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CYTOCHROME *C* – CARDIOLIPIN INTERACTIONS: EXTENDED LIPID ANCHORAGE REVISITED

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Resonance energy transfer (RET) from antrylvinyl-labeled (AV) lipids to the heme moiety of cytochrome *c* (cyt *c*) has been employed to assess the molecular level details of cyt *c* interactions with negatively charged lipid membranes composed of phosphatidylcholine (PC) and its mixture with 10 or 20 mol % of cardiolipin (CL). At the lowest ionic strength used here (20 mM) RET profiles from neutral (AV-PC) and anionic (AV-CL) donor were virtually indistinguishable, suggesting that the peculiarities of cyt *c* association with lipid membranes containing different donors are identical. In contrast, elevating ionic strength up to 40 and 60 mM resulted in expected decrease of energy transfer efficiency in the case of AV-PC containing liposomes, but not for those with AV-CL where RET exhibited an unexpected enhancement with increasing ionic strength. Monte Carlo analysis of the results obtained allowed us to attribute this untypical behavior to the transition of CL into extended lipid conformation. The revealed peculiarities of cyt *c* – CL interactions are of great interest not only from the viewpoint of regulating cyt *c* electron transfer and apoptotic propensities but also for elucidating the general mechanisms by which membrane functional activities can be modulated by protein-lipid interactions.

KEY WORDS: cytochrome *c*, cardiolipin, extended lipid conformation, ionic strength

ВЗАИМОДЕЙСТВИЕ ЦИТОХРОМА *C* С КАРДИОЛИПИНОМ: ВЫТЯНУТАЯ КОНФОРМАЦИЯ ЛИПИДА СНОВА В ФОКУСЕ

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С помощью метода индуктивно-резонансного переноса энергии (ИРПЭ) с антрилвинил-меченых (АВ) липидов на гемовую групу цитохрома *c* исследованы молекулярные детали связывания белка с модельными липидными мембранами, состоящими из фосфатидилхолина (ФХ) и его смесей с 10 или 20 мол. % кардиолипина (КЛ). При ионной силе 20 мМ профили ИРПЭ для нейтрального (АВ-ФХ) и анионного (АВ-КЛ) донора были одинаковыми, указывая на то, что особенности взаимодействия цитохрома *c* с липидными мембранами, содержащими разные доноры, идентичны. В отличие от этого, повышение ионной силы до 40 и 60 мМ приводило к предсказываемому уменьшению эффективности переноса энергии в случае с АВ-ФХ и неожиданному усилению ИРПЭ в случае с АВ-КЛ. Анализ полученных результатов методом Монте-Карло позволил интерпретировать наблюдаемые эффекты в рамках перехода КЛ в вытянутою конформацию. Полученные данные важны не только с точки зрения регулирования биологических функций цитохрома *c*, но и в контексте фундаментального исследования общих механизмов модулирования функциональной активности мембран белок-липидными взаимодействиями.

КЛЮЧОВІ СЛОВА: цитохром *c*, кардиолипин, вытянутая конформация, ионная сила.

ВЗАЄМОДІЯ ЦИТОХРОМУ *C* З КАРДІОЛІПІНОМ: ВИТЯГНУТА КОНФОРМАЦІЯ ЛІПІДУ ЗНОВ У ФОКУСІ

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За допомогою індуктивно-резонансного переносу енергії (ІРПЕ) з антрилвініл-мічених (АВ) ліпідів на гемову групу цитохрому *c* було досліджено молекулярні деталі зв'язування білка з модельними ліпідними мембранами, що складалися з фосфатидилхоліну (ФХ) та його сумішей із 10 або 20 мол. % кардіоліпіну (КЛ). При іонній силі 20 мМ профілі ІРПЕ для нейтрального (АВ-ФХ) та аніонного (АВ-КЛ) донору не відрізнялися один від одного, вказуючи на те, що особливості взаємодії цитохрому *c* з ліпідними мембранами із різними донорами ідентичні. На противагу цьому, підвищення іонної сили до 40 або 60 мМ призводило до передбачуваного зменшення ефективності переносу енергії у випадку з АВ-ФХ та неочікуваного посилення ІРПЕ у випадку з АВ-КЛ. Аналіз отриманих результатів методом Монте-Карло дозволив інтерпретувати спостережувані ефекти у рамках переходу КЛ у витягнуту конформацію. Отримані дані важливі не тільки з точки зору регулювання біологічних функцій цитохрому *c*, але й у контексті фундаментального дослідження загальних механізмів модулювання функціональної активності мембран білок-ліпідними взаємодіями.

