluorescence were calculated from the decay times and pre-exponential factors using the equation[12]

$$\langle\tau\rangle = \frac{\alpha_1\tau_1^2 + \alpha_2\tau_2^2 + \alpha_3\tau_3^2}{\alpha_1\tau_1 + \alpha_2\tau_2 + \alpha_3\tau_3} \quad (3)$$

Results

The fluorescence of the dansyl group is known to be sensitive to the microenvironment in which the fluorophore is localized.[12] The membrane properties of cholesterol are critically dependent on the phase of the membrane.[2] We therefore chose to monitor the fluorescence characteristics of DChol in vesicles made of POPC, DPPC, and DPPC containing 40 mol % cholesterol, as these vesicles represent liquid-disordered (fluid), gel, and liquid-ordered phase membranes, respectively.[23] Whereas the lipid acyl chains are ordered and extended in the all-trans conformation in the gel phase, they are fluid and disordered in the liquid-disordered phase. The liquid-ordered phase exists above a threshold level of cholesterol for binary lipid mixtures. For DPPC membranes containing 40 mol % cholesterol, it is evident from the temperature−composition phase diagram that the only phase that exists is the liquid-ordered phase (i.e., no coexisting phases).[23,24] This phase is characterized by acyl chains that are extended and ordered (as in the gel phase) but that exhibit high lateral mobility similar to that observed for the liquid-disordered phase. The fluorescence emission spectra of DChol in these vesicles are shown in Figure 2. The emission maximum[25] of DChol in liquid-disordered-phase POPC vesicles was found to be 498 nm when excited at 335 nm. In gel-phase DPPC vesicles, the emission maximum of DChol displayed a blue shift toward lower wavelength (~7 nm) and was found to be 491 nm. This could be due to a more compact arrangement of the lipid acyl chains in the gel phase, which results in less water penetration than in the liquid-disordered phase.[26] In vesicles made of DPPC and 40 mol % cholesterol (liquid-ordered phase), the emission maximum of DChol was similar to that observed in the gel phase (491 nm).

Red-edge excitation shift (REES) represents a powerful approach that can be used to directly monitor the environment and dynamics around a fluorophore in a complex biological system.[27−29] A shift in the wavelength of maximum fluorescence emission toward higher wavelengths, caused by a shift in the excitation wavelength toward the red edge of absorption band, is termed REES. This effect is mostly observed with polar fluorophores in motionally restricted environments where the dipolar relaxation time for the solvent shell around a fluorophore is comparable to or longer than its fluorescence lifetime. REES arises because of slow rates of solvent relaxation (reorientation) around an excited-state fluorophore, which depends on the motional restriction imposed on the solvent molecules (or the dipolar environment, as in green fluorescent protein[30]) in the immediate vicinity of the fluorophore. Utilizing this approach, it is possible to probe the mobility parameters of the environment itself (represented by the relaxing solvent molecules) using the fluorophore merely as a reporter group. Further, because the ubiquitous solvent for biological systems is water, the information obtained in such cases comes from the otherwise "optically silent" water molecules. Whereas other fluorescence techniques provide information about the fluorophore itself, REES provides information about the relative rates of solvent relaxation that cannot be obtained by other techniques. This makes REES extremely useful, given that hydration plays a crucial modulatory role in a large number of important cellular events such as protein folding[31] and lipid−protein interactions.[32] The shifts in the maxima of fluorescence emission of DChol in vesicles of varying phase as a function of excitation wavelength are shown in Figure 3a. As the excitation wavelength is changed from 335 to 400 nm, the emission maximum of DChol displays a shift toward longer wavelengths in all cases. The emission maximum is shifted from 498 to 516 nm in liquid-disordered-phase POPC vesicles, from 491 to 501 nm in gel-phase DPPC vesicles, and from 491 to 504 nm in liquid-ordered-phase DPPC membranes containing 40 mol % cholesterol. These shifts correspond to REES of 18 nm for liquid-disordered-phase vesicles, with corresponding REES values of 10 and 13 nm for gel-phase and liquid-ordered-phase vesicles, respectively (see Figure 3b). Such a dependence of emission spectra on the excitation wavelength is characteristic of the red-edge effect. Observation of REES in these cases implies that the dansyl group of DChol is localized in motionally restricted environments in these membranes, possibly at the membrane interface (see later). Importantly, although REES in liquid-disordered- and gel-phase membranes was reported previously,[33,34] this is the first report of REES in liquid-ordered membranes.

