

Influence of cholesterol and ergosterol on membrane dynamics using different fluorescent reporter probes

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Abstract

Ergosterol is an evolutionary precursor of cholesterol and is the major sterol present in lower eukaryotes. Although detailed biophysical characterization of the effect of cholesterol on membranes is well documented, the effect of ergosterol on the organization and dynamics of membranes is still at a very early stage. We have monitored the effect of cholesterol and ergosterol on the dynamic properties of both fluid (POPC) and gel (DPPC) phase membranes utilizing fluorescent reporter probes pyrene and TMA-DPH. These results show, for the first time, the important differences on the effect of cholesterol and ergosterol in short-range ordering (reported by TMA-DPH) and long-range dynamics (reported by pyrene). In addition, pyrene vibronic peak intensity ratio provides information on polarity of the microenvironment experienced by the probe. These novel results are relevant in the context of membrane domains in ergosterol-containing organisms such as *Drosophila* which maintain a low level of sterol compared to higher eukaryotes.

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Cholesterol is an essential constituent of eukaryotic membranes and plays a crucial role in membrane organization, dynamics, function, and sorting [1]. It is proposed to maintain a laterally heterogeneous distribution of lipids and proteins on the plasma membrane due to its putative role in the formation and maintenance of domains such as lipid rafts [1–3]. Such membrane domains have been implicated in important cellular functions such as signal transduction [4] and entry of pathogens into the cell [5].

Cholesterol, the most representative sterol present in vertebrate membranes, is the end product of the long and multi-step sterol biosynthetic pathway [6]. Konrad Bloch

speculated that the sterol biosynthetic pathway parallels sterol evolution (the “Bloch hypothesis”). According to this hypothesis, cholesterol has been selected over a very long time scale of natural evolution for its ability to optimize certain physical properties of eukaryotic cell membranes with regard to biological functions [6]. Cholesterol precursors should therefore have properties that gradually support cellular function of higher organisms as they progress along the pathway toward cholesterol. Defects in cholesterol biosynthetic pathway have been identified with several inherited metabolic disorders [7]. Comparative studies of the effects of cholesterol and its evolutionary precursors on membranes therefore assume significance. An important evolutionary precursor of cholesterol is ergosterol, the major sterol component present in lower eukaryotes such as certain protozoa, yeast, and other fungi, and in insects such as *Drosophila* [6]. The chemical structure of ergosterol differs from that of cholesterol in having two additional double bonds (at positions C₇ and C₂₂) and a methyl group at C₂₄ of the side chain. Both structural

Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, dipalmitoyl-*sn*-glycero-3-phosphocholine; LUV, large unilamellar vesicle; MLV, multilamellar vesicle; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; TMA-DPH, 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene.

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features appear relatively late during ergosterol biosynthesis in response to some specialized requirements related to the physiology of organisms containing ergosterol as the major sterol [6]. Interestingly, lipid rafts have been isolated from organisms such as yeast [8] and *Drosophila* [9] which have ergosterol as their major sterol component.

Although detailed biophysical characterization of the effect of cholesterol on membranes is well documented [1], the effect of ergosterol on the organization and dynamics of membranes is just beginning to be addressed [10–12]. We recently monitored the effect of cholesterol and ergosterol on the dynamic properties of both fluid (POPC) and gel (DPPC) phase membranes utilizing the environment-sensitive fluorescent membrane probe DPH [10]. Our results showed differential effects of ergosterol and cholesterol toward membrane dynamics. DPH, which is a rod-like molecule, partitions into the interior of the bilayer. However, its precise location and orientation in the membrane is not known, since DPH does not localize at a unique location in the membrane. In addition, since the membrane is considered as a two-dimensional anisotropic fluid, any possible change in membrane order may not be uniform and restricted to a unique location in the membrane. It is therefore important to monitor the change in membrane order at more than one region in the membrane in order to obtain a comprehensive understanding of any change in membrane (lipid) dynamics. Moreover, one of us has previously shown that stress such as heat shock can induce anisotropic changes in membrane order, i.e., the change in membrane order was different when monitored in different regions in adult rat liver cell plasma membranes [13]. It is for this reason that we wanted to explore the differential effects of ergosterol and cholesterol on membrane dynamics using TMA-DPH. TMA-DPH is a derivative of DPH with a cationic moiety attached to the para position of one of the phenyl rings [14]. The amphipathic TMA-DPH is oriented in the membrane bilayer with its positive charge localized at the lipid-water interface. Its DPH moiety is localized at ~ 11 Å from the center of the bilayer and reports the interfacial region of the membrane [15]. In contrast to this, the average location of DPH has been shown to be ~ 8 Å from the center of the bilayer [15].

