BACTERIORHODOPSIN AS AN ELECTROGENIC PROTON PUMP: RECONSTITUTION OF BACTERIORHODOPSIN PROTEOLIPOSOMES GENERATING $\Delta\psi$ AND ΔpH

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1. Introduction

In 1971 Stoeckenius et al. [1,2] described the retinal-containing protein in an extreme halophilic microorganism, *Halobacterium halobium,* the molecular weight, amino acid composition and properties of which were found to be similar to those of rhodopsin of higher animals. The study of the functions of this protein, called bacteriorhodopsin, led the authors to suggest [3] that it operates as a light-dependent H^+-pump creating a transmembrane electrochemical H^+ potential which can then be utilized for ATP synthesis by a chemiosmotic energy coupling mechanism [4]. In fact, they obtained some indications of a light-induced pH gradient across the bacterial membrane and found an increase in the intracellular ATP level and a decrease in the respiration rate on illumination of intact cells of *H. halobium.* Racker and Stoeckenius [5] succeeded in incorporating bacteriorhodopsin into liposome membranes. The particles obtained were shown to take up H^+ ions in a light-dependent, uncoupler-sensitive fashion. Incorporation of beef-heart mitochondrial ATPase into the same liposomes made the system capable of ADP photophosphorylation by inorganic phosphate.

In this paper, we shall report data demonstrating the generation of an electric potential difference $(\Delta\psi)^*$ and a pH gradient (ΔpH) by bacteriorhodopsin. To this end, proteoliposomes, reconstituted from bacteriorhodopsin and mitochondrial phospholipids, were studied. The penetrating anion phenyldicarbaundecaborane (PCB^-) and atebrin were used as probes for $\Delta\psi$ and ΔpH, respectively. It was found that illumination of bacteriorhodopsin proteoliposomes gives rise to PCB^- uptake which indicates that a transmembrane $\Delta\psi$ is generated (positive inside the proteoliposomes). Turning off the light caused extrusion of the PCB^- taken up under illumination. Atebrin fluorescence was shown to be strongly decreased by illumination of proteoliposomes, evidence of the formation of a transmembrane ΔpH (acid inside the proteoliposome). Parallel measurements of pH of the incubation medium showed an alkalinization. In the dark, both the atebrin fluorescence and the external pH returned to initial levels. All light-induced responses were sensitive to trichlorocarbonylcyanidephenylhydrazone (CCCP).

2. Methods

A strain of an extreme halophile first isolated from the Aral sea was used as a source of bacteriorhodopsin [6]. Study of this microorganism showed that it is very close to the strain of *H. halobium* without gas vacuoles, that was recently placed at our disposal by the kindness of Dr. W. Stoeckenius.

The properties of the bacteriorhodopsin isolated by our group [6] were found to be quite similar to those reported by Stoeckenius et al. [1–3].

Purple membrane sheets (fragments of the *H. halobium* cell membrane containing bacteriorhodopsin as the only protein) were isolated according to Oesterhelt and Stoeckenius [1]. Their spectrum showed the absorption maximum of bacteriorhodopsin (fig. 1).

* *Abbreviations:* $\Delta\mu_{H^+}$ – electrochemical H^+ potential difference; $\Delta\psi$ – electric potential difference; CCCP, 2,4,6-trichlorocarbonylcyanidephenylhydrazone; PCB^-, phenyldicarbaundecaborane anion.

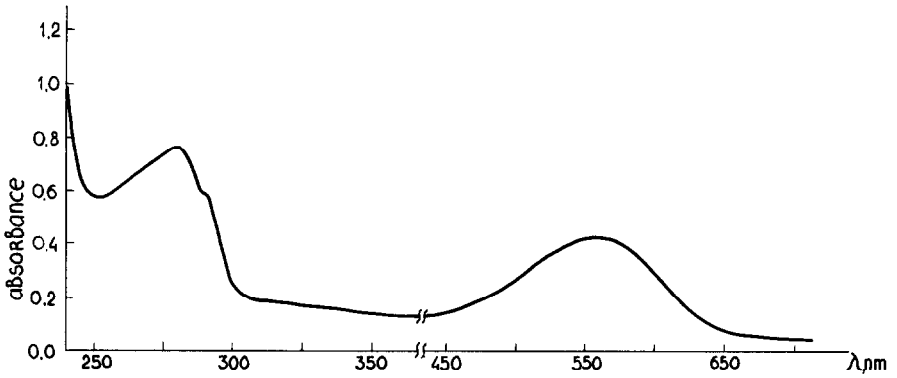

Fig. 1. Absorption spectrum of bacteriorhodopsin membranes. The mixture contained 0.27 mg membrane protein per ml, the optical path was 1 cm.

Mitochondrial phospholipids were prepared after Folch et al. [7].

