

Functions of a New Photoreceptor Membrane

(halophilism/photosynthesis/chemiosmotic energy coupling/proton transport/rhodopsin)

DIETER OESTERHELT AND WALTHER STOECKENIUS

Institute für Biochemie, Chemisches Laboratorium der Universität München, München; and Cardiovascular Research Institute and Department of Biochemistry and Biophysics, University of California, San Francisco, Calif. 94143

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ABSTRACT The purple membrane of *Halobacterium halobium* contains only one protein, bacteriorhodopsin, which closely resembles the visual pigments of animals. Light flashes cause a rapid transient shift of its absorption maximum from 560 to 415 nm. This shift is accompanied by release and uptake of protons. Respiring cells acidify the medium in the dark; if they contain purple membrane their O₂ consumption is reduced in the light. Starved or anaerobic cells containing purple membrane, in the absence of any apparent source of energy, generate and maintain a proton gradient across the cell membrane as long as they are exposed to light. We postulate that the light-generated proton gradient arises from a vectorial release and uptake of protons by bacteriorhodopsin, which is suitably oriented in the cell membrane and under continuous illumination oscillates rapidly between the long- and short-wavelength form. Preliminary results indicate that the gradient in *H. halobium* plays the central role in energy coupling attributed to such electrochemical gradients by Mitchell's chemiosmotic theory.

The extreme halophile *Halobacterium halobium* synthesizes cell membrane that contains a rhodopsin-like protein, bacteriorhodopsin. It forms distinct patches with a hexagonal lattice structure in the plane of the membrane. The patches have been isolated and show a strong absorption band near 560 nm. They have been termed the purple membrane (1, 2).

Photoreactions similar to those in rhodopsin have been observed in bacteriorhodopsin. Illumination causes a transient decrease in the absorption maximum near 560 nm with a corresponding increase in absorption near 415 nm. This finding suggests the possibility that the purple membrane functions as a photoreceptor. We, therefore, investigated light responses in halobacteria and found: phototaxis; ATP synthesis; and changes in O₂ consumption, purple membrane biosynthesis, and proton translocation. We report here on the last three effects, which suggest that the purple membrane may function as an energy-coupling membrane for light.

A photocoupling* function of purple membrane appeared more likely than a photosensing* function, because under optimal conditions nearly half of the total surface area of the cells may be occupied by purple membrane. A photocoupling function does, of course, not exclude a simultaneous photosensing function (3).

* We use photoreceptor as the general term comprising both photocouplers and photosensors. A photosensing function implies that light absorption triggers a process that derives its required energy from other sources. A photocoupling function implies that the light energy absorbed is used as the driving force for such a process, for instance ion translocation or ATP synthesis.

MATERIALS AND METHODS

Growth of *H. halobium* and isolation of purple membrane have been described (4-6). To inhibit purple membrane synthesis, we added diphenylamine to cells growing in the dark (7). Purple membrane content of cells was measured as described (1).

pH was measured on cells suspended in basal salt solution (growth medium without nutrients; ref. 4). For anaerobic conditions a thermostated measuring cell (Rank Bros., Nottingham, England) and a pH electrode (Ingold no. 401) were used. For aerobic conditions a similar cell was constructed that allowed gas exchange through a hydrophobic Millipore filter (Millipore Corp., Bedford, Mass., RAHPO2500) between the cell suspension and an exterior gas chamber. Cells were starved with constant aeration for 20-30 hr in basal salt solution. When O₂ consumption had dropped to 50% or less, aliquots were transferred to the measuring cell in which they could be maintained for several days. O₂ consumption was also measured in a continuously recording Warburg apparatus (8). For illumination, quartz-iodine lamps of 250-1,000 W were used in combination with cut-off filters.

Reversible bleaching of isolated purple membrane in ether-saturated solution has been described (9). In other bleaching experiments a special single-beam spectrophotometer (10) and a flash spectrophotometer, designed and constructed by R. A. Cone, were used.

Freeze-fracturing techniques have been described (2, 5). To obtain an estimate of the proportion of purple membrane in electron micrographs of freeze-fractured cells, the areas of purple membrane and other surface membrane ("red membrane"; ref. 5) were cut out and weighed separately for A and B faces.

