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Cytochrome *c* location in phosphatidylcholine/cardiolipin model membranes: resonance energy transfer study

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Abstract

Resonance energy transfer between lipid-bound fluorescent probe 3-methoxybenzanthrone as a donor and heme group of cytochrome *c* as an acceptor has been examined to ascertain the protein disposition relative to the surface of model membranes composed of phosphatidylcholine and cardiolipin (10, 50 and 80 mol%). The model of energy transfer in membrane systems has been extended to the case of donors distributed between the two-bilayer leaflets and acceptors located at the outer monolayer taking into account the donor and acceptor orientational behavior. Assuming specific protein orientation relative to the membrane surface and varying lateral distance of the donor–acceptor closest approach in the range from 0 to 3.5 nm the limits for possible heme distances from the bilayer midplane have been found to be 0.8–3 nm (10 mol% CL), 0–2.6 nm (50 mol% CL), and 1.4–3.3 nm (80 mol% CL).

Keywords: Resonance energy transfer; Cytochrome *c*; Protein–lipid complexes; Protein bilayer location

1. Introduction

Cytochrome *c*, a component of the mitochondrial electron transport chain, is proved to be a protein whose functioning strongly depends on the nature of its interactions with membrane lipids [1,2]. These interactions play an essential role in determining the conformation of cyt *c*, its bilayer location and orientation of heme groups [3–5]. Numerous studies suggest that the cyt *c* binding to a lipid bilayer involves several steps: (i) formation of electrostatic contacts and hydrogen bonds between the amino acid side chains and phospholipid headgroups [6–8]; (ii) conformational changes of the protein molecule coupled with structural reorganization of the lipid bilayer [9–14]; and (iii) hydrophobic protein–lipid interactions originating either from the penetration of the non-polar amino acid residues in the membrane hydrocarbon region [7,15] or incorporation of the lipid acyl chain into the hydrophobic cavity in the protein molecule [16–18]. Despite abundance of information on the mechanisms of cyt *c* association with lipids a lot of features of the binding process remain unclear. In particular, the disposition of the protein molecule relative to the lipid–water inter-

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face is not yet unequivocally ascertained. A variety of physical methods provide evidence for cyt *c* insertion in the membrane interior, but so far there is a significant lack of detailed knowledge of the factors controlling this process and its quantitative characteristics. Specifically, little is known about the depth of cyt *c* bilayer penetration. One attempt to answer this question was made in our previous works [19,20]. To gain insight into the structure of the cyt *c*–lipid model system we examined the resonance energy transfer (RET) between the membrane-embedded fluorescent probes as donors and the heme group of cyt *c* as the acceptor. The RET efficiency is known to depend on the donor–acceptor separation, the donor quantum yield, overlap of the donor emission and acceptor absorption spectra and relative orientation of the donor and acceptor transition dipoles (orientation factor) [21,22]. The accuracy of the RET-based distance estimation is primarily determined by adequacy of the theoretical model employed in analyzing the experimental data and validity of the assumptions concerning the orientation factor value. The problem of the orientation factor is regarded as the main limitation of the RET method [22]. In the majority of RET models the orientation factor is considered as being distance-independent, attaining certain dynamically or statically averaged values. However, in two-dimensional membrane systems where chromophores are located in the plane, there exists certain distribution of donor–acceptor distances and relative orientations of the donors and acceptors. As a consequence, the orientation factor becomes dependent on the donor–acceptor separation. This peculiar feature of energy transfer in membranes has not been taken into account in our previous RET investigation of cyt *c* location in the lipid bilayer. To obtain more adequate structural information on the cyt *c*–lipid model system the goals of the present study were: (i) to extend the model of energy transfer in membrane systems by introducing the orientation factor as a function of the donor–acceptor separation and orientational distributions of the donor emission and acceptor absorption transition moments; and (ii) to characterize the location of cyt *c* in the lipid bilayers composed of PC and CL, the latter being a putative constituent of the protein binding site in the inner mitochondrial membrane [23].

2. Materials and methods

2.1. Experimental

Egg yolk PC and beef heart CL were purchased from Biolek (Kharkiv, 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). Bovine heart cyt *c* (oxidized form) and thiourea were obtained from Reakhim (Russia). MBA was from Zonde (Latvia).

