BIOENERGETIC ROLE OF HALORHODOPSIN IN *HALOBACTERIUM HALOBIUM* CELLS

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1. Introduction

Bioenergetic functions in the membranes of *Halobacterium halobium* depend on respiration in the dark, and on two retinal pigments during illumination. The better known of these is bacteriorhodopsin [1], which is found in crystalline arrays (patches) covering as much as 50% of the cell surface. Bacteriorhodopsin is a light-dependent pump for protons, and it has been proposed [2–4] that the pH difference and particularly the electrical potential, which is created during illumination by proton extrusion across the cytoplasmic membrane, is the energizing force for ATP synthesis. Illumination will indeed support the growth of bacteriorhodopsin-containing *H. halobium* under strictly anaerobic conditions [5].

The second retinal pigment, present in smaller amounts in the halobacteria, is halorhodopsin. This pigment is a light-driven electrogenic pump for Na^+ [6–8], and its spectral characteristics are somewhat different [9,10] from those of bacteriorhodopsin. The presence of this pump is most easily recognized in cell envelope vesicles prepared from *H. halobium* strains lacking bacteriorhodopsin. Illumination of such vesicles results in uncoupler-facilitated passive proton uptake until a pH difference (acid inside) is balanced by a membrane potential (negative inside), so that the protonmotive force is zero [8]. Appropriately prepared vesicles from wild-type, bacteriorhodopsin-containing cells show both active and passive proton movements upon illumination [7,8], the former appearing as efflux, the latter as influx. Proton conductors will tend to suppress proton efflux but enhance the influx. It appears therefore, that when present, both pumps are oriented in the outward direction in these membranes.

It has been known from the earliest studies [11] that illumination of whole *H. halobium* cells produces both pH rise and pH drop in the medium. In [12] it was a strain of *H. halobium* largely or completely lacking bacteriorhodopsin exhibited pH rises during illumination, which were not followed by a pH drop as in wild-type cells. The proton uptake was accompanied by ATP synthesis. Action spectra identified a pigment shifted in absorption to about 590 nm, but the presence of large amounts of red carotenoids made the interpretation of this result ambiguous. Nevertheless, after finding some differences between the behaviour of bacteriorhodopsin and the pigment responsible for photophosphorylation in their strain, it was proposed that the pH rise and ATP synthesis is associated with a special kind of bacteriorhodopsin [12].

These findings and hypotheses now need to be re-examined in light of the recent discovery of halorhodopsin. From preliminary experiments, it was suggested [8] that the pH rise and drop in the *H. halobium* cell suspensions (as in vesicles) reflect passive and active proton movements due to the activities of halorhodopsin and bacteriorhodopsin, respectively. This idea implies that ATP synthesis during illumination should occur in response to the membrane potential created by Na^+ extrusion by halorhodopsin. This report contains evidence relevant to this question, and makes use of new strains of *H. halobium* which we have isolated. To date, only a limited number of *H. halobium* mutants have been available for bioenergetic studies. The wild-type strains contain both bacteriorhodopsin and halorhodopsin, and large (strain R1) or smaller (strains S9 and R_1M_1) quantities of red carotenoids. The carotenoids badly interfere with spectrophotometric measurements of the bacterial rhodopsins. Bacteriorhodopsin-deficient, but halorhodopsin-containing

Abbreviations: CCCP, carbonyl cyanide metachlorophenylhydrazone; TPP^+, tetraphenylphosphonium cation

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strains are also available in red pigmented (strain RlmR [12,13]) and carotenoid-free (strain ET-15 [9,10]) form. Finally, retinal-deficient mutants which also lack carotenoids have been described (strain RlmW [14]; strain 5002-7 [9]) but these are able to synthesize both bacterio-opsin and halo-opsin. We have searched for and found *H. halobium* strains, which lack carotenoids and bacteriorhodopsin but contain amounts of halorhodopsin increased over the small amounts [9] found in ET-15. One of the strains described (strain L-07) is deficient in retinal synthesis, and offers the possibility of manipulating halorhodopsin synthesis by controlled addition of retinal. A retinal-deficient, but bacterio-opsin-containing strain, W-296, which lacks carotenoids, was a gift from G. Weidinger.

2. Materials and methods

Halobacterium halobium was grown at 40°C with shaking, in the medium of [15]. The cells were harvested in middle logarithmic growth phase and suspended in 'basal salts' to 2 or 3 mg protein/ml.

