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Structure of cytochrome *c* complexes with phospholipids as revealed by resonance energy transfer

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Abstract

Resonance energy transfer between a series of lipid-bound fluorescent probes as donors and the heme group of cytochrome *c* as acceptor has been used to obtain structural information on the protein complexes with model membranes, composed of phosphatidylcholine and cardiolipin. Analysis of experimental data in terms of the model of energy transfer in two-dimensional systems provides further evidence for preferential cytochrome *c* orientation with respect to the lipid bilayer and penetration of the protein into the membrane interior.

Keywords: Fluorescence energy transfer; Cytochrome *c*; Protein-lipid complex

1. Introduction

Model systems consisting of cytochrome *c* (cyt *c*), the peripheral protein of the inner mitochondrial membrane, and lipid monolayers or vesicles are presently extensively investigated in two main aspects. The first aspect involves gaining insight into molecular mechanisms and functional consequences of cyt *c* association with a lipid component of the mitochondrial membrane [1–3], while the other one concerns elucidating the general features of protein-lipid interactions, manifested themselves in the formation of cyt *c* complexes with lipids [4–6]. A substantial body of evidence obtained to date suggests that the process of cyt *c* binding to the lipid bilayer includes the following main steps: (i) protein adsorption on the membrane surface via electrostatic interactions and hydrogen bonding, (ii) conformational changes of the protein molecule, (iii)structural alterations of the lipid phase, (iv) partial or full insertion of the protein into the membrane interior [7,8]. Under physiological conditions, cyt *c* carries a net positive charge ([8,9]), determining a high affinity of the protein to lipid bilayers containing negatively charged phospholipids. One of such phospholipids, cardiolipin (CL), attracts a particular interest as putative component of cyt *c* binding sites at the inner mitochondrial membrane [1]. Two mechanisms are assumed to be responsible for cyt *c* adsorption to the lipid bilayer surface [4,9,10]. The first mechanism involves electrostatic binding of positively charged amino acid residues such as lysine or arginine to a deprotonated headgroup of negatively charged phospholipids, while the other one consists of the formation of hydrogen bonding between the protein side groups and protonated phospholipid headgroup. Association of cyt *c* with the bilayer surface is followed

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by structural changes of the protein involving alterations of the conformational state of the heme group and loosening or unfolding of the overall protein structure [11–14]. Such changes in cyt *c* conformations are supposed to promote its penetration into the membrane interior with subsequent hydrophobic interactions between non-polar residues of a polypeptide chain and hydrocarbon chains of the lipids [8,14]. It should be noted, however, that cyt *c* insertion in the membrane inner region can be mediated not only by protein conformational alterations, but also by modification of the lipid organization. Particularly, in CL-containing model membranes, cyt *c* can induce the formation of non-bilayer structures, followed by the protein trapping into aqueous cylinders spanning the bilayer [15]. All these findings suggest that the process of cyt *c* association with lipids must not be interpreted within the framework of a simplified electrostatic mechanism, postulated for peripheral membrane proteins. Electrostatic interactions are usually considered to be non-specific, depending on the net charge of the protein and the surface potential of the lipid bilayer, while hydrogen bonding, that requires complementarity between the contact regions in the protein-lipid complex, accounts for specific interactions between the protein and lipids. However, it seems likely that not only the net charge but also the charge distribution on the surface of the protein molecule can determine the character of the protein-lipid interactions. For instance, the clustering of positively charged groups in the protein surface may lead to appearance of an area, providing the most thermodynamically favorable contacts with the lipid bilayer. This may enhance the specificity of electrostatic binding, that, like hydrogen bonding, can be the reason for a certain preferential orientation of the protein molecule with respect to the bilayer surface. Elucidating such an aspect of the protein-lipid interactions requires structural information on the protein complexes with lipids. One approach to obtain such information is based on examining resonance energy transfer (RET) between fluorophores, bound to lipids and intrinsic protein chromophores, particularly a heme group. A previous study of RET between fluorescent probe 3-methoxybenzanthrone (MBA) and the heme group of cyt *c* suggests that there exists preferential protein orientation relative to the lipid bilayer, composed of phosphatidylcholine (PC) and CL. The main goal of the present work was to obtain further evidence for the specific disposition of cyt *c* in complexes with CL-containing model membranes by monitoring RET between donors, localized in the lipid phase and heme group of cyt *c*. A series of donors being employed includes MBA, *N*,*N*′-bis-hexamethylenrhodamine (RH), rhodamine 6G (R6G), 4-(dimethylaminostyryl)-1-dodecylpiridine *n*-toluenesulfonate (DSP-12), 4-(dimethylaminostyryl)-1-methylpiridine *n*-toluenesulfonate (DSM).

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). Bovine heart cyt *c* and R6G were obtained from Reakhim (Russia). MBA, DSM and DSP-12 were from Zonde (Latvia). RH was kindly supplied by Victorova E.N. (Optical Institute, St. Petersburg, Russia). 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 [16]. 1 ml of the ethanol lipid solution containing appropriate amounts of PC and CL was injected into 13 ml 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 [17].