Unilamellar phospholipid vesicles composed of PC and 10, 50 or 80 mol% of CL were prepared by the ethanol injection method [24]. Fluorescence measurements were performed with Signe spectrofluorometer (Latvia). MBA emission spectra were excited at 440 nm. Excitation and emission slit widths were set at 5 nm. Fluorescence intensity measured in the presence of cyt *c* at the maximum of MBA emission spectra (540 nm) was corrected for reabsorption and inner filter effects. To choose the most appropriate method of correction, we compared the theoretical and empirical correction coefficients. Theoretical coefficients were calculated as [20,25]:

$$k=\frac{(1-10^{-A_o^{ex}})(A_o^{ex}+A_a^{ex})}{(1-10^{-(A_o^{ex}+A_a^{ex})})A_o^{ex}}\,\frac{(1-10^{-A_o^{em}})(A_o^{em}+A_a^{em})}{(1-10^{-(A_o^{em}+A_a^{em})})A_o^{em}} \quad (1)$$

where A_o^{ex}, A_o^{em} are the donor optical densities at the excitation and emission wavelengths in the absence of acceptor ($A_o^{ex}\sim 0.04$, A_o^{em} was negligible), A_a^{ex}, A_a^{em} are the acceptor optical densities at the excitation and emission wavelengths, respectively. The heme concentrations used in the RET experiments fall in the range 1.2–5.1 μM (10 mol% CL), 1.4–6.2 μM (50 mol% CL), and 1.2–7.1 μM (80 mol% CL). These concentrations correspond to the following limits of the acceptor optical densities: 10 mol% CL—$A_a^{ex}\sim 0.02$–0.1, $A_a^{em}\sim 0.01$–0.06; 50 mol% CL—$A_a^{ex}\sim 0.03$–0.12, $A_a^{em}\sim 0.02$–0.07; 80 mol% CL—$A_a^{ex}\sim 0.02$–0.14, $A_a^{em}\sim 0.01$–0.08.

Theoretical correction coefficients (k_{th}) calculated from Eq. (1) using the above A_o and A_a values were estimated to be: 10 mol% CL—k_{th} ~ 1.05–1.21; 50 mol% CL—k_{th} ~ 1.06–1.26; 80 mol% CL—k_{th} ~ 1.05–1.3. The validity of these estimates was assessed by comparing the k_{th} values with the empirical correction coefficients (k_{em}). Empirical correction was based on the fact that cyt *c* very weakly binds to lipids at the ionic strengths above 0.5 M [15]. This allowed us to determine the k_{em} values as the ratio of the donor fluorescence intensities measured at the ionic strength of 0.6 M in the absence and presence of the protein. The k_{em} estimates derived in such a way were found to be: 10 mol% CL—k_{em} ~ 1.06–1.29; 50 mol% CL—k_{em} ~ 1.07–1.4; 80 mol% CL—k_{em} ~ 1.06–1.41. Thus, the difference between k_{th} and k_{em} values appeared to be rather small. Finally, k_{em} values were used for obtaining corrected donor fluorescence intensities.

Critical distance of energy transfer between MBA and the heme group of cyt *c* (R_0) calculated as described previously [20] was found to be 3.3 nm. To prevent protein-induced lipid peroxidation all RET experiments were conducted in the presence of antioxidant thiourea (100 mM).

2.2. Theory

The results of RET measurements were quantitatively interpreted in terms of the model of energy transfer in two-dimensional systems [26] extended here by taking into account relative orientations of donors and acceptors. Donors are assumed to be uniformly distributed in two planes confined to the outer and inner bilayer leaflets while acceptors are supposed to be located only at the outer membrane side (Fig. 1). In this case relative quantum yield of a donor (Q_r) can be represented as a sum of two terms corresponding to energy transfer from the outer (Q_{r1}) and inner (Q_{r2}) donor planes:

$$Q_r = 0.5(Q_{r1} + Q_{r2}) = 0.5\left(\int_0^\infty \exp[-\lambda](I_1(\lambda))^{N_1}\mathrm{d}\lambda + \int_0^\infty \exp[-\lambda](I_2(\lambda))^{N_2}\mathrm{d}\lambda\right) \quad (2)$$

$$I_1(\lambda) = \int_{\sqrt{(d_c-0.5d_t)^2+R_e^2}}^{R_d} \exp\left[-\lambda\kappa^2(R)\left(\frac{R_0^r}{R}\right)^6\right] \times\left(\frac{2R}{R_d^2-(d_c-0.5d_t)^2-R_e^2}\right)\mathrm{d}R \quad (3)$$

$$I_2(\lambda) = \int_{d_c+0.5d_t}^{R_d} \exp\left[-\lambda\kappa^2(R)\left(\frac{R_0^r}{R}\right)^6\right] \times\left(\frac{2R}{R_d^2-(d_c+0.5d_t)^2}\right)\mathrm{d}R \quad (4)$$