In this paper, we have monitored the effect of cholesterol and ergosterol on the dynamic properties of both fluid (POPC) and gel (DPPC) phase membranes utilizing the location-specific fluorescent membrane probe TMA-DPH. In addition, we have used the hydrophobic membrane probe pyrene to gain further insight into the effect of cholesterol and ergosterol on membrane dynamics. The fluorescence emission spectrum of pyrene is sensitive to environmental polarity [16]. Pyrene also forms excimers with very different fluorescence characteristics and the ratio of excimer/monomer is known to be dependent on membrane dynamics [17,18]. Our results using both TMA-DPH and pyrene as reporter probes show differential effects of ergosterol and cholesterol toward membrane dynamics.

Materials and methods

Materials. DPPC and POPC were obtained from Avanti Polar Lipids (Alabaster, AL, USA). DMPC, cholesterol, ergosterol, TMA-DPH and pyrene were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All lipid stock solutions were made in methanol except ergosterol which was dissolved in ethanol. Phospholipids were checked for purity and assayed as described earlier [10]. Concentration of stock solution of TMA-DPH and pyrene in methanol was estimated from the respective molar absorption coefficient (ϵ) of $80,000 \text{ M}^{-1} \text{ cm}^{-1}$ at 350 nm for TMA-DPH [19] and $54,000 \text{ M}^{-1} \text{ cm}^{-1}$ at 335 nm for pyrene [20].

Sample preparation. Multilamellar vesicles (MLVs) of either POPC or DPPC containing increasing concentrations (0–50 mol%) of sterol (cholesterol or ergosterol) and 1 mol% TMA-DPH were prepared. For this, 100 nmol of total lipid (phospholipid and sterol) and 1 nmol of TMA-DPH were mixed well and dried under a stream of nitrogen while being warmed gently (~ 35 °C). After the lipids were dried further under a high vacuum for at least 3 h, they were hydrated (swelled) by adding 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 MLVs. The buffer was always maintained at a temperature well above the phase transition temperature of the phospholipid used as the vesicles were made. Thus, the lipids were swelled at a temperature of 40 °C for POPC and 60 °C for DPPC samples. The samples were freeze-thawed five times by cycling the samples in liquid nitrogen and in a water bath maintained at 60 °C to ensure solute equilibration between trapped and bulk solutions. Background samples were prepared in the same way except that TMA-DPH was not added to them. The optical density of the samples measured at 358 nm was ~ 0.15 which rules out any possibility of scattering artifacts in the polarization measurements. Samples were kept in dark for 1 h before measuring fluorescence. All the experiments were carried out with multiple sets of samples at room temperature (~ 23 °C).

For experiments involving pyrene, large unilamellar vesicles (LUVs) of 100 nm diameter of either POPC or DPPC containing increasing concentrations (0–50 mol%) of sterol (cholesterol or ergosterol) and 1 mol% pyrene were prepared. For this, 640 nmol of total lipid (phospholipid and sterol) and 6.4 nmol of pyrene were mixed well and dried under a stream of nitrogen while being warmed gently (~ 35 °C). Homogeneous MLVs were prepared as described above. LUVs of 100 nm diameter were prepared by the extrusion technique using an Avestin Liposofast Extruder (Ottawa, Ontario, Canada) as previously described [21]. Samples were incubated in dark for 12 h at room temperature (~ 23 °C) for equilibration before measuring fluorescence. Background samples were prepared the same way except that pyrene was not added.

Steady state fluorescence measurements. Steady state fluorescence polarization of samples containing TMA-DPH were carried out as described earlier with a Hitachi F-4010 spectrofluorimeter [10]. For measuring pyrene fluorescence, samples were excited at 335 nm. Excitation and emission slits were set at 5 nm. The excimer to monomer fluorescence intensity ratio was determined by measuring fluorescence intensity at the monomer (393 nm) and excimer (480 nm) peaks. The ratio of the first (373 nm) and third (384 nm) vibronic peak intensities (I_1/I_3) was monitored from pyrene emission spectra.

