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Supporting Material

Cytochrome c-lipid interactions: new insights from resonance energy transfer

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SUPPORTING MATERIAL

Binding model

Binding of cyt *c* to phospholipid membranes was analyzed in terms of the adsorption model allowing for area exclusion and electrostatic effects. The employed approach is based on Gouy-Chapman double-layer theory and scaled particle (SPT) model developed by Chatelier and Minton (S1) and further extended by Minton (S2) to take into account the possibility of multiple adsorbate conformations. SPT formalism is currently regarded as providing the most adequate description of excluded area interactions between the adsorbing protein molecules. Importantly, SPT expressions derived for the case of multiple conformations of bound protein appear to be applicable to treating protein association with heterogeneous surfaces where binding sites differ in their size and free energy of adsorption. In other words, if a protein adsorbs onto lipid bilayer surface containing two types of binding sites (i.e., there exist two populations of bound protein), the activity coefficient of a spherical ligand adsorbed in a particular conformation *i* or associated with the site of *i-th* type is given by:

$$\gamma_{1,2} = \frac{1}{1-\langle \rho a \rangle} \exp\left(\frac{a_{1,2}\langle \rho \rangle + s_{1,2}\langle \rho s \rangle / 2\pi}{1-\langle \rho a \rangle} + \frac{a_{1,2}}{4\pi}\left[\frac{\langle \rho s \rangle}{1-\langle \rho a \rangle} \right]^2 \right) \tag{S1}$$

$$a_{1,2} = n_{1,2} S_L, \qquad \rho_{1,2} = \frac{B_{1,2}}{L_{out} S_L}, \qquad s_{1,2} = 2\sqrt{\pi n_{1,2} S_L} \tag{S2}$$

$$\langle \rho \rangle = \frac{B_1 + B_2}{L_{out} S_L}, \quad \langle \rho a \rangle = \frac{n_1 B_1 + n_2 B_2}{L_{out}}, \quad \langle \rho s \rangle = \frac{2\sqrt{\pi}\left(B_1 \sqrt{n_1} + B_2 \sqrt{n_2} \right)}{L_{out} \sqrt{S_L}} \tag{S3}$$

where $\rho_{1,2}$, $a_{1,2}$, $s_{1,2}$ are the surface number density, area and circumference of the footprint of species *i*, $B_{1,2}$ – concentration of bound protein, $n_{1,2}$ – number of lipid molecules per binding site, L_{out} is the concentration of accessible lipids related to total lipid concentration (L) as $L_{out} = 0.5L$, S_L is the mean area per lipid molecule taken here as 0.65 nm^2 for PC and 1.2 nm^2 for CL (S3).

The adsorption isotherm can be described by the following equations:

$$K_{a1}(P - B_1 - B_2) = \rho_1 \gamma_1, \qquad K_{a2}(P - B_1 - B_2) = \rho_2 \gamma_2 \tag{S4}$$

where P – total protein concentration, $K_{a1,2}$ – association constant.

The choice of the model assuming two types of binding sites was dictated by the fact that at neutral pH there exist two CL populations, viz. deprotonated (DP) and partially protonated (HP). These species represent two distinct binding sites for cyt *c* with different energy of binding and association constant (K_{a1}^0 and K_{a2}^0, respectively). Peculiar protonation behavior of CL stems from its unique structure with two acyl chains and glycerol-phosphate per each monomer linked through a single glycerol head.

The equilibrium binding constant is generally represented as consisting of electrostatic (K_{el}) and non-electrostatic or intrinsic (K^0) terms: $K_a = K_{el} K^0$. Electrostatic component of

binding constant, dependent on electrostatic surface potential, environmental conditions (pH, ionic strength), and degree of surface coverage by a protein is given by:

$$K_{el} = \exp\left(-\frac{d}{dN_P}\left[\frac{\Delta F_{el}(N_P)}{k_B T} \right] \right) \tag{S5}$$

where T is the temperature, k_B is Boltzmann's constant, and ΔF_{el} is the total gain in electrostatic free energy, being a function of the number of adsorbed protein molecules, $N_P = B_a N_A$:

$$\Delta F_{el}(N_P) = F_{el}^{s}(N_P) - F_{el}^{s}(0) - N_P F_{el}^{P} \tag{S6}$$

where F_{el}^{s} and F_{el}^{P} are the electrostatic free energies of a membrane and a protein, respectively. The electrostatic free energy of a spherical protein molecule with effective charge +*ze* and uniform charge distribution can be written as (S4):

$$F_{el}^{P} = \frac{z^2 e^2}{2\varepsilon r_{cyt}(1 + \kappa_d r_{cyt})} \tag{S7}$$

with r_{cyt} standing for the protein radius, *e* the elementary charge, N_A Avogadro's number, ε the dielectric constant, *c* the molar concentration of monovalent ions, and κ_d the reciprocal Debye length.

