# REKONSTRUCTION OF CYTOCHROME $b_6$f-COMPLEX INTO ARTIFICIAL LIPID MEMBRANES

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## ABSTRACT

Cytochrome $b_6$f is known as plastoquinol reductase (EC1.10.99.1). It transfers electrons between photo system 2 (PSII) and photo system 1 (PSI) via plastocyanines. Often proteins are reconstructed in liposomes to study their properties. Present work reported similar experiments.

Cytochrome $b_6$f was isolated and purified from poplar leaves (Populus nigra, var. Italica).

Used liposomes were fluorescently marked and their electric charge was controlled.

In neutral liposomes approximately 10% of $b_6$f-complex spontaneously penetrates into the membranes. In charged liposomes the reconstruction decreases significantly.

0.4% n-dodecyl β-d maltoside (DDM) increased the reconstitution to 40%. Simple procedure for removing of this detergent was also experienced.

***Key words**: Reconstruction, Membrane protein, Unilamellar vesicles.*

## INTRODUCTION

Multisubunit oligomeric protein cytochrome $b_6$f complex (Cyt $b_6$f) is classified as plastoquinol-plastocyanin reductase (EC1.10.99.1). It is a dimeric transversal membrane complex, isolated from different species as cyanobacterium and green algae [6], chloroplasts of spinach and pea [9, 3] and other plants. Data available for spinach had shown that the complex is consisted of four main subunits: cytochrome $b_6$ (24.2 kD), cytochrome f (32 kD), Rieske iron-sulfur protein (19.1 kD) and subunit IV (17.4 kD) (13, 3). Cyt $b_6$f is a key complex in the photosynthesis. It transfers electrons from PSII to PSI and participate in the formation of transmembrane electrochemical gradient, transferring protons from the stromal to the inner lumenal space of the thylakoid membrane [6]. Structure and functioning of Cyt $b_6$f are highly dependent of the presence of lipids. Many authors emphasize their important role in the structure [5, 4, 9, 2]. During the procedures of isolation of Cyt $b_6$f from its natural membrane environment, the complex looses many constituent lipids. Experiments demonstrate that the activity of the complex could be recovered by addition of lipids. However, single work describes reconstitution of Cyt b6f in phospholipid vesicles [12]. Liposomes were formed from a mixture of Cyt $b_6$f and lecithin under sonication.

In the present work we report reconstruction in preliminary formed phospholipid bilayer membranes with controlled composition and electrical charge. Liposomes were constructed in presence of fluorescent marker1-palmitoyl-2-[12-[(7-nitro-2-1, 3-benzoxadiazol-4-yl) amino] hexanoyl] –sn-glycero-3-phosphoholine ($NBD_{12}PC$). Neutral lecithin liposomes, negatively charged with additive of phosphatidylglycerol (PG) and positively charged with stearyl amine (SA) liposomes were used.

Cyt $b_6$f from poplar leaves *(Populus nigra, var.Iitalica*) was for a first time isolated in our lab [8]. Used in the present work preparations were obtained by similar, but improved chromatographic procedures without detergents [7], giving highly purified Cyt $b_6$f-complex.

## MATERIALS AND METHODS

Egg yolk lecithin was purchased 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. 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 (DDM) was from Sigma, USA and Bio-Beads from Bio-Rad, USA. All other

chemicals were of analytical grade. Polycarbonate Track-Etch membranes with pore size of 100 nm, used for extrusion, were purchased from Whatman, Germany.

Cyt $b_6f$ subcomplexes were prepared from fresh leaves of poplar (*Populus nigra, var. Italica*) following the method of Dimitrov et al. [7]. In order to prove the presence of the heme proteins of Cyt $b_6f$ subcomplexes absorbance spectra were recorded at room temperature on Specord UV-Vis spectrophotometer (Germany).

Fluorescent liposomes were prepared by a modified procedure of Hub et al. [11].

Neutral liposomes were constructed from a mixture of lecithin and $NBD_{12}PC$ (100:1 mol/mol) in chloroform. Solvent was evaporated in a rotary vacuum evaporator, keeping the flask in a water bath at 40 $^0$C, and then the solvent were additionally evaporated for 2 hours 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 to gain complete hydration. Hydrated lipids were gently shacked to obtain spherical unilamellar liposomes with different diameters. These suspensions of liposomes were extruded to size of 100 nm liposome diameter. Positively charged fluorescent liposomes were prepared by the same manner but in the initial mixture were added 5% stearyl amine. Negatively charged liposomes were obtained by the addition of 5% phosphatidyl glycerol (instead of SA).

