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ORIGINAL PAPER

Examining Protein-Lipid Interactions in Model Systems with a New Squarylium Fluorescent Dye

Valeriya M. Ioffe · Galyna P. Gorbenko · Anatoliy L. Tatarets · Leonid D. Patsenker · Ewald A. Terpechnig

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Abstract The applicability of newly synthesized squarylium dye Sq to probing the changes in physical characteristics of lipid bilayer on the formation of protein-lipid complexes has been evaluated. Lipid vesicles composed of zwitterionic phospholipid phosphatidylcholine (PC) and its mixtures with positively charged detergent cetyltrimethylammonium bromide (CTAB), anionic phospholipid cardiolipin (CL), and cholesterol (Chol) were employed as lipid component of model membrane systems while protein constituent was represented by lysozyme (Lz). Fluorescence intensity of Sq was found to decrease on Lz association with lipid bilayer. This effect was observed in all kinds of model systems suggesting that Sq is sensitive to modification of lipid bilayer physical properties on hydrophobic protein-lipid interactions. It was found that Sq spectral response to variations in Chol content depends on relative contributions of electrostatic and hydrophobic components of Lz-membrane binding.

V. M. Ioffe · G. P. Gorbenko
Department of Biological and Medical Physics, V.N. Karazin Kharkov National University,
4 Svobody Sq., Kharkov 61077, Ukraine

A. L. Tatarets · L. D. Patsenker
STC "Institute for Single Crystals" of the National Academy of Sciences of Ukraine,
60 Lenin Ave., Kharkov 61001, Ukraine

E. A. Terpechnig
SETA BioMedicals,
LLC, 22555 Nadine Circle 239, Torrance, California 90505

V. M. Ioffe (✉)
32-90 Geroyev Truda St., Kharkov 61146, Ukraine
e-mail: vioffe@yandex.ru

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Introduction

One of the most intriguing features of biological membranes involves a complex relationship between physical state and function of these highly organized multi-component systems [1, 2]. A diversity of membrane processes is crucially dependent on physical properties of lipid bilayer, such as surface charge, dipole potential, fluidity, degree of hydration, phase transition behavior, etc. Accumulating evidence indicates that these properties can be modulated by a wide variety of factors, particularly, by protein-lipid interactions [3–7]. To address this problem a lot of powerful physical techniques have been employed, with fluorescent probe approach being one of the most informative [8]. Despite rapidly growing number of newly synthesized fluorophores, their potential in gaining information on membrane physical state still remains rarely evaluated. Specifically, this concerns long-wavelength squarylium probes belonging to the class of cyanines. Our previous study revealed one prospective squarylium probe (below referred to as Sq) distinguished by high photostability, low quantum yield in buffer solutions and its significant increase when bound to the model membranes [9]. This allowed us to quantify probe partitioning into the lipid bilayers composed of egg yolk phosphatidylcholine (PC) and its mixtures with anionic phospholipid cardiolipin (CL), cholesterol (Chol) and positively charged detergent cetyltrimethylammonium bromide (CTAB). In the present study these lipid systems have been employed to evaluate the applicability of Sq (Fig. 1) to monitoring the change in physical properties of lipid bilayer on protein binding. Lysozyme (Lz), a small globular protein (MW 14 600 Da), known for its

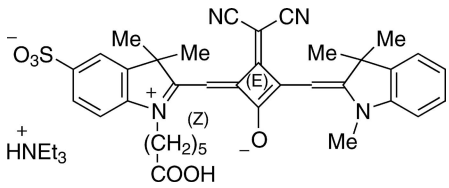

Fig. 1 Structure of squarylium dye

antimicrobial, antitumor and immune activities, has been chosen as a component of the model protein-lipid systems. Lysozyme disrupts bacteria by hydrolyzing the mucopolysaccharide portion of the cell wall [10, 11]. A number of recent studies provide strong support to the idea that not only catalytic but also lipid-associating abilities of Lz may play essential role in its bactericidal action [12]. Due to its cationic (pI 10.8) and hydrophobic properties Lz is capable of forming both electrostatic and hydrophobic contacts with lipids [13–18]. Despite extensive research efforts the mechanisms of Lz effect on the membrane structure and physical state remain poorly understood. Gaining further insight into this problem is of great interest not only for identifying the determinants of its bactericidal action but also for elucidating general principles of protein-lipid interactions.