$$\kappa_d = \sqrt{\frac{8\pi e^2 N_A c}{\varepsilon k_B T}} \tag{S8}$$

In terms of the Gouy-Chapman double layer theory the electrostatic free energy of a membrane of area $S_m = S_L L_{out}$ is given by (S5):

$$F_{el}^{s} = \frac{2k_B T S_m}{e}\left(\sigma \sinh^{-1}\left(\frac{\sigma}{a} \right) - \sqrt{a^2 + \sigma^2} + a \right); \qquad a = \sqrt{2\pi^{-1}\varepsilon c N_A k_B T} \tag{S9}$$

where σ is the surface charge density determined by the mole fraction of CL (f_{CL}), the degree of its ionization (α), and the extent of neutralization of membrane charge by the adsorbed protein:

$$\sigma = \frac{-e}{S_m}\left(\alpha f_{CL} L_{out} - zB_a \right) \tag{S10}$$

Considering CL as a dibasic acid, α can be written as:

$$\alpha = 2\alpha_P + \alpha_{HP} \tag{S11}$$

where α_P and α_{HP} are the fractions of deprotonated and partially protonated species, respectively:

$$\alpha_P = \frac{K_1 K_2}{K_1 K_2 + K_1 [H^+]_b \exp\left(\frac{-e\psi_0}{k_B T} \right) + \left([H^+]_b \exp\left(\frac{-e\psi_0}{k_B T} \right) \right)^2},$$

$$\alpha_{HP} = \frac{\alpha_P [H^+]_b \exp\left(\frac{-e\psi_0}{k_B T}\right)}{K_2} \tag{S12}$$

here K_1 are K_2 are CL ionization constants, $[H^+]_b$ is the bulk proton concentration, ψ_o is electrostatic surface potential of a membrane related to the surface charge density as:

$$\psi_o = \frac{2k_B T}{e} \sinh^{-1}\left(\frac{\sigma}{a}\right) \tag{S13}$$

Numerical solution of the set of Eqs. S1 – S13 yields theoretical isotherms that were fitted to the experimental data.

Resonance energy transfer model

The results of RET measurements were quantitatively analyzed in terms of the model of energy transfer in 2D systems formulated by Fung & Stryer (S6) and extended in our previous studies to allow for distance dependence of the orientation factor (S7,S8). Cyt *c* – lipid systems were treated as containing one donor plane and two acceptor planes located at distances d_1 and d_2 from membrane center. Anthrylvinyl fluorophores employed here as donors are attached to terminal methyl groups of acyl chains of both outer and inner monolayers. Due to the high mobility of these groups, AV moieties located at the outer and inner bilayer leaflets seem to be indistinguishable, so that the donor plane can be regarded as coinciding with the bilayer midplane while two populations of the bound protein (i.e., associated either with deprotonated or partially protonated CL species) were considered as being confined to two acceptor planes. In this case, relative quantum yield of the donor is given by:

$$Q_r = \int_0^\infty \exp(-\lambda) \exp\left[-\left(\frac{B_1}{S_m} S_1(\lambda) + \frac{B_2}{S_m} S_2(\lambda)\right)\right] d\lambda \tag{S14}$$

$$S_1(\lambda) = \int_{d_1}^\infty \left[1 - \exp\left(-\lambda \kappa_1^2(R) \left(\frac{R_o^r}{R}\right)^6\right)\right] 2\pi R dR \tag{S15}$$

$$S_2(\lambda) = \int_{d_2}^\infty \left[1 - \exp\left(-\lambda \kappa_2^2(R) \left(\frac{R_o^r}{R}\right)^6\right)\right] 2\pi R dR \tag{S16}$$

where R is the donor-acceptor separation, $\lambda = t/\tau_d$; τ_d is the lifetime of excited donor in the absence of acceptor. By representing Förster radius as $R_o = \left[\kappa^2(R)\right]^{1/6} \cdot R_o^r$, it follows that

$$R_o^r = 979\left(n_r^{-4} Q_D J\right)^{1/6} \qquad J = \frac{\int_0^\infty F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda}{\int_0^\infty F_D(\lambda) d\lambda} \tag{S17}$$

here n_r is the refractive index of the medium (n_r=1.37), Q_D is the donor quantum yield which was estimated to be ca. 0.8 for all types of membranes using 9,10-diphenyl anthracene as