Fluorescent spectroscopy was applied by mixing of 2.3 ml of 20 mM phosphate buffer, pH 7 and 0.2 ml suspension of the respective fluorescent liposomes in a quartz fluorescence cuvette and measurements were made on a spectrofluorometer Jobin Yvon JY3 D (France). Spectrofluorometer is completed with temperature adapted cuvette holders. The temperature of the liposome suspensions into the quartz cuvettes was adjusted to 40 $^0$C using water-flow thermostat and temperature controlled with thermocouple thermometer Omega, Newport, Germany with resolution 0.1 $^o$C, allowing performing the experiments above the phase transition of the used lipids. Phase transition of egg yolk lecithin membranes is 39 $^o$C (10) and 23 $^o$C for phosphatidylcholine [17].

$B_6f$-subcomplex was added consequently into the cuvette by portions of 20 µl to final quantity of 240 µl and final protein concentration up to 0.4 µM. Fluorescence of $NBD_{12}PC$-labeled liposomes was excited at 460 nm and emission spectra scanned in the range of 486 to 638 nm. All the fluorescence spectra were digitalized by analog-to-digital converter; model NI-USB6008, National Instruments, USA and operating with "LabView" software. Digital data bases were then processed by Origin 7.0 (OriginLab, Northampton, MA, USA) software 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 absence of protein.

Quenching efficiency of $b_6f$-complex on the fluorescence was analyzed according to the Stern-Volmer equation for collisional dynamic quenching [1]

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

where $F_o$ is the fluorescence in absence of protein and F 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

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

and that is the concentration at which Cyt $b_6f$ quenches 50% of the fluorophores.

In another set of experiments the soft detergent n-dodecyl-β-D-maltoside was added beforehand to the liposome suspension to a final concentration in the cuvette 0.4%. $B_6f$-subcomplex was added to 0.15µM concentration and fluorescence spectra were measured in time intervals 5, 15, 30, 45 and 60 minutes. Detergent was removed by 1 h incubation with 15 mg BioBeads per 1 mg DDM. After centrifugation the supernatant was free of DDM [16].

**RESULTS AND DISCUSSION**

Gel electrophoresis of the used $b_6f$ fraction had shown that it contains the four main components of the complex, Cyt f, Cyt $b_6$, Rieske protein and Subunit IV. The "purity index", ratio

of absorption at 404 to 280 nm, was over 1.1, demonstrating the presence of hems in the complex, so we can expect that the complex will be functionally active.

Constructed liposomes possessed good fluorescent intensities, enough for spectral measurements. The fluorescent group of the used liposome marker $NBD_{12}PC$ locates in the region of the 12-th carbon atom of the lipid tails, approximately of 18 Å from the membrane surface [15], the most hydrophobic regions of the membrane. Cyt $b_6f$ complex penetrates there and collisionaly quenches the fluorescence. In Fig. 1 are present the fluorescent spectra of the liposomes in absence and presence of $b_6f$, which demonstrate decreasing intensities with the increase of the $b_6f$ concentration in the liposome suspension.

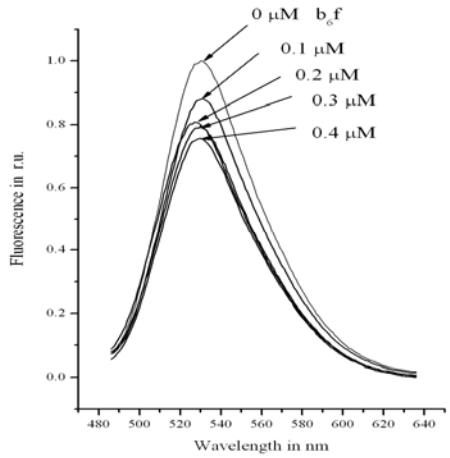


Fig. 1. Fluorescence spectra of $NBD_{12}PC$-marked liposomes in absence and presence of 0.1, 0.2, 0.3 and 0.4 μM $b_6f$-complex. All spectra were for 12 different concentrations of $b_6f$, but here are present only four. Spectra were normalized to the highest intensity in absence of quencher.

These experiments clearly demonstrated that $b_6f$ penetrates deeply into the membranes, reaching the fluorescent group of $NBD_{12}PC$, located in the hydrophobic membrane regions and quenching their emission. Analysis of the spectra had shown that approximately 10% of the fluorophores were quenched, evidencing that about 10% of the $b_6f$-constituents are able to reconstruct in the membrane and the complex is active to penetrate into the membrane. Used approach allows us to analyze the kinetic of this process by the model of Stern-Volmer [1]. In Fig. 2 are present the calculated by equation (2) graphs for the quenching process in neutral, positive and negative charged liposomes.