Materials and methods

Materials

Egg yolk phosphatidylcholine and beef heart cardiolipin were purchased from Biolek (Kharkov, Ukraine). Both phospholipids gave single spots by thin layer chromatography in the solvent system chloroform:methanol:acetic acid:water, 25:15:4:2, v/v. Chicken egg white lysozyme, CTAB and Chol were from Sigma (St. Louis, MO, USA). Sq was synthesized as described previously [19, 20].

Preparation of lipid vesicles

Unilamellar lipid vesicles composed of pure PC and PC mixtures with (a) 10, 20 or 40 mol% CL; (b) 5 or 10 mol% CTAB; (c) 10, 30 or 60 mol% Chol; (d) 10 mol% CL and 10, 30 or 60 mol% Chol were prepared by the extrusion method [21]. The thin lipid films were obtained by evaporation of lipids' ethanol solutions and then hydrated with 1.2 ml of 5 mM Na-phosphate buffer (pH 7.4). Thereafter lipid suspensions were extruded through a 100 nm pore size polycarbonate filter. The phospholipid concentration was determined according to the procedure of Bartlett [22]. The dye-liposome mixtures were prepared by adding the proper amounts of the probe stock solutions in buffer to liposome suspension. The probe and protein concentrations were determined spectrophotometrically, using extinction coefficients $\varepsilon_{662} = 2.1 \times 10^5\ M^{-1}\ cm^{-1}$ [20] and $\varepsilon_{280} = 3.8 \times 10^4\ M^{-1}\ cm^{-1}$, respectively.

Fluorescence measurements

Fluorescence measurements were performed at 20 °C with CM 2203 spectrofluorimeter equipped with magnetically stirred, thermostated cuvette holder (SOLAR, Belarus). Excitation wavelength was 630 nm. Excitation and emission slit widths were set at 5 nm.

Computer-assisted structural analysis

Three-dimensional crystal structure of hen egg white lysozyme was generated by WebLab ViewerPro Trial37 software using the Protein Data Bank file [23; PDB entry 1HEW].

Results and discussion

The Sq partitioning into the lipid phase was followed by more than 10-fold fluorescence intensity increase, with emission maximum being shifted from 674 nm in buffer to about 710 nm in the liposome medium [9]. Fig. 2 shows typical fluorescence spectra of lipid-bound probe measured in the absence of Lz and at varying protein concentration from 0.3 to 1.9 μM. The binding of Lz to the model membranes resulted in a marked decrease of Sq fluorescence intensity, the magnitude of this effect being varied depending on liposome composition (Fig. 3). A question arises whether the observed fluorescence decrease may be caused by the probe exit from the vesicles and transfer to the protein. To answer this question, it seemed reasonable to quantitatively describe the dye partitioning into the lipid system. On the addition of Lz the dye redistribution between liposomal, aqueous and protein phases may occur. Denoting the probe concentrations free in bulk aqueous solution, incorporated into the model membranes and bound to Lz as Z_f, Z_L and Z_P, respectively, the coefficients of dye partitioning in the lipid (K_{PL}) and protein (K_{PP}) phases can be written as:

$$K_{PL} = \frac{Z_L V_w}{Z_f V_L}$$

$$K_{PP} = \frac{Z_P V_w}{Z_f V_P} \quad (1)$$

here V_w, V_L and V_P are the volumes of the aqueous, lipid and protein phases, respectively.