Figure 4 shows the variation in steady-state anisotropy of DChol in membranes of varying phase. Fluorescence anisotropy is correlated to the rotational diffusion rate of membrane-embedded probes, which is sensitive to the packing of lipid fatty acyl chains.[12] This is because fluorescence anisotropy depends on the degree to which the probe is able to reorient after excitation, and probe reorientation is a function of local lipid

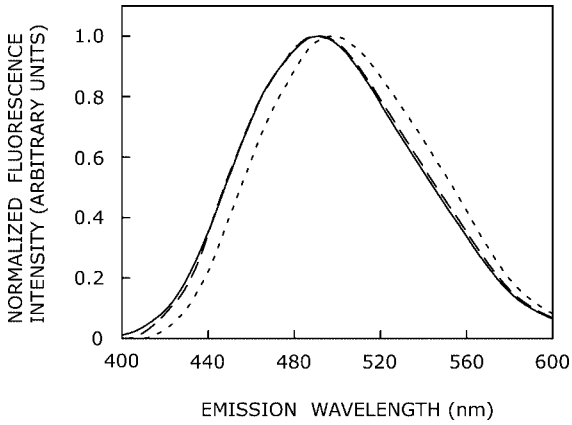

Figure 2. Fluorescence emission spectra of DChol in POPC membranes (- - -), in DPPC membranes (—), and in DPPC membranes containing 40 mol % cholesterol (— — —). The spectra are intensity-normalized at the respective emission maxima. Measurements were carried out at room temperature (~23 °C). The excitation wavelength used was 335 nm. The ratio of DChol to total lipid was 1:100 (mol/mol), and the total lipid concentration was 0.43 mM in all cases. See Experimental Section for other details.

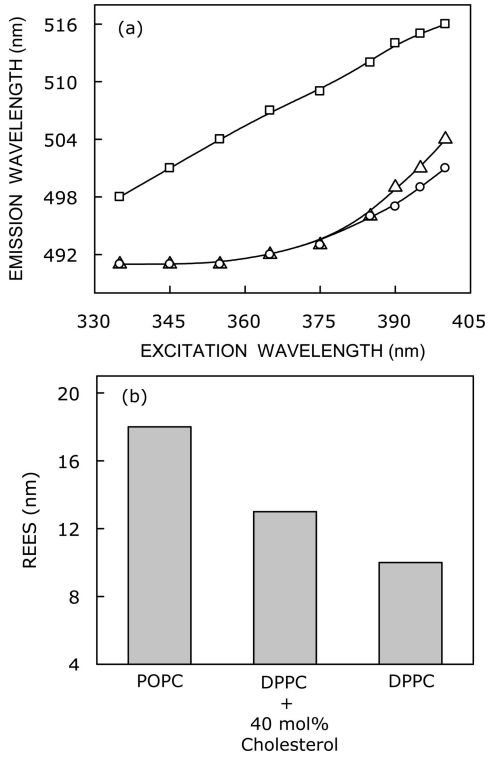

Figure 3. (a) Effect of changing excitation wavelength on the wavelength of maximum emission for DChol in POPC membranes (□), DPPC membranes (○), and DPPC membranes containing 40 mol % cholesterol (Δ). The lines joining the data points are provided merely as viewing guides. (b) Comparison of the magnitude of REES of DChol in various membranes. All other conditions are as in Figure 2. See Experimental Section for other details.

packing. As seen from Figure 4, the anisotropy characteristics in gel-phase DPPC vesicles and liquid-ordered-phase DPPC−cholesterol vesicles are more or less same. This is not surprising given that the packings of acyl chains are similar (extended and ordered) in the two cases. The anisotropy is markedly reduced in fluid-phase POPC vesicles, indicating a higher degree of freedom for the fluorophore to reorient because of the relatively flexible packing in the liquid-disordered phase.