The reconstitution procedure was essentially the same as that used earlier by Racker's and our groups for preparation of proteoliposomes containing ATPase [8–10], cytochrome oxidase [9–13] or NADH dehydrogenase [14]. A 2% cholate solution in 0.15 M KCl of beef-heart mitochondrial phospholipids (50 mg/ml) and purple membranes (0.8 mg protein/ml) were mixed and then dialyzed against 0.15 M KCl for 18 hr at 0–2°C to remove the cholate, according to the procedure of Racker and Stoeckenius [5]. Proteoliposomes reconstituted during dialysis were centrifuged at 226 000 *g* for 60 min, suspended in the solution containing 0.3 M sucrose and 5 mM Tris–citrate, pH = 6.25, and stored at 0°C.

To detect the membrane potential in the bacteriorhodopsin proteoliposomes, the PCB^- method was used [15,16]. It has been previously shown that PCB^-, an anionic penetrant of artificial [16,17] and mitochondrial [18] membranes, was taken up by proteoliposomes if their interior was positively charged, and was extruded if it was negatively charged [9,10]. As a probe for transmembrane ΔpH, atebrin fluorescence measurements were used. Fluorescence of this penetrating dye is high at alkaline and neutral pH's. Acidification results in a decrease in fluorescence. Atebrin as a weak base can be accumulated down a pH gradient into vesicles when the pH in their interior is lower than in the incubation medium. Atebrin accumulation in the acidic compartment can be shown up by a fluorescence decrease [19]. Atebrin fluorescence was excited by light of 365 nm and measured at 539 nm.

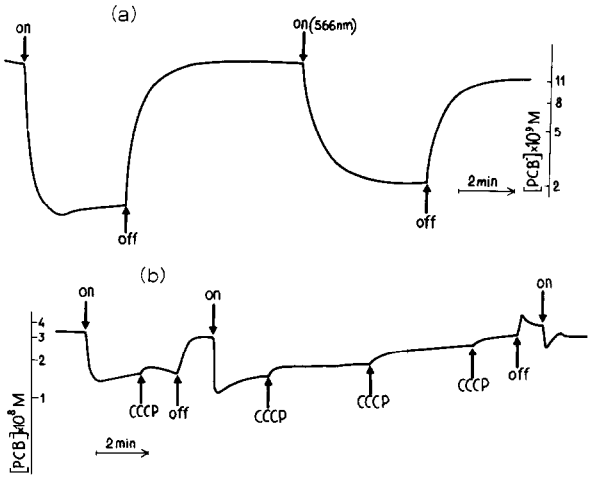

Fig. 2. The light-induced PCB^- responses in bacteriorhodopsin proteoliposomes. Incubation mixture: 0.3 M sucrose; 5 mM Tris–citrate, pH 6.25; proteoliposomes (0.08 mg protein/ml). Concentrations of CCCP: 1st, 2nd and 3rd additions, 3×10^{-8} M; 4th addition, 1×10^{-7} M.

3. Results and discussion

The results of a typical PCB^- experiment with bacteriorhodopsin proteoliposomes are given in fig. 2. It is demonstrated (fig. 2A) that the turning on of white light gives rise to PCB^- uptake by proteoliposomes. In

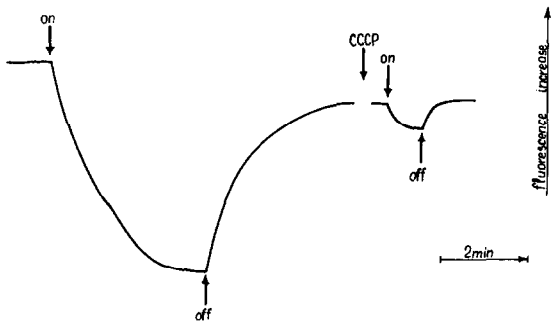

Fig. 3. The light-induced atebrin fluorescence changes in bacteriorhodopsin proteoliposomes. Incubation mixture: 0.3 M sucrose; 5×10^{-6} M atebrin; 3×10^{-2} M KNO_3; proteoliposomes (0.16 mg protein/ml). Addition: 1×10^{-6} M CCCP.

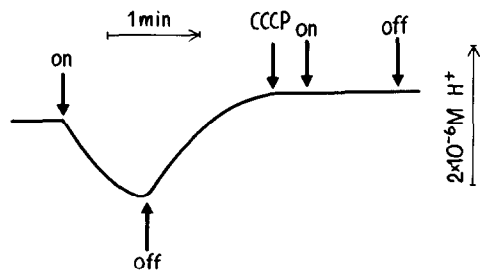

Fig. 4. The light-induced pH changes in the incubation mixture with the bacteriorhodopsin proteoliposomes. Incubation mixture as in fig. 3 but atebrin was omitted.

the dark, the whole of the PCB^- taken up during illumination is extruded. Then green light (566 nm interference filter) was turned on. It is seen that green light, whose maximum coincides with that of bacteriorhodopsin absorption, induces a PCB^- response of almost the same magnitude as that of the white light. The slower PCB^- response to green light is most probably due to its lower intensity.

