BOUNDARY LIPID IN PROTEOLIPID–LIPID RECOMBINANTS REVEALED BY FLUORESCENCE ENERGY TRANSFER AND SPIN PROBE STUDY

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1. Introduction

Very much attention has been paid to the investigation of the protein–lipid interactions in biological membranes as a way of understanding the lipid neighbourhood influence upon the membrane enzymes and (understanding) of the mechanisms of membrane structures stabilization [1,2]. There are publications on a number of membrane proteins [3–8] and intact membranes [7,9–11] in which the existence of the lipid layer(s) surrounding protein molecules with the decreased fatty acid chain mobility have been proposed. It is believed that the exchange time of these lipids with free lipid phase is 10^{-8} s $\leqslant \tau_{exchange} < 10^{-5}$ s while it is diminished as compared with that in pure lipid ($\sim 10^{-7}$ s) [10]. This dynamic formation of the lipid molecules with the infinite lifetime was called 'boundary lipid'.

However, there exists a point of view [12,13] that the above-mentioned data could be satisfactorily explained as a result of the lipid entrapment in the protein network of natural or reconstituted membrane followed by a decrease of the mobility of the lipid molecules [14–17]. According to this opinion the boundary lipid content (mol/mol protein) must decrease with decreasing of the protein concentration in membrane [17], while according to 'boundary lipid' conception it should be constant. However, the available methods do not allow the estimation of the boundary lipid content in systems with low protein/lipid ratio. Thus, the main reason for discussion being the rather high protein–lipid ratio and integral physical methods (ESR, NMR, DSC, Raman spectroscopy) used to detect the structural non-homogeneity of lipids.

Here we have used the 'local' approach that permitted us to estimate the degree of lipid immobilization in the vicinity of the integral protein(s) in the membrane. Energy transfer from the hydrophobic proteins (extracted from *Micrococcus lysodeikticus* membranes and reconstructed with their lipids) onto fluorescent probe pyrene was found to allow direct comparison of the excimer-forming probe mobility in the total bilayer and the area adjacent to protein molecules ($r \leqslant R_0 = 27$ Å; R_0, characteristic energy transfer distance (Förster's radius)) irrespective of the protein concentration in the system. Spin exchange in the system containing paramagnetic 16-NS probe (16-nitroxyl stearate (2-(14-carboxytetra-decyl-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl)) was also utilized to characterize boundary layer(s). These data indicate that there is the decrease of lipid mobility around the hydrophobic protein(s) in proteolipid recombinants. The lipid mobility gradient becomes higher on decreasing protein/lipid ratio which implies the conception of the 'boundary lipid' with up to 3.3 mg lipid/mg protein.

2. Experimental

Cytoplasmic membranes were isolated from *M. lysodeikticus* cells in [18]. Hydrophobic proteins (PL, proteolipid) and lipids were extracted from wet membranes with chlorophorm–methanol (2:1. v/v), 200 ml/1 g dry mass; and PL was collected as an interphase on the addition of 0.034% $MgCl_2$ (pH 7.4) to the equal volume of the centrifuged extract. Both PL and lipids were kept at –20°C under N_2 before use.

Samples for fluorescence spectroscopy were prepared as follows stock solutions of PL were mixed with appropriate amounts of lipids and ethanol solution of pyrene (Sigma). The solvent was removed by

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nitrogen gas, followed by desiccating under vacuum for 2 h. The samples were hydrated with 40 mM Tris–HCl buffer (pH 7.4) with 1 mM $MgSO_4$ and sonicated at 0°C for 3 min. Spin probe 16-NS(SYVA) was incorporated into liposomes or proteoliposomes by the addition of ethanol solution to the buffered suspension of the structures (20 mM phosphate, 1 mM $MgSO_4$, pH 7.0).

Protein content of the vesicles was determined as in [19].