2.3. Fluorescence measurements

Fluorescence measurements were performed with a Signe spectrofluorimeter (Latvia). Emission spectra of fluorescent probes were excited at 440 nm (MBA), 460 nm (DSM, DSP-12), 490 nm (R6G, RH). Excitation and emission slit widths were set at 5 nm. The fluorescence intensity measured in the presence of cyt *c* was corrected for reabsorption and

inner filter effects using the following coefficients [18,19]:

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

where A is the donor absorbance in the absence of the protein, A_s is the total absorbance of the sample at excitation or emission wavelengths. Quantum yields of the donors in liposomal suspensions were estimated using a fluorescein solution as standard [20]. The critical distance of energy transfer (R_o, nm) was calculated as [18]:

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

where J is the overlap integral, n_r is the refractive index of the medium ($n_r = 1.37$), K^2 is an orientation factor ($K^2 = 2/3$), Q_D is the donor quantum yield. Concentrations of donors employed in RET experiments were (μM): 6 (MBA), 2 (R6G), 2 (RH), 3 (DSM), 2 (DSP-12). Cyt *c* was used in the oxidized state. To prevent protein-induced lipid peroxidation, the anti-oxidant thiourea was added to the liposomal suspensions in a concentration of 100 mM.

2.4. Theory

The theoretical background for analyzing RET in membranes is provided by a number of models, elaborated for two-dimensional systems [21–26]. In the present study, the results of RET measurements were quantitatively interpreted in terms of the modified model of Wolber and Hudson [22], as described in more detail previously [27]. Donors are assumed to be uniformly distributed in two planes, localized in the outer and inner bilayer leaflets, while acceptors are supposed to be situated only on the outer side of membranes. According to the model employed, the relative quantum yield of donor (Q_r) is given by:

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

$$I_1(t) = \int_{R_e}^{R_d} \exp[-\lambda (R_0/R)^6] W_1(R) dR \quad (4)$$

$$I_2(t) = \int_{R_e}^{R_d} \exp[-\lambda (R_0/R)^6] W_2(R) dR \quad (5)$$

where Q_D, Q_{DA} are 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, $W_1(R)dR$, $W_2(R)dR$ are the probabilities of finding acceptor in the annulus between radii R and $R+dR$ 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 energy transfer is insignificant. If the concentration of acceptors per unit area is equal to C_a^s, N can be written as:

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

If the acceptors are assumed to be situated on the outer side of the membrane and separated from a nearest plate of donors by a distance d_a, the expression for $W_1(R)$ is given by:

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

By denoting the distance separating donor planes, d_t, and considering the case when donors are localized deeper than acceptors, $W_2(R)$ can be written as:

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

If acceptors are situated deeper than donors, $W_2(R)$ is given by:

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

3. Results

RET investigation of cyt *c* complexes with phospholipids included the following main steps: (i) assessment of the quantum yield and critical distance of energy transfer for various donors, (ii) a binding study, aimed at determining the amount of cyt *c* associated with liposomal membranes, (iii) obtaining and analyzing dependencies of the relative quantum yield on the surface acceptor concentration.

3.1. Characterization of donors

To test the conclusions drawn in the previous study of RET between MBA and heme of cyt *c* [27] and to obtain more reliable structural information, the present work was focused on analysis of data sets, combining the results of RET measurements for a series of donors. All donors employed are rather well-characterized with respect to their spectral properties and localization in the lipid bilayer [20]. DSM and R6G, possessing a positive charge at neutral pH, reside at the lipid-water interface, orienting in such a way that a positively charged nitrogen atom is located in the vicinity of phosphate groups, while the aromatic ring is situated in the polar region of the bilayer. DSP-12, differing from DSM by the hydrocarbon chain, consisted of 12 carbons, penetrates to a greater extent into the membrane interior, localizing presumably at the boundary of polar and non-polar parts of the lipid phase. A similar localization is also supposed for neutral hydrophobic probes RH and MBA, situated in the glycerol backbone region between hydrophilic headgroups of phopholipids and non-polar acyl chains. The quantum yield of all donors bound to liposomal membranes, except R6G, appears to be ca. two orders of magnitude greater as compared to that in buffer solution. Therefore, the contribution of free probe to the measured fluorescence intensity seems to be negligibly small. For R6G, the values of the quantum yield in buffer and suspension of liposomes were comparable, though fluorescence spectra of the dye exhibit a marked (ca. 10 nm) blue shift, suggesting its effective association with lipids. In view of this, special experiments have been performed, aimed at evaluating the degree of probe binding to liposomes. It appeared that at R6G and lipid concentrations, employed in RET measurements, the contribution of free probe to the total fluorescence intensity does not exceed 2%.

Presented in Table 1 are the quantum yields of the donors employed and critical distances of energy transfer, calculated according to Eq. 2. For DSM and DSP-12, quantum yield exhibits a marked dependence on the CL content of liposomal membranes, while for the other probes, such a dependence appears to be less pronounced (RH, MBA) or insignificant (R6G).

