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EFFECT OF LIGHT-ADAPTATION ON THE PHOTOREACTION OF BACTERIORHODOPSIN FROM *HALOBACTERIUM HALOBIUM*

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SUMMARY

Light-induced formation of the 410 nm intermediate was investigated on dark- and light-adapted bacteriorhodopsin. The amplitude of the light-induced absorption increase at 410 nm of the light-adapted bacteriorhodopsin was twice as large as that of the dark-adapted bacteriorhodopsin. The amount of protons released from bacteriorhodopsin in response to illumination was also enhanced by light-adaptation. The degree of the enhancement was independent of the temperature in the dark-adaptation. The relation between these photochemical events and the isomeric configurations of retinal is discussed.

INTRODUCTION

When grown in the light and under low oxygen tension, the extreme halophile *Halobacterium halobium* produces specialized regions in its cell membrane. These patches, known as the purple membranes, contain a single protein, bacteriorhodopsin, arrayed in a two-dimensional crystalline lattice [1–6]. Bacteriorhodopsin functions as a light-driven proton pump across the membrane [7] and the produced proton gradient is utilized to synthesize ATP [8–11], or serves as a driving force for the transport of amino acids [12].

Bacteriorhodopsin undergoes a light-induced reaction cycle. Several intermediates in the cycle have been identified [13–17], among which a relatively long-lived intermediate, the 410 nm intermediate, is believed to play a central role in the function of proton transport. Linked to the formation of the 410 nm intermediate, approximately one proton per cycling bacteriorhodopsin is released in the aqueous phase [18].

In the dark, bacteriorhodopsin exists in a stable dark-adapted form [19] with an absorption maximum around 560 nm originating from retinal chromophore [1]. After exposure to light, it becomes another quasi-stable form termed light-adapted bacteriorhodopsin [19]. When light-adapted, the peak shifts to around 570 nm and increases in absorption by about 10 %. Retinal extracted from the light-adapted bacteriorhodopsin is reported to have the all-*trans* configuration and the dark-adapta-

tion process involves isomerization of the retinal to the 13-*cis* configuration [19–22]. The present paper reports that the dark-adapted bacteriorhodopsin exists as a mixture of about equal amounts of all-*trans* and 13-*cis* forms, and only the bacteriorhodopsin which contains all-*trans* retinal takes part in the proton transporting photoreaction cycle through the 410 nm intermediate.

MATERIALS AND METHODS

Cells were grown and purple membranes were isolated following standard methods [23]. Isolated purple membranes were suspended in distilled water and stored in the dark at 4 °C.

Absorption spectra were obtained using a Shimadzu UV-200 or a Shimadzu MPS-50L spectrophotometers. Light-induced formation of the 410 nm intermediate was observed by means of a single-beam spectrophotometer equipped with an actinic light source. The actinic light was from a 650 W quartz halogen lamp through a 10 cm water layer and an orange filter (Toshiba, V-055). Short time illumination of the sample was realized using a shutter for a camera (COPAL, No. 0). A combination of bandpass filters (Hoya, B-440 and V-390) was placed in front of the photomultiplier in order to protect it from scattered light. Output signals from the photomultiplier were memorized on a transient recorder (datalab, Model DL905) and recorded on a chart recorder (YEW, Model 3047).

Light-induced changes of pH were followed with a glass combination electrode (Beckman, No. 39183) attached to a Beckman expandomatic pH-meter (Model SS-2) connected to a chart recorder (Hitachi, Model 056). The light-sensitive area of the electrode was shaded and the direct perturbation of the actinic light on the electrode was completely avoided.

Molarities of bacteriorhodopsin were based on millimolar absorption coefficient of 63 $mM^{-1} \cdot cm^{-1}$ at 570 nm [24].

RESULTS

When kept in the dark, the light-adapted bacteriorhodopsin gradually reverses to the dark-adapted form. Fig. 1 shows the difference spectra between the light-adapted and the dark-adapted bacteriorhodopsin. The time course of the dark-adaptation can be followed by measuring the absorption at 590 nm, where the absorption difference is the largest.

The time courses of the dark-adaptation were followed at various temperatures at pH 8. Suspensions of purple membrane were light-adapted completely by illuminating with a 150 W projector lamp for 3 min. After turning off the light, the change in absorption at 590 nm was followed by a spectrophotometer. The activation energy was calculated to be 24 kcal/mol from the Arrhenius plot of the kinetic data. The half time of the decay of the absorption difference was 21.8 min at 35 °C which was nearly the same as that reported by Oesterhelt et al. [19]. At 4 °C, it becomes about 30 h as calculated by extrapolating the plot.

