LIGHT-DEPENDENT PROTON AND RUBIDIUM TRANSLOCATION IN MEMBRANE VESICLES FROM HALOBACTERIUM HALOBIUM

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SUMMARY

A procedure for the isolation of membrane vesicles after sonication of Halobacterium halobium is described. Upon illumination these vesicles took up rubidium. This process was stimulated 3 to 7 fold by valinomycin, and inhibited by uncouplers of oxidative phosphorylation or by nigericin. In the light, these vesicles extruded protons. However, on addition of low concentrations of uncoupler the direction of proton movement was reversed. All proton movements were abolished by high concentrations of uncoupler or by nigericin. These observations suggest that part of the vesicle population was inverted and less sensitive to uncouplers.

Halobacterium halobium, grown in the light at low oxygen concentrations, produce purple membrane patches which contain a single protein, bacteriorhodopsin (1,2,3). It was concluded from in vivo studies that this protein acts as a light-driven proton pump (3). Moreover, it was observed (4) that on illumination of starved cells, concomitantly with efflux of protons, there was an increase in the intracellular ATP concentration. Purified membrane preparations of bacteriorhodopsin have been incorporated into liposomes, which in contrast to the intact bacteria took up protons (5,6,7). In the presence of the mitochondrial ATPase complex these inside-out vesicles catalyzed in the light, the formation of ATP (5,6). We describe in this communication the preparation of right side-in vesicles which catalyze light-dependent efflux of protons and uptake of rubidium.

Methods

Halobacterium halobium S_9 (kindly given to us by Dr. H. G. Khorana) was grown batchwise in 2 liter Erleneyers filled with 1 liter of medium with slow reciprocal shaking at approximately 37°C. The flasks were illuminated with four fluorescent lamps from above. The growth medium contained per 1000 ml: NaCl 250 g, $MgSO_4$, 9.75 g, KCl 2.0 g, $CaCl_2.2H_2O$, 0.265 g and bacteriological peptone 10 g. After filtration through soft filter paper the pH of the medium was adjusted to pH 7.2 with NaOH.

Light-driven proton translocation was measured at room temperature with 50-150 µg of membrane protein as described (6) except that the medium was 3 M KCl. Rubidium uptake experiments were performed in glass test tubes containing membrane vesicles (400 to 800 µg of protein) and 4 M NaCl in a final volume of 0.6 ml. Valinomycin, when present, was used at 2 µg per assay. The tubes were incubated at room temperature in the dark for 5 min. The reaction was started by the addition of 1.8 µmoles of KCl and approximately 2 x 10^6 cpm carrier-free $^{86}RbCl$. The tubes were placed in a shaking waterbath at room temperature. Three lamps of 200 W each were turned on and the light was passed through a 10 cm pathlength waterfilled heat filter and a yellow plastic filter-sheet (Edmond Scientific Filter #809). Under these conditions the light intensity in the tubes was approximately 6 x 10^5 ergs/cm^2sec. Aliquots (0.1 ml) were withdrawn, and filtered on 0.45 µm millipore filters, washed with 4 M NaCl, dried and counted in a gas flow counter.

Protein was determined according to Lowry et al. (8). Bacteriological peptone was obtained from Wilson diagnostics, Inc. (Glenwood, Ill.). Carrier-free $^{86}RbCl$ was purchased from ICN (Cleveland, Ohio).

Results and Discussion

Preparation of membrane vesicles and bacteriorhodopsin--Halobacterium halobium was grown, under the conditions described under Methods, to the early stationary phase ($A_{660} \simeq 1.7$). This took about 7 days when the flasks were inoculated from a slant. The cells were collected by centrifugation for

20 min at 10,000 x g and were washed once in Basal salts medium (the growth medium without peptone), and finally suspended in the Basal salts medium at about 5% of the volume of the culture medium. When not processed immediately the cells were stored at 4°C. The bacterial suspension was diluted with an equal volume of 4 M NaCl and sonicated in 30 ml batches for 2 min in a 10 KH_3 Ratheon Sonifier at maximal output. After centrifugation at 13,000 x g for 15 min, the supernatant was centrifuged in a 50 Ti rotor at 133,000 x g for 45 min. The precipitate was washed once and resuspended in 4 M NaCl at 25% of the volume of the washed bacterial suspension. Harvesting and all following preparative steps were performed at 4°. The vesicles stored at 4° were stable for over 8 months with respect to light-induced proton efflux. All experiments on vesicles were performed with this preparation. In contrast to intact bacteria these vesicles did not catalyze oxygen uptake either in the absence or presence of glycerol. The preparation was also used for the isolation of bacteriorhodopsin. For this purpose the vesicles were resuspended in distilled water and exposed twice to 1% deoxycholic acid (neutralized with NaOH, pH 8.0). After centrifugation (133,000 x g, 15 min) the purple precipitate was washed three times with distilled water and resuspended in distilled water at a protein concentration of 2 to 4 mg/ml. This bacteriorhodopsin preparation is essentially pure exhibiting a single major band in acrylamide gel electrophoresis and is reconstitutively active in the sonication system described previously (6).

