Light-driven proton transport of bacteriorhodopsin incorporated into long-term stable liposomes of a polymerizable sulfolipid

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The chromoprotein bacteriorhodopsin from *Halobacterium halobium* has been incorporated into liposomes made of a fully synthetic, polymerizable lipid. Bacteriorhodopsin is found to be active in these polymer liposomes. The advantage in the use of such polymer systems concerning long-term stability in comparison with liposomes made of natural lipid is demonstrated.

Bacteriorhodopsin *Light-driven proton pump* *Liposome* *Polymerizable synthetic lipid* *Long-term stability*

1. INTRODUCTION

Incorporation of purified membrane proteins into model membranes is a biochemical approach to the elucidation of their function. The use of reconstituted model systems such as liposomes from phospholipids is restricted by their lack of long-term stability. A method to overcome this problem is the application of polymerizable synthetic lipids [1].

The membrane protein used for our reconstitution experiments is the chromoprotein bacteriorhodopsin. In the cell membrane of *Halobacterium halobium* bacteriorhodopsin (M_r 26000) is arranged in semicrystalline domains, the purple membrane patches [2]. The protein acts as a light-driven proton pump. Upon illumination with visible light (λ = 570 nm) it undergoes a photochemical cycle, which is accompanied by a vectorial proton transport across the cell membrane. In reconstituted systems, due to this effect, protons are taken up by the proteoliposomes whereas intact Halobacteria cells pump the protons to the outside. This difference is caused by the inverse orientation of bacteriorhodopsin in reconstituted liposomes [3]. The proton transport is indicated by a pH shift in the outer medium.

Here we report on the incorporation and function of purple membrane (PM) fragments in liposomes prepared from soybean lecithin and a polymerizable, synthetic sulfolipid (fig.1). The application of the polymerizable lipid to the study of liposome-bound membrane proteins was introduced in [4].

Abbreviations: PM, purple membrane; BR, bacteriorhodopsin

2. MATERIALS AND METHODS

Halobacterium halobium R_1M_1 cells were grown in a 10 l fermenter at 40°C according to [5]. Growth conditions for optimal PM synthesis were as in [6]. The isolation of PM-patches was performed according to [7]. The final purification procedure of the PM fragments was modified by applying a 30–40% sucrose density gradient. The polymerizable sulfolipid was a generous gift by H. Koch (Mainz). Soybean lecithin was purchased from Sigma (St Louis MO).

Proteoliposomes were prepared by presonication of lipid suspensions in 150 mM KCl with a microtip. The pH of the almost clear solution was adjusted to 7.0. After addition of the PM

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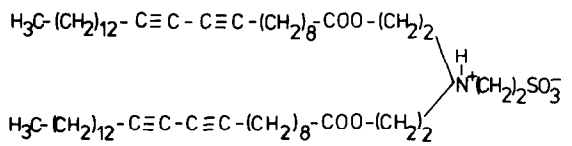

Fig.1. Structure of the polymerizable diacetylenic sulfolipid.

fragments the incorporation was achieved by further ultrasonication. This procedure was carried out under nitrogen, at 60°C in the case of the sulfolipid and at 0°C in the case of soybean lecithin.

Energization of the BR liposomes was achieved by illumination with a 250 W projector lamp (1.2×10^3 J/m^{2}s). The resulting pH shift was measured with a micro pH-electrode (EA 147, Metrohm) connected to a highly sensitive ion meter (E 600, Metrohm) equipped with a Labograph E 580 (Metrohm). The liposome suspension was magnetically stirred in a thermostated cuvette. All experiments were performed at 37°C. The sulfolipid proteoliposomes were polymerized by UV-irradiation with a Philips low pressure mercury lamp (254 nm; 0.1 J/m^{2}s) at 6 cm.