КЛЮЧОВІ СЛОВА: цитохром *c*, кардіоліпін, витягнута конформація, іонна сила.

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The molecular details of interactions between two major mitochondrial membrane components, water-soluble protein cytochrome *c* (cyt *c*) and anionic lipid cardiolipin (CL), remains for a long time in the main focus of a good deal of studies [1-3]. Inextinguishable interest to this problem is determined by significant physiological relevance of cyt *c* – CL complexation. Specifically, the biological consequences of this process were shown to embrace the following aspects: i) cyt *c* – mediated electron transport, ii) programmed cell death (apoptosis) triggered by protein release from mitochondria into cytosol, iii) lipid-induced formation of cyt *c* amyloid fibers [4-6]. A number of works indicates that cyt *c* – CL binding includes several important stages: i) protein adsorption onto lipid bilayer surface, ii) conformational changes of cyt *c* coupled with structural perturbations of a lipid bilayer, iii) protein insertion into membrane hydrophobic core [7-9]. Along with this, some aspects specific only for cyt *c* – CL association have been reported. Accordingly, the existence of two CL binding sites on cyt *c,* namely, A- and C-site, has been proposed. The former one facilitates electrostatic contacts between positively charged protein patches and CL anionic headgroups. The latter one is involved in hydrophobic interactions between cyt *c* and lipid fatty acyl chains. An alternative "extended lipid anchorage" model of hydrophobic interactions has been proposed according to which lipid acyl chains point to the opposite directions from the headgroup producing straight angle of 180° [10]. In such an orientation one acyl chain remains within the bilayer interior, while the other extends outwards and accommodates in the protein hydrophobic channel located near heme crevice. Although being clearly identified, this abnormal protein-lipid conformation needs precise sophisticated methods of detection. Among the diversity of experimental techniques used for examination of protein-lipid interactions, the utility and versatility of resonance energy transfer (RET) for resolving spatial details of proteolipid complexes are indubitable. In the present study we applied RET from antrylvinyl-labeled PC (AV-PC) or CL (AV-CL) to the heme moiety of cyt *c* to detect and characterize the extended lipid conformation adopted by CL upon cyt *c* binding to the model lipid membranes composed of phosphatidylcholine (PC) and CL with molar fractions of anionic lipid 10 and 20 mol%.

MATERIALS AND METHODS

Bovine heart cardiolipin, horse heart cyt *c* (oxidized form), NaCl, HEPES and EDTA were purchased from Sigma (St. Louis, MO, USA). 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine was from Avanti Polar Lipids (Alabaster, AL). Fluorescent lipids, 1-acyl-2-(12-(9-anthryl)-11-trans-dodecenoyl)-sn-glycero-3-phosphocholine (AV-PC), and 1-acyl-2-(12-(9-anthryl)-11-trans-dodecenoyl)-sn-glycero-3-phospho-1-rac-cardiolipin (AV-CL) were synthesized as described in detail elsewhere [11]. Large unilamellar lipid vesicles were prepared by the extrusion method. AV-PC or AV-CL (0.26 and 0.13 mol% of total lipid, respectively) were added to the mixture of PC and CL prior to the solvent evaporation. To incorporate cytochrome *c* into the lipid bilayers, prepared liposome suspensions were incubated with protein of required concentration for 15 minutes at room temperature. Steady-state fluorescence spectra were recorded with LS-50B spectrofluorometer equipped with a magnetically stirred, thermostated cuvette holder (Perkin-Elmer Ltd., Beaconsfield, UK). AV-PC and AV-CL emission spectra were recorded with 367 nm excitation wavelength. Excitation and emission slit widths were set at 5 nm. Hereafter, liposomes containing 10 or 20 mol% CL are referred to as CL10 or CL20, with the subscript denoting the type of energy donor (AV-PC or AV-CL). The datasets obtained at different ionic strength are referred to as CL10/I20 or CL20/I40, where the figure after slash stands for the salt concentration in mM.

RESULTS AND DISCUSSION

In an attempt to identify the extended lipid conformation we based our experimental strategy on the analysis of the differences in RET profiles from AV-PC and AV-CL. The main idea of our approach was to detect lipid anchorage against the background of other processes occurred in protein-lipid systems employed here. Speaking in more detail, if there is no difference between $CL_{AV\text{-}PC}$ and $CL_{AV\text{-}CL}$ systems, it may be supposed that the peculiarities of cyt *c* association with lipid membranes containing different donors are identical. Otherwise, the conclusion about specific interactions between the protein and liposomes with different donors can be made.

