THE LOW pH SPECIES OF BACTERIORHODOPSIN

Structure and proton pump activity

Kinko TSUJI and Kurt ROSENHECK
Department of Membrane Research, The Weizmann Institute of Science, Rehovot, Israel

Received 8 December 1978

1. Introduction

The purple membrane from *Halobacterium halobium* shows a reversible colour change from purple to blue around pH 3 [1,2]. The blue species has a broad absorption band with a maximum at ~600 nm. This species may be considered to be a protonated species of bacteriorhodopsin (bR) stabilized in the acidic environment [3].

The flash photolysis of the blue species, incorporated into the matrix of a polyacrylamide gel has been studied [3] and it was suggested that the blue species may be one of the protonated intermediates which appear in the normal photoreaction. This species was assigned [4] to phototransient *O*, also on the basis of flash photolytic studies.

If, indeed, the acid-stabilized species is one that appears transiently during the normal photocycle, it is to be expected that the H^+ pump [5] would respond to the altered conditions. Furthermore, it is of interest to determine some of the structural characteristics of such a stabilized phototransient, which would normally be difficult to measure during its short lifetime. The data presented herein indicate that in the purple–blue transition:

(i) bR undergoes only slight structural rearrangements;

(ii) The structure becomes less stable and can be irreversibly bleached;

(iii) The efficiency of the H^+ pump is strongly decreased.

2. Materials and methods

H. halobium M1 strain was grown, as in [6], using a modified growth medium [7]. Purple membranes were isolated from the cells of the *Halobacterium halobium*, according to [8]. bR concentration in the purple membrane suspension was determined on the basis of $\epsilon = 63\,000\ M^{-1}\ cm^{-1}$ at 570 nm [9].

Proteoliposomes were prepared, according to [10], from purple membrane and soybean lipid; the mixture of 3 ml purple membrane suspension (~40 nmol/bR) in 1 M NaCl and about 1 mg soybean lipid was sonicated for 1 h in a bath-type Lab. Supplies sonicator.

Absorption spectra, in the visible region, were measured with a Cary 15 spectrophotometer, and circular dichroism (CD) with a Cary 60 spectropolarimeter and 6002 CD attachment, using quartz cells of 0.5 mM lightpath for the ultraviolet region and 1.0 cm lightpath for the visible region.

2.1. *Bleaching procedure*

Purple membrane suspensions ($\sim 1.4 \times 10^{-5}$ M bR), at various pH-values in a cubic cell of 1.0 cm lightpath, were steadily illuminated with a slide projector (R. L. Lamp, 210 W, GAF, Belgium). The distance between the light source and the cell was 15 cm. Suspensions were stirred magnetically during illumination.

2.2. *Measurement of the proton pump activity*

Light-induced pH changes in reconstituted proteoliposomes were measured according to [11]. The initial pH of the proteoliposome suspension was

 Elsevier/North-Holland Biomedical Press

adjusted by adding a small quantity of 0.1 M HCl or 0.1 M NaOH. Then, at least 10 min after addition of HCl or NaOH, the suspension was illuminated by a slide projector with halogen lamp, Widiscope (AB Wiktors Mekaniska, Sweden) and pH changes were measured at 25°C with a Radiometer GK 2321 C combined glass electrode connected to a Radiometer 64 pH-meter. The output was recorded on a high-speed Varian A-25 recorder. The recorder scale was calibrated by addition of 5 μl 10 mM HCl to the suspension.

3. Results

The absorption spectra of purple membrane suspensions at varying pH-values were similar to those reported in [4]. Upon lowering the pH from neutral to 3.0, the absorption maximum shifts from 560 nm for the dark-adapted state or 570 nm for the light-adapted state [12] to ~600 nm. Absorption at 630 nm and 540 nm are plotted versus pH in fig.1. The from-purple–blue transition occurs smoothly and reversibly between pH 4.0 and pH 3.3 at room temperature. Below pH 3.0, purple membranes form large aggregates, the suspension being more turbid than at neutral pH. It should be mentioned that the difference in the absorption maxima of the dark-adapted and the light-adapted forms can be observed at pH as low as 3.7; however, at pH 3.3 the spectra of the two forms are identical.