3. Results

3.1. *Estimation of lateral mobility gradient in proteolipid–lipid recombinants with fluorescent probe pyrene: Effect of gramicidin S*

The position of λ^{max} = 330 nm in the fluorescence spectrum of PL indicates that tryptophanyls are responsible for the main portion of the emission [20]. In the presence of pyrene the energy of excited protein chromophores is transfered onto the pyrene localized in the vicinity of the PL molecules ($r \leqslant R_0 = 27$ Å [21]). As calculated from the spectra (fig.1a) 87% of the energy emitted by pyrene originate from the tryptophanyls excited at λ_{ex} = 282.5 nm. It is now well established that excited pyrene molecules are associated with unexcited ones to form excimers in a diffusion-dependent manner [22] excimers being responsible for the structureless broad band in the fluorescence spectrum centered at 470 nm (fig.1b). The lateral mobility of the pyrene molecules can be assessed [22] by the excimer (λ^{max} = 470 nm) to monomer (λ^{max} = 391 nm) fluorescence intensity ratio (α). It should be emphasized that reduced excimer formation at λ_{ex} = 286 nm as compared to excimer formation at λ_{ex} = 338 nm, where all of the probe molecules may be excited (fig.1b), is due to lower pyrene lateral mobility in the vicinity of the PL structure assuming of the even probe distribution in the membrane.

The typical results on the alteration of the excimerization of the whole population of the probe molecules and the ratio of excimerization parameters on different excitation wavelengths as a function of protein increase in the reconstituted system are presented in fig.2. There is evident linear relationship between the protein content and the parameter of the probe lateral mobility gradient $\alpha_{338}/\alpha_{286}$, as predicted by the eq. [4] (see legend to fig.2) on the assumption of the constancy of mol boundary lipid/mol protein(s) ratio. Boundary lipid content is thus 3.3 mg/mg protein.

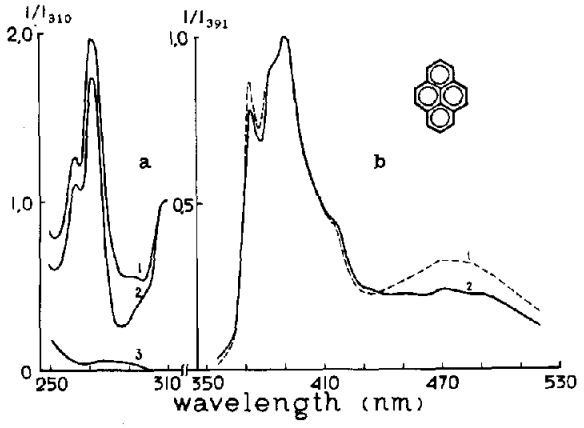

Fig.1. Corrected excitation and emission spectra of pyrene. (a) Excitation spectra of 10 μM pyrene in proteoliposomes (curve 1), liposomes (curve 2) and excitation spectrum of proteolipid tryptophanyls (curve 3) using 391 nm as emission wavelength. The final concentrations of proteoliposomes (protein/lipid = 0.21) and liposomes were 0.1 mg lipid/ml. Curves 1 and 2 are normalized to pyrene fluorescence intensity I at excitation wavelength 310 nm where light absorbance by protein chromophores does not occur. (b) Normalized emission spectra of 10 μM pyrene in proteoliposomes using 338 nm (curve 1) or 286 nm (curve 2) as respective excitation wavelengths. The final concentration of proteoliposomes (protein/lipid = 0.13) was 0.1 mg lipid/ml. Sample temperature was 30°C. All measurements were done in 0.5 cm path cuvettes in a Hitachi MPF-4 spectrofluorimeter operated in a 'Ratio' mode.

Membranotropic decapeptide gramicidin S ionically interacting with the phospholipids was shown to disturb protein–lipid interactions in *M. lysodeikticus* membranes and suggested to induce the aggregation of membrane proteins [23,24]. At moderate concentration (0.2–0.4 mg/mg lipid) gramicidin S does not induce substantial alterations of pyrene mobility and the probe lateral mobility gradient in the system employed but at the elevated concentration (0.6 mg/mg lipid) lipid non-homogeneity was drastically reduced (table 1).

3.2. *A comparative spin probe study*

Another approach to the exploration of the boundary lipid layer(s) is based on the assumption of the lowered spin probe solubility in the vicinity of the PL structure as compared with the free liquid–crystalline phase. Thus one can expect that the modification of the ESR spectrum of the hydrophobic spin probe 16-NS due to the spin exchange as a function of the

