УДК 577.37

RESONANCE ENERGY TRANSFER STUDY OF HEMOGLOBIN BINDING TO MODEL LIPID MEMBRANES

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Submitted 31 May, 2010

Accepted 17 June , 2010

In the present study fluorescence resonance energy transfer (FRET) technique was employed to obtain the information about the structure of hemoglobin (Hb) complexes with model lipid membranes of different composition. For this purpose three membrane probes, 3-methoxybenzanthrone (MBA), 4-dimethylaminochalcone (DMC) and 6-propionyl-2-dimethylaminonaphthalene (Prodan) were assessed as possible donors for heme moiety of the protein. Model membranes were composed of zwitterionic lipid phosphatidylcholine (PC), anionic lipid cardiolipin (CL) and cholesterol (Chol). FRET measurements were interpreted in terms of the model of energy transfer in two-dimensional systems proposed by Fung and Stryer and further extended by Davenport et al. No FRET was observed between Prodan and Hb because Prodan under the employed experimental conditions was not distributed into the lipid bilayer. In the case of DMC, Hb-induced oxidative processes in the lipid phase hampered the estimation of Hb location in a lipid bilayer. Therefore, structural analysis of Hb-lipid complexes was carried out using MBA as a donor. First, the donor quantum yield, Förster radii and fluorescence anisotropy of the probes have been measured. Second, the amount of Hb bound to model membranes was estimated in terms of the lattice models of large ligand adsorption to lipid bilayers allowing for the possibility of protein insertion into membrane interior. Finally, the distance from acceptor plane to the bilayer center and the depth of Hb penetration into lipid bilayer were calculated. It was assumed that protein binds to membranes in the form of dimers and penetrates into the membrane interior. In neutral liposomes Hb penetrates only to the depth of lipid headgroups. The observed higher extent of Hb penetration into Chol containing bilayer as compared to PC liposomes may be a consequence of specific Hb-Chol interaction. In the case of PC/CL liposomes Hb was found to insert in the non-polar membrane region. Taking into account the possibility of forming the inverted hexagonal structures in the presence of CL, it cannot be excluded that Hb being entrapped in such structures, translocates through the membrane. If this phenomenon takes place, deeper Hb penetration into lipid bilayer might be expected. The obtained results can be useful for exact characterization of Hb binding to the membranes.

KEY WORDS: hemoglobin, model membranes, protein-lipid complexes, fluorescence energy transfer.

ИССЛЕДОВАНИЕ СВЯЗЫВАНИЯ ГЕМОГЛОБИНА С МОДЕЛЬНЫМИ ЛИПИДНЫМИ МЕМБРАНАМИ МЕТОДОМ РЕЗОНАНСНОГО ПЕРЕНОСА ЭНЕРГИИ

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В данной работе метод индуктивно резонансного переноса энергии (ИРПЭ) был применен для исследования структуры комплексов гемоглобина (Hb) с модельными липидными мембранами различного состава. Для достижения цели в качестве доноров применялись три мембранных зонда 3-метоксибензантрон (МБА), 4-диметиламинохалкон (ДМХ) и 6-пропионил-2-диметиламинонафтален (Продан), а акцептором выступала гемовая группа Hb. Модельные мембраны состояли из цвиттерионного липида фосфатидилхолина (ФХ), анионного липида кардиолипина (КЛ) и холестерина (Хол). Данные ИРПЭ интерпретировались в рамках модели переноса энергии в двумерных системах, предложенной Фангом и Страером и позднее расширенной Дэвенпорт и др. Между Проданом и Hb не наблюдалось переноса энергии вследствие того, что при используемых экспериментальных условиях зонд практически не распределялся в липидную фазу. В случае ДМХ окислительные процессы, инициированные Hb, затруднили определение положения гемоглобина на липидном бислое. Таким образом, анализ структуры Hb-липидных комплексов проводился с применением МБА в качестве донора. Вначале были измерены квантовый выход, анизотропия флуоресценции доноров и радиусы Ферстера. Затем проводилась оценка связанного с мембранами Hb в рамках решеточных моделей адсорбции больших лигандов, учитывающих проникновение белка в бислой. Затем рассчитывали расстояние плоскости акцепторов до центра бислоя и глубину проникновения белка в мембрану. Высказано предположение, что Hb связывается с мембранами в виде димеров и проникает во внутреннюю область бислоя. В нейтральных липосомах Hb проникает только на глубину липидных головок. Возможно, большая глубина проникновения белка в бислой, содержащий Хол, по сравнению с ФХ мембранами является

