ELSEVIER

Biophysical Chemistry 81 (1999) 93–105

Biophysical Chemistry

www.elsevier.nl/locate/bpc

Resonance energy transfer study of hemoglobin complexes with model phospholipid membranes

Galina P. Gorbenko*

Department of Physics and Technology, Kharkov State University, Svoboda Sq. 4, Kharkov 310077, Ukraine

Received 26 March 1999; received in revised form 17 June 1999; accepted 17 June 1999

Abstract

By examining the resonance energy transfer between fluorescent probes, embedded in the lipid bilayer (4-(dimethylaminostyryl)-1-methylpiridine, 4-(dimethylaminostyryl)-1-dodecylpiridine, *N*,*N*′-bishexamethylenrhodamine, rhodamine 6G) as donors, and the heme group of hemoglobin as acceptor, the structure of the protein complexes with the model membranes composed of phosphatidylcholine and cardiolipin was characterized. Quantitative interpretation of the experimental data was performed in terms of the model of energy transfer in two-dimensional systems, using a set of parameters including the distance of closest approach between donor and acceptor, the vertical separation of donor planes, the acceptor distance from the donor plane and the orientation factor. The limits for the heme distance from the lipid bilayer center and the depth of the protein penetration in the membrane interior were estimated. The results obtained suggest that the depth of hemoglobin insertion into liposomal membranes decreases upon increasing CL content in the lipid bilayer.

Keywords: Hemoglobin; Liposomes; Protein–lipid complexes; Fluorescence energy transfer

1. Introduction

It is becoming increasingly apparent that the structural features of the protein–lipid complexes are determined by a number of factors, including, in particular, surface topography of the protein's amino acid residues, total hydrophobicity of the protein, chemical nature of the lipid's headgroups and acyl chains, etc. [1,2]. One approach to elucidating these factors and gaining further insight into their relative significance in controlling the protein binding to a lipid bilayer is based on the employment of the model protein–lipid and peptide–lipid systems [3–6]. These systems frequently contain non-membrane water soluble proteins, characterizing by a high affinity for the lipids.

* Department of Physics and Technology, Kharkov State University, Tobolskaya 52–52, 310072 Kharkov, Ukraine.

0301-4622/99/$ - see front matter
PII: S0301-4622(99)00085-X

One of the proteins, being extensively utilized in the model studies, is hemoglobin (Hb) [7,8]. A substantial body of the data available in the literature are suggestive of multi-step process of Hb interaction with lipids, including: (i) surface adsorption of the protein via electrostatic or hydrogen bonding; (ii) conformational alterations in the protein molecule; (iii) structural reorganization of the lipid bilayer; and (iv) the protein penetration into the membrane interior [9–13]. The relative contributions of these steps may differ with varying the experimental conditions and the composition of the lipid bilayer. The model systems, containing Hb, seem to be relevant not only in further understanding the general principles of the protein–lipid interactions, but also in elucidating specific features of Hb binding to the lipid bilayer, being of interest in view of Hb propensity to interact with the inner surface lipids of erythrocyte membrane [9].

In our previous study an attempt has been made to characterize the structure of Hb complexes with the model membranes by monitoring the resonance energy transfer (RET) between the lipid-bound fluorescent probe (3-methoxybenzanthrone) and the heme group of Hb [14]. The main goal of the present work was to obtain further structural information on the system Hb–liposomes using a series of fluorescent probes as donors and the heme group of Hb as acceptor. A lipid component of the systems studied was represented by zwitterionic phospholipid phosphatidylcholine (PC) and negatively charged phospholipid cardiolipin (CL). A series of donors being used involves 4-(dimethylaminostyryl)-1-methylpiridine *n*-toluenesulfonate (DSM), 4-(dimethylaminostyryl)-1-dodecylpiridine *n*-toluenesulfonate (DSP-12), *N*,*N*′-bishexamethylenrhodamine (RH), rhodamine 6G (R6G).

2. Materials and methods

2.1. Chemicals

Egg yolk PC and beef heart CL were purchased from Bakpreparat (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). R6G was obtained from Reakhim (Russia). DSM, DSP-12 were from Zonde (Latvia). RH was kindly supplied by E.N.Victorova (Optical Institute, Sanct-Petersburg, Russia). Horse hemoglobin and thiourea was purchased from Reanal (Hungary).

2.2. Preparation of liposomes

A stock suspension of unilamellar phospholipid vesicles was prepared by the method of Batzri and Korn [15]. One ml of the ethanol lipid solution containing appropriate amounts of PC and CL was injected into 13 ml of 5 mM sodium-phosphate buffer, pH 7.4 under continuous stirring. Ethanol was then removed by dialysis. The phospholipid concentration was determined according to the procedure of Bartlett [16].

2.3. Fluorescence measurements

Fluorescence measurements were performed with Signe spectrofluorimeter (Latvia). The emission spectra of the fluorescent probes were upon excitation at 460 nm (DSM, DSP-12) or 490 nm (R6G,RH). Excitation and emission slit widths were set at 5 nm. Fluorescence intensity measured in the presence of Hb was corrected for the reabsorption and inner filter effects using the following coefficient [17,18]:

$$k = \frac{(1 - 10^{-A})A_s}{(1 - 10^{-A_s})A} \quad (1)$$

where A is the donor absorbance of in the absence of the protein, A_s is the total absorbance of the sample at excitation or emission wavelengths. The quantum yields of the donors in liposomal suspensions were estimated using fluorescein solution as standard [19]. The critical distance of energy transfer (R_o, nm) was calculated as [17]:

$$R_0 = 979(K^2 n_r^{-4} Q_d J)^{1/6} \quad (2)$$

where J is the overlap integral (in $cm^6 \cdot M^{-1}$), n_r

is the refractive index of the medium ($n_r = 1.37$), K^2 is the orientation factor, Q_D is the donor quantum yield. Concentration of the donors employed in the RET experiments were (in μM): 2 (R6G), 2 (RH), 3 (DSM), 2 (DSP-12). Hb was used in oxidized state. The protein concentration was found spectrophotometrically, using the extinction coefficient $E_{407} = 5.66 \cdot 10^5\ M^{-1}\ cm^{-1}$ for Hb tetramer [20]. To prevent the protein-induced lipid peroxidation an antioxidant thiourea was added to the liposomal suspensions in the concentration of 100 mM.

