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Penetration of Lysozyme and Cytochrome C in Lipid Bilayer: Fluorescent Study

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Abstract Lysozyme and cytochrome c (CytC) are well-investigated proteins. Their specific interactions with lipid membranes, however, keep surprising secrets. Lysozyme destroys bacterial membrane; CytC binds hydrophobically to alkyl chains of the membrane lipid tails, indicating that both proteins are able to interact directly with the inner membrane components, especially with the fatty acyl chains of membrane lipids. The degrees of integration, depth of localization in the hydrophobic interior of different types of model membranes, and the type of interaction of lysozyme and CytC with surrounding lipids were investigated by fluorescent spectroscopy. Three different fluorescent markers, located at approximately 6.5, 9, and 18 Å into the lipid bilayer, were used. In addition, liposomes were designed as electrically neutral or positively or negatively charged to unravel the importance of the net electrical charge for lipid/protein interaction. CytC penetrates deeper into the lipid bilayer in comparison with lysozyme, and data are discussed in the terms of Stern–Volmer quenching of fluorescence.

Keywords Cytochrome c · Fluorescent probes · Liposomes · Lysozyme · Protein penetration · Quenching of fluorescence

Liposomes are widely used as models for investigation of biological membranes (New 1999). In pharmacology liposomes are often applied as drug targets and drug transporters (Tashjian et al. 2008). Among the various types of liposomes, unilamellar bilayer vesicles are preferred as model systems because they have flexible, cell-like surfaces and are suitable for numerous applications (Stamouli et al. 2003; Richter et al. 2007).

Our first step was to create a handle procedure for preparation of stable liposomes with controlled fluorescent and electrical properties. The procedure was based on the work of Hub et al. (1982). Small amounts of fluorescent lipids were added to the initial lipid mixture for liposome formation. Obtained vesicles were visible by fluorescent microscope. Using the technique of fluorescent spectroscopy it is possible to observe fine changes in the membrane surrounding of the fluorophores under the influence of different physical factors or chemical agents.

An important factor for the physical and chemical properties of the liposomes is their net electrical charge. In order to obtain different degrees of positive or negative electrical charge, we added different amounts of positively or negatively charged phospholipids or fatty acids before the liposome formation.

The next step was to obtain stable liposomes. For this purpose an apparatus for extrusion of the vesicles was constructed on the basis of the original ideas of Subbarao et al. (1991) and MacDonald et al. (1991). A detailed description of the apparatus is given below.

Experiments were carried out with lysozyme and CytC to investigate their interactions with the model membranes. Lysozyme, a cationic protein with a small molecular weight of 14.6 kDa, has been completely characterized in respect to its primary, secondary, and tertiary structure (Blake et al. 1965). It hydrolyzes the glycosidic linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine in bacterial cell wall and destroys the structural integrity of the membrane (Salton 1957). Lysozyme is able also to

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penetrate into lipid bilayer; this process is dependent on electrostatic and hydrophobic interactions (Kimelberg 1976; Sessa and Weissmann 1970; Gorbenko et al. 2007), often leading to aggregation and fusion of negatively charged and neutral liposomes (Posse et al. 1994). These properties make lysozyme a strong bactericidal agent.

CytC is a soluble cationic protein with a small size, 12 kDa, that shuttles electrons from glucose through a series of pumping proteins to oxygen, finally forming water molecules. However, it has been established that CytC plays an important role in the apoptotic process (Liu et al. 1996). Under special signals CytC passes through the mitochondrial membrane to the cytosol, where it associates with the protein Apaf-1 and forms apoptosomes (Goodsell 2004). This complex activates the protein-cutting caspases to begin the process of apoptosis. The binding domain of CytC includes a heme complex and few positively charged lysines (Bernad et al. 2004). Interactions of CytC with liposomes are dependent on electrostatic and hydrophobic binding with the lipids. Rytomaa and Kinnunen (1995) have proposed two distinct acidic phospholipid binding sites in CytC; A-site recognizes deprotonated and C-site protonated phospholipids. The authors suggest a mechanism of attachment of CytC to lipid membrane on the basis of the so-called extended lipid conformations, in which two acyl chains of the lipid are pointed in opposite directions from the head group. Others (Quinn and Dawson 1969; Morse and Deamer 1973; Pinheiro 1994; Salamon and Tollin 1996a, b; Zuckermann and Heimburg 2001) have described hydrophobic interactions of CytC and lipid membranes, proposing the existence of a hydrophobic cavity of the protein.

In the present work we studied the penetration of the small cationic proteins lysozyme and CytC into phospholipid membranes and assessed how deep they can insert into the lipid bilayer.