Quenching coefficient for the neutral liposomes $K^N_{sv}$ was 0.27 ± 0.01 $\mu M^{-1}$. Following equation 2 one can calculate that half of fluorophores groups could be quenched with 3.7 μM $b_6f$-complex. Respective quenching in positively-charged liposomes $K^+_{sv}$ was 0.22 ± 0.02 $\mu M^{-1}$ and 4.5 μM $b_6f$-complex need for half quenching and finally for the negative liposomes $K^-_{sv}$ was 0.03 ± 0.01 $\mu M^{-1}$ and half-quenching requires respectively 33.3 μM $b_6f$. These data demonstrate that the charge of the liposomes hinders reconstruction of $b_6f$ into the membrane. This could be explained with electrostatic interactions between $b_6f$ and liposome surface. B6f has a net negative charge [8] and electrostatic attractions of the positively-charged surface of liposomes detain $b_6f$ molecules at the surface, being obstructive the complex to penetrate into the deep membrane regions. However this effect is week in comparison with the

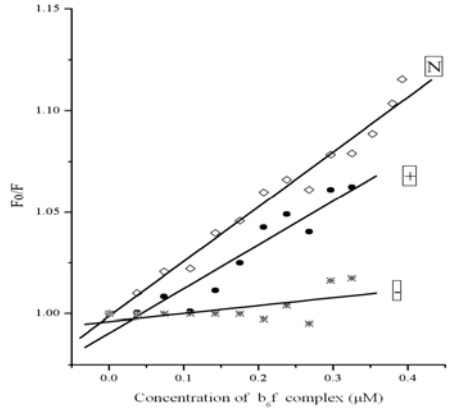


Fig.2. Stern-Volmer graphs of quenching by $b_6f$-complex in neutral (N), positively (+) and negatively (-) charged fluorescent liposomes. Experimental points were measured for the maximal fluorescence intensities in presence of 12 different concentrations of $b_6f$ (n=12).

Negatively charged liposomes, where the spontaneous reconstruction in the membranes is practically blocked by the electrostatic repulsion between $b_6f$ and charged liposomes. Similar data we obtained in previous experiments with lipid monolayers [14].

Degree of reconstruction is possible to increase with application of detergents [16]. In Fig. 3 are present the results of similar experiments.

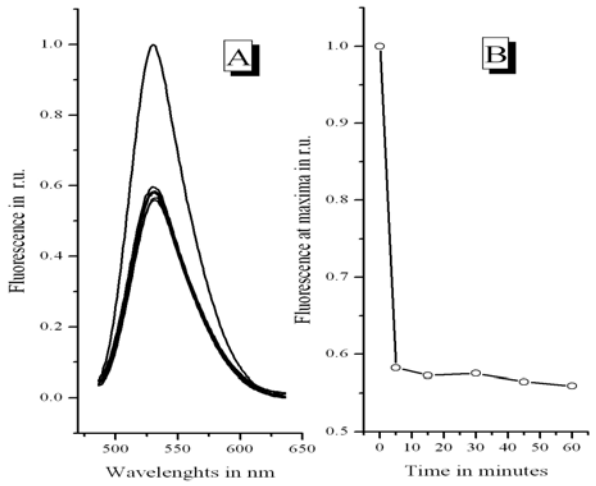


Fig. 3. Panel A: Fluorescent spectra of liposomes in presence of 0.2 % DDM. Highest spectrum was recorded in absence of $b_6f$ and next lowered spectra in presence of 0.15 μM $b_6f$ after 5, 15, 30, 45 and 60 minutes after their addition. Panel B: Time dependent fluorescence intensities after the addition of Cyt $b_6f$

Effectiveness of the reconstruction increased 4 times, reaching about 40% (Panel A). Results in Panel B demonstrate that the process is comparatively fast and after 5-th minute extend penetration is practically absent.

## CONCLUSIONS

- ✓ Cyt $b_6f$ from poplar is able spontaneously reconstructed in artificial lipid membranes.
- ✓ It is functionally active to reacts with differently charged liposomes.
- ✓ Negative charges of liposomes block the reconstruction.
- ✓ Weak detergent in minimal concentration increases the spontaneous reconstruction nearly four times.

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