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EFFECTS OF FLUORESCAMINE MODIFICATION ON LIGHT-INDUCED H^+-MOVEMENT IN RECONSTITUTED PURPLE MEMBRANE OF HALOBACTERIA

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SUMMARY

The reconstituted purple membrane of halobacterium halobium was treated with amine-specific reagents, fluorescamine, NBD-Cl, DNFB and pyridoxal phosphate. Only the fluorescamine modification of bacteriorhodopsin has significant effects on the light-induced H^+ movement. Analysis of the kinetic data shows an enhancement of the decay of the proton gradient under illumination, while the initial proton pumping rate and the decay constant in the dark remain unchanged. The results suggest the existence of a light-triggered proton pathway which is susceptible to fluorescamine modification, and which is different from the pathway for proton pumping.

Bacteriorhodopsin in the cell membrane of halobacteria functions as a proton pump to convert light energy into a transmembrane electrochemical potential (1,2). It has been concluded, from flash spectrometry, that the protonation state of the Schiff base and the conformation of the retinal isomers in bacteriorhodopsin are important elements in the light-driven transmembrane proton pumping (3). A change in the pK_a of the Schiff base nitrogen owing to a twist around the C_{14}-C_{15} single bond of the chromophore (4), and a light-induced movement of the Schiff base which changes its contact from one proton channel to another (5), could be the mechanisms of the pump operation. Changes in pK_a of protein groups may also be involved in the proton pump cycle (6,7). Those investigations have not established if there is a particular mechanism, other than the nonspecific proton leak of the membrane,

Abbreviations: Fluorescamine, 4-phenylspiro[furan-2(3H), 1'-phthalan]-3,3'-dione; NBD-Cl, 7-chloronitrobenzo-2-oxa-1,3-diazole; DNFB, 2,4-dinitrofluorobenzene; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, SDS, sodium dodecyl sulfate.

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for the regulation of the proton pump activity in the reconstituted purple membrane. A recent report (8) demonstrates that, in the broken chloroplast system, there is a light-dependent proton leak pathway, which seems to be sensitive to the conformational state of the coupling factor CF_1-CF_o complex. The present Communication provides evidence for the existence, in the reconstituted purple membrane, of a light-dependent proton leak pathway which is different from the pathway for proton pumping.

MATERIALS AND METHODS

A published procedure (9) was followed to grow and harvest the Halobacterium halobium R_1, and to isolate the purple membrane containing bacteriorhodopsin. SDS-acrylamide gel electrophoresis revealed that the purple membrane contained only one protein of MW 26,000. The composition of this protein, as revealed by amino acid analysis, was the same as that obtained in other laboratories (1). Our preparation contained no histidine nor cysteine residues. The concentration of bacteriorhodopsin was determined from the absorbance at 560 nm (ε = 54000 M^{-1} cm^{-1}). Bacteriorhodopsin was reconstituted into egg yolk phosphatidylcholine vesicles by the octylglucoside dilution method (10). A 2 mL-solution containing 1 to 2 mg of bacteriorhodopsin and 2.5% of octylglucoside was mixed with a 2 mL-solution containing 40 mM vesicles, 75 mM KCl and 1 mM Hepes (pH 8.0). The mixture was incubated for 3 hr at 0° C, and was then diluted 20 times in volume with 150 mM KCl. 2 mL-Aliquots of this solution were used for measurements of the light-induced H^+ movement. The modification of the reconstituted purple membrane by various reagents was carried out prior to the illumination step. The light source was a slide projector with a 500 W halogen lamp. The intensity of the actinic light was controlled by the voltage applied to the lamp. The suitably filtered light was focused on the suspension of reconstituted membrane in a modified Gilson-Medical oxygen chamber equipped with a thermo-jacket for circulation of constant-temperature water. Control of the on-off illumination was achieved by means of the projector shutter. Measurements of the H^+ movement, and the buffering capacity of the sample were carried out as described (11,12). All reagents were of maximum obtainable purity.

RESULTS

Kinetics of the Light-Induced H^+ Movement in Reconstituted Purple Membrane.