Materials and Methods

Fluorescent probe 4-(4-(dihexadecylamino)styryl)-*N*-methylpiridinum iodide (DiA) was purchased from Molecular Probes, USA; egg yolk lecithin from Merk, Germany; stearyl amine (SA) from Fluka, Germany; and phosphatidylglycerol (PG) (1,2-dimyristoyl-*sn*-glycero-3-phospho-(1′-rac-glycerol), sodium salt, 14:0 PG) from Avanti, USA. We also purchased 1-palmitoyl-2-[6-[(7-nitro-2-1, 3-benzoxadiazol-4-yl)amino]hexanoyl]-sn-glycero-3-phosphoholine (NBD_6PC) and 1-palmitoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl) amino] hexanoyl]-sn-glycero-3-phosphoholine ($NBD_{12}PC$) from Avanti, USA. Hen egg white lysozyme was purchased from Sigma, USA, and horse heart CytC from Fluka, Germany. All other chemicals were of analytic grade. Polycarbonate Track-Etch membranes with a pore size of 100 nm, used for extrusion, were purchased from Whatman, Germany.

Preparation of Neutral Fluorescent Liposomes by a Modified Procedure of Hub et al. (1982)

A total of 4.95 ml of 1 mM solution of lecithin were mixed in a round-bottom flask with 0.05 ml of 1 mM DiA, 1 mM NBD_6PC, or 1 mM $NBD_{12}PC$ in chloroform, and solvents were evaporated in a rotary vacuum evaporator, keeping the flask in a water bath at 40°C. The solvents were additionally evaporated for 2 h under high vacuum. Hydration of the obtained lipid film was made by addition of 5 ml 20 mM phosphate buffer pH 7.0 and incubated overnight at 40°C for complete hydration. Hydrated lipids were gently shacked to obtain spherical unilamellar liposomes with different diameters. These suspensions of liposomes were extruded to size of liposomes of 100 nm. By these means we obtained three types of neutral fluorescent liposomes: DiA, NBD_6PC, and $NBD_{12}PC$ labeled.

The obtained fluorescent liposomes were observed with a fluorescent microscope.

Preparation of Positively Charged Fluorescent Liposomes

A total of 5, 10, and 20 mol% positively charged liposomes were obtained by the addition of 0.25, 0.5, and 1 ml of 1 mM chloroform solution of stearyl amine, respectively, to 4.75, 4.5, and 4 ml of the mixture of lecithin and DiA, NBD_6PC, or $NBD_{12}PC$, described for the neutral liposomes. SA is well known as a cationic fatty primary amine and charges the liposomes positively.

By these means we obtained nine types of charged liposomes: 5, 10, and 20 mol% charged DiA labeled; 5, 10, and 20 mol% charged NBD_6PC labeled; and 5, 10, and 20 mol% charged $NBD_{12}PC$ labeled.

Preparation of Negatively Charged Liposomes

Analogously, nine types of 5, 10, and 20 mol% of negatively charged liposomes were obtained by the addition of 1 mM solution of phosphatidyl glycerol instead of SA.

Apparatus for Liposome Extrusion

The operating element was a Nuclepore polycarbonate track–etched membrane with a diameter of 25 mm (Whatman, Germany), with different pore sizes from 1 to 5000 nm. In the present work we used membranes with a 100-nm pore size. A single membrane was fixed between two Teflon holders situated into a disjointable cylindrical

duralumin fundamental cell. Two 5-ml glass syringes were stacked on both ends of the Teflon holders and fixed to duralumin syringe holders with screws. All of these metal elements were mounted on a 20-mm-thick duralumin plate that in turn was mounted on the central body of the apparatus. Electrical heating elements were situated directly under the basic plate and were able to heat all the metal elements to a definite temperature in the range of room temperature to 80°C. In the present study the extrusion was performed at 45°C. Heating was controlled by an automatic thermocontroller.

Microscopic Characterization of the Liposomes

Fluorescent liposomes were controlled by microscopic observation with a fluorescent Axiovert 25 microscope with source lamp HBO-50 (Karl Zeiss, Germany) and a digital camera (VSS1000, Inray Solution Ltd., Bulgaria). The size of the liposomes was measured by a Burker chamber (Fein Optic, Germany). Digital images were analyzed by ImageJ software (http://rsb.info.nih.gov/ij/) to measure the size of the liposomes.

