Journal of Bioenergetics and Biomembranes, Vol. 10, Nos. 1/2, 1978

Spectral Transitions in Purple Membranes from *Halobacterium halobium*. I. Effect of Preliminary Illumination on Photochemical Processes

N. N. Vsevolodov and L. N. Chekulaeva

Institute of Biological Physics, Academy of Sciences of the USSR, Poustchino, Moscow Region, USSR

Received November 15, 1977

Abstract

The effect of preliminary illumination of purple membranes by yellow light on the difference spectra of short-lived intermediates has been studied. It has been found that changes of the optical density of two of these intermediates, which have the maxima of the difference spectrum at 412 nm and 650 nm, coincides well with the kinetics of the known reversible transitions of the main band of the purple membrane absorption (560 570), i.e., 13-*cis-trans* transitions. The changes at 412 nm and 650 nm are proportional to the concentration changes of the all-trans retinal and 13-cis retinal, respectively. It was concluded that the formation of the short-lived 412 and 650 intermediates occurs in different photochemical cycles. The pH is found to affect the formation of the 650 intermediate.

Introduction

A lipochromoprotein complex, the properties of which resemble visual rhodopsin, was discovered in the cell membrane of bacteria *H. halobium*. This complex was called bacteriorhodopsin, by Oesterhelt and Stoeckenius

0145-479X/78/0400-0013$05.00/0

[1]. Henderson [2] has shown that bacteriorhodopsin molecules in the purple membrane are centered all in one plane and arranged in the form of a hexagonal crystal lattice with three molecules in each cluster. The complex dissociates when it is removed from the membrane [1], therefore all the experiments with bacteriorhodopsin as a complex are carried out on membrane suspensions in different solvents. A peculiar feature of bacteriorhodopsin is the ability for multiple cyclic transformations induced by light. Oesterhelt and Hess [3] and Stoeckenius and Lozier [4] have shown that during a photochemical cycle protons are transferred through the cell membrane, and as a result energy is stored in the form of a proton gradient [5] and then, according to Mitchell's theory, is used for the ATP synthesis. The participation of the bacteriorhodopsin in photophosphorylation was demonstrated in a reconstituted system by Racker and Stoeckenius [6].

At present, the proton transfer through the purple membrane is considered to be caused by the appearance and the decay of the intermediate products of the photochemical cycle. However, it is not yet determined what intermediates are related to the proton transfer through the membrane. At first it was considered that only a short-lived product with the absorption band at 412 nm took part in this transfer [3]. Later a short-lived product discovered in the long-wave spectrum region (640 nm) [7] was supposed to possess the same function. In one of the recent papers by Lozier et al. [8], an attempt was made to characterize all the intermediates of the photochemical cycle which are known at present. The main difficulty is that the form and the position of the absorption bands, the kinetics of appearance and decay, and other characteristics of the cycle intermediates are greatly affected by the experimental conditions (pH, medium, temperature, preliminary illumination, etc.). In this paper we have studied the effects of the preliminary illumination and pH of the medium on the spectral and kinetic characteristics of the intermediates H412 and $H650^{x}$.[1]

The latter is suggested to be identical to the intermediate at 640 nm as described by Dencher and Wilms [7]. As will be shown below, the maximum of absorption band of this intermediate can be localized in the spectral range 620–680 nm.

Materials and Methods

The purple membranes were isolated from *Halobacterium halobium* by the method of Oesterhelt and Stoeckenius [1]. The aqueous suspension of

[1]All intermediates are designated by H. The figure denotes the maximum of the absorption band. The symbol "x" indicates that the maximum belongs to the difference spectrum.

purple membranes was not buffered. pH was changed by adding HCl and NaOH and tested before and after the experiments. The temperature of the samples in all experiments (except the temperature-dependent experiments) was 19–22°C. Absorption spectra and slow changes (half-time $>10^{-1}$ sec) of difference spectra of the optical density or transmittance were registered by spectrophotometers SF-10 (USSR) and Specord UV VIS (GDR). For measurements of the fast spectral changes (half-time $<10^{-1}$ sec) a flash photolysis apparatus was used [9]. The duration of a flash was 6 μsec, power -10–20 MW.