$$N_1 = \pi C_a^s(R_d^2-(d_c-0.5d_t)^2);\ N_2 = \pi C_a^s(R_d^2-(d_c+0.5d_t)^2) \quad (5)$$

here Förster radius is represented as $R_0^6 = \kappa^2(R)\cdot(R_0^r)^6$, $R_0^r = 979(n_r^{-4}Q_D J)^{1/6}$, $\kappa^2(R)$ is the orientation factor, Q_D is the donor quantum yield, n_r is the refractive index of the medium ($n_r = 1.37$), J is the spectral overlap integral calculated by numerical integration, $\lambda = t/\tau_d$, τ_d is the lifetime of an excited donor in the absence of acceptors, d_c is the acceptor distance from the bilayer midplane, d_t is the separation between donor planes, R_e is the minimum horizontal separation of donor and acceptor (minimum lateral distance between the application points of the donor emission and

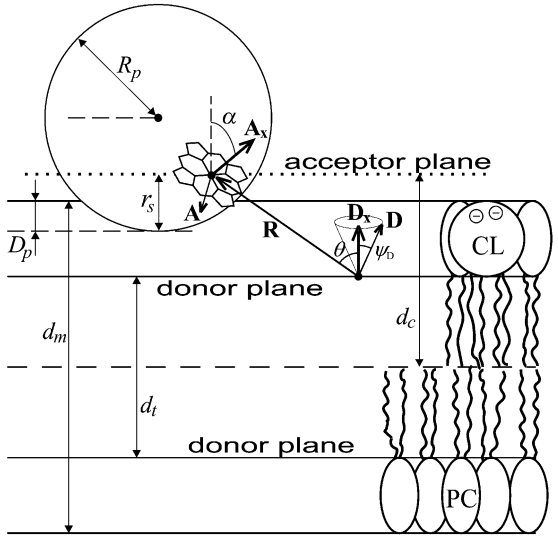

Fig. 1. Scheme of spatial and orientational relationships between donor and acceptor planar arrays in a lipid bilayer.

acceptor absorption transition moments); d_m is the membrane thickness (see Fig. 1), C_a^s is the acceptor concentration per unit area related to the molar concentrations of lipids (L) and bound acceptor (B): $C_a^s = B/(0.5L)\times(f_{PC}S_{PC}+f_{CL}S_{CL})$, f_{PC}, f_{CL} are the mole fractions of PC and CL; S_{PC}, S_{CL} are mean areas per PC and CL headgroups, respectively ($S_{PC}=0.65$ nm^2, $S_{CL}=1.2$ nm^2) [27]. The values of C_a^s have been determined in a separate series of binding experiments as described in more detail elsewhere [19,20].

Assuming that donor emission and acceptor absorption transition moments are axially distributed within a cone with half-angles $\psi_{D,A}$, dynamically averaged value of the orientation factor is given by [21]:

$$\kappa^2 = \kappa^{x2} d_D^x d_A^x + 1/3(1-d_D^x) + 1/3(1-d_A^x) + \cos^2\Theta_D d_D^x(1-d_A^x) + \cos^2\Theta_A d_A^x(1-d_D^x) \quad (6)$$

$$\kappa^{x2} = (\sin\Theta_D \sin\Theta_A \cos\Phi - 2\cos\Theta_D \cos\Theta_A)^2 \quad (7)$$

where Θ_D and Θ_A are the angles between the donor or acceptor cone axis and the vector joining donor and acceptor (**R**); Φ is the angle between the planes containing the cone axes and vector **R**, d_D^x and d_A^x (below referred to as d_D and d_A) are axial depolarization factors

$$d_{D,A}^x = 3/2\langle\cos^2\psi_{D,A}\rangle - 1/2 \quad (8)$$

related to the steady-state r and fundamental r_0 anisotropies of donor and acceptor [21]:

$$d_{D,A}^x = \pm(r_{D,A}/r_{0D,A})^{1/2} \quad (9)$$

In the case of acceptors distributed over a plane the orientation factor appears to be a function of the donor–acceptor separation (R) or the angle θ between vector **R** and normal to bilayer surface ($\mathbf{D}_x$), since $R=|d_c \pm 0.5d_l|\sec\theta$ (Fig. 1). The formalism for describing energy transfer between donors and acceptors whose transition moments are symmetrically distributed about axes parallel to the bilayer normal has been developed by Davenport et al. [28]. In the present paper this formalism has been extended to a more complex case, taking into account the orientational behavior of the heme transition moment in the cyt *c* molecule. We have assumed that in the lipid-bound state cyt *c* adopts certain specific orientation relative to the membrane surface, so that normal to the porphyrin plane ($\mathbf{A}_x$) makes an angle α with the bilayer normal $\mathbf{D}_x$ (Fig. 1). Donor emission and acceptor absorption transition dipoles are considered to be axially symmetrically distributed about $\mathbf{D}_x$ and $\mathbf{A}_x$, lying on the surface of the cones with half-angles ψ_D and ψ_A, respectively. Following the averaging procedure proposed in Dale et al. [21] and applying Soleillet's theorem to describe depolarization of the transition moments one obtains:

$$\kappa^2(\theta) = d_D^x d_A^x d_\alpha (3\cos^2\theta - 1)^2 + \frac{1}{3}(1-d_D^x) + \frac{1}{3}(1-d_A^x d_\alpha) + \cos^2\theta(d_D^x - 2d_D^x d_A^x d_\alpha + d_A^x d_\alpha) \quad (10)$$

where

$$d_\alpha = 3/2\cos^2\alpha - 1/2 \quad (11)$$

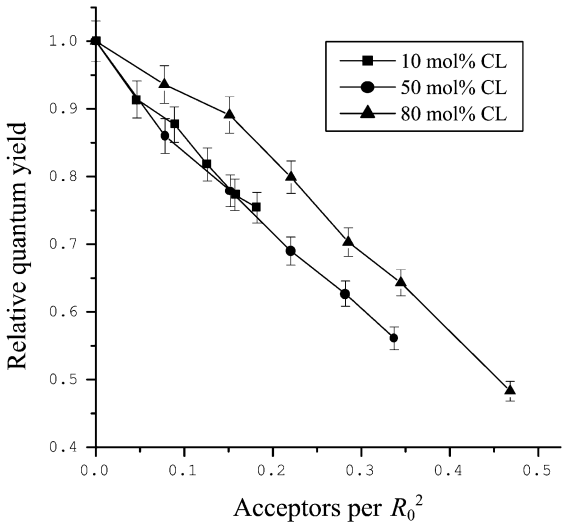

Fig. 2. The relative quantum yield of MBA as a function of acceptor surface concentration. Lipid concentration, mM—0.7 (10% CL), 0.4 (50% CL), 0.3 (80% CL). MBA concentration was 4 μM.

3. Results

Shown in Fig. 2 are the experimental dependencies of the MBA relative quantum yield on the surface concentration of cyt *c* heme groups. To

Table 1
Structural parameters of cytochrome *c*–lipid complexes

R_e	PC:CL (9:1, mol:mol)				PC:CL (1:1, mol:mol)				PC:CL (1:4, mol:mol)			
	d_c	D_P^{min}	D_P^s	D_P^{max}	d_c	D_P^{min}	D_P^s	D_P^{max}	d_c	D_P^{min}	D_P^s	D_P^{max}
0	3.0	0	0.2	2.3	2.6	0.3	0.6	2.7	3.3	0	0	2.0
1.5	2.5	0.4	0.7	2.8	2.2	0.7	1.0	3.1	2.9	0	0.3	2.4
2.0	2.2	0.7	1.0	3.1	1.9	1.0	1.3	3.4	2.6	0.3	0.6	2.7
2.5	1.8	1.1	1.4	3.5	1.5	1.4	1.7	3.8	2.2	0.7	1.3	3.1
3.0	1.3	1.6	1.9	4.0	0.9	2.0	2.3	4.4	1.8	1.1	1.7	3.5
3.5	0.8	2.1	2.4	4.5	$\rightarrow 0$				1.4	1.5	2.1	3.9

All values are given in nanometers.

extract quantitative information on the heme bilayer location the results of RET measurements were analyzed in terms of the above energy transfer model. As seen from the relationships (6,10) application of this model requires knowing the depolarization factors d_D, d_A and d_α. The value of d_D was calculated from Eq. (9). Steady-state anisotropy measurements yielded r_D of approximately 0.05 for MBA bound to PC/CL model membranes regardless of their CL content. The fundamental anisotropy was taken from the data reported elsewhere (0.28 at $\lambda_{ex}=440$ nm [30]). By substituting these anisotropy values in Eq. (9) one obtains $d_D=0.4$. In d_A determination we used linear dichroism data indicating that transition moment of cyt *c* lies within the porphyrin plane [5]. In this case $\psi_A=\pi/2$ and $d_A=-0.5$.

