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INFLUENCE OF CHOLESTEROL CONCENTRATION ON BACTERIORHODOPSIN PHOTOCYCLE

The photocycle of the membrane protein bacteriorhodopsin in Dipalmitoylphosphatidylcholine(DPPC)/Cholesterol membranes with various cholesterol concentrations has been studied using the time-resolved spectroscopy method. The temperature dependences of the rate constants of bacteriorhodopsin transitions between transient states are shown to satisfy the Eyring equation. It is proved that the growth of the cholesterol concentration in the DPPC membrane accelerates the bacteriorhodopsin photocycle.

K e y w o r d s: laser photolysis, lipid membranes, photocycle, bacteriorhodopsin, cholesterol.

1. Introduction

Lipid membranes are an important component of living organisms, because they regulate plenty of the processes that are required to maintain the viability of every cell and cell organelles. That is why a considerable number of works on biophysics is devoted to the study of phase transitions in lipid membranes and their dependence on the temperature and pressure [1–4], the structural characteristics of various lipid phases [5, 6], the influence of various impurities on the properties of membranes [7–11], as well as to the measurement of the dynamic characteristics of single- and multicomponent membranes [12, 13].

One of the most important functions of the lipid membrane is to provide the functioning of membrane proteins. It is the lipid membrane that is assumed to control the activity of proteins [14], in particular, by changing its phase state and composition. The study of lipid-protein interactions is a rather challenging task, because it helps to better understand the functioning mechanisms of membrane proteins.

One of the most wide-spread components of the eukaryotic cell membranes is cholesterol, the fraction of which reaches 20–30 mol% [15]. An excess of cholesterol in the body leads to the development of gallstone disease, upsets metabolism, and promotes the formation of various depositions in the organism. Recent studies testify that it is excess cholesterol that is actively involved in the formation of amyloid plaques giving rise to Alzheimer's disease [15, 16]. From the viewpoint of the lipid membrane structure, cholesterol plays a role of a peculiar modifier of the lipid phase [15, 17–19]. An increase of its amount in the membrane leads to a denser packing of molecules in the lipid bilayer [20].

With regard for the role of cholesterol in the life of living organisms, we decided to consider its influence on the functioning of membrane proteins. The membrane protein bacteriorhodopsin (BR), one of the most studied photoactive proton pumps [21], was chosen as the object of research. Proton pumping occurs as a result of the series of BR conformational tran-

sitions. In the course of this process, five intermediate forms of BR are distinguished. Each of them is characterized by the light absorption with a characteristic maximum at a certain wavelength. The photocycle measurement (times of the protein staying in each transient form) is a direct method of determining the influence of external conditions on the protein functioning [22–24].

This work is devoted to the application of the laser flash photolysis method to study the photocycle of the BR embedded into Dipalmitoylphosphatidylcholine (DPPC) vesicles with various cholesterol concentrations in them.

2. Materials and Methods

2.1. Sample preparation

To study the dependence of the BR kinetics on the lipid composition in the surrounding membrane, four samples of DPPC-based vesicles with different cholesterol contents were prepared: 0, 6.5, 18, and 35 mol%. DPPC and cholesterol were obtained from the Avanti Polar Lipids Co. and used without additional purification. The total lipid mass (DPPC and cholesterol) was 20 mg in each sample. The resulting lipid mixtures were dissolved in 1 ml of trifluoroethanol with a small chloroform admixture (less than 1%) in order to obtain homogeneous lipid mixtures. The solutions were thoroughly stirred and kept at room temperature for several hours. Afterward, the organic solvents were evaporated until lipid films were formed in each of the four reservoirs. Then, the samples were held for two days in a vacuum chamber to ultimately remove the organic solvents from them.

For the coarse purification of BR, a phosphate buffer solution with the acidity of pH = 6.94 and containing the sodium salt with a concentration of 20 mmol/l – those parameters are natural for BR – was prepared. For the purification, the protein solution (with a protein concentration of about 8 mg/ml) was washed three times with the phosphate buffer and subjected to the 90-min centrifugation at an acceleration of 20000 g. The lipid residues of the native membrane were removed from the protein solution by adding the detergent octyl-beta-glucoside (OBG) to the latter and repeating the centrifugation procedure.

Hence, after the purification, we obtained a protein solution in which BR molecules were solubilized by the detergent OBG. This solution was added to a vessel containing the previously dried lipid films. In each of the four samples, the molar ratio between the total amounts of lipids and protein was 300 : 1. In order to remove the detergent from each sample, the non-polar adsorbent Bio-Beads SM-2 Resin (Bio-Rad, USA) was used. It is widely applied for the selective removal of organic substances from aqueous systems. The adsorbent mass was 20 times the detergent mass in each solution.