Slow spectral changes were induced by continuous illumination of the sample with a 1-kW incandescent lamp. The optical system for illumination was the same both for flash and continuous illumination. In the latter case an additional 25-cm water filter was used. The intensity of incident light was 20 mW/cm^{-2}. The spectral region of excitation was cut off by a combination of glass filters BS-10, GS-18, SS-11 which had a summary transmittance coefficient of about 90% in the range of 540 nm. Samples of the purple membrane suspensions kept in darkness for no less than 48 hours will be called "dark samples," those illuminated by yellow light for 2 min will be called "light samples." Slow spectral changes in the light samples attained 95% saturation. After 20 min illumination slow spectral changes were not observed. In the other cases the conditions of illumination will be indicated specially.

To measure the difference absorption spectra of dark samples the following procedure was used. The purple membrane suspension in aqueous solution (25–30 cm^3) was kept in darkness for 48 hours. A 2-cm^3 cell was filled with the suspension under scattered weak red light. The cell was then illuminated by two or three yellow flashes and two or three points of the difference spectrum were registered. After that the sample was considered to be bleached and was replaced by a new dark sample. Measurements were carried out in succession within the interval from 370 to 750 nm in both directions. The results of the measurements were averaged. The time of filling the cell and placing it into the chamber was the same for all measurements of the same series. The procedure for the light samples was the same with the only difference being that before the sample was placed into the chamber it was illuminated for 2 min by yellow light.

Results

The maximum of the main absorption band of the purple membrane suspension of a dark sample is located at 560 nm, and that of the light

sample at 570 nm. Under illumination the maximum reversibly shifts from 560 to 570 nm, which points to isomerization of 13-cis retinal to all-trans [1, 10].

Figure 1 represents a difference spectrum which shows the formation of H412 after illumination of both dark and light samples by a yellow flash. The maximum of the band in the negative area of the difference spectrum is at 580 ± 2 nm, and not at 570 nm as was previously reported [9, 11]. The preliminary illumination affected the position of the maximum at neither 580 nm nor 412 nm. Changes of the optical density at 580 and 412 nm are twice as high for the light samples than for the dark ones (Fig. 1, curves L and D).

Figure 2 represents oscillograms of the absorption changes of a light sample in a long-wave region after a yellow flash. The oscillograms show that in conformity with Fig. 1 (curve L) the negative absorption caused by band 580^x decreases gradually with the increase of the wavelength, while the positive absorption which we ascribe to the intermediate $H650^x$ increases and reaches the maximum at 680 nm (Figs. 2a, b, c). For the dark sample (Fig. 1, curve D) the spectral changes are similar but the maximum of the absorption band of the $H650^x$ is located at 620 nm. This indicates that under illumination the maximum shifts from 620 nm to the red region.

The masking effect of band 580^x does not allow us to determine with high precision the form and the amplitude of the absorption band of $H650^x$ in the difference spectrum. However, the effect of light on the position of the band is obvious. Moreover, the value of the optical density change of $H650^x$ decreases proportionately to the duration of illumination. After 20 min of

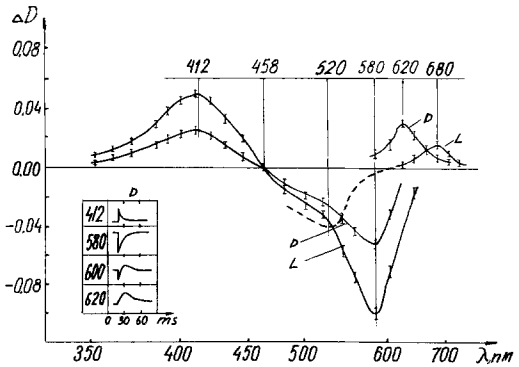


Figure 1. Difference spectra of absorption changes in the aqueous suspension of purple membranes after the flash of yellow light, pH 6.7. L and D represent light sample and dark sample, respectively. For the dark sample, the kinetic curves are given for four wavelengths (nm).