следствием специфического взаимодействия Hb с Хол. В случае липосом, содержащих КЛ, белок проникает в неполярную область мембраны. Кроме того, если принять во внимание возможность формирования инвертированных гексагональных структур в присутствии КЛ, нельзя исключить тот факт, что при попадании Hb в такие структуры он может проникать через мембрану. В таком случае можно допустить более глубокое проникновение белка в бислой. Полученные данные могут быть полезными для детальной характеризации связывания Hb с мембранами.
КЛЮЧЕВЫЕ СЛОВА: гемоглобин, модельные мембраны, белок-липидные комплексы, резонансный перенос энергии.

ДОСЛІДЖЕННЯ ЗВ'ЯЗУВАННЯ ГЕМОГЛОБІНУ З МОДЕЛЬНИМИ ЛІПІДНИМИ МЕМБРАНАМИ МЕТОДОМ РЕЗОНАНСНОГО ПЕРЕНОСУ ЕНЕРГІЇ

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В данній роботі метод індуктивно резонансного переносу енергії (ІРПЕ) був використаний для дослідження структури комплексів гемоглобіну (Hb) з модельними ліпідними мембранами різного складу. У якості донорів застосовувались три мембранні зонди 3-метоксибензантрон (МБА), 4-діметиламінохалкон (ДМХ) та 6-пропіоніл-2-діметиламінонафтален (Продан), а акцептором виступала гемова група Hb. Модельні мембрани складалися з цвіттеріонного ліпіду фосфатидилхоліну (ФХ), аніонного ліпіду кардіоліпіну (КЛ) та холестерину (Хол). Дані ІРПЕ інтерпретувались у рамках моделі переносу енергії в двовимірних системах, запропонованої Фангом і Страєром та пізніше розширеної Девенпорт та ін. Між Проданом та Hb не спостерігалось переносу енергії внаслідок того, що за застосованих експериментальних умов зонд практично не розподілявся у ліпідну фазу. У випадку ДМХ окиснювальні процеси, ініційовані Hb, ускладнили визначення положення білка у ліпідному бішарі. З огляду на це, аналіз структури Hb-ліпідних комплексів проводився із застосуванням МБА у якості донора. Спочатку були проведені вимірювання квантового виходу донорів, анізотропії флуоресценції зондів та радіуси Фьорстера. Потім проводилася оцінка зв'язаного з мембранами Hb у рамках решіткових моделей адсорбції великих лігандів, які враховують проникнення білка у бішар. Потім розраховувались відстань площини акцепторів до центру бішару та глибина проникнення білка у мембрану. Було висловлено припущення, що Hb зв'язується з мембранами у вигляді димерів та проникає у внутрішню область мембрани. У нейтральних ліпосомах Hb проникає тільки на глибину ліпідних голівок. Можна припустити, що більша глибина проникнення білка у бішар, який містить Хол, у порівнянні з ФХ мембранами, може бути наслідком специфічної взаємодії Hb з Хол. У випадку ліпосом, які містять КЛ, білок проникає у неполярну область мембрани. Крім того, якщо взяти до уваги можливість формування інвертованих гексагональних структур в присутності КЛ, не можна виключити, що при включенні Hb у такі структури, білок може проникати через мембрану. У такому випадку можна припустити більш глибоке проникнення білка у бішар. Отримані дані можуть бути корисними для детальної характеризації зв'язування Hb з мембранами.
КЛЮЧОВІ СЛОВА: гемоглобін, модельні мембрани, білок-ліпідні комплекси, резонансний перенос енергії