3.2. Binding of cyt c to liposomes

As follows from the statements of the model used, adequate quantitative interpretation of the results of RET measurements in membrane systems requires knowing the surface acceptor concentration (C_a^s) being proportional to the molar concentration of bound protein (B). Estimation of B was made within the framework of the simplest binding model (Langmuir isotherm), using an approach described in detail in the previous work [27]. The underlying assumption of this approach is that the lipid-induced decrease of cyt *c* absorbance in the Soret band (ΔA_{407}) is proportional to the concentration of bound protein (B):

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

Where a is a coefficient of proportionality. Denoting the total protein and lipid concentrations by P_o and L_o, respectively, the association constant (K_b) can be written as:

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

where n is the number of lipid molecules constituting a protein binding site on the membrane surface. Rearranging Eqs. 10 and 11, one obtains:

Table 1
Some characteristics of donors, employed in resonance energy transfer measurements

CL, mol%	Quantum yield					Critical distance of energy transfer, nm				
	DSM	DSP	R6G	NRH	MBA	DSM	DSP	R6G	NRH	MBA
10	0.07	0.19	0.89	0.18	0.12	2.83	3.23	4.77	3.42	3.67
20	0.10	0.17	0.89	0.15	0.12	3.01	3.22	4.76	3.32	3.67
40	0.13	0.15	0.90	0.16	0.11	3.18	3.15	4.77	3.36	3.68
60	0.13	0.13	0.90	0.17	0.11	3.20	3.05	4.78	3.39	3.68
90	0.13	0.12	0.90	0.17	0.09	3.18	3.01	4.78	3.39	3.68

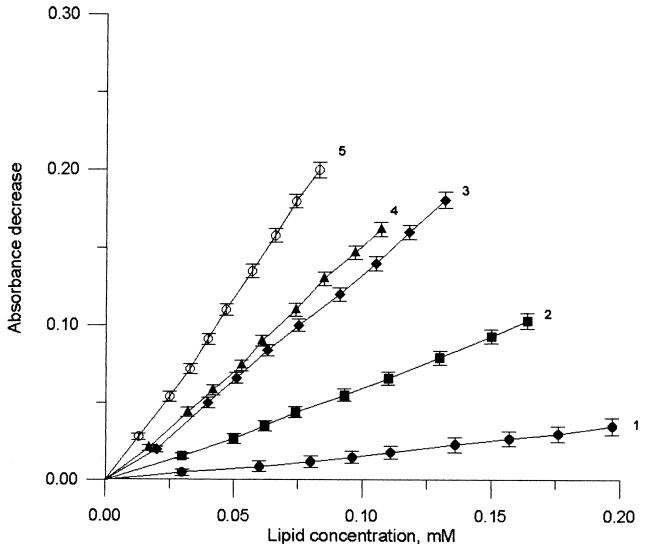

Fig. 1. Decrease of cyt *c* absorbance at 407 nm in liposomal suspensions as a function of the lipid concentration. CL content, mol%: 1, 10; 2, 20; 3, 40; 4, 60; 5, 90. The protein concentration was 5.4 μM.

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

Eq. 12 was used for determining binding parameters (K_b, n) and the coefficient of proportionality (a) for each of the lipid-protein systems studied. Initial estimates of these parameters, derived from the double reciprocal plots [20,27], were subsequently iteratively improved in the fitting procedure, based on comparison of the experimental value of L_o with that calculated according to Eq. 12. Fig. 1 shows typical plots of ΔA_{407} versus L_o, analyzed in terms of the aforementioned approach. Quantitative characteristics of cyt *c* binding to liposomes obtained in this way (Table 2) were used for calculation of the concentration bound protein according to the relationship stemming from Eq. 11:

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

The surface acceptor concentration was then estimated as follows:

$$C_a^s = \frac{N_A B}{S_L} \quad (14)$$

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

where N_A is the Avogadro number f_{PC}, f_{CL} are mol fractions of PC and CL, respectively, S_{PC}, S_{CL} are mean areas per lipid molecule, taken to be 0.65 nm^2 [28] and 1.2 nm^2 [29], respectively. To represent the concentration of bound acceptor in units commonly used for 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 seems to be of importance to note that the lipid-induced decrease of cyt *c* absorbance at the Soret band is usually interpreted in terms of conformational changes of the protein, originating from its capability to cause lipid peroxidation [30]. Taking into account that any alteration in the heme absorbance would give rise to a change of R_o values, depending on the spectral overlap of donor and acceptor, all RET measurements were performed in the presence of anti-oxidant (thiourea). On the contrary, in the binding studies, no anti-oxidant was added to the suspension of liposomes.

Another question noteworthy concerns the validity of K_b and n values, obtained according to the simplest binding model described by a Langmuir isotherm. Although such an approach is rather convenient, it seems to be inadequate in analyzing protein-lipid systems. As indicated in a lot of studies, a more

Table 2
Parameters of cyt *c* binding to liposomes

Cardiolipin content, mol%	K_b, ($\times 10^5$ M^{-1})	n	a ($\times 10^4$)
10	6.2 ± 1.1	103 ± 25	4.8 ± 1.2
20	7.1 ± 1.2	51 ± 11	8.1 ± 1.9
40	7.6 ± 1.2	47 ± 9	16 ± 4
60	0.6 ± 0.1	14 ± 3	21 ± 3
90	0.9 ± 0.2	15 ± 3	23 ± 3