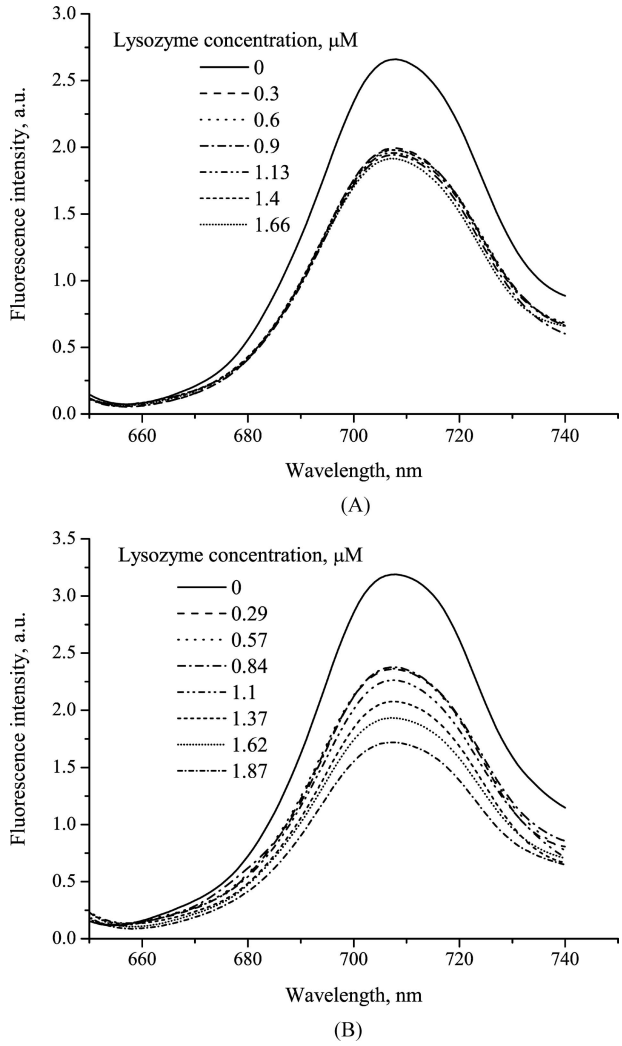

Fig. 2 Fluorescence spectra of Sq in PC (A) and PC:CL (9:1) (B) model membranes measured at varying lysozyme concentration. The lipid concentrations were 0.5 mM for PC and 0.73 mM for PC:CL vesicles. Probe concentration was 0.08 μM

The fraction of the lipid-bound dye is given by:

$$X = \frac{Z_L}{Z_L + Z_P} \tag{2}$$

or by combining Eqs. (1) and (2) one obtains

$$X = \frac{K_{PL}}{K_{PL}V_L + K_{PP}V_P} \tag{3}$$

The volume of lipid phase may be calculated as follows:

$$V_L = N_A C_L \sum v_i f_i \tag{4}$$

where C_L is the molar lipid concentration, f_i is mole fraction of the ith bilayer constituent, v_i is its molecular volume taken as 1.58, 3, 0.74 and 1.1 nm^3 for PC, CL, Chol and CTAB,