standard, J is the overlap between the donor emission ($F_D(\lambda)$) and acceptor absorption ($\varepsilon_A(\lambda)$) spectra. When the donor emission and acceptor absorption transition moments are symmetrically distributed within the cones about certain axes $\boldsymbol{D_x}$ and $\boldsymbol{A_x}$, distance-dependent orientation factor is given by:

$$\kappa^2_{1,2}(R) = d_D d_A\left(3\left(\frac{d_c \mp 0.5d_t}{R}\right)^2 - 1\right) + \frac{1-d_D}{3} + \frac{1-d_A}{3} + \left(\frac{d_c \mp 0.5d_t}{R}\right)^2 \left(d_D - 2d_D d_A + d_A\right) \quad \text{(S18)}$$

$$d_{D,A} = \left\langle d^x_{D,A}\right\rangle\left(\frac{3}{2}\cos^2\alpha_{D,A} - \frac{1}{2}\right) \qquad \left\langle d^x_{D,A}\right\rangle = \left(\frac{3}{2}\cos^2\psi_{D,A} - \frac{1}{2}\right) \quad \text{(S19)}$$

where $\psi_{D,A}$ are the cone half-angles, $\alpha_{D,A}$ are the angles made by $\boldsymbol{D_x}$ and $\boldsymbol{A_x}$ with the bilayer normal $\boldsymbol{N}$. The axial depolarization factors $\left\langle d^x_D\right\rangle$ and $\left\langle d^x_A\right\rangle$ are related to the experimentally measurable steady-state (r) and fundamental (r_0) anisotropies of donor and acceptor (S9):

$$d^x_{D,A} = \pm\left(r_{D,A} / r_{0D,A}\right)^{1/2} \quad \text{(S20)}$$

Monte Carlo simulation

The results of RET measurements suggesting lateral redistribution of CL and PC molecules upon cyt *c* binding were treated using a Monte Carlo (MC) approach. Positions of donors and acceptors were generated randomly in a square cell assuming periodic boundary conditions to avoid edge effects. The relative quantum yield averaged over all donors was calculated from fluorophore coordinates as:

$$Q_r = \frac{1}{N_D}\sum_{j=1}^{N_D}\left[1 + \sum_{i=1}^{N_{AC}}\left(\frac{R_o^r \kappa^2(r_{ij})}{r_{ij}}\right)^6\right]^{-1} \quad \text{(S21)}$$

where N_D, N_{AC} stand for the number of donors and acceptors, respectively, r_{ij} represents the distance between j th donor and i th acceptor. The simulation procedure was repeated for at least 1000 fluorophore configurations until the standard deviation in Q_r was < 2%. The simulation algorithm was tested by comparing the data acquired from Fung & Stryer and the Monte Carlo calculation schemes. The results from analytical and numerical simulation approaches turned out to be in good agreement.

While analyzing the case of protein-induced domain formation we assumed that total number of disk-shaped domains (N_{dm}) is equal to the number of membrane-bound protein molecules (B_a), i.e. $N_{dm} = B_a N_A$, N_A is Avogadro's number. Total number of lipid molecules and the number of CL (N^{dm}_{CL}) and PC (N^{dm}_{PC}) molecules in domains can be calculated as:

$$N^{dm}_L = N^{dm}_{CL} + N^{dm}_{PC} = \frac{B_a N_A \pi r^2_{dm}}{S_L}; \qquad N^{dm}_{CL} = f_{CL} k N^{dm}_L; \qquad N^{dm}_{PC} = \left(1 - f_{CL}k\right)N^{dm}_L \quad \text{(S22)}$$

where k is the ratio of CL concentrations in the protein-affected region (adsorption disk-shaped domain of radius r_{dm}) at nonrandom and random distribution of charged lipids, $B_a = B_1 + B_2$. For molar fraction of donors (AV-PC or AV-CL) f_D, total number of AV-PC or AV-CL molecules in outer monolayer is given by:

$$N_{AV-PC}^{tot} = N_{AV-CL}^{tot} = L_{out} f_D N_A \quad \text{(S23)}$$

Given that the fraction of CL (f_{CL}^{dm}) and PC (f_{PC}^{dm}) in domains is equal to

$$f_{CL}^{dm} = \frac{B_a N_A f_{CL} k\pi r_{dm}^2}{L_{out} N_A f_{CL} S_L} = \frac{B_a k\pi r_{dm}^2}{L_{out} S_L}; \quad f_{PC}^{dm} = \frac{B_a \pi r_{dm}^2 (1 - f_{CL} k)}{L_{out} S_L (1 - f_{CL})} \quad \text{(S24)}$$

the number of AV-CL molecules in domain (N_{AV-CL}^{dm}) and non-domain (N_{AV-CL}^{ndm}) regions can be expressed as:

$$N_{AV-CL}^{dm} = N_{AV-CL}^{tot} f_{CL}^{dm} = \frac{B_a k\pi r_{dm}^2 N_A f_D}{S_L}; \qquad N_{AV-CL}^{ndm} = L_{out} N_A f_D - N_{AV-CL}^{dm} \quad \text{(S25)}$$