Fluorescence Resonance Energy Transfer (FRET), is an increasingly popular method used to measure the distances between two fluorophores. Resonance energy transfer occurs only over very short distances, typically within 10 nm, and involves the direct transfer of excited state energy from the donor fluorophore to an acceptor. A donor chromophore, initially in its electronic excited state, may transfer energy to an acceptor chromophore through nonradiative dipole–dipole interaction. In most applications, the donor and acceptor dyes are different, and FRET can be detected by measuring the fluorescence of the acceptor or quenching of the donor fluorescence [1].

The resonance transfer of energy between molecules, or between constituents of a large molecule, plays a central role in many areas of modern biochemistry and biophysics. FRET is an important technique for investigating a variety of biological phenomena which produce changes in molecular proximity. It is a useful tool to quantify intra- and intermolecular interactions in biological systems, such as protein-lipid, protein-DNA interactions, and protein conformational changes. Since fluorophores can be employed to specifically label biomolecules and the distance condition for FRET is of the order of the diameter of most biomolecules, FRET is often used to determine when and where two or more biomolecules interact within their physiological surroundings [2, 3].

The validity of distance estimates derived from FRET crucially depends on the adequacy of the employed theoretical model. This model must take into account peculiar properties of investigated systems, for example specific features of the donor and acceptor distribution or

putative mutual orientation of donor-acceptor pairs. This technique proves to be especially valuable in exploring molecular organization of biological membranes, particularly, in ascertaining the structure of protein-lipid complexes.

In the present study FRET was employed to gain molecular-level insights into the structure of hemoglobin complexes with model lipid membranes of different composition. Hemoglobin (Hb) is essential for the oxygen transport in vertebrates and some bacteria. It is the main component of red blood cells whose inner membrane surface has two types of binding sites for this protein. First, high affinity sites localize on the inner segment of band 3, while second, not well characterized low affinity sites, are thought to involve phospholipids [4]. The existence of low affinity sites indicates that Hb interactions with phospholipid bilayers may play important role in oxygen carrying processes. Elucidation of general principles of Hb-lipid binding is crucial not only for understanding physiological function of Hb, but also for certain applied aspects. For example, Hb, encapsulated into the phospholipid vesicles, is a base for development of blood substitutes [5]. Likewise, Hb in various organic films is used in biosensor devices [6]. Despite numerous studies [7-9] exact structure of Hb-lipid complexes so far remains poorly characterized. To obtain structural information about these complexes three membrane probes 3-methoxybenzanthrone (MBA), dimethylaminochalcone 4-dimethylaminochalcone (DMC) and 6-propionyl-2-dimethylaminonaphthalene (Prodan) were recruited as donors, while heme moiety of the protein served as an acceptor.

MATHERIALS AND METHODS

Fluorescent probes 3-methoxybenzanthrone (MBA), 4-dimethylaminochalcone (DMC) were from Zonde (Latvia), 6-propionyl-2-dimethylaminonaphthalene (Prodan) was from Sigma (Germany). Horse hemoglobin in methemoglobin form was purchased from Reanal (Hungary), butylated hydroxytoluene (BHT) was from Merck (Germany). Egg yolk phosphatidylcholine (PC), beef heart cardiolipin (CL) and cholesterol (Chol) were purchased from Biolek (Kharkov, Ukraine). All chemicals were of analytical grade. Lipid vesicles were formed using the extrusion technique [10]. The thin lipid film was obtained by evaporation of lipids' ethanol solutions and then hydrated with 1.2 ml of 5 mM Na-phosphate buffer (pH 7.4). Lipid suspension was extruded through a 100 nm pore size polycarbonate filter. Hb concentrations was determined spectrophotometrically using the extinction coefficient $\varepsilon_{Hb}^{406}=1.415\times10^5$ $M^{-1}cm^{-1}$.