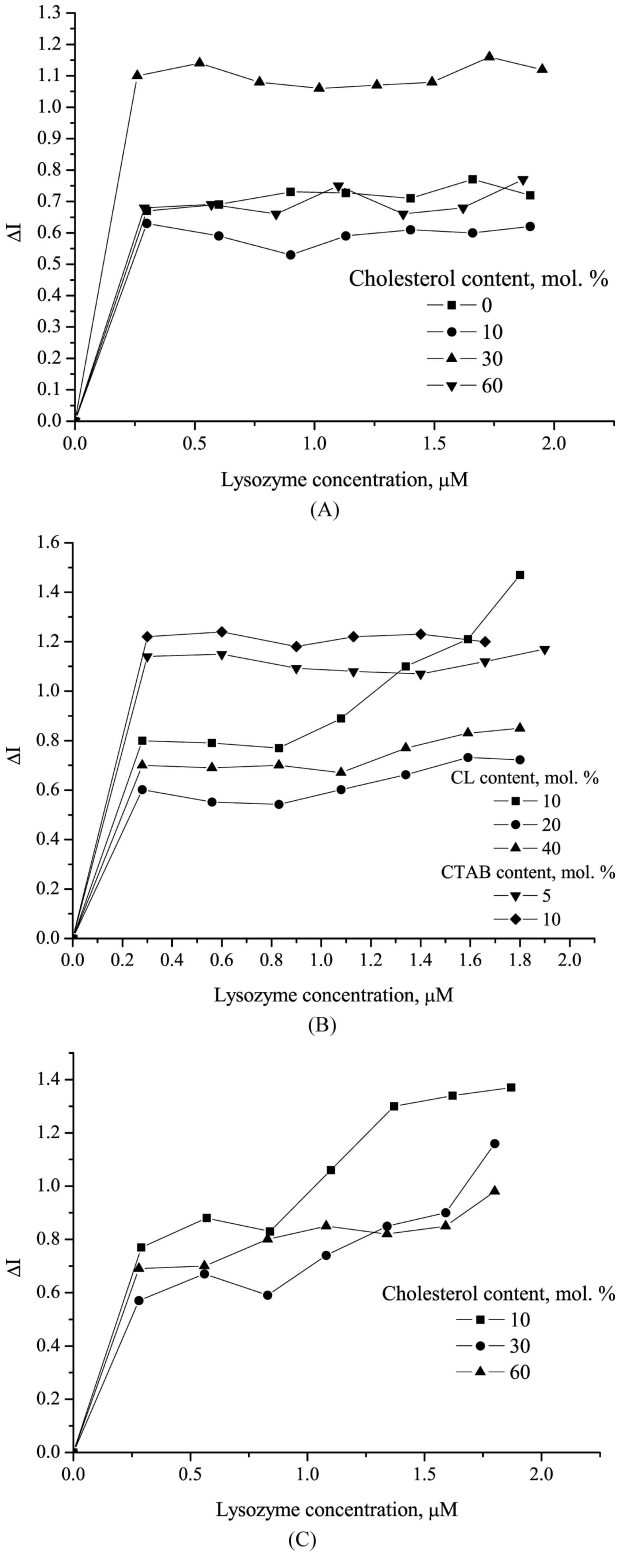

Fig. 3 Changes in Sq fluorescence intensity upon lysozyme binding to PC:Chol (A), PC:CTAB and PC:CL (B), and PC:CL:Chol (C) model membranes. ΔI stands for the difference between Sq fluorescence measured in the absence of protein ($I^{LP}_{meas}(0)$) and at certain protein concentration C_P ($I^{LP}_{meas}(C_P)$), $\Delta I = I^{LP}_{meas}(0) - I^{LP}_{meas}(C_p)$

respectively [24]. Since cholesterol exerts a condensing effect on the lipid bilayer, in PC:Chol and PC:CL:Chol model membranes v values were reduced by the factors 1.3 and 1.2 for PC and CL, respectively [24].

The volume of the protein phase can be calculated as:

$$V_P = N_A C_P \nu_P \tag{5}$$

where C_P stands for the protein molar concentration, and ν_P is the volume of fully hydrated Lz molecule taken as 2.46 nm^3 [25].

Given that under the employed experimental conditions V_L and V_P are much less than the total volume of the system, $V_W \approx V_t = 1\,dm^3$.

By examining the fluorescence intensity change (ΔI_m) on the probe association with lipids or protein as a function of lipid or protein concentration the values of K_{PL} and K_{PP} can be derived from the following relationship:

$$\Delta I_m = I - I_W = \frac{K_P V (I_{max} - I_W)}{1 + K_P V} \tag{6}$$

here I is the fluorescence intensity measured in the lipidic or protein environment, I_W is the probe fluorescence in buffer, I_{max} is the limit fluorescence in liposome or protein phase, V denotes the volume of lipid (V_L) or protein (V_P) phase, K_P corresponds to the probe partition coefficient either into the vesicles (K_{PL}) or Lz (K_{PP}). Based on the results of the dye fluorometric titration with liposomes or Lz (data are not shown), we have determined the partition coefficients. The value of K_{PL} are presented in our previous paper [9], while K_{PP} was found to be 3.62×10^3.

