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Evaluation of cytochrome *c* affinity to anionic phospholipids by means of surface plasmon resonance

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ABSTRACT

We attempted to evaluate the affinity of the anionic phospholipids to cytochrome *c* by means of surface plasmon resonance (SPR) technique and to correlate it with the cytochrome *c* active site alterations and peroxidase activity. Our experiments showed a strong interdependence between the phospholipid fatty acid saturation degree, the active site structure alterations and peroxidase activity of the cytochrome *c* phospholipid complex. Cytochrome *c* peroxidase activity and Trp59 fluorescence increase in the sequence of phosphatidyl choline (PC) → phosphatidylserine (PS) → cardiolipin (CL) → phosphatidic acid (PA). The association constant (K_a) increased in the sequence PC → PA → PS → CL. The SPR spectroscopy data shows that K_a is independent of lipid saturation degree, but correlates with phospholipid negative charge value.

1. Introduction

Interaction of phospholipids with cytochrome *c* is a crucial step in apoptosis development, resulting in acquiring by cytochrome *c* a peroxidase activity [1,2]. This activity varies depending on the type of lipid and is due to the rearrangements in the protein active site [3]. The main event in cytochrome *c* transformation into a peroxidase is the cleavage of the heme iron-Met80 bond that facilitates the access of hydrogen peroxide to heme and its oxidation to Compound I [4–7].

Recently, it was shown that high ionic strength media can inhibit the transformation of the cytochrome *c* into a peroxidase [7]. This observation proves that electrostatic interaction of lipid and cytochrome *c* is of primary importance to this process. The main goal of the present paper was to clarify the mechanism of anionic phospholipid–cytochrome *c* interaction and evaluate the dissociation constants of these complexes. Relationship of phospholipid–cytochrome *c* affinity and rearrangement of the protein active site and its peroxidase activity was the second goal of the present study. To evaluate the dissociation constants of the phospholipid–cytochrome *c* complex we used the SPR technique [8–10]. To study the transformations of the cytochrome *c* active site we have applied the fluorescent assay of Trp59. The peroxidase activity of the phospholipid–cytochrome *c* complex was estimated by means of luminol-dependent chemiluminescent assay.

2. Materials and methods

2.1. Materials

2.1.1. Reagents

The following substances were used in this study: horse heart cytochrome *c* (Cat. No. C7752 Sigma, USA); sodium dodecyl sulfate (Cat. No. L6026 Sigma, USA); phospholipids: palmitoylstearoyl phosphatidylcholine (PSPC, Cat. No. 850456P), distearoyl phosphatidylserin (DSPS, Cat. No. 840029P), dioleoyl phosphatidylserin (DOPS, Cat. No. 840035P), distearoyl phosphatidic acid (DSPA, Cat. No. 830865P), dioleoyl phosphatidic acid (DOPA, Cat. No. 840875P), tetramyristoyl cardiolipin (TMCL, Cat. No. 750332P), tetraoleoyl cardiolipin (TOCL, Cat. No. 710335P) – all from Avanti Polar Lipids, USA.

Abbreviations: SPR, surface plasmon resonance; PC, phosphatidyl choline; PSPC, palmitoylstearoyl phosphatidylcholine; PS, phosphatidylserine; DSPS, distearoyl phosphatidylserine; DOPS, dioleoyl phosphatidylserine; CL, cardiolipin; TMCL, tetramyristoyl cardiolipin; TOCL, tetraoleoyl cardiolipin; PA, phosphatidic acid; DSPA, distearoyl phosphatidic acid; DOPA, dioleoyl phosphatidic acid, K_a, association constant

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2.1.2. Liposome preparation

To prepare the liposomes phospholipid was dissolved in Folch reagent (chloroform/methanol/water, 2:1:0.5, v/v) and mixed with equimolar amount of PSPC. Solvents were removed by argon bubbling for 10 min. Than, phosphate buffer pH 7.4 (10 mM or 150 mM) was added into the sample tubes and hardly stirred to get the crude lipid suspension. Than, these suspensions were sonicated by ultrasonic homogenizer SONOPULS HD 2200 (Bandelin, Germany) (20 s with 10% pulsation) and than homogenized by LipoFast extruder with 0.1 μm membrane filter (Avestin, Canada) to form slightly opalescent fine liposome suspension [11]. The final phospholipid concentration in the sample was 2.5 mM. Fresh liposomes were immediately used for injection into the biosensor.