Light-dependent proton translocation--Upon illumination the membrane vesicles extruded protons into the medium (Fig. 1). When the light was turned off, protons were taken up. This cycle could be repeated numerous times. The effect of pH on the light-dependent proton extrusion by these vesicles is shown in Fig. 2. At pH 3.5 the extent was about 6-fold higher than at pH 6.0. The extent of proton extrusion varied considerably from batch to batch between 4 to 16 ng atoms H^+ per mg protein at pH 5.8. Proton extrusion was virtually abolished by

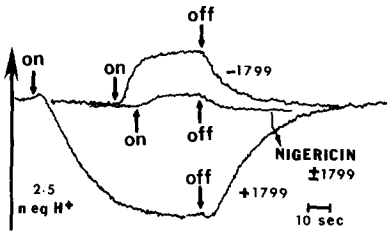

Figure 1. Light-driven proton translocation by vesicles isolated from H. halobium. Membrane vesicles (120 µg of protein) were assayed for light-dependent proton translocation in 1 ml of 3 M KCl (6). The pH was adjusted to 5.75 and at the end of the experiments the response of the pH meter was standardized by addition of 2.5 nmoles of HCl. Nigericin (2 µg) and 1799 (10^{-5}M) were added where indicated.

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2 µg/ml nigericin. Stimulations up to 2-fold could be observed with DCCD*(100 µM) or valinomycin (2 µg/ml), but these effects also varied greatly. In the presence of the uncoupler 1799*(10^{-5} M) the light-dependent proton extrusion was reversed and this effect was also abolished by nigericin (2 µg/ml) (Fig. 1) as well as by high concentrations of 1799 (5×10^{-4} M). A similar reveral was observed with the uncoupler FCCP*(2×10^{-6} M). A possible explanation for this phenomenon is that there may be two populations of vesicles, one right side-in and one inside-out. In this case, the data shown in Fig. 1 are the resultant of opposite light-dependent proton movements in the two types of vesicles. If this is correct, the inside-out vesicles are more resistant to uncouplers and exhibit slower kinetics of proton uptake. Two lines of evidence support this explanation. The ratio of light-dependent proton extrusion/light-dependent proton uptake in the presence of uncoupler was high in preparations with a high Rb^+ transport activity and low for these which had low Rb^+ transport activity. One would expect only the right side-out vesicles to actively take up Rb^+ in exchange for

*Abbreviations used: DCCD, N,N'-dicyclohexylcarbodiimide; 1799, bis-(hexafluoroacetonyl)-acetone; FCCP, p-trifluoromethoxycarbonylcyanide phenylhydrazone.

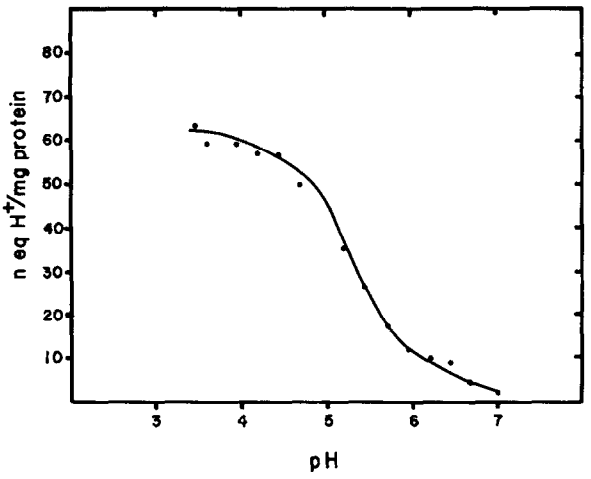

Figure 2. The effect of pH on light-driven proton translocation in vesicles from H. halobium. Membrane vesicles (140 µg of protein) were assayed for light-dependent proton extrusion as a function of the external pH. The pH was adjusted to 3.44 with 0.1 N HCl. After each measurement of the response to light, the buffering capacity was measured with known pulses of HCl. The pH was increased by sequential addition of small amounts of 25 mM KOH.

H^+. Moreover we have isolated vesicle populations by differential centrifugation, which displayed light-dependent, nigericin-sensitive proton uptake in the absence of uncoupler. Addition of uncoupler dramatically enhanced this effect. This vesicle population was apparently still heterogenous but significantly enriched in inside-out vesicles.

Light-dependent Rb^+ uptake--Halobacteria grow usually in an environment with high sodium present (4 M) and relatively low potassium. It was shown by Ginsburg et al. (9) that the predominant cation inside the bacteria is potassium. These authors suggested that potassium is a permeant ion in the Halobacteria, and that the cation is bound somehow within the cell. We observed that in intact bacteria, Rb^+ uptake is energy dependent and that there is a rapid efflux in the