Fluorescence measurements were performed using CM 2203 and Perkin Elmer spectrofluorimeters. Emission spectra of fluorescent probes were recorded with excitation wavelength 420 nm (MBA, DMC) and 350 nm (Prodan). Excitation and emission slit widths were set at 5 nm. Quantum yields of the MBA and DMC in liposomal suspensions were estimated using their solutions in dimethylformamide as standard [11], quantum yields of Prodan was estimated using Rhodamine 101 solution as standard [1]. Fluorescence intensity measured in the presence of Hb was corrected for reabsorption and inner filter effects using the following coefficients [1]:

$$k_{corr}=10^{(A_{ex}+A_{em})/2} \quad (1)$$

where A_{ex} and A_{em} is the acceptor absorbance at the excitation and emission wavelengths, respectively. At Hb concentrations employed in our experiments (0 – 2 μM) the correction coefficient calculated from Eq. 1 does not exceed 2.5. To assess the validity of Eq. 1, theoretical correction coefficient was complemented by determination of the empiric correction coefficient. We found that there was no energy transfer between Prodan and Hb. Further investigation showed that under the employed experimental conditions (Prodan

concentration – 6.9 μM, lipid concentration – 80 μM, Prodan partition coefficient into PC liposomes – 6288) only 11% of the probe appears to partition into the lipid bilayer. Consequently, Prodan and Hb do not approach each other to the distances which permits FRET, so that energy transfer in this system is absent. In this case the decrease of the probe emission is caused by the inner filter effect and empiric correction coefficient can be calculated as relation of the initial probe fluorescence to the probe fluorescence in the presence of Hb. The obtained empiric coefficients deviate from theoretical coefficients by no more than 10%. This implies that Eq. 1 can be used for the correction of fluorescence data.

To quantitatively interpret the results of FRET measurements, the model of energy transfer in two-dimensional systems proposed by Fung and Stryer [12] and further extended by Davenport et al [13] has been employed. If the system contains one donor plane located at a distance d_c from the membrane center and two acceptor planes separated by a distance d_t, the following relationships hold:

$$F(t) = F(0)\exp(-t/\tau_0)\exp\left(-C_a^s\left[S_1(t)+S_2(t)\right]\right) \tag{2}$$

$$S_1(\lambda) = \int_{|d_c-0.5d_t|}^{\infty}\left[1-\exp\left(-\lambda\kappa_1^2(R)\left(\frac{R_0^r}{R}\right)^6\right)\right]2\pi R dR \tag{3}$$

$$S_2(\lambda) = \int_{d_c+0.5d_t}^{\infty}\left[1-\exp\left(-\lambda\kappa_2^2(R)\left(\frac{R_0^r}{R}\right)^6\right)\right]2\pi R dR \tag{4}$$

$$\kappa_{1,2}^2(R) = d_D d_A\left(3\left(\frac{d_c \mp 0.5d_t}{R}\right)^2 - 1\right) + \frac{1-d_D}{3} + \frac{1-d_A}{3} + \left(\frac{d_c \mp 0.5d_t}{R}\right)^2\left(d_D - 2d_D d_A + d_A\right) \tag{5}$$

$$d_{D,A} = \sqrt{r_{D,A}/r_{0D,A}}$$

where S_1 and S_2 are the quenching contributions describing energy transfer to the outer and inner acceptor planes, respectively, $r_{D,A}$ and $r_{0D,A}$ are steady-state anisotropies and fundamental anisotropies of the donor and acceptor, κ^2 is an orientation factor, $F(0)$ is the initial fluorescence intensity, τ_d is the lifetime of the excited donor in the absence of acceptor, $\lambda = t/\tau_d$, R_0 is the Förster radius, and C_a^s is the concentration of acceptors per unit area that depends on molar concentrations of lipid accessible to acceptor (L_0) and bound acceptor (B):