3. RESULTS

A typical experimental curve for the light-driven proton uptake in monomeric sulfolipid and soybean lecithin proteoliposomes is shown in fig.2. On illumination, bacteriorhodopsin energizes the proton transfer across the membrane. The magnitude of the resulting pH shift in the medium depends on several experimental parameters, in particular on the sonication time, the amount of lipid and the protein : lipid ratio. In the case of soybean lecithin a maximal pH shift was obtained at a lipid : protein ratio of 10–15 : 1 (w/w) whereas a 4 : 1 (w/w) ratio was optimal in the case of the sulfolipid. The light-driven proton uptake increased with the time of sonication. The maximal effect was achieved after 12 min sonication with soybean lecithin and after 6 min with the sulfolipid. The maximum proton uptake under steady state condition typically reached 6 mol H^+/mol BR for liposomes from either lipid. Polymerization of the monomeric lipids in the BR liposomes changed the characteristic of the pH change–time diagrams (fig.3). The pH no longer rises to a constant value

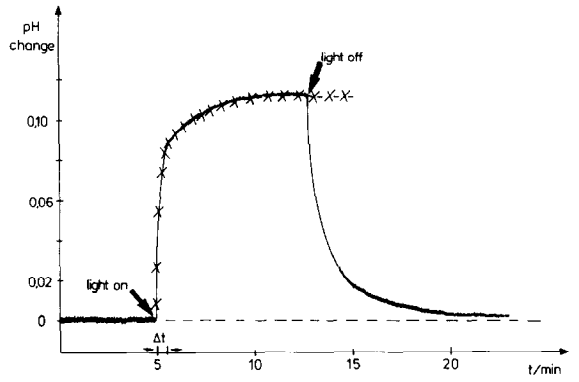

Fig.2. Typical pH change–illumination time curve for soybean lecithin and monomeric sulfolipid BR liposomes. The figure shows the illumination of soybean lecithin BR liposomes with visible light (150 μg BR/ml, 1.8 mg lecithin/ml); (×—×) theoretical curve; S_1 = 0.091, k_1 = 0.075 ± 0.002; S_2 = 0.037, k_2 = 0.0067 ± 0.0001.

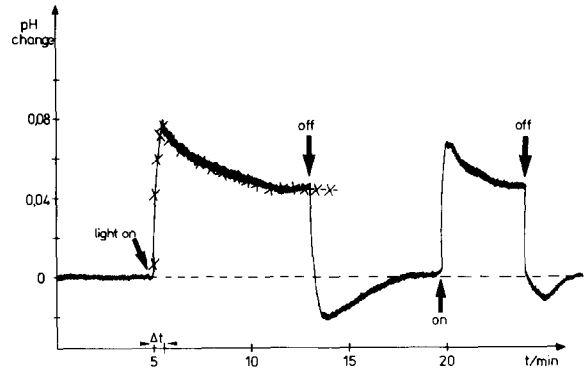

Fig.3. Proton pumping activity of BR in polymerized sulfolipid liposomes (150 μg BR/ml, 0.6 mg sulfolipid/ml): (×—×) theoretical curve; S_1 = 0.193, k_1 = 0.091 ± 0.001; S_2 = −0.115, k_2 = 0.07 ± 0.001; S_3 = −0.0385, k_3 = 0.005 ± 0.0002.

(no steady state characteristic; e.g., no plateau zone). After a fast pH shift caused by BR action a second effect occurs that is opposite to the initial direction of proton transport. Thus only the superimposition of both effects is measurable. When the light is turned off a fast pH drop occurs which is followed by a much slower pH increase. Both effects are reversible and lead to the pH value measured at the beginning of the experiment. Pure polymerized sulfolipid liposomes (without BR) also show a slow, but constant pH drop after illumination. This result suggests that the slow ef-

fect is due to a light-induced change of pK'-values of the polymeric sulfolipid. Absorption spectra indicate that the absorption band of the red polymer shows overlapping with the BR absorption [4]. Therefore, the use of optical filters does not allow a separation of both opposite effects. The pH change–time diagrams can be described mathematically by the following equation (according to [8]):

$$\mathrm{pH}(t) = \sum_{i}^{n} S_i\,(1-\exp(-k_i t)) + \sum_{j}^{m} P_j t$$

t = irradiation time
S_i = max. pH shift caused by process i
k_i = time constant of process i
P_j = terms to describe photolysis (can be neglected in the present case)