Results

The change in fluorescence polarization of TMA-DPH with increasing sterol concentration is shown in Fig. 1. Fluorescence polarization measured using probes such as TMA-DPH is correlated to the rotational diffusion of membrane embedded probes [22], which is sensitive to the packing of lipid fatty acyl chains and sterols. This is due to the fact that fluorescence polarization depends on the degree to which the probe is able to reorient after

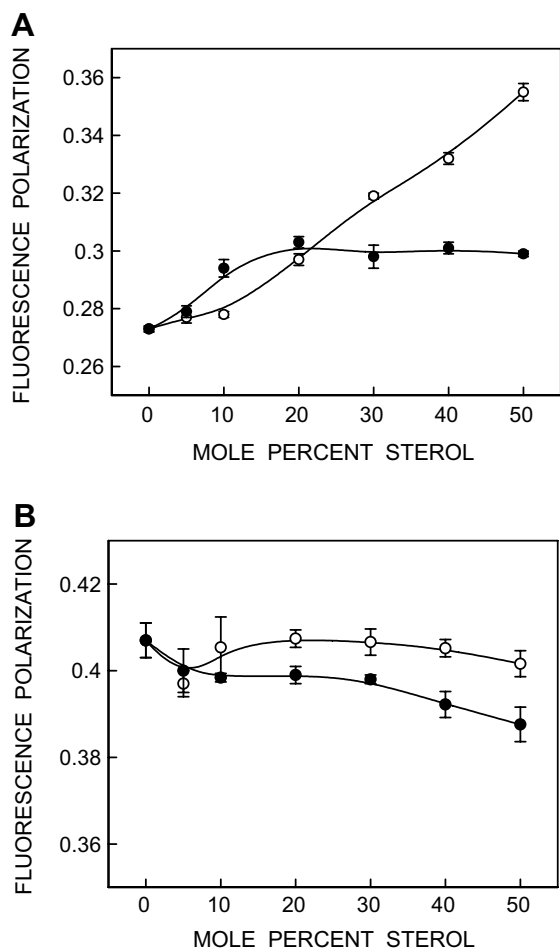


Fig. 1. Fluorescence polarization of TMA-DPH in (A) POPC and (B) DPPC membranes as a function of increasing concentrations of cholesterol (○) and ergosterol (●). The ratio of TMA-DPH to total lipid was 1:100 (mol/mol) and total lipid concentration was 66.6 μ M in all cases. The excitation wavelength used was 358 nm and emission was monitored at 430 nm. Measurements were carried out at room temperature (~ 23 °C). The data points shown are means \pm SE of at least three independent measurements.

excitation, and probe reorientation is a function of local lipid packing. Fig. 1A shows that with increase in cholesterol concentration, the TMA-DPH polarization in fluid POPC membranes shows a continuous increase up to the highest concentration of cholesterol used. Thus, there is considerable (30%) increase in polarization when 50 mol% of cholesterol was incorporated in POPC membranes. This could possibly indicate that the membrane interfacial region (where the DPH moiety in TMA-DPH is localized) becomes more ordered (rigid) with increasing concentration of cholesterol when added to membranes in the fluid phase. This is consistent with our earlier results using DPH in fluid phase POPC membranes [10]. However, the reported increase in polarization of DPH was much higher.

In contrast to what was observed in case of cholesterol containing POPC membranes, the fluorescence polarization of TMA-DPH in POPC membranes shows an increase

(11%) only up to ~ 20 mol% when ergosterol was used as the sterol component (see Fig. 1A). At higher concentrations of ergosterol, the polarization values stabilize with increasing ergosterol concentration up to 50 mol%. This suggests that ergosterol does not influence the membrane interfacial order and dynamics in fluid membranes beyond a certain concentration, in agreement with our previous results using DPH as a probe [10]. The effect of ergosterol therefore appears to be markedly different than that of cholesterol on the dynamics (order) of fluid phase POPC membranes, irrespective of the membrane region probed. To the best of our knowledge, this is the first report describing this unique effect of ergosterol using TMA-DPH fluorescence. It is important to note here that the polarization values determined remained identical even after dilution of membrane samples indicating the absence of any scattering artifacts [23].

Fig. 1B shows the corresponding changes in TMA-DPH polarization when the sterols were incorporated in the gel phase DPPC membranes at room temperature (~ 23 °C). In DPPC membranes containing increasing amounts of cholesterol, the polarization values remain essentially invariant up to 50 mol% cholesterol. Upon incorporation of ergosterol in gel phase DPPC membranes, there is a very small (3%) reduction in polarization (see Fig. 1B). Interestingly, TMA-DPH polarization values for each ergosterol concentration appear to be lower than the corresponding polarization value for cholesterol. This is in contrast to what we earlier observed with DPH as a probe where polarization values for ergosterol-containing membranes were higher than the corresponding values for cholesterol-containing membranes in gel phase DPPC membranes [10].