RESULTS

Biosynthesis of Purple Membrane. Usually synthesis of purple membrane occurs when cell density becomes high and O₂ concentration low (1, 11, 12). Cells synthesize purple membrane already in early growth if O₂ concentration in the culture is kept low (Fig. 1A). Near air saturation we observed no synthesis of purple membrane (Fig. 1B). Low O₂ concentrations do not only trigger the synthesis but are necessary for continued formation of purple membrane (Fig. 2). Light stimulates synthesis; purple membrane content of cells grown at low O₂ concentration typically is 6- to 7-times higher in the light than in the dark (11).

Freeze-fracture preparations have shown that purple membrane maximally occupies about 50% of the total cell mem-

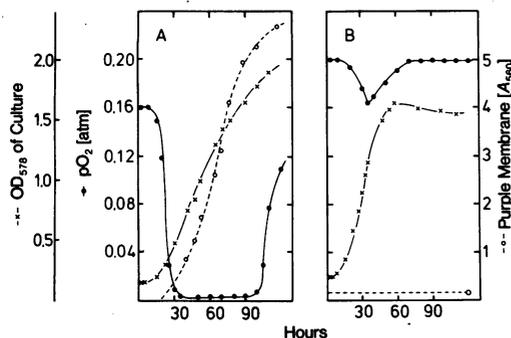


FIG. 1. Synthesis of purple membrane. (A) 120 liters of air per hr, stirring at 300 rpm; (B) 180 liters of air per hr, 500 rpm. Growth in suspension culture in peptone medium at 39°. For determination of purple membrane, see ref. 1.

brane area; this estimate entails the assumption that tangential fractures occur with equal probability in purple membrane and red membrane. It may actually be too low a value, because tangential fractures are seen far less frequently in isolated purple membrane than in red membrane. In any case, it is clear that the cells can convert a substantial part of their total surface membrane into purple membrane. Electron micrographs further show purple-membrane patches relatively homogeneously distributed over all cells.

Bleaching of Isolated Purple Membrane. Purple membrane can reversibly be bleached by light. The absorption maximum near 560 nm decreases and a simultaneous increase of absorption occurs near 415 nm. At room temperature this effect can only be observed with flash spectroscopy because the 415-nm form reverts spontaneously within a few milliseconds to the 560-nm form; formation of the 415-nm form is even faster (Cone, R. A., Fein, A. & Stoekenius, W., unpublished). The bleached form can also be observed if the purple membrane is frozen in liquid nitrogen during illumination (Lozier, R. H. & Stoekenius, W., unpublished; Fig. 3) or if ether is added to the membrane suspension. Ether retards the return to the unbleached form sufficiently to maintain the membrane in the bleached state with relatively low light intensities and at room temperature. In the dark the purple complex regenerates with

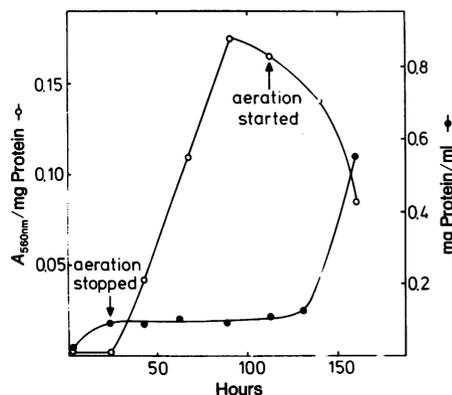


FIG. 2. Synthesis of purple membrane at low O_2 concentration. Cessation of aeration inhibits growth and initiates purple membrane synthesis. Resumption of aeration arrests purple membrane synthesis and initiates growth again. Growth conditions as in Fig. 1. Protein determined in washed cells by a micro-Kjeldahl technique.

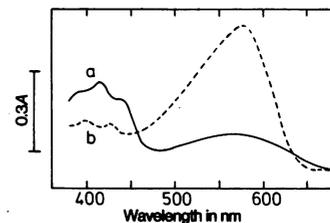


FIG. 3. Bleaching of isolated purple membrane. Isolated purple membrane was frozen in liquid nitrogen during illumination (a) and in the dark (b). In the light the absorption at 570 nm decreases; absorption increases near 415 nm. The isosbestic point is near 460 nm.

a half-time of 13 sec. The absolute quantum yield for bleaching in ether is 0.79 (9).