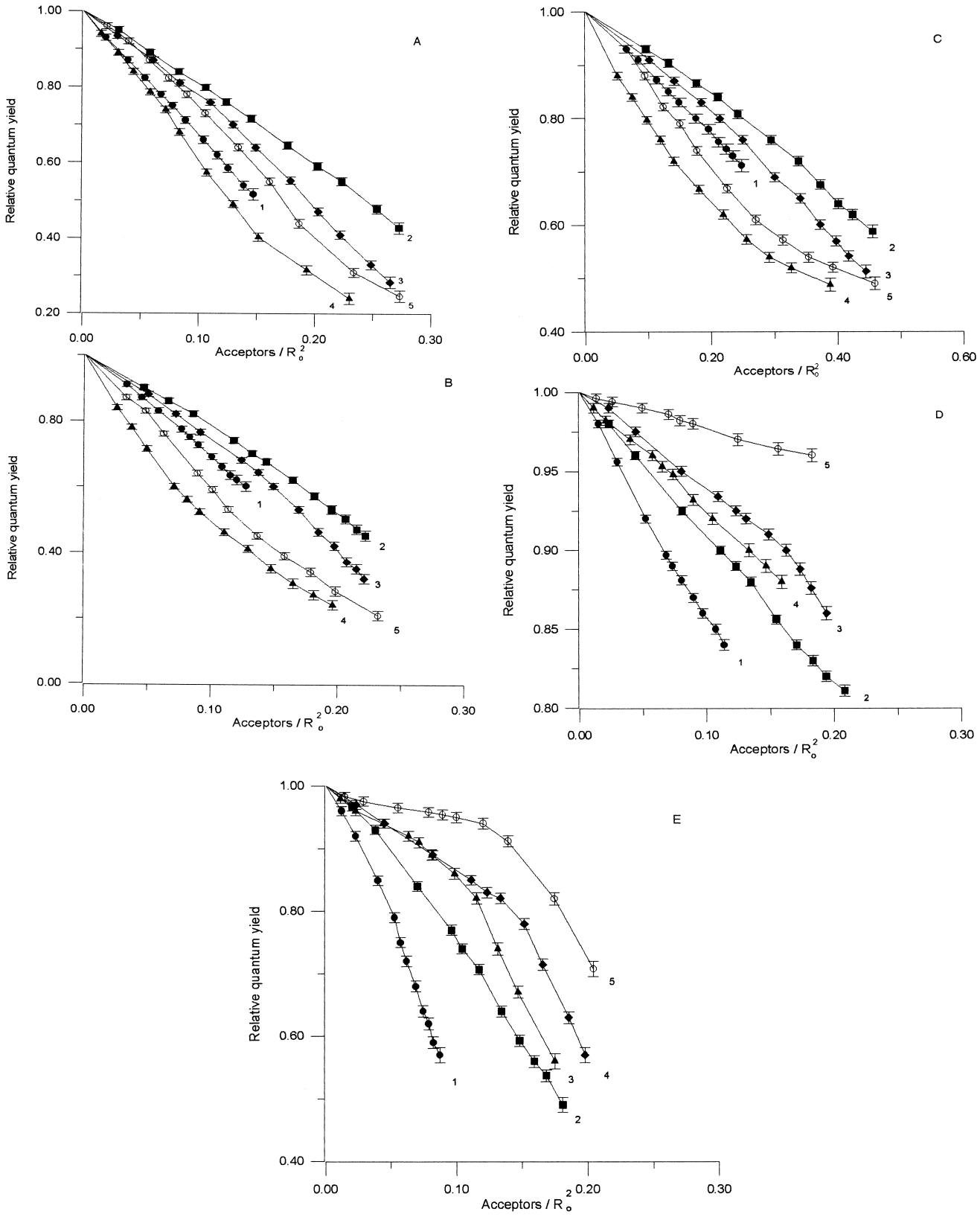
A
B
C
D
E
Relative quantum yield
Acceptors / R$_o^2$
1.00
0.80
0.60
0.40
0.20
0.00
0.10
0.20
0.30
0.95
0.90
0.85
1
2
3
4
5

Fig. 2. The relative quantum yield of donors versus the concentration of heme groups of cyt *c*, bound to liposomes: A, MBA; B, RH; C, R6G; D, DSP-12; E, DSM. CL content, mol%: 1, 10; 2, 20; 3, 40; 4, 60; 5, 90. Lipid concentration, mM: 1, 0.42; 2, 0.35; 3, 0.28; 4, 0.23; 5, 0.18.
←

correct description of the protein binding to the lipid bilayer is provided by the models of non-localized adsorption of large ligands to the surface [6,31,32]. However, within the framework of the present work, application of the simplest binding scheme seems to be reasonable, since parameters K_b and n are used only for evaluation of the concentration of bound protein, being independent of the model employed.

3.3. Resonance energy transfer studies

Fig. 2 shows the plots of the relative quantum yield of the donors versus the concentration of heme groups of the protein bound to liposomal membranes. A further data analysis was aimed at obtaining the sets of parameters R_e, d_a and d_t, giving the best fit of experimental Q_r values (Q_r^e) to Eqs. 3–9. The fitting procedure involved minimization of the function:

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

where Q_r^t is the Q_r value derived from numerical integration of Eq. 3, n_a is the number of acceptor concentrations employed in RET measurements. Parameters d_t and R_e were allowed to vary in the ranges, consistent with the real dimensions of the lipid bilayer and protein molecule. For donors located at the boundary between polar and non-polar regions of the membrane (DSP-12, RH, MBA), the values of d_t were chosen to be close to the thickness of the hydrocarbon core, being ca. 2–2.4 nm [28]. Given that the size of the hydrophilic region of the lipid bilayer is ca. 1 nm, for DSM and R6G, situated in the vicinity of phospholipid headgroups, d_t limits were taken to be 3–3.6 nm. Parameter R_e, characterizing the minimum possible distance between the centers of the probe and heme, equals the sum of the donor radius, being ca. 0.5–0.6 nm [20], and the distance from the heme center to the protein surface (r_h). A molecule of cyt *c* is known to be a spheroid with dimensions $3\times3.4\times3.4$ nm and a heme group located in the center of the protein, ca. 1.5 nm from its surface [33]. Thus, for a native protein molecule R_e will be ca. 2 nm. However, as indicated above, cyt *c* binding to the lipid bilayer is accompanied by significant alterations in the protein conformation, involving changes in the environment and coordination of the heme group and structural reorganization of the polypeptide chain [34–36]. In view of this, it seemed reasonable to vary R_e in a rather wide range from 1.5 to 3.5 nm.