Fluorescent Spectroscopy

A total of 2.3 ml of 20 mM phosphate buffer, pH 7, and a 0.2-ml suspension of the respective fluorescent liposomes were mixed in a quartz fluorescence cuvette, and measurements were made with a spectrofluorometer (Jobin Yvon JY3 D, France). The spectrofluorometer was completed with temperature-adapted cuvette holders. The temperature of the liposome suspensions into the quartz cuvettes was adjusted to 40°C using a water-flow thermostat, and temperature was controlled with a thermocouple thermometer (Omega, Newport, Germany) with a resolution of 0.1°C, allowing us to perform the above experiments with the phase transition of the lipids we used. Phase transition of egg yolk lecithin membranes was 39°C (Ekman and Lundberg 1978) and 23°C for phosphatidylcholine (van Dijck et al. 1978). Proteins, lysozymes, or CytC were then added into the cuvette in 20-µl portions to a final quantity of 240 µl and a final protein concentration of up to 8 µM. Fluorescence of DiA-labeled liposomes was excited at 474 nm and emission spectra were scanned in the range of 500–680 nm. Fluorescence of the labeled with NBD particles was excited at 460 nm and emission spectra scanned in the band of 480 to 640 nm. All the spectra were digitalized by an analog-to-digital converter (model NI-USB6008, National Instruments, USA) and operated by LabView software. Digital databases were then processed by Origin 7.0 software (OriginLab, USA) to obtain all the fluorescent spectral parameters. Spectra were corrected for the blank fluorescence as well as for dilution and finally normalized to the initial fluorescence in the absence of proteins.

Analysis of Fluorescence Quenching by Proteins

Lysozyme and CytC penetrate into the liposome membranes and quench the fluorescence of DiA by collisions with their fluorophores. CytC also quenches the fluorescence of NBD_6PC and $NBD_{12}PC$. The degree and character of quenching were dependent on the location of fluorophores and the electrical charge of the studied liposomes.

Quenching efficiency was analyzed according to the Stern–Volmer equation for collisional dynamic quenching (Lakowicz 2006):

$$F_o/F = 1 + K_{sv} \times [Q] \quad (1)$$

where F_o is the fluorescence in the absence of protein, F is the fluorescence in the presence of protein, K_{sv} is the Stern–Volmer constant, and Q is the concentration of the protein.

When $F_o/F = 2$, then

$$[Q] = 1/(K_{sv}). \quad (2)$$

This is the concentration of protein quenching 50% of the fluorophores.

However, in many cases the experimental data have shown mixed dynamic and static quenching of the liposome fluorescence, where the static component is a result of any binding of protein groups to the lipid fatty acid chains. In these cases we have used the modified Stern–Volmer equation:

$$F_o/(F_o - F) = (1/f) \times (1/K_{sv}) \times (1/[Q]) + (1/f), \quad (3)$$

where f is the part of the initial fluorescence of fluorophores accessible for the quenching of the penetrating into the membranes proteins.

In all cases fluorescent intensities were corrected for dilution in the process of adding increasing concentrations of protein. Spectral parameters were calculated by Origin 7.0 software.

Results

Fluorescent Probes

Figure 1 presents the molecular structures of the fluorescent probes used in the present study. Fluorescent group of DiA (Fig. 1a) locates in the interfacial membrane region at the border between the phospholipid head and its fatty acid chain (Kachel et al. 1998; Huber et al. 1999; Moyano et al. 2008). These authors demonstrate that the approximate depth of location of DiA from the membrane surface is 6.5 Å. A second fluorescent probe, NBD_6PC (Fig. 1b), possesses a fluorescent group at the sixth position of the