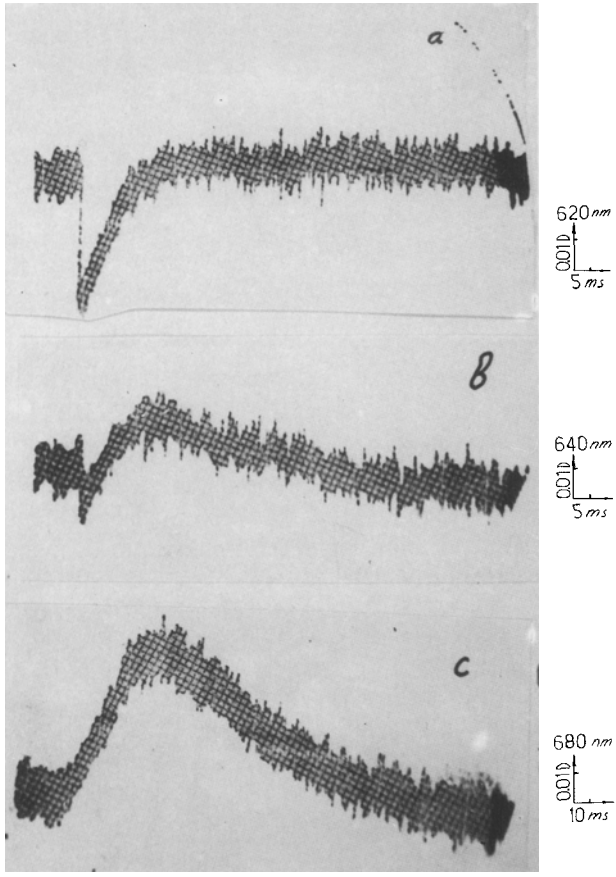


Figure 2. Oscillograms of the optical density changes in the aqueous suspension of purple membranes (light samples) at pH 6.7 after a yellow flash. Curves are given for three wavelengths.

continuous preliminary illumination of the sample, $H650^{x}$ was not detected in the spectral region up to 750 nm while the amplitude of the H412 absorption band was maximal.

A "light minus dark" difference spectrum, shown in Fig. 3, reflects the light-induced reversible transition of the absorption band from 560 nm to 570 nm, i.e., the transition of 13-cis retinal to all-trans retinal. In the dark sample the contents of 13-cis and trans retinal isomers were approximately equal [1, 10, 17].

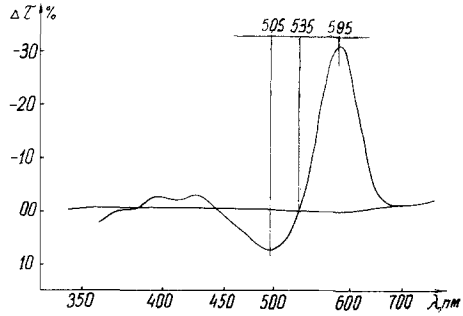


Figure 3. "Light minus dark" difference spectra of the samples at pH 7.0 after 20 min of illumination of one of them. An increase of the absorption at 595 nm occurs in the light sample.

The absence of an isosbestic point between $H650^x$ and band 580^x indicates that the formation of $H650^x$ proceeds due to the decay of an unknown intermediate. It is assumed that this intermediate is identical to the intermediate with the absorption band 520 nm described in the paper by Lozier [8]. This assumption was indirectly confirmed since band 620 nm appeared when negative band 580^x of the difference spectrum of a light sample was subtracted (graphically) from the main absorption band 570 nm. The result is shown in Fig. 1 by a broken line.

All the measurements, the results of which are given above, were carried out at pH 7.0. In the dark sample the change of the optical density at 620 nm is insignificant at this pH value. Acidification leads to a sharp increase of the absorption changes of $H650^x$ but at pH 3.5 the absorption changes rapidly decrease due to the transition of H570 into the long-wave form [1] (Fig. 4).

Figure 5 represents the different transmittance spectra of acidified and neutral samples. It is seen that a spectral form with the band in the long-wave region of the spectrum appears in acidified samples.

Discussion

Each cluster of the purple membrane consisting of three molecules of bacteriorhodopsin surrounded by lipids should probably be considered as a whole chromoprotein complex, the characteristics of which can differ considerably from those of a separate bacteriorhodopsin molecule. At different intensities of continuous illumination a cluster can exist in one of the four steady forms: (1) all the three retinals of a cluster are in a 13-cis form; (2) two retinals are in a 13-cis and one is in a trans form; (3) two retinals are in a

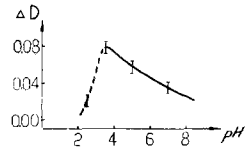


Figure 4. Dependence on pH of the H650^{x} absorption changes at 620 nm in the dark sample.

