BACTERIORHODOPSIN: A LIGHT-DRIVEN PROTON PUMP IN *HALOBACTERIUM HALOBIUM*

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When grown under low oxygen tension in the light, *Halobacterium halobium* produces distinct patches in its plasma membrane which can be isolated by differential and sucrose density gradient centrifugation after lysis of the cells by dialysis against distilled water (Oesterhelt and Stoeckenius, 1974). These membrane fragments, which contain 75% protein and 25% lipid by weight, have been named purple membrane because of their characteristic color. This color is due to the protein bacteriorhodopsin, the only protein found in these membranes; it was so named by analogy with the visual pigment rhodopsin, because it contains retinal bound by a Schiff base linkage to an amino group of a lysine residue and shows a broad absorption maximum at 570 nm (Oesterhelt and Stoeckenius, 1971).

Isolated purple membrane, when incorporated into lipid vesicles, has been shown to act as a light-driven proton pump, and when mitochondrial ATPase is also incorporated, photophosphorylation can be demonstrated (Racker and Stoeckenius, 1974). In intact cells, photophosphorylation (Danon and Stoeckenius, 1974), light-induced pH changes (Oesterhelt and Stoeckenius, 1973) and light-induced inhibition of respiration (Oesterhelt and Stoeckenius, 1973) have been observed and all of these effects have action spectra closely corresponding to the absorption spectrum of bacteriorhodopsin (R. A. Bogomolni, R. A. Baker, R. H. Lozier, and W. Stoeckenius, in preparation).

These facts suggest that the purple membrane functions in vivo to supplant oxidative phosphorylation as an energy source. They also strongly support a chemiosmotic mechanism for energy transduction.

The proton pumping of the purple membrane suggests that bacteriorhodopsin undergoes a light-induced cyclic reaction involving a proton release on one side of the membrane and proton uptake on the opposite side. A light-induced transient shift of the absorption maximum to 412 nm and a concomitant release and uptake of protons have been observed in the purple membrane (Oesterhelt and Stoeckenius, 1973; Oesterhelt and Hess, 1973). A simple two-component system cannot explain the release and

uptake of a proton on opposite sides of the membrane and other intermediates must occur in a light-induced reaction cycle to effect a net translocation of protons across the membrane. We have investigated the light-induced reaction cycle of bacteriorhodopsin in isolated purple membrane and in *H. halobium* cells by low-temperature and flash spectroscopy[1]; several spectroscopically distinct intermediates have been identified (Stoeckenius and Lozier, 1974).

Bacteriorhodopsin exists in a stable dark-adapted (*DA*) form with an absorbance maximum at 558–560 nm (bR^{DA}_{560}) and a metastable light-adapted (*LA*) form with an absorbance maximum at 568–570 nm (bR^{LA}_{570}) (Stoeckenius and Lozier, 1974). Retinal extracted from bR^{DA}_{560} and bR^{LA}_{570} are reported to be the 13-cis and all trans isomers, respectively (Jan, 1974). The dark reaction $bR^{LA}_{570} \xrightarrow{k_{DA}} bR^{DA}_{560}$ is much too slow to account for the proton pumping rate observed in whole cells. Because bR^{LA}_{570} is the predominant form under physiological conditions, we have studied it in greatest detail. The spectrum of bR^{LA}_{570} at −196°C (footnote 2) and the effect of actinic irradiation at −196°C on bR^{LA}_{570} are shown in Fig. 1. Actinic 500 nm irradiation of bR^{LA}_{570} at −196°C causes a red shift of the absorption spectrum. Subsequent actinic irradiation with 650 nm light regenerates the initial spectrum. Actinic irradiation at wavelengths between 500 nm and 650 nm result in photostationary states intermediate between curves 1 and 2 in Fig. 1, with the same isosbestic point at 590 nm (data not shown). Apparently the photoproduct of bR^{LA}_{570} is stable at −196°C and can be reconverted to bR^{LA}_{570} by light. The ratio of bR^{LA}_{570} to its photoproduct is about 1 after 500 nm irradiation; the absorption spectrum of the photoproduct at −196°C has been calculated and is found to peak at 610 nm (Stoeckenius and Lozier, 1974). When a sample containing the red-shifted photoproduct is warmed slowly in the dark, the red-shifted photoproduct decays through two intermediates with relatively large blue shifts (Fig. 2) and ultimately returns to the initial form. Preliminary spectra of the first photoproduct and of the two subsequent thermal intermediates have been reported (Stoeckenius and Lozier, 1974). Their absorbance maxima occur at 610, 550, and 415 nm, respectively, and they have been named intermediates *K*, *L*, and *M*.