Fig. 2 shows the fluorescence emission spectra of pyrene in fluid phase POPC and gel phase DPPC membranes, and also in presence of cholesterol and ergosterol. A characteristic feature of the structured emission spectra is the maxima at 373, 384, and 393 nm. This type of structured vibronic band intensities, displayed by fluorophores such as pyrene [16] and dehydroergosterol [24], are known to be environmentally sensitive. This property has previously been effectively used for elucidating microenvironments of pyrene [18]. The ratio of the first (373 nm) and third (384 nm) vibronic peak intensities (I_1/I_3) in pyrene emission spectra provides a measure of the apparent polarity of the environment. An increase in the ratio is indicative of increased polarity. Fig. 3A shows the change in the ratio of vibronic peak intensities (I_1/I_3) in pyrene emission spectra in fluid phase POPC membranes with increasing sterol concentration. Increasing sterol concentration in general resulted in a decrease in the peak intensity ratio which could imply a reduction in apparent polarity experienced by the fluorophore (pyrene). However, this effect stabilizes in case of ergosterol beyond 20 mol%, while the decrease in apparent polarity in case of cholesterol continues beyond this point. It is interesting to note that this is the same concentration threshold (~ 20 mol%) beyond which

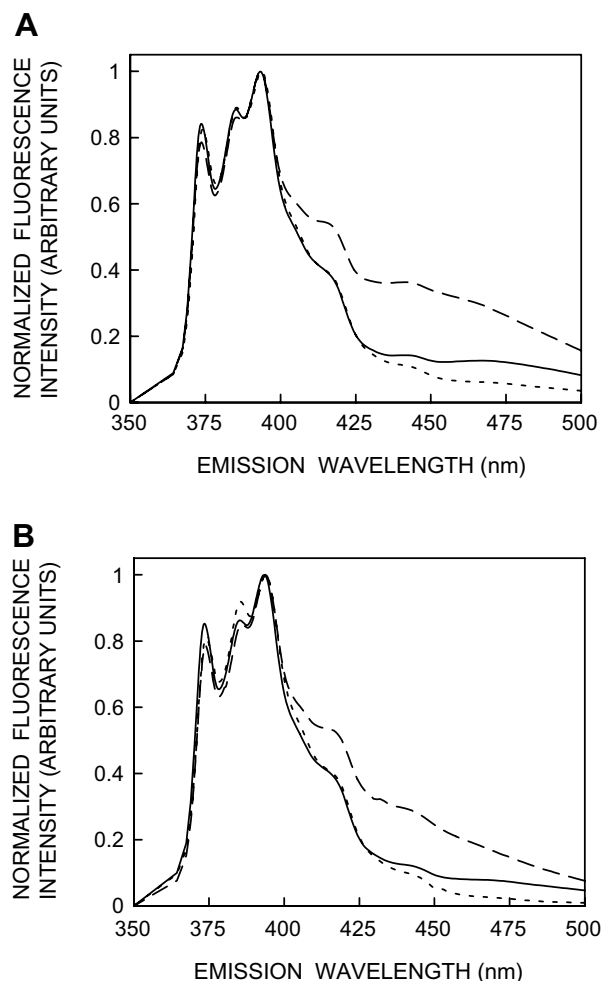


Fig. 2. (A) Fluorescence emission spectra of pyrene in POPC membranes (—), POPC membranes containing 50 mol% cholesterol (----), and POPC membranes containing 50 mol% ergosterol (---). (B) Fluorescence emission spectra of pyrene in DPPC membranes (—), DPPC membranes containing 50 mol% cholesterol (----), and DPPC membranes containing 50 mol% ergosterol (---). The spectra are intensity-normalized at the respective emission maxima. Measurements were carried out at room temperature ($\sim 23^\circ\text{C}$). The excitation wavelength used was 335 nm. The ratio of pyrene to total lipid was 1:100 (mol/mol) and total lipid concentration was 0.43 mM in all cases.

