PHOTOCHEMICAL REACTION OF BACTERIORHODOPSIN

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1. Introduction

Halobacterium halobium utilizes light both as a stimulus for phototaxis [1] and as an energy source for creating a proton gradient across the cell membrane which in turn produces ATP [2]. The photoreceptive pigment of this bacterium was first investigated by Oesterhelt and Stoeckenius [3] who showed that it is the only protein lying in so-called 'purple membrane' of the cell. Since this pigment, like visual pigments, has retinal as a prosthetic group [3] it is called 'bacteriorhodopsin'. There are two forms of this pigment, dark-adapted bacteriorhodopsin (bR^D, λ_{max}: 558 nm) and light-adapted bacteriorhodopsin (bR^L, λ_{max}: 568 nm). While Lozier et al. [4] and Kung et al. [5] have reported on the photochemical reactions of the light-adapted form, the photochemical reactions of the dark-adapted pigment have not been studied.

We investigated the photochemical reaction of these forms of bacteriorhodopsin at liquid nitrogen temperatures.

2. Materials and methods

Halobacterium halobium was cultured and the purple membrane was prepared according to the method described by Oesterhelt and Stoeckenius [6]. Purple membrane, isolated by sucrose density gradient centrifugation, was dialyzed against distilled water. After centrifugation (40 000 × *g*, 40 min), the precipitated pellet was suspended in distilled water and glycerol added to a final concentration of 75%. Using the cryostat described by Yoshizawa [7], low temperature absorption spectra were measured with a Hitachi 323 recording spectrophotometer. A 2KW Xenon lamp was used for irradiation. The wavelengths of irradiation were selected by interference filters and/or glass cut-off filters.

3. Results

Light-adapted bacteriorhodopsin (bR^L) in the purple membrane suspended in 75% glycerol showed its absorption maximum (λ_{max}) near 565 nm at room temperature. After incubation in the dark for more than seven hours at room temperature, bR^L fully converted to bR^D and the λ_{max} shifted to 558 nm. The half-time of the dark conversion of bR^L to bR^D in water–glycerol (1:3, v/v) was about 25 min at 21°C. The rate was slowed down by cooling. After bR^L was formed by irradiating bR^D at 500 nm for 10 min (curve 1 in fig.1) at 0°C it remained in the dark for two hours; no conversion of bR^L to bR^D was observed.

Further irradiation resulted in no additional spectral changes, so we used the 10 min irradiation at 500 nm for preparation of bR^L. At 0°C, bR^L and bR^D showed their λ_{max} at 572 nm and 563 nm respectively (curves 1 and 2 in fig.1 top and bottom).

Upon cooling bR^D to −190°C, its λ_{max} shifted to 570 nm and its absorbance rose (curve 1 in fig.2 top).

When bR^D at −190°C was irradiated at 500 nm, the spectrum shifted to longer wavelengths and the absorbance in the longer wavelength region rose. This spectral shift (curve 1–7 in fig.2 top) formed a clear isosbestic point (581 nm). Curves 7–9 showed a photosteady state, which was designated as State^D-I. Warming State^D-I to 0°C gave curve 3 (fig.1 top), which intersected at the isosbestic points (532 nm and