The estimation of d_α was based on the assumption that cyt *c* possesses specific lipid-binding site responsible for certain preferable orientation of the protein molecule with respect to bilayer surface. According to the literary data this site is likely to contain clustered lysine residues, particularly, Lys_{72} and Lys_{73} [16]. Thus, one can assume that positively charged nitrogen atoms of Lys_{72}, Lys_{73} and one of the adjacent clustered lysines (86, 87, 88, 79, 39, 53, 55) lie in the protein–lipid contact plane. Being parallel to the membrane surface this plane makes an angle α with the porphyrin ring. Using crystallographic coordinates of cyt *c* [29] we have found that for the aforementioned lysine residues the angle α lies between 58 and 82° (depending on the third lysine added to Lys_{72} and Lys_{73} in the contact plane definition). These values were used in calculating d_α according to Eq. (11).

The estimates of d_D, d_a and d_α were further employed in analyzing the quenching profiles presented in Fig. 2 within the framework of the model described by Eqs. (2)–(5) and (10). This analysis was aimed at estimation of the heme distance from the bilayer center (d_c) by the fitting procedure involving minimization of the function:

$$f=\frac{1}{n_a}\sum_{i=1}^{n_a}(Q_r^e-Q_r^t)^2 \qquad (12)$$

where Q_r^e is the experimental Q_r value, Q_r^t is the relative quantum yield calculated by numerical integration of Eqs. (2)–(5) and (10), n_a is the number of acceptor concentrations employed in the RET experiments. In the data fitting parameter d_c was optimized while parameters d_t and R_e were held constant. Since hydrophobic fluorescent probe MBA is supposed to be located at the boundary between polar and non-polar membrane regions, distributing between the outer and inner bilayer leaflets [30], the values of d_t were chosen to be close to the thickness of hydrocarbon core, being approximately 2.4 nm [27]. Parameter R_e characterizing minimum lateral donor–acceptor distance was varied in the limits 0–3.5 nm. When $R_e=0$ the protein does not exclude any area in the outer donor plane while at the greater R_e values cyt *c* is supposed to pierce this plane. The upper R_e limit was taken to be consistent with the size of cyt *c* molecule.

Shown in Table 1 are the estimates of heme distance from the bilayer midplane providing the best fit of experimental quenching profiles. The *f* values derived in the data treatment fall in the range from 1.5×10^{-4} to 3×10^{-3}.

4. Discussion

As a first step in analyzing the results presented here it is important to justify the assumptions underlying the approach by which quantitative information on the structure of the cyt *c*–lipid model system was extracted from the RET studies. The main point of these assumptions concerns the existence of the preferential orientation of cyt *c* with respect to the membrane surface. The idea that the cyt *c* molecule contains specific lipid-binding site was put forward as early as 1971 [3] and developed in a series of further works [5,16,17]. The rationale of this idea comes from crystallographic studies that recovered such structural features of cyt *c* as segregation of acidic and basic groups and arrangement of non-polar amino acids residues into the two hydrophobic 'channels' connecting the heme crevice with the protein surface [3]. The opening of each of these channels is surrounded by a cluster of basic groups pertaining to lysine residues. This fact has led a number of authors to propose that one of such positively charged clusters is involved in cyt *c* binding to mitochondrial lipids, particularly, to negatively charged CL. Recent studies have provided evidence for the existence of two distinct acidic phospholipid binding sites in the cyt *c* molecule, denoted as A- and C-sites [16,17]. The A-site is thought to account for electrostatic interactions between cyt *c* and deprotonated phospholipids, while the C-site is responsible for the protein association with protonated phospholipids via hydrogen bonding. Given the peculiarities of the cyt *c* structure it has been suggested that the A-site contains clustered basic residues, specifically, the invariant lysines located at positions 72 and 73 [16]. Thus, it is highly probable that Lys_{72} and Lys_{73} are involved in cyt *c* binding to the PC/CL model membranes examined in the present study. This allowed us to suppose that there exists a particular protein–lipid contact plane containing Lys_{72} and Lys_{73}. To describe this fact mathematically, an angle between the contact plane and porphyrin ring (α) was introduced in the RET model and calculated from crystallographic data. The existence of specific protein orientation relative to the bilayer surface implies that this angle is identical for all acceptors involved in energy transfer.

However, it is evident that the above α estimates must be considered as a rough approximation to the real situation since structural features of the protein free in solution cannot be strictly derived from crystallographic data. In view of this it seemed of importance to ascertain to what extent RET efficiency depends on the angle α. Interestingly, for d_D and d_A values derived in the present study theoretical curves Q_r (C_a^s) and, as illustrated in Fig. 3, parameters recovered from the curve-fitting procedure, were found to exhibit no marked dependence on α. This means that the approach proposed here does not permit answering the question of what site at the protein surface is responsible for the formation of protein–lipid contacts.