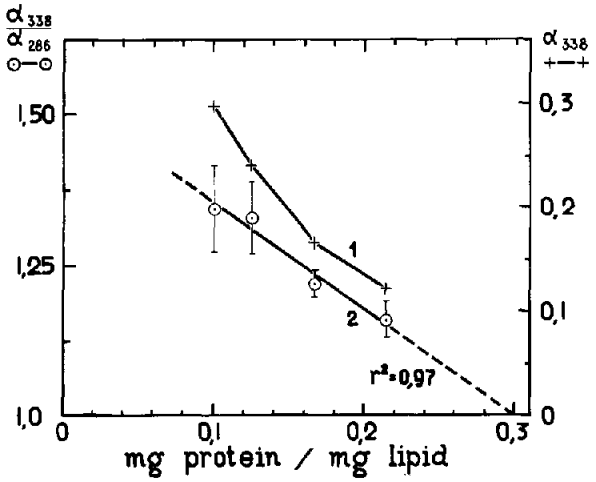

Fig.2. Dependence of pyrene excimerization parameter α_{338} (curve 1) and lateral probe mobility gradient $\alpha_{338}/\alpha_{286}$ (curve 2) on protein content in proteoliposomes: (1) typical results on excimerization of 10 μM pyrene in proteoliposomes (0.1 mg lipid/ml); (2) dependence constructed by a linear regression of data points (mean ± SD from 5 separate expt); r, correlation coefficient of these points to line. α_{338} and α_{286}, fluorescence intensity ratio I_{470}/I_{391} in emission spectra of pyrene using 338 nm or 286 nm as respective excitation wavelength:

$$\alpha_{338} = \frac{I^{470(f)} + I^{470(b)}}{I^{391(f)} + I^{391(b)}} \quad (1)$$

where $I^{\lambda(f)}$ and $I^{\lambda(b)}$ are fluorescence intensities of the free and localized in the boundary layer(s) probe molecules, respectively, at the wavelength of registration λ. Equation (1) may be re-written:

$$\alpha_{338} = \frac{\alpha^{(f)} I^{391(f)} + \alpha^{(b)} I^{391(b)}}{I^{391(f)} + I^{391(b)}} \quad (2)$$

$\alpha^{(f)}$ and $\alpha^{(b)}$ are the values of α in liposomes and in the boundary layer(s), respectively. It can be normalized: $I^{391(f)} + I^{391(b)} = 1$. If we assume that $I^{391(b)} = K\,[P]/[L]$ where K = proportionality constant between fluorescence probe intensity and protein/lipid ratio [P]/[L], then:

$$\alpha_{338} = \alpha^{(f)} - K\,[P]/[L] \times [\alpha^{(f)} - \alpha^{(b)}] \quad (3)$$

and designing $\alpha_{286} = \alpha^{(b)}$ we obtain:

$$\frac{\alpha_{338}}{\alpha_{286}} = \frac{\alpha^{(f)}}{\alpha^{(b)}} - K\frac{[P]}{[L]}\left[\frac{\alpha^{(f)}}{\alpha^{(b)}} - 1\right] \quad (4)$$

The meaning of the proportionality constant K is revealed when $\alpha_{338} = \alpha_{286}$ that is when all the lipid falls under the influence of PL, then $K = [L]_{boundary}/[P]$, and this opens the way for calculation of $[L]_{boundary}$. Note that the parameter $\alpha_{338}/\alpha_{286}$ does not depend on probe/lipid ratio.

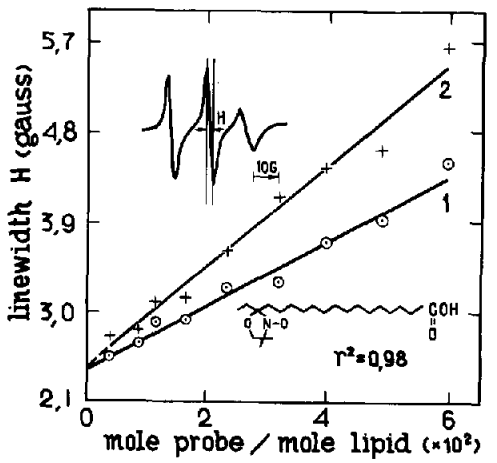

Fig.3. Dependence of middle component linewidth H in ESR spectra of spin probe 16-NS on probe concentration in liposomes (1) and proteoliposomes (2). The final concentrations of proteoliposomes (protein/lipid 0.25) and liposomes were 6.4 mg lipid/ml. The insert shows the ESR spectrum of the spin-probe (5×10^{-3} mol/mol lipid) in proteoliposomes. Sample temperature was 24°C. ESR spectra were taken using a RE-1301 (USSR) spectrometer. Equation (5) [26] was used to evaluate the phenomenon under study:

$$H = H_0 + \Delta H \quad (5)$$

where: H, total linewidth; H_0, linewidth in the case no spin exchange; ΔH, line broadening due to the exchange interactions ($K_{ex} \cdot C$) which are much larger than dipole–dipole interactions in the case of the high probe mobility [26]; K_{ex}, exchange constant; C, spin probe concentration in the membrane.