2.4. Theory

In analyzing the RET occurring in membranes the models developed for the two-dimensional systems appear to be most appropriate [21–26]. One of such models, proposed by Wolber and Hudson [22], provides a formalism for the description of RET between the donors and acceptors, randomly distributed in a plane. Given that fluorescent probes, bound to a lipid bilayer, usually tend to partition between the outer and inner leaflets, in our previous study an attempt has been made to extend the model of Wolber and Hudson to the case of donors, localized at both sides of the membrane [14]. As a result, the following expression for the relative quantum yield of the donor was obtained:

$$Q_r = 0.5\left(\int_0^\infty \exp[-\lambda](I_1(t))^N \mathrm{d}\lambda + \int_0^\infty \exp[-\lambda](I_2(t))^N \mathrm{d}\lambda \right) \quad (3)$$

$$I_1(t) = \int_{R_e}^{R_d} \exp\left[-\lambda(R_o/R)^6\right] W_1(R)\mathrm{d}R \quad (4)$$

$$I_2(t) = \int_{R_e}^{R_d} \exp\left[-\lambda(R_o/R)^6\right] W_2(R)\mathrm{d}R \quad (5)$$

where Q_D, Q_{DA} are the donor quantum yields in the absence and presence of acceptors, respectively, $\lambda = t/\tau_d$, τ_d is the lifetime of an excited donor in the absence of acceptors, $W_1(R)\mathrm{d}R$, $W_2(R)\mathrm{d}R$ are the probabilities of finding acceptor in the annulus between radii R and $R + \mathrm{d}R$ at the outer and inner donor planes, respectively, R_e is the distance of closest approach between donor and acceptor, N is the number of acceptors within the disc of radius R_d, beyond which the energy transfer is insignificant (in analyzing the data R_d was put equal to $3R_o$). If the concentration of acceptors per unit area is equal to C_a^s, N can be written as:

$$N = \pi R_d^2 C_a^s \quad (6)$$

Fig. 1 illustrates the possible modes of the disposition of donor and acceptor planes in the lipid bilayer, assuming that acceptors are localized at the outer side of membrane. Denoting the distance separating the acceptor plane from a nearest donor plane by d_a, $W_1(R)$ can be written as:

$$W_1(R) = \frac{2R}{R_d^2 - R_e^2 - d_a^2} \quad (7)$$

If the donors are situated deeper than acceptors, and donor planes are separated by a distance d_t, $W_2(R)$ is given by:

$$W_2(R) = \frac{2R}{R_d^2 - R_e^2 - (d_t + d_a)^2} \quad (8)$$

Alternatively, when acceptors are localized deeper than donors, $W_2(R)$ equals:

$$W_2(R) = \frac{2R}{R_d^2 - R_e^2 - (d_t - d_a)^2} \quad (9)$$

3. Results

Quantitative interpretation of the results of RET measurements in terms of the modified model of Wolber and Hudson [Eqs. (3)–(9)] requires knowing the critical distance of energy

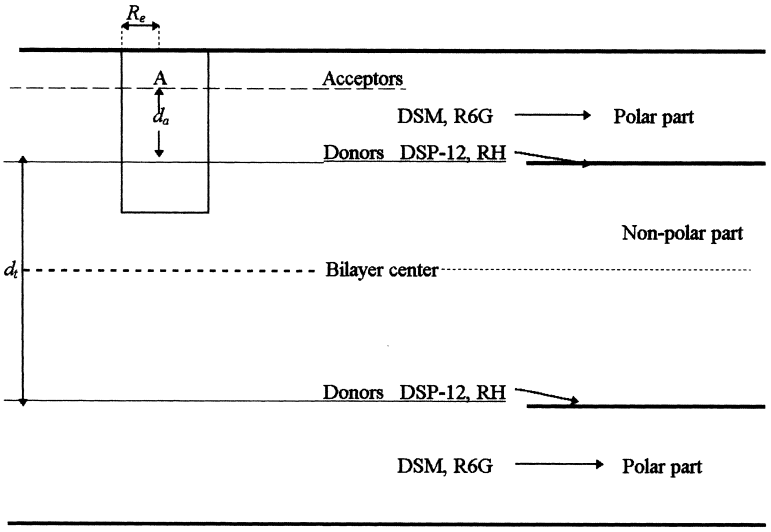

Fig. 1. Schematic representation of the localization of donor and acceptor planes in the lipid bilayer.

transfer (R_o), the possible limits for separation of donor planes (d_t) and the surface acceptor Concentration (C_a^s). In view of this the main steps of the present study were directed toward: (i) determination of quantum yields and R_o values for the donor employed; (ii) estimation of the amount of heme groups bound to the liposomal membranes; and (iii) examination of the RET and quantitative characterization of the heme position relative to the lipid bilayer.

3.1. Donor location in model membranes

To date, the properties of the fluorescent probes, employed in the present work as donors, are rather well studied [19]. DSM and its more hydrophobic derivative DSP-12, containing additional hydrocarbon chain (of 12 carbons), possess at neutral pH positive charge and reside presumably in the polar part of lipid bilayer, in the vicinity of phosphate groups (DSM) or at the boundary between hydrophilic and hydrophobic regions (DSP-12). Another positively charged probe, R6G is also supposed to be situated at the lipid–water interface, penetrating, to some extent, in the polar membrane region. Neutral hydrophobic probe RH, like DSP-12, is located presumably between the glycerol backbone and acyl chains of lipid molecules. It should be noted, that the diameter of the probes, being approximately 1–1.2 nm [19], is comparable with the size of the polar portion of lipid bilayer, so that the concept 'donor plane' seems to account for the position of the centers of donor molecules.

The binding of all fluorescent probes, being used, except R6G, to the liposomal membrane is accompanied by the substantial increase of their quantum yields (approx. two orders of magnitude), so that unbound probe appears not to contribute significantly to the measured fluorescence intensity. The quantum yield of R6G in buffer solution was found to be comparable with that in the lipid bilayer, while the changes in the probe fluorescence spectra (blue shift approx. 10 nm) provide arguments in favor of its effective association with lipids. Given this observation, control experiments were focused on examining the extent of R6G binding to liposomes. It was established that under the experimental conditions employed in the RET studies the contribution of free probe to the total fluorescence is negligible.