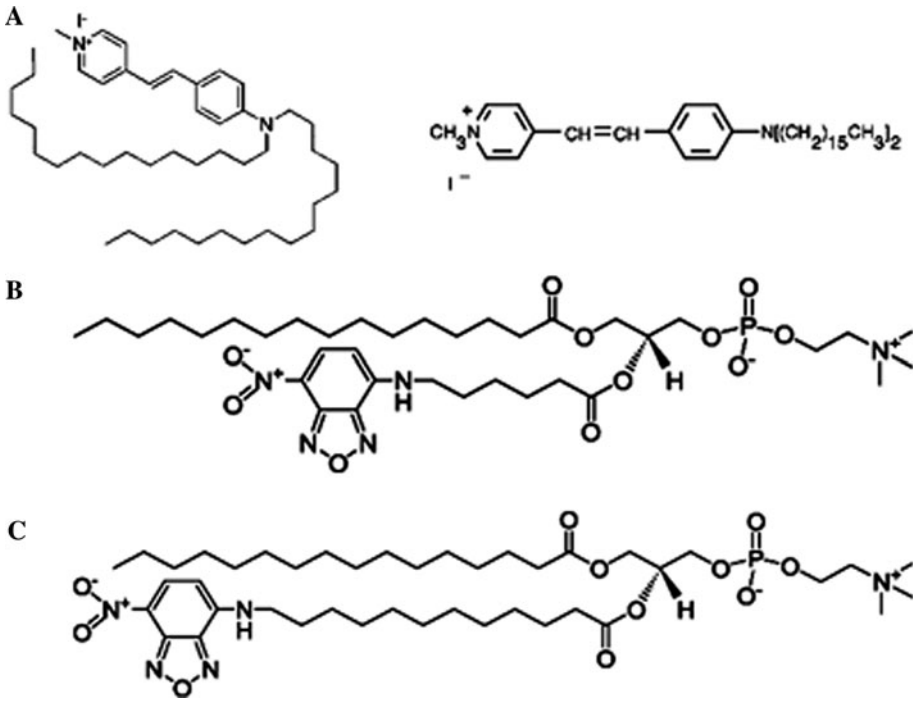

Fig. 1 Molecular structures of the used three fluorescent probes. **a** 4-(4-[Dihexadecylamino]styryl)-*N*-methylpiridinum iodide (DiA). **b** 1-Palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl) amino]hexanoyl]-*sn*-glycero-3-phosphoholine (NBD_6PC). **c** 1-Palmitoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino] hexanoyl]-sn-glycero-3-phosphoholine ($NBD_{12}PC$)

carbohydrate chain of the lipid. Approximate depth is about 9 Å. In the deepest hydrophobic zones are located the fluorophores of $NBD_{12}PC$ (Fig. 1c). These depths were calculated keeping in mind the fact that the distance between the carbon atoms of the fatty acid lipid chains is 1.5 Å (Kachel et al. 1998).

Incorporation of these three fluorescent probes into the lipid bilayer allows us to measure the degree of fluorescence quenching by proteins and to determine how deep the investigated proteins are able to penetrate the membrane.

Fluorescent Liposomes

We used diluted liposome suspensions with a lipid content 0.09 mM in the experiments. In these suspensions, the light scattering was minimal, in the range of 1% (data not shown) and in the interval of 460–470 nm. Where the fluorescence was excited the extinction coefficient was 0.27, and in the region of the maximal emission around 525 nm the extinction was under 0.21. These parameters indicate that the suspensions we used were convenient for fluorescence analysis.

In a series of pilot experiments we ascertained that the minimal appropriate concentration of fluorescent lipids in the membranes is 1 fluorescent molecule per 100 lipids.

Microscopic Characterization of Suspensions

The size of liposomes before the extrusion was highly variable. Using the grids of a Burker chamber, the size of liposomes was measured by converting the distances in pixels. Liposome size was in the range of 0.9 ± 0.01 to 5.86 ± 0.23 μm ($n = 10$).

After extrusion, liposomes were visible as homogeneous small fluorescent nanoparticles with a size of 100 nm.

Fluorescence Quenching

Fluorescence spectra of the suspensions were scanned for every concentration of the protein added. The highest fluorescence was emitted in absence of protein. Figure 2 presents a family of spectra of fluorescent liposomes, labeled with the fluorescent phospholipid NBD_6PC. Addition of increasing concentrations of CytC leads to fluorescence quenching.

Similar families of spectra were obtained for the other used 20 types of fluorescent liposomes, neutral or charged (data not shown).

Every spectrum was analyzed by Origin 7.0 software, and the parameters of maximal intensity, area under the fluorescent spectrum, position of the maximum, and half-width of the spectrum were calculated. Stern–Volmer graphs were plotted and fitted to Eqs. 1 or 3 to calculate Stern–Volmer constants of quenching and to analyze the type of quenching.

Figure 3 presents typical results for quenching of DiA-labeled neutral liposomes. Lysozyme quenches the fluorescence linearly with increase of concentration, demonstrating collisional dynamic mechanism (described in Lakowicz 2006) (Fig. 3a), while CytC quenches the

