TWO POSSIBLE ROLES OF BACTERIORHODOPSIN; A COMPARATIVE STUDY OF STRAINS OF *HALOBACTERIUM HALOBIUM* DIFFERING IN PIGMENTATION

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SUMMARY

The light-induced changes in pH and ATP level were compared for cell suspensions between strains of *Halobacterium halobium* differing in pigmentation after growth under the same conditions. Upon illumination, red cells which contained no detectable amount of bacteriorhodopsin showed only a pH increase, which, in the case of purple cells containing bacteriorhodopsin, was followed by a spontaneous pH decrease during illumination. Pre-incubation of cells at 75° for 5 min depressed the pH increase in both cells. Pre-illumination of cells with hydroxylamine depressed the pH decrease in purple cells. Whenever the pH increase was observed, the cellular ATP level increased. The presence of a bacteriorhodopsin different from that in the purple membrane is postulated.

Oesterhelt and Stoeckenius (1) reported that the purple membrane in *Halobacterium halobium* pumps protons outward (resulting in a decrease in pH of the cell suspension) under illumination, causing a proton gradient across the cell membrane. Danon and Stoeckenius (2) reported that these cells synthesize ATP utilizing this proton gradient. The purple membrane is also considered to be essential for transport of metabolites (3) and phototaxis (4). However, upon illumination, the pH of an intact cell suspension transiently increases then decreases (1,5) and ATP synthesis appears to take place in the phase of the pH increase (5). It thus seems unlikely that ATP is synthesized directly using the proton gradient revealed as the pH decrease.

We have isolated a strain of *H. halobium* which apparently has no detectable bacteriorhodopsin and a strain which has bacteriorhodopsin. Using these strains, we compared the light-induced changes in pH of intact cell suspensions as well as in the cellular ATP level. The results strongly suggest that the light-induced pH increase is closely related to ATP synthesis and that some

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bacteriorhodopsin in a special environment participates in ATP synthesis and differs from the majority that form the purple membrane.

MATERIALS AND METHODS

Halobacterium halobium R_1 (a kind gift of Prof. M. Masui of Osaka City Univ.; the strain originated from Dr. D. J. Kushner of Univ. of Ottawa) was used otherwise noted. Two kinds of colonies, red (red cells) and purple (purple cells), were obtained from an agar plate. Each strain was grown for 7-10 days at 37-40° under a fluorescent lamp (20 W) in a medium containing 25% NaCl, 0.2% KCl, 1% $MgSO_4 \cdot 7H_2O$, 0.02% $CaCl_2$, 0.3% sodium citrate, and 0.33% polypeptone (Daigo-eiyo Co.) at pH 7.4 under constant stirring for appropriate aeration. Bacteriorhodopsin content was estimated to be about 1.4 nmoles/mg protein in purple cells by the method described by Danon and Stoeckenius (2) using a molar extinction coefficient of 63 $mM^{-1}cm^{-1}$ at 570 nm (6). No bacteriorhodopsin was detected in red cells by the same method. Protein concentrations were determined by the Lowry procedure.

Cells in a stationary phase were harvested and suspended in the basal salt solution (the growth medium without nutrients). A cell suspension was placed in a jacketed container at 37° under nitrogen at a concentration of 1-3 mg protein/ml and illuminated with a 750 W projector through a cutt-off filter (>500 nm). Changes in pH were measured with a pH meter (Hitachi-Horiba, Model F-7) with a combined electrode. The pH traces were calibrated by adding HCl to the cell suspension. Portions of cell suspensions were pipetted out during the pH measurement, and cellular ATP levels under a given anaerobic condition were assayed by the luciferin/luciferase method (7).

In some experiments, cells were pre-incubated in a water bath at 75° for 5 min (the heat treatment), or pre-illuminated in the presence of 0.4M hydroxylamine (pH 7) at 37° for 2 hr (the NH_2OH treatment) according to the method of Oesterhelt *et al.* (8). To measure the action spectrum, interference filters were used after light energy had been corrected for each filter using a photometer (United Detector Tech. Inc., Model PIN-10F).