probe concentration in liposomes will differ from that in proteoliposomes and this appeared to be a fact (fig.3). Rotational correlation time of the spin probe in liposomes is identical with that in proteoliposomes ($\tau_c = 1.1 \times 10^{-9}$ s) and the noticable difference of spin–spin interactions in these two systems (at constant temperature) can be interpreted as a function of alterations of the efficient probe concentration, which in turn may be due to the probe accumulation in the liquid–crystalline area of the membrane [25]. If so, boundary lipid content can be calculated as:

$$[L]_{boundary} = [L]_{liposome}\left(1 - \frac{tg\beta_1}{tg\beta_2}\right) \quad (6)$$

where: $[L]_{boundary}$, mol boundary lipid in proteoliposomes; $[L]_{liposome}$, mol lipid in liquid–crystalline phase in liposomes; β_1 and β_2, angles formed by the

Table 1
Gramicidin S action on pyrene excimerization parameter α_{338} and lateral probe mobility gradient $\alpha_{338}/\alpha_{286}$ in reconstructed system

mg antibiotic / mg lipid	0	0.2	0.4	0.6
α_{338}	0.266 + 0.045	0.294 ± 0.034	0.266 ± 0.045	0.208 ± 0.045
$\alpha_{338}/\alpha_{286}$	1.345 ± 0.073	1.410 ± 0.107	1.419 ± 0.066	1.143 ± 0.122

Gramicidin S in desired amounts was introduced as ethanol solution before evaporation of organic solvents (see section 2). The final concentration of proteoliposomes (protein/lipid = 0.1) was 0.1 mg lipid/ml. See legend of fig.2 for details of calculation of α_{338} and $\alpha_{338}/\alpha_{286}$. The data presented are mean values ± SD from 4 separate expt. Sample temperature was 30°C

lines 1 and 2 and abscissa in fig.3. The amount of the boundary lipid calculated from eq. (6) (1.4 mg/mg protein) is half as much as that derived from pyrene fluorescence measurements and can be explained by uneven distribution of the spin probe in both free and protein-perturbed lipid phase. Alternative explanation of the increased efficiency of the spin exchange in proteoliposomes through the concentration of the probe in the boundary layer(s) can be ruled out on the ground that the effectiveness of the spin exchange was measured from a high-mobility component of the spectrum, while a low-mobility component was negligible due to low protein/lipid ratio.

4. Discussion

Reduced excimerization of the fluorescent probe in the area adjacent to the protein(s) as compared with that in free liquid phase can be explained by viscosity increase and (or) decrease of the probe concentration in the area. Experiments done with the spin probe also indicate that in a sence a foreign molecule may be expelled from the boundary layer(s) thus once more emphasizing special properties of the layer(s) in which lipid molecules are hindered in their motion (spin probe 16-NS is distributed in the membrane in favour of liquid–crystalline domains [25]). Thus, both types of experiments reveal perturbed lipids in the vicinity of the protein(s) with properties corresponded to boundary lipid.

It should be stressed that in our study unlike in [3,8–11] the form of the ESR signal was not important for the lipid gradient estimation (the evaluation of the immobilized component requires larger protein concentrations). Moreover lateral gradient as measured in the pyrene study reached its maximum at the smallest protein/lipid ratio when the possibility of protein–protein contacts and subsequent lipid entrappment is minimized. On assumption of α-helical structure of hydrophobic proteins with of M_r 9000, 14 000 and 18 000 [24], their *trans*-bilayer orientation and side-to-side interaction of lipids and proteins in proteoliposomes we can calculate that three molecular layers of the lipid experience the influence of the protein, though it may well be that lipid forms one layer with molecules parallel the membrane surface with the minimal lifetime of the boundary shell $\sim 10^{-7}$ s as determined from the excited-state lifetime of the pyrene molecules.

Structural difference of the free and boundary lipids may play its role in prevention or regulation of protein aggregation in the biological membranes [1]. Gramicidin S reduces this structural difference and inactivates and sensibilizes membrane proteins to cross-linking by bifunctional reagent which is thought to indicate their lateral rearrangement [24].

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