The rate of the dark-adaptation as a function of pH was examined (Fig. 2). The temperature was maintained at 30 °C. In the range between pH 7 and pH 8.5, the rate was virtually independent of pH. But in the acidic and alkaline region, a strong

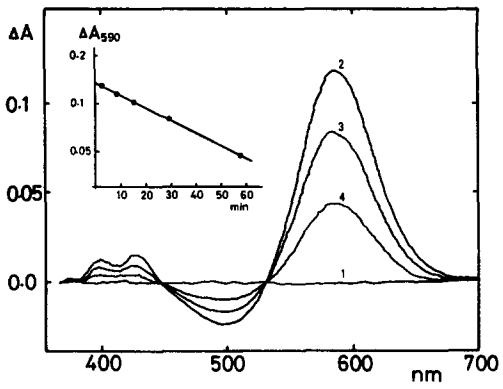

Fig. 1. Time dependence of the difference spectra between the light-adapted and dark-adapted bacteriorhodopsin. Spectra were taken using a dark-adapted sample as reference at 30 °C. Recording was started (1) before illumination and (2) 1 min, (3) 16 min and (4) 56 min after turning off the light. Bacteriorhodopsin concentration was 15 μM in 0.2 M KCl, 20 mM Tris · HCl pH 8. Insert: Time course of the absorption difference at 590 nm.

dependence on pH was observed. As for the kinetics, the dark-adaptation was a first-order process at neutral and alkaline region. In the acidic region, however, it deviated from first-order. In this report, the rate of the dark-adaptation at acidic pH was defined by measuring the time required for the absorption difference to become 1/e of the initial value. As the rate of the dark-adaptation becomes very large at acidic region, the light-adapted state is difficult to handle at low pH without lowering the temperature of the sample. At neutral and alkaline pH, on the other hand, the dark-adaptation process is so slow that it took a long time to obtain a dark-adapted sample. To get a sample equilibrium at 4 °C and at pH 8, for example, it is necessary to keep it in the dark for about a week.

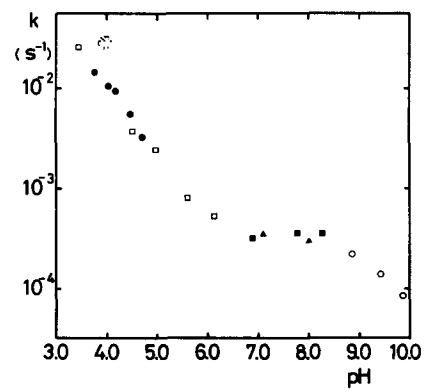

Fig. 2. The rate of the dark-adaptation as a function of pH. Temperature was maintained at 30 °C. Bacteriorhodopsin concentration was 15 μM in (▲) 0.2 M KCl, 20 mM Tris · HCl, (○) 0.2 M KCl, 20 mM glycine, (■) 10 mM KCl, 1 mM Tris · HCl, (□) 10 mM KCl, 1 mM sodium acetate and (●) 10 mM KCl, 1 mM glycine.

Light quanta absorbed by purple membrane bring about at least two effects on the bacteriorhodopsin. One is the so-called light-adaptation [19] which is in essence the isomerization of retinal from 13-*cis* to all-*trans* configuration. The process is relatively slow and the produced state is quasi-stable. The other is a reaction cycle which comes to an end in a time as short as several ms at physiological temperature [17]. The cycle contains several short-lived intermediates, among which the so-called 410 nm intermediate is the most easy to observe. Light-induced formation of the 410 nm intermediate was investigated on the dark-adapted and light-adapted bacteriorhodopsin. The energy of the actinic light was made so small that no serious light-adaptation was caused by the actinic light itself. Fig. 3 compares the absorption increase of the dark-adapted and light-adapted bacteriorhodopsin at 410 nm induced by an actinic light pulse. Suspension of purple membrane was kept in the dark for 4 h at 30 °C for complete dark-adaptation of the membrane. The dark-adapted sample was quickly cooled to 4 °C and the amplitude of the absorption increase at 410 nm induced by an actinic light pulse, ΔA^{D}_{410}, was measured. After measuring ΔA^{D}_{410}, the sample was light-adapted by illuminating with the shutter kept open. And again, the light-induced absorption change, ΔA^{L}_{410}, was measured for the light-adapted sample. The amplitude of the light-induced absorption increase of the light-adapted form, ΔA^{L}_{410}, was about twice as large as that of the dark-adapted form, ΔA^{D}_{410}. The decay of the absorption change followed good first-order kinetics and the lifetimes of both forms were almost the same. The extent of the light-adaptation caused by an actinic light pulse was about 0.1 % as estimated from the quasi-stable absorption increase around 400 nm (see Fig. 1).