Presented in Table 1 are the values of critical distance of energy transfer between various fluorescent probes and the heme group of Hb, calculated according to Eq. (2) for the lipid–protein systems, differing in the liposome composition.

Table 1
Critical distances of energy transfer[a]

CL (mol%)	R_o (nm)			
	DSM	DSP-12	R6G	RH
10	2.83	3.23	4.77	3.42
20	3.01	3.22	4.76	3.32
40	3.18	3.15	4.77	3.36
60	3.20	3.05	4.78	3.39
90	3.18	3.01	4.78	3.39

[a]Calculated according to Eq. (2) assuming that $K^2 = 0.67$.

3.2. Hemoglobin association with liposomes

To assess the concentration of the surface bound heme groups an approach, described in more detail in the previous work [14], has been employed. This approach is based on the assumption that the decrease of Hb absorbance in Soret band, occuring upon the formation of protein–lipid complexes, is proportional to the concentration of bound protein (B):

$$\Delta A_{407} = aB \tag{10}$$

where a is a coefficient of proportionality, ΔA_{407} is the absorbance change in Soret band maximum (407 nm). According to the simplest binding model (Langmuir isotherm) association constant (K_b) can be represented as:

$$K_b = \frac{B}{(P_o - B)(L_o/n - B)} \tag{11}$$

where P_o, L_o are the total protein and lipid concentrations, respectively, n is the number of lipid molecules per molecule of bound protein at saturation. Eq. (11) can be rearranged to give:

$$P_o = \Delta A_{407}\left(\frac{1}{a} + \frac{n}{K_b(L_o a - n\Delta A_{407})}\right) \tag{12}$$

Eq. (12) has been used in obtaining the binding characteristics (K_b, n) and the coefficient of proportionality (a) for the lipid–protein systems under study. Initial estimates of these parameters, derived from the double reciprocal plots [14,19], were iteratively improved by the data fitting, based on the comparison of P_o values, determined spectrophotometrically, with those calculated from Eq. (12). Fig. 2 shows typical dependencies of ΔA_{407} on P_o, being treated in terms of this approach. The binding parameters, derived in such a way (Table 2) were subsequently used for the estimation of the concentration of the bound protein according to the relationship:

$$B = 0.5\Big[P_o + L_o/n + 1/K_b - \sqrt{(P_o + L_o/n + 1/K_b)^2 - 4P_o L_o/n}\Big] \tag{13}$$

The surface acceptor concentration was then calculated as follows:

$$C_a^s = \frac{N_A B}{S_L} \tag{14}$$

$$S_L = N_A L_o(f_{\mathrm{PC}} S_{\mathrm{PC}} + f_{\mathrm{CL}} S_{\mathrm{CL}}) \tag{15}$$

where N_A is Avogadro number, f_{PC}, f_{CL} are the mole fractions of PC and CL, respectively, S_{PC}, S_{CL} are the mean areas per lipid molecule, taken to be 0.65 nm^2 and 1.2 nm^2 [27,28], respectively. To represent the concentration of bound acceptor

Table 2
Parameters of hemoglobin binding to liposomes

Cardiolipin content (mol%)	K_b ($\times 10^5$ M^{-1})	n	a ($\times 10^5$)
10	2.5 ± 0.7	216 ± 52	5.2 ± 1.2
20	2.1 ± 0.5	116 ± 31	3.6 ± 0.8
40	2.6 ± 0.7	130 ± 34	9.1 ± 2.2
60	5.3 ± 1.4	170 ± 42	10 ± 2.4
90	5.5 ± 1.2	83 ± 21	9.2 ± 2.1

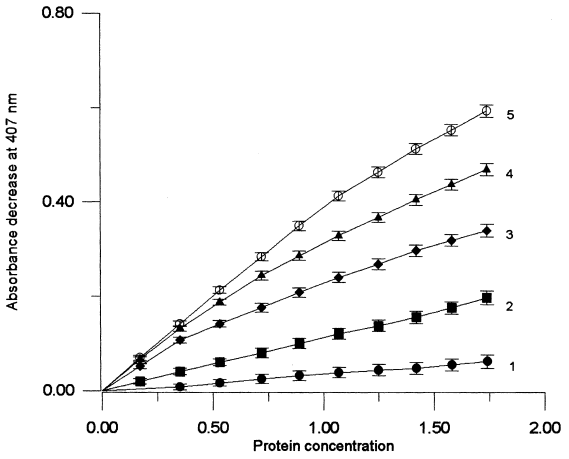

Fig. 2. Decrease of hemoglobin absorbance at 407 nm in liposomal suspensions as a function of protein concentration (μM). CL content, mol%: 1–10, 2–20, 3–40, 4–60, 5–90. Lipid concentration, mM: 1–0.17, 2–0.59, 3–0.46, 4–0.38, 5–0.29.

in units, commonly used for the two-dimensional systems (i.e. a number of acceptors per R_o^2) the C_a^s values were multiplied by R_o^2.

It is noteworthy that the reduction in Hb absorbance observed upon its binding to lipids, is thought to originate from the protein capability to induce lipid peroxidation, which, in turn, leads to the substantial alterations in the structural state of heme group [10–13]. Given that any change in the heme absorbance would affect the spectral overlap of donor and acceptor and, hence, the critical distance of energy transfer, all RET measurements were performed under conditions, preventing lipid peroxidation (by adding antioxidant thiourea), while the binding studies were carried out in the absence of antioxidant.

It is also of importance to bear in mind, that parameters K_b and n, being relevant for the calculation of the amount of bound protein within the scope of the present study, do not reflect, however, the actual quantitative characteristics of Hb association with liposomes, since the simplest binding model is believed to be inadequate in considering the lipid–protein systems [29,30].