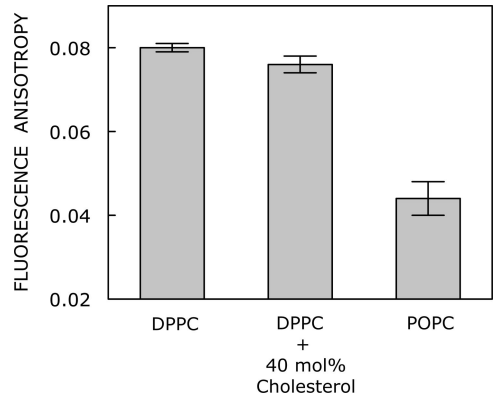

Figure 4. Fluorescence anisotropy of DChol in various membranes. The excitation wavelength used was 335 nm. Emission was monitored at 491 nm for DPPC membranes and DPPC membranes containing cholesterol and at 498 nm for POPC membranes. Measurements were carried out at room temperature (~23 °C). Data shown are means ± standard errors of at least three independent measurements. All other conditions are as in Figure 2. See Experimental Section for other details.

In addition to the dependence of fluorescence emission maxima on the excitation wavelength, fluorescence anisotropy is known to depend on the excitation wavelength in motionally restricted media.[35] Because of strong dipolar interactions with the surrounding solvent molecules, there is a decreased rotational rate of the fluorophore in the relaxed state. On red-edge excitation, a selective excitation of this subclass of fluorophores occurs. Because of strong interactions of the polar solvent molecules with this subclass of fluorophores in the excited state, one might expect these "solvent-relaxed" fluorophores to rotate more slowly, thereby increasing the anisotropy. The excitation anisotropy spectra (i.e., a plot of steady-state anisotropy vs excitation wavelength) of DChol in membranes of varying phase are shown in Figure 5a. The anisotropy of DChol in these membranes shows an increase as the excitation wavelength increases from 335 to 400 nm in all cases. Figure 5b shows the variation in steady-state anisotropy of DChol in membranes of varying phase as a function of wavelength across its emission spectrum. As can be seen from the figure, there is decrease in anisotropy with increasing emission wavelength in all cases. The lowest anisotropy is observed toward longer wavelengths (red edge), where emission from the relaxed fluorophores predominates. We should mention here that the anisotropy of DChol in bulk 70% (v/v) ethanol essentially remains invariant over the same range of excitation and emission wavelengths. Taken together, these results reinforce our previous conclusion that the dansyl group in DChol is localized in a motionally restricted region of the membrane in these cases.

Fluorescence lifetime serves as a reliable indicator of the local environment in which a given fluorophore is localized.[36] In addition, it is well-known that the fluorescence lifetime of the dansyl moiety is sensitive to its local environment.[37−39] A typical decay profile of DChol in DPPC membranes with its triexponential fitting and the statistical parameters used to check the goodness of the fit is shown in Figure 6. Table 1 lists the lifetimes of DChol in membranes of varying phase. All fluorescence decays obtained for membrane-bound DChol could be fitted well with a triexponential function. We chose to use the mean fluorescence lifetime as an important parameter for describing the behavior of membrane-bound DChol because it is independent of the method of analysis and the number of exponentials used to fit the time-resolved fluorescence decay. The mean fluorescence lifetimes of DChol in membranes of