2.2. Methods

2.2.1. Luminol-enhanced chemiluminescence

The intensity of cytochrome *c* peroxidase activity was measured by means of luminescence assay on KhLM-3 chemiluminometer (Bikap, Russia). Phospholipids with various characteristic groups and saturated and unsaturated fatty acid residues (TMCL, TOCL, DSPA, DOPA, DSPS, DOPS, PSPC and surfactant SDS) were used in our study. Hydrogen peroxide (0.1 mM) and luminol (0.5 mM) were used as substrates of peroxidase reaction. All solutions were prepared using phosphate buffer 10 mM, pH 7.4.

2.2.2. Fluorescence of cytochrome c Trp59

The fluorescence of cytochrome *c* Trp59 was measured by means of Hitachi-4000 spectrofluorimeter (Hitachi, Japan). In our experiments the same set of phospholipids as in chemiluminescent assay was used (TMCL, TOCL, DSPA, DOPA, DSPS, DOPS, PSPC and surfactant SDS). Trp59 fluorescence was measured at λ_{ex} – 279 nm and λ_{em} – 335 nm. All solutions were prepared using phosphate buffer 10 mM, pH 7.4. To take into account the effect of scattering the fluorescence intensity was calculated as a difference of 335 nm and 300 nm signal.

2.2.3. Surface plasmon resonance

The anionic phospholipid–cytochrome *c* affinity was measured with Biacore 3000 instrument (Biacore, Sweden). All experiments were made with lipid biosensor chips L1 (Biacore). Biosensor chips used in experiment were processed before the phospholipid immobilization: at first they were subjected to repeated washing with CHAPS (20 mM) (10 μl/min for 5 min) and than with 30% ethanol washing (5 μl/min for 3 min). In our experiments, the biosensor chip channel #4 always contained PSPC and was used as a reference channel, the channels #2 and #3 contained various phospholipids (such as phosphatidylserine (PS), phosphatidic acid (PA) and cardiolipin (CL)). All lipids were immobilized by injection of liposome suspension with 10 μl/min flow rate for 30 min. After the liposome injection the 10 mM NaOH solution was introduced for 1 min at a 10 μl/min flow rate to remove loosely bound structures, form lipid layer and stabilize the baseline. At the end of phospholipid immobilization procedure the BSA (1 mg/ml) was added to the analyte (10 μl/min flow rate for 20 min) to seal the gaps in the phospholipid layer on the chip channel surface to prevent nonspecific binding. After the BSA injection the solution of NaOH (10 mM) was introduced twice for 1 min at 10 μl/min flow rate for final stabilization of the baseline. The immobilization of anionic phospholipids on the negatively charged biosensor chip surface at low ionic strength was practically impossible. To solve this problem, NaCl (0.5 mM) was added to phospholipids before the immobilization. Finally, after the phospholipid immobilization was completed, the cytochrome *c* titration was performed. The cytochrome *c* injection was done with 5 μl/min flow rate and 6 min duration. After each experiment all channels of optical chip were washed by 10 mM NaOH (10 μl/min for 2 min) to remove the cytochrome *c* traces.

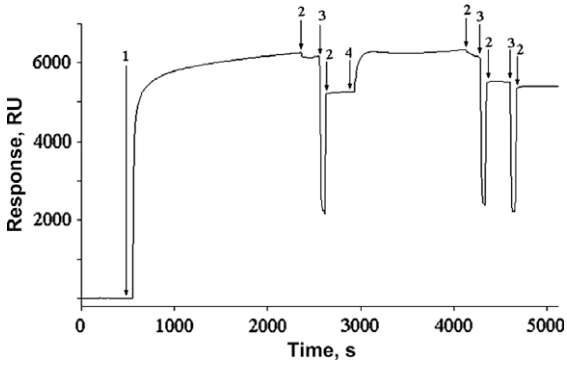

Fig. 1. Immobilization of TOCL liposomes for the generation of a lipid layer on a lipophilic biosensor L1 chip surface. Arrows indicate injections: 1 – 2.5 mM TOCL; 2 – PBS (pH 7.4; 2.7 mM KCl, 137 mM NaCl, 10 mM phosphate buffer); 3 – 10 mM NaOH; 4 – 1 mg/ml BSA. Ordinate – the intensity of the plasmon resonance signal (relative units). After lipid immobilization was completed cytochrome *c* solution was introduced to the flow to measure the association constants.