3.3. Resonance energy transfer measurements

Fig. 3 shows the plots of relative quantum yield as a function of the surface concentration of the heme groups, bound to liposomes, differing in CL content. These data were analysed in terms of the aforementioned model, in order to obtain the sets of parameters R_e, d_t, d_a, providing the best fit of experimental Q_r values (Q_r^e) to Eq. (3). The fitting procedure involved the minimization of the function:

$$f = \frac{1}{n_a} \sum_{i=1}^{n_a} (Q_r^e - Q_r^t)^2 \tag{16}$$

where Q_r^t is the Q_r value calculated by numerical integration of Eq. (3), n_a is the number of acceptor concentrations used in the RET experiments. The possible limits for d_t and R_e were chosen by considering the size of model membranes and Hb molecule and taking into account the aforementioned assumptions concerning the localization of donors in the lipid bilayer. As illustrated in Fig. 1, the most reasonable choice for d_t limits seems to be approximately 2.0–2.4 nm in the case of DSP-12 and RH, and approximately 3.0–3.6 nm for DSM and R6G, given that the thickness of the hydrophobic core of lipid bilayer is approximately 2 nm [27]. The distance of closest approach (R_e) equals the sum of donor radius, taken to be approximately 0.5–0.6 nm [19], and the distance from heme center to the protein surface (r_h). The dimensions of Hb molecule, containing four subunits, are known to be $6.4 \times 5 \times 5$ nm, with the heme groups located in the vicinity of the protein surface [31]. In the range of the relatively low protein concentrations, employed in the RET studies (0.3–1.5 μM), Hb tends to dissociate into dimers [7]. Thus, it seems probable that Hb binds to liposomal membranes in the form of dimers with the average diameter of approximately 2.5 nm. Since the orientation of subunits with respect to bilayer surface is unknown, one cannot exclude, in principle, two extreme possibilities, corresponding to the cases of heme location in the proximity of lipid-binding site ($R_e \approx 1.1$ nm, with heme radius approximately 0.6 nm), or at the maximum distance from the contact area in the protein–lipid complex ($R_e \approx 3$ nm).

Another important question arising in inter-

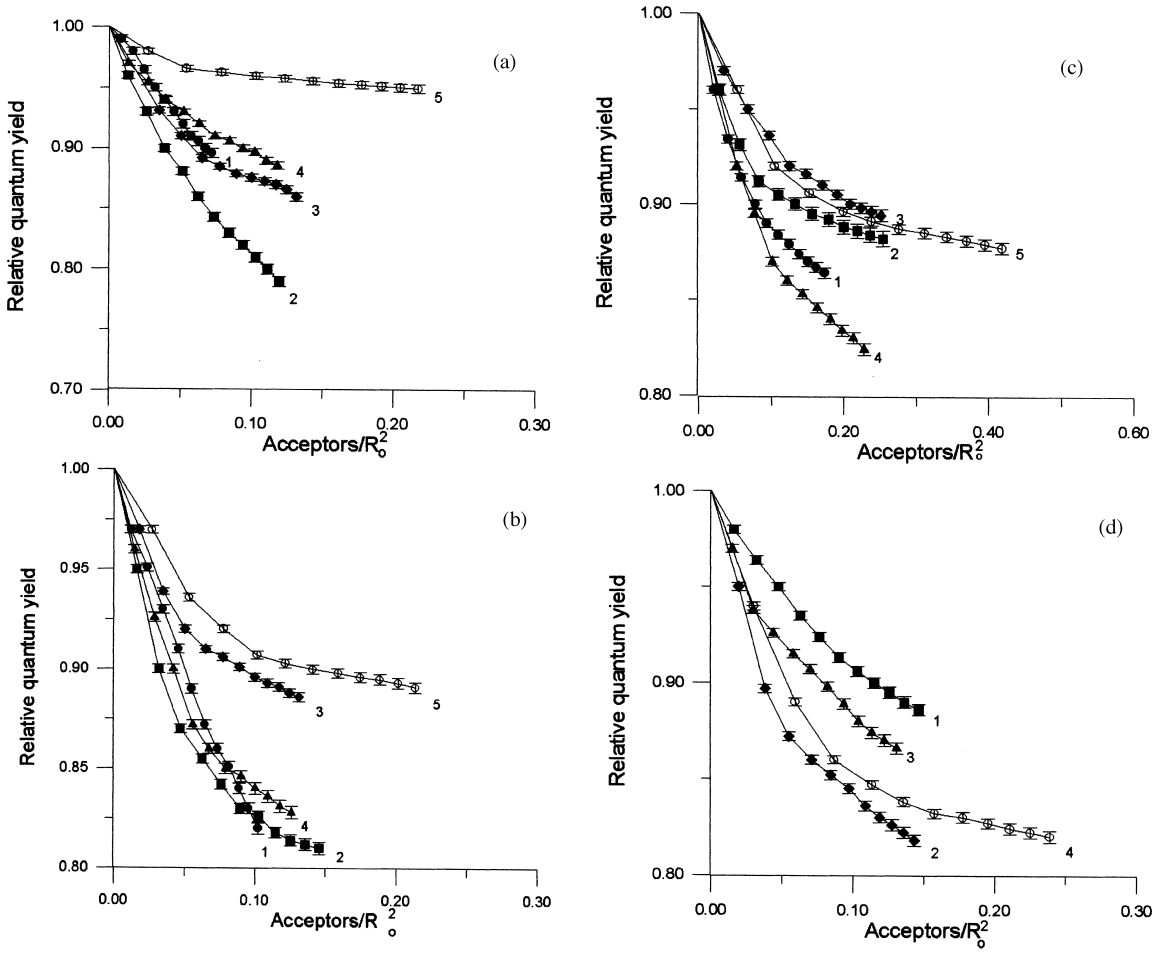

Fig. 3. Relative quantum yield of donors vs. concentration of heme groups of hemoglobin, bound to liposomes: A-DSM; B-DSP-12; C-R6G; D-RH. CL content, mol%: 1–10, 2–20, 3–40, 4–60, 5–90 (A,B,C); 1–20, 2–40, 3–60, 4–90 (D). Lipid concentration, mM: 1–0.42, 2–0.35, 3–0.28, 4–0.23, 5–0.18.

