Genetics and Plant Physiology – 2013, Volume 3 (1–2), pp. 27–41

Available online at http://www.ifrg-bg.com

# FLUORESCENT STUDY OF THE SPONTANEOUS INSERTION OF CYTOCHROME $B_6F$ INTO LIPID BILAYERS

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Received: *05 January 2013* Accepted: *28 January 2014*

**Summary:** The aim of the present study was to investigate the ability of cytochrome $b_6f$ complex to be inserted spontaneously into artificial lipid membranes with different physical properties using fluorescent methods. Artificial membranes were constructed as fluorescent liposomes with controlled composition, electrical charge and size. Fluorescent membrane probes were 7-nitro-2-1, 3-benzoxadiazol-4-yl (NBD)-labeled phosphatidyl choline whose fluorophores were situated in different depths of the lipid bilayer. Electrical charges of the liposomes were modified by adding 5 mol% negatively or positively charged lipids during the liposome formation. The size of liposomes was fixed to 100 nm by extrusion through polycarbonate Track-Etch membranes.
The process of insertion of cytochrome $b_6f$ complex into the lipid bilayers was measured and analyzed by fluorescence quenching caused by the collision of the protein groups with the fluorophores (NBD) into the membrane. Slight differences in the quenching Stern-Volmer constants were registered for the different depths in the bilayer. Spontaneous insertion of cytochrome $b_6f$ complex reached up to 9 % of their surrounding concentration in neutral and negatively charged liposomes. Positive electrical charges of the membranes inhibited significantly the incorporation. The spontaneous insertion of cytochrome $b_6f$ complex into the lipid bilayers was enhanced 5-fold by treatment of liposomes with low concentrations of the weak detergent n-dodecyl β-D-maltoside (DOM) that was removed by Bio-Beads before application of the protein.
These cytochrome $b_6f$-enriched liposomes possessed observable reductase activity.

**Citation:** Zlatanov I., N. Terezova, S. Stoichev, M. Dimitrov, 2013. Fluorescent study of the pontaneous insertion of cytochrome $b_6f$ into lipid bilayers. *Genetics and Plant Physiology*, 3(1–2): 27–41.

**Keywords:** Cytochrome $b_6f$; fluorescent liposomes; spontaneous protein insertion; Stern-Volmer constants.

**Abbreviations:** Cyt $b_6f$ – cytochrome $b_6f$-complex; PSII and PSI – photosystem II and photosystem I; SA – stearyl amine; PG – phosphatidylglycerol; $NBD_6PC$ – 1-palmitoyl-2-[6-[(7-nitro-2-1, 3-benzoxadiazol-4-yl)amino]hexanoyl]-sn-glycero-3-phosphoholine; $NBD_{12}PC$ – 1-palmitoyl-2-[12-[(7-nitro-2-1, 3-benzoxadiazol-4-yl)amino]hexanoyl]-sn-glycero-3-phosphoholine; DOM – n-dodecyl β-D-maltoside.

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## INTRODUCTION

Cytochrome $b_6f$-complex is a key enzyme in the photosynthetic chain of the green plants and some bacteria. It is classified as plastoquinol-plastocyanin reductase (EC1.10.99.1) and participates in the process of electron transfer between PSII and PSI by the mediation of plastocyanins. This large oligomeric complex consolidates 2 monomers, everyone consisting of 8 or 9 subunits. (Whitelegge et al., 2002; Baniulis et al., 2011). General subunits of cyt $b_6f$-complex providing its oxidoreductase activity are PetA: cytochrome f-32 kDa, PetB: cytochrome $b_6$-24.2 kDa, PetC: Rieske-iron-sulfur protein-19.1 kDa and PetD: subunit IV-17.4 kDa (Cramer et al., 1992; Baniulis et al., 2011). In the complex are included also a number of small proteins such as PetG-4.2 kDa, PetL-3.5 kDa, PetM-3.97 kDa and PetN-3.2 kDa, which play the role of hydrophobic "sticks" between the subunits (Whitelegge et al., 2002; Cramer et al., 2006), as well as associated prosthetic groups like chlorophyll a, β-carotene and heme $c_n$. The mass of the whole complex isolated from spinach is 286.5 kDa (Baniulis et al., 2011), from algae and bacteria - 217 kDa.

Important components of the complex are also specific lipids, inserted in the native structures. Hasan et al. (2011) reported about the existence of 8 lipid binding sites per monomer of cyanobacterial cyt $b_6f$ and emphasized on their location into important functional regions of the complex. Rytomaa and Kinnunen (1995) suggested the existence of an "extended lipid conformation" for cytochrome C able to crosslink some protein residues through lipid acyl chains-to-membrane phospholipids.