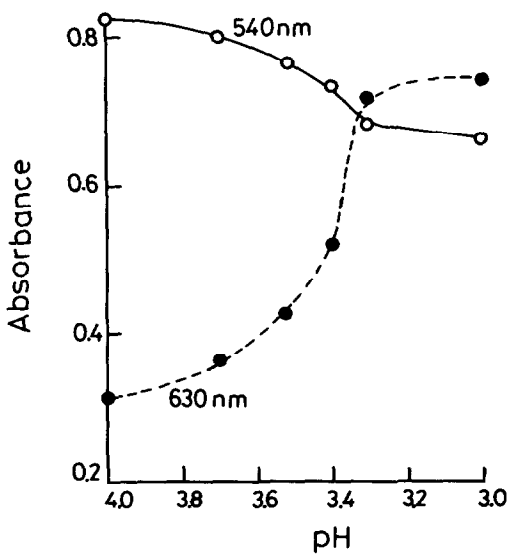
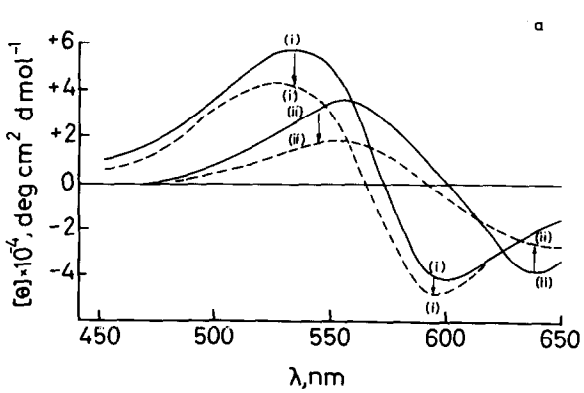


Fig.1. pH dependence of A_{540} (○) and A_{630} (●) of purple membrane suspensions. pH was adjusted with 0.1 N HCl.

The CD spectrum of bacteriorhodopsin in the visible region (fig.2a) is composed of a negative and a positive band of unequal strengths at pH 6.0, as well as at pH 3.0. Both peaks are shifted to longer wavelengths in the latter case, reflecting the red shift in the absorption spectrum mentioned above. The asymmetry in the spectrum has been attributed to scattering distortions [13]. In order to reduce the scattering effect, the spectra of the sonicated samples were measured. As can be seen, after 10 min sonication, the spectrum at pH 6.0 is almost symmetric due to an increase in $[\theta]$ of the negative band and a decrease in the positive band, and the crossover shifts to a shorter wavelength, as in [13]. At pH 3.0, both the negative and the positive bands become smaller after sonication. This change is not reversed by raising the pH, indicating that the chromophore is to some extent broken down by sonication. On the other hand, in the ultraviolet region (fig.2b) sonication raises the values of $[\theta]$ both at pH 6.0 and at pH 3.0. This is a consequence of the reduction of scattering artefacts, which usually decrease the ellipticities and distort the CD spectra [14]. The corrected spectra yield quantitative estimates of protein secondary structure [14]. The $[\theta]$ at 222 nm, which can be taken as a rough index of the amount of α-helical structure [15], is about 10% less at pH 3.0 than at pH 6.0.

When illuminating purple membrane suspensions at low pH, the pigment is bleached irreversibly. The kinetics of the bleaching process is shown in fig.3.



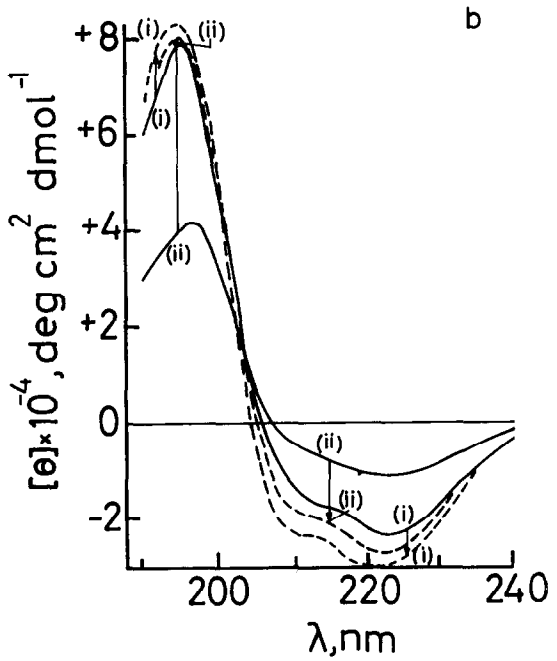


Fig.2. CD spectra of purple membrane in the visible (a) and ultraviolet (b) regions. (——) before sonication; (- - - -) after 10 min sonication. (i) pH 6.0; (ii) pH 3.0.

At pH 3.7, bleaching takes place slowly and is accompanied by an increase from pH 3.7–4.2. At pH 3.2, the suspension is bleached more quickly and no significant change in pH is observed. Long-term illumination at pH 6.0 induces neither irreversible bleaching nor pH changes.