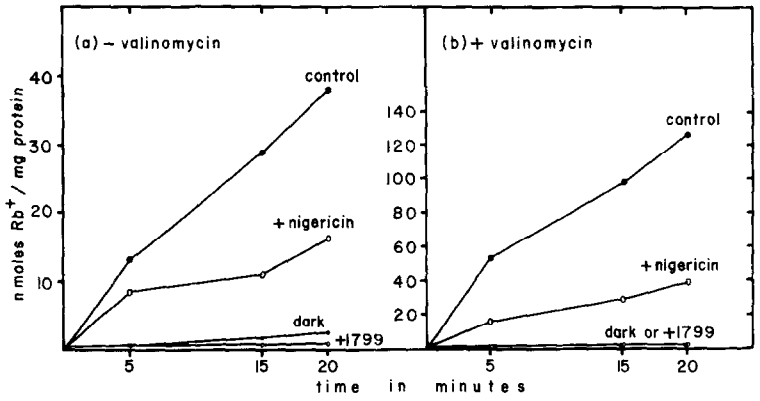

Figure 3. Light-dependent rubidium uptake in membrane vesicles from H. halobium. Experiments were performed with 470 µg protein in the presence or absence of valinomycin as described under Methods. Valinomycin (2 µg), nigericin (1.2 µg) and 1799 (8 x 10^{-5}M) were included during the pre-incubation period in the experiments indicated in the figure.

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dark in the presence of an uncoupler and nigericin. Fig. 3 shows that upon illumination rubidium was taken up by the vesicles and that this process was completely light dependent and abolished by the uncoupler 1799 (8 x 10^{-5} M). Nigericin at 2 µg/ml inhibited about 60%. Similar results were obtained when the unlabeled KCl was replaced by RbCl. In membrane vesicles of E. coli, valinomycin appears to be required for rubidium or potassium uptake (10,11). Fig. 3 shows that valinomycin markedly stimulated rubidium uptake in membrane vesicles of H. halobium. The extent of stimulation again varied with the batches of vesicles, ranging from 3-7 fold. The Rb^+ uptake in the presence or absence of valinomycin was sensitive to nigericin and uncoupler (Fig. 3). About 30 to 50% of the Rb^+ transport activity of the intact cells was recovered in the vesicles when tested in the absence of valinomycin. Attempts to isolate an ionophore for Rb^+ from these vesicles have thus far been unsuccessful.

In the dark, efflux of the rubidium occurred from the membrane vesicles,

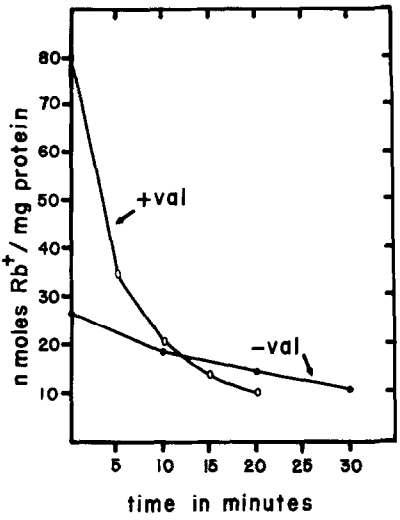

Figure 4. Efflux of rubidium from membrane vesicles of H. halobium in the dark. Membrane vesicles (470 μg of protein) were incubated in the light in the presence or absence of valinomycin (2 μg) for 20 min. At this point efflux was started by turning off the light. At the indicated times 0.1 ml samples were filtered, washed, dried and counted as described under Methods.

both in the presence or in the absence of valinomycin (Fig. 4), but much more rapidly in its presence.

Finally, MacDonald and Lanyi (12) observed that vesicles prepared from H. halobium according to the procedure described here, catalyze a light- and sodium-dependent uptake of ^{14}C-leucine. This observation is of particular interest, because under these experimental conditions, leucine transport could not be detected in the intact bacteria (R. MacDonald, personal communication).

These and our experiments illustrate the usefulness of the right side-out vesicle preparation described in this communication for the study of translocation of solutes in the absence of respiratory energy.

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References

1. Oesterhelt, D. and Stoeckenius, W. (1971) Nature New Biol., 233, 149-152.
2. Blaurock, A.E. and Stoeckenius, W. (1971) Nature New Biol., 233, 152-155.
3. Oesterhelt, D. and Stoeckenius, W. (1973) Proc. Natl. Acad. Sci. USA, 70. 2853-2857.
4. Danon, A. and Stoeckenius, W. (1974) Proc. Natl. Acad. Sci. USA, 71, 1234-1238.
5. Racker, E. and Stoeckenius, W. (1974) J. Biol. Chem., 249, 662-663.
6. Racker, E. (1973) Biochem. Biophys. Res. Commun., 55, 224-230.
7. Racker, E. and Hinkle, P.C. (1974) J. Membr. Biol. 17, 181-188.
8. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem., 193, 265-275.
9. Ginsburg, M., Sachs, L. and Ginsburg, B.Z. (1971) J. Membr. Biol., 5, 78-101.
10. Bhattacharyya, P., Epstein, W. and Silver, S. (1971) Proc. Natl. Acad. Sci. USA, 68, 1488-1492.
11. Lombardi, F.J., Reeves, J.P. and Kaback, H.R. (1973) J. Biol. Chem. 248, 3551-3565.
12. MacDonald, R.E., and Lanyi, J.K., Biochemistry, submitted for publication.