Accompanying with the light-induced formation of the 410 nm intermediate, protons are released in the medium from bacteriorhodopsin. Light-induced proton release from bacteriorhodopsin was measured by a glass combination electrode as a change of pH of the medium. Suspension of dark-adapted purple membrane was replaced in a cuvette kept at 7 °C, and the changes of pH of the medium in response to a train of light pulses were measured. As the rate of the dark-adaptation is very small

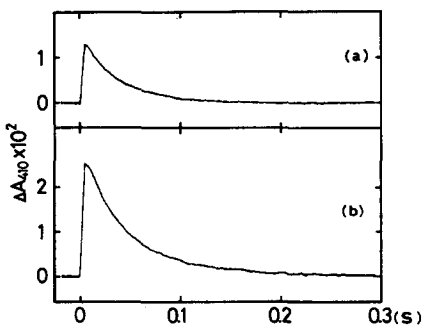

Fig. 3. Enhancement of the light-induced absorption change at 410 nm caused by light-adaptation. A light-induced absorption change at 410 nm was measured for the dark-adapted and light-adapted bacteriorhodopsin. Protein concentration was 26 μM in 0.2 M KCl, 20 mM potassium phosphate pH 8. The sample was dark-adapted by storing in the dark for 4 h at 30 °C. The dark-adapted sample was replaced in a cuvette kept at 4 °C and the light-induced absorption change at 410 nm was measured (a). Then the sample was light-adapted and the light-induced absorption change at 410 nm was measured again (b). Actinic light pulse was 4 ms in duration and 670 mW/cm^2 intensity.

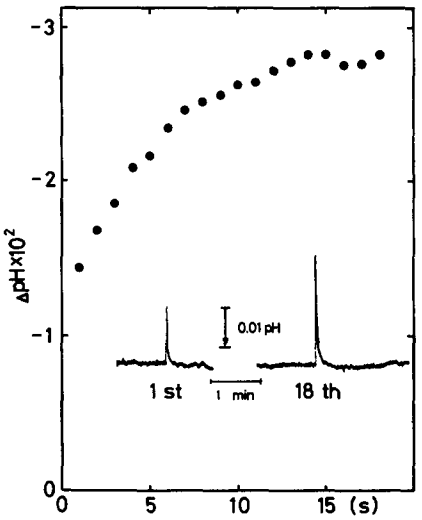

Fig. 4. Enhancement of the light-induced proton release from bacteriorhodopsin caused by light-adaptation. Suspension of purple membrane was dark-adapted by storing in the dark at room temperature overnight. The dark-adapted sample was replaced in a vessel under dim red light. The vessel was a temperature controlled cylindrical one (12 mm internal diameter) and the volume of the sample was 2.5 ml. The sample was illuminated by a train of light pulses and the proton release induced by each pulse was measured with a glass combination electrode as a change of pH of the medium. The membrane suspension was stirred in order to light-adapt all pigments. An actinic light pulse was 1 s in duration and 750 mW/cm^2 intensity. Bacteriorhodopsin concentration was 48 μM in 0.2 M KCl. Measurement was made at 7 °C and at pH 7.7 under an Ar atmosphere. Abscissa: Total time of illumination. Ordinate: The changes of pH induced by 1 s light pulses. Insert: The output signals of the pH meter for the 1st and the 18th light pulses.

at this temperature, successive light pulses have an accumulative effect of light-adaptation on the membrane. It is seen in Fig. 4 that the changes of pH induced by an actinic light pulse is increased by light-adaptation. As calibration with standard HCl pulses showed no difference in the buffering capacity between the dark-adapted and light-adapted purple membrane, we can say that the observed enhancement of the light-induced change of pH is the result of the enhancement of the amount of protons released from purple membrane.

Fig. 5 shows the light-induced difference spectra around 410 nm of the dark-adapted and light-adapted purple membrane suspensions. The absorption changes for the light-adapted form were measured three times at each wavelength and averaged. For the dark-adapted form, however, the measurement was only once at each wavelength since the measurement itself caused a small light-adaptation of the sample. It is seen that the isosbestic points and the profiles of the difference spectra are much the same.