Given that fluorescence of the bound Sq is much higher than that of the free probe, the measured fluorescence intensity can be written as:

$$I_{meas} = a_L Z_L + a_P Z_P \tag{7}$$

where a_L and a_P are the molar fluorescence of probe bound to the liposomes or protein, respectively. It can be easily shown that:

$$X = \frac{I_{meas} - I^P}{I^L - I^P} \tag{8}$$

where $I^L = a_L Z_{tot}$ and $I^P = a_P Z_{tot}$, Z_{tot} is total concentration of the probe. The relationship (8) was used to recover $X(C_P)$ dependencies from the experimental data, with the values of a_L and a_P being derived from the results of fluorimetric titration. These dependencies were compared to $X(C_P)$ curves calculated from Eq. (3). As seen from Fig. 4, there exists noticeable discrepancy between theoretical and experimental curves. This implies that the probe redistribution between the lipid and protein phases cannot be considered as the only reason for the protein-induced fluorescence decrease.

In the absence of Lz the fluorescence intensity (I^L_{meas}) can be represented as a function of a_L and K_{PL}:

$$I^L_{meas} = \frac{Z_{tot} a_L K_{PL} V_L}{1 + K_{PL} V_L} \tag{9}$$

Addition of the protein replenishes the value of the detected fluorescence with two terms:

$$I^{LP}_{meas} = \frac{Z_{tot}(a^*_L K^*_{PL} V_L + a^*_P K^*_{PP} V_P)}{1 + K^*_{PL} V_L + K^*_{PP} V_P} \tag{10}$$

where asterisk allows for the possibility that the partition coefficients and molar fluorescence in the lipidic and protein environment are influenced by protein-lipid interactions. In terms of the modern theories of membrane electrostatics partition coefficient can be represented as consisting of electrostatic and nonelectrostatic terms [26 , 27]:

$$K_{PL} = K^{el}_{PL} \cdot$$
$$K^{nel}_{PL} = \exp\left(\frac{\{ze\psi_{el} + w_{Born} + w_h + w_n + w_d\}}{kT}\right) \tag{11}$$

where ze is the dye charge; ψ_{el} is the membrane Coulomb potential; w_{Born} is the free energy of charge transfer between the media having different dielectric constants; w_n is the neutral energy term determined by hydrophobic, van der Waals and steric factors; w_h is the contribution controlled by the degree of membrane hydration; w_d is the term dependent on the membrane dipole potential (ψ_D), k is Boltzmann constant, T is absolute temperature.

Because of the strong cross-correlation between the parameters contributing to I^{LP}_{meas} value and complexity of the process of Lz-membrane binding it is hardly possible to establish an unambiguous relation between the protein-induced fluorescence decrease and the changes in the probe partition coefficients and molar fluorescence. However, based on available information on the molecular details of protein-lipid interactions we succeeded in recovering the factors which may influence the spectral characteristics of the examined squarylium probe under the employed experimental conditions (Fig. 5).

Several lines of evidence indicate that Lz is capable of interacting with liposomes both electrostatically and hydrophobically [13–18]. Lipid chemical nature, charge of polar headgroups, structural and dynamical features of acyl chains control the interplay between electrostatic and hydrophobic components of lysozyme membrane binding. In this regard it is tempting to relate the physicochemical properties of the employed modulators of the bilayer physical