Cyt $b_6f$ is a transmembrane complex with hydrophilic loops exposed to the stroma and lumen of thylakoid membranes. The large hydrophobic surface regions inside the membrane are tightly bound to the surrounding lipids. So, the extraction of the complex from the membrane is very difficult and researchers are constrained very often to apply different strong detergents to remove the complex from the thylakoid (Hurt and Hauska 1981; Hurt et al., 1982; Doyle and Yu, 1985; Adam and Malkin, 1987; Cramer et al., 1992; Huang et al., 1994; Chain and Malkin, 1995; Breyton et al., 1997; Schoepp et al., 2000; Yan et al., 2001; Whitelegge et al., 2002; Lezhneva et al., 2008; Hasan et al., 2011; Sang et al., 2011; Szymanska et al., 2011) However, detergents extract the structural lipids inside the complex as well. Moreover, in many cases detergent molecules replace these structural lipids, and then it is difficult to reconstitute back the isolated complex into its native structure (Adam and Malkin, 1987).

In order to avoid the above mentioned problems we used in our experiments extraction and purification procedures without the application of detergents following the method published earlier by Donchev et al. (2004) and Dimitrov et al. (2009). These authors reported for the first time about the isolation of cyt $b_6f$ complex from poplar leaves (*Populus Nigra, var. Italica*) and named by them RF3 fraction.

To investigate the role of lipids and their contribution to the structural organization and functioning of cyt $b_6f$ complex, a number of researchers have reported on the incorporation of cyt $b_6f$ into artificial lipid membranes (Hurt and Hauska, 1981; Hurt et al., 1982; Doyle and Yu, 1985; Adam and Malkin, 1987; Schoepp, 2000; Szymanska et al.,

2011). Charvolin et al. (2009) have even attempted to immobilize cyt $b_6f$ on solid surfaces as a bio-chip-system.

An original approach to investigate the cyt $b_6f$ unique potentials was reported by Mulkidjanian (2010). The isolated native cyt $b_6f$ complex was inserted into inside-out vesicles of the inner cellular membranes obtained by disruption of cells of phototrophic *Rodobacter capsulatus*. However, the question whether the obtained data are relevant for thylakoid membranes remains still open.

In most of the cited above investigations concerning the restoration of cyt $b_6f$-complex, it was performed with lipid micelles or vesicles. In the present study we used a different approach to investigate the cyt $b_6f$ integration into the lipid bilayer of liposomes with strictly defined size and composition, constructed by the modified method of Hub et al. (1982). Fluorescent labeled lipids in a ratio 1:100 were added to the mixture of phospholipids. Fluorescent residues were positioned in a different depth into the lipid bilayer, giving the opportunity to obtain spectral information from the different regions of the membrane. The fluorescence emitted by the probe was quenched by cyt $b_6f$ when it penetrated into the lipid bilayer. Processes are described by the Stern-Volmer equation (Lakowicz, 2006) and the fluorescence spectra give us lavish information for the protein-to-lipid contacts. Detailed information about the used fluorescent liposomes can be found in the paper of Zlatanov and Popova (2011).

Large unilamellar vesicles obtained by the method of Hub et al. (1982) however, are not very stable and we applied the extrusion method through 100 nm pored polycarbonate membranes (Olson et al., 1997) to obtain stable liposome suspensions (Guichardon et al., 2005) using the apparatus described earlier by Zlatanov and Popova (2011).

To unravel the effect of membrane electrostatics on the spontaneous insertion of cyt $b_6f$-molecules into the lipid bilayer, three types of liposomes were formed: neutral, negatively and positively charged. The modification of the electrical charge on the surface of liposomes was achieved by insertion of controlled quantities of negatively or positively charged lipids.
Here we report also on the approach to enhance the degree of cyt $b_6f$ complex integration into the bilayer by using a pre-treatment of liposomes with the weak detergent n-dodecyl β-D-maltoside.

The main goal of the present study was to answer the following questions: i). How do the physical properties of the membrane, like lipid composition and electrical charge, influence the spontaneous insertion of cyt $b_6f$ into a lipid bilayer? ii). Is it possible to improve the spontaneous insertion of cyt $b_6f$ by preliminary detergent treatment of the liposomes? and iii). How can free of detergent cyt $b_6f$-enriched liposomes be obtained?

If it will be possible to achieve significant enrichment in liposomes, it could be any little step ahead for a future composition of a photosynthesizing biochip.

## MATERIALS AND METHODS

### Materials

Egg yolk lecithin was purchased from Merck, Germany; stearyl amine (SA) from Fluka; Germany and phosphatidylglycerol (PG) (1, 2-dimyristoyl-*Sn*-glycero-3- phos-

pho-1'-rac-glycerol), sodium salt, (14:0 PG) from Avanti, USA. 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 ($NBD_{12}PC$) were from Avanti, USA. N-Dodecyl β-D-maltoside (DOM) and decylplastoquinon was from Sigma-Aldrich, USA and Bio-Beads from Bio-Rad, USA. Plastocyanin was isolated in our laboratory following the procedure of Dimitrov et al. (2010, 2010a). Polycarbonate Track-Etch membranes with pore size of 100 nm, used for extrusion, were purchased from Whatman, Germany. All other chemicals were of analytical grade.

