QUANTITATIVE ASPECTS OF ENERGY CONVERSION IN HALOBACTERIA

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1. Introduction

The retinal protein complex bacteriorhodopsin together with lipid forms a specialized area within the cell membrane of Halobacteria which is called purple membrane [1]. Under growth conditions favourable for bacteriorhodopsin synthesis, the purple membrane may cover more than 50% of the total cell membrane area [2].

Bacteriorhodopsin functions as a proton pump converting light energy into the energy of an electrochemical proton gradient across the cell membrane. Halobacteria are aerobic organisms, thus respiration provides an alternative source of energy for the production of the electrochemical proton gradient. The gradient energy is then utilized to drive various bioenergetic processes such as ATP synthesis [3–6], ion exchange [7] and amino acid uptake (see [8]).

If Halobacteria are grown in the presence of the alkaloid, nicotine synthesis of retinal is blocked but bacterio-opsin is still formed [9]. Addition of retinal to these cells results in reconstitution of bacteriorhodopsin and restoration of its bioenergetic function [10]. However, newly formed bacteriorhodopsin is not found in purple membrane but occurs in another cell membrane fraction which is called the brown membrane [9]. An energy requiring process then induces bacteriorhodopsin to form the crystalline array which is found within the purple membrane [11].

In this paper we determine the quantitative relationships in halobacterial energy conversion and the efficiency of bacteriorhodopsin which is a function of the different physio-chemical environment within the brown and the purple membrane.

2. Material and methods

2.1. *Strain and culture conditions*

Halobacterium halobium cells were grown in shaking cultures under limited aeration as described in [6]. In some experiments 1 mM nicotine was added to the cultures. If not indicated otherwise *H. halobium* strain R_1M_1 [12] lacking gas vacuoles and bacterioruberine was used.

2.2. *Determination of the ATP/O_2 ratio*

Cells were suspended in basal salt [6] and kept anaerobically at room temperature for 3–5 h. Different amounts of oxygen-saturated basal salt were added and the change in ATP level determined [6]. The oxygen content of basal salt is 47 nmol/ml at 25°C and atmospheric pressure as calculated from the Bunsen absorption coefficient and Henry's law. All experiments were carried out at 25°C.

2.3. *Action spectrum and quantum requirement of H^+-extrusion*

Cells were suspended in basal salt and 2.5×10^{-4} M phloretin added in order to block ATP synthesis [6]. Monitoring of pH and the experimental set-up for the determination of the action spectrum and the quantum requirement were as already described [6].

2.4. *Calculation of rates of H^+-extrusion and change in membrane potential*

The average dimensions of a halobacterial cell under our growth conditions are 5 μm in length and 0.6 μm in diameter (1.4×10^{-12} ml). The cell volume can be directly estimated from cell count and total cell volume of a given suspension [13]. One ml cell

suspension of an A_{578} 5.5 has a cell volume of 7.5 μl and a protein content of 2.75 mg protein. These values are taken from [13]. The dielectric constant ϵ and the thickness of the halobacterial cell membrane are taken as those of a lipid bilayer (ϵ 3, thickness 60 Å). From these figures an electric capacity of 4.45×10^{-14} F for the bacterial cell is calculated. The dependence of the number of cycles per bacteriorhodopsin molecular on light intensity is described in [14]. The turnover number (light saturation) is about 200 s^{-1} at room temperature in the intact cell. From measurements of the bacteriorhodopsin/H^+ ratio under light saturation of the system we conclude that one proton is translocated per cycle (U. Fischer, unpublished observation). Protein was determined by the biuret method [15] and the bacteriorhodopsin concentration in whole cells was measured as described in [6]. Using the above parameters, proton extrusion per unit time and changes in membrane potential can be calculated and compared with the measured rates of photophosphorylation.

$$V/s = \frac{\text{Protons/BR·s} \times \text{BR molecules/cell} \times 1.6\cdot10^{-19}\text{Cb}}{\text{capacity of the cell}}$$

BR = Bacteriorhodopsin

$1.6\cdot10^{-19}$Cb = 1 elementary charge

Comparison of photophosphorylation mediated by bacteriorhopdopsin in brown and in purple membrane.

Cells grown in the presence of nicotine [16] were transferred to basal salt containing 1 mM nicotine and 0.2% alanine. One-third of the cell suspension served as a control. In two-thirds bacteriorhodopsin was reconstituted by addition of 13-*cis* retinal and then divided into two parts. One sample was kept aerobically for 6 h at 40°C, the other, anaerobically in the dark. Bacteriorhodopsin concentration was determined by difference spectroscopy using an unreconstituted cell suspension as reference. The localization of bacteriorhodopsin in the different membrane fractions (brown and purple membrane, respectively) of the two samples was demonstrated by sucrose density-gradient analysis of the cell lysates [9].