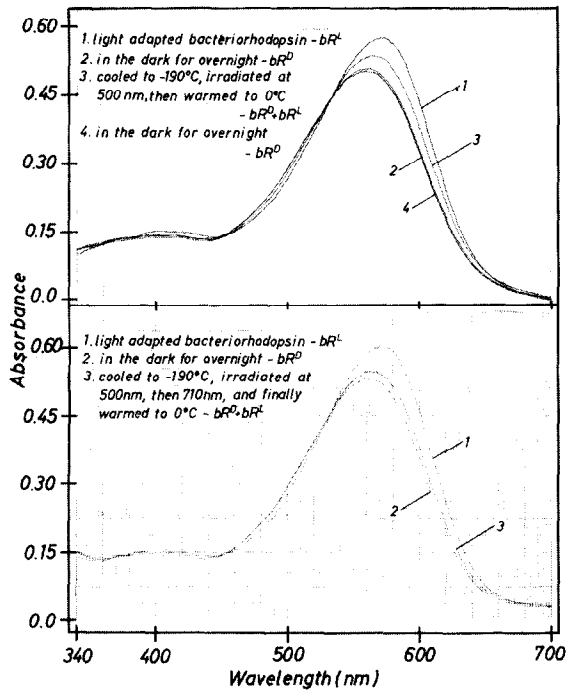


Fig.1. Interconversion between dark-adapted and light-adapted bacteriorhodopsins by irradiation at liquid nitrogen temperatures. All the spectra were recorded at 0°C. Top: curve 1: Light-adapted bacteriorhodopsin (bR^L), produced by irradiating the purple membrane (in 75% glycerol) at 500 nm for 10 min at 0°C. Curve 2: Dark-adapted bacteriorhodopsin (bR^D), produced by incubating bR^L (curve 1) in the dark at room temperature overnight. Curve 3: After conversion of bR^D (curve 2) to a photosteady state by irradiation at 500 nm at liquid nitrogen temperature, the sample was warmed to 0°C. This spectrum represents a mixture of bR^D and bR^L. Curve 4: The sample (curve 3) was incubated in the dark overnight at room temperature. The mixture (curve 3) changed to bR^D. Bottom: curves 1 and 2 are bR^L and bR^D respectively, which were produced in a similar manner to that used above. Curve 3: After conversion of bR^D to photosteady state by irradiation at 500 nm, the sample was reirradiated with longer wavelengths of light (> 670 nm) at liquid nitrogen temperatures for 42 min and then warmed to 0°C. This spectrum represents a mixture of bR^D and bR^L.

450 nm) of bR^D and bR^L (curve 1 and curve 2 in fig.1 top). Thus, a part of the photosteady state mixture was converted to bR^L by warming. Upon dark adaptation, curve 3 (fig.1 top) reverted to the spectrum of the original bR^D (curve 4 in fig.1 top).

When $State^D$-I (curve 1 in fig.2 middle) was irradiated at 710 nm, the absorbance near 570 nm rose and that near 630 nm decreased. This spectral change had an isosbestic point at 594 nm. Curves 7 and 8 (fig.2 middle) show another state which is