Another important problem encountered in RET studies consists in the correct choice of the value of the orientation factor (K^2) upon R_o calculation Eq. 2. Coefficient K^2, commonly used in the analysis of RET data (0.67), corresponds to a random reorientation of the donor emission and acceptor absorption moments during the emission lifetime. However, if the position of acceptor or donor is characterized by a certain preferential orientation relative to the lipid bilayer, the K^2 value would differ from 0.67. Such a situation may take place when the formation of cyt *c* complexes with a membranes is mediated by specific lipid binding sites. In this case, orientation of the heme dipole cannot be considered as random. In addition, a restricted mobility of fluorescent probes in the lipid phase may narrow the range of possible orientations for donor dipole, thus increasing the difference between the actual K^2 value and the isotropic one (0.67). In principle, K^2 can alter from 0 to 4, the limits corresponding to perpendicular and parallel orientations of donor and acceptor dipoles [18]. Taking into account the results of the previous RET study suggesting that for the donor-acceptor pair MBA-heme of cyt *c*, the K^2 value differs from 0.67 [27], in analyzing the data presented here, K^2 was allowed to vary in the range 0–4.

4. Discussion

Analysis of the results of RET measurements, performed with a series of donors, yields numerous sets of parameters, d_a, d_t, R_e, K^2, providing a satisfactory