Surface densities of AV-CL in domain (δ_{AV-CL}^{dm}) and non-domain (δ_{AV-CL}^{ndm}) regions are given by:

$$\delta_{AV-CL}^{dm} = \frac{N_{AV-CL}^{dm}}{B_a N_A \pi r_{dm}^2} = \frac{f_D k}{S_L}; \quad \delta_{AV-CL}^{ndm} = \frac{N_{AV-CL}^{ndm}}{L_{out} S_L N_A - B_a N_A \pi r_{dm}^2} = \frac{f_D \left(L_{out} S_L - k B_a \pi r_{dm}^2\right)}{S_L \left(L_{out} S_L - B_a \pi r_{dm}^2\right)} \quad \text{(S26)}$$

Analogously, for AV-PC one obtains:

$$N_{AV-PC}^{dm} = N_{AV-PC}^{tot} f_{PC}^{dm} = \frac{B_a \pi r_{dm}^2 N_A f_D (1 - f_{CL} k)}{S_L (1 - f_{CL})}; \qquad N_{AV-PC}^{ndm} = L_{out} N_A f_D - N_{AV-PC}^{dm} \quad \text{(S27)}$$

$$\delta_{AV-PC}^{dm} = \frac{N_{AV-PC}^{dm}}{B_a N_A \pi r_{dm}^2} = \frac{f_D (1 - f_{CL} k)}{S_L (1 - f_{CL})}; \; \delta_{AV-PC}^{ndm} = \frac{f_D}{S_L (1 - f_{CL})} \left(1 - \frac{f_{CL} \left(L_{out} S_L - k B_a \pi r_{dm}^2\right)}{L_{out} S_L - B_a \pi r_{dm}^2}\right) \quad \text{(S28)}$$

Eqs. S26 and S28 were used to calculate the number of donors in domain and non-domain regions for a square cell with the side length taken as $10 R_o$ (here $R_o = 0.67 R_o^r$). The number of acceptors was determined by multiplying protein surface density (C_a^s) by the cell square (S_c) ($N_{AC} = C_a^s S_c$). The simulation program was scripted in Mathcad 2001 Professional.

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Table S1

Binding and structural parameters of cyt *c* interaction with PC/CL model membranes

Parameter	CL2.5			CL5			CL10			CL20		
	Ionic strength, mM			Ionic strength, mM			Ionic strength, mM			Ionic strength, mM		
	20	40	60	20	40	60	20	40	60	20	40	60
K_1^0, μM^{-1}	$120^{\pm 32}$	$500^{\pm 145}$	$2000^{\pm 564}$	$14^{\pm 3.1}$	$50^{\pm 10.5}$	$200^{\pm 48}$	$10^{\pm 2.6}$	$30^{\pm 7.4}$	$50^{\pm 10.5}$	$10^{\pm 2.7}$	$30^{\pm 7.2}$	$30^{\pm 8.4}$
K_2^0, μM^{-1}	$900^{\pm 262}$	$820^{\pm 234}$	$740^{\pm 212}$	$50^{\pm 12}$	$39^{\pm 15.2}$	$27^{\pm 12}$	$10^{\pm 2.6}$	$8.2^{\pm 2.2}$	$7^{\pm 2.1}$	$9^{\pm 2.7}$	$7.1^{\pm 2.4}$	$5.8^{\pm 2.8}$
d_1, nm	$3.5^{\pm 0.97}$	$3.0^{\pm 0.89}$	$2.9^{\pm 0.91}$	$4.1^{\pm 0.94}$	$3.3^{\pm 0.66}$	$3.0^{\pm 0.72}$	$4.1^{\pm 1.1}$	$3.5^{\pm 0.77}$	$3.0^{\pm 0.63}$	$4.1^{\pm 1.1}$	$3.8^{\pm 0.9}$	$3.5^{\pm 0.9}$
d_2, nm	$3.2^{\pm 0.91}$	$2.9^{\pm 0.82}$	$2.7^{\pm 0.75}$	$3.9^{\pm 0.82}$	$3.0^{\pm 0.6}$	$2.7^{\pm 0.65}$	$3.9^{\pm 1}$	$3.1^{\pm 0.68}$	$2.8^{\pm 0.59}$	$3.9^{\pm 1.05}$	$3.6^{\pm 0.8}$	$3.1^{\pm 0.8}$