preting the results of RET measurements concerns the validity of the orientation factor value, used in the R_o calculation. Usually K^2 is assumed to be 0.67, the value, characterizing random reorientation of the donor emission and acceptor absorption moments. It is noteworthy that K^2 can range from 0 to 4, the lower and upper limits correspond to perpendicularly oriented and parallel donor and acceptor dipoles, respectively [17]. The significance of orientational effects in determining the RET efficiency is considered in a number of studies [21–24]; and [32]. It is clear from such consideration, that if there exists certain preferential orientation of donor and acceptor dipoles, the assumption of K^2 being equal to 0.67 may be the reason for significant ambiguities in the data analysis. This K^2 value is valid for the isotropic and dynamic averaging conditions, when the donor and acceptor molecules are rapidly tumbling and their transition dipoles can adopt all orientations in a time short compared with the transfer time. However, in the membranes, being highly anisotropic systems, the donors have limited freedom of motion and the isotropic condition is hardly satisfied. The estimation of K^2 bounds for donor–acceptor

pairs examined in the present work appears to be hardly feasible task, both in experimental and theoretical aspects. In view of this, in analyzing the results of RET studies K^2 was allowed to vary from the minimum to maximum possible values. Analysis of the experimental data within the framework of aforementioned approach yields numerous sets of the parameters d_t, R_e, K^2, d_a, giving satisfactory fit of the measured dependencies $Q_r(C_a^s)$ to those calculated according to Eqs. (3)–(9). Based on the acceptor distance from the nearest donor plane (d_a), one can estimate heme distance from the lipid bilayer center (d_c):

$$d_c = 0.5\, d_t \pm d_a \tag{17}$$

where '+' corresponds to the case when donors are localized deeper than acceptors, while '−' characterizes the opposite case. The relationships between parameters d_c and K^2, obtained for various kinds of liposomes, are shown in Fig. 4. It may be seen that the minimum d_c values, being equal to zero, are observed at certain minimum K^2 value (K^2_{min}), while the maximum d_c (d_c^{max}) corresponds to $K^2 = 4$. Note that upon varying d_t in the limits, indicated above, the changes in d_c do not exceed 0.2 nm. Since parameter d_c is invariant over a series of donors, it seems reasonable to assume that the true d_c value falls in the range, being common for all donors, and actual d_c^{max} equals the least of d_c^{max} values, derived for a given kind of liposomes. As follows from Fig. 4, the lowest d_c are obtained with DSM, therefore

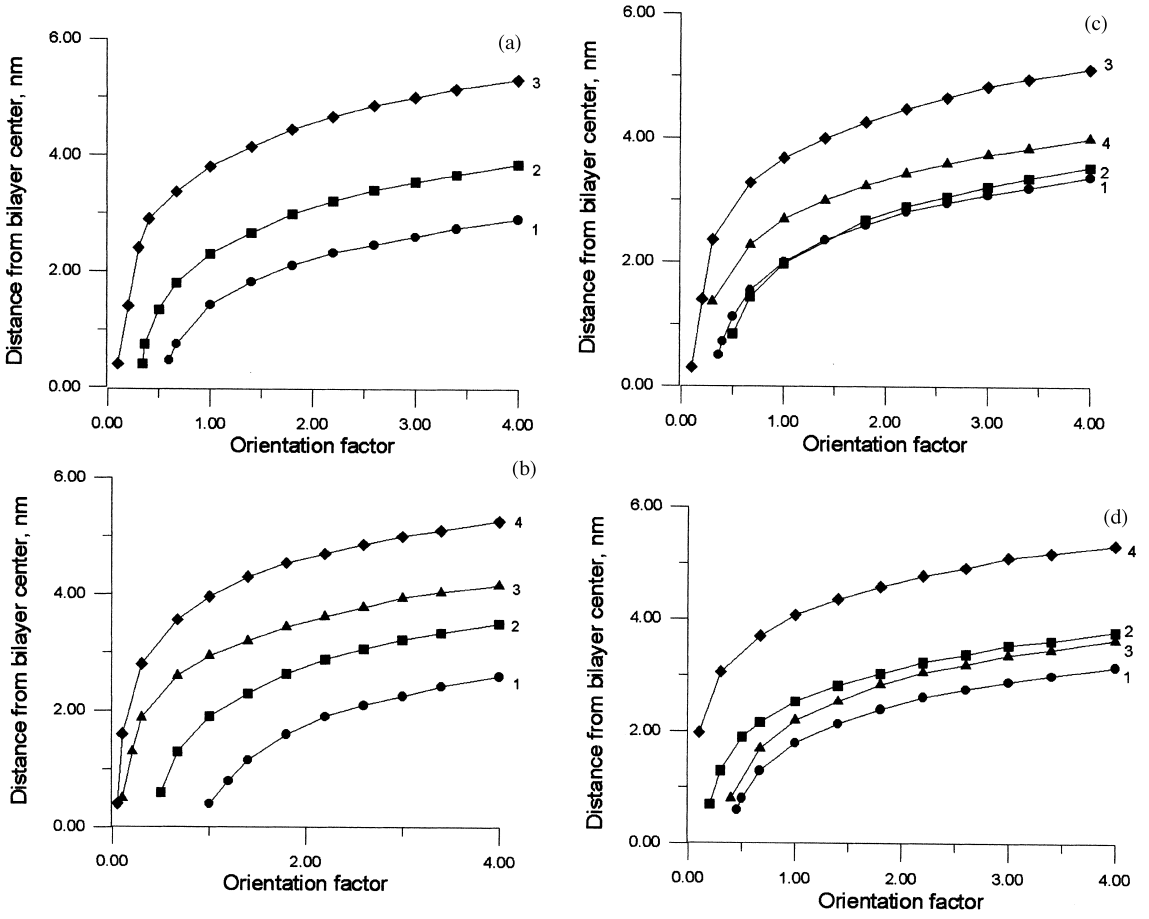

Fig. 4. Distance of heme group of hemoglobin from the lipid bilayer center as a function of orientation factor: CL content, mol%: a-10; b-20; c-40; d-60; e-90. Donors: 1-DSM, 2-DSP-12, 3-R6G, 4-RH.

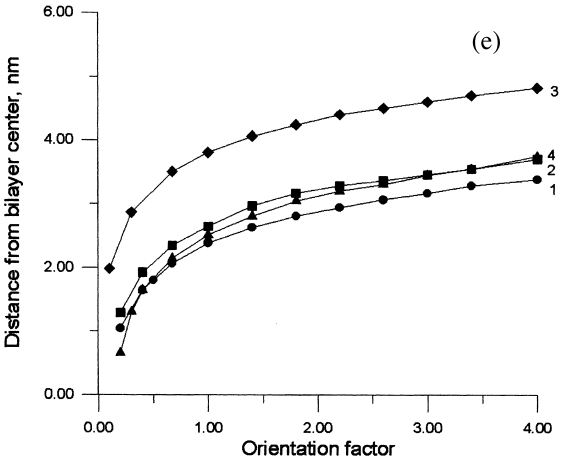

Fig. 4. (*Continued*).

the further data treatment was focused on this donor. Fig. 5 illustrates dependencies of $d_c^{\max}$ on R_e, derived for DSM from analyzing the results of RET measurements, while Fig. 6 shows the typical relationships between parameters d_c, R_e and K^2, obtained for liposomes differing in CL content.