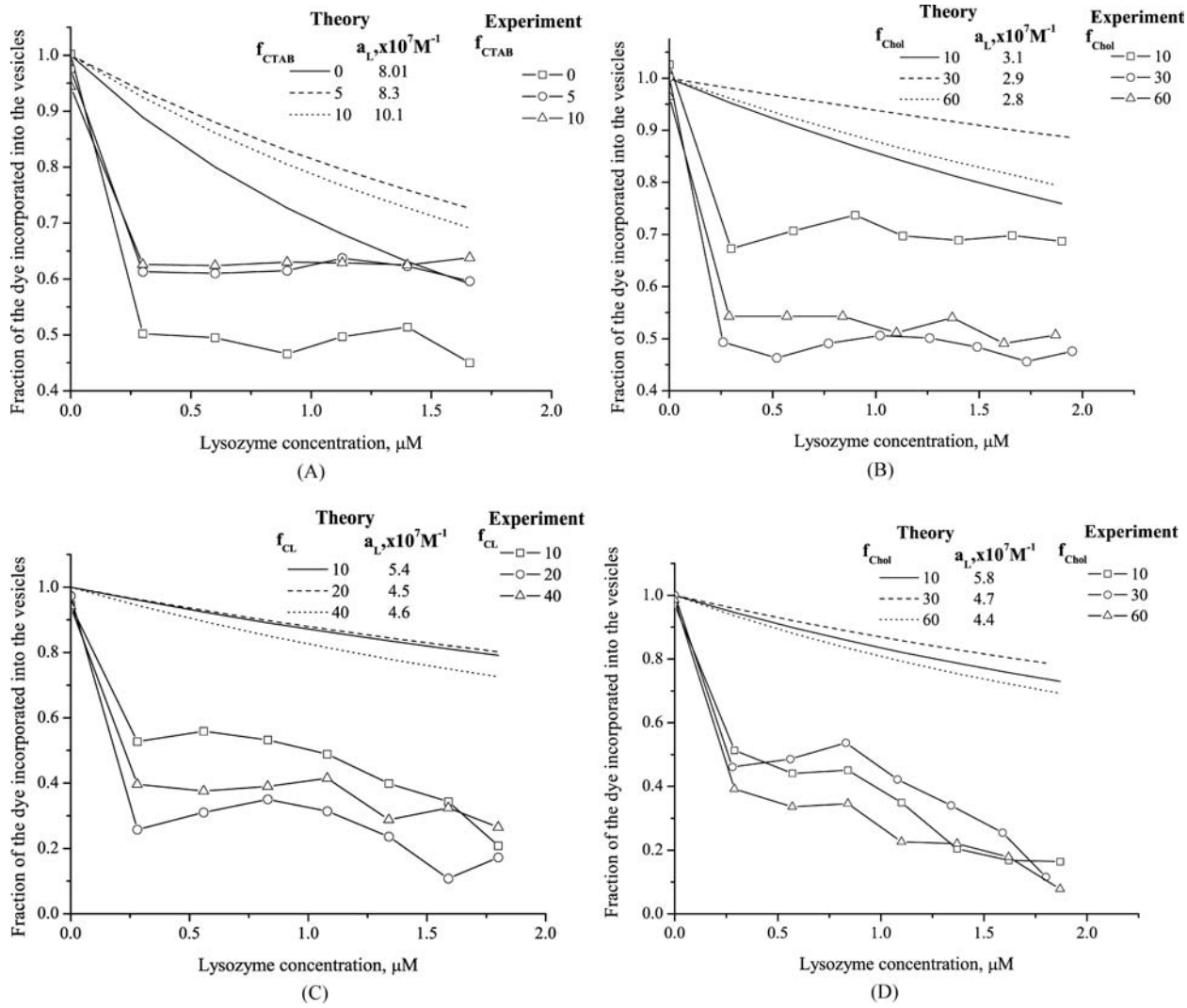

Fig. 4 Distribution of Sq between the lipidic and protein phases: PC:CTAB (A), PC:Chol (B), PC:CL (C) and PC:CL:Chol (D) model membranes. f corresponds to the mole percent of the additives. Dye molar fluorescence in the protein phase a_P was taken as 2.6×10^7 M^{-1}

state (CTAB, CL and Chol) with the mode of protein-lipid interactions.

Firstly, following the scheme presented in Fig. 5, it seems reasonable to analyze the consequences of lysozyme-lipid interactions which are common for all types of membranes, regardless of the differences in charge and chemical nature of their constituents. Since the ground state dipole moment of Sq is rather high (*ca*. 6.5 D), it seems energetically unfavorable for the probe to penetrate into hydrophobic bilayer region. This dye is most probably located at the lipid-water interface being oriented parallel to the membrane surface.