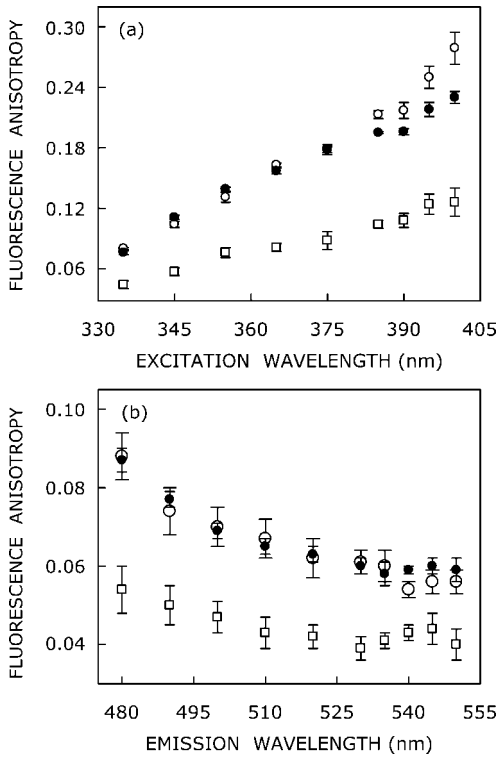

Figure 5. Fluorescence anisotropy of DChol in POPC membranes (□), DPPC membranes (○), and DPPC membranes containing 40 mol % cholesterol (●) as a function of (a) excitation and (b) emission wavelengths. For a, the anisotropy values were recorded at an emission wavelength of 491 nm for DPPC and DPPC membranes containing cholesterol, whereas emission was monitored at 498 nm for POPC membranes. For b, the excitation wavelength used was 335 nm in all cases. Data shown are means ± standard errors of at least three independent measurements. All other conditions are as in Figure 2. See Experimental Section for other details.

varying phase were calculated from the data in Table 1 using eq 3 and are shown in Figure 7a. The mean fluorescence lifetime of DChol in liquid-disordered-phase POPC membranes was found to be ~13.2 ns. The mean fluorescence lifetimes of DChol in gel- (DPPC) and liquid-ordered- (DPPC−cholesterol) phase membranes are longer, with values of ~15.6 and 14.6 ns, respectively. It has been previously reported that the fluorescence lifetime (radiative decay rate constant) of the dansyl group is reduced with increasing polarity of the medium in which the fluorophore is embedded.[38] The longer mean fluorescence lifetimes of DChol in DPPC and DPPC−cholesterol vesicles could therefore be due to the relatively compact organization of the lipid fatty acyl chains in the gel and liquid-ordered phases, which results in less water penetration.

To ensure that the anisotropy values measured for membrane-bound DChol (Figure 4) were not influenced by lifetime-induced artifacts, the apparent (average) rotational correlation times were calculated using Perrin's equation[12]

$$\tau_c = \frac{\langle\tau\rangle r}{r_o - r} \quad (4)$$

where r_o is the limiting (fundamental) anisotropy of the dansyl group (in the absence of any other depolarizing processes such as rotational diffusion), r is the steady-state anisotropy, and $\langle\tau\rangle$ is the mean fluorescence lifetime taken from Figure 7a. Although Perrin's equation is not strictly applicable to this system, it is assumed that this equation will apply to a first approximation, especially because we used mean fluorescence lifetimes for the analysis of multiple component lifetimes. The values of the apparent rotational correlation times, calculated in this way using an r_o value of 0.321,[40] are shown in Figure 7b. As is evident from the figure, the apparent rotational correlation times show a trend similar to that observed in Figure 4. This shows that the observed changes in anisotropy values are largely free from lifetime-induced artifacts.