TMA-DPH polarization stabilizes when ergosterol is incorporated in fluid phase POPC membranes (see Fig. 1A). Fig. 3B shows the corresponding changes in the ratio of pyrene vibronic peak intensities (I_1/I_3) when the sterols were incorporated in the gel phase DPPC membranes. Interestingly, incorporation of both cholesterol and ergosterol in gel phase DPPC membranes led to an almost identical reduction in the peak intensity ratio up to 20 mol%. Beyond this point, there is a considerable increase in I_1/I_3 ratio at higher concentrations of ergosterol, while increasing concentrations of cholesterol led to further decrease in the ratio (see Fig. 3B).

Another commonly used parameter related to pyrene fluorescence is the excimer/monomer fluorescence intensity ratio [17,18]. This parameter is indicative of the extent of

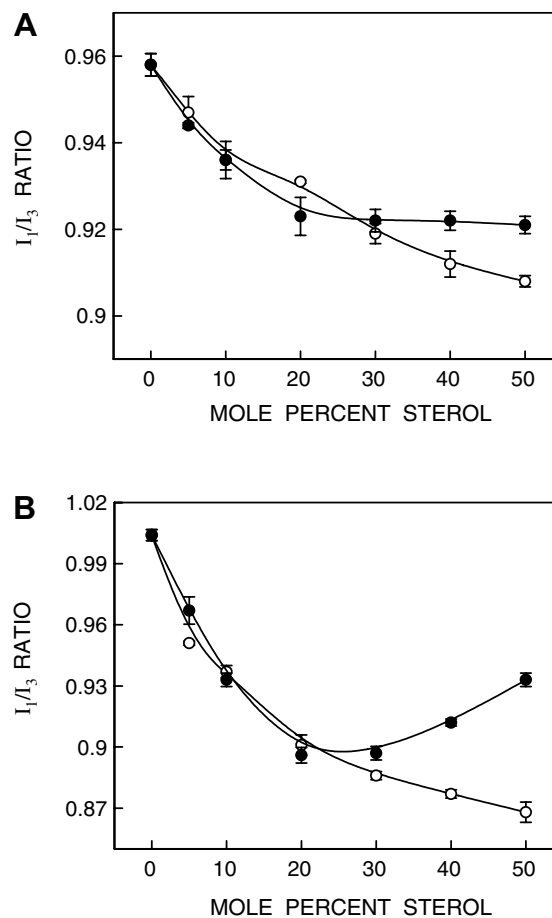


Fig. 3. Change in fluorescence peak intensity ratio of pyrene (I_1/I_3) in (A) POPC and (B) DPPC membranes as a function of increasing concentrations of cholesterol (\circ) and ergosterol (\bullet). The data points shown are means \pm SE of at least three independent measurements. All other conditions are as in Fig. 2.

pyrene excimerization which is believed to depend on the monomer lateral distribution and dynamics (diffusion) in the membrane. Nonetheless, the exact mechanism of excimerization is not clear [25]. Diffusion in membranes is commonly described in terms of the free volume model [26]. The free volume framework is a semi-empirical approach based on statistical mechanical considerations of density fluctuations in the lipid bilayer. According to this model, transient voids that are created in the lipid bilayer by such density fluctuations are filled by the movement of neighboring lipid molecules into the void. It is generally agreed that the greater the free volume available for pyrene, the higher the level of excimer formation [18]. Fig. 4A shows the excimer/monomer ratio in fluid phase POPC membranes with increasing sterol concentration. There appears to be a marked difference in the extent of excimer formation in case of cholesterol and ergosterol. The excimer/monomer ratio shows considerable enhancement up to 20 mol% ergosterol beyond which it shows a plateau. The excimer/monomer ratio shows a small and steady decrease in case of cholesterol (see Fig. 4A). In case of the gel phase DPPC membranes also, the excimer/monomer ratio shows