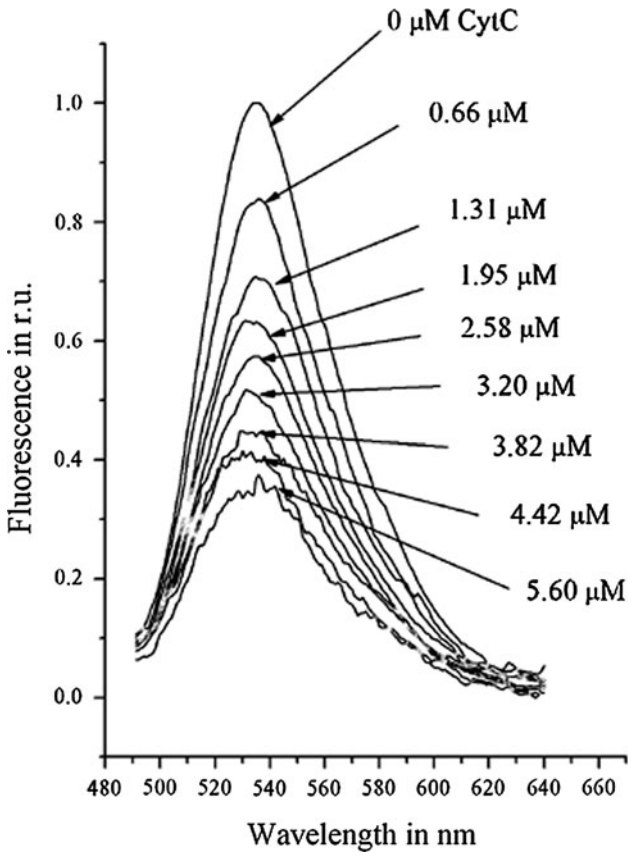

Fig. 2 Typical family of fluorescent spectra of NBD_6PC-containing liposomes, quenched by increasing concentrations of CytC. Fluorescence was excited at 460 nm and emission registered in the region of 480–640 nm. Emission maxima were at about 534 nm. The highest spectrum is of liposomes without CytC. The next lower spectra are in the presence of CytC at concentrations 0.66, 1.31, 1.95, 2.58, 3.2, 3.82, 4.42, and 5.6 μM, respectively. All spectra presented were obtained after correction procedures and normalization of the instrumental spectra to the maximal fluorescence in the absence of protein

fluorophores emission by mixed dynamic and static interactions (defined in Lakowicz 2006) (Fig. 3b). These differences result in the use of Eq. 1 to plot the Stern–Volmer graph for lysozyme quenching (Fig. 3c) and respectively Eq. 3 for CytC (Fig. 3d).

Similar graphs were plotted for all the quenching experiments in the present study, but it is not possible to present all of them here. We calculated the Stern–Volmer constants; the rest of the spectral parameters are summarized Tables 1, 2, 3.

Fluorescence Quenching in DiA-labeled Liposomes

In Table 1 are arranged the spectral characteristics in the presence of lysozyme. The protein quenches the fluorescence in neutral and charged liposomes, demonstrating the ability to penetrate to approximately 6.5 Å into the lipid membrane, where the fluorescent probe DiA is located (Kachel et al. 1998). In the neutral liposomes, penetration of lysozyme shifts the fluorescence to the blue region (about 20 nm) and simultaneously narrows the spectral half-widths. In neutral and positively charged liposomes, quenching is dynamic, showing direct collisional interactions with DiA (Lakowicz 2006). Efficiency of quenching diminishes (see data for K_{sv}) with the accumulation of positive charges, and theoretically the concentration of lysozyme has to grow more than 100 times to reach 50% quenching effectiveness.

In the negatively charged liposomes, the character of quenching is mixed dynamic and static. The static component is probably a result of binding to the fluorophores. It is discernible from data in Table 1 that efficiency of quenching diminishes with increase of negative charge.

In charged liposomes, these findings are valid only for cases with low concentrations of lysozyme. At over 3 μM, the lysozyme causes spontaneous aggregation of the liposomes, and the fluorescence of the samples falls sharply. Aggregation was highly dependent on the liposome charges. Over 20% of negatively charged liposomes aggregate even at very low concentrations of lysozyme, and suspensions soon turbided.

In Table 2 are presented the parameters of quenching by CytC. The most effective was the quenching in neutral liposomes, where 74% of the fluorophores were accessible for quenching by CytC. Slight blueshift (3 nm) of the spectra and 26% increase of their half-widths were observed, demonstrating accumulation of CytC molecules around DiA.

In charged liposomes, the quenching was dynamic, and no changes were observed in the half-widths of spectra; shifts of maxima were missing.

Negative charges lowered the quenching constants K_{sv} more than twice with respect to neutral liposomes. With the increase of negative charges, quenching constants increase, demonstrating better accessibility to DiA.

In positively charged liposomes, efficiency of quenching diminishes with the degree of charging, and quenching constants decrease. Blueshift and increase of half-widths show accumulation of CytC around DiA, resulting in tighter packing of molecules in this region of the membrane.

NBD_6PC-labeled Liposomes

In NBD_6PC-labeled liposomes, the fluorophores are situated at about 9 Å deep in the membranes, approximately equally distanced from the most hydrophilic and most hydrophobic zones of the membrane.

The experiments clearly demonstrate that lysozyme is not able to quench the fluorescence in this zone.