$$C_a^s = \frac{B}{L_0\sum f_i A_{Li}} \tag{6}$$

where f_i and A_{Li} are the mole fractions and mean areas per molecule of lipid species constituting the membrane. The relative quantum yield of the donor is given by:

$$Q_r = \frac{Q_{DA}}{Q_D} = \frac{1}{\tau_d}\int_0^{\infty}\frac{F(t)}{F(0)}dt = \int_0^{\infty}\exp(-\lambda)\exp\left[-C_a^s\left(S_1(\lambda)+S_2(\lambda)\right)\right]d\lambda \tag{7}$$

The Förster radius is related to the donor quantum yield (Q_D) and the overlap between the donor emission ($F_D(\lambda)$) and acceptor absorption ($\varepsilon_A(\lambda)$) spectra [1]:

$$R_0 = 979\left(\kappa^2 n_r^{-4} Q_d J\right)^{1/6} \qquad J = \frac{\int_0^{\infty} F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda}{\int_0^{\infty} F_D(\lambda)d\lambda} \tag{8}$$

where n_r is the refractive index of the medium (n_r = 1.37), κ^2 is an orientation factor that was taken as 0.67 in calculating the R_0^r value.

RESULTS AND DISCUSSION

Analysis of the results of FRET measurements in terms of Fung and Stryer model included several steps. (1) Calculation of critical distance of energy transfer, which requires determination of the donor quantum yield in different model membranes. (2) Estimation of the amount of heme groups bound to liposomal membranes. (3) Quantitative interpretation of FRET data aimed at estimation of the heme position relative to the lipid-water interface, provided that donor location is known.

Table 1

Some characteristics of donors, employed in resonance energy transfer measurements

System	Quantum yield		Critical distance of energy transfer, nm		Fluorescence anisotropy	
	MBA	DMC	MBA	DMC	MBA	DMC
PC	0.05	0.08	1.9	2.6	0.024	0.140
PC:CL 10% CL	0.05	0.07	1.8	2.5	0.028	0.138
PC:Chol 10% Chol	0.06	0.08	1.8	2.5	0.022	0.142
PC:Chol 30% Chol	0.06	0.07	1.8	2.5	0.028	0.130

To date the properties of the fluorescent probes, employed in the present work as donors are rather well characterized. The neutral hydrophobic probes DMC and MBA reside in the interfacial region of the membrane between hydrophilic headgroups of phospholipids and nonpolar acyl chains [14]. Quantum yield of the donors, bound to liposomal membranes appears to be *ca*. two orders of magnitude greater than that in a buffer solution, therefore the contribution of free probe to the measured fluorescence intensity seems to be negligibly small.

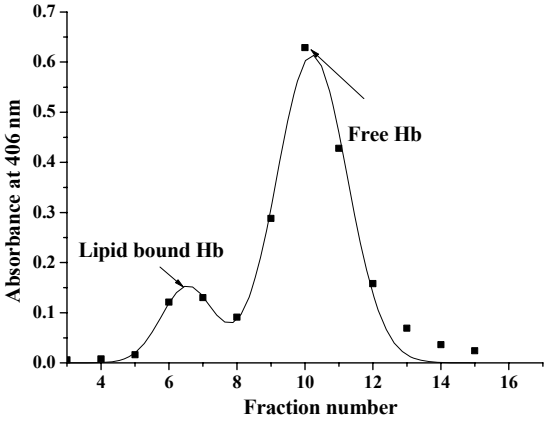

Fig. 1. Elution profile of hemoglobin-liposome mixture. Lipid concentration was 1 mM. First peak corresponds to the protein-liposome complexes and the second peak – to the protein free in solution

Presented in Table 1 are the quantum yields of the employed donors, critical distances of energy transfer, calculated according to Eq. (8), and fluorescence anisotropy. Fundamental anisotropies were taken as 0.34 for MBA and 0.39 for DMC [11]. For both probes quantum yield appears to be virtually independent of membrane composition.