RESULTS AND DISCUSSION

As has been reported (1), the pH of the purple cell suspension at the initial pH around 7 transiently increased upon illumination and decreased to a steady level within about 2 min (Fig. 1a). After the illumination was stopped, the pH decreased then increased to the original level. The red cell suspension showed a monophasic pH increase upon illumination and a monophasic decrease in the dark (Fig.1b). These changes were repeatedly observed with light and dark cycles. Red cells showed no pH decrease with prolonged illumination or at various initial pH values (between 5 and 8). The pH decrease in a purple cell suspension is ascribed to bacteriorhodopsin in the purple membrane which pumps protons out of the cell under illumination (1), and lack of the pH decrease in a red cell suspension agrees with the observation that

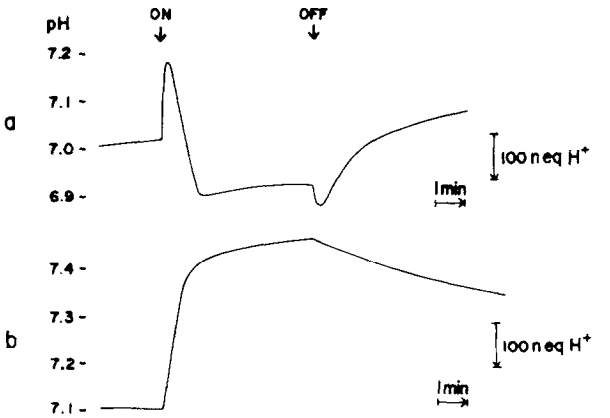

Fig. 1 Light-induced pH changes in cell suspensions of two strains of *H. halobium*. Cells were suspended in the basal salt solution (5 ml) in a jacketed container at 37° under nitrogen and illuminated about 30 min later when the pH of the suspension became steady. (a) Purple cells (1.7 mg protein /ml). (b) Red cells (1.7 mg protein/ml) which apparently contain no bacteriorhodopsin.

red cells have no detectable bacteriorhodopsin.

When the cells were pre-incubated at 75° for 5 min, the light-induced transient pH increase in the purple cell suspension disappeared and the pH decrease was apparently stimulated (Fig. 2a). The light-induced pH increase in the red cell suspension was also lost after this heat treatment (Fig. 2b), and thus no pH response was observed upon illumination. The absorption spectra of lysates of both cells were almost the same before and after the heat treatment. When the cells were pre-illuminated in the presence of NH_2OH for bleaching bacteriorhodopsin (8), the pH decrease was drastically depressed in the purple cell suspension and only the pH increase was observed (Fig. 2c). The absorption spectrum of the lysate of these NH_2OH-treated purple cells became similar to that of red cells due to the disappearance of bacteriorhodopsin. The light-induced pH changes in a red cell suspension were not significantly altered by the NH_2OH treatment (Fig. 2d). These results support the idea that the pH decrease under illumination is caused by the purple membrane (1), and suggest that the pH increase is due to another mechanism common to both purple and red cells.

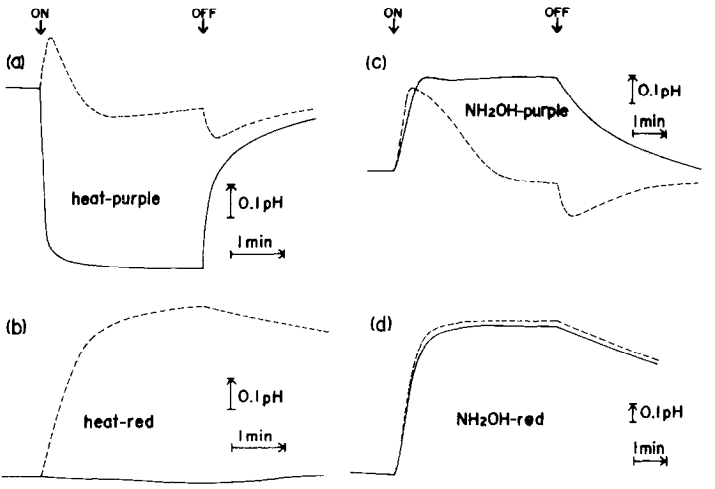

Fig. 2 Effect of various treatments on the light-induced pH changes. The pH change was measured as for Fig. 1 at the initial pH 7.1 ± 0.3. Purple or red cells (1.8 - 3.4 mg protein/ml) were pre-incubated (a), (b) at 75° for 5 min (heat treatment) or (c), (d) at 37° for 2 hr in the presence of NH_2OH (0.4M) under illumination (NH_2OH treatment). Broken lines refer to the pH changes of untreated cells.