Flash spectroscopy indicates that the same intermediates also occur at or near ambient temperature and allows us to obtain the relevant kinetic data. Rapid transmission changes of purple membrane suspensions at 1°C induced by a short laser flash are shown for four wavelengths on three time scales in Fig. 3. At the time of the laser flash, a time-unresolved (< 1 μs) red shift (increase in transmission at 500 and 580 nm; decrease in transmission at 660 nm) is observed which is followed by a blue shift (increase in transmission at 660 and 580 nm; decrease in transmission at 500 nm) with a half time of 4.5 μs. The second time scale shows a second blue shift (increase in transmission at 500, 580, and 660 nm; decrease in transmission at 420 nm) with a half-time

[1] The initial observation of this reaction cycle including intermediates K, L, and M was made in collaboration with Dr. R. A. Cone in his laboratory using both isolated purple membrane and intact bacteria.

[2] The absorption maximum of bR^{LA}_{570} at −196°C occurs at 575 nm. Similarly, the absorption maximum of the 412 nm complex is shifted to 415 nm at −196°C (Stoeckenius and Lozier, 1974).

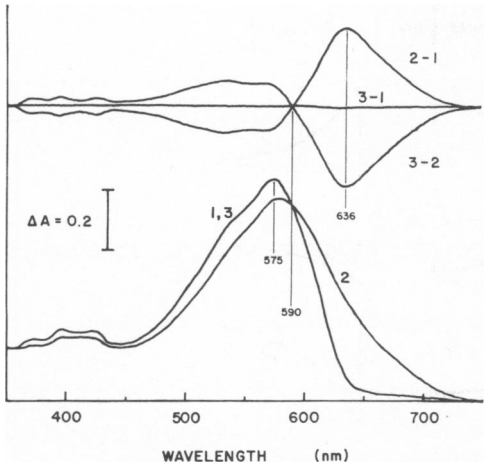


FIGURE 1

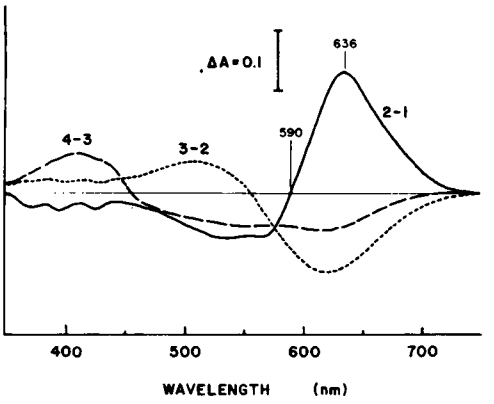


FIGURE 2

FIGURE 1 Absorption spectra of purple membrane at −196°C. Purple membrane containing ~10 nmol of bacteriorhodopsin suspended in 0.4 ml distilled water was light adapted with 500 nm light at room temperature, then frozen in the dark to −196°C in the vertical cuvette and Dewar system described by Butler (1972), and its absorption spectrum was recorded (curve 1). The sample at −196°C was then irradiated with saturating 500 nm light and its spectrum was recorded again (curve 2). Irradiating with 650 nm light at −196°C regenerated the initial spectrum (curve 3). Difference spectra between the 500 nm irradiated and unirradiated sample (curve 2-1), between the 650 nm irradiated and the 500 nm irradiated sample (curve 3-2) and between the 650 nm irradiated and unirradiated sample (curve 3-1) are also shown. Spectrum 2 represents a mixture of bR_{570}^{LA} and its photoproduct K_{610}^{LA}.

FIGURE 2 Difference spectra calculated from the transient absorption changes observed during warming of a purple membrane sample containing the photoproduct K_{610}^{LA} (see Fig. 1). Curve 2-1, low temperature light-induced difference spectrum (see curve 2-1 of Fig. 1); curve 3-2, difference spectrum between the spectrum measured at ca. −100°C and the spectrum measured after the low temperature irradiation; curve 4-3, difference spectrum between the spectrum measured at ca. −50°C and the −100°C spectrum.

of 300 μs. The last time scale shows a decay to the initial transmission value with an apparent half-time of about 100 ms. However, a small transient overshoot of the base line can be seen at 660 nm. This suggests that another intermediate may exist between the 412 and 570 complexes. Such an intermediate can be clearly demonstrated at higher temperature (Fig. 4). At 40°C a large transient overshoot of the baseline occurs at 660 nm. The difference spectra for the initial red shift and the two subsequent blue shifts (Fig. 5) are remarkably similar to the light-induced red shift and subsequent blue shifts observed in the low temperature experiment (Fig. 2).