Light-dependent pH changes were measured in a cylindrical cuvette, thermostatted at 30°C. Sample volume was 6 ml; the cell suspension was purged throughout the experiment with a slow stream of nitrogen. Illumination was with a Perkeo 511 slide projector, 150 W (full intensity) lamp, through a GG 495 or an OG 515 (Schott) filter. Light intensity at the cuvette was 50 mW . cm^{-2}. An Ingold combination glass electrode was used, and the pH trace was followed on a strip chart recorder.

Photophosphorylation was measured in the same cuvette as the pH changes, but aliquots of 50 μl were taken, and ATP levels determined as in [16]. Buffering was either at pH 7.0 (11.5 mM sodium citrate) or at pH 7.8 (20 mM Tris maleate).

Illumination of cells during growth was in a Warburg vessel, as in [22]. Illumination was with a 1000 W Xenon lamp through water, $CuSO_4$ solution and a GG 455 cut-off filter. Energy input (in vessel) was 2.3 W.

3. Results and discussion

We searched for mutants of *H. halobium* strain S9 deficient in bacteriorhodopsin and carotenoids after UV irradiation by visual inspection of colonies grown on agar plates. Two such strains were found, labeled L-07 and L-33. Strain L-07 did not respond to illumination, while L-33 exhibited large pH increases upon illumination.

Fig.1 shows the responses of *H. halobium* strains L-33 and L-07. The proton conductor, CCCP, causes a 5–6-fold increase in the light-dependent alkalinization observed in suspensions of L-33 cells. It seems likely that the uncoupler acts by providing increased proton permeability, so as to permit the electrical and chemical potential differences for protons to balance one another. This interpretation is supported by the observation that addition of the permeant cation TPP^+ virtually completely eliminates the pH change (fig.1A). In a separate experiment we showed that TPP^+ abolished the pH increase also in the absence of CCCP and no acidification occurred, indicating the absence of active proton extrusion in these cells.

Although L-07 does not respond to illumination (fig.1B), growth in the presence of added retinal restores a normal light-dependent alkalinization to the cells. We conclude therefore, that this mutant is defective in retinal synthesis, but not in the synthesis of halo-opsin. When retinal is added to cell envelope membranes prepared from this strain, a difference spectrum develops, whose band at 588 nm is indicative of halorhodopsin [17]. Cell envelope vesicles prepared from L-07 grown with retinal are not distinguishable from L-33 vesicles.

Spectrophotometric estimates [17] of halorhodopsin in cell envelope vesicles from L-33 yielded about 0.5 nmol/mg protein, which is 4-times higher than estimated for the best strain so far, ET-15 [9]. Strain L-07 appears to be deficient in retinal as well, and therefore offers the possibility to reconstitute halorhodopsin with retinal, without interference of bacteriorhodopsin, as exist in other strains, such as RlmW [14].

ATP synthesis by *H. halobium* strain L-07 is shown in fig.2. This strain produces no ATP upon illumination under anaerobic conditions, but will phosphorylate during respiration. Growth in the presence of retinal restores photophosphorylation to these cells (fig.2), as it does light-dependent pH changes (fig.1). Illumination of L-33 cells caused ATP synthesis similar to those shown in fig.2 for L-07 cells.

The initial rate of ATP synthesis was followed during illumination of L-33 cells through narrow bandwidth interference filters. The resulting action spec-