Next step of the study involved the calculation of the amount of membrane bound Hb. To this end, the isotherms of Hb adsorption to the model membranes were obtained using size-exclusion chromatography on Toyopearl HW-60F gel suitable for the separation of lipid bound and free Hb (Fig. 1). The binding curves were analyzed in terms of the lattice model of large ligand adsorption to membranes allowing for the possibility of protein insertion into bilayer interior (Eqs. 9) [15].

$$K_a \cdot F = \frac{B}{L}\left(1+n_2\frac{B}{L}\right)^a\left(1-n_1\frac{B}{L}\right)^{-n_1}\left(1-n_1\frac{B}{L}+n\frac{B}{L}\left(\frac{z-2}{z}-\frac{2}{n\cdot z}\right)\right)^c,$$

$$a=-\frac{\frac{n_2}{n}\left(1-n\left(\frac{z-2}{z}-\frac{2}{n\cdot z}\right)\right)}{1-\frac{z-2}{z}+\frac{2}{n\cdot z}}, \quad c=\frac{\left(\frac{n_1}{n}-\frac{z-2}{z}+\frac{2}{n\cdot z}\right)(n-1)}{1-\frac{z-2}{z}+\frac{2}{n\cdot z}}, \qquad (9)$$

where K_a – association constant, F – concentration of Hb free in solution, B – concentration of lipid-bound Hb, L – lipid concentration, n_1, n_2 – the number of lipid molecules constituting the binding sites for surface-bound and bilayer-inserted Hb, respectively, $n=n_1+n_2$ – total number of lipids per bound protein, $z = 6$ – coordination number for hexagonal lattice. The recovered binding parameters presented in Table 2, were further used for estimation of the concentration of membrane-bound Hb.

Table 2

Thermodynamic parameters of Hb-lipid binding

System	n	K_a, M^{-1}
PC	17±3	$(1.8\pm0.2)\times10^4$
PC:CL (10 mol% CL)	19±4	$(4.5\pm0.9)\times10^3$
PC:Chol (10 mol% Chol)	20±3	$(1.7\pm0.6)\times10^4$
PC:Chol (30 mol% Chol)	31±6	$(1.8\pm0.4)\times10^4$

Shown in Fig.2 is the relative quantum yield of MBA *vs.* concentration of Hb. The further data analysis was aimed at estimating the average distance between heme groups and bilayer center (d_c), giving the best fit of the experimental quantum yield to Eqns (2) – (8). The recovered d_c values are presented in Table 3. For the donors, located at the boundary between polar and nonpolar membrane regions the values of d_t were chosen to be close to the thickness of hydrocarbon core, being *ca*. 3 nm. The estimation of Hb dimer size using WebLab Pro Trial software revealed that protein dimer is a globule with dimensions 5.4×3.8×4.5 nm. Given that the width of lipid monolayer is *ca*. 2.3 nm, the distance of acceptor plane from the lipid bilayer center in the limiting case of the surface location of Hb in the lipid bilayer must be approximately 4 nm. However, d_c values obtained with DMC as a donor are higher than this limiting value. It seems probable that in this case Hb-induced oxidative processes in the lipid phase contribute to the decrease of DMC fluorescence, thereby making d_c estimation incorrect. In the absence of antioxidant (BHT) DMC fluorescence exhibited abrupt decrease. Presumably, BHT addition was not able to suppress all oxidative processes in the model membranes and DMC-bilayer interactions were followed by the prolonged quenching kinetic. Therefore, there exists inconsistency between the experimental and theoretical FRET curves. It can be assumed that high d_c values arise from DMC discoloring by the lipid oxidative products. On the contrary, in the case of MBA no oxidative processes were registered in the presence of BHT.