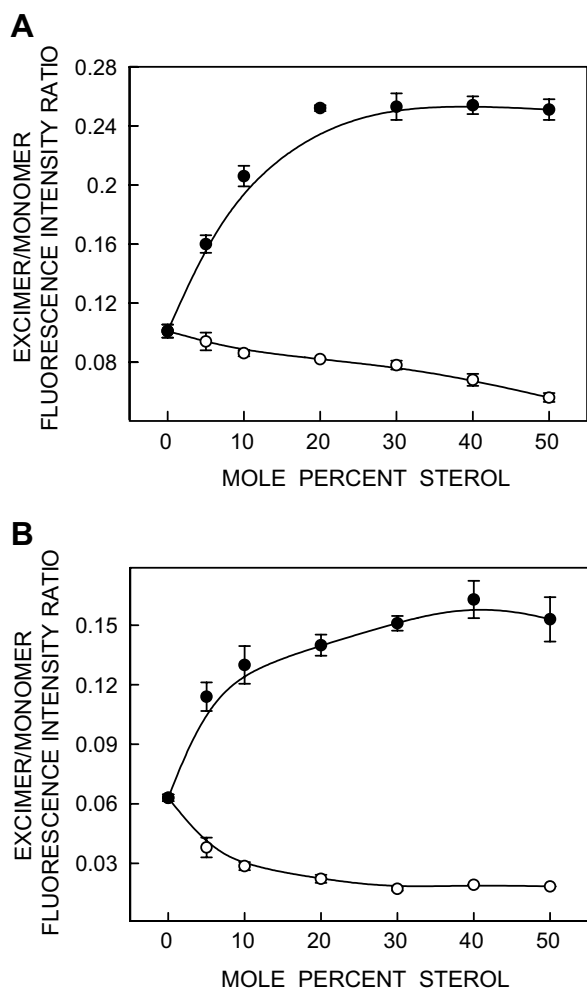


Fig. 4. Pyrene excimer (480 nm)/monomer (393 nm) fluorescence intensity ratio in (A) POPC and (B) DPPC membranes as a function of increasing concentrations of cholesterol (○) and ergosterol (●). The data points shown are means \pm SE of at least three independent measurements. All other conditions are as in Fig. 2.

an increase in case of ergosterol and decrease in case of cholesterol, although the ratio stabilizes beyond 30 mol% cholesterol. Assuming excimer/monomer ratio to be indicative of free volume in the membrane bilayer [18], ergosterol appears to increase the free volume up to 20 mol% in fluid POPC bilayers. In contrast to this, cholesterol appears to slightly reduce the free volume. The interpretation of the change in excimer/monomer ratio in gel phase DPPC membranes (Fig. 4B) is difficult since diffusion in the gel phase is known to be influenced by sub-microscopic linear defects formed at the interstices of relatively homogeneous gel phase regions of the membrane [26,27]. The regions of defects are characterized by greater disorder and enhanced diffusion rates.

Discussion

While cholesterol represents the major sterol species ubiquitously present in mammalian cells, ergosterol, is

found in lower eukaryotes such as fungi and protozoan parasites such as *Leishmania* and *Trypanosoma*. It is methylated at its side chain and it has been postulated that this specialized structural feature has been chosen by yeast during sterol evolution for enhancing membrane disorder as an alternative to synthesis of unsaturated fatty acids [6]. Although insects such as *Drosophila* cannot synthesize sterols and require a dietary source [28], the predominant membrane sterol in *Drosophila* is ergosterol [9]. Ergosterol has been recently shown to be crucial for targeting of plasma membrane proteins in yeast [29]. In addition, one of us has recently shown that ergosterol mutants of the pathogenic yeast *Candida albicans* show drug resistance which could be related to the organization, dynamics, and specific interaction of membrane ergosterol with sphingolipids in the plasma membrane [30]. Likewise, drug resistance in *Leishmania donovani* has been correlated to ergosterol biosynthesis and resulting changes in membrane dynamics [31]. The understanding of ergosterol organization in membranes is therefore crucial.

An interesting aspect for fluid membranes containing ergosterol is that a number of experimentally observed parameters (based on fluorescence and NMR measurements) appear to display change up to \sim 20 mol% (Figs. 1A, 3A, 4A [10,12,32]). This is an interesting observation since the ergosterol content of the plasma membrane of ergosterol-containing organisms such as *Drosophila* has previously been shown to be in this range [9]. Taken together, the results from experiments using pyrene as a probe provide novel information on the possible difference in the organization and dynamics of cholesterol and ergosterol in fluid and gel phase membranes. Interestingly, pyrene has recently been shown to be localized in the interfacial region of the membrane [33], the same region where the DPH moiety of TMA-DPH is localized [15]. Our results bring out important differences on the effect of sterols in short-range ordering (reported by fluorescence polarization of probes such as DPH and TMA-DPH) and long-range dynamics (from pyrene excimer/monomer measurements). In addition, the ratio of vibronic peak intensities provide information on polarity of the microenvironment, possibly arising due to variations in the extent of water penetration. In summary, our results using different fluorescent reporter probes show that the effect of ergosterol on membrane organization and dynamics is markedly different than that of cholesterol. These results are relevant in the context of membrane domains in ergosterol-containing organisms such as *Drosophila* which maintain a low level of sterol compared to higher eukaryotes.

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