**Isolation of cyt $b_6f$ from poplar leaves**

Cyt $b_6f$ sub-complexes were isolated and prepared from 5 kg fresh leaves of poplar (*Populus nigra, var. Italica*) by the procedure described by Dimitrov et al. (2009) without using detergents. The presence of the heme proteins in cyt $b_6f$ complexes was proved by the absorbance spectra of the eluates recorded at room temperature on a Specord UV-VIS spectrophotometer (Germany). Polyacrylamide gel electrophoresis (PAGE) was performed as described by Donchev et al. (2004) and the purity indexes calculated from the ratio of absorbance at 404 nm (heme Soret peak) to 278 nm (protein peak) were above 1. Gel electrophoresis of the used cyt $b_6f$ fraction, called RF3 (Dimitrov et al., 2009) showed that it contained the four main components of the complex, PetA:Cyt f, PetB:Cyt $b_6$, PetC:Rieske protein and PetD:Subunit IV, as well as some minor components. The molecular mass of the dimmer was approved to be 220 kDa. The "purity index", ratio of absorption at 404 to 278 nm, was 1.1. Measurements of the reductase activity demonstrated that the isolated complex was functionally active.

**Reductase activity assay**

Reductase activity was determined by the procedure of Wood and Bendall (1976), see also Grey and Phillips (1982). Briefly, a small quantity of plastocyanin was preliminary oxidized by addition of $K_3[Fe(CN)_6]$ and the excess of oxidant was then removed by filtration through Sephadex G25 column. The assay mixture was prepared in 10 mM 2-(N-morpholino)-ethane sulphonate buffer, equilibrated to pH 6.2 with NaON (MES-NaOH buffer). 90 mM NaCl was added to reach an ionic strength of 0.1. The reaction mixture was composed of 0.1 % digitonin, 1.5 μM oxidized plastocyanin and 0.1 μM Cyt $b_6f$-complex. Before and after the addition of 40 μM decylplastoquinon the absorbance of the sample was measured at 595 nm on a double beam spectrophotometer Specord UV-VIS, Germany for 30 min at room temperature. Just the same reaction mixture was used for the assay of the Cyt $b_6f$-enriched non-fluorescently labeled liposomes, but instead of 0.1 μM cyt $b_6f$-complex were added liposome suspensions, containing approximately 0.01 μM cyt $b_6f$. The activities of the free and liposome-inserted cyt $b_6f$ preparations were compared on the time-related graphs for the absorbance at 595 nm.

**Preparation of fluorescent liposomes with controlled size and electrical charges**

Neutral fluorescent liposomes were prepared by a modified procedure of

Hub et al. (1982), described previously (Zlatanov and Popova, 2011). Briefly, 5 mg lecithin and $NBD_6PC$ or $NBD_{12}PC$ (100:1, mol/mol) in chloroform were mixed and solvent was evaporated in a water bath at 40°C to obtain thin film on the flask bottom. Traces of chloroform were additionally removed under high vacuum for 4 h. Hydration of the dry lipid films was performed by the addition of 5 ml 20 mM phosphate buffer (pH 7.0) and incubated at room temperature overnight to gain complete hydration. Hydrated lipids were gently shacked to obtain multilamellar liposomes with different diameters followed by extrusion through polycarbonate filters with 100 nm pores to get unilamellar liposomes with defined size.

Fluorescent groups of the used probes, $NBD_6PC$ and $NBD_{12}PC$, are located in the region of $6^{th}$ and $12^{th}$ carbon atom of the lipid tails, in different depths of the membrane of about 9 Å and 18 Å from the membrane hydrophilic surface, respectively (Zlatanov and Popova, 2011) calculated according to Kachel et al. (1998). The quenching of their fluorescence by cyt $b_6f$ demonstrates the ability of the complex to be inserted into the highly hydrophobic region of the lipid membrane.

The used lipid suspensions were diluted to 0.09 mM lipid content and provided to possess good optical parameters, very low light scattering, less than 1 %, as well as low extinction coefficients in the region of excitation and emission of the fluorescence. The constructed liposomes demonstrated a good fluorescent signal suitable for spectral measurements. Extruded to 100 nm liposomes were stable for 5 days at 4°C, but experiments were performed only with freshly prepared ones.

As referent preparations were also produced non-fluorescent liposomes by the same procedure.

Positively charged fluorescent liposomes were prepared by the same procedure but to the initial lipid mixture was added 5 mol% stearyl amine (SA).