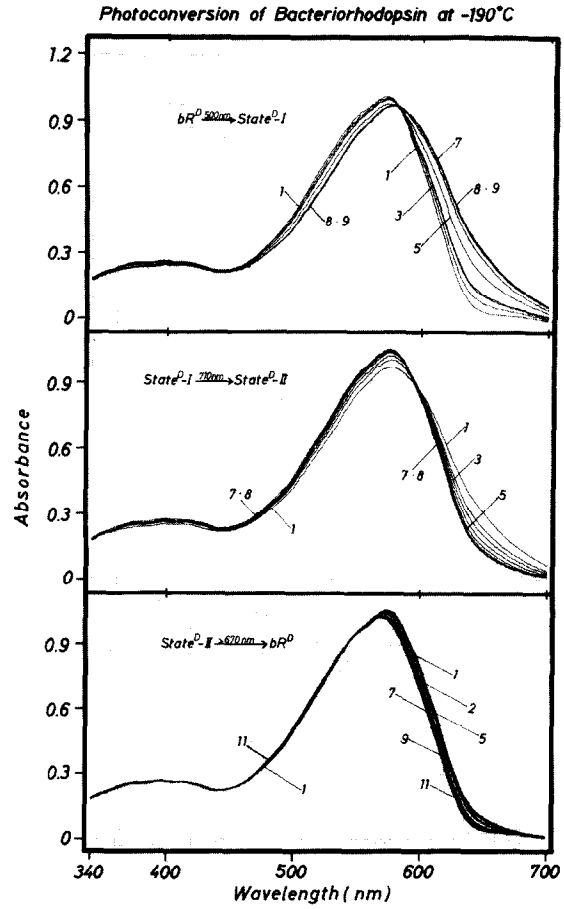


Fig.2. Course of interconversions among dark-adapted bacteriorhodopsin and two kinds of photosteady states at −190°C. Top: The photoconversion of bR^D to $State^D$-I. Curve 1: bR^D, produced by incubating the purple membrane (in 75% glycerol) in the dark at room temperature overnight. Curves 2–9: Products of irradiation of bR^D at 500 nm for 10,10,20,40,80,160,320 and 640 s. Curves 8 and 9 represent a photosteady state ($State^D$-I). Middle: The photoconversion of $State^D$-I to $State^D$-II. Curve 1: $State^D$-I (redrawn from curve 9 in the top figure). Curves 2–8: Products of irradiation of $State^D$-I at 710 nm for 10,10,20,40,80,160 and 320 s. Curve 8 shows $State^D$-II. Bottom: The photoconversion of $State^D$-II to bR^D. Curve 1: $State^D$-II (redrawn from curve 8 in the middle figure). Curves 2–11: Products of irradiation of $State^D$-II with light of wavelengths longer than 670 nm for 10,10,20,40,80,160,320,640,1280 and 2560 s. Curve 11 represents bR^D.

designated as State^D-II. Warming State^D-II produced a mixture of bR^D and bR^L (curve 3 in fig.1 top). So, in a similar manner to the above observation, a part of State^D-II also was converted to bR^L. As expected, this sample (curve 3 in fig.1 bottom) also reverted to bR^D by dark incubation at room temperature overnight.

When State^D-II was irradiated with light of wavelengths longer than 670 nm, the absorbance at λ_{max} decreased, the λ_{max} shifted slightly to shorter wavelengths and an isosbestic point was seen at 544 nm (fig.2 bottom). The final spectrum (curve 11 in fig.2 bottom) coincided with that of the original bR^D (curve 1 in fig.2 top).

The series of spectral changes described above strongly suggests that there are at least two distinct states – State^D-I and State^D-II – which can be produced by irradiating bR^D at liquid nitrogen temperatures. These two states (State^D-I and State^D-II) and bR^D are interconvertible by light. Since the photoproducts contained in State^D-I and State^D-II have λ_{max} at longer wavelengths than bR^D, they are similar to the batho-intermediate of visual pigments (see below).

Next, the photoreactions of bR^L were examined. When bR^L was cooled to −190°C, the absorbance at λ_{max} rose. A shoulder appeared in the shorter wavelength region near 550 nm, and some smaller bands (fine structure) appeared near 400 nm (curve 3 in fig.3 bottom). In the spectrum of bR^D at liquid nitrogen temperature, a similar shoulder was identified but the fine structure could not be observed (curve 1 in fig.2 top). Absorption by carotenoids in the purple membrane probably contributed wholly or in part to these shoulders [8]. Upon irradiation of bR^L with 500 nm light at liquid nitrogen temperature, the absorbance at λ_{max} decreased, the absorbance near 635 nm rose, and the λ_{max} shifted slightly to longer wavelengths (fig.4 top). The isosbestic point of this spectral change was at 594 nm*. This photosteady