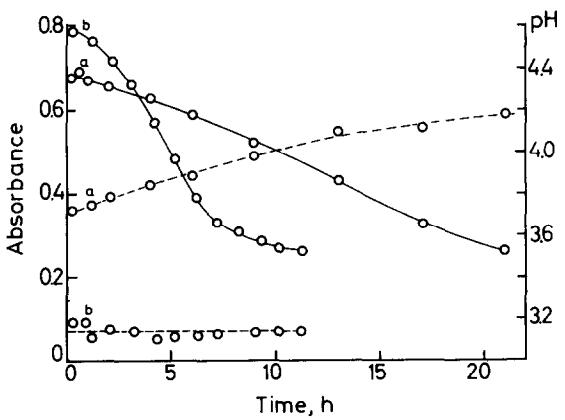


Fig.3. Kinetics of bleaching (——) and concomitant pH changes (- - -). Initial pH values were 3.7 (a) and 3.2 (b).

Since bleaching is a relatively slow process (fig.3), the light-induced $\Delta[H^+]$ in reconstituted proteoliposomes incorporating bacteriorhodopsin can still be measured at low initial pH values. These measurements are carried out during much shorter time intervals [11]. H^+ uptake by the proteoliposomes occurs during the 'light-on' process in the pH region from 5.5–2.8, and in the dark, i.e., the 'light-off' process, the pH returns to the original value. Figure 4 shows the dependence of $\Delta[H^+]$ in the 'light-on' process on the initial pH. $\Delta[H^+]$ decreases almost linearly with decreasing pH of the suspension, to ~33% of that at high pH.

The kinetics of both the 'light-on' and the 'light-off' phases, at low pH, could be resolved into two first-order processes, similar to those at neutral pH [11,16]. However, the rate constants are much higher. Thus, the rate constant for the fast process, k_1 (on), and that for the slow process, k_2 (on), at pH 3.5, are found to be 0.40 s^{-1} and 0.12 s^{-1}, respectively, compared to 0.13 s^{-1} and 0.026 s^{-1} at neutral pH [17]. Similarly, k_1 (off) and k_2 (off) are 0.18 s^{-1} and 0.05 s^{-1}, respectively, compared to 0.09 s^{-1} and 0.01 s^{-1} at neutral pH [17]. These values did not show any further change upon lowering the pH to 3.0.

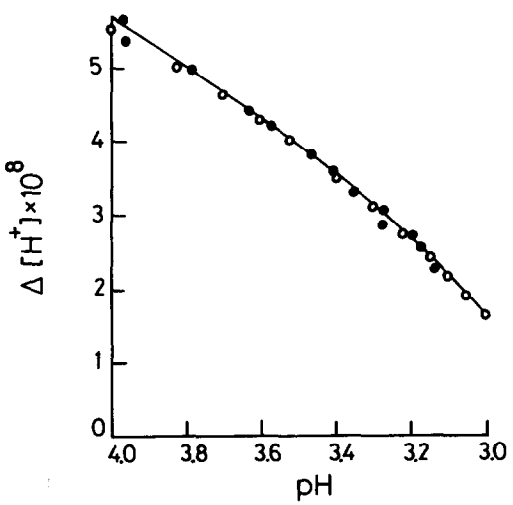


Fig.4. Amplitude of light-induced $\Delta[H^+]$ of reconstituted proteoliposome suspensions at various initial pH values. Initital pH was changed from 5.5–2.8 by adding 0.1 M HCl (○) and then from 2.8–5.5 by adding 0.1 M NaOH (●).

4. Discussion

The spectral transition from the purple to the blue form (fig.1) reflects in all probability the protonation of one or several groups of the bR chromophore. The nearly symmetrical CD spectrum in the visible region has been interpreted as being due to exciton interaction [18] between the chromophores in the hexagonal array of protein trimers [19]. The low pH blue species shows these same characteristic bands shifted to longer wavelengths (fig.2a), suggesting that exciton interaction due to the trimer still exists at pH 3.0. The CD spectra in the ultraviolet region (fig.2b) indicate that the α-helical conformation of bR is not very significantly altered at this low pH; the decrease of the α-helix content is ≤10%. Therefore, the structure of the blue bR molecule, and its arrangement in the membrane, seems to be essentially similar to that of the purple pigment at neutral pH. The spectral shift is thus likely to stem from slight local rearrangements and/or the above-mentioned protonation of groups in the vicinity of the retinal chromophore.