3. Results and discussion

3.1. *Relation of ATP/O_2 ratio to quantum yield of photophosphorylation*

Figure 1b shows changes in cellular ATP content of a cell suspension as a function of amount of oxygen added. The ATO/O_2 ratio calculated from these data is 1 ± 0.2. This ratio appears to be rather low compared with the ATP/O_2 ratio of 6 found in mitochondria. The measurements shown in fig.1A exclude the possibility that ATP hydrolysis accounts

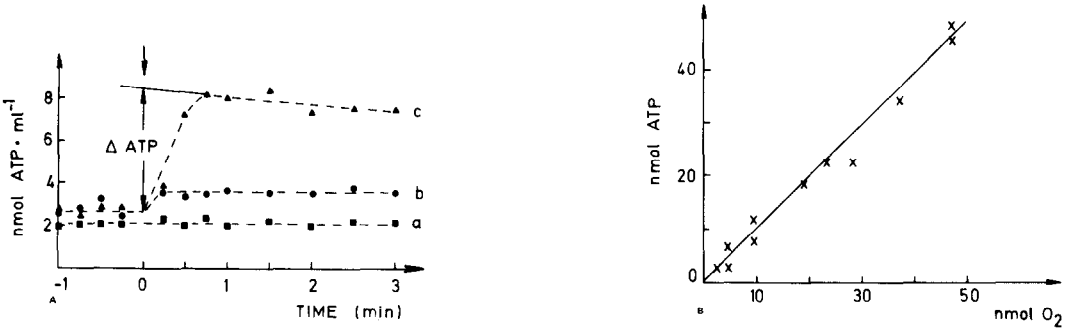

Fig.1. Determination of ATP/O_2 ratio. (A) For each experiment 5 ml buffered cell suspension (basal salt with 12.5 mM Tris–maleate, pH 6.5, 2.75 mg protein/ml) were kept anaerobically in the dark at room temperature for 4 h. Different amounts of air-saturated basal salt were added at time zero and samples for ATP determination were taken at indicated times. The ATP values were corrected for the change in volume. (–■–) Control, addition of 0.5 ml nitrogen saturated basal salt; (–●–) 0.1 ml air-saturated basal salt; (–▲–) 0.8 ml air-saturated basal salt. (B) The difference in the ATP level before and after addition of air-saturated basal salt was determined as shown in fig.1A and plotted against amount of oxygen added.

for the low ratio observed in Halobacteria. Addition of small amounts of oxygen causes small changes in the ATP level which then remains constant for the duration of the experiment (—●— fig.1A). Even when the increase in ATP level is large (—▲— fig.1A) the following decrease is very slow and the ATP/O_2 ratio obtained by extrapolation is 1.

An alternative explanation for the low ATP/O_2 ratio would be the competitive consumption of proton-gradient energy by processes other than ATP synthesis such as ion exchange, amino acid uptake or flagellae movement. If this were the case, one would expect a low quantum yield for halobacterial photophosphorylation. Indeed we observe that 22 quanta must be absorbed by bacteriorhodopsin in order to generate one molecule of ATP [6].

Experiments involving inhibition of respiration by light allow the determination of a quantitative relationship between respiration and light energy conversion [17].

Absorption of 24 quanta is required to prevent the consumption of 1 molecule of oxygen. Thus, the ATP/O_2 ratio can be predicated:

$$(1)\ \frac{\text{Photons}}{O_2} = 24$$

$$(2)\ \frac{\text{Photons}}{\text{ATP}} = 22$$

$$(1) : (2) = (3)\ \frac{\text{ATP}}{O_2} = 1.1$$

This value of 1.1 is in good agreement with the experimental data obtained from fig.1 and makes it very likely that other energy requiring processes compete with ATP synthesis in the cell. A somewhat lower value of 9–15 photons/ATP was measured for the *Halobacterium halobium* R_1 strain (R. Bogomolni, personal communication). This might well be due to a different proportion of the various energy consuming processes in the different strains.

3.2. *Quanta requirement and action spectrum of proton extrusion*

Phloretin treatment of cells leads first to inhibition of ATP synthesis and then to the disappearance of the transient alkalinization [6]. Such cells, which immediately acidify the medium were used in the experiments. Figure 2 shows that the initial rate of proton extrusion is a linear function of light intensity