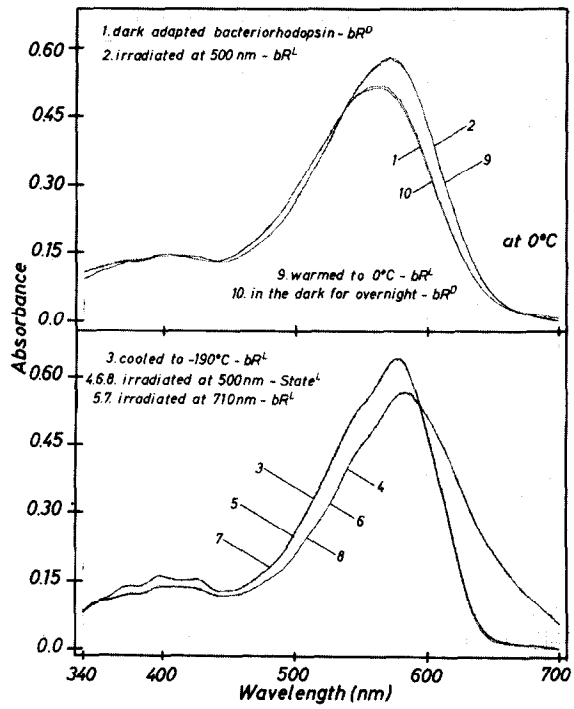


Fig.3. Photoconversion of light-adapted bacteriorhodopsin at liquid nitrogen temperatures. Top: All the spectra were measured at 0°C. Curve 1: bR^D, produced by incubating the purple membrane (in 75% glycerol) in the dark at room temperature overnight. Curve 2: bR^L, formed by irradiating bR^D at 0°C for 10 min. Curve 9: bR^L, formed by warming State^L (curve 8) to 0°C. Curve 10: bR^D, formed by incubating bR^L (curve 9) in the dark at room temperature. Bottom: All the spectra was measured at −190°C. Curve 3: bR^L (curve 2) was cooled to −190°C. Curves 4, 6 and 8: State^L, formed by irradiating bR^L (curve 3) at 500 nm for 320 s. Curves 5 and 7: bR^L, formed by irradiating State^L at 710 nm for 1280 s.

state is designated as State^L. On irradiating State^L at 710 nm, the spectrum reverted to the original bR^L with an isosbestic point at the same wavelength (fig.4 bottom) as the conversion of bR^L to State^L (fig.4 top). No spectral change was observed by further irradiation with light at wavelengths longer than 670 nm. Thus, only one state, that is one intermediate, is produced by irradiating bR^L at liquid nitrogen temperature. Lozier et al. have called this intermediate resembling bathorhodopsin of visual pigment, K [4]. The sample was then irradiated alternately by 500 nm and at 710 nm light. State^L and bR^L are

*J. Hurley and T. Ebrey (private communication) have found very small deviations from a perfect isosbestic point and have tentatively suggested that these may be related to the small changes in absorption spectra of bR^L due to altered interaction with nearest neighbors (exciton interaction) when the nearest neighbors are changed from bR^L to the batho-product by irradiation.

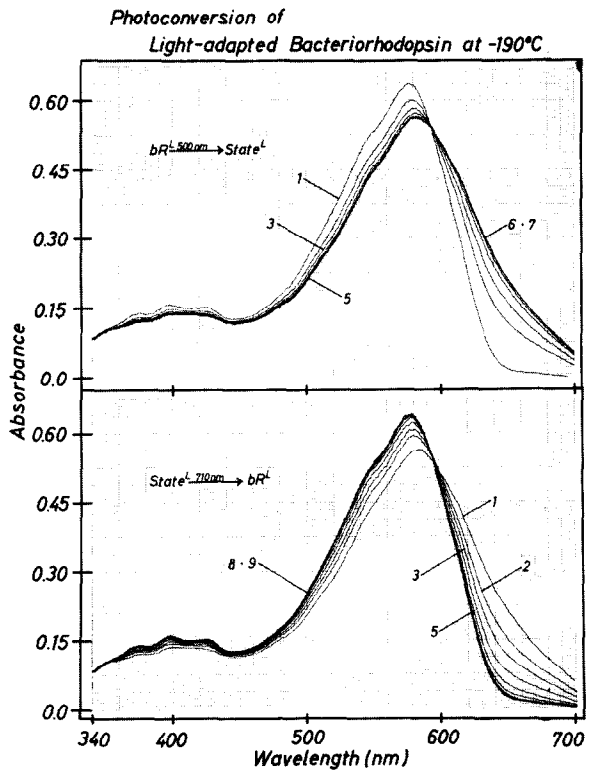


Fig.4. Course of photoconversion of light-adapted bacteriorhodopsin at −190°C. Top: The photoconversion of bR^L to $State^L$. Curve 1: bR^L (redrawn from curve 3 in fig.3 bottom). Curves 2–7: Products of irradiation of bR^L at 500 nm for 5,5,10,20,40 and 160 s. The curve 6 and 7 represents a photosteady state ($State^L$). Bottom: The photoconversion of $State^L$ to bR^L. Curve 1: $State^L$ (redrawn from the final spectrum in the above figure). Curves 2–9: Products of irradiation of $State^L$ at 710 nm for 10,10,20,40,80,100, 320 and 640 s. The final spectrum (curve 9) represent bR^L.

