LIGHT-STIMULATED OXYGEN UPTAKE BY VESICLES CONTAINING CYTOCHROME *c* OXIDASE AND BACTERIORHODOPSIN

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1. Introduction

Photophosphorylation in *Halobacterium halobium* is dependent on the presence of bacteriorhodopsin. The chromoprotein is thought to function in this system as a light-dependent proton pump [1].

The properties of bacteriorhodopsin can be studied in vitro after its reconstitution with phospholipids into vesicles. Illumination of such vesicles leads to alkalinization of the medium. However, the maximal extent of the pH change is rather small: at most 5–8 protons per molecule of bacteriorhodopsin are taken up from the medium [2,3]. This finding could be explained by a model in which bacteriorhodopsin undergoes a light-dependent conformational change, leading to p*K* shifts and binding of protons.

To eliminate the latter possibility, we have studied the influence of light and bacteriorhodopsin in a membrane where transmembrane electron movements take place [4], namely through cytochrome *c* oxidase. Our finding that light stimulates the electron transport in this system can be most easily explained by a model in which the membrane potential, generated by transmembrane electron transport, is compensated by a transmembrane transport of protons, catalysed by bacteriorhodopsin under the influence of light. This finding parallels the finding by Oesterhelt and Krippahl [5] of an inhibition by light of respiration in whole cells of *Halobacterium halobium.*

Abbreviations: 1799, α,α′-bis (hexafluoroacetonyl)acetone; valino., valinomycin.

2. Materials and methods

Bacteriorhodopsin [2], cytochrome *c* oxidase [6], cytochrome *c* [7] and soy-bean phospholipids [8] were isolated as described in the literature. Protein was determined according to Lowry et al. [9].

Valinomycin was a gift from Eli Lilly Co. The uncoupler 1799 was a gift from Dr P. G. Heytler.

The procedures used for preparation of proteoliposomes are indicated in the legends to the figures. Sucrose density gradient centrifugation was carried out as described before [2].

Oxygen uptake was measured with a Clark oxygen electrode in a glass vessel, thermostatted with a 2-cm jacket through which a 0.1% $CuSO_4$ solution was circulating. In a final volume of 1.5 ml, 15 μmol potassium phosphate, 30 nmol cytochrome *c* and 7.5 μmol potassium ascorbate (final pH 7.0) were mixed. As a control for autoxidation of ascorbate, readings of oxygen consumption were taken for 5 min before addition of the proteoliposomes.

The effect of: (i) 5.6 μl 1799 (10 mM in ethanol) plus 2 μl valinomycin (1 mg/ml in ethanol) or (ii) illumination with a 500 W slide projector at a distance of 40 cm was tested.

3. Results and discussion

Analysis on a sucrose density gradient of a mixture of proteoliposomes, reconstituted according to the cholate dialysis procedure in the presence of both bacteriorhodopsin and cytochrome *c* oxidase, reveals the segregation of two types of vesicles (fig.1). The

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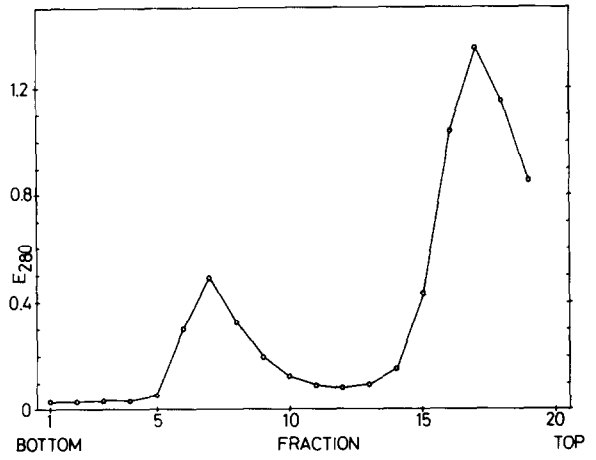

Fig.1. Pattern of mixed cytochrome *c* oxidase-bacteriorhodopsin liposomes on a linear sucrose density gradient. 40 mg soybean phospholipids in chloroform were evaporated to dryness on a rotatory evaporator. The lipids were suspended in 10 mM potassium phosphate, pH 7.2, plus 2% cholate and finally 400 μg cytochrome *c* oxidase (40 mg/ml in 50 mM Tris-SO_4 plus 5% cholate) and 1 mg bacteriorhodopsin (4 mg/ml in aqua dest.) were added; final volume, 2 ml. The mixture was dialysed overnight at 4°C against 2 litres 10 mM potassium phosphate, pH 7.2, with one change after 2 h. After this, sucrose density gradient centrifugation on a linear 45–20% (w/w) sucrose gradient was carried out as described previously [2]. After centrifugation the E_{280} of the eluted fractions was measured.