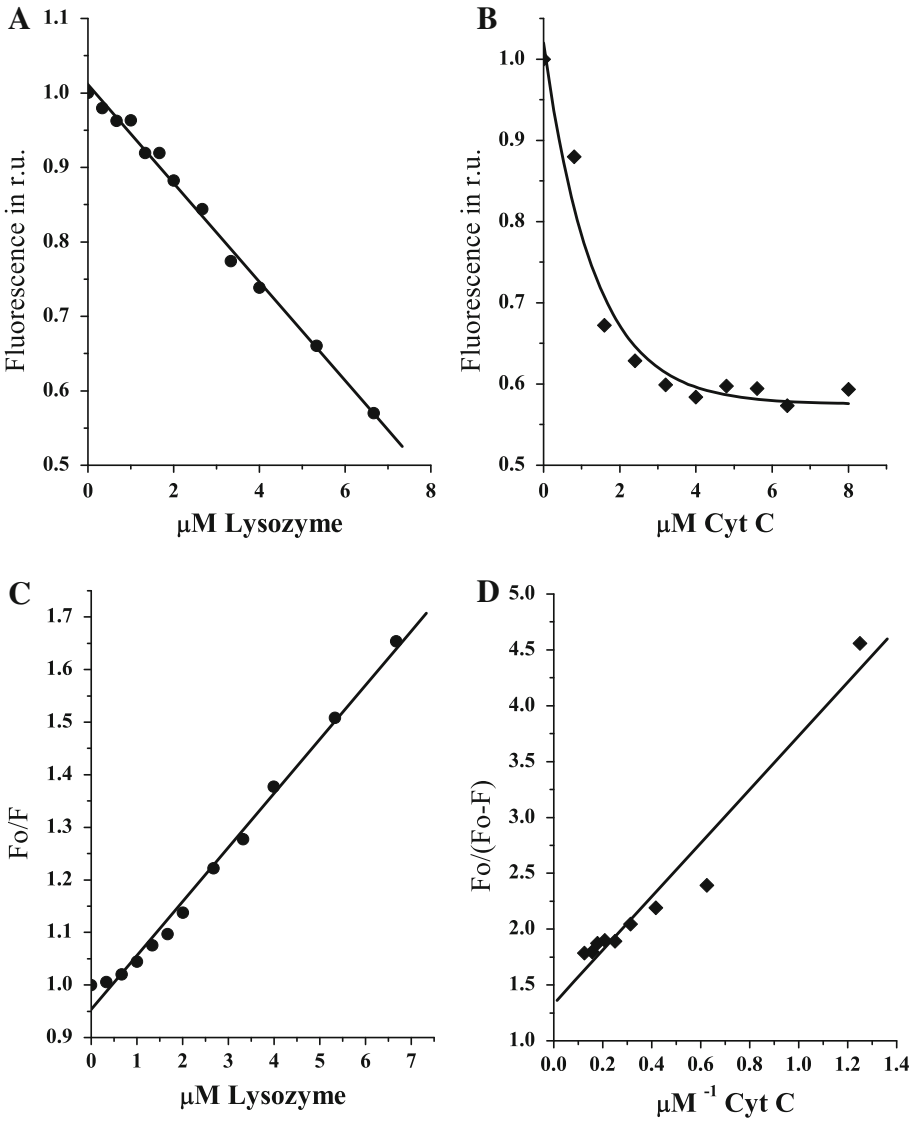

Fig. 3 Quenching of the fluorescence of DiA-labeled neutral liposomes by lysozyme and CytC. Data are presented in relative units (r.u.). **a** Linear quenching by lysozyme of the fluorescence at the spectral maxima. **b** Nonlinear quenching by CytC. **c** Stern–Volmer graph, calculated by Eq. 1 for dynamic quenching by lysozyme. **d** Stern–Volmer graph of quenching by CytC by Eq. 3 for mixed dynamic and static quenching

CytC, on the other hand, quenches this fluorescence; a significant Stern–Volmer constant of 0.363 ± 0.018 μM^{-1} and 2.75 μM are required for 50% quenching of the fluorescence.

Hence, it was interesting to test CytC-influenced fluorescence quenching in the deepest hydrophobic zones.

$NBD_{12}PC$-labeled Liposomes

In the case of $NBD_{12}PC$-labeled liposomes, fluorophores are situated in the deepest hydrophobic zones, approximately 18 Å from the membrane surface. Table 3 lists the quenching parameters. In neutral and negatively charged liposomes, the quenching was dynamic, but in positively charged ones, the character of quenching turns to mixed dynamic and static, revealing that part of CytC molecules closely touch to the fluorescent marker. In all cases, shifts of fluorescence maxima and changes in the spectral half-widths were absent.