At the final stage, four samples – aqueous systems containing lipid vesicles with embedded BR protein molecules – with different cholesterol concentrations were obtained. To homogenize the samples, they were extruded through a filter with a pore diameter of 100 nm. It was done to obtain vesicles with small sizes, which are transparent in the visible light spectral interval.

2.2. Laser flash photolysis

The laser flash photolysis method consists in measuring the time dependence of the light transmittance through a sample at a certain wavelength. The research was performed on a specially designed installation, the schematic diagram of which is shown in Fig. 1.

A quartz cuvette with the sample was mounted on a special holder (Quantum, Norway). Inside the latter, a given temperature was maintained with an accuracy of 0.05 °C. To excite the BR photocycle, a Nd : YAG laser Brilliant B (Quantel, France) equipped with an optical parametric oscillator MagicPrism (Opotec, Carlsbad, USA) was used. It generated a laser beam 6 mm in diameter and a pulse duration of 4 ns. The light wavelength can be varied from 400 to 700 nm. As a source of probing radiation, a 75-W Hamamatsu xenon lamp (Japan) in combination with an MSH-150 monochromator (LOT, Germany) was used. The detector system included a photoelectronic multiplier R12829 and two DSO-X4022A oscilloscopes (Keysight) with a bandwidth of 200 MHz.

3. Results and Their Discussion

To study the dependence of the characteristic features in the bacteriorhodopsin kinetics on the composition content in lipid membranes, four liposome samples with different cholesterol concentrations were prepared. Each sample was measured using the laser