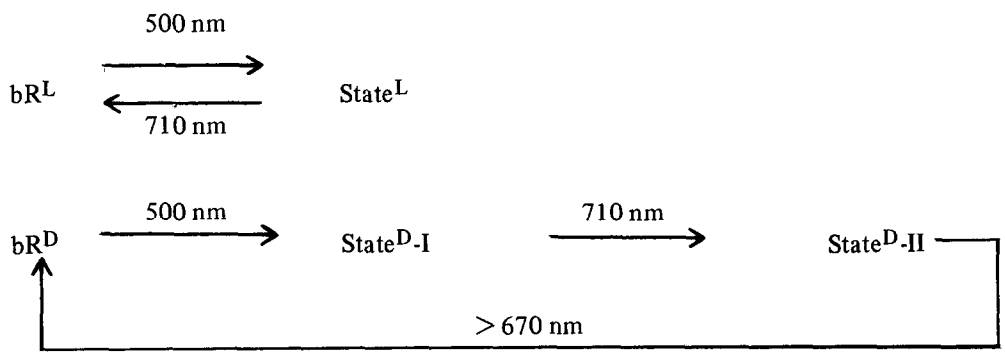
perfectly interconvertible by light (curves 3–8 in fig.3 bottom). The spectrum produced by warming $State^L$ to 0°C (curve 9 in fig.3 top) was identical with the spectrum of bR^L (curve 2 in fig.3 top); it changed to that of bR^D (curve 10 in fig.3 top) after incubating in the dark at room temperature. Thus, the intermediate contained in $State^L$ reverts to bR^L by warming. This reversion is different from the conversion of the intermediates produced from bR^D (in $State^D$-I and $State^D$-II) to bR^L by warming.

4. Discussion

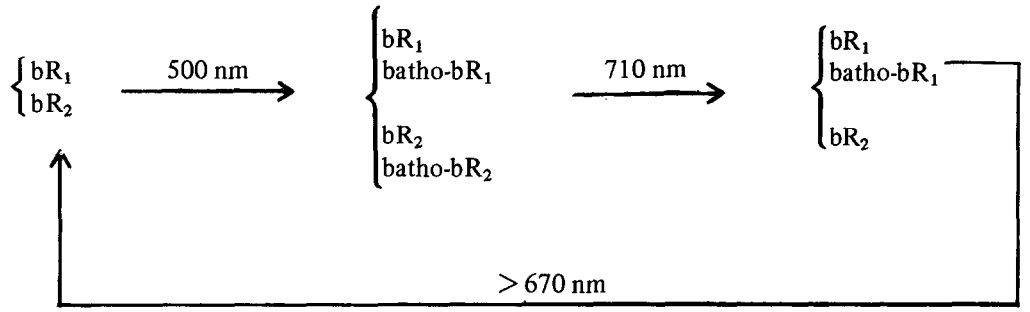
There is some controversy about which isomer of retinal is bound to the protein in the dark- and light-adapted states. Though all workers agree that the light-adapted chromophore is all-*trans* retinal, Oesterhelt et al. [9] reported that the dark-adapted form of the pigment has a mixture of 13-*cis* and all-*trans* retinal as a chromophore, while Jan [10] found only 13-*cis* retinal. More recent work (Maeda, Iwasa and Yoshizawa, unpublished observations and Petti et al. [11]) have supported the original findings of Oesterhelt.

In the case of the homogeneous species (bR^L), only one photosteady state, ($State^L$), is produced by irradiation at liquid nitrogen temperature. This photosteady state consists of the original pigment and its photoproduct (batho-intermediate).

Unlike bR^L two different states can be produced by irradiating bR^D at liquid nitrogen temperature.



This may be explained as follows:

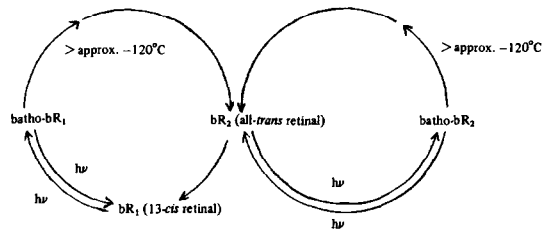


bR^D is a mixture of two species (bR_1 and bR_2), which have different isomeric forms of the chromophores and have slightly different absorption spectra. Now we will call bR having a 13-*cis* retinal as a chromophore, bR_1, and the other having all-*trans* retinal as a chromophore, bR_2. When bR^D (a mixture of bR_1 and bR_2) is irradiated at 500 nm at liquid nitrogen temperature, batho-bR_1 and batho-bR_2 are produced respectively, which are the bathochromic products of the bR. That is, the photosteady state mixture contains batho-bR_1 and batho-bR_2 as well as unconverted bR_1 and bR_2. Since one of the bR^D, say bR_2, has its absorption band at a longer wavelength than the other bR_1, batho-bR_2 may have its absorption band at a longer wavelength than batho-bR_1. Thus, when $State^D$-I is irradiated at 710 nm, the batho-bR_2 absorbs more light than batho-bR_1. Batho-bR_2 is therefore preferentially photoconverted back to the original pigment, bR_2. We suppose that irradiation of $State^D$-II with the light of wavelengths longer than 670 nm results in the conversion of the remaining batho-bR_1 into the original bR_1.

Because bR_2 has all-*trans* retinal as its chromophore, it seems reasonable to assume that this all-*trans* pigment is identical with the all-*trans* pigment from bR^L. We have some specific evidence for this identification. The difference spectrum between $State^D$-I and $State^D$-II, which is presumably the difference spectrum between bR_2 and batho-bR_2 (curve 1 and curve 8 in fig.2 middle) is similar to that between bR^L and $State^L$ (curve 3 and curve 4 in fig.3 bottom). The latter difference spectrum which can be regarded as that between bR^L and batho-bR^L, suggests that bR_2 and bR^L are the same all-*trans* species.

The intermediate present in $State^D$-II (batho-bR_1) is converted to bR^L (that is bR_2) by warming. The photoconversion of bR_1 (13-*cis* form) to bR^L or bR_1 (all-*trans* form) via batho-bR_1 suggests that for this species the primary photoreaction may be the photoisomerization of the double bond between C_{13} and C_{14} (i.e., 13-*cis* → *trans*). Moreover, although batho-bR_2 reverts to bR^L (bR_2) by warming, the spectral change in the conversion from bR_2 to batho-bR_2 is very similar to that observed in the conversion of a visual pigment to its batho-intermediate; this photochemical reaction is also a *cis–trans* isomerization of a double bond of retinal [12] so the conversion of bR_2 to batho-bR_2 may be some kind of photoisomerization such as a *cis–trans* isomerization of a double or single bond of retinal or a *syn–anti* isomerization of C=N bond at Schiff base. (A similar suggestion has been made by Rosenfeld et al. [13].)

From the above experimental results, the following reaction cycles for bacteriorhodopsin are proposed:



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References

[1] Hildebrand, R. and Dencher, N. (1975) Nature 257, 46–48.
[2] Danon, A. and Stoeckenius, W. (1974) Proc. Natl. Acad. Sci. USA 71, 1234–1238.
[3] Oesterhelt, D. and Stoeckenius, W. (1971) Nature 233, 149–152.
[4] Lozier, R. H., Bogomolni, R. A. and Stoeckenius, W. (1975) Biophys. J. 15, 955–962.
[5] Kung, M. C., DeVault, D., Hess, B. and Oesterhelt, D. (1975) Biophys. J. 15, 907–911.
[6] Oesterhelt, D. and Stoeckenius, W. (1974) in: Methods in Enzymology, XXXI, Biomembranes (Fleischer, S. and Packer, L. eds) pp. 667–678, Academic Press, New York.
[7] Yoshizawa, T. (1972) in: Handbook of Sensory Physiology, VII/2, Photochemistry of Vision (Dartnall, H. J. A. ed) pp. 146–179, Springer-Verlag, Berlin.
[8] Becher, B. and Cassim, J. Y. (1975) Prep. Biochem. 5, 161–178.
[9] Oesterhelt, D., Meentzen, M. and Schumann, L. (1973) Eur. J. Biochem. 40, 453–563.
[10] Jan, Lilly Yeh (1974) Vision Res. 15, 1081–1086.
[11] Petti, M., Yudd, A., Nakanishi, K. and Stoeckenius, W. (1976) submitted to Biochemistry.
[12] Yoshizawa, T. and Wald, G. (1963) Nature 197, 1279–1286.
[13] Rosenfeld, T., Honig, B., Ottolenghi, M., Hurley, J. and Ebrey, T. (1976) Pure Appl. Chem. in press.