To establish further that the observed increase of the light-induced absorption change is due to isomerization of the retinal, the relation between the percent light-adaptation and the amplitude of the light-induced absorption change at 410 nm was investigated. A suspension of light-adapted purple membrane was stored in the dark and the decrease of absorption at 590 nm accompanying the dark-adaptation was

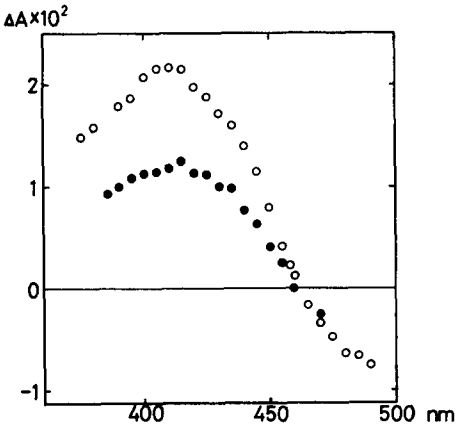

Fig. 5. Light-induced difference spectra around 410 nm of the dark-adapted and light-adapted bacteriorhodopsin. Conditions and measurement procedures were the same as in Fig. 4. (●) Dark-adapted sample. (○) Light-adapted sample.

followed at 25 °C. The percent light-adaptation η was defined as

$$\eta(t) = \frac{(A_{590}(t) - A_{590}(\infty))}{(A_{590}(0) - A_{590}(\infty))} \times 100$$

where $A_{590}(0)$ and $A_{590}(\infty)$ denote absorption at 590 nm of light-adapted and dark-adapted purple membrane suspensions, respectively, and t is the time elapsed after moving into the dark. In parallel with the above experiment, the amplitude of the light-induced absorption change at 410 nm, $\Delta A_{410}(t)$, induced by a 30 ms actinic light pulse was measured as a function of t. Along with the process of the dark-adaptation, $\Delta A_{410}(t)$ became smaller and a good linear relation between $\eta(t)$ and $\Delta A_{410}(t)$ was observed. The ratio between the amplitude of the light-induced absorption change of the light-adapted form and that of the dark-adapted form, $\Delta A_{410}(0)/\Delta A_{410}(\infty)$, was nearly equal to 2.

DISCUSSION

The chromophores extracted from the light-adapted bacteriorhodopsin are reported to be all-*trans* retinal. Concomitant with dark-adaptation, isomerization of the retinal to 13-*cis* configuration takes place. Oesterhelt et al. [19], from the result of thin-layer chromatography, reported that the retinal extracted form the dark-adapted bacteriorhodopsin is a mixture of 13-*cis* and all-*trans* configurations. On the other hand, as a result of the same analytical method, Jan [20] reported that the dark-adapted bacteriorhodopsin contains predominantly 13-*cis* retinal. Very recently, based on high-pressure liquid chromatography [21] and a reconstitution experiment [22], Dencher et al. reported that the dark-adapted bacteriorhodopsin contains about equal amounts of 13-*cis* and all-*trans* retinal. Maeda et al. (Maeda, A., Iwasa, T., Schichida, Y. and Yoshizawa, T., unpublished work) also, using high-pressure liquid chromatography, obtained a result suggesting that the dark-adapted bacterio-

rhodopsin is a 1 : 1 mixture of 13-*cis* and all-*trans* forms. Investigation of the photoreaction of bacteriorhodopsin reconstituted from retinal-free protein [25] and a mixture of 13-*cis* and all-*trans* retinal (Ohno, K., Takeuchi, Y. and Yoshida, M., unpublished work) strongly suggested that only the bacteriorhodopsin with all-*trans* retinal attends the photoreaction cycle through the 410 nm intermediate. As described in Results, the dark-adapted bacteriorhodopsin formed the 410 nm intermediate in response to illumination. The amount was about a half of that from light-adapted bacteriorhodopsin. Thus we propose that the dark-adapted bacteriorhodopsin contains about equal amounts of all-*trans* and 13-*cis* forms, and that only the bacteriorhodopsin with all-*trans* retinal takes part in the proton transporting photoreaction cycle through the 410 nm intermediate. This conclusion is consistent with the observation by Dencher et al. [21, 22] but not with the chromatographic analysis reported in [20].