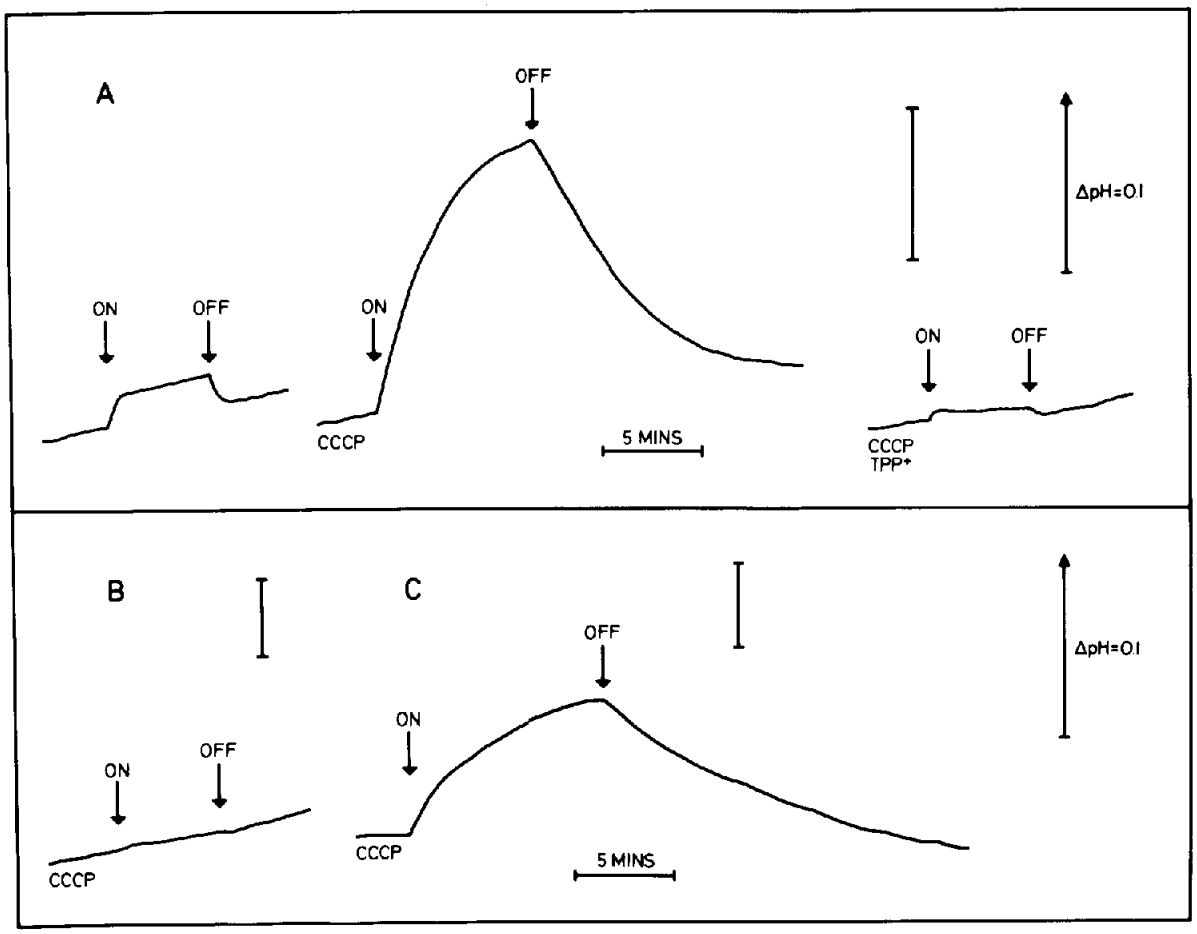

Fig.1. Light-dependent pH changes in suspensions of *H. halobium* strain L-33 and L-07 cells. The cells were suspended in 5 ml basal salt solution to 2 mg protein/ml, and the pH recorded at 30°C as in section 2. In all experiments, the initial pH was 7.5 ± 0.05. (A) Strain L-33 cells (middle logarithmic growth phase) illuminated without addition, with 20 μM CCCP and 20 μM CCCP and 250 μM TPP^+. At the pH used, addition of the inhibitors in the dark caused little or no pH change in the cell suspension. (B) Strain L-07 cells (middle logarithmic growth phase) illuminated with 20 μM CCCP. (C) Strain L-07 cells, grown for 1.5 days in the presence of 5 μM *trans*-retinal (from early to middle logarithmic phase) and illuminated with 20 μM CCCP. The unlabeled vertical bars refer to pH changes upon addition of 200 nequiv. HCl.

trum, given in fig.3 is consistent with the 588 nm absorption maximum of bacteriorhodopsin. We conclude from these results that in the strains used illumination-dependent proton uptake and ATP synthesis are both attributable to halorhodopsin. Both processes are probably induced by the increased membrane potential due to the electrogenic extrusion of Na^+, as proposed in [8]. This also explains light inhibition of respiration we observed in L-33 cells and L-07 cells supplemented with retinal (not shown).