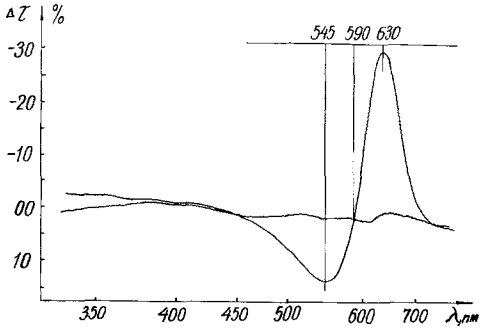


Figure 5. "Acidified minus neutral" difference spectra of absorption changes in the aqueous suspension of purple membranes for the dark samples (pH 2.5 and 7.0). An increase of absorption at 630 nm occurs in the sample at pH 2.5.

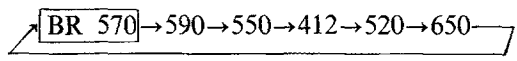
trans form and one is in a 13-cis form; (4) all the three retinals are in a trans form.

The first form corresponds to an ideal dark sample, and the fourth to a sample under continuous saturating light; the second and the third are intermediate forms.

There are no direct experiments proving the existence of these forms. Indirect evidence of the existence of the first and the fourth forms is provided by experiments with thin-layer chromatography [10, 12]. Close proximity of the molecules in the cluster permits their interaction. An excited molecule can transfer part of its energy to two (or one) neighbors. As a result the cluster state will not be the same as if two or three molecules were excited simultaneously. The variety of spectral forms of the intermediates could probably be explained by the features of the cluster composition.

According to our results the content of H412 in a sample is maximal for a cluster in the fourth form and is equal to zero for the first form. On the contrary, the content of H650^{x} must be maximal for the first and zero for the fourth form. When all the retinal was in the trans form after 20 min of continuous preliminary illumination (the fourth form of the cluster) only the H412, but not the H650^{x} absorption was detected and it had the maximal

value. The $H650^x$ absorption was maximal in dark samples and decreased during illumination. This means that H412 cannot be a precursor of $H650^x$. According to the scheme by Lozier [8] the formation of intermediates in the photochemical cycle proceeds in the following way:



Our data suggest that this scheme should be modified since the formation of $H650^x$ and H412 seems to occur in different photochemical cycles.

We have observed only the three last intermediates of this scheme (H412, $H520^x$, $H650^x$) under normal conditions (Fig. 1). These intermediates were called "fast" since their time of transition was less than 10^{-1} sec. At continuous illumination slow (greater than 10^{-1} sec) spectral forms appear (Fig. 3) i.e., H410–430 and $H595^x$ [11]. After a flash (10 kW/cm^2) changes of the optical density in the region of 412 nm were approximately 0.04 D, and in the region of 410–430 nm approximately 0.0004 D. This means that the duration of the flash was insufficient for the slow forms to arise in considerable amount.

We suggest that after a yellow flash the photochemical transitions of bacteriorhodopsin proceed simultaneously in three different ways, as shown in Fig. 6, where (1) and (2) are the fast photochemical cycles, corresponding to the modified scheme of Lozier [8]. Cycle (3) corresponds to the slow photochemical cycle [11] caused by photoisomerization of 13-cis retinal to all-trans retinal.

The purple membrane is one of the most rigid membranes. However, in this membrane fast photochemical processes proceed even at low temperature [14]. Spectral changes can be caused by changing temperature or pH and are often similar by the form and position of the difference spectra to the changes caused by light. The reason for such a high sensitivity to external effects, despite the rigidity of the membrane as a crystal lattice, seems to be a high mobility of chromophores in the cluster.

Acknowledgements

The authors are grateful to Dr. V. P. Skulachev, Dr. A. M. Shkrob, and Dr. A. P. Kostikov for their interest in this work and for valuable discussions.

This study was carried out within the framework of the scientific project "Rhodopsin" organized by the USSR Academy of Sciences and Moscow State University and supervised by Academician Y.A. Ovchinnikov.