Negatively charged liposomes were obtained by the addition of 5 mol% phosphatidyl glycerol (instead of SA).

The size of the fluorescent liposomes before and after the extrusion was controlled microscopically, using a fluorescent microscope Axiovert 25, Karl Zeiss with a source lamp HBO-50, Germany and digital camera VSS1000, Inray Solution Ltd, Bulgaria. The size of the liposomes was measured by a Bürker chamber (Fein Optic, Germany). Digital images were analyzed by using the ImageJ software (http://rsb.info.nih.gov/ij/) for measurements of the size of liposomes.

**Fluorescent spectroscopy analyses**

All fluorescent experiments were performed using a spectrofluorometer Jobin Yvon JY3D (France) equipped with temperature control device. Fluorescent liposomes were dissolved in 20 mM phosphate buffer (pH 7.0) at a final concentration 0.08 mg lipid/ml in a final volume of 2.5 ml. All experiments were performed at 36°C, above the phase transition temperature of the lipids and under continuous stirring. Temperature in the cuvette was controlled with a thermostat U-10, Germany, and thermocouple thermometer (Omega, Newport, Germany) with resolution 0.1°C.

Cyt $b_6f$-complex was added

consecutively into the thermo stated cuvette containing the liposomes by portions of 20 µl to a final quantity of 240 µl and a final protein concentration up to 0.37 µM. Fluorescence of $NBD_n$PC-labeled liposomes was excited at 459 nm and emission spectra recorded in the range of 486 to 636 nm. All fluorescent spectra were digitalized by an analog-to-digital converter (model NI-USB6008, National Instruments, USA) and then operated with "LabView" software (National Instruments, Austin, TX, USA). Obtained digital data were analyzed by Origin 7.0 (OriginLab, Northampton, MA, USA) software to obtain all fluorescent spectral parameters.

**Corrections of spectra and Stern-Volmer parameters**

All fluorescent spectra were corrected for the blank fluorescence, for bleaching effect, as well as for dilution and finally normalized to the initial fluorescence in the absence of protein. In a separate experiment the fluorescent spectra of the initial liposome suspension were measured but instead of cyt $b_6f$, pure buffer was added by 20 µl portions and spectra were recorded after each addition. Data were used to determine the linear correction function:

$$Q = 1 + K_n^{corr} \times V_n \qquad (1)$$

where Q is the quantum yield (the area under the spectrum) and $K_n^{corr}$ is the correction constant. The area under the measured spectra was simultaneously corrected for bleaching effect and dilution.

The fluorescence quenching efficiency of cyt $b_6f$ complex was analyzed by the Stern-Volmer equation for collision dynamic quenching (Lakowicz 2006):

$$Q_o/Q = 1 + K_{sv} \times [\text{Cyt } b_6f] \qquad (2)$$

where $Q_o$ and Q are the quantum yields in the absence or presence of protein, respectively. $K_{sv}$ is the Stern-Volmer constant and [Cyt $b_6f$] is the concentration of the protein.

When $Q_o/Q = 2$, then

$$[\text{Cyt } b_6f] = 1 / (K_{sv}) \qquad (3)$$

This is the concentration of cyt $b_6f$-induced quenching to 50% of the fluorophores.

Usually for determination of $Q_o$ and Q the fluorescence intensities at the maxima of the spectra are taken. However, in our experiments we always calculated the area under the spectra and used it as $Q_o$ and Q in Eq. 2.

**Detergent treatment of liposomes and removal of the detergent by Bio-Beads**

Three liposome samples, S1, S2 and S3, each containing 5 ml diluted to 0.09 mM lipid content of neutral liposomes were used. Liposomes in S1 and S2 were fluorescently labeled, but in S3 they were non-labeled. Fluorescence spectra of all samples were recorded. The purpose was all of them to go under equal light bleaching. This approach was applied in the procedures described below. An aliquot of 25 µl DOM from a stock solution was added to samples S1 and S3 to a final concentration of 8 µM (0.4%). Only buffer was added to S2. After 30 min of incubation, to S1 and S3 Bio-Beads were added to a final concentration of 50 mg per mg DOM (Rigaud et al., 1997). An equal volume of buffer was added to S2. All samples were incubated at room temperature for 2 h under continuous shaken, followed by centrifugation for 1 min at 1000 r.p.m. Supernatants from S1 and S3 were carefully removed and placed in fluorescent cuvettes. S2 was used as a

reference against Bio-Beads treatment and showed the absence of liposome sediment. The fluorescent spectra showed a slight loss of intensity of S1 compared with S2, evidencing that the treatment with Bio-Beads did not damage the liposomes. Afterwards Cyt $b_6f$-complex was added to S1 and S3 to a final concentration of 0.073 μM. An equal volume of buffer was added to S2. Fluorescent spectra of S1 and S2 were recorded 5, 15, 30, 45 and 60 min after protein addition to S1. Spectra of S2 were used for correction of the bleaching effect. S3 was used for reductase activity assay of the cyt $b_6f$-enriched liposomes.