Bleaching of isolated purple membrane in ether-saturated basal salt solution causes a drop of pH in the suspension, which also reverses in the dark (Fig. 4, a). Changes of about 0.2 pH unit are observed at 0.1 mM purple complex concentration and pH 6.8. This light-induced pH effect is insensitive to carbonylcyanide *m*-chlorophenylhydrazone and other uncouplers (Fig. 4, b) that abolish the pH effect in whole cells, as will be shown below. Spectral and pH effects in isolated membrane have about the same time course (9). Purple membrane apparently releases and rebinds protons during a bleaching cycle. We assume that the same release and uptake of protons occurs also in the absence of ether because the spectroscopic changes appear to be identical; however, the fast reversal of bleaching has so far prevented a direct demonstration of the pH effect in the absence of ether.

Bleaching of Purple Membrane in Intact Bacteria. This effect is difficult to demonstrate because of the relatively low concentration of the membrane in cell suspensions, the strong light scattering, and the presence of other pigments. Only the transient increase in absorption at 415 nm has been observed (Cone, R. A., Fein, A. & Stoekenius, W., unpublished). This absorbance decays with the same time constant as in isolated membrane, however, the strong Soret band of the cytochromes present makes an unequivocal interpretation difficult. Low-temperature spectroscopy allows us to demonstrate bleaching in intact cells. Spectra of samples frozen in the light and in the dark show a decrease of purple complex absorption around 585 nm and a corresponding increase around 415 nm (Fig. 5). This observation demonstrates in intact bacteria the same spectral changes observed in isolated purple membrane.

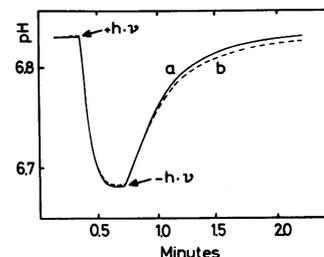


FIG. 4. pH changes upon illumination of isolated purple membrane. Purple membrane in water at 0.5 mM purple complex concentration diluted with 5 volumes of basal salt solution and saturated with ether at room temperature. Illumination through an orange filter (Schott OG 4) causes a drop in pH, which is reversed in the dark (a); 0.8 mM carbonylcyanide *m*-chlorophenylhydrazone has no significant effect on the reaction (b).

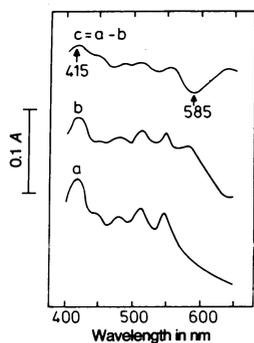


FIG. 5. Bleaching of purple membrane in intact bacteria. 3.5×10^9 cells per ml of *H. halobium* R₁L₃, which contains reduced amounts of bacterioruberin; peaks at 480, 512, and 547 nm are due to this pigment. One aliquot is illuminated with orange light for 1 min and then frozen in the light (*a*) or in the dark (*b*). Difference spectrum *a* - *b* (*c*). Prior illumination at room temperature, which shifts the absorption spectrum of the purple complex to slightly longer wavelength with a very slow return in the dark, (1), was used in *b* to give comparable samples. The peak in the difference spectrum appears at 585 nm rather than 570 nm because low temperature causes a small shift to longer wavelength.