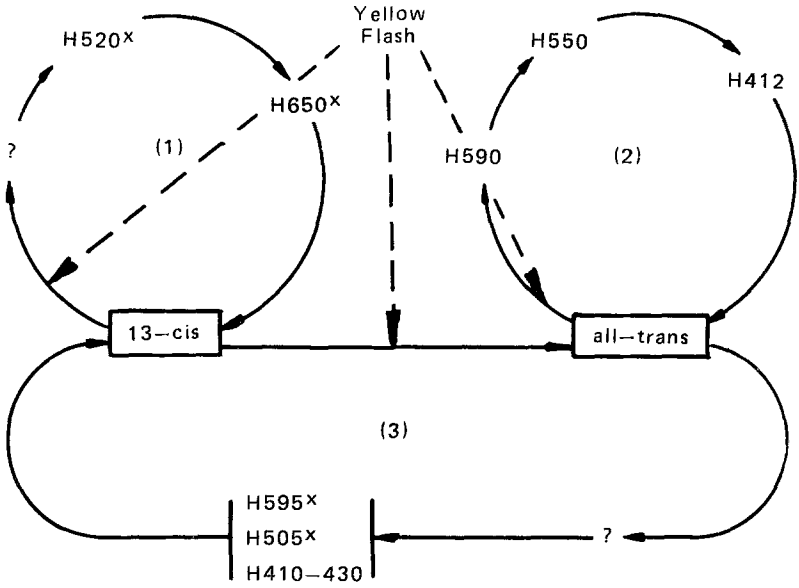


Figure 6. Scheme of photochemical transitions of bacteriorhodopsin (BR) after a yellow flash. The time of the turnover of the cyles: (1) ~100 msec, (2) ~10 msec, (3) ~10 min.

Note

This paper was already prepared for publication when we received the preprint by Dencher et al. [17], which was kindly sent to us by Dr. Dencher. We would like to note that some conclusions of our paper are in agreement with the results obtained by Dencher et al. and, in particular, cycle (2) (Fig. 6). Dencher et al. succeeded to reconstitute the purple membrane containing only 13-cis retinal, which corresponds to cluster 1 (the ideal dark sample) in our paper. H412 was completely absent from this membrane and was formed only when the trans retinal appeared. Experimentally, this corroborates our assumption that the formation of H412 occurs only in cycle (2). As for ^{610}C, we suppose that this is the same intermediate which we have designated as H650^x.

References

1. D. Oesterhelt and W. Stoeckenius, *Nature New Biol.*, **233** (1971) 149–152.
2. R. Henderson and P. N. T. Unwin, *Nature*, **257** (1975) 28–32.
3. D. Oesterhelt and B. Hess, *Eur. J. Biochem.*, **37** (1973) 316–326.

4. W. Stoeckenius and R. Lozier, *J. Supramol. Struct.*, **2** (1974) 769–774.
5. L. Drachev, V. Frolov, A. Kaulen, E. Liberman, S. Ostroumov, V. Plakunova, A. Semenov and V. Skulachev, *J. Biol. Chem.*, **251** (1976) 7059–7065.
6. E. Racker and W. Stoeckenius, *J. Biol. Chem.*, **249** (1974) 662–663.
7. N. Dencher and M. Wilms, *Biophys. Struct. Mechanism*, **1** (1975) 259–271.
8. R. Lozier, R. Bogomolni and W. Stoeckenius, *Biophys. J.*, **15** (1975) 953–962.
9. N. N. Vsevolodov, G. T. Rikhireva and A. P. Kostikov, *Biofizika*, **19** (1974) 942–946.
10. D. Oesterhelt, M. Meentzen and L. Schuhmann, *Eur. J. Biochem.*, **40** (1973) 453–463.
11. N. N. Vsevolodov and L. P. Kayushin, *Studia Biophys.*, **59** (1976) 81–87.
12. L. Y. Jan, *Vision Res.*, **15** (1975) 1081–1086.
13. K. J. Kaufmann, P. M. Rentzepest, W. Stoeckenius and A. Lewis, *Biochem. Biophys. Res. Comm.*, **68** (1976) 1109–1115.
14. F. F. Litvin, S. P. Balashov and V. A. Sineschekov, *J. Bioorganicheskaja chimija*, **1** (1975) 1767–1776.
15. W. V. Sherman, M. A. Slifkin and S. R. Caplan, *Biochem. Biophys. Acta*, **423** (1976) 238–248.
16. M. C. Kung, D. Devault, B. Hess and D. Oesterhelt, *Biophys. J.*, **16** (1975) 907–911.
17. N. A. Dencher, C. N. Rafferty and W. Sperling, Preprint, *Institut für Neurobiologie*, Jülich, West Germany, December 1976.