4. Discussion

As can be seen in Fig. 4, at K^2, corresponding to a random reorientation of the donors and acceptors (0.67), d_c values, obtained with various donors, in the most cases appreciably differ. Such a finding suggests that the true value of K^2, at least for some of the donor–acceptor pairs being used are distinct from the isotropic one. As indicated above, the main reason for this may be the existence of specific lipid-binding sites at the surface of Hb molecule, leading to a particular heme disposition relative to the lipid bilayer. However, one should bear in mind, that in Hb tetramers or dimers symmetrical disposition of hemes leads to a degeneracy of the acceptor transition moment, thus providing an additional source of uncertainty in the K^2 value.

In this respect of great interest are the early studies of cytosol Hb in erythrocytes, performed by Eisinger and Flores [33–35]. By examining the RET between fluorescent probes (*n*-(9-anthroyloxy) stearic acids), embedded into a lipid portion of erythrocyte membranes, as donors, and the hemes of cytosol Hb as acceptors, these authors have made an attempt to assess possible bounds for the orientation factor. In considering the tetramer Hb molecule bound to the membrane, it has been found, that the contributions of symmetrically situated heme groups into the average K^2 value are mutually compensated, so that the resulting K^2 range appears to be rather small, even if Hb should have specific binding sites with particular heme orientation. It seems noteworthy that the estimation of K^2 bounds can, in principle, be made on the basis of spectroscopic information on the orientation of transition moments relative to the plane of porphyrin ring, and X-ray data on the heme position in the protein molecule. Such an approach has been elaborated in a series of works by Gryczynski et al. [36,37], in examining the energy transfer between the tryptophan residue and the heme group of Hb . Unfortunately, within the scope of the present work any narrowing of K^2 bounds for the donor–acceptor pairs examined appears to be impossible, mainly because of the lack of reliable information regarding the orientation of the donor transition moments. In addition, despite the X-ray

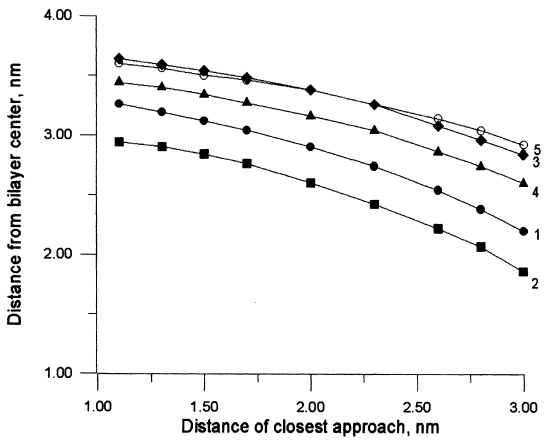

Fig. 5. Plots of the maximum possible distance of hemoglobin heme from the lipid bilayer center vs. R_e, obtained with $K^2 = 4$ for DSM. CL content, mol%: 1-10, 2-20, 3-40, 4-60, 5-90.

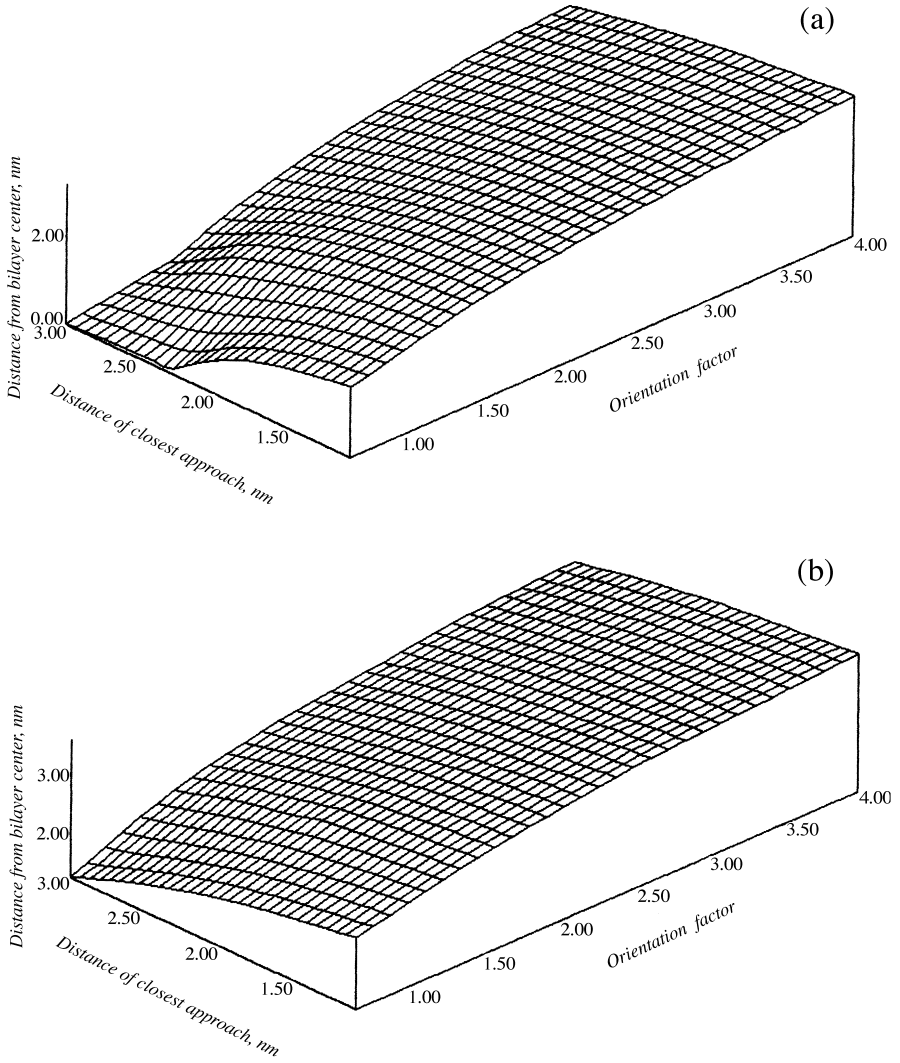

Fig. 6. Typical relationships between heme distance from the lipid bilayer center, distance of closest approach between donor and acceptor and orientation factor. CL content, mol%: A-10, B-90.