In order to obtain the comprehensive picture of the process under study, the relative quantum yield (Q_r) of donor (AV-PC or AV-CL) was measured as a function of acceptor (cyt *c*) concentration upon varying the ionic strength (20, 40 and 60 mM) and CL content (10 or 20 mol%). Fig. 1 represents the quenching profiles of AV fluorescence in the presence of cyt *c* acquired at different ionic strength. As seen from the figure, at ionic strength 20 mM ($I = 20$ mM) the efficiencies of energy transfer from neutral (AV-PC) and anionic (AV-CL) donor are virtually indistinguishable, suggesting that the processes occurring in both $CL_{AV\text{-}PC}$ and $CL_{AV\text{-}CL}$ systems are similar. In contrast, increasing ionic strength gives rise to the modification in curve behaviour.

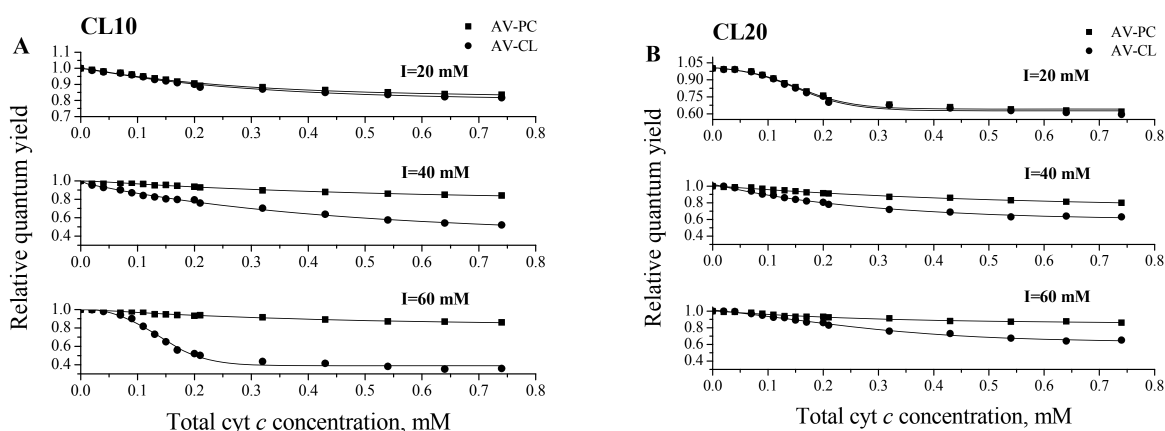

Fig. 1. Comparison of AV-PC and AV-CL quenching profiles obtained in CL10 (A) and CL20 (B) liposomes at different ionic strength. Lipid concentration was 20 μM. Solid lines are presented to guide the reader's eye.

Conceptually, rise in monovalent ion concentration would attenuate electrostatic protein-lipid interactions thereby resulting in overall decrease of energy transfer efficiency. This was the case for AV-PC containing liposomes, but not for those with AV-CL where RET exhibited an unexpected enhancement with increasing ionic strength. As a consequence, difference between the relative quantum yields of AV-PC and AV-CL (ΔQ_r) did increase with ionic strength, this effect being most pronounced in CL10 membranes (Fig. 1). The first evident factor that could explain this finding is local lipid lateral redistribution upon cyt *c* adsorption onto the surface of oppositely charged membranes – CL molecules moving towards the membrane bound protein to minimize the electrostatic free energy of complexation. Factors that would facilitate the preferential interactions of protein with anionic lipids can be identified as: a) presence of clusters of positively charged amino acid residues on the protein surface allowing one protein molecule to interact simultaneously with several anionic lipids; b) conformational flexibility of a polypeptide chain to promote the formation of protein conformer in which the distance between positive charges and anionic lipid headgroups is minimal; c) physiological stimuli providing the screening of electrostatic