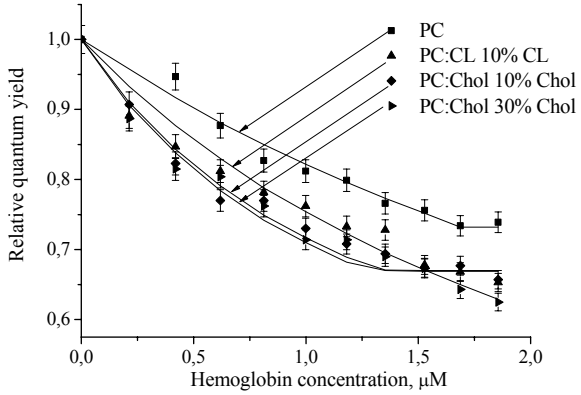

Fig. 2. Relative quantum yield of MBA. Lipid concentration was 80 μM. MBA concentration was `1.4 μM. BHT concentration was 1 μM

Table 3

Hemoglobin location in the lipid bilayer

System	MBA		DMC
	Distance from acceptor plane to the bilayer center, nm	Depth of Hb penetration into bilayer, nm	Distance from acceptor plane to the bilayer center, nm
PC	3.5	0.4	4.7
PC:CL 10% CL	2.9	1.2	4.0
PC:Chol 10% Chol	3.0	0.9	4.3
PC:Chol 30% Chol	3.0	0.9	5.7

Hemoglobin (Hb), although being a soluble protein, demonstrates high affinity for lipid bilayer. Initial Hb-membrane interaction is caused by electrostatic coupling [16]. However, this interaction is weak because protein under physiological conditions is practically uncharged (its isoelectric point ~7.4). Weak electrostatic binding is followed by stronger nonpolar protein-bilayer interaction. Under the experimental conditions employed here (Hb concentration does not exceed 2 μM) Hb tends to dissociate into dimers [17]. This process results in the exposure of hydrophobic regions that include 10 nonpolar (Tyr_{42}, Leu_{91}, Val_{93}, Pro_{95}, Val_{96}, Tyr_{140}, Val_1, Leu_2, Ala_5, Leu_{100}), 6 polar (Thr_{38}, Thr_{41}, Ser_3, Ser_{130}, Thr_{134}, Thr_{137}) 5 charged aminoacids (Arg_{92}, Asp_{94}, Asp_6, Lys_{99}, Arg_{141}) on α-subunit and 3 nonpolar (Pro_{36}, Trp_{37}, Val_{98}), 2 polar (Gln_{39}, Asn_{102}) and 4 charged aminoacids (Arg_{40}, His_{97}, Asp_{99}, Glu_{101}) on β-subunit. It is likely, that the non-polar region exposed upon Hb dissociation into dimers is responsible for Hb ability to interact with lipids hydrophobically. This phase of Hb-lipid complexation is followed by protein conformational changes and exposure of additional hydrophobic regions on the protein surface which facilitate protein insertion into bilayer interior. Hb penetration into lipid bilayer in turn causes membrane perturbations [16, 18, 19]. If lipid bilayer contains anionic lipids Hb-membrane complexation can be finished by the protein destruction and dissociation of heme-globin complex. In this case heme would reside in the lipid bilayer, while globin would dissociate into the aqueous phase [20].