Membrane penetration depth represents an important parameter in the study of membrane structure and organization.[41,42] Knowledge of the precise depth of a membrane-embedded group or molecule often helps define the conformation and topology of membrane probes and proteins. In addition, properties such as polarity, fluidity, segmental motion, ability to form hydrogen bonds, and extent of solvent penetration are known to vary in a depth-dependent manner in the membrane. To gain an overall understanding of the orientation and location of membrane-bound DChol, the penetration depth of the dansyl group in DOPC membranes was determined. The membrane penetration depth of the dansyl group was calculated by the parallax method[14] using the equation

$$z_{cF} = L_{c1} + \{[(-1/\pi C)\ln(F_1/F_2) - L_{21}^2]/2L_{21}\} \quad (5)$$

where z_{cF} is the depth of the fluorophore from the center of the bilayer, L_{c1} is the distance of the center of the bilayer from the shallow quencher (Tempo-PC in this case), L_{21} is the difference in depth between the two quenchers (i.e., the transverse distance between the shallow and deep quenchers), and C is the two-dimensional quencher concentration in the plane of the membrane (molecules/Å^2). Here, F_1/F_2 is the ratio of F_1/F_o and F_2/F_o, in which F_1 and F_2 are the fluorescence intensities in the presence of the shallow quencher (Tempo-PC) and the deep quencher (5-PC), respectively, both at the same quencher concentration C; F_o is the fluorescence intensity in the absence of any quencher. All of the bilayer parameters used were the same as described previously (for further details about these parameters, see ref 14). Our results show that the depth of penetration of the dansyl group of DChol, on average, was ~15.6 Å (see Figure 8) from the center of the bilayer. (In principle, this distance represents the distance between the center of the bilayer and the transition dipole of the dansyl group.) This suggests that the dansyl group in DChol is localized at the interfacial region of the membrane.

Discussion

Although a number of fluorescent analogues of cholesterol have been developed, each one has certain advantages and limitations.[9,10] As mentioned earlier, fluorescent sterols offer a powerful approach for studying cholesterol behavior in membranes for a number of reasons. In general, there are two types of fluorescent sterols that mimic cholesterol in membranes: (i) intrinsically fluorescent sterols such as dehydroergosterol (or cholestatrienol) and (ii) cholesterol probes with chemically labeled fluorophores. Each class of fluorescent sterols has specific merits and demerits. The members of the first class are naturally occurring fluorescent cholesterol analogues found in yeast and fungi and can be considered to be close analogues of cholesterol. However, their use has been limited because of their unfavorable fluorescence properties such as low quantum yield and rapid bleaching. Sterol analogues of the second class exhibit superior fluorescence characteristics, making their detection possible even when present in low concentrations. They carry fluorescent reporter groups that could be bulky. Nonetheless, fluorescent sterols labeled with the 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) group (22-NBD-cholesterol)[43] or the dansyl group (DChol)[11] have been previously to reliably mimic the biochemistry