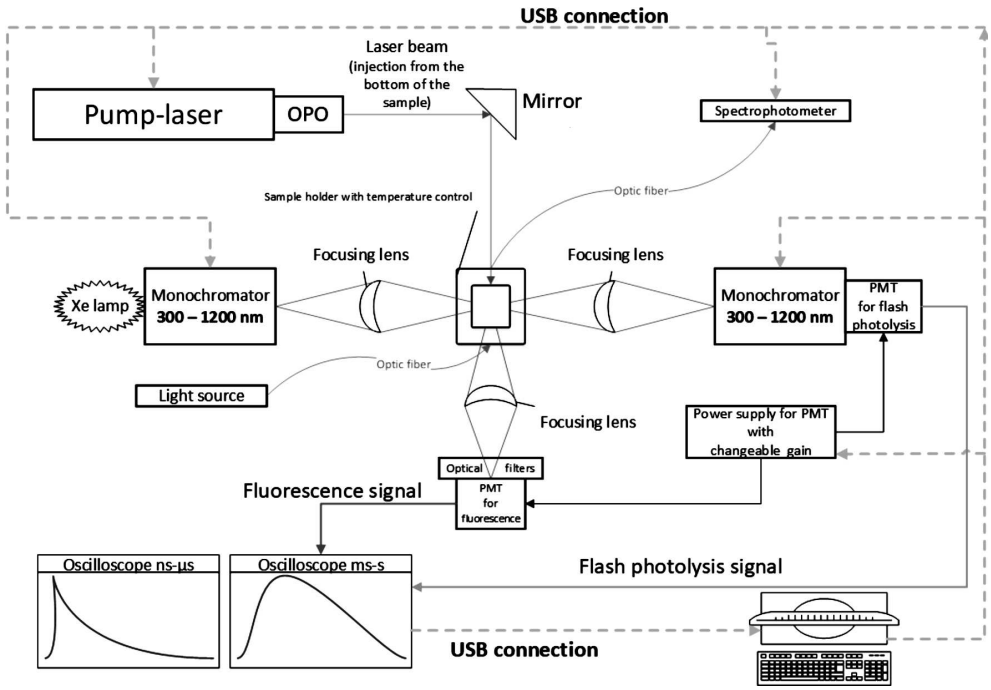

Fig. 1. Schematic diagram of the laser photolysis installation

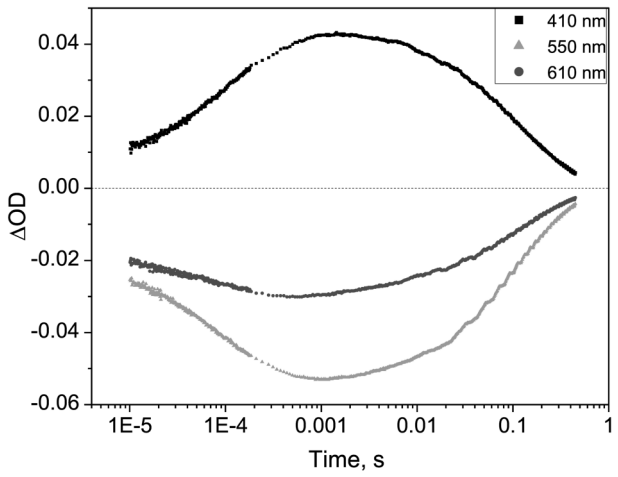

Fig. 2. Time dependences of the optical density changes of the bacteriorhodopsin sample with the 35% cholesterol concentration in the membrane at a temperature of 10 °C for various probing radiation wavelengths: 410 (squares), 550 (triangles), and 610 nm (circles)

flash photolysis method within the wavelength interval of probing radiation from 330 to 730 nm with an increment of 10 nm. The measurements were performed at temperatures of 10, 20, 30, and 40 °C. In order to reduce the statistical error, the time dependence of the light transmittance at every examined wavelength was independently measured 25 times for each sample. As a result, a set of 41 experimental dependences for the change of the optical density of the protein solution in time was obtained for each sample.

Figure 2 demonstrates the time dependences of the optical density ΔOD_λ obtained for the bacteriorhodopsin sample with a 35% cholesterol concentration in the membrane. The dependences were measured at a temperature of 10 °C and various probing radiation wavelengths: 410 (squares), 550 (triangles), and 610 nm (circles). The magnitudes of the rate constants k_i for the transitions between the transient BR states, the set of ΔOD_λ values at each temperature was approximated by the sum of five exponential functions (according to the number of transient BR states),

$$\Delta OD_\lambda(t) = \sum_{i=1}^{5} A_{i\lambda} e^{-k_i t}. \qquad (1)$$

The experimental data were analyzed with the help of the software MEXFIT [25]. The results obtained were tested by comparing the model curves with the

corresponding experimental results. This procedure is described in work [21] in more details.

As a result of fitting the experimental data that were measured for each sample at a given temperature, a set of rate constants describing the BR photocycle was obtained. An analysis of the rate constant dependences on the inverse temperature $1/T$ testifies that the rate constants change according to Eyring's law

$$\ln(k_i) = \ln\frac{R}{N_A h} + \frac{\Delta S_i}{R} - \ln\frac{1}{T} - \frac{1}{T}\frac{\Delta H_i}{R}, \qquad (2)$$

where R is the universal gas constant, h Planck's constant, N_A the Avogadro number, and ΔS_i and ΔH_i are the activation entropy and enthalpy, respectively. In Fig. 3, an example of this dependence is exhibited for the rate constants of the bacteriodopsin sample with the 35% concentration of cholesterol in the membrane. The right-hand axis of the plot gives the transition half-time τ_i, which is related to the transition rate constant τ_i by the formula $\tau_i = (\ln 2)/k_i$.

When comparing the experimental time dependences of the changes in the optical densities obtained for the samples with various cholesterol contents, one can see that the duration of the BR photocycle decreases, as the cholesterol content in the membrane increases. In Table, the half-times of BR transitions between the transient states at a temperature of 20 °C are quoted for various concentrations of cholesterol in the membrane. According to the literature data [21], the duration of the photocycle of BR in its natural membrane does not exceed 25 ms at a temperature of 20 °C. At the same time, the duration of the BR photocycle in a DPPC membrane in the absence of cholesterol increases to 120 ms (see Table, column τ_5). The growth of the cholesterol concentration in the membrane accelerates the BR photocycle, so that if the cholesterol content equals 35%, the BR transition into the latest transient state occurs during 70 ms.

It should be noted that cholesterol does not enter the composition of bacterial membranes, i.e. it is not a natural component of the BR protein. Bearing this fact in mind, we may assert with a high degree of confidence that the acceleration of the BR photocycle is not associated with the direct interaction between BR and cholesterol. Therefore, the main reason for the photocycle kinetics to change is a modification

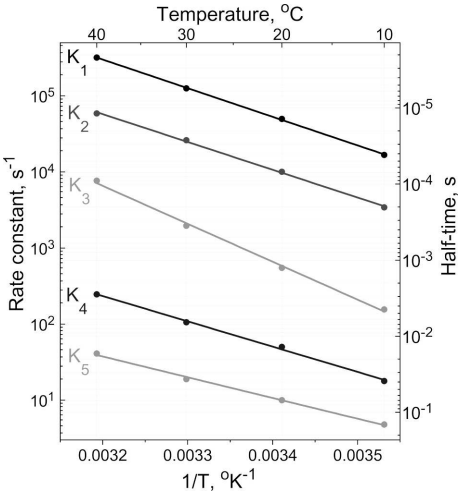

Fig. 3. Dependences of the rate constants for the transitions between the transient states of the bacteriodopsin sample with a cholesterol concentration of 35% on the inverse temperature

in the structure of the membrane itself invoked as a result of the cholesterol adding.