The next point noteworthy concerns the rationales underlying the choice of the bounds for distance of closest approach of donors and acceptors (R_e). As indicated above, this parameter is sensitive to the magnitude of the lipid-induced conformational changes of the protein molecule. A number of studies provide evidence for destabilization of cyt *c* tertiary structure on the protein association with anionic phospholipids [11,12,31]. Solid state ^{2}H-NMR [12] and FTIR [31] studies revealed loosening and reversible unfolding of the cyt *c* molecule at the lipid–water interface. An increased susceptibility of cyt *c* to denaturation by urea was observed on the protein binding to lipid micelles [34]. All these findings suggest that the lipid-bound state of cyt *c* bears a resemblance to a molten globule state occurring as an intermediate on the protein denaturation [35]. Although the molten globule state is featured by the increased intramolecular mobility and loosening of the overall protein structure, an increase of the hydrodynamic radius of the protein molecule does not exceed 20% [35]. According to X-ray data cyt *c* can be approximated by a spheroid with dimensions $3\times3.4\times3.4$ nm [3,29]. Taking the heme radius to be 0.6 nm and supposing that the average size of cyt *c* to change by a factor of no more than 1.2 it follows that on the protein association with lipids maximum possible distance of the heme center from the protein surface is approximately 3 nm. Given that MBA radius is approximately 0.6