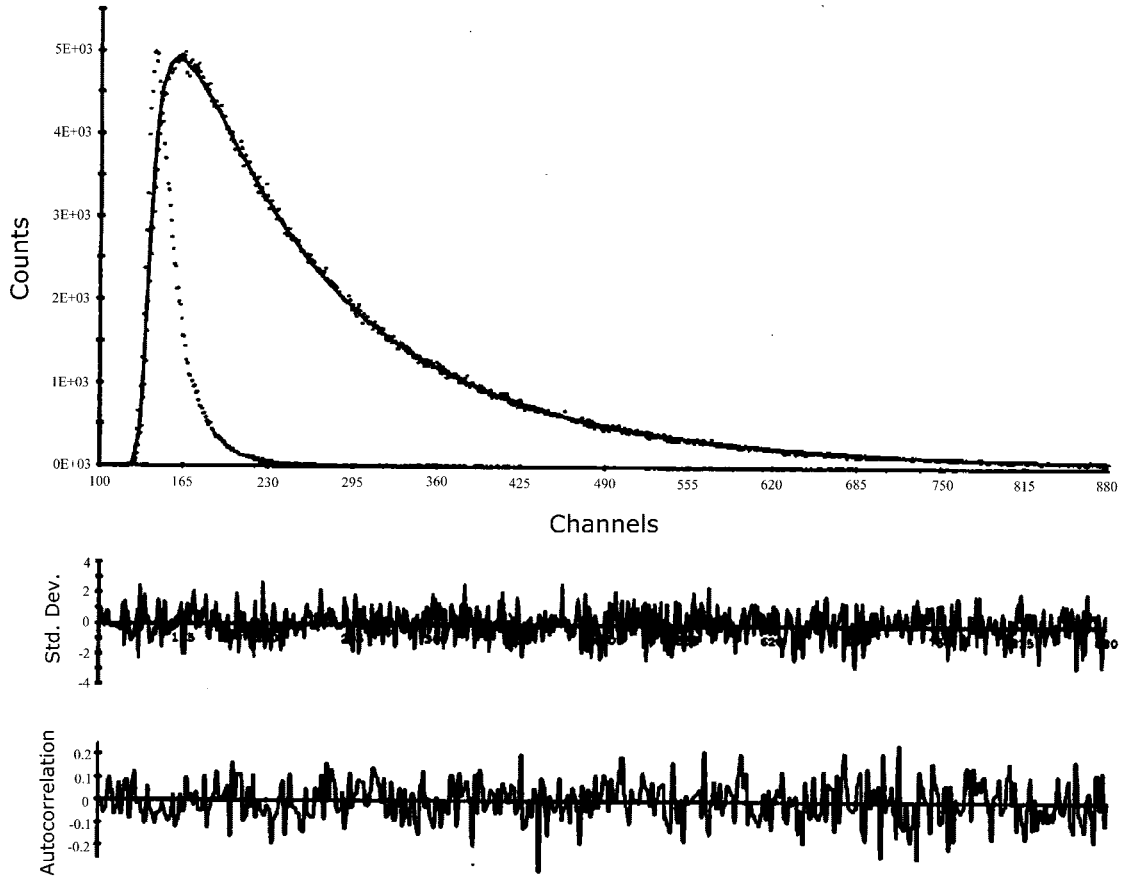

Figure 6. Time-resolved fluorescence intensity decay of DChol in DPPC membranes. The excitation wavelength was at 335 nm, and emission was monitored at 491 nm. The sharp peak on the left is the lamp profile. The relatively broad peak on the right is the decay profile, fitted to a triexponential function. The two lower plots show the weighted residuals and the autocorrelation function of the weighted residuals. All other conditions are as in Figure 2. See Experimental Section for other details.

TABLE 1: Representative Fluorescence Lifetimes of DChol in Membranes[a]

	α_1	τ_1 (ns)	α_2	τ_2 (ns)	α_3	τ_3 (ns)
POPC	0.23	5.41	0.09	0.42	0.68	14.28
DPPC	0.21	8.18	0.07	0.30	0.72	16.75
DPPC + 40 mol % cholesterol	0.25	8.41	0.08	0.13	0.67	15.83

[a] Excitation wavelength was 335 nm. Emission monitored at 491 nm for DPPC membranes and DPPC membranes containing cholesterol and at 498 nm for POPC membranes. Concentration of total lipid was 0.43 mM, and ratio of DChol to total lipid was 1:100 (mol/mol). See Experimental Section for other details.

and cell biology of cholesterol in living cells. Members of this class of fluorescent sterols are appropriate for pulse-chase experiments and imaging in living cells. For DChol, the excellent photophysical properties of the dansyl group (see above) combined, with a relatively small size (compared to other fluorophores), make it a suitable fluorescent sterol for studies using biological and model membranes.

In this work, we have monitored the organization and dynamics of DChol in membranes of varying phase, i.e., liquid-disordered, gel, and liquid-ordered phases, utilizing fluorescence-based approaches including the REES approach and the parallax method for depth analysis. Our results show that the fluorescence emission maximum, anisotropy, and lifetime of DChol are dependent on the phase of the membrane. Interestingly, DChol exhibits significant REES effects that appear to be dependent on the phase of the membrane. This indicates that the dansyl group in DChol is localized in a motionally restricted environment in these membranes. In addition, we report, for the first time, REES in liquid-ordered-phase membranes using DChol as a probe. Interestingly, it was recently reported that the liquid-ordered phase has hydration characteristics different from those of the liquid-disordered and gel phases.[44] An important criterion for a fluorophore to exhibit REES is a relatively large change in dipole moment upon excitation.[27,28] The dansyl group is particularly suitable in this respect, as it was previously shown that this group undergoes a large dipole moment change upon excitation.[45] The motionally restricted environment of the dansyl group in the membrane is further supported by wavelength-dependent anisotropy changes.