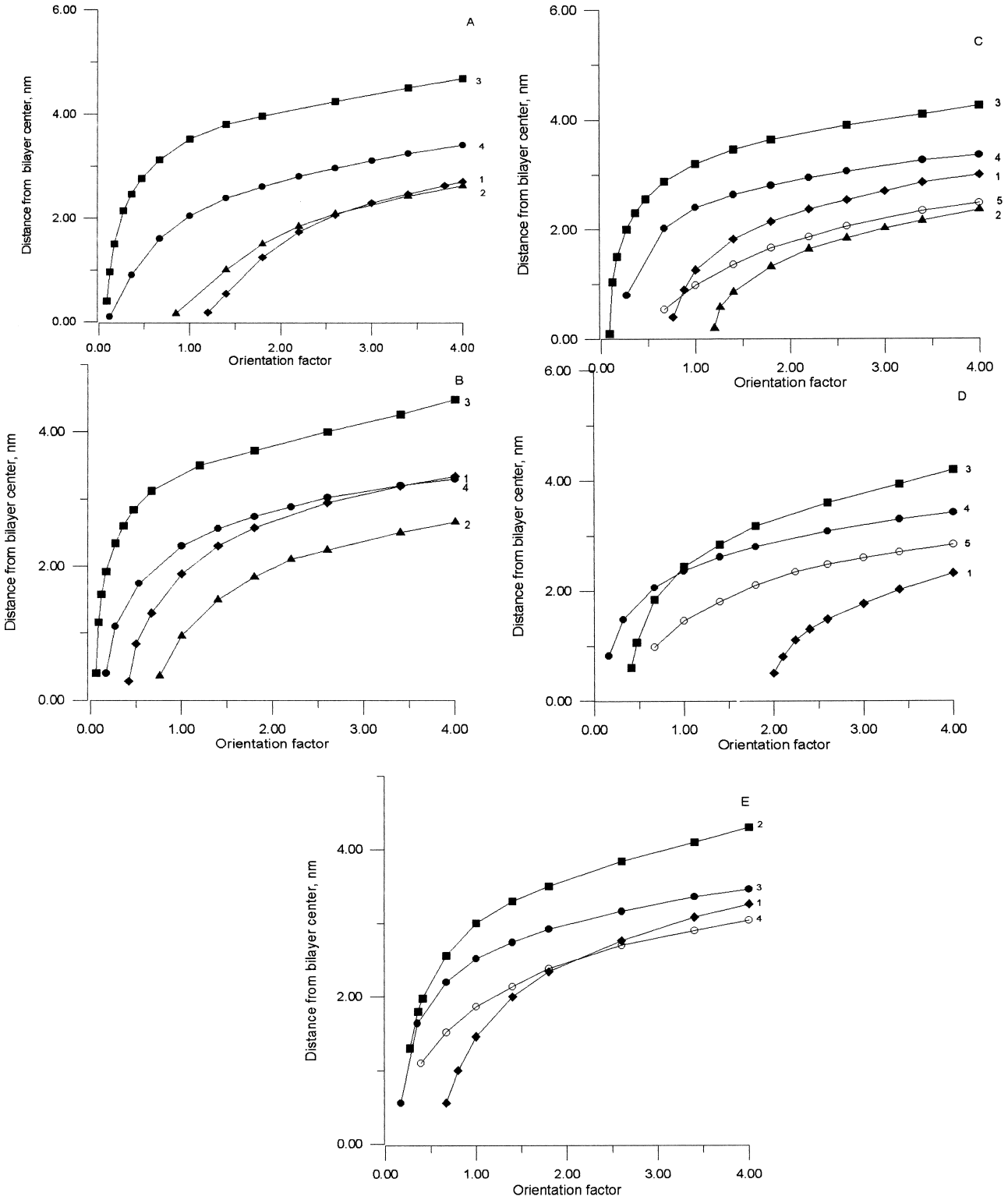

Fig. 3. The distance of cyt *c* heme from the lipid bilayer center as a function of the orientation factor: CL content, mol%: A, 10; B, 20; C, 40; D, 60; E, 90. Donors: 1, MBA; 2, RH; 3, R6G; 4, DSP-12; 5, DSM (A–C); 1, MBA; 2, R6G; 3, DSP-12; 4, DSM (D, E).

fit of experimental dependencies $Q_r(C_a^s)$ to those calculated within the framework of the model employed. The data obtained were used subsequently to estimate the heme distance from the bilayer midplane (d_c) as follows:

$$d_c = 0.5d_t + d_a \quad (17)$$

when donors are situated closer to the membrane center than acceptors and

$$d_c = 0.5d_t - d_a \quad (18)$$

in the opposite case.

Fig. 3 illustrates the relationships between d_c and K^2 obtained with $R_e = 2$ nm, the value being most probable for an unmodified protein structure. It appeared that for each of the donors used, there exists a certain minimum K^2 value (K^2_{min}), corresponding to $d_c = 0$, and maximum d_c value (d_c^{max}), derived for $K^2 = 4$. In the case of random reorientation of donors and acceptors, one should expect the intersection of the plots d_c (K^2), obtained for different donors, in the point corresponding to $K^2 = 0.67$ and the actual d_c value. The absence of such an intersection for the curves, presented in Fig. 3, may be considered as an additional argument in favor of a specific orientation of the heme group of cyt *c* with respect to the lipid bilayer. Another support for this assumption comes from the findings indicating that for some donors (MBA, RH), minimum possible K^2 values for most of the protein-lipid systems studied exceed 0.67 (Table 3). In view of this, it seems of importance to emphasize that the idea concerning preferential cyt *c* orientation in complexes with phospholipids was put forward in the early RET study of Teissie [37]. Considering K^2 as optimizing parameter in the data fitting led to the conclusion that cyt *c* associated with liposomes, composed of PC and phosphatidic acid, adopts a specific position relative to the lipid bilayer. Such a behavior of cyt *c* in complexes with lipids may originate from peculiarities of its structure. According to X-ray data, on the surface of the cyt *c* molecule, acidic and basic groups are segregated into two positively charged patches with a negative patch between them [33]. Involvement of one of the positively charged patches in the interactions with lipids may account for specific protein disposition with respect to the membrane surface.

It seems noteworthy that in some cases (DSM, 10 and 20 mol% CL; RH, 60 and 90 mol% CL), data fitting Eqs. 3–9 was unsuccessful, being indicative of a contribution of other factors, besides RET, in the changes of the donor quantum yield. For DSM, this may be the competition between the protein and probe for the binding sites on the bilayer surface, that was found to take place at a low CL content. Competitive binding of DSM and cyt *c* to liposomes would lead to a decrease of donor fluorescence, being independent of RET. In the case of RH, the decrease of Q_r with increasing C_a^s turned out to be more drastic than that predicted by the model. As demonstrated by Wolber & Hudson [22], one reason for such a Q_r behavior may be the formation of a complex between donor and acceptor. Given that cyt *c*, bound to liposomes, can adopt an unfolded conformation, one cannot exclude, in principle, the possibility of RH binding to the heme group of the protein.

Since parameter d_c is invariant for a given kind of

Table 3
The limits of orientation factor (K^2_{min}), heme distance from the lipid bilayer center (d_c^{max}) and the depth of protein penetration in the membrane interior (d_P^{min}), recovered from the analysis of RET data assuming that $R_e = 2$ nm

Donor	10 mol% CL		20 mol% CL		40 mol% CL		60 mol% CL		90 mol%CL	
	K^2_{min}	d_c^{max}	K^2_{min}	d_c^{max}	K^2_{min}	d_c^{max}	K^2_{min}	d_c^{max}	K^2_{min}	d_c^{max}
MBA	1.2	2.7	0.4	3.3	0.7	3	1.4	2.3*	0.6	3.3
RH	1.3	2.6*	0.8	2.7*	1.2	2.4*	[a]	[a]	[a]	[a]
R6G	0.1	4.9	0.06	4.5	0.1	4.3	0.4	4.2	0.2	4.4
DSP-12	0.3	3.6	0.2	3.3	0.1	3.4	0.1	3.4	0.1	3.5
DSM	[a]	[a]	[a]	[a]	0.5	2.5	0.4	2.8	0.2	3*
d_P^{min}, nm	1.2		1.1		1.4		1.5		0.8	

The values of d_c^{max} are given in nm.
[a] Unsuccessful fitting.

liposomes, it seems reasonable to assume that the most probable d_c values fall in the region of overlap and the actual d_c^{max} will be equal to the least of the values derived for various donors. As can be seen from Table 3, for liposomes, containing 10, 20 and 40 mol% CL, the minimum d_c^{max} was recovered with RH and for liposomes, containing 60 or 90 mol% CL, with MBA and DSM, respectively. The results of RET measurements, performed with these donors, were subsequently analyzed to obtain dependencies of d_c on R_e. Fig. 4 shows such dependencies for maximum possible d_c values, estimated for liposomes, differing in CL content, while Fig. 5 illustrates relationships between parameters d_c, R_e and K^2.