In fig. 2B the effect of low concentrations of the protonophorous uncoupler CCCP on the PCB^- response is shown. One can see that 2×10^{-7} M CCCP almost completely reverses the light-induced PCB^- uptake. In another experiment (not shown) 1×10^{-6} M, CCCP was found to prevent any light effect on the PCB^- level.

The response of atebrin fluorescence in bacteriorhodopsin proteoliposomes is given in fig. 3. It is seen that illumination results in a decrease in fluorescence. In the same conditions, a small but measurable alkalinization of the incubation medium occurs (fig. 4) which confirms the data of Racker and Stoeckenius [5].

It is obvious that the above decrease in fluorescence cannot be the result of the change in the extravesicular pH which is shifted, under illumination, to the alkaline side. So, a pH change inside the proteoliposomes, but not outside them must be responsible for the fluorescence changes observed. This conclusion was confirmed by experiments with CCCP which was found to abolish the light-induced fluorescence and pH changes (see figs. 3 and 4).

The above data lead to the conclusion that bacteriorhodopsin proteoliposomes are competent in generating an electrochemical H^+ potential ($\Delta\bar{\mu}_{H^+}$) in both $\Delta\psi$ and ΔpH form. Since bacteriorhodopsin is the only protein species in the purple membrane sheets used for reconstitution of the proteoliposomes, it can be assumed that bacteriorhodopsin functions as an electrogenic proton pump converting light energy into $\Delta\bar{\mu}_{H^+}$. Utilization of $\Delta\bar{\mu}_{H^+}$ by a reversible H^+-ATPase localized in membrane areas other than the purple sheets may be supposed to be responsible for the light-dependent ATP synthesis in *H. halobium* cells [3].

Certain problems concerning the principles of assembly of lipoprotein membranes arise in connection with the above data. One question is why do bacteriorhodopsin H^+ pumps in proteoliposomes carry protons preferentially from the outside to the inside? Maybe, this is a consequence of the volume difference of the extra- and intravesicular compartments. If formation of closed phospholipid vesicles is assumed to be the first step of the proteoliposome reconstitution, and bacteriorhodopsin incorporation the second, the outer surface of the vesicles may bind more protein than the inner, perhaps because the amount of bacteriorhodopsin available to the inner surface is limited by the amount present in the intravesicular compartment. Another reason for the proteoliposome assymmetry may be the difference in areas of the inner and outer surfaces of the reconstituted vesicles.

In conclusion, bacteriorhodopsin proteoliposomes seem to be the simplest experimental system for studying the electric generators of coupling membranes.

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References

[1] Oesterhelt, D. and Stoeckenius, W. (1971) Nature New Biol. 233, 149.
[2] Blaurock, A.E. and Stoeckenius, W. (1971) Nature New Biol. 233, 152.
[3] Oesterhelt, D. and Stoeckenius, W. Proc. Natl. Acad. Sci. U.S. (in press).
[4] Mitchell, P. (1966) Chemiosmotic Coupling in Oxidative and photosynthetic phosphorylation (Glynn Research, Bodmin).
[5] Racker, E. and Stoeckenius, W. (in press).
[6] Kayushin, L.P., Lasareva, A.V., Sibeldina, L.A., Chekulaeva, L.N., Richireva, G.T., Kostikov, A.V. and Vsevolodov, N.N., Biofizika (1973) (in press).
[7] Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497.
[8] Kagawa, Y. and Racker, E. (1971) J. Biol. Chem. 246, 5477.
[9] Jasaitis, A.A., Nemeček, I.B., Severina, I.I., Skulachev, V.P. and Smirnova, S.M. (1972) Biochim. Biophys. Acta 275, 485.
[10] V.P. Skulachev (1972) FEBS Symp. 28, 371.
[11] E. Racker and A. Kandrash (1971) J. Biol. Chem. 246, 7069.
[12] Hinkle, P.C., Kim, J.J. and Racker, E. (1972) J. Biol. Chem. 247, 1338.
[13] Racker, E. (1972) J. Membrane Biol. 10, 221.
[14] Ragan, I. and Racker, E. J. Biol. Chem. (in press).
[15] Liberman, E.A., Topaly, V.P., Tsofina, L.M., Jasaitis, A.A. and Skulachev, V.P. (1969) Nature 222, 1076.
[16] Grinius, L.L., Jasaitis, A.A., Kadziauskas, J.P., Liberman, E.A., Skulachev, V.P. Topali, V.P., Tsofina, L.M. and Vladimirova, M.A. (1970) Biochim. Biophys. Acta 216, 1.
[17] Liberman, E.A. and Topali, V.P. (1969) Biofizika 14, 452.
[18] Jasaitis, A.A., La Van Chu and Skulachev, V.P. (1973) FEBS Letters 31, 241.
[19] Barsky, E.L., Bonch-Osmolovskaya, E.A., Ostroumov, S.A., Samuilov, V.D. and Skulachev, V.P., Biochim. Biophys. Acta (submitted).