data on the heme position within Hb crystals been available, they cannot be used directly in considering the protein behavior in a solution. Furthermore, as indicated in a number of studies, the heme structural state may substantially alter upon the formation of protein–lipid complexes. Although all these factors significantly complicate the quantitave interpretation of the results of RET measurements, some valuable estimates can be drawn from the mentioned above relationships between the parameters d_c, K^2 and R_e. Provided that R_e and d_t do not depend appreciably on the liposome composition, from Figs. 5 and 6 it follows that the distance of heme group from the bilayer center exhibits nonmonotonous dependence on CL content (f_{CL}). An increase of f_{CL} from 10 to 20 mol% leads to some decrease in d_c, while further growth of f_{CL} from 20 to 40 mol% and higher is accompanied by the pronounced increase in d_c. These findings are consistent with those obtained in the previous work by monitoring the RET between MBA and heme of Hb

[14]. As can be seen in Table 3, the maximum d_c values, derived with $R_e = 1.1$ nm and $K^2 = 4$, fall in the range 2.9–3.6 nm. If the heme center is localized at vertical distance r_h^* from the protein surface area, being in contact with lipids, the depth of the protein penetration into the lipid bilayer interior (d_p) can be defined as follows:

$$d_p = 0.5 d_m - (d_c - r_h^*) \quad (18)$$

where d_m is the thickness of the lipid bilayer, that is known to be approximately 4.6 nm [27]. Since it seems difficult to make any assumptions concerning the position of lipid-binding site on the protein surface, the minimum r_h^* value ($r_h^{\min}$) can be taken 0.6 nm (heme radius), while maximum $r_h^*(r_h^{\max})$ would be approximately 2 nm (for Hb dimers with average radius approx. 1.3 nm). Based on these estimates one can determine the lower ($d_P^{\min}$) and the upper ($d_P^{\max}$) limits for the depth of Hb insertion into the liposomal membrane:

$$d_p^{\min} = 0.5\, d_m + r_h^{\min} - d_c^{\max} \quad (19)$$

$$d_p^{\max} = 0.5\, d_m + r_h^{\max} - d_c^{\min} \quad (20)$$

The values of $d_P^{\min}$ derived in this way for extreme and intermediate R_e are presented in Table 3. Note that the maximum penetration depth ($d_P^{\max}$), calculated with $d_c^{\min} = 0$, equals 4.3 nm for all kinds of liposomes. Assuming that the true K^2 value for donor–acceptor pair DSM-heme is not far from isotropic one (0.67) and from the estimate of Eisinger et al. (0.5) [35], the limits for $d_P^{\min}$, obtained with $R_e^{\min} = 1.1$ nm and $R_e^{\max} = 3$ nm (Table 3) may be considered as arguments in favor of Hb penetration into the membrane interior. A lot of data available in the literature are indicative of Hb capability to hydrophobic interactions with lipids. Such a capability is supposed to account for the substantial increase of bilayer ion permeability in the presence of Hb [7,38], the alterations in the thermotropic behavior of lipids [39], strong association of Hb with neutral lipids [7]. Total hydrophobicity of Hb is similar to that of other water soluble proteins [8], but the possibility of the exposure of nonpolar faces upon Hb dissociation into dimers or separate subunits may substantially increase the protein affinity for the hydrophobic portion of the lipid bilayer. Another factor, that may enchance the hydrophobic interactions between Hb and lipids, consists in the conformational changes of the protein molecule, followed by the exposure of apolar residues on

Table 3
The limits of orientation factor ($K^2_{\min}$) and the depth of hemoglobin penetration in the membrane interior ($d_P^{\min}$, nm)

CL content (mol%)	$K_{\min}{}^2$	R_e (nm)	$K^2 = 0.67$		$K^2 = 2.6$		$K^2 = 4$	
			d_c	$d_P{}^{\min}$	d_c	$d_P{}^{\min}$	d_c	$d_P{}^{\min}$
10	0.5	1.1	1.6	1.3	2.8	0.1	3.3	0
		2	0.7	2.2	2.5	0.4	2.9	0
		3	0	2.9	1.6	1.3	2.2	0.7
20	0.9	1.1	[a]		2.5	0.4	2.9	0
		2	[a]		2.1	0.8	2.6	0.3
		3	[a]		1.1	1.8	1.9	1
40	0.3	1.1	2.1	0.8	3.3	0	3.6	0
		2	1.5	1.4	3	0	3.4	0
		3	$\rightarrow 0$	> 2.9	2.3	0.6	2.8	0.1
60	0.4	1.1	1.9	1	3.1	0	3.4	0
		2	1.3	1.6	2.8	0.1	3.2	0
		3	$\rightarrow 0$	> 2.9	2.1	0.8	2.6	0.3
90	0.1	1.1	2.5	0.4	3.4	0	3.6	0
		2	2.1	0.8	3.1	0	3.4	0
		3	1.2	1.7	2.6	0.3	2.9	0

[a] Minimum K^2 value exceeds 0.67.

the protein surface. In particular, negatively charged phospholipids, including CL, are reported to induce considerable destabilization of Hb structure [9–13]. It seems noteworthy that not only the protein conformational alterations, but also the structural reorganization of the lipid bilayer can be responsible for the protein penetration into the membrane. This concerns, particularly, the possibility of the formation of non-bilayer structures such as inverted micelles or hexagonal H_{II} phase, leading to the protein trapping into the aqueous cylinders, spanning the bilayer [40]. There exists some evidence for the formation of these structures upon Hb binding to the model membranes, composed of PC and phosphatidylethanolamine [41], but for the systems, containing PC and CL such information is still lacking. Since the data presented here indicate that the maximum depth of Hb penetration in the lipid bilayer is approximately 4.3 nm, (the value, corresponding to practically full insertion of Hb dimers into the membrane interior), one cannot rule out, in principle, the possibility of the formation of the non-bilayer structures in the systems being examined.