Lysozyme membrane binding was reported to cause displacement of the solvent followed by dehydration of bilayer surface [28, 29]. Removal of water enhances the interactions between lipid molecules and increases ordering of lipid headgroups [30]. Restrictions imposed by increased packing density of lipid headgroups inhibit the probe photoisomerization [31, 32] and hinder its rotation, the rate of which depends on free volume of the probe microenvironment [33]. Likewise, bilayer dehydration can alter configuration of H-bond networks between phospholipid headgroups [30], thus affecting the dye spectral properties by modifying its hydrogen bonding to the solvent cage. It is naturally to expect that these processes would lead to increase in Sq fluorescence intensity (resulted from a_L increase) [8, 34]. However, since the examined squarylium dye is water-soluble, lysozyme-induced membrane dehydration may result in K_{PL} decrease, thus preventing the probe from partitioning into lipid phase. If K_{PL} contribution to I^{LP}_{meas} dominates over that of a_L, fluorescence decrease will be observed.

Another consequence of lysozyme-lipid interactions which may account for K^{nel}_{PL} decrease involves the changes in membrane surface pressure (π). This parameter could be

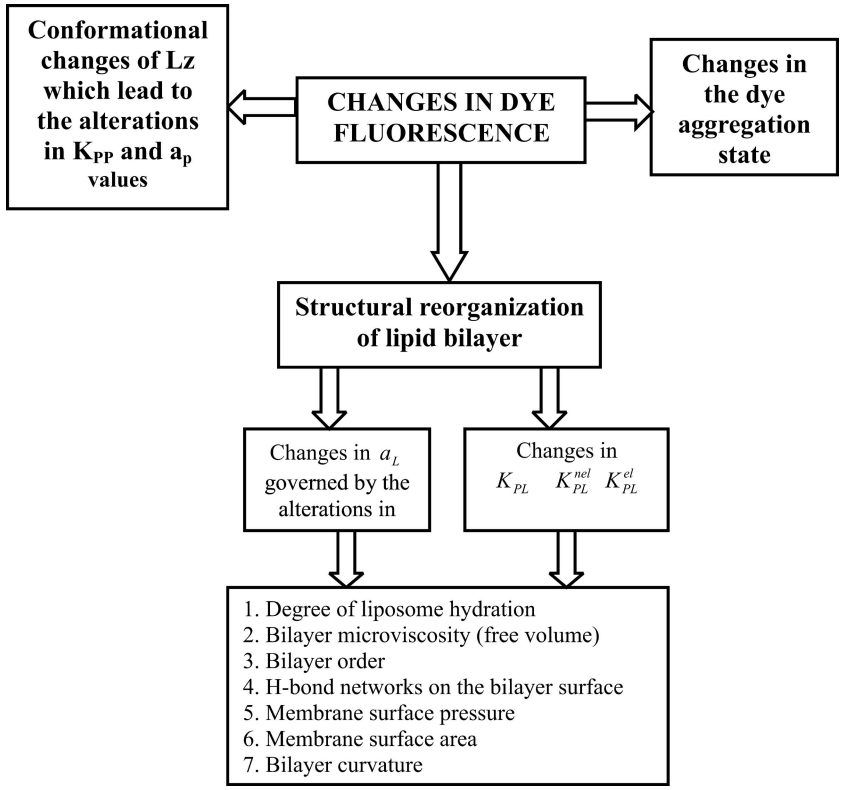

Fig. 5 Schematic illustration of possible contributions to spectral response of squarylium dye

of significance in controlling the dye partitioning into lipid phase. Monolayer experiments revealed lipid surface pressure to increase on Lz addition [35 , 36]. It has been shown that π of liposomes, thereby bringing about the reduction of bilayer free volume [37]. This phenomenon may also contribute to decrease of Sq membrane partitioning.