Data analysis was done by applying the BIAevaluation v.4.1 software (Biacore AG). Apparent association constant (K_a) was calculated from dependence of equilibrium biosensor signal (R_{eq}) on cytochrome *c* concentration by nonlinear fitting of the primary sensograms data using the equation:

$$R_{eq} = K_A C R_{max}/(1 + K_A C), \quad (1)$$

where R_{eq} is the equilibrium biosensor signal at given concentration of cytochrome *c* (values of R_{eq} were obtained from primary sensograms), R_{max} the maximal value of biosensor signal R_{eq} for the given quantity of immobilized phospholipids (the capacity of given phospholipid-modified chip surface), *C* the concentration of cytochrome *c* in solution, K_A the apparent association constant (reciprocal to apparent K_D, $K_A = 1/K_D$). Lines in Fig. 3 are approximation curves generated by BIAevaluation software using Eq. (1) and experimental data obtained.

3. Results

3.1. SPR investigation of cytochrome c–anionic phospholipid dissociation constants

3.1.1. Titration by cytochrome c

To study the association of cytochrome *c* with anionic phospholipids we attached the lipid on the biosensor chip and let the cytochrome *c* to move along the lipid layer. The procedure of tetraoleoyl cardiolipin (TOCL) immobilization on the surface of optical chip L1 is presented in the Fig. 1. The phospholipid association kinetics reaches its saturation within 30 min. Replacement of the lipid-containing analyte with buffer solution resulted in the decrease of the signal value due to the removal of loosely attached phospholipids. To seal the gaps not containing phospholipids on the biochip surface a BSA solution was used.

The affinity of cytochrome *c* to the biochip attached phospholipid was evaluated by making the cytochrome *c* molecules to float over the phospholipid layer. In the Fig. 2 the sensogram of various cytochrome *c* concentrations (10–300 μM) is presented. It can be seen that the higher the cytochrome *c* concentration the more cytochrome *c* is binded to the TOCL attached to the biochip. These experiments were carried out to estimate the equilibrium affinity constants of phospolipid-cytochrome *c* complex. Similar experi-

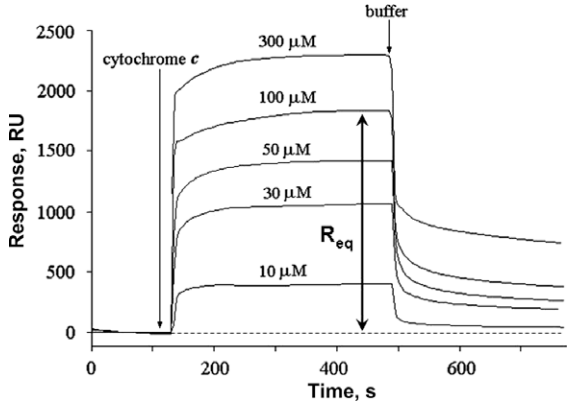

Fig. 2. Sensorgrams of cytochrome *c* binding to TOCL. Representative sensograms of L1 immobilized TOCL titration by increasing concentrations of cytochrome *c*. The specific binding profiles of the cytochrome *c* to the immobilized TOCL were obtained after subtracting the control flow cell with immobilized PSPC signal from the TOCL response signal.

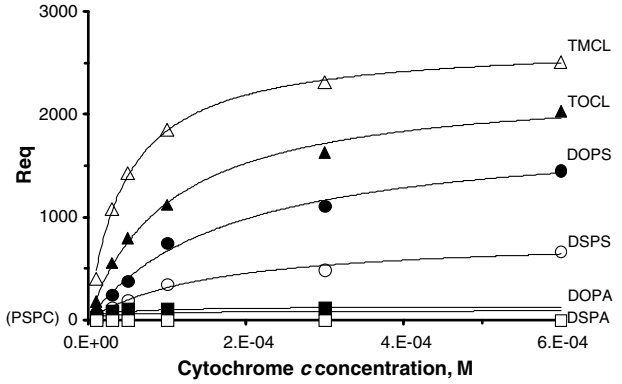

Fig. 3. Effect of cytochrome *c* on the SPR signal intensity (R_{eq}) in various phospholipids. Each point represents the difference of cytochrome *c* association (R_{eq}) between the reference channel (PSPC) and sample channels (TMCL (△); TOCL (▲); DSPS (○); DOPS (●); DSPA (□); DOPA (■)). Cyt *c* (10 μM) in phosphate buffer was used.