repulsion between charged lipids – increasing salt concentration and decreasing pH. To account for lipid demixing effect quantitatively, we developed Monte Carlo simulation procedure, the validity of which has been demonstrated in our previous study [12]. Briefly, AV-CL donors were considered as being randomly distributed within disk-shaped domains of radius r_{dm} centered at each acceptor's location. Our ultimate goal was to determine characteristic domain size, i.e. dimensions of the protein-affected region where CL concentration is k times higher than that for a random lipid distribution. However, this basic model turned out to be inapplicable for satisfactory fitting of the experimental curves at $I = 40$ mM and $I = 60$ mM – the relative error of calculation was found to be $\delta > 15$ % with theoretical value being every time much higher than the experimental one. For the sake of comparison, reconciliation of the CL10/I20 data with theoretical computation in terms of protein-lipid association model which does not account for lipid demixing effect, corresponds to $\delta < 2$ %. A question arises what are the reasons for the observed discrepancy between simulation and experimental data acquired at $I = 40$ and $I = 60$ mM? To answer this question, we put forward and tested several hypotheses concerning the possible factors which may substantially enhance the energy transfer at increasing salt concentration. Among them are imperfection of theoretical models, aggregation of membrane-bound cyt *c*, protein-induced lipid peroxidation, transition of CL into extended lipid conformation. Intriguingly, only the last conjecture was found to be correct, i.e. only adoption by CL the extended lipid anchorage is the main process controlling the revealed RET enhancement in $CL_{AV\text{-}CL}$ membranes at elevated ionic strength. This phospholipid frustration is specific for CL and is dictated by amphiphile tendency to minimize the bending stress created by high negative curvature. It was supposed that strong electrostatic interactions between a network of positively charged amino acid residues (Lys_{72}, Lys_{73} and Lys_{86}) and deprotonated (DP) cardiolipin in the case of A-site, and H-bonds between Asn_{52} and partially protonated (HP) lipid in the case of C-site, hold the acyl chain in the protein groove [10,13,14]. If one assumes that CL adopts such a conformation and acyl tail bearing the AV chromophore protrudes out of a membrane entering the hydrophobic cavity of cyt *c*, the enhancement of energy transfer might be expected due to the reduction of donor-acceptor separation distance.

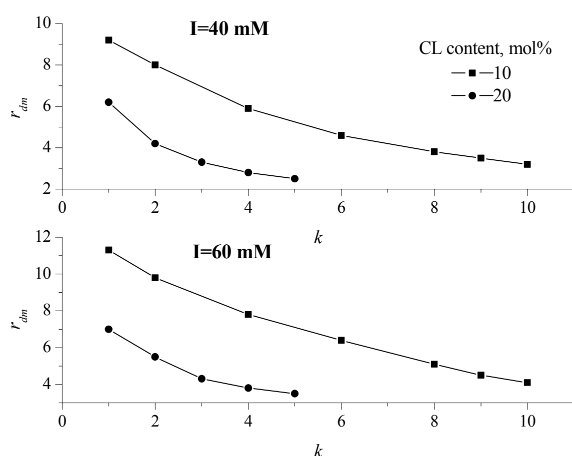

Fig. 2. Membrane domain characteristics recovered from the combined "lipid demixing + extended lipid anchorage" Monte-Carlo model for CL10 (■) and CL20 (●) systems at ionic strength 40 (upper panel) and 60 mM (lower panel).

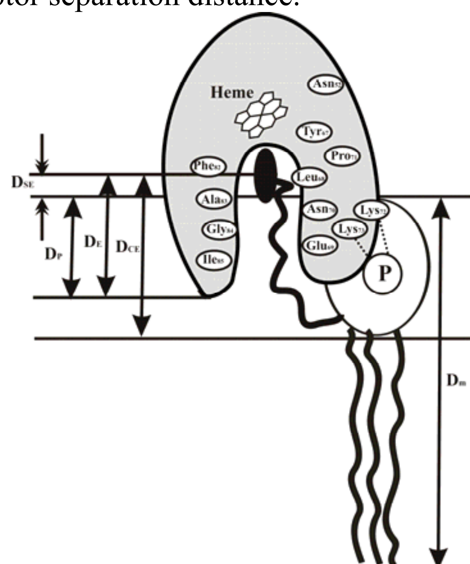

Fig. 3. Schematic presentation of tentative topography of extended lipid conformation. Highlighted are amino acid residues constituting cyt *c* hydrophobic channel. *P* stands for CL phosphate. Black oval represents AV moiety. See text for explanation.