An important issue for cholesterol analogues is the orientation of the molecule with respect to the membrane.[46,47] Analysis of the membrane penetration depth shows that the dansyl group of DChol is localized at ~15.6 Å from the center of the bilayer, i.e., in the interfacial region of the membrane (Figure 8). This depth of the dansyl group in DChol is in very good agreement with the location of cholesterol in fluid-phase membranes, as determined by the neutron diffraction method,[48] reinforcing the fact that DChol is a faithful analogue of cholesterol. We conclude that DChol could potentially be used for future studies involving model and biological membranes.

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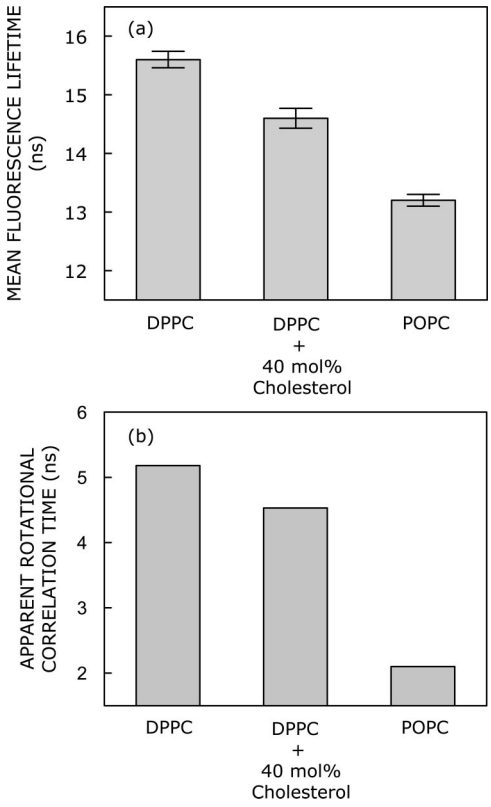

Figure 7. (a)Mean fluorescence lifetimes of DChol in various membranes. Mean fluorescence lifetimes were calculated using eq 3. The excitation wavelength used was 335 nm. Emission was monitored at 491 nm for DPPC membranes and DPPC membranes containing cholesterol and at 498 nm for POPC membranes. Data shown are means ± standard errors of at least three independent measurements. All other conditions are as in Figure 2. See Experimental Section for other details. (b) Apparent rotational correlation times of DChol in membranes. Apparent rotational correlation times were calculated from fluorescence anisotropy values of DChol from Figure 4 and mean fluorescence lifetimes from panel a using eq 4. See text for other details.

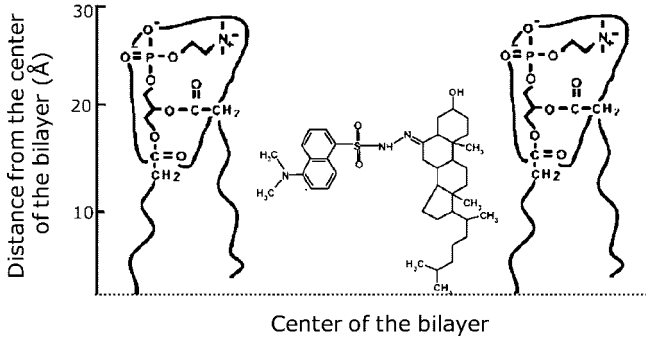

Figure 8. Schematic representation of one-half of the membrane bilayer showing the localization of DChol in phosphatidylcholine membranes. The horizontal line at the bottom indicates the center of the bilayer.

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