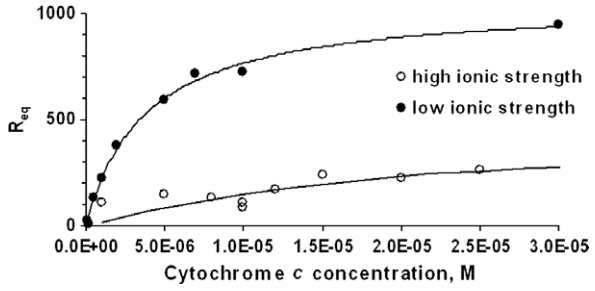

Fig. 4. Effect of high (150 mM) and low (10 mM) ionic strength on the SPR signal intensity (R_{eq}) of TOCL in phosphate buffer. Sample contained TOCL and PSPC attached on the biochip and Cyt *c* (10 μM) in phosphate buffer.

ments were performed for all phospholipids under the study: TMCL, DOPS, DSPS, DOPA, DSPA, DOPC and PSPC.

To calculate the K_a of the cytochrome *c*-phospholipid complex we used the R_{eq} and R_{max} values of each cytochrome *c*-phospholipid couple (see Fig. 3).

The K_a values for all phospholipid–cytochrome *c* complexes were calculated using the Eq. (1):

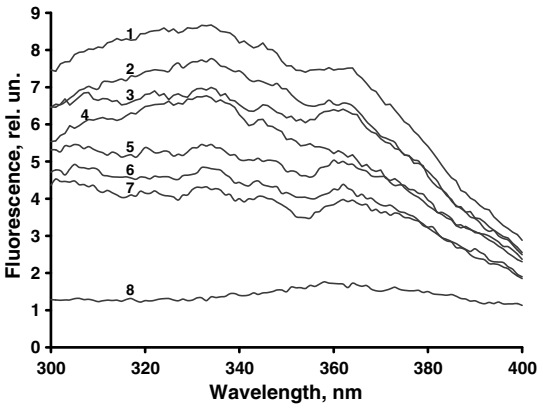

Fig. 5. Fluorescence spectra of cytochrome *c* Trp59 in the presence of unsaturated phospholipids: 1 – TOCL (250 μM) + PSPC (250 μM) + Cyt *c* (10 μM); 2 – DOPA (250 μM) + PSPC (250 μM) + Cyt *c* (10 μM); 3 – TMCL (250 μM) + PSPC (250 μM) + Cyt *c* (10 μM); 4 – DOPS (250 μM) + PSPC (250 μM) + Cyt *c* (10 μM); 5 – DSPA (250 μM) + PSPC (250 μM) + Cyt *c* (10 μM); 6 – DSPS (250 μM) + PSPC (250 μM) + Cyt *c* (10 μM); 7 – PSPC (500 μM) + Cyt *c* (10 μM); 8 – Cyt *c* (10 μM). All samples contained phosphate buffer pH 7.4 (10 mM). λ_{ex} = 279 nm, λ_{em} = 335 nm.

$$K_a(\text{CytC-DSPS}) = (6.31 \pm 1.51) * 10^3\ \text{M}^{-1};$$
$$K_a(\text{CytC-DOPS}) = (5.63 \pm 1.35) * 10^3\ \text{M}^{-1};$$
$$K_a(\text{CytC-DSPA}) = (2.16 \pm 0.60) * 10^3\ \text{M}^{-1};$$
$$K_a(\text{CytC-DOPA}) = (2.22 \pm 0.53) * 10^3\ \text{M}^{-1};$$
$$K_a(\text{CytC-TMCL}) = (2.15 \pm 0.62) * 10^4\ \text{M}^{-1};$$
$$K_a(\text{CytC-TOCL}) = (9.78 \pm 2.35) * 10^3\ \text{M}^{-1}$$

It is seen that the K_a values increase in a sequence DSPA → DOPA → DOPS → DSPS → TOCL → TMCL. All K_a values of the cytochrome *c* complexes studied are located in the region from $2 * 10^3$ to $2 * 10^4$. It can be concluded that the K_a values of these cytochrome *c*-phospholipid complexes vary depending on the characteristic group of the phospholipid rather than on the saturation degree of the fatty acid residue.