Indeed, allowing for this phenomenon in MC simulation by varying the zeta coordinate of donor and introducing the additional parameter D_{CE} standing for the distance between bilayer center and the plane of donors adopting the extended conformation provided satisfactory fit of experimental results for CL10 and CL20 liposomes. Approximation of the experimental data by the combined "domain + extended lipid conformation" MC model yielded the radius of CL-enriched areas to fall in the ranges 3.2 – 9.2 ($CL10_{AV\text{-}CL}$/I40), 2.5 – 6.2 ($CL20_{AV\text{-}CL}$/I40), 4.1 – 11.3 ($CL10_{AV\text{-}CL}$/I60) and 3.5 – 7 nm ($CL20_{AV\text{-}CL}$/I60), depending on *k* (Fig. 2). These estimates suggest that domain radius (taken at certain *k*) increases with ionic strength and decreases with CL content. Yet, for $CL2.5_{AV\text{-}CL}$ and $CL5_{AV\text{-}CL}$ systems the above data treatment strategy proved unsuccessful suggesting the involvement of other factors. Denoting the depth of protein penetration in the bilayer, the distance between membrane surface and plane of donor in the extended conformation, and membrane half-width by D_P, D_{SE} and D_m (Fig. 3), respectively, the depth at which CL acyl chain impales cyt *c* (D_E) can be roughly estimated as $D_E = D_P + D_{SE} = D_P + D_{CE} + D_m$. The bounds for the depth of protein insertion into lipid bilayer can be found by considering two cyt *c* orientations with the heme lying above or below the molecule center. Then, the lower and upper limits for D_P are given by $D_P^{\min,\max} = D_m - d_{1,2} + R_P \mp r_t$ where d_1 and d_2 are acceptor distances from membrane center, $R_P \approx 2.1$ nm is protein effective radius in the lipid-bound state, $r_t \approx 0.7$ nm is displacement of heme group off the protein center. Notably, this expression may acquire more defined form if one addresses the recent work of Kalanxhi and Wallace [10] in which a mechanistic model for the extended lipid anchorage of cyt *c* to CL-containing membranes was proposed. This model supposes that ionic contacts between phosphate group and Lys_{72}, Lys_{73} anchor CL in cyt *c* hydrophobic channel created by two non-polar polypeptide patches (residues Tyr_{67} – Pro_{71} and Phe_{82} – Ile_{85}). Such cyt *c* alignment, represented schematically in Fig. 3, implies that heme moiety is located above the center of protein molecule and thus the upper limit of D_P should be considered. Furthermore, the above orientation suggests that Asn_{52} is more than 1 nm distant from the phosphate group of the lipid. Given that the length of H-bond is typically ~0.2 nm, hydrogen bonding between the protein and HP lipid species (characteristic of C-site) is unlikely, suggesting that in extended lipid conformation CL binds to cyt *c* mainly via the A-site. Accordingly, the depth of cyt *c* membrane penetration can be calculated from equation $D_P = D_m - d_1 + R_P - r_t$, with $D_m \approx 2.3$ nm. Evaluated in such a way the sets of parameters characterizing cyt *c* disposition relative to lipid-water interface are summarized in Table 1. These quantitative estimates suggest that ionic strength – induced RET enhancement may arise from favoring of extended lipid conformation.

Table 1. Parameters characterizing cyt *c* location at lipid-water interface

Parameter	**I = 40 mM**		**I = 60mM**	
	CL10	**CL20**	**CL10**	**CL20**
D_P, nm	$0.3^{\pm 0.02}$	$0.1^{\pm 0.007}$	$0.7^{\pm 0.04}$	$0.4^{\pm 0.03}$
D_{CE}, nm	$2.7^{\pm 0.4}$	$3.0^{\pm 0.7}$	$2.4^{\pm 0.4}$	$2.9^{\pm 0.1}$
D_E, nm	$0.7^{\pm 0.06}$	$0.8^{\pm 0.09}$	$0.8^{\pm 0.09}$	$1.0^{\pm 0.12}$

Clustering of CL molecules creates gradients of curvature and line tension along the membrane surface. Tending to compensate this energy cost, CL-enriched regions undergo

structural transformations. The route by which the system further reduces the interaction energy seems to involve CL transition into extended conformation. It's noteworthy in this regard that conditions facilitating the extended anchorage implicate increasing bilayer curvature and charge, and decreasing the headgroup size. These factors are believed to allow stronger electrostatic cyt *c* – lipid interactions resulting in more pronounced conformational changes of both protein (greater extent of unfolding and wider opening of a crevice) and lipid that facilitate the insertion of acyl chain in cyt *c* interior. The augmented ability of CL to adopt the extended conformation with lifting the concentration of monovalent ions originates, apparently, from the alterations in polarity near the membrane surface. From a thermodynamic point of view, to insert into the protein, lipid molecule should overcome the barrier associated with passage of acyl chain through the highly polar bilayer surface. Rising salt concentration brings about a drop in the dielectric constant at the lipid-water interface and, as a consequence, reduction in interfacial polarity which makes the accommodation of CL chain in cyt *c* groove more energetically favorable.

CONCLUSIONS

Overall, the results obtained strongly suggest that cyt *c* binding to the lipid membranes induces the transition of CL molecules into extended lipid conformation. The factors controlling such transition were found to be milieu conditions (i.e. ionic strength) and molar content of anionic lipid. The revealed peculiarities of cyt *c* – CL interactions are of great interest not only from the viewpoint of regulating cyt *c* electron transfer and apoptotic propensities but also for elucidating the general mechanisms by which membrane functional activities can be modulated by protein-lipid interactions.

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