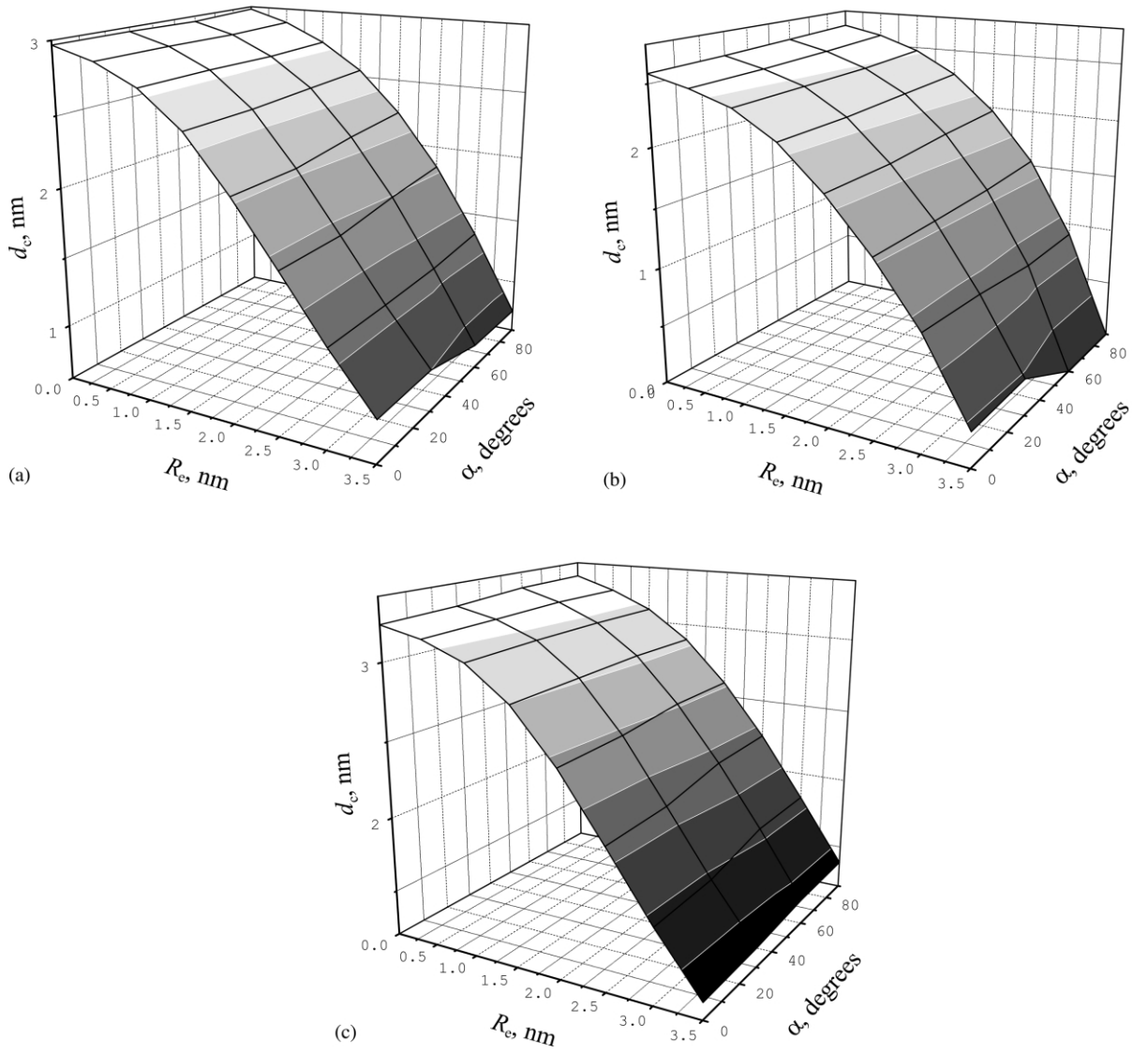

Fig. 3. Three-dimensional diagrams illustrating the relationships between the heme distance from the bilayer center (d_c), lateral distance of closest approach between donor and acceptor (R_e) and the angle between the porphyrin plane and membrane surface (α). CL content, mol%: A, 10; B, 50; C, 80.

nm [36], the reasonable choice for the maximum possible lateral distance between the acceptor and donor centers seems to be 3.5–4 nm.

In the following it seems of importance to consider the factors that may affect the randomness of the donor or acceptor distribution thereby rendering the above analysis untenable. These include: (i) the formation of negatively charged lipid clusters on the binding of cyt *c* to bilayers composed of neutral and anionic phospholipids [1,32]; and (ii) aggregation of the lipid-bound protein [33]. Lipid clustering phenomena are analyzed in several theoretical models proposed by Mosior and McLaughlin [37], Denisov et al. [38], Heimburg et

al. [39], May et al. [40]. A series of models have been developed to consider the case of acceptor exclusion from the protein aggregate [41]. The concepts of fractal geometry have been used to describe energy transfer from the lipid domain to the protein aggregate [42].

In the model systems lipid clustering or protein aggregation are observed under the strict experimental conditions. The magnitude of these effects depends on the surface concentration of the bound peptide or protein. For instance, fluorescence imaging microscopy study of the domain-forming properties of the basic peptide pentalysine revealed that in the DOPC/DOPS (9:1) bilayer lipid clusters are formed in the restricted region of peptide concentrations—from 0.1 to 2 mM ($L=0.1$ mM) [38]. Likewise, the size of lipid domains was demonstrated to change as the peptide concentration increased. The domains enriched in acidic phospholipids were observed on the binding of cyt *c* to PC/PA (19:1) model membranes [43]. It was found that the size and extent of domain formation depend on cyt *c* concentration, ionic strength and membrane composition. In the presence of 10 μM of cyt *c* ($L=0.5$ mM) small multiple domains were observed. As far as the aggregation of lipid-bound cyt *c* is concerned, the magnitude of this effect was demonstrated to be crucially dependent on temperature [31,33]. IR-studies of cyt *c*–CL systems provided no evidence for the protein aggregation at the temperatures below 30 °C [31].

As indicated above, the RET measurements discussed here have been performed at room temperature (25 °C) with the protein concentration varying from 1 to 7 μM and lipid concentration of 0.7 mM (10 mol% CL), 0.4 mM (50 mol% CL) and 0.3 mM (80 mol% CL). Under these conditions the concentration of the protein free in solution appeared to be negligibly small, i.e. the concentration of bound protein (B) was close to the total protein concentration (P). This was proven in the separate series of binding and RET experiments conducted with lipid concentrations varied from 0.1 to 0.5 mM and protein concentration lying between 1 and 6 μM. The quenching efficiency was found to decrease as the lipid concentration increases. Furthermore, taken together all 18 measured Q_r values yielded a single exponential-like curve $Q_r\ (P/L)$ with the deviation of some points lying within the experimental uncertainty. These findings suggest that $B \sim P$ within the employed ranges of P and L since if B were dependent on L we would observe scattered experimental points instead of the quenching profile identical for all B/L ratios used in the experiment. The fact that being plotted vs. P/L (rather than vs. B/L) Q_r values are concentrated near to a single curve with no abnormalities could be interpreted as indicating that the quenching pattern does not undergo marked changes as the surface concentration of bound protein increases. In other words, within the employed range of cyt *c* concentrations the randomness of the donor or acceptor distribution is unlikely to be considerably disturbed. On the contrary, if chromophore distribution becomes non-random the quenching conditions will change with varying protein concentration so that the quenching profile $Q_r\ (P/L)$ might be expected to be non-exponential. Based on the above arguments we have concluded that in the system under study the extent of lipid clustering or protein aggregation is rather small, i.e. these phenomena do not manifest themselves under the experimental conditions of our RET measurements.