## RESULTS

### Fluorescent quenching and spectra corrections

Cyt $b_6f$ complex spontaneously diffuses into the lipid bilayer and collisionaly quenches the fluorescence emitted by the fluorophores NBD-groups. In Fig. 1A a typical family of non-corrected fluorescent spectra in the absence or presence of cyt $b_6f$ is presented which demonstrates decreasing intensities with the increase of the cyt $b_6f$ concentration. The first spectrum on the left side was taken in the absence of protein and it had

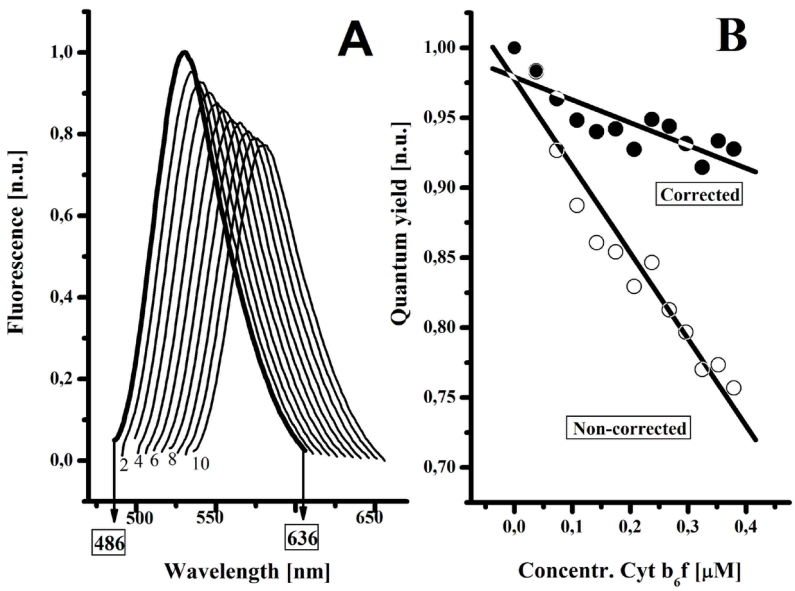


**Figure 1.** Fluorescence quenching during the Cyt $b_6f$ spontaneous insertion into liposome bilayer presented as changes in the quantum yield Q in normalized units (n.u.). Panel A - a typical family of non-corrected fluorescent spectra of $NBD_{12}PC$-labeled liposomes, quenched by increasing concentrations of Cyt $b_6f$. Bolded spectrum was experimentally obtained in the absence of Cyt $b_6f$ under excitation at 459 nm and fluorescence emission in the band of 486 to 636 nm. The emission maximum was at 530.5 nm. This spectrum was used as a base for the normalization calculations. Next 10 spectra (not bolded) were scanned at identical conditions after a gradual addition of Cyt $b_6f$ to the samples at concentrations ranging from 0.037 to 0.37 μM. These spectra are presented in the panel as right-shifted through 5 nm graphs. Panel B - the significance of the correction procedures for dilution and bleaching of the samples. Graphs were built from the spectral data in panel A for the quantum yield Q (area under the spectra).

the highest intensity as well as the highest area under spectrum. Fluorescence was excited at 459 nm and scanned from 486 to 638 nm. The maximum was at 530.5 nm. The next 10-step quenched spectra were obtained in the presence of increasing concentrations of Cyt $b_6f$, from 0.037 to 0.37 μM, under the same conditions. The spectra included in Fig 1A were right-shifted with 5, 10, 15 …50 nm from their real position into 486 to 638 nm band for better visualization. Detailed analysis of all spectra obtained with $NBD_6PC$- or $NBD_{12}PC$-containing liposomes, neutral, negatively and positively charged, showed neither shifts of the spectral maxima nor changes in the half-width during titration with cyt $b_6f$. This fact clearly demonstrated that the process of spontaneous insertion of cyt $b_6f$ caused dynamical, but not static quenching of the fluorophores emission. In other words Cyt $b_6f$ components did not bind to the membrane lipids.

Graphical presentation of the quenching effect of cyt $b_6f$ is given in Fig. 1B, demonstrating the significant influence of the dilution and bleaching of the sample on the non-corrected data. The correction function was experimentally obtained by the procedure described in "Materials and Methods", resulting in a [Cyt $b_6f$] value of 0.00094 μM.

The analysis of the experimental data presented in Fig. 1B clearly demonstrates that cyt $b_6f$ was spontaneously inserted deeply into the bilayer, reaching the fluorescent groups, located in the hydrophobic regions of the membrane and quenched their emission. The corrected data showed that approximately 9% of the fluorophores were quenched, evidencing that the cyt $b_6f$ constituents were able to insert into the bilayer and the complex was active to diffuse well.