Light Effects on pH and Respiration in Cell Suspensions. Respiring cells of *H. halobium*, similar to other prokaryotic cells (13), acidify the medium. Cell suspensions in basal salt solution have a pH between 6.2 and 7.2. Aliquots transferred to the closed measuring cell become rapidly anaerobic; simultaneously the pH drops and rises again briefly. After about 10 min it has reached a nearly stable value but continues to increase very slowly. This quasistable state is used as the basis for the study of light effects. Upon illumination with wavelengths absorbed by the purple membrane, the pH, after a transient rise, drops 0.1–0.2 pH units to a new stable level. In the dark, the pH returns to the original level. This cycle can be repeated several times (Fig. 6A). The light-to-dark pH transition has at least one fast and one slow kinetic component. Carbonylcyanide *m*-chlorophenylhydrazine or other uncouplers, such as dinitrophenol and *p*-trifluoromethoxyphenylhydrazine, increase the proton permeability of membranes (14–16). If they are added at the time of return to dark condition, the pH rise is accelerated about 40 times compared to the fast component (Fig. 6B) and subsequent illumination shows that the pH effect is now abolished. This result implies that illumination generates a pH and/or electrical gradient across the cell membrane. The final pH after addition of the chlorophenylhydrazine is higher than the original dark level. Apparently a pH gradient—with the same sign as the one induced by illumination—existed before illumination, and its slow relaxation is responsible for the quasistable state. The preexisting as well as the light-induced gradient are collapsed by uncouplers. We also tentatively explain the initial transient increase in pH as a light-induced relaxation of this gradient. It does not occur in starved cells.

Aerated cells in basal salt solution slowly cease to consume O₂. In a chamber that allows diffusion of O₂ into the cell suspension, the pH of the suspension increases slowly and reaches a stable level when O₂ consumption approaches zero. Illumination then causes within about 5 min a drop of about 0.05–0.1 pH unit to a new stable level. In the dark the pH rises again to the original level. If the cells used still consume some O₂

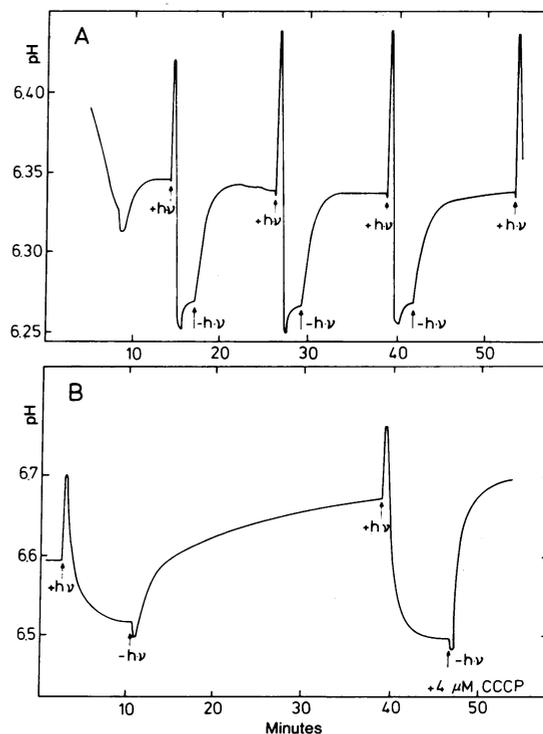


FIG. 6. pH changes in anaerobic cell suspensions. (A) Late log-phase cells resuspended in basal salt at 5-times their original concentration show reversible pH changes upon illumination. (B) Same conditions as in A. The rise from the light to dark level shows two components. Carbonylcyanide *m*-chlorophenylhydrazine (CCCP) causes a large increase in the rate of the rise and an increase in the final pH level.

when the suspension is illuminated, a reversible reduction of O₂ consumption is observed (Fig. 7). The changes in respiration are slower than the pH changes. These light effects are completely inhibited by 4 μM carbonylcyanide *m*-chlorophenylhydrazine and dinitrophenol or *p*-trifluoromethoxyphenylhydrazine added before illumination (not shown). Light inhibition of respiration of up to 30% has also been recorded in an automatic Warburg apparatus (Krippahl, G. & Oesterhelt, D., in preparation) (Fig. 8). In cells with diphenylamine inhibited purple membrane synthesis the O₂ consumption is not sensitive to the illumination (not shown).

At equal purple membrane concentration, the light-induced increase of proton concentration in cell suspensions exceeds by a factor of at least 20 the increase observed when isolated purple membrane is fully bleached. The light-induced pH change in cell suspensions cannot, therefore, be explained simply as the result of a release of protons by purple membrane. Light apparently induces a net outward proton translocation† from the cells. The gradient is sensitive to chlorophenylhydrazine and other uncouplers, which have no effect on the pH changes observed in isolated purple membrane (Fig. 4). A cyclic vectorial release and uptake of protons by purple membrane in illuminated cells would account for generation of the pH gradient across the cell membrane.