The overall proton pump process can be expressed as a superposition of various events with different time constants and S_i-values. In the case of soybean lecithin proteoliposomes (fig.2) two exponential terms are necessary for the construction of a theoretical curve (×—×) which fits the experimental one. For a description of the pH change–time diagram as shown in fig.3 (the proton pump process in polymeric sulfolipid proteoliposomes) the introduction of a third exponential term is required. The resulting theoretical curve (×—×) is shown in fig.3. The S_i-values and time constants (k_i) are given in the legends to fig.2,3.

We suggest that the first term is largely determined by the overall proton pump process due to BR action [8] whereas the subsequent terms are in addition also controlled by the leakiness of the liposomes for ions [10] and by the photochemical reactivity of the polymer. The magnitude of the k_i-values indicates, however, that the first process (described by S_1 and k_1) is initially significantly faster than the subsequent processes. Thus a comparison of the differently shaped pH change–time diagrams for soybean lecithin and polymeric sulfolipid proteoliposomes can be made on the basis of the pH changes observed during the first 30 s of illumination. We have compared these initial pH changes produced by monomeric and polymeric BR liposomes.

The dependence of the magnitude of the initial pH shift on the concentration of BR in liposomes (given as μg BR/ml at a constant [lipid] is shown in fig.4. Up to 25 μg BR/ml the soybean lecithin and the sulfolipid proteoliposomes show a comparable linear increase of pH. At higher concentrations of BR the proton pumping activity is reduced for both types of proteoliposomes, but the reduction is somewhat stronger for the polymeric sulfolipid proteoliposomes. A possible explanation for these findings was given in [8]. The authors suggest that in proteoliposomes rich in BR the formation of large aggregates of two-dimensional hexagonal arrays of BR molecules could be favoured. These structures likely introduce constraints on the conformational changes accompanying the proton pump process.

The experimental curves shown in fig.2,3,5,6 were obtained with 50–150 μg BR/ml (see the legends also). In addition, however, experiments have also been performed with 8–30 μg BR/ml. The observed pH change–illumination time relationships were principally the same as those for the higher BR concentrations.

In fig.5 the effect of UV-irradiation on the pumping activity of BR liposomes is shown. This photoinactivation is likely due to the content of BR in tyrosin and tryptophan [9]. At 0.1 J/m^{2}s the half-life of BR in soybean lecithin liposomes is 2 h and > 3 h for sulfolipid liposomes. UV-irradiation for 10 min leads to a 5% decrease of pumping activity in sulfolipid BR liposomes (no effect in lecithin liposomes). The UV-irradiation time for

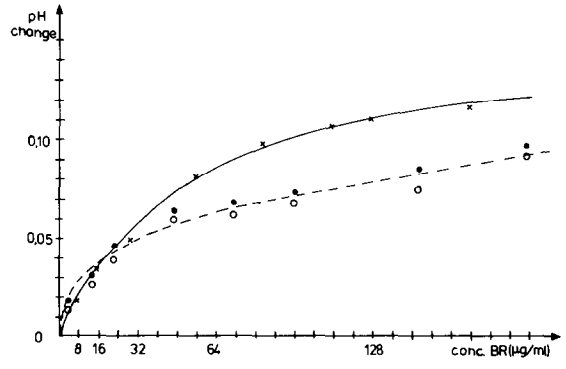

Fig.4. Dependence of pH change on [BR] in liposomes (μg BR/ml): (×) soybean lecithin BR liposomes (1.8 mg lecithin/ml); (●) monomeric sulfolipid BR liposomes (0.6 mg sulfolipid/ml); (○) polymeric sulfolipid BR liposomes (0.6 mg sulfolipid/ml).