The reconstituted purple membrane has an inverted orientation of bacteriorhodopsin; therefore, the membrane takes up protons upon illumination. Figure 1 shows that, under constant light intensity and a nullified membrane potential (K^+ and valinomycin), the proton uptake by the membrane reaches a steady state. When the light is turned off, those protons leak back to the medium at a

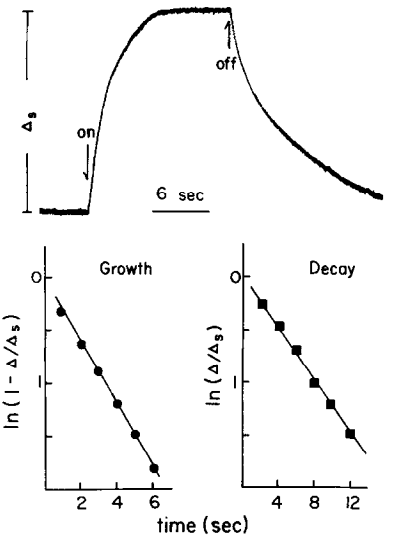

Figure 1. KINETICS OF H^+ MOVEMENT ASSOCIATED WITH RECONSTITUTED PURPLE MEMBRANE. Purple membrane was reconstituted from bacteriorhodopsin and egg yolk phosphatidylcholine as described. The upper part of the figure represents an actual trace of the light-induced proton uptake and of the proton release in the subsequent dark stage. In a volume of 2.0 mL containing 150 mM KCl, 3 µg of valinomycin, and 100 µg of bacteriorhodopsin with pH carefully adjusted to 5.0 before illumination, the extent of proton uptake (Δ_S) was about 130 nmol of H^+ per mg of protein when the actinic light intensity was 190 J m^{-2} sec^{-1}. The applicability of equations (1) and (2) to the kinetics of H^+ movement is shown in the lower part of the figure. The determined k_L, k_D, and R_o are 0.362, 0.131 (sec^{-1}), and 2824 nmol H^+/min.mg, respectively.

slightly lower rate. In agreement with the results of Ho et.al. (8), the growth phase of the proton movement obeys the empirical equation: $\ln(1-\Delta/\Delta_S) = -k_L t$ (1), where Δ and Δ_S are the extent of proton uptake at time t of illumination and at the steady state, respectively, while k_L is the rate constant for the proton leak under illumination. The release of protons in the dark obeys the first-order decay equation: $\ln(\Delta/\Delta_S = -k_D t$ (2), where k_D is a light-independent, although composition-dependent (9), decay constant. The initial rate of proton pumping, R_o, is obtained from equation: $R_o = k_L \Delta_S$ (3).

We observed that the rate constant k_L increases, while k_D remains unchanged, as the intensity of the light is increased. Since k_D is included in k_L, the quantity 'k_L-k_D' describes the actual path of the light-dependent

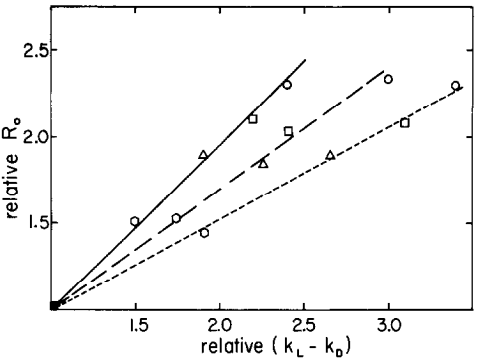

Figure 2. EFFECT OF LIGHT INTENSITY ON THE KINETIC PARAMETERS OF THE H^+ MOVEMENT IN THE ABSENCE AND IN THE PRESENCE OF FLUORESCAMINE. The reconstituted purple membranes were treated with 0 (——), 15 (– –), and 30 x 10^{-5} M (---) of fluorescamine before illumination. The light-induced H^+-movement of the treated system was analyzed in the same manner described in Figure 1. The correlation between R_o and 'k_L-k_D' was determined for different light intensities by assigning to the R_o and 'k_L-k_D' obtained under 80 J m^{-2} sec^{-1} illumination the value of one (■) for all systems. The relative values of R_o and 'k_L-k_D' at 105 (○), 135 (△), 170 (□), and 195 J m^{-2} sec^{-1} (◇) were then plotted. The values for k_L, k_D, and R_o for ——, – –, and --- under 80 J m^{-2} sec^{-1} illumination were 0.231, 0.130 sec^{-1}, and 1238 nmol H^+/min.mg; 0.288, 0.125 sec^{-1}, and 1185 nmol H^+/min.mg; 0.320, 0.131 sec^{-1}, and 1255 nmol H^+/min.mg, respectively. The slopes of ——, – –, and --- are 1.0, 0.82, and 0.69 respectively.

proton decay in the reconstituted membrane. The initial rate of proton pumping, R_o, should be directly related to the primary photochemical cycle of bacteriorhodopsin. The top line in Figure 2 shows that, in untreated purple membrane, an increase in actinic light intensity results in an increase in R_o and 'k_L-k_D' to the same extent. This indicates that the pathway of the light-dependent proton decay is tightly regulated by the proton pumping that is associated with the photochemical cycle.