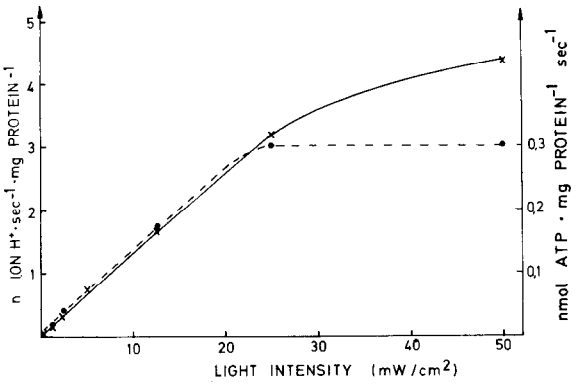

Fig.2. Rate of proton extrusion and photophosphorylation at different light intensities. For the rate of proton extrusion (—X—) 10 ml cell suspension (unbuffered basal salt, initial pH 6.6, 1.7 mg protein/ml) containing $2.5 \cdot 10^{-4}$ M phloretin were first illuminated for 10 min at a light intensity of 50 mW/cm², then kept in the dark until the pH reached a stable level. The cell suspension was then sequentially illuminated (2–3 min) at the indicated light intensities with dark periods in between. From the initial decrease of pH with time the velocity of proton extrusion was determined and plotted against light intensity. The rate of photophosphorylation (—○—) was determined as described [6] under identical conditions except that phloretin was omitted. All experiments were carried out at 25°C under nitrogen.

up to 25 mW/cm². The measured values of absolute light intensity and the absorption of the sample allow calculation of photons absorbed per proton released. Our experimental data for the quanta requirement of proton extrusion $h\nu/H^+$ vary between 1.6 and 2.5 corresponding to a quantum yield of $H^+/h\nu$ 0.4–0.6 (table 1 and [18]). This value comes close to the quantum yield of the photochemical cycle which was measured on isolated purple membranes (0.8) [19]. The action spectrum of proton extrusion was obtained by a set of experiments already shown in fig.2 but at different wavelengths. As expected, and as already demonstrated for photophosphorylation [3,6] and light inhibition of respiration (G. Krippahl, unpublished result and [18]), the action spectrum of proton extrusion also [18] is identical with the absorption band of the bacteriorhodopsin chromophore (fig.3a). However, this is only found for the strain R_1M_1 lacking the carotenoid bacterioruberine. Taking the action spectrum of R_1 cells which synthesize bacterioruberine as the predominant carotenoid (fig.3b) broken line,

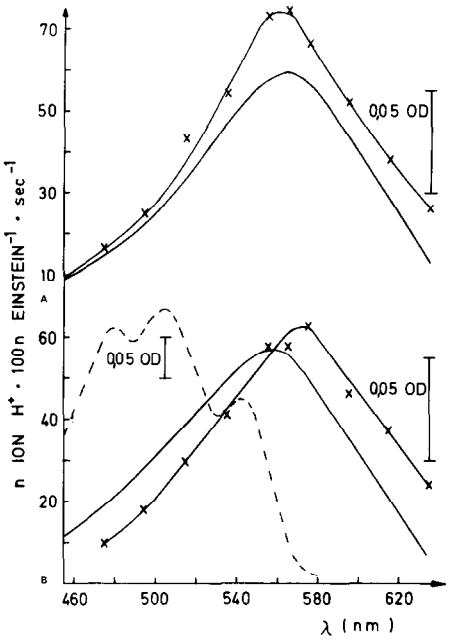

Fig.3. Action spectrum of proton extrusion and visible absorption spectrum of bacteriorhodopsin. (3A) *H. halobium* R_1M_1. (3B) *H. halobium* R_1. 10 ml cell suspension with $2.5 \cdot 10^{-4}$ M phloretin (2 mg protein/ml) was illuminated with light of indicated wavelengths and initial rate of proton extrusion determined. The measured rates (crosses) were proportional to light intensity at all wavelengths and normalized to a light flux of 100 nEinstein/s. The light absorption of bacteriorhodopsin (solid lines) in the cell suspension was determined as described [6]. The light absorption of bacterioruberine (dashed lines) in the cells was determined by difference spectroscopy of R_1 cells against R_1M_1 cells.

see [9] for comparison of R_1, and R_1M_1 spectra), a discrepancy between the action spectrum for proton extrusion and the absorption band of bacteriorhodopsin at wavelengths shorter than 550 nm is found (fig.3b). Obviously light absorbed by bacterioruberine cannot reach bacteriorhodopsin by energy transfer but is lost as heat. This seems to exclude the role of carotenoids as accessory pigments in halobacterial light energy conversion and leaves the question of carotenoid function in these bacteria unanswered.

3.3. *Correlation of proton extrusion and ATP synthesis*

The H^+/ATP ratio can be calculated from the initial rate of proton extrusion in the presence of phloretin (fig.2) and the initial rate of photophosphorylation at the same light intensity. It should be stressed that this H^+/ATP ratio has nothing to do with the mechanistic H^+/ATP ratio of the ATP-synthesizing enzyme system in the cell membrane. Provided that phloretin only inhibits phosphorylation and does not drastically influence the proton extrusion process the experimentally measured H^+/ATP ratio can be related to the set of parameters given in the above section to check for their mutual fit. A significant influence of phloretin on bacteriorhodopsin itself is not likely because the drug has no apparent effect on the quantum yield for proton extrusion (0.5 as we found, compared with 0.6, in absence of the drug [18]).