Unfortunately, the data obtained do not allow to draw any unambiguous conclusions regarding the role of the membrane composition (particularly CL content) in determining the heme localization in the protein-lipid complex. This is caused, in particular, by the possibility of varying degrees of protein conformational changes with an increasing CL content, leading to different R_e values for various liposomes. Besides, one cannot rule out that the heme orientation relative to the bilayer, contributing to the K^2 value, depends on the fraction of CL in liposomal membranes. To elucidate these questions, further studies are needed.

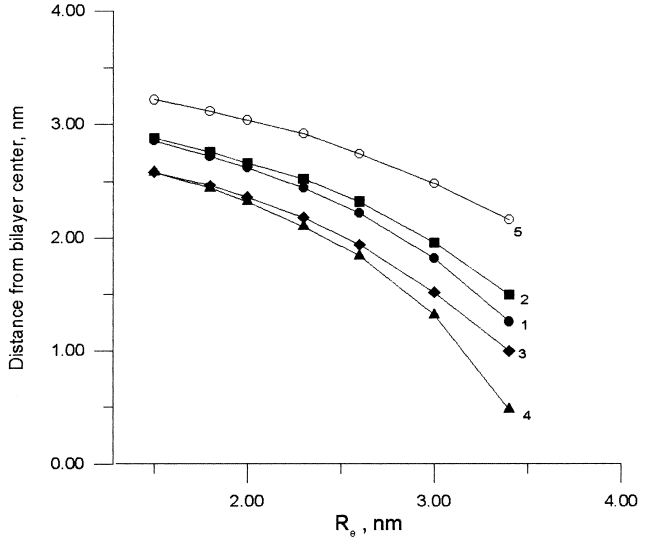

Fig. 4. Plots of the maximum possible distance of cyt *c* heme from the lipid bilayer center versus R_e. CL content, mol%: 1, 10; 2, 20; 3, 40; 4, 60; 5, 90. Donors: 1–3, RH; 4, MBA; 5, DSM.

Supposing that heme is localized in the center of the protein molecule, on the basis of d_c estimates, one can determine the depth of the protein penetration in the membrane interior (d_P):

$$d_P = 0.5d_m - (d_c - r_h) \quad (19)$$

where d_m is the thickness of the lipid bilayer, being ca. 4.6 nm [28]. If no appreciable conformational changes of cyt *c* occur, the heme distance from the protein surface (r_h) would be ca. 1.5 nm. However, a lot of evidence indicates that negatively charged phospholipids, including CL, induce loosening or partial unfolding of the cyt *c* molecule [34–36]. This destabilization of the protein structure, originating, presumably, from its electrostatic interaction with lipids, is thought to be followed by exposure of non-polar amino acid residues accounting for the protein insertion into the membrane interior [8,14]. It seems probable that conformation of cyt *c* in complexes with phospholipids resembles the molten globule state, being intermediate upon the protein denaturation. Although this state is characterized by a drastically increased intermolecular mobility of the protein and overall loosening of its tertiary structure, the size of the globule does not alter significantly (an increase of the hydrodynamic radius of the protein does not exceed 20%) [38]. Given these observations, the reasonable choice for the possible r_h range appears to be ca. 1.5–2 nm. Thus, using Eq. 19, one can estimate the lower (d_P^{min}) and upper (d_P^{max}) limits for the depth of cyt *c* penetration into the membrane interior:

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

$$d_P^{max} = 0.5d_m + r_h^{max} - d_c^{min} \quad (21)$$

Presented in Table 3 are the estimates of d_P^{min}, derived for d_c^{max}, being equal to the values marked by an asterisk. Note that d_P^{max} in all cases equals 4.3 nm ($d_c^{min} = 0$). Taking into account that the size of the polar region of the lipid bilayer is ca. 1 nm [28], the results obtained appear to be indicative of partial or full penetration of cyt *c* in the membrane interior. These data are in agreement with some observations reported in the literature. In particular, examination of cyt *c* interaction with dipalmitoylphosphatidylcholine by Raman spectroscopy has shown that the pro-