Since the light-adapted bacteriorhodopsin contains only all-*trans* retinal, the present study provides an analytical method for estimation of the isomeric composition of a given state by measuring the photochemical response ΔA_{410} using the light-adapted sample as a standard. We investigated the temperature dependence of the isomeric composition of retinal in the dark-adapted bacteriorhodopsin. Each of the purple membrane suspensions stored in the dark at 35 °C for 3 h, at 15 °C for 24 h, and at 4 °C for 10 days was replaced in a cuvette kept at 4 °C and $\Delta A^{D}_{410}/\Delta A^{L}_{410}$ measured as described above. The ratios were always close to 0.5 and no significant temperature dependence was observed. Thus we are led to a conclusion that the dark-adapted bacteriorhodopsin exists as a mixture of about equal amounts of all-*trans* and 13-*cis* forms over a wide range of temperature, hence the enthalpy change of bacteriorhodopsin associated with the isomerization of retinal must be close to zero. According to the recent experiment by Dencher et al. [22], the enthalpy change is not exactly zero. They report that high temperature slightly favours the all-*trans* form.

It is concluded that only the bacteriorhodopsin with all-*trans* retinal acts as a light-driven proton pump. A question remains unsolved about the biological function of the 13-*cis* type bacteriorhodopsin.

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REFERENCES

1 Oesterhelt, D. and Stoeckenius, W. (1971) Nat. New Biol. 233, 149–152
2 Blaurock, A. E. and Stoeckenius, W. (1971) Nat. New Biol. 233, 152–155
3 Henderson, R. and Unwin, P. N. T. (1975) Nature 257, 28–32
4 Henderson, R. (1975) J. Mol. Biol. 93, 123–138
5 Blaurock, A. E. (1975) J. Mol. Biol. 93, 139–158
6 Unwin, P. N. T. and Henderson, R. (1975) J. Mol. Biol. 94, 425–440
7 Oesterhelt, D. and Stoeckenius, W. (1973) Proc. Natl. Acad. Sci. U. S. 70, 2853–2857
8 Danon, A. and Stoeckenius, W. (1974) Proc. Natl. Acad. Sci. U. S. 71, 1234–1238

9 Racker, E. and Stoeckenius, W. (1974) J. Biol. Chem. 249, 662–663
10 Yoshida, M., Sone, N., Hirata, H., Kagawa, Y., Takeuchi, Y. and Ohno, K. (1975) Biochem. Biophys. Res. Commun. 67, 1295–1300
11 Kagawa, Y., Ohno, K., Yoshida, M., Takeuchi, Y. and Sone, N. (1977) Fed. Proc. 36, 1815–1818
12 MacDonald, R. E. and Lanyi, J. K. (1975) Biochemistry 14, 2882–2889
13 Stoeckenius, W. and Lozier, R. H. (1974) J. Supramol. Struc. 2, 769–774
14 Dencher, N. and Wilms, M. (1975) Biophys. Struct. Mech. 1, 259–271
15 Kung, M. C., Devault, D., Hess, B. and Oesterhelt, D. (1975) Biophys. J. 15, 907–911
16 Lozier, R. H., Bogomolni, R. A. and Stoeckenius, W. (1975) Biophys. J. 15, 955–962
17 Sherman, W. V., Korenstein, R. and Caplan, S. R. (1976) Biochim. Biophys. Acta 430, 454–458
18 Lozier, R. H., Niederberger, W., Bogomolni, R. A., Hwang, S. and Stoeckenius, W. (1976) Biochim. Biophys. Acta 440, 545–556
19 Oesterhelt, D., Meenzen, M. and Schumann, L. (1973) Eur. J. Biochem. 40, 453–463
20 Jan, L. Y. (1975) Vision Res. 15, 1081–1086
21 Dencher, N. A., Carl, P. and Sperling, W. (1976) Proceedings of 10th International Congress of Biochemistry, p. 270, Abstr.
22 Dencher, N. A., Rafferty, Ch. N. and Sperling, W. (1976) Berichte der Kernforschungsanlage Jülich Nr. 1374
23 Oesterhelt, D. and Stoeckenius, W. (1974) in Methods in Enzymology (Fleisher, S. and Packer, L., eds.), Vol. 31. Biomembranes (part A), pp. 667–678, Academic Press, New York
24 Oesterhelt, D. and Hess, B. (1973) Eur. J. Biochem. 37, 316–326
25 Oesterhelt, D., Schumann, L. and Gruber, H. (1974) FEBS Lett. 44, 257–261