The simplest scheme which accounts for the observed transmission changes is a linear cyclic reaction involving five spectrally distinct components:

$$bR \xrightarrow[h\nu]{k_0} K \xrightarrow{k_1} L \xrightarrow{k_2} M \xrightarrow{k_3} 0 \xrightarrow{k_4} bR$$

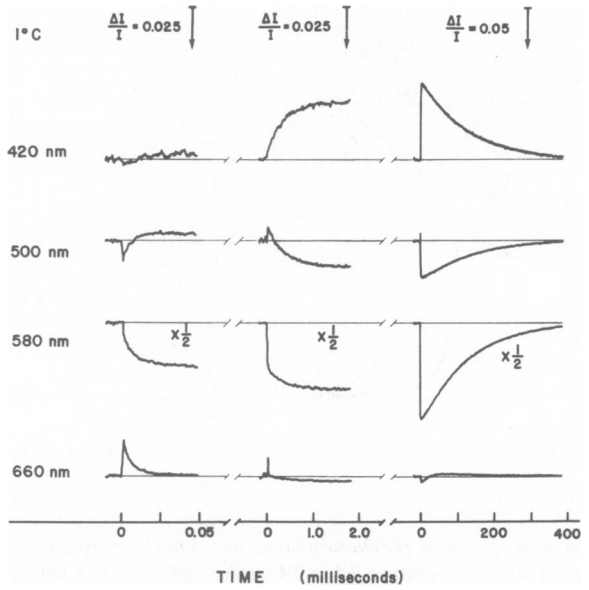
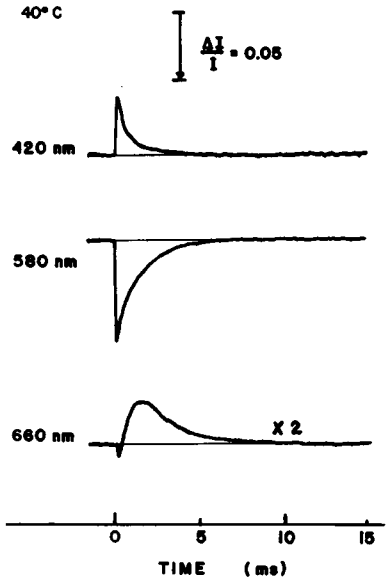


FIGURE 3 Transient transmission changes of purple membrane samples in distilled water at 1°C after exposure to a laser flash. Bacteriorhodopsin in concentration, 20 μM; measuring beam pathlength, 1.0 cm. First two time scales: average of transients obtained from 256 flashes at 562 nm; 0.5 mJ per flash 300 ns duration, 10 flashes per second. Last time scale: average of 64 flashes at 532 nm; 0.5 mJ per flash 100 ns duration, 2 flashes per second. The smaller absorption changes observed on the first two time scales may be due to the more rapid flash repetition rate which did not allow complete relaxation of the sample between flashes.

The spectra of the intermediates K through O have been calculated from the transmission changes on the basis of this scheme where k_1 through k_4 are first order rate constants. We find constants $k_0 \gg k_1 \gg k_2 \gg k_3$ (see Fig. 3) and can thus obtain k_1 and k_2 from semilog plots of the absorbance changes vs. time. Constants k_3 and k_4 are of comparable magnitude and have been estimated by assuming first order kinetics and adjusting approximate values to fit the transmission changes at long wavelengths at 40°C. The amount of bacteriorhodopsin cycling was estimated from the maximum transmission changes that occur at the time when most of the cycling pigment is in the M form and assuming the absorption of the M form is negligible at 590 nm. The spectra so obtained are shown in Fig. 6. Intermediates K, L, and M are clearly similar to the intermediates resolved by the low temperature experiments[3] (Stoeckenius and Lozier, 1974). Intermediate O was not observed at low temperatures. The maximum accumulation of O depends on the ratio of its formation and decay constants. This ratio systematically decreases from a value greater than 1 at 40°C to a value less than one at 1°C (Lozier, Bogomolni, and Stoeckenius, in preparation), explaining our failure to observe this intermediate in the low temperature experiments.

[3] As with bR and M, the calculated spectra of the K intermediate is red shifted at −196°C.