The action spectra for the pH increase in an untreated red cell and in an NH_2OH-treated (pH decrease-lost) purple cell suspension agreed with each other, revealing a maximum at around 580-600 nm (Fig. 3A). The action spectrum for the pH decrease in a heat-treated (pH increase-lost) purple cell suspension (the strain R_1M_1 which contains considerably less carotenoids than R_1 was used) showed a maximum at around 560 nm and fairly well corresponded to the absorption spectrum of bacteriorhodopsin as expected (Fig. 3B). The pH decrease in a heat-treated purple cell suspension was partially depressed by addition of heat-treated red cells which showed no light-induced pH changes. This depression was especially remarkable at wavelengths shorter than 550 nm probably because of the shielding effect of carotenoids in the added heat-treated red cells (Fig. 3B), and resulted in an apparent shift of the maximum in the action spectrum (normalized to 100% at the maximum) to a longer wavelength. This shifted spectrum of R_1M_1 resembled the action spectrum for the pH decrease in a heat-treated purple (R_1) cell suspension. If purple (R_1) or

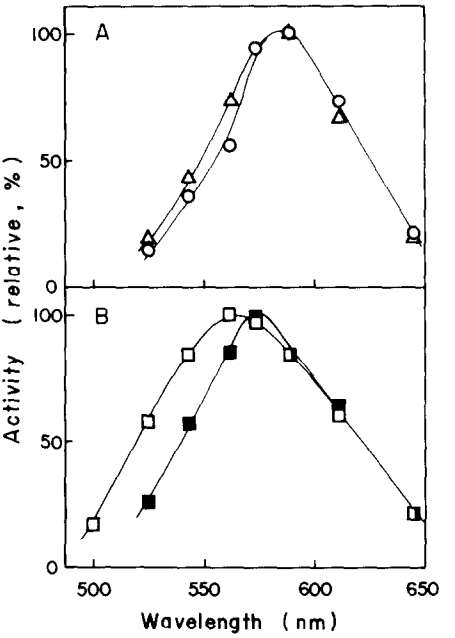

Fig. 3 Action spectra for the initial rates of pH changes. The pH change was measured as for Fig. 1 upon illumination at a given wavelength (half band width ≈ 20 nm) at the initial pH 7.0 ± 0.3. The initial rate showed a linear relation with the light intensity at each wavelength measured. The initial rates were normalized to 100% at the maximum. (A) pH increase: (○) untreated red cells (1.4 mg protein/ml), (△) NH_2OH-treated purple cells (3.1 mg protein /ml), (B) pH decrease: (□) heat-treated purple cells (0.19 mg protein/ml), (■) heat-treated purple cells (0.17 mg protein/ml) + heat-treated red cells (1.08 mg protein/ml). Each treatment was carried out as given in Fig. 2.

red cells contain a lesser amount of red pigments, the maximum of the action spectrum for the pH increase can be expected to shift to a shorter wavelength. Therefore, bacteriorhodopsin most likely participates not only in the pH decrease (1) but also in the pH increase (see below), although some unidentified pigments might be responsible for the pH increase.

Dark and light ATP levels of the cells were compared after various treatments (Table I). A high ATP level was achieved and maintained under illumination by untreated red cells as well as by purple cells. Dark levels of ATP of both purple and red cells after the heat treatment were greatly reduced and no ATP was synthesized in the light. On the other hand, ATP levels of both purple and red cells rose under illumination even after the NH_2OH treatment

Table I Dark and light levels of cellular ATP before and after various treatments. The ATP levels were assayed by the luciferin/luciferase method (7). Portions of cell suspension were sampled at intervals in the dark or light. The steady reading of the ATP levels is shown in nmoles/mg protein. Treatment, if any, was carried out as given in Fig. 2. The direction of the light-induced pH change (ΔpH) is also shown (↑ increase, ↓ decrease, ↑↓ increase followed by decrease, - no change). The values in each of the four frames were obtained from one cell preparation each.

	Purple			Red		
	Dark	Light	ΔpH	Dark	Light	ΔpH
Untreated	1.4	3.8	↑↓	0.7	3.4	↑
Heat-treated	0.4	0.3	↓	0.2	0.2	-
Untreated	2.8	7.0	↑↓	1.4	3.6	↑
NH_2OH-treated	0.8	4.7	↑	0.8	2.6	↑

which lowered the ATP levels in the dark. These results strongly suggest a parallelism between the light-induced pH increase and ATP synthesis.

The above results lead to a postulation that bacteriorhodopsin, which has been suggested to participate in the pH increase, is also responsible for ATP synthesis. This bacteriorhodopsin differs from that abundant in the purple membrane, which causes the pH decrease, in the following points: 1) it functions in much smaller quantities, which are still sufficient for maintaining a high ATP level under an anaerobic, light condition; 2) it is much less reactive to NH_2OH presumably owing to its localization or environment or both; 3) it is located in a system susceptible to heat, which would be involved in ATP synthesis. Some evidence in favor of the presence of bacteriorhodopsin in red cells has been accumulated. Red cells grown in a medium containing nicotine, an inhibitor of retinal synthesis (10), completely lost the activities of both light-induced pH increase and ATP synthesis, but regained them on an addition of retinal. Detailed results will be published elsewhere.

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