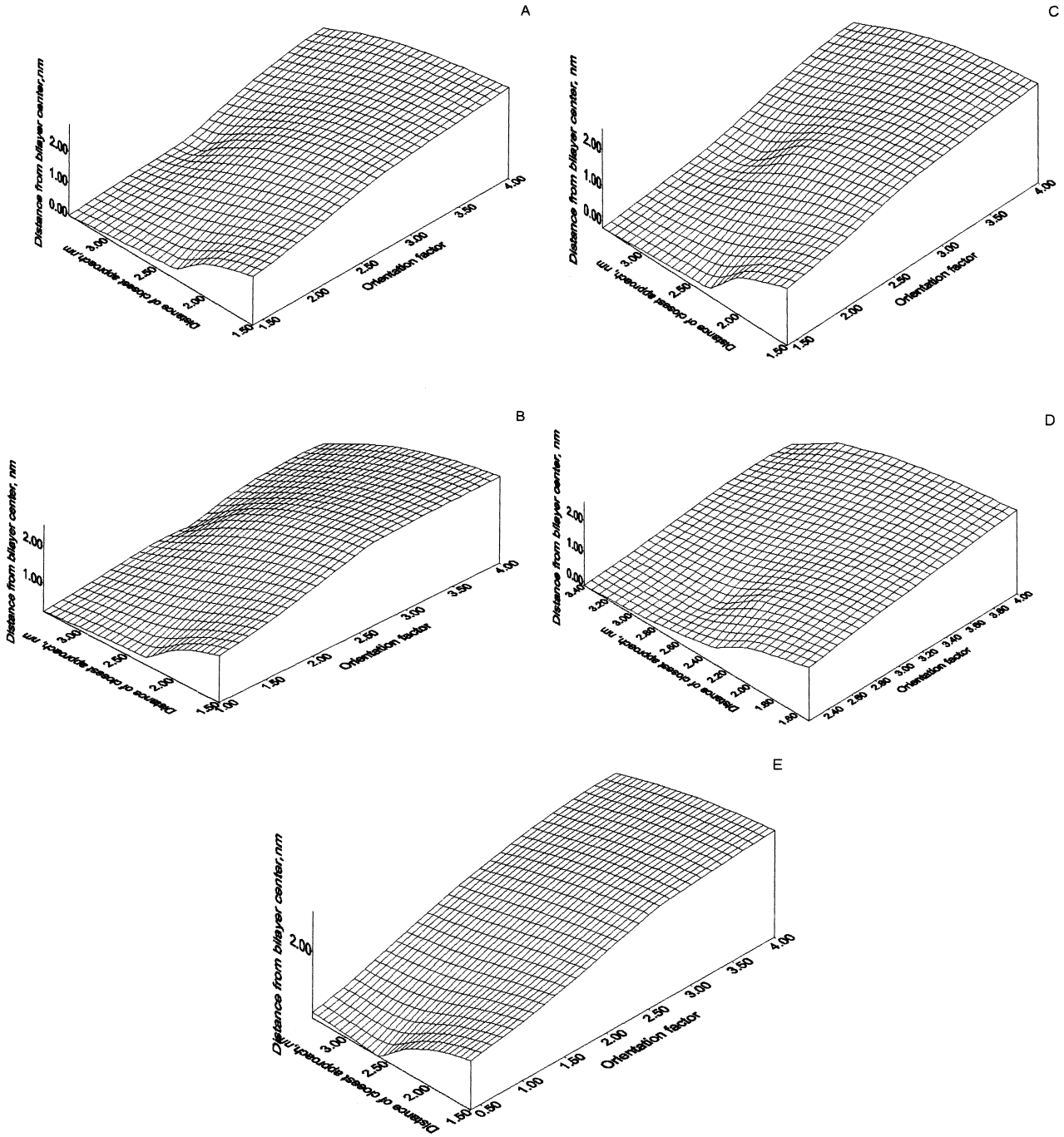

Fig. 5. Three-dimensional plots illustrating the relationships between the heme distance from the bilayer center, the distance of closest approach between donor and acceptor and the orientation factor. CL content, mol%: A, 10; B, 20; C, 40; D, 60; E, 90. Donors: A–C, RH; D, MBA; E, DSM.

tein can penetrate into the acyl chain region [39]. Other arguments in favor of such a possibility have been provided by calorimetric studies of cyt *c* complexes with dioleoylphosphatidylglycerol [40]. Using surface plasmon resonance spectroscopy to investigate cyt *c* binding to lipid membranes, composed of PC and CL, Salamon and Tollin have obtained evidence for the protein unfolding and subsequent hydrophobic interactions with lipids [8]. In this context, it seems of importance to emphasize that the

protein unfolding is not the only possible reason for cyt *c* penetration into the membrane interior. As demonstrated in comprehensive studies of de Kruijff et al. [15], cyt *c* association with CL-containing model membranes can induce the formation of non-bilayer structures, particularly inverted micelles and a hexagonal H_{II} phase. Such a modification of the lipid organization is thought to be accompanied by protein incorporation into the aqueous cylinders spanning the bilayer. The data presented here, being suggestive of the full protein insertion in the liposomal membrane, do not exclude, in principle, the possibility of formation of non-bilayer structures.

In summary, the results of this study provide further evidence for a specific orientation of cyt *c*, associated with model membranes composed of PC and CL. The capability of cyt *c* to adopt a particular position in complexes with phospholipids can be attributed to a peculiar distribution of amino acid residues on the protein surface. It seems probable that a certain spatial arrangement of the protein side groups leads to the formation of a site, providing the most thermodynamically favorable contacts with lipids. Specificity of cyt *c* binding to the lipid bilayer, originating, presumably, from electrostatic protein-lipid interactions and hydrogen bonding, may have both structural and functional consequences. Quantitative interpretation of the results of RET measurements suggests that cyt *c* can partially or fully insert into the membrane interior. Possible lower and upper limits for the depth of protein penetration in the lipid bilayer were ascertained to be 0.8 and 4.3 nm, respectively. A specific orientation of cyt *c* in complexes with phospholipids and its ability to penetrate in the membrane interior may significantly contribute to the protein functioning as a component of the mitochondrial electron transport chain.

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