The used approach allowed us to analyze the kinetics of this process by the model of Stern-Volmer (Lakowicz, 2006). The calculated by Eq. 2 graphs for the quenching process in neutral, negatively and positively charged liposomes, fluorescently labeled with $NBD_{12}PC$ are presented in Fig. 2. The graphs for $NBD_6PC$-labeled liposomes were similar and were not shown here. In all cases the graphs were straight lines evidencing again dynamical but not static quenching. The process of insertion was practically identical for neutral and negatively charged liposomes. However, positive charges on the membrane surface significantly hampered the insertion of cyt $b_6f$ into the lipid bilayer.

The Stern-Volmer coefficients calculated by these data (Eq.2) and concentration for 50% quenching (Eq. 3) are shown in Table 1. In all cases the Stern-Volmer constants for $NBD_{12}PC$ liposomes were higher than those for $NBD_6PC$, indicating better diffusion in the most hydrophobic zones. Statistical analysis based on the Student's *t*-test showed that the measured constants and 50%-accessibility concentrations were not significantly different at a level of confidence 90%. So we can only suggest a tendency for differences in these parameters for $NBD_6PC$ and $NBD_{12}PC$-liposomes.

## Insertion in Detergent-treated Liposomes

The experimentally obtained fluorescent spectra of sample S1 treated as described in Materials and Methods, are presented in Fig. 3A. Spectrum 1 was recorded in the absence of cyt $b_6f$.

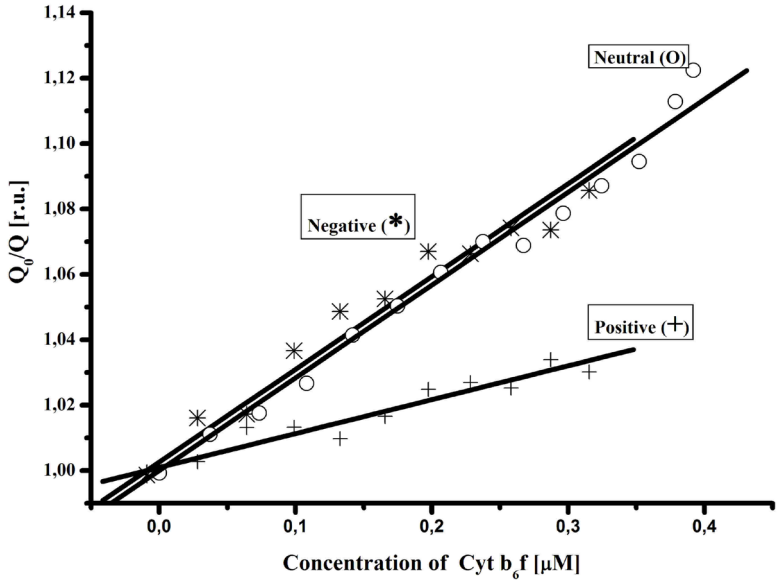


**Figure 2.** Stern-Volmer quenching graphs of $NBD_{12}PC$-labeled liposomes during Cyt $b_6f$ spontaneous insertion in neutral (○), negatively (*) and positively (+) charged liposomes. Experimental points represent results of three measurements (n=3) and standard errors did not exceed 2%.

**Table 1.** Quenching parameters of fluorescently labeled liposomes in the presence of Cyt $b_6f$ as a quencher.

| Type of liposomes | Stern-Volmer constants [$\mu M^{-1}$] | Accessibility of quencher [μM] |
|---|---|---|
| Neutral liposomes | | |
| $NBD_6PC$ labeled | 0.230 ± 0.06 | 50% at 4.35 ± 0.18 |
| $NBD_{12}PC$ labeled | 0.284 ± 0.06 | 50% at 3.52 ± 0.14 |
| Detergent-treated membranes | 10.000 | 50% at 0.10 |
| 5 mol% negatively charged | | |
| $NBD_6PC$ labeled | 0.240 ± 0.07 | 50% at 4.17 ± 0.21 |
| $NBD_{12}PC$ labeled | 0.284 ± 0.03 | 50% at 3.52 ± 0.18 |
| 5 mol% positively charged | | |
| $NBD_6PC$ labeled | 0.098 ± 0.05 | 50% at 10.2 ± 0.87 |
| $NBD_{12}PC$ labeled | 0.104 ± 0.01 | 50% at 9.61 ± 0.78 |

Note: Stern-Volmer constants were calculated according to Eq. 2. Accessibility of quencher to the fluorophores was calculated by Eq. 3.