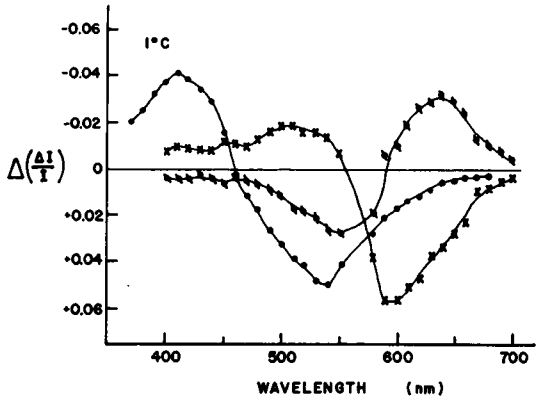


FIGURE 4

FIGURE 5

FIGURE 4 Flash-induced transient transmission changes of purple membrane (containing ~10 μM bacteriorhodopsin in 0.1 M potassium phosphate buffer, pH 7) at 40°C. Average of 256 flashes at 532 nm, 10 flashes per second.

FIGURE 5 Difference spectra of transient transmission changes calculated from data of Fig. 3 and additional experiments at other wavelengths. ✎, transmission immediately after flash minus transmission before flash; X, change after 40 μs minus initial change; •, change after 2 ms minus transmission at 40 μs.

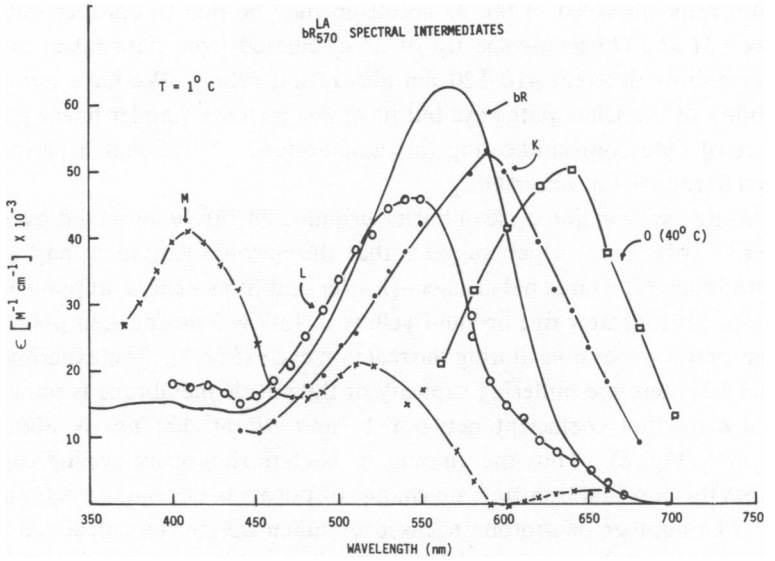


FIGURE 6 Spectra of intermediates K^{LA}_{590}, L^{LA}_{550}, M^{LA}_{412}, O^{LA}_{640}, and the spectrum of bacteriorhodopsin (bR^{LA}_{570}). Spectra of intermediates are calculated from data obtained at 1°C except for O, which is calculated from data taken at 40°C. The apparent peak at 520 nm in the spectrum of M^{LA}_{412} is possibly a contribution of N^{LA}_{520} (see text).

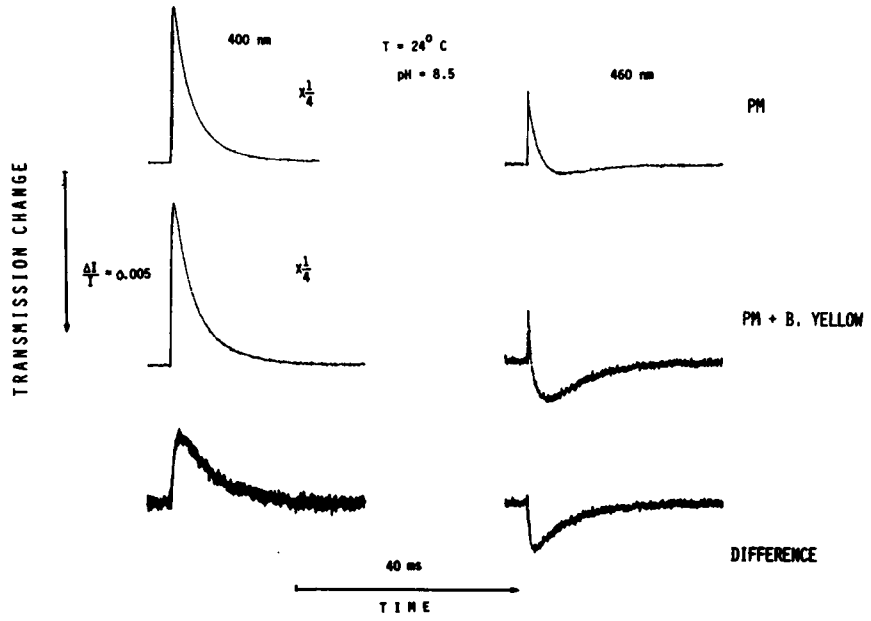


FIGURE 7 Transient transmission changes of brilliant yellow during the bacteriorhodopsin photoreaction cycle. The two upper rows show the transmission changes of purple membrane with and without indicator dye, the third row the difference between these, i.e., the transmission change of the dye.