Stern–Volmer constants in negatively charged liposomes are lower than in neutral liposomes and decrease slightly with the charge increase, demonstrating a decrease in the quenching effectiveness during the increase of the negative charge of the membrane. On the contrary, in positively charged liposomes, the quenching effectiveness of CytC grows sharply with charge, and in the investigated interval, this increase was more than 30 times. This effect can be explained by extensive binding of CytC to the hydrophobic lipid tails.

Discussion

In the present work we use the term “neutral membranes” for liposomes containing fluorescent probe DiA. However,

Table 1 Quenching and spectral parameters of DiA-labeled fluorescent liposomes in the presence of lysozyme as a quencher

mol% of charge	Type of quench	Stern Volmer constant μM^{-1}	Accessibility to quencher	Shift of spectral maxima	Spectral half-width
Neutral liposomes					
0	Dynamic	0.103 ± 0.003	50% at 9.7 μM	Blueshift 20 nm	Tendency to narrowing
Negatively charged liposomes					
5	Dynamic and static	0.222 ± 0.005	82.4%	No change	No change
10	Dynamic and static	0.210 ± 0.012	68.3%	No changes	No changes
15	Dynamic and static	0.202 ± 0.020	63.3%	No changes	No change
Positively charged liposomes					
5	Dynamic	0.113 ± 0.002	50% at 8.85 μM	No change	No change
10	Dynamic	0.016 ± 0.001	50% at 62.50 μM	No change	No change
20	Dynamic	0.0084 ± 0.0005	50% at 119.00 μM	No change	No change

Stern–Volmer constants were calculated by Eq. 1 for the dynamic quenching and Eq. 3 for the mixed (dynamic and static) quenching. Accessibility of the quencher to the fluorophores was calculated by Eq. 2 for the dynamic quenching. The mixed quenching data for f in Eq. 3 are presented as percentages. Spectral shifts and spectral half-widths were calculated from the fluorescent spectra. All data are a result of three experiments ($n = 3$)

Table 2 Quenching and spectral parameters of DiA-labeled fluorescent liposomes in the presence of cytochrome c as a quencher

mol% of charge	Type of quench	Stern Volmer constant μM^{-1}	Accessibility to quencher	Shift of spectral maxima	Spectral half-width
Neutral liposomes					
0	Dynamic and static	0.571 ± 0.08	74%	Blueshift 3 nm	26% increase
Negatively charged liposomes					
5	Dynamic	0.21 ± 0.014	50% 4.78 μM	No change	No change
10	Dynamic	0.127 ± 0.005	50% 4.50 μM	No change	No changes
20	Dynamic	0.312 ± 0.05	50% 3.20 μM	No changes	No changes
Positively charged liposomes					
5	Dynamic	0.145 ± 0.002	50% 6.89 μM	Blueshift 4 nm	10 nm increase
10	Dynamic	0.086 ± 0.005	50% 11.63 μM	Blueshift 4 nm	9 nm increase
20	Dynamic	0.053 ± 0.002	50% 18.87 μM	Blueshift 5 nm	3 nm increase

Constants and parameters are calculated as described in Table 1

DiA is positively charged, and the membrane is slightly positively charged to about 1 mol%.

Our results indicate that both investigated proteins, lysozyme and CytC, are able to quench the fluorescence of DiA as a result of their ability to penetrate into the membrane (Tables 1, 2). The character of the quenching was different for the two proteins. Although lysozyme quenches the fluorescence dynamically, CytC also possesses a static component, demonstrating its ability to form complexes with the membrane lipids. This was probably the reason its effectiveness for quenching ($K_{sv} = 0.571$, Table 2) was more than 5 times higher than for lysozyme ($K_{sv} = 0.103$, Table 1).

Both proteins are relatively small, with similar molecular weights, which facilitates their good diffusibility in the membrane. Both of them also have a net positive charge (Gorbenko et al. 2007; Goodsell 2004), but they succeed in overcoming the electrostatic repulsion forces and collide with DiA. In addition to the electrostatic interactions, CytC is able to interact hydrophobically with the alkyl chains of the lipids (Morse and Deamer 1973; Pinheiro 1994; Quinn and Dawson 1969; Salamon and Tollin 1996a, b; Zuckermann and Heimburg 2001). Most likely these interactions are responsible for the static component of the quenching of DiA fluorescence.