In [5] it was reported that illumination permits the growth of bacteriorhodopsin containing *H. halobium* cells under anaerobic conditions, i.e., when no other source of energy is available. Continuous illumination of these cells causes recirculation of protons, and in the steady-state the cell interior is somewhat alkaline relative to the exterior. In contrast, bacteriorhodopsin negative strains which contain halorhodopsin accumulate protons during continuous illumination, and under anaerobic conditions could not be able to export them. The continued acidification of the cytoplasm thus predicted is probably not consistent with normal growth. Indeed, it was found in [18] that continued light-dependent ATP synthesis in bacterio-

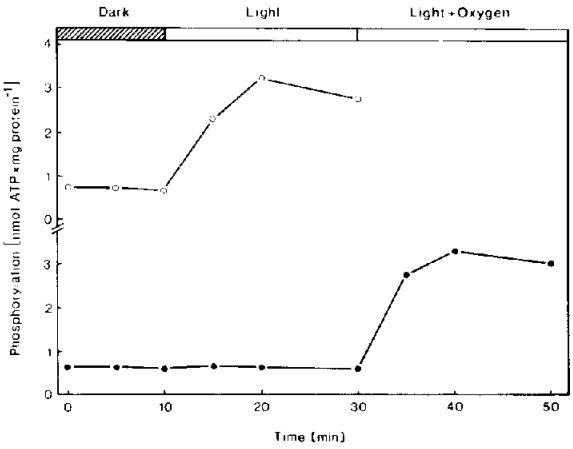

Fig.2. Photophosphorylation and oxidative phosphorylation in *H. halobium* strain L-07 cells, before and after growth with retinal. Lower trace (•): cells grown without retinal. Upper trace (○): cells grown 1.5 days with 5 μM retinal (added in isopropanol). Anaerobic conditions were maintained with a stream of nitrogen until aerobic conditions were desired; at the time indicated a stream of air was introduced. Temperature, 30°C; protein, 2 mg/ml, basal salt solution with 11.5 mM sodium citrate at pH 7.0.

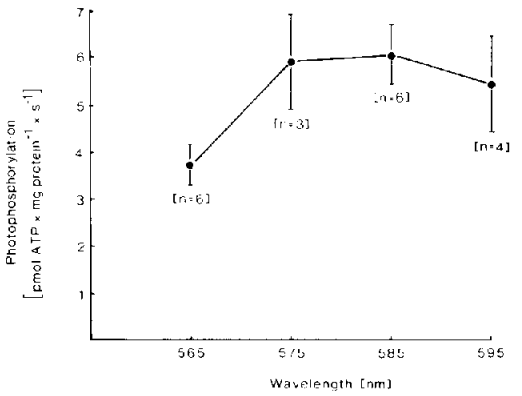

Fig.3. Initial rate of photophosphorylation in *H. halobium* strain L-33 cells as a function of wavelength. Monochromatic light was selected with Anders interference filters, half-band width 5 nm. Photon flux was adjusted to 5 nmol . cm^{-2} . s^{-1} with neutral density filters. The mean spherical attenuance factor [21] of the sample, decreasing from 595–565 nm by <2% has not been corrected for. Conditions as in fig.2, but protein 3 mg/ml, and buffer 20 mM Tris maleate (pH 7.8). The bars indicate standard error of the mean; the number of determinations is given in parenthesis.

rhodopsin-deficient cells was seen only in the presence of triphenyl tin, which can act as a OH^-/Cl^- exchange. In table 1, results are presented on the ability of different *H. halobium* strains to grow under such conditions. All strains grew in the dark with aeration and none of them grew in the dark with nitrogen alone. Strains containing either bacteriorhodopsin (S9) or bacterio-opsin with added retinal (W-296) did grow with light as the only energy source. In contrast, strains lacking bacteriorhodopsin, but containing either halorhodopsin (L-33).or halo-opsin with added retinal (L-07) did not grow under illumination without oxygen. Neither did that strain grow, which lacked retinal (W-296), but was not supplemented. It appears therefore that sustained growth is not supportable by photophosphorylation dependent on halorhodopsin alone. Whether or not anaerobic growth under illumination will be seen in strains containing bacteriorhodopsin without halorhodopsin is, at present, a conjecture.