The authors of work [26] studied the influence of bacteriodopsin on the temperature of the main phase transition in phospholipid membranes making use of the differential scanning calorimetry method. It was shown that if bacteriodopsin is added to dimeristoylphosphatidylcholine (DMPC) membranes, the temperature of the main phase transition in the membrane increases by 5 °C. The cited authors explain this phenomenon from the viewpoint of the ratio between the size of the hydrophobic part of bacteriodopsin and the lipid membrane thickness. The hydrophobic part of bacteriodopsin has a larger thickness, which results in an artificial growth of the membrane thickness around the protein. But this growth

Comparison of half-times of bacteriodopsin transitions into transient states at a temperature of 20 °C for various concentrations of cholesterol in the membrane

Cholesterol concentration, mol%	τ_1, μs	τ_2, μs	τ_3, μs	τ_4, μs	τ_5, μs
0	1.5	60	1300	27 000	120 000
6.5	1.5	70	840	23 000	100 000
18	1.6	70	8500	16 000	82 000
35	1.4	7	1300	14 000	70 000

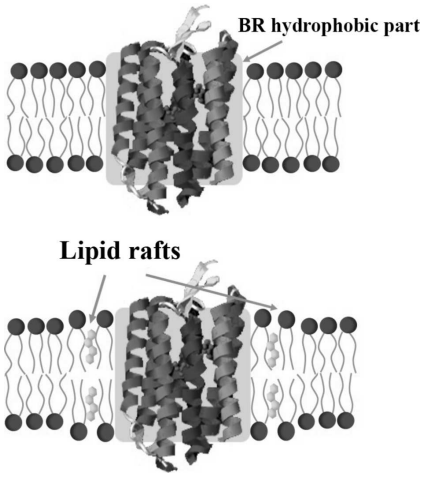

Fig. 4. Bacteriorhodopsin in the lipid membrane of DPPC in the absence of cholesterol (top panel) and the growth of the membrane thickness around the protein due to the formation of domains (bottom panel)

is possible only provided that the lipid tails are elongated as much as possible. Therefore, for the tails of lipid molecules located around the protein to melt, more energy is required than for molecules not adjacent to the protein. The cited authors marked that the growth of the main phase transition temperature in the presence of bacteriodopsin was also observed for DPPC-based membranes. However, the temperature difference was smaller, because the DPPC-based membranes are thicker than the DMPC ones.

Since the described lipid-protein interaction is rather strong, it evidently affects not only the lipid but also the protein. Therefore, the deceleration of the BR photocycle in the absence of cholesterol can be explained by the thickness difference between the DPPC lipid bilayer and the hydrophobic part of the protein (Fig. 4, top panel).

On the other hand, the addition of even a small amount of cholesterol to the membrane brings about the formation of domains in it [13], whose thickness is larger than the thickness of single component membrane [20]. The arrangement of the domains around the BR molecules reduces the local difference between the thicknesses of the hydrophobic part of protein and the membrane, thus reducing the strength of the lipid-protein interaction (Fig. 4, bottom panel) and accelerating the BR photocycle. Since the results of our studies correspond to a BR photocycle averaged over the whole ensemble of molecules in the solution (rather than the photocycle of a single BR molecule), a gradual increase of the cholesterol concentration leads to a gradual increase of the BR photocycle rate.

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ВПЛИВ КОНЦЕНТРАЦІЇ ХОЛЕСТЕРИНУ НА ФОТОЦИКЛ БАКТЕРІОРОДОПСИНУ

Р е з ю м е

В роботі за допомогою методу спектроскопії з розділенням в часі проведено дослідження фотоциклу мембранного білка бактеріородопсину в залежності від концентрації холестерину в мембрані дипальмітоїфосфатидилхоліну (ДПФХ). Показано, що температурні залежності констант швидкостей переходів бактеріородопсину між проміжними станами задовольняють рівняння Ейрінга. Доведено, що додавання холестерину в мембрану ДПФХ приводить до прискорення фотоциклу бактеріородопсину.