The 520 nm peak observed in the *M* spectrum may be due to another intermediate (*N*) between *M* and *O* because spectra of *M* calculated from data taken at different temperatures show different 410–520 nm absorption ratios. We have not ruled out the possibility of branched pathways but have assumed the simpler linear pathway in the absence of data contraindicating this assumption. Additional experiments will be required to resolve this uncertainty.

The light-induced reaction cycle of bacteriorhodopsin can be observed over a broad pH range ($3 < pH < 12$) which suggests that the purple membrane may be able to pump protons against a large pH gradient (Lozier and Stoeckenius, unpublished). We have used the pH indicator dye brilliant yellow to follow light-induced pH changes in purple membrane suspensions during the reaction cycle (Fig. 7). The experiments were done at pH 8.5 where the buffering capacity of the purple membrane is minimal. The differential extinction coefficient between *M* and *bR* at 400 nm is about 25,000 $1\ mol^{-1} \cdot cm^{-1}$ (Fig. 6). Thus the amount of bacteriorhodopsin cycling can be calculated from the maximal transmission change of purple membrane at 400 nm (Fig. 7, top left). The number of protons released or taken up can be calculated from the transmission changes due to the indicator dye (lower kinetic traces) plus the absorbance change observed when a known amount of acid is added. It appears that one proton per molecule of pigment cycling is released into the aqueous phase with a time

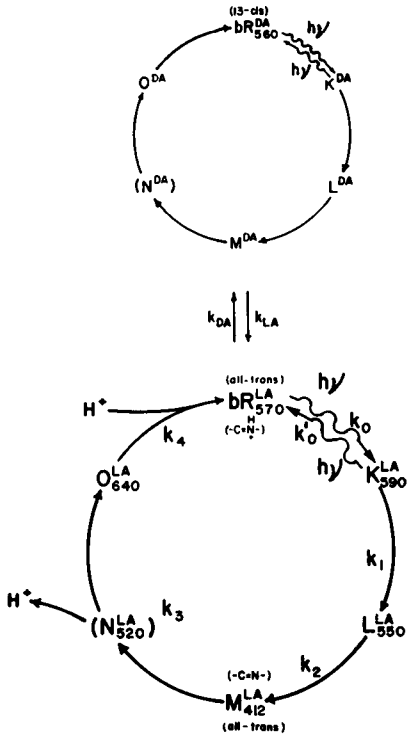


FIGURE 8 Scheme for photochemical and thermal reactions of bacteriorhodopsin. The point(s) of connection between the bR^{DA}_{560} and bR^{LA}_{570} cycles have not been established.

constant slightly longer than the appearance of M and one proton is subsequently taken up, probably in the $N \rightarrow bR$ step.

The photoreaction cycle of bacteriorhodopsin is summarized in the scheme shown in Fig. 8. The dark-adapted pigment, bR^{DA}_{560}, appears to undergo a light-induced reaction cycle similar to that of bR^{LA}_{570} (Lozier and Stoeckenius, unpublished), and a small fraction of the cycling pigment may be converted to the bR^{LA}_{570} pigment in every cycle. bR^{LA}_{570} returns slowly to bR^{DA}_{560} in the dark. Light absorbed by bR^{LA}_{570} converts it to K^{LA}_{590}. K^{LA}_{590} decays through thermal intermediates L^{LA}_{550}, M^{LA}_{412}, and O^{LA}_{640} and returns to bR^{LA}_{570}. Another intermediate, N^{LA}_{520}, may occur between M^{LA}_{412} and O^{LA}_{640}. A proton appears in the aqueous phase after the rise of M^{LA}_{412} and a proton is taken up in the last step of the cycle. Resonance Raman spectroscopy has shown that the retinylidene-opsin Schiff base is protonated in the bR^{LA}_{570} complex and unprotonated in the M^{LA}_{412} complex (Lewis et al., 1974). However, the Schiff base is relatively inaccessible from the medium and neither the proton release nor uptake appears to occur directly from the Schiff base. We envisage that the mechanism of light-induced proton translocation may involve a chain of acid/base groups in or on the protein through which a proton can pass across the membrane. A light-induced reversible pK change in one of these

groups could translocate protons against a gradient across the membrane. The Schiff base is the most likely candidate for this role.

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