3.1.2. Effects of ionic strength

Fig. 4 shows that R_{eq} values depend on the ionic strength of the medium. It can be found that for all cytochrome *c* concentrations used the K_a values at high ionic strength were manifold less than those at low ionic strength. For instance, the K_a value for TOCL at

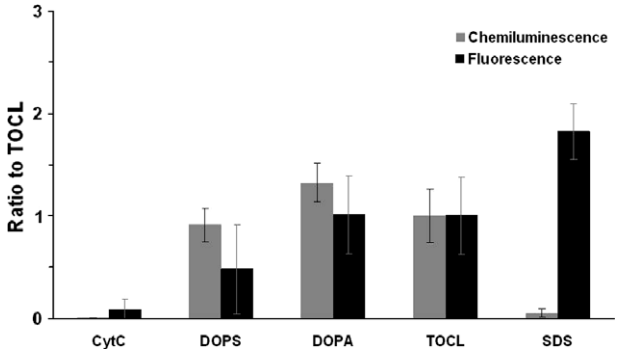

Fig. 6. The Tyr-59 fluorescence (black columns) was estimated as integral fluorescence for 240 s. TOCL; DOPS; DOPA or SDS were used in concentration 0.25 mM. Cytochrome *c* was used in concentration of 10 μM. The lipid/protein ratio was 25. Gray columns denote the peroxidase activity of cytochrome *c* in the presence of phospholipids. The same concentrations of cytochrome *c* and phospholipids were used for these experiments. Luminol – 500 μM and H_2O_2 – 100 μM were used in experiments. The cytochrome *c* without any lipid was used as a control. The sample of TOCL was used as 100% standard.

high ionic strength (150 mM) were – $(9.78 \pm 2.35) * 10^3\ M^{-1}$ and for low ionic strength (10 mM) were $(5.25 \pm 1.15) * 10^5\ M^{-1}$. The explanation of this fact may be that cytochrome *c*–phospholipid interaction is mainly a result of electrostatic interaction between the phospholipid characteristic group and a protein.

3.2. Studies of active site alterations and peroxidase activity

3.2.1. Trp59 displacement as evidenced by fluorescence

The rearrangements of the cytochrome *c* active site induced by anionic phospholipid interaction involve the displacement of the Trp59 amino acid residue and concomitant change in fluorescence intensity. We've measured the fluorescence of Trp59 in cytochrome *c*–phospholipid complexes and compared it with the K_a values obtained from the SPR assay. It was found that all the cytochrome *c*–phospholipid complexes with saturated fatty acid residues under the study gave almost no effect on the Trp59 fluorescence. In contrast, the cytochrome *c*–phospholipid complexes with unsaturated fatty acid residues showed a dramatic increase in the fluorescence intensity as compared to that of cytochrome *c* in the absence of phospholipids (Fig. 5). The cytochrome *c* Trp59 fluorescence growth increased in the row DOPS → DOPA → TOCL. These experimental results are presented in the Fig. 5.

3.2.2. Peroxidase activity modulation by phospholipids

Rearrangements in the cytochrome *c* active site induced by phospholipids may affect not only cytochrome *c* Trp59 fluorescence, but also the peroxidase activity of the complex. Notably, the interaction of cytochrome *c* with saturated phospholipids practically does not affect the peroxidase activity of the complex (data not shown). On the other hand, the interaction of cytochrome *c* with phospholipids with unsaturated fatty acid residues showed the significant increase in peroxidase activity (Fig. 6). It can be seen that peroxidase activity grows in a sequence DOPS → TOCL → DOPA.

4. Discussion

It is known that apoptotic reactions can be induced by the interaction of anionic phospholipids with cytochrome *c*, which initiates its active site rearrangements resulted in the increasing of peroxidase activity. The question to be answered is if there is a correlation between cytochrome *c*-phospholipid affinity (evaluated as K_a) and phospholipid ability to modify the cytochrome *c* active site. In our experiments we have measured the K_a value surface plasmon resonance (SPR), the Trp59 fluorescence and the peroxidase activity (chemiluminescent assay) of the cytochrome *c*–phospholipid complexes. In these complexes we varied the phospholipid characteristic group phosphatidylcholine (PC), PS, PA and CL and the saturation degree of the phospholipid fatty acid residue (stearic, palmitic, oleic and mirystic acids).

It was found that saturated and unsaturated anionic phospholipids (DSPS; DOPS; DSPA; DOPS; TMCL; TOCL) can bind to the cytochrome *c* almost to the same extent. On the other hand, saturated phospholipids affect cytochrome *c* active site (and peroxidase activity) several-fold weaker than unsaturated. The data presented correlates with that published by Kapralov et al. [4] proving the maximal effectiveness of TOCL and DOPA in destabilization of cytochrome *c* tertiary structure and stimulation its peroxidase activity.

Presented results clearly evidence that enhancement of cytochrome *c* peroxidase activity is a complicated process, involving phospholipid binding, based on the electrostatic and hydrophobic interactions and chemical reactivity of the lipids.

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