The data we present here demonstrate that both proteins cause a blueshift of the fluorescent emission as a result of tighter packing of the regions where DiA is located. The protein molecules are probably able to move locally just in these regions. In addition, CytC increases the spectral half-widths. This is most likely the effect of hydrophobic binding with the lipids (Bernad et al. 2004), resulting in

Table 3 Quenching and spectral parameters of $NBD_{12}PC$-labeled fluorescent liposomes in the presence of cytochrome c as a quencher

mol% of charge	Type of quench	Stern Volmer constant μM^{-1}	Accessibility to quencher	Shift of spectral maxima	Spectra half-width
Neutral liposomes					
0	Dynamic	0.506 ± 0.005	50% at 1.98 μM	No change	No change
Negatively charged liposomes					
5	Dynamic	0.422 ± 0.014	50% at 2.37 μM	No change	No change
10	Dynamic	0.420 ± 0.015	50% at 2.38 μM	No change	No changes
20	Dynamic	0.415 ± 0.02	50% at 2.4 μM	No changes	No changes
Positively charged liposomes					
5	Dynamic and static	1.37 ± 0.23	51.14%	No change	No change
10	Dynamic and static	5.656 ± 0.50	21.96%	No change	No change
20	Dynamic and static	31.46 ± 5.23	17.42%	No change	No change

Constants and parameters are calculated as described in Table 1

widening of the vibrational sublevels in the emission spectra.

The efficiency of quenching by lysozyme of negatively charged DiA liposomes diminishes with an increase in the charge (Table 1) as a result of the electrostatic interaction of lysozyme with the negatively charged heads of phosphatidylglycerol, and fewer quencher molecules can collide with DiA. An increase of the negative charge of the membrane surface soon leads to a critical point where smaller concentrations of lysozyme provoke aggregation (Gorbenko et al. 2007; Posse et al. 1994). This was the reason we used low lysozyme concentrations—less than 3 μM—with negatively charged liposomes in our experiments.

On the other hand, in negative liposomes, CytC quenches DiA dynamically, and Stern–Volmer constants increased gradually (Table 2) with the increase of charges. This fact principally demonstrates the different penetration of CytC into membranes in comparison with lysozyme.

Obviously in the surface membrane regions where DiA is located, CytC quenches fluorescence dynamically without formation of complexes with the lipids. This reveals the better diffusibility of CytC in comparison with lysozyme.

The results for the positively charged liposomes, where CytC quenches DiA fluorescence more effectively than lysozyme, were similar. Comparing the data in Table 1 for lysozyme to Table 2 for CytC, one can see that CytC causes a blueshift of the spectra and increases spectral half-widths, whereas lysozyme does not perturb these parameters.

In conclusion, CytC diffuses in this zone more freely than lysozyme, packs the lipids locally, and widens the vibrational sublevels of the spectra.

Results with deeper-situated probes have shown an ability of CytC to penetrate freely to the deepest regions of the membrane. Although lysozyme penetrates only into most hydrophilic regions and is able to interact violently with DiA, CytC is able to quench NBD_6PC significantly, with a Stern–Volmer constant 0.363 ± 0.018 μM^{-1}. Even in these middle zones CytC does not have static quenching, thus evidencing absence of hydrophobic bonds with lipids.

Data for the deepest located fluorophores $NBD_{12}PC$, presented in Table 3, show Stern–Volmer constants in these zones similar to constants in Table 2, indicating that CytC is able to effectively quench the fluorescence in both hydrophilic and hydrophobic zones.

Data for the deepest zones (Table 3) demonstrate, however, that an increase of negative charges is able to suppress the decrease in the quenching constants. This phenomenon is probably a result of the electrostatic interactions of CytC with the negatively charged phosphatidylglycerol head groups on the membrane surface, forcibly detaining part of the CytC molecules into the surface membrane slices.

Concerning the positively charged liposomes, the situation is generally different: a significant static component of the quenching appears (Table 3). As a result, CytC molecules enter more freely into the deepest zones and bind hydrophobically with the lipid tails (Pinheiro 1994; Salamon and Tollin 1996a; Zuckermann and Heimburg 2001). Under these conditions CytC reaches enough concentration in this zone to generate long-life existing complexes with the lipids. Under these conditions, the quenching constants increase more than 30 times (Table 3).

From the present work, we conclude that fluorescent liposomes are a suitable model for comparative studies of different protein properties. The experiments with lysozyme and CytC revealed unexpected behavior in the lipid bilayer. Nevertheless, both proteins have similar molecular weights, 12 kDa for CytC and 14.6 kDa for lysozyme, with a net positive charge, both of them interacting electrostatically with membrane lipids. The present study clearly

shows that the mobility of CytC into the lipid bilayer is many times higher than that of lysozyme.

The present investigation contributes to our understanding of the uncommon properties of these proteins in the bactericidal properties of lysozyme and of the observed participation of CytC in the process of controlled apoptosis.

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