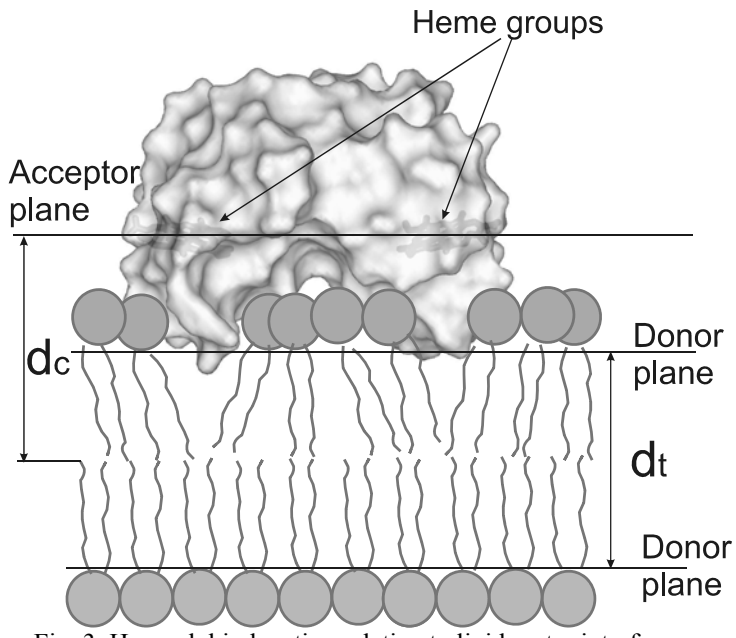

Fig. 3. Hemoglobin location relative to lipid-water interface

In our case d_c values, obtained with MBA as a donor, proved to be greater than bilayer half-width (2.3 nm). This implies that acceptor (heme) locates outside the lipid bilayer. Thus, one can assume, that heme-globin dissociation in our case does not occur. Release of the heme from globin was observed only in the lipid bilayers with high content of negatively charged lipids [16, 20]. It seems probable, that concentration of anionic lipid (CL) in the

model membranes explored here, is insufficient for protein destruction. Taking into account that the distance from heme plane to Hb-lipid contact region is about 1.6 nm, we can estimate the depth of the protein penetration into bilayer: $d_p = 1.6 - (d_c - 2.3)$. As seen from Table 3, in neutral liposomes Hb penetrates only to the depth of lipid headgroups (the size of polar membrane region about 0.8 nm). This finding is in agreement with other investigations. Hb binding to PC membranes makes them permeable, promotes water penetration into polar regions of the bilayer, but does not obviously change membrane conformation [21] and Hb does not interact with non-polar membrane core [22]. Chol in lipid bilayers prevents bilayer perturbations [19], Hb conformational changes and its penetration into hydrophobic membrane core [18]. In addition, it was reported that Chol influence on oxidative processes in Hb-membrane system is connected with Chol ability to form complexes with Hb [23]. It seems likely that higher d_p value for Chol liposomes as compared to PC liposomes is a consequence of this specific protein-Chol interaction. Hb penetration in the lipid bilayer containing CL was deeper than in neutral liposomes (Fig. 3). In this case Hb penetrates in the non-polar membrane region. Such event is facilitated by two facts. First, interaction between Hb and anionic lipids causes protein conformational changes which render Hb capable of inserting in the inner membrane regions. Second, specific CL packing facilitates protein penetration in the lipid bilayers from aqueous phase. In addition, CL in the presence of some proteins can form inverted hexagonal structures [24]. Being entrapped in such structures Hb can permeate through the membrane [25] and there is a probability of protein binding to both inner and outer layers of the model membranes. Provided that Hb locates inside and outside the model membrane, d_c value would be equal 2.4 nm that corresponds to the deeper penetration of Hb into the acyl chain region of the membrane (d_p is ~ 1.5 nm).

CONCLUSIONS

Resonance energy transfer study of hemoglobin binding to model lipid membranes allowed us to conclude that:

1. In phosphatidylcholine model membranes the depth of the protein penetration into membrane interior was minimal (0.4 nm).
2. In cholesterol-containing bilayer hemoglobin was found to insert to the level of carbonyl groups (0.9 nm), presumably, due to specific cholesterol-hemoglobin interactions.
3. In the model membranes composed of phosphatidylcholine and cardiolipin hemoglobin penetrates to nonpolar membrane region (d_p is 1.2 nm).

The results obtained may prove useful for understanding the nature of hemoglobin-lipid interactions and its physiological implications, development of artificial blood on the basis of liposome encapsulated hemoglobin and design of biosensor devices.

This work was supported by the grant # 4534 from the Science and Technology Center in Ukraine and grant № F28.4/007 from Fundamental Research State Fund.

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