† We shall follow Mitchell (18) and use the term effective proton translocation or simply proton translocation to describe transport of protons across the cell membrane in one direction or transport of hydroxyl ions in the opposite direction, or both.

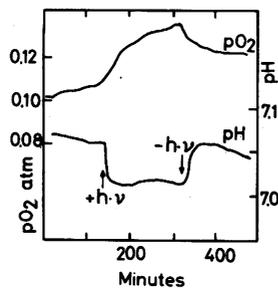


Fig. 7. pH changes and O_2 consumption in aerobic cell suspensions. Late log-phase cells were washed twice in basal salt solution. Aeration was through a Millipore filter. Illumination causes a reversible drop in pH and rise in pO_2 . The difference from Fig. 6, a lack in the transient rise in pH, is typical for starved cells.

Halobacteria are obligate aerobes (17). Cells suspended in basal salt solution under anaerobic conditions or starved cells have no apparent energy source to drive the proton translocation unless light energy absorbed by the bacteriorhodopsin is used for this process. If this assumption is correct, the rate of pH change upon illumination and the difference between the dark and light level should depend on light intensity and purple membrane concentration. Qualitatively this is borne out because *H. halobium* cells grown in the presence of diphenylamine or at high O_2 have little purple membrane and show a proportionately decreased pH effect (Fig. 9). The extent of the pH effect depends on the light intensity and can be saturated at high intensities. The exact quantitative relations have not been established.

DISCUSSION

The results presented suggest that purple membrane may be a photocoupler. This rather startling conclusion has to be qualified. While there is no other obvious energy source available in starved and anaerobic cells, the possibility exists that the cells may be able to use certain substrates in the light but not in the dark. This could either occur through photoconversion of nonmetabolizable compounds into substrates for energy metabolism or photoactivation of an enzyme controlling a metabolic pathway. Respiration would be excluded by the anaerobic experiments unless cells in the light could use

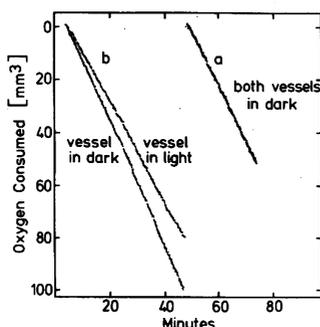


Fig. 8. Respiration of cells in the light and the dark. 10-times concentrated late log-phase cells in fresh medium were transferred to Warburg flasks with 0.2 ml of 20% KOH in the center well, and gassed with O_2 . Both vessels are compared to the same control vessel containing medium only. Temperature, 25°. (b) One vessel was illuminated through Schott filter OG 4. O_2 consumption in the illuminated vessel is 79% of consumption in the dark vessel.

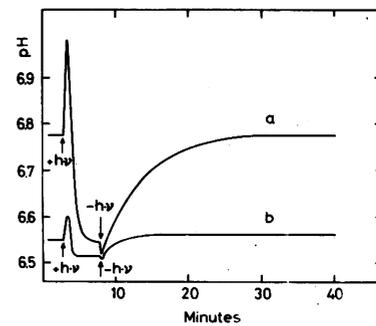


Fig. 9. Comparison of pH effect in cells with different amounts of purple membrane. Conditions as in Fig. 6. (a) Cells with large amounts of purple membrane; (b) cells grown in diphenylamine with greatly reduced amount of purple membrane.

alternative electron acceptors, such as nitrate or sulfate. Participation of purple membrane would still be required, because the effect is dependent on its presence and on light of a wavelength absorbed by purple membrane. Substrate photoconversion might still be a photocoupling process. Enzyme activation would require either that bacteriorhodopsin is the enzyme or that energy-transfer intermediates are present. In either case the dependence on purple membrane concentration and the large amount of bacteriorhodopsin that is present and held in a rigid lattice in the cell membrane make this an unlikely assumption.