Taken together, the results of the present study can be summarized as follows. Resonance energy transfer between a series of fluorescent probes, embedded in the lipid bilayer, as donors, and the heme group of Hb as acceptors, appears to be relevant to the structural characterization of the protein–lipid complexes. A set of parameters employed in the data analysis involves a distance of closest approach between donor and acceptor (R_e), the vertical separation of donor planes (d_t), the acceptor distance from a nearest donor plane (d_a) and the orientation factor (K^2). Varying d_t and R_e in the limits, consistent with the dimensions of the protein and the model membranes yields possible limits for the heme distance from the lipid bilayer midplane and the depth of the protein penetration into the membrane interior. The data obtained suggest that the extent of Hb penetration in the lipid bilayer decreases with increasing CL content in the liposomal membranes. Although the present work provides some evidence for the particular heme orientation in the complexes with lipids, for the definitive conclusions concerning the existence of specific lipid-binding sites at the surface of Hb molecule further studies are needed.

Acknowledgements

I am very grateful to Dr Ole G. Mouritsen (Technical University of Denmark, Lyngby, Denmark), Dr Derek Marsh, (Max-Planck Institute fur Biophysikalische Chemie, Gottingen, Germany), Dr Paavo Kinnunen (University of Helsinki, Finland), Dr Zygmunt Gryczynski (Department of Biochemistry, University of Maryland, USA) and Dr Stephen H.White (University of California, Irvine, USA) for providing me with the reprints of papers. I have also benefited greatly from the valuable remarks of the referee.

References

[1] O Mouritsen, Curr. Opin. Colloid Interface Sci. 3 (1998) 78.
[2] M. Sankaram, D. Marsh, in: A. Watts (Ed.), Protein–Lipid Interactions, Elsevier, Amsterdam, 1993, pp. 127–162.
[3] G. Schwarz, Biophys. Chem. 58 (1996) 67.
[4] J. Kleinschmidt, D. Marsh, Biophys. J. 73 (1997) 2546.
[5] A. Ladokhin, M. Selsted, S. White, Biophys. J. 72 (1997) 1762.
[6] N. Hellman, G. Schwarz, Biochim. Biophys. Acta 1369 (1998) 267.
[7] L. Bossi, S. Alema, P. Calissano, E. Marra, Biochim. Biophys. Acta 375 (1975) 477.
[8] H. Kimelberg, Mol Cell. Biochem. 10 (1976) 171.
[9] N. Shaklai, J. Yguerabide, H. Ranney, Biochemistry 16 (1977) 5593.
[10] Y. Shviro, I. Zilber, N. Shaklai, Biochim. Biophys. Acta 687 (1982) 63.
[11] S.V. Bondarenko, I.P. Ushakova, A.F. Levit, G.A. Serebrennikova, R.P. Evstigneeva, Bioorg. Khim. 11 (1985) 1385.
[12] J. Szebeni, E. Lorio, H. Hauser, H. Winterhalter, Biochemistry 24 (1985) 2827.
[13] E. Marva, R. Hubbel, Blood 83 (1994) 242.
[14] G. Gorbenko, Biochim. Biophys. Acta 1409 (1998) 12.
[15] S. Batzri, E. Korn, Biochim. Biophys. Acta 298 (1973) 1015–1019.
[16] G. Bartlett, J. Biol. Chem. 234 (1959) 466–472.
[17] J.R. Lakowicz, Principles of Fluorescent Spectroscopy, Plenum Press , New York, 1983.
[18] A.A. Bulychev, V.N. Verchoturov, B.A. Gulyaev, Current methods of biophysical studies, Vyschaya shkola, Moscow, (1988).

[19] G.E. Dobretsov, Fluorescent Probes in the Studies of Cells, Membranes and Lipoproteins, Nauka, Moscow, 1989.
[20] R. Benesch, E. Benesch, S. Yung, Anal. Biochem. 55 (1973) 245.
[21] T. Estep, T. Thompson, Biophys. J. 26 (1979) 195.
[22] P. Wolber, B. Hudson, Biophys. J. 28 (1979) 197.
[23] T. Dewey, G. Hammes, Biophys. J. 32 (1980) 1023.
[24] B. Snyder, E. Freire, Biophys. J. 40 (1982) 137.
[25] M. Doody, L. Sklar, H. Pownall, J. Sparrow, A. Gotto, L. Smith, Biophys. Chem. 17 (1983) 139.
[26] A. Kleinfeld, M. Lukacovic, Biochemistry 24 (1985) 1883.
[27] V.G. Ivkov, G.N. Berestovsky, Dynamic Structure of Lipid Bilayer, Nauka , Moscow, 1981.
[28] R. Rand, S. Sengupta, Biochim. Biophys. Acta 255 (1972) 484.
[29] S. Stankowski, Biochim. Biophys. Acta 777 (1984) 167.
[30] R. Chatelier, A. Minton, Biophys. J. 71 (1996) 2367.
[31] H. Muirhead, J. Cox, L. Mazzarella, M. Peruz, J. Mol. Biol. 28 (1967) 117.
[32] R. Dale, J. Eisinger, W. Blumberg, Biophys. J. 26 (1979) 161.
[33] J. Eisinger, J. Flores, Biophys. J. 37 (1982) 6.
[34] J. Eisinger, J. Flores, Biophys. J. 41 (1983) 367.
[35] J. Eisinger, J. Flores, R. Bookhin, J. Biol. Chem. 259 (1984) 7169.
[36] Z. Gryczynski, S. Beretta, J. Lubkowski, A. Rasynska, I. Gryczynski, E. Bucci, Biophys. Chem. 64 (1997) 81.
[37] Z. Gryczynski, J. Lubkowski, E. Bucci, Meth. Enzymol. 278 (1997) 538.
[38] P. Calissano, S. Alema, G. Rusca, Biochim. Biophys. Acta 255 (1972) 1009.
[39] D. Papahadjopoulos, M. Moscarello, E. Eylar, T. Isac, Biochim. Biophys. Acta 401 (1975) 317.
[40] B. de Kruijff, P. Cullis, A. Verkleij et al., In Progress in Protein–Lipid Interactions, Elsevier Science Publisher, 1985, 89.
[41] V.V. Chupin, I.P. Ushakova, S.V. Bondarenko et al., Bioorg. Khim. 8 (1982) 1275.