Whatever the rearrangements, that do occur, they significantly reduce the stability of the bR structure. Thus, blue bR is irreversibly bleached under conditions, under which the purple pigment is not affected. As described above, it is bleached photochemically, i.e., by long-time illumination (fig.3), and mechanically, i.e., by sonication (fig.2b). Recent observations (K.T., K.R., unpublished results) indicate that it is bleached also by applying an electrical field impulse to the membrane suspension, such as that used in the measurement of the electric dichroism [20]. Mechanical shearing stresses and electric field gradients may be expected to induce displacements of chain segments and/or chromophore relative to one another, which may be sufficient to cause disruption of the blue complex. Long-time illumination may have a similar, and cumulative, effect, if the mechanism of the proton pump comprises the protein conformational transition proposed [17], by virtue of which one or more chain segments may undergo translocation. The uptake of protons, at pH 3.7 (fig.3), may in that case be due to the unmasking of some further protonable sites during this process. At pH 3.2, however, all such sites may already be saturated and, therefore, no irreversible pH change occurs. There may of course be alternative explanations for the photochemical bleaching not involving a conformational transition; however, the similarity in the effects of the 3 widely-differing treatments remains striking.

Since protonation and deprotonation equilibria play an important role in the light-driven H^+ pump of the purple membrane [21], it was to be expected that the pump activity would be sensitive to the high acidity of the medium. This was indeed found to be the case, and is expressed in changed rate constants for both 'light-on' and 'light-off' processes, but primarily in the vastly-decreased pump efficiency (fig.4). These results may be ascribed to interference with either the transport chain or the efficiency of the photocycle. It is reasonable to assume that stabilization of a protonated intermediate could interfere with any subsequent stages of either of these two, during which deprotonated species would normally appear. The absence of an intermediate corresponding to unprotonated M_{412}, under similar high acidity conditions, was noted [3].

The protonated species was assigned [4] to the phototransient *O*. However, one should be aware of the fact that the absorption spectrum of this species ([4], and our results) is almost identical with that of phototransient *K* [21], both as regards the wavelength of the maximum and the bandwidth. Stabilization of the *K* intermediate could account for the absence of an M_{412} species at low pH, noted [3].

Acknowledgement

This work wa supported by a grant from the Stiftung Volkswagenwerk.

References

[1] Oesterhelt, D. and Stoeckenius, W. (1971) Nature 233, 149–152.
[2] Neugebauer, D. (1975) Diplomarbeit, Wurzburg.
[3] Lozier, R. H., Chae, Q., Mowery, P. C. and Stoeckenius, W. (1978) in: Energetics and Structure of Halophilic Microorganisms (Caplan, S. R. and Ginzburg, M. eds) pp. 297–301, Elsevier/North-Holland Biomedical Press, Amsterdam, New York.

[4] Moore, T. A., Edgerton, M. E., Parr, G., Greenwood, C. and Perham, R. (1978) Biochem. J. 171, 469–476.
[5] Oesterhelt, D. and Stoeckenius, W. (1973) Proc. Natl. Acad. Sci. USA 70, 2853–2857.
[6] Danon, A. and Stoeckenius, W. (1974) Proc. Natl. Acad. Sci. USA 71, 1234–1238.
[7] Eisenbach, M., Cooper, S., Garty, H., Johnstone, R. M., Rottenberg, H. and Caplan, S. R. (1977) Biochim. Biophys. Acta 465, 599–613.
[8] Oesterhelt, D. and Stoeckenius, W. (1974) Methods Enzymol. 31A, 667–668.
[9] Oesterhelt, D. and Hess, B. (1973) Eur. J. Biochem. 37, 316–326.
[10] Racker, E. (1973) Biochem. Biophys. Res. Commun. 55, 224–230.
[11] Eisenbach, M., Bakker, E. P., Korenstein, R. and Caplan, S. R. (1976) FEBS Lett. 71, 228–232.
[12] Stoeckenius, W. and Lozier, R. H. (1974) J. Supramol. Struct. 2, 769–774.
[13] Brith-Lindner, M. and Rosenheck, K. (1977) FEBS Lett. 76, 41–44.
[14] Schneider, A. S., Schneider, M.-J.T. and Rosenheck, K. (1970) Proc. Natl. Acad. Sci. USA 66, 793–798.
[15] Greenfield, B. and Fasman, G. D. (1969) Biochemistry 8, 4108–4116.
[16] Hwang, S.-B. and Stoeckenius, W. (1977) J. Membr. Biol. 33, 325–350.
[17] Eisenbach, M., Garty, H., Bakker, E. P., Klemperer, G., Rottenberg, H. and Caplan, S. R. (1979) Biochemistry, in press.
[18] Heyn, M. P., Bauer, P. J. and Dencher, N. A. (1975) Biochem. Biophys. Res. Commun. 67, 897–903.
[19] Henderson, R. (1975) J. Mol. Biol. 93, 123–138.
[20] Tsuji, K. and Rosenheck, K. (1979) Electro-optic and Dielectric Studies on Macromolecules and Colloids, Conf. Proc., in press.
[21] Lozier, R. H., Bogomolni, R. A. and Stoeckenius, W. (1975) Biophys. J. 15, 955–962.