Table 1
Anaerobic growth of different *Halobacterium halobium* strains[a]

Strain	Phenotype[b]	Density before incubation	Air, dark	Density after 60 h	
				Nitrogen, light	Nitrogen, dark
S_9	$BO^+HO^+Ret^+$	0.10	0.70	0.34	0.10
W_{296}	$BO^+HO^+Ret^-$	0.11	1.10	0.11	0.11
W_{296} (+ retinal)	$BO^+HO^+Ret^-$	0.19	1.00	0.36	0.19
L-33	$BO^-HO^+Ret^+$	0.04	1.05	0.04	0.04
L-07 (+ retinal)	$BO^-HO^+Ret^-$	0.09	1.05	0.09	0.09

[a] The cells were grown for 5 days and then diluted 10-fold with fresh medium; 3 ml were shaken in a Warburg vessel, and the cell density was measured as turbidity at 578 nm [5]. When retinal was present, it was added in isopropanol to a final concentration of about 5 μM.
[b] BO, bacterio-opsin; HO, halo-opsin; Ret, retinal, it is assumed that strains S_9 and W_{296} are HO^+

It is difficult to assess quantitatively the contribution of halorhodopsin to light-induced membrane potential changes and photophosphorylation in wild-type cells, because the cellular content of halorhodopsin and the quantum yield of sodium translocation are not known. Although both halorhodopsin and bacteriorhodopsin act as light-driven ion pumps and mediate phosphorylation, only bacteriorhodopsin can remove protons which accumulate in the cellular interior. On the other hand, halorhodopsin as a sodium pump is an alternative driving force for Na^+/K^+ exchange, which simulates the combined action of bacteriorhodopsin and the Na^+/H^+ antiporter. The three catalysts together, however, will fulfill, in a balanced action, all the bioenergetic functions of the halobacterial cell under varying physiological conditions. In addition, it should be noted that additional functions, such as phototaxis [19,20], have been attributed to the retinal proteins of the halobacteria.

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References

[1] Oesterhelt, D. and Stoeckenius, W. (1971) Nature New Biol. 233, 149–152.
[2] Danon, A. and Stoeckenius, W. (1974) Proc. Natl. Acad. Sci. USA 71, 1234–1238.
[3] Oesterhelt, D. (1975) Ciba Found. Symp. 31, 147–167.
[4] Danon, A. and Caplan, S. R. (1976) Biochim. Biophys. Acta 423, 133–140.
[5] Hartmann, R., Sickinger, H.-D. and Oesterhelt, D. (1980) Proc. Natl. Acad. Sci. USA 77, 3821–3825.
[6] Lindley, E. V. and MacDonald, R. E. (1979) Biochem. Biophys. Res. Commun. 88, 491–499.
[7] MacDonald, R. E., Greene, R. V., Clark, R. D. and Lindley, E. V. (1979) J. Biol. Chem. 254, 11831–11838.
[8] Greene, R. V. and Lanyi, J. K. (1979) J. Biol. Chem. 254, 10986–10994.
[9] Lanyi, J. K. and Weber, H. J. (1980) J. Biol. Chem. 255, 243–250.
[10] Weber, H. J. and Bogomolni, R. A. (1981) Photochem. Photobiol. 33, 601–608.
[11] Oesterhelt, D. and Stoeckenius, W. (1973) Proc. Natl. Acad. Sci. USA 70, 2853–2857.
[12] Matsuno-Yagi, A. and Mukohata, Y. (1977) Biochim. Biophys. Res. Commun. 78, 237–243.
[13] Matsuno-Yagi, A. and Mukohata, Y. (1980) Arch. Biochem. Biophys. 199, 297–303.
[14] Mukohata, Y., Sugiyama, Y., Kaji, Y., Usukura, J. and Yamada, E. (1981) Photochem. Photobiol. 33, 593–600.
[15] Oesterhelt, D. and Stoeckenius, W. (1974) Methods Enzymol. 31, 667–678.
[16] Wagner, G., Hartmann, R. and Oesterhelt, D. (1978) Eur. J. Biochem. 89, 169–179.
[17] Lanyi, J. K. (1981) Methods Enzymol. in press.
[18] Mukohata, Y. and Y. Kaji (1981) Arch. Biochem. Biophys. 208, 615–617.
[19] Narurka, V. and Spudich, J. L. (1981) Biophys. J. 33, 218a.
[20] Wagner, G. and Linahrdt, R. (1981) Mol. Biol. Biochem. Biophys. in press.
[21] Hartmann, K. M. and Cohnen-Unser, I. (1972) Ber. Deutsch. Bot. Ges. 85, 481–551.
[22] Oesterhelt, D. and Krippahl, G. (1973) FEBS Lett. 36, 72–76.