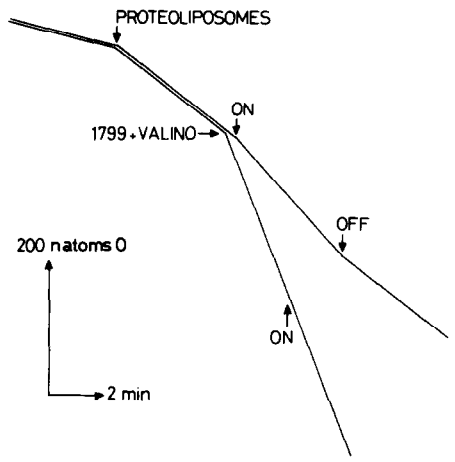

Fig.2. Stimulation by an uncoupler or by illumination of ascorbate oxidation in mixed bacteriorhodopsin-cytochrome *c* oxidase liposomes. 40 mg soybean phospholipids in chloroform were evaporated to dryness by rotatory evaporation and suspended in 1.8 ml 10 mM potassium phosphate, pH 7.2, containing 2 mg bacteriorhodopsin, by shaking on a Vortex mixer with the addition of three glass beads. This suspension was sonicated in a M.S.E. ultrasonifier for 30 times 15 s, with 45 sec-intervals without sonication, at 20 kHz; 4 μm amplitude, 0°C, and under argon atmosphere. Finally 0.2 ml 20% cholate, pH 7.2, was added plus 400 μg cytochrome *c* oxidase (40 mg/ml in 50 mM Tris-SO_4 with 5% cholate) and the solution was dialysed overnight against 2 litres 10 mM potassium phosphate, pH 7.2, with one change after 2 h. The activity of the cytochrome *c* oxidase in the suspension of these mixed proteoliposomes was measured as described in Materials and methods, using 5 μg cytochrome *c* oxidase.

peak with the lowest density contains bacteriorhodopsin liposomes and in this fraction virtually no cytochrome *c* oxidase activity could be demonstrated. The second peak contains cytochrome *c* oxidase vesicles without detectable amounts of bacteriorhodopsin.

Since we found that cytochrome *c* oxidase is sensitive to denaturation upon sonication, a procedure was developed in which first sonicated bacteriorhodopsin liposomes were prepared into which subsequently cytochrome *c* oxidase was incorporated by incubation in the presence of cholate [10]. The final mixture was dialysed to remove the cholate, thereby increasing the factor by which an uncoupler can stimulate the rate of ascorbate oxidation in the presence of the vesicles.

The oxidation of ascorbate by proteoliposomes, reconstituted in this way, can be stimulated either by an uncoupler of oxidative phosphorylation or by illumination (fig.2). In the first case, a 'respiratory control' of about 3 and in the second case of about 1.5 can be calculated. The effect of the two stimulants is not additive.

The difference between the two values may be explained in several ways. One possibility is that the stimulation by bacteriorhodopsin is less efficient because there is a partial 'misorientation' of the bacteriorhodopsin molecules in the membrane. A second possibility is that, even with this procedure, there is an extensive segregation of the protein components into different vesicles (cf. fig.1). Analysis of this mixture on a sucrose gradient shows in this case a single peak. However, this evidence is not conclusive since sonicated bacteriorhodopsin liposomes

Table 1
Temperature dependence of the respiratory control ratio, induced by the addition of uncoupler or by illumination of the mixed proteoliposomes

Temperature	Oxygen uptake (μatoms O/min per mg protein)		
	No addition	Illuminated	+ 1799 + Valinomycin
10°C	1.6	3.8 (2.4)	4.3 (2.7)
15°C	2.4	4.8 (2.0)	8.9 (3.7)
20°C	3.6	5.8 (1.6)	10.8 (3.1)
25°C	5.4	7.0 (1.3)	13.5 (2.5)
30°C	7.4	8.9 (1.2)	19.2 (2.6)

Mixed proteoliposomes (5 μg cytochrome *c* oxidase), prepared as described in the legend to fig. 2, were tested for oxygen uptake at the temperatures indicated, as described in Materials and methods. The rate of oxygen uptake was corrected for the autoxidation of ascorbate. The stimulation factors are given in brackets.

and cytochrome *c* oxidase liposomes have approximately the same density (cf. ref. [2]).

The temperature dependence of the respiratory control in these proteoliposomes was determined. As can be seen in table 1, the stimulation by light of the oxygen uptake is more pronounced at lower temperature. In contrast, the stimulation by uncoupler is much less dependent on the temperature in the range tested.

Apparently, the passive permeability towards protons of the proteoliposomes is less at lower temperature. On the other hand, the extra oxygen uptake induced by activating the bacteriorhodopsin is approximately constant. This results in a greater factor of stimulation at lower temperature. A similar effect for the uncoupler may be masked by a decrease in the mobility of the uncoupler at lower temperature.

The increase in oxygen uptake, induced by illumination of the mixed proteoliposomes, corresponds to the transport of one electron – and hence one proton – per molecule of bacteriorhodopsin per second. This is much lower than the turnover number of bacteriorhodopsin calculated by Oesterhelt [11]. However, our value is a minimal estimate of the proton-translocating activity of bacteriorhodopsin.

In any case, during one experiment more than 300 protons per molecule of bacteriorhodopsin are transported into the vesicles upon illumination, proving that the protein acts as a true catalyst in the transmembrane proton movement.

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