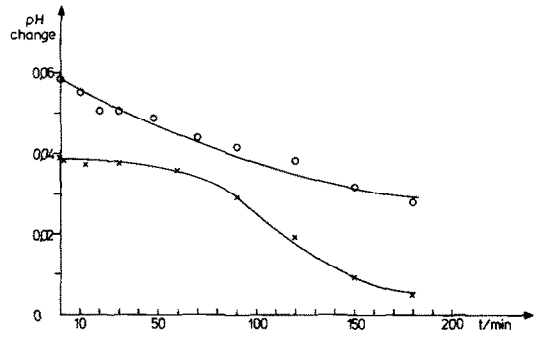

Fig.5. Effect of UV-irradiation on the proton pumping activity of BR liposomes: (×) soybean lecithin BR liposomes (16 μg BR/ml; 1.6 mg lecithin/ml); (○) sulfolipid BR liposomes (50 μg BR/ml; 0.6 mg sulfolipid/ml).

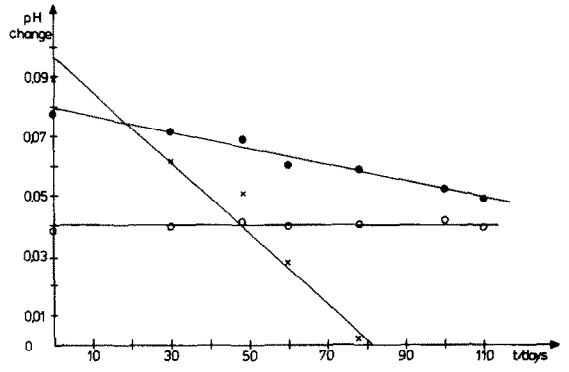

Fig.6. Long-term stability of BR liposomes. The samples were stored at 4°C. Measurements were performed at 37°C: (×) soybean lecithin BR liposomes (100 μg BR/ml; 1.6 mg lecithin/ml); (●) monomeric sulfolipid BR liposomes (100 μg BR/ml; 0.6 mg sulfolipid/ml); (○) polymeric sulfolipid BR liposomes (50 μg BR/ml; 0.6 mg sulfolipid/ml).

polymerization was therefore limited to 10 min at 0.1 J/m^{2}s.

Finally, one of the most interesting questions was the long-term stability of the polymeric sulfolipid proteoliposomes. The pumping activity of BR in liposomes was repeatedly measured over 3 months (fig.6). In the case of soybean lecithin the proton pumping activity had decreased to zero after 80 days. This effect was in part also due to bacterial infection as could be revealed by microscopic investigations. The activity of BR in monomeric sulfolipid liposomes decreased continuously to ~1/3rd of the initial activity (after 3 months) whereas the activity of polymeric sulfolipid BR liposomes remained completely unchanged during that period.

4. DISCUSSION

These data demonstrate the incorporation of a light-driven proton pump (bacteriorhodopsin) into liposomes made from a fully synthetical and polymerizable sulfolipid. During its UV-induced polymerization the initially colorless liposome suspension becomes red because the diacetylenic sulfolipid is converted into a conjugated polymer system (polyene). The mechanism of these reactions is as yet unknown.

The polymer interferes with the visible light-dependent proton pumping activity of BR because in its excited state the polymer releases some protons into the medium, whereas BR pumps protons from the medium into the liposomes. Polymeric sulfolipid BR liposomes show a distinctly lower pumping activity than monomeric sulfolipid BR liposomes because the BR is partially inactivated by the UV-dose that is required for polymerization. The difference between the UV inactivation of BR (see fig.5) in soybean lecithin and sulfolipid liposomes is likely due to the interference of the polymerization reactions and physical protection by the chromophore in the case of sulfolipid liposomes.

The investigations of the stability of polymeric sulfolipid BR liposomes clearly show their pronounced rigidity and long-term stability and suggest that they are superior to monomeric BR liposomes in many experimental procedures.

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