One more probable reason for the observed changes in Sq fluorescence in lysozyme-lipid systems relates to the change in bilayer local curvature. Zuckermann and Heimburg suggested a general mechanism for adsorption and insertion of soluble proteins onto membranes. They postulated that adsorbed proteins form a two-dimensional gas on the bilayer surface which generates a lateral pressure on the membrane [38] thus locally changing its curvature. Such membrane "squeezing" near the area of protein-lipid contact may perturb bilayer order, affecting in such a way dye partitioning into lipid phase.

It should also be noted that changes in membrane surface pressure and local curvature can cause reorientation of water dipoles coupled with rearrangement of the hydrogen bond network. This in turn can alter the dye bonding to the solvent and affect configurational movement of the network, i.e. relaxation of the network configuration with time [39]. Clearly, this process may exert influence on the quantum yield of membrane-bound probe.

Last but not least possible mechanism behind the observed decrease of Sq fluorescence which does not depend on membrane type is the formation of dye aggregates. Self-association of bound probe can be promoted by increase of its local concentration in interfacial region.

One should bear in mind that all the above considerations concerning the possible reasons for changes in $K_{\mathrm{PL}}^{\mathrm{nel}}$ and a_{L} are valid for all types of liposomes. However, it is evident that differences in chemical nature and physical properties of membrane constituents may provide specific mechanisms for the observed effects, particularly those involving electrostatic phenomena and protein conformational changes (Fig. 5).

As illustrated in Fig. 3 the changes in Sq fluorescence induced by Lz in various types of liposomes are similar in sign, but somewhat differ in magnitude and character of fluorescence decrease (ΔI) dependency on the protein concentration.

The fact that Sq fluorescence decreases on Lz addition to uncharged liposomes (PC and PC:Chol), as well as to positively and negatively charged lipid vesicles containing CTAB or CL, respectively, strongly suggests that the probe responds to the alterations in membrane physical state resulting from nonpolar interactions between lipid acyl chains and protein nonpolar patches. These patches may be exposed on partial unfolding on the protein molecule at lipid-water interface [40, 41]. Lz is rather rigid protein whose structure is stabilized by four disulfide bonds [42]. No convincing evidence for Lz unfolding on membrane binding has been obtained yet.

Computer-assisted analysis of Lz crystal structure revealed the existence of hydrophobic patch on the protein surface. One can assume that this patch including the residues Ile58, Leu75, Cys76, Ile78, Ala82, Leu83, Ala90 and Ile98, is involved into the hydrophobic interactions of Lz molecule with side chains of phospholipids. This patch is located close to the protein active site which is supposed to contain amino acid residues associating with lipid headgroups [11, 13]. Notably, Lz-lipid binding via hydrophobic patch is likely to occur in all lipid systems examined here. The assumption that Sq is sensitive to the hydrophobic protein-lipid interactions is also corroborated by the observation that ΔI exhibits more pronounced dependence on Chol concentration compared to those on CL or CTAB (Fig. 3). As seen in Fig. 3, A, at Chol content around 30 mol% which is reported to be critical for membrane transition from liquid-disordered to liquid-ordered phase [43, 44] ΔI experiences dramatic increase. However, this effect was not observed when 10 mol% CL was incorporated into PC:Chol membranes (Fig. 3 C) to enhance electrostatic component of Lz-lipid binding. These findings suggest that Sq sensitivity to Chol effects depends on relative contributions of electrostatic and hydrophobic components of protein-lipid interactions.

To summarize, the present study was undertaken to estimate sensitivity of squarylium probe to the different modes of protein-lipid interactions. The main conclusion reached in the present study is that the dye in question is largely sensitive to modification of the membrane structure and physical state caused by hydrophobic protein-lipid interactions. However, electrostatic component of protein-membrane binding can be essential in modulating spectral responses of Sq probe, as was demonstrated for PC:CL:Chol membranes. Intriguing property of Sq to respond to Chol ordering effect opens new attractive field of inquiry.

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