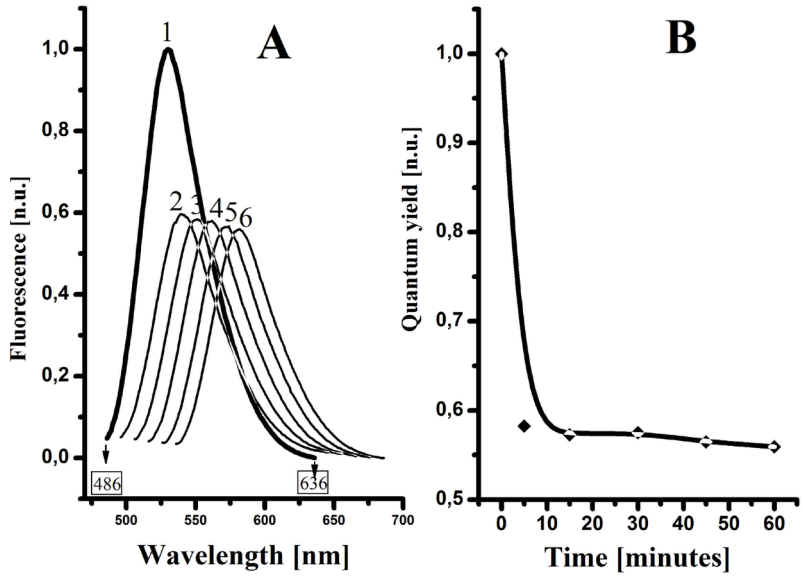


**Figure 3.** Influence of preliminary detergent treatment of liposomes on the spontaneous insertion of Cyt $b_6f$ into the lipid bilayer. Panel A - non-corrected fluorescent spectra of detergent-treated $NBD_{12}PC$-labeled liposomes. Fluorescence was excited and emission registered as described in Fig. 1. Bolded spectrum is of liposomes in the absence of Cyt $b_6f$. Next 5 spectra are obtained in the presence of 0.073 μM Cyt $b_6f$ 5, 15, 30, 45 and 60 min after protein addition. Their graphs are right-shifted trough 10 nm for better visualization. Panel B - time-dependent quenching of the fluorescence. Experimental points represent results of three measurements (n=3) and standard errors did not exceed 2%.

Spectrum 2 was registered 5 min after the addition of cyt $b_6f$ to 0.073 μM. Spectra 3 to 6 were scanned 15, 30, 45 and 60 min after the addition of cyt $b_6f$. The presented spectra 2 to 6 were again right-shifted as in Fig. 1A for a better visualization. It is easy to observe the strong quenching effect of cyt $b_6f$. Fig. 3B demonstrates that 5 min incubation with cyt $b_6f$ was enough to decrease the maximal fluorescence intensity to 0.5755 followed by a slow decrease with time. Using the correction procedure the insertion of cyt $b_6f$ was calculated as 42 % quenching, which was 4.7 times higher than that (9%) in the previous experiments in the absence of detergent.

**Reductase activity**

The absorbance spectra in the region of 595 nm of the liposome probe S3 after Bio Beads treatment showed that the activity of spontaneously inserted in liposomes cyt $b_6f$ was about 8% of the initial one. Cyt $b_6f$ activity inserted in DOM-treated liposomes reached 35% of the initial one, measured in free cyt $b_6f$ fractions.

## DISCUSSION

The large hydrophobic regions of the big protein complex cyt $b_6f$ are tightly built in the interior of the thylakoid membranes, thus making its extraction a serious problem. Procedures for solubilization of

the thylakoid membranes and extraction of cyt $b_6f$ in most cases include drastic invasion with NaBr and strong detergents, which are able to reduce the activity of important enzymes (Szymanska et al., 2011) or to remove important lipids from the native protein structures (Hasan et al., 2011).

On the other hand, extracted and solubilized protein structures undergo complex conformational changes to orient their less hydrophobic or hydrophilic residues to the new water surrounding. In the presence of detergents isolated proteins can bind their molecules and include them into its structures (Adam and Malkin, 1987) that may result in the loss of their native composition.

Having in mind all these complications for the isolation and purification of cyt $b_6f$, we used a procedure without application of detergents. The so prepared fraction of cyt $b_6f$ demonstrated the ability to reorganize the molecules of surface Langmuir monolayer phospholipids in specific structures (Jordanova et al., 2009). The nature of these structures was dependent on the electrical charge of the lipids. The ability of the cyt $b_6f$ fraction to bind lipids was recently confirmed with the most frequently found in the thylakoid membranes monogalactosyldiacylglycerol in experiments with surface films (Georgiev et al., 2012).