We also suggest that purple membrane, through cyclic light-induced conformational changes of its bacteriorhodopsin, directly converts absorbed light energy into a proton gradient and presumably also an electric potential difference across the membrane analogous to observations in other prokaryotic cells, mitochondria, and chloroplasts (13, 19). What mechanism creates the pH gradient? Formation of a pH gradient through an electron-transport chain analogous to the mechanism postulated for chloroplasts (19) should be considered. We think this is unlikely. The membrane patches contain only one protein, and the electron acceptors of a membrane-bound electron transport chain would have to be located at the periphery of the patches, which have an average diameter of at least 0.5 μm . The distance of about 60 \AA between protein molecules argues against energy transfer between chromophores, and preliminary results of experiments on decay of induced dichroism (Cone, R. A., Fein, A. & StoECKENIUS, W., unpublished) appear to exclude it. Intramembrane light-driven redox reactions cannot be excluded; they would, however, require the additional assumption of diffusible electron donors and/or acceptors. So far we have no evidence for such a mechanism and we prefer the simpler explanation that the light-induced reversible deprotonation observed *in vitro* operates as a vectorial process *in vivo* and generates the gradient. In other words: we propose that the purple membrane—presumably bacteriorhodopsin itself—acts as a light-driven proton pump. The release and uptake of protons during the bleaching cycle and the fast reversible bleaching in intact cells strongly suggest this possibility. It entails only the assumption that this reaction occurs as a vectorial process across the cell membrane, but would require that all protein molecules that are in the same conformational state are identically oriented in the direction normal to the plane of the membrane. Morphological data bear this out. Freeze-frac-

turing of the dark-adapted membrane leaves all protein attached to the cytoplasmic side which, together with the arrangement of the protein in the planar hexagonal lattice, shows that the protein has the required orientation (2, 20). A direct pumping action of bacteriorhodopsin in the purple membrane therefore fits all observations and requires the fewest additional assumptions.

The interpretation of our results necessarily leads to a consideration of Mitchell's chemiosmotic theory (19), which states that in respiration and photosynthesis a series of redox reactions is used to generate the proton gradient and its associated electrical potential across the membrane. We propose that in *H. halobium* absorption of light quanta causes a transient conformational change in one membrane protein which, through the accompanying vectorial release and uptake of protons, generates the gradient. The utilization of the energy stored in the gradient presumably is the same as proposed by the chemiosmotic theory. A membrane-bound ATPase has been found in *H. halobium* (21), which may function in ATP synthesis driven by the gradient. ATP synthesis in anaerobic cells in the light but not in the dark has been demonstrated (Danon, A. & Stoeckenius, W., in preparation); we expect, however, that transport processes across the cell membrane may also be driven directly by the electrochemical gradient in analogy to observations in other prokaryotic cells (13). *H. halobium* apparently has two alternative possibilities to create the gradient: respiration and light absorption. If our interpretation of purple-membrane function is correct, the central role of the pH gradient for energy conversion becomes obvious.

If the purple membrane functions as indicated here, it would be, beside the thylakoid membrane, the only other known photocoupling membrane. This hypothesis throws a new light on photoreceptor evolution. Apparently membranes containing rhodopsin-like proteins as well as chlorophyll-containing membranes function both as photocouplers and photosensors, but the main evolutionary trend has apparently been toward photosensing in the case of retinal-protein complexes and toward photocoupling in the case of chlorophylls. Why this should be so is an intriguing question.

Purple membrane can easily be obtained in pure form and large quantities; detailed chemical and structural data are available. In addition to its significance in problems of photosynthesis, evolution, and the chemiosmotic theory, it may be the best available model to study an ion pump. Because of its similarity to the visual pigments, the results obtained may also be relevant for the physiology of vision.

Note Added in Proof. Since this manuscript was submitted for publication we have also demonstrated light-driven proton

translocation in a model system. It consists of vesicles formed from purified lipids into which purple membrane has been incorporated (Racker, E. & Stoeckenius, W., submitted for publication). These experiments confirm the conclusion that the purple membrane functions as a light-driven proton pump and further reduce the probability that other mechanisms are responsible for light energy conversion in *H. halobium*.

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