Effects of Fluorescamine Modification on the Light-Induced H^+ Movement. Treatment of the reconstituted purple membrane with fluorescamine in the absence of actinic light does not alter the kinetic expressions which govern the light-induced H^+ movement; i.e., equations (1), (2) and (3) also hold for the membrane modified by fluorescamine in the dark. The lower lines in Figure 2 show that, in the modified membrane, an increase in the intensity of actinic light does not affect the constant k_D but causes an increase

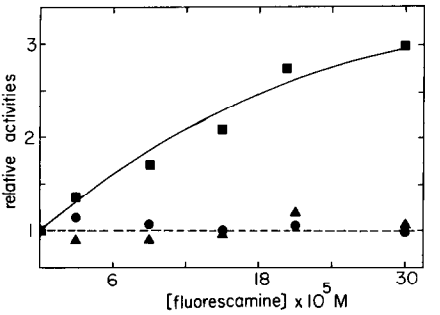

Figure 3. EFFECT OF FLUORESCAMINE ON THE KINETIC PARAMETERS OF H^+ MOVEMENT ON RECONSTITUTED PURPLE MEMBRANE. Reconstituted purple membrane was treated with various concentrations of fluorescamine before illumination by 135 J m^{-2} sec^{-1} actinic light. The subsequent light-induced H^+ movement was analyzed as described. The 'k_L-k_D', k_D, and R_o of untreated system are 0.186, 0.130 sec^{-1}, and 2300 nmol H^+/min.mg. respectively, and are assigned the value of 1 in the Figure. ■, ●, and ▲ represent the relative 'k_L-k_D', k_D, and R_o, respectively.

in both 'k_L-k_D' and R_o. The increase is greater for 'k_L-k_D' than for R_o. Figure 3 depicts results obtained with the modified membrane at one constant light intensity. It is apparent that modification of the membrane by increasing concentrations of fluorescamine in the dark, followed by illumination, induces an enhancement of 'k_L-k_D', but has no significant effect on k_D or R_o. In Figure 3, the amount of bacteriorhodopsin is kept constant.

From the linear relationship between R_o and 'k_L-k_D' observed with the untreated and the modified membranes in Figure 2, and the data of Figure 3, we conclude that neither the primary proton pumping action nor the coupling between this action and the light-induced pathway of proton decay are affected by modification of the membrane by fluorescamine in the dark. Therefore, the enhancement of 'k_L-k_D' upon modification is probably the result of an impairment of the light-dependent proton decay pathway. The phospholipids used for reconstitution of the purple membrane in the present work do not contain $-NH_2$ groups. Hence, fluorescamine should react primarily with lysine residues of the protein. Our amino acid analysis show that, even at the highest concentration of fluorescamine utilized in Figure 3, approximately one lysine residue per bacteriorhodopsin molecule underwent modification.

Behavior of the Reconstituted Purple Membrane Toward Other Reagents. The effects described above appear to result from a covalent modification of the protein by fluorescamine, rather than from an effect of the products of fluorescamine hydrolysis. Thus, when fluorescamine was allowed to hydrolyze over a 10 minute-period in the assaying solution prior to the addition of reconstituted purple membrane, no significant changes in the H^+ movement were observed.

The following amine-reagents were also tested in the reconstituted purple membrane and proved to have no significant effect on the kinetic parameters of the H^+ movement: 1 mM NBD-Cl (up to 120 minutes), 0.5 mM DNFB (up to 90 minutes), and 8.0 mM pyridoxal phosphate (up to 30 minutes).

DISCUSSION

This investigation suggests the existence of an inherent H^+ leak pathway, which is physically different from the pathway associated with proton pumping during the primary photochemical cycle (1,6) in bacteriorhodopsin. However, the operation of this inherent H^+ leak pathway ("on-off") is under the regulation of events associated with the light-induced proton pumping. Apparently, one $-NH_2$ group in a lysine residue is involved in the regulation of the H^+ transport via the leak pathway, and this amino group is accessible to fluorescamine before the activation of the proton pumping cycle. Since, in the reconsitituted purple membrane, bacteriorhodopsin has an inverted orientation, and since fluorescamine is a surface labeling reagent (13,14), it would appear that this particular amino group is situated on the side that is associated with the C-terminal end of the protein, on the basis of the known orientation of the C-terminal end in the native membrane (1).

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