In the present work, new results evidencing the ability of cyt $b_6f$ to diffuse spontaneously into lipid bilayers, which are accepted as a good model of biological membranes, are discussed (New, 1999). Inserting into the interior of the lipid bilayer, the molecules of cyt $b_6f$ reach the hydrophobic regions where the fluorophores of the labeled phosphatidylcholine, carbon atoms $C_6$ and $C_{12}$ are situated. Part of Cyt $b_6f$ molecules come close to the excited fluorophores and quench their fluorescence. The experimental results clearly demonstrated that the complex penetrated easily in the shallower 9 Å and best in the deepest 18 Å from the surface hydrophobic regions of the membrane and this process was linearly dependent on the concentration of the complex (Fig. 1B). In accordance with the Stern-Volmer collisional coupling model the linearity indicated that there was no binding between cyt $b_6f$ and the lipids. On the other hand, the lack of changes in the half-widths and wavelength shifts of the fluorescent spectra (data not shown) manifested that the complex was able to self-reorganize into the hydrophobic lipid surrounding. These results indicated that the complex preserved its native properties to a great extent after the isolation procedure. The analysis of the experimental results showed that about 9 mol% of the cyt $b_6f$ molecules penetrated into the lipid bilayer without side influences and evidenced for the first time the principal opportunity for cyt $b_6f$ to diffuse spontaneously into the membranes. The calculated Stern-Volmer parameters presented in Table 1 showed that in neutral liposomes cyt $b_6f$ was better self-organized in the most hydrophobic regions where $NBD_{12}PC$ fluorophores were situated 18 Å from the hydrophilic surface in comparison with the upper regions ($NBD_6PC$), 9 Å from the surface. Accessibility concentrations for the most hydrophobic regions were lower than those for the shallower hydrophobic region (Table 1), evidencing that the complex assumed more convenient conformation in the most hydrophobic surrounding.

The process of spontaneous diffusion depends also on the electrical charge of the lipid bilayer. Poplar cyt $b_6f$ possesses a net negative charge (Donchev et al., 2004). Our results showed that the negative charge of the liposomes did not influence the diffusion in comparison with the neutral liposomes. One possible speculation is that the conformation of the complex in water environment buries his surface negative charges and some positively charged amino-acid residues predominate on the cyt $b_6f$ surface. In this case electrostatic repulsion with the negative surface of the vesicles did not affect the diffusion. If such an assumption is correct, it is easy to explain the decrease of the penetration into positively charged liposomes (Fig. 2). In this case the electrostatic attraction detained the negatively charged complex to the positively charged surface and cumbered the insertion of cyt $b_6f$ to the deepest membrane regions.

The parameters of Stern-Volmer for electrically charged liposomes again confirmed the preference of cyt $b_6f$ to diffuse better into the most hydrophobic zones of the bilayer (Table 1).

Many investigators have reported more effective approaches for reconstitution of cyt $b_6f$ in lipid-enriched systems (Hurt and Hauska, 1981; Hurt et al., 1982; Doyle and Yu, 1985; Adam and Malkin, 1987; Cramer et al., 1992; Huang et al., 1994; Chain et al., 1995; Breyton et al., 1997; Schoepp et al., 2000; Yan et al., 2001; Whitelegge et al., 2002; Girard et al., 2004, Lezhneva et al., 2008; Hasan et al., 2011; Sang et al., 2011; Szymanska et al., 2011). In the present work, we also report an attempt to enhance the process of spontaneous diffusion applying a moderate effect of the week non-ionic detergent DOM, which would help to loosen the packing of lipids and open gaps on the vesicle surface. With the aim to avoid insertion of DOM molecules into cyt $b_6f$ structures, the liposome suspension was treated with Bio-Beads before the addition of cyt $b_6f$. The pore size of the polystyrene adsorbent Bio-Beads was 9 nm, large enough to adsorb the small molecules of DOM. Extruded liposomes (100 nm) remained in the supernatant, clear of free detergent molecules.

The spontaneous diffusion of cyt $b_6f$ in these detergent-treated liposomes reached more than 40 mol% (Fig. 3). The apparent Stern-Volmer parameters presented in Table 1 confirmed the improved more than 11 times diffusion of cyt $b_6f$ in detergent-treated membranes.

The speed of spontaneous diffusion is also an important parameter. This process needed 5 min to reach 39 mol% of enrichment of the vesicles with cyt $b_6f$ (Fig. 3B). Prolonged to 60 min incubation enhanced the cyt $b_6f$-enrichment of liposomes with only 3 mol% to 42 mol%. These results showed that the diffusion process was quite fast.

It is important finally to point out that all experiments in this work were performed at 36°C, above the phase transition of the lipids used.

In conclusion, here we report a new approach for obtaining clear cyt $b_6f$-enriched lipid membranes possessing reductase activity, suitable also for planar systems which are more usable for bio-technological applications.

## ACKNOWLEDGMENTS

This study was partly supported by the Bulgarian National Fund for Scientific Investigations, Contract B1519/2005.

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