Fig. 3 shows how the heme distance from the bilayer midplane, being the optimizing parameter in the data fitting, depends on the fixed parameters R_e and α. It seemed of interest to ascertain what bilayer positions of the protein are consistent with the recovered d_c values. Denoting the vertical distance from the acceptor plane to the protein surface being in contact with lipids as r_s the depth of the protein bilayer penetration can be calculated from $D_p=0.5d_m-(d_c-r_s)$. Taking the radius of the porphyrin ring to be 0.6 nm and assuming that association of cyt *c* with lipids causes the average diameter of the protein to increase by not greater than 20% (from 3 to 3.6 nm, as on the transition to molten globule state) the limits for r_s would be $r_s^{\min}\cong 0.6$ nm and $r_s^{\max}\cong 3$ nm. Then, the lower and upper estimates for the depth of the cyt *c* bilayer penetration can be obtained as follows:

$$D_P^{\min}=0.5d_m-d_c+r_s^{\min};$$
$$D_P^{\max}=0.5d_m-d_c+r_s^{\max} \quad (13)$$

where d_m is the bilayer thickness that for the model membranes is known to be approximately 4.6 nm [44]. Derived in such a way D_P limits (Table 1) correspond to the two extreme cases when the porphyrin ring is oriented perpendicularly to the bilayer surface and located in the hemispheres of the protein globule facing the lipid or aqueous phase, respectively. Additionally, a more unequivocal D_P estimate (D_P^s) can be obtained in terms of the above assumption as to the involvement of Lys_{72} and Lys_{73} in the formation of protein–lipid contacts:

$$D_P^s = 0.5d_m - d_c + r_s^s; \quad r_s^s \cong R^*_P - r_t \sin\alpha \qquad (14)$$

where r_t is the distance between the center of the protein globule and application point of the heme transition moment, R^*_P is the average radius of the cyt *c* molecule in the lipid-bound state. Taking R^*_P to be 1.8 nm, r_t is 1.1 nm [5], and $\alpha \approx \pi/3$ one obtains that r_s^s is approximately 0.9 nm. As seen in Table 1, most of D_P estimates are suggestive of cyt *c* penetration in the membrane interior. Importantly, these estimates do not exclude the possibility of practically full insertion of the cyt *c* molecule in the lipid bilayer. Such a possibility is corroborated by NMR data indicating that in CL-containing bilayers cyt *c* can bring about the rearrangement of lipid molecules into inverted micelles and the hexagonal H_{II} phase [14]. On the formation of such structures the protein molecule, bearing net positive charge, appears to be entrapped into the interior of aqueous cylinders spanning the membrane or in the polar core of inverted micelles. This mechanism of cyt *c* bilayer penetration does not require any significant redistribution of the polar and non-polar amino acid residues.

On the other hand, lipid-induced conformational changes of the protein involving loosening or unfolding of cyt *c* structure [9–11,13] are supposed to promote exposure of non-polar protein areas accounting for cyt *c* insertion into the membrane core [7]. However, it should be noted that there exists another mechanism of hydrophobic protein–lipid interactions that is not associated with the penetration of cyt *c* in the hydrocarbon region. This mechanism involves extension of the lipid tails in the opposite directions from the headgroup and further accommodation of one acyl chain within the non-polar cavity of cyt *c* [17]. Such changes in the structural state of lipid molecules are hardly probable to occur without partial penetration of cyt *c* in the bilayer interior, up to the level of initial carbons of acyl chains. The results presented here are consistent with this possibility.

In summary, it seems of importance to emphasize that the estimates obtained for the depth of cyt *c* bilayer penetration strongly depend on the choice of the putative lipid-binding site on the protein surface. Taking into account one of the models of cyt *c* association with anionic phospholipids [17] we have analyzed the results of RET measurements on the assumption that this site contains clustered lysine residues, specifically, Lys_{72} and Lys_{73}. The main advantage of the RET model employed in this study over those used previously [19,20] consists in a more adequate analysis of orientational behavior of donors and acceptors. However, this model appears to be inappropriate in treating the case of non-random chromophore distribution that may originate from the formation of lipid domains or cyt *c* aggregation. Further RET-based structural characterization of cyt *c*–lipid systems requires the acquisition of unambiguous data by the proper choice of experimental conditions and application of more sophisticated theoretical approaches.

5. Nomenclature

cyt *c*: cytochrome *c*
PC: phosphatidylcholine
CL: cardiolipin
MBA: 3-methoxybenzanthrone
RET: resonance energy transfer
DOPC: dioleoylphosphatidylcholine
DOPS: dioleoylphosphatidylserine
PA: phosphatidic acid

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