Furthermore the light-induced changes of membrane potential and pH differences in phloretin-treated cells are similar to those in *N,N'*-dicyclohexylcarbodiimide (DCCD)-treated ones [20]. DCCD applied in low concentrations is known to be a specific inhibitor of the ATP synthase.

The experimental results of table 1 compare quantum requirement of proton extrusion and photophosphorylation at varying light intensities. The H^+/ATP ratio was calculated to be 10. Therefore two more experimentally determined ratios:

$$(4)\ \frac{\text{Photons}}{H^+} = 2$$

$$(5)\ \frac{H^+}{\text{ATP}} = 10$$

are available to quantitatively relate the 4 parameters, O_2, H^+, photons, ATP of the cellular system to one another. A H^+/O_2 ratio of 10.2 was reported in the literature [21] and fits our experimental results very well.

Furthermore the data presented on table 2 allow the calculation of the number of photochemical cycles per bacteriorhodopsin per second necessary to account for minimal and maximal rates of photophosphorylation in a cell (table 1). From the values one can easily estimate that the photochemical cycle in bacteriorhodopsin has only to operate at 1–2% of its maximal speed (about 200 cycles. s^{-1} releasing 1 proton/cycle) to boost the rate of photophosphorylation to its maximum. The minimal observable rate of photophosphorylation can be produced by bacteriorhodopsin operating at about 0.01% of maximal rate.

Table 1
Quantum requirement of proton extrusion and photophosphorylation

a	b	c	d	e	f	g	h	i
Exp.	Wavelength of light (nm)	Light intensity (%)	Absorption (nEinstein/s)	H^+ (nion/s)	$\frac{h\nu}{H^+}$	ATP (nmol/s)	$\frac{h\nu}{ATP}$	$\frac{H^+}{ATP}$
I	575	25	4.8	3.0	1.6	–	–	–
		50	9.9	5.7	1.7	0.4	25	14
		100	18.7	9.2	1.9	0.85	22	11.5
II	565	25	6.2	2.5	2.5	–	–	–
		50	11.7	5.25	2.2	0.5	23	10.5
		100	23.6	9.5	2.5	1.15	20	8

Cells from two different cultures were used. Light absorption (column d) and rate of photophosphorylation (g) were determined as in [6]. The rate of proton extrusion (column e) was determined with phloretin-treated cells as described in fig.2. The numbers in columns f, h, i were computed from the values in d, e, g

At maximal rate bacteriorhodopsin would electrogenically create a membrane potential of 300 mV within the first cycle as can be calculated from the figures given in Materials and methods. At 1% a rate of approx. 300 mV/100 ms is calculated. From the measured number of protons extruded per cell at this rate (table 2) a membrane potential of 360 mV/100 ms can be computed which fits the theoretically calculated membrane potential very well.

3.4. *Efficiency of bacteriorhodopsin in different environments of the cell membrane*

Cells grown in the presence of nicotine continue to synthesize bacterio-opsin and reconstitute bacteriorhodopsin upon addition of retinal [9]. Newly formed bacteriorhodopsin is first found in the brown membrane. An energy-dependent step then converts bacteriorhodopsin to the purple membrane state. Distinct properties of bacteriorhodopsin are found depending on its membrane environment. In brown membrane this protein does not form a crystalline lattice (R. Henderson, personal communication), it is able to rotate in the plane of the membrane but is not in a monomeric form [22]. In contrast, in purple membrane trimers of bacteriorhodopsin form a crystalline lattice and are not capable of rotation [23,24]. Since photophosphorylation can start within minutes after addition of retinal [10] whereas conversion of

Table 2

a	b	c	d	e	f
Light intensity (mW/cm^2)	Rate of photophosphorylation ($nmol \cdot s^{-1} \cdot mg\ protein^{-1}$)	Rate of proton extrusion ($nion \cdot s^{-1} \cdot mg\ protein^{-1}$)	Rate of proton extrusion per cell (H^+/s)	Bacteriorhodopsin content ($nmol \cdot mg\ protein^{-1}$)	Protons released per molecule bacteriorhodopsin (H^+/s)
25	0.3	3.3	$1 \cdot 10^6$	1.2	2.7
0.25	0.027	0.027	$8.7 \cdot 10^3$	1.2	0.023

The rate of photophosphorylation (column b) proton extrusion (column c) and bacteriorhodopsin content (column e) were determined as described in [6] and fig.3. The protein content of a single cell is $5.2 \cdot 10^{-10}$ mg (see Materials and methods). Rate of proton extrusion per cell (column d) is obtained from column c multiplied by the protein content of the cell and Avogadro's number. The number of proton released per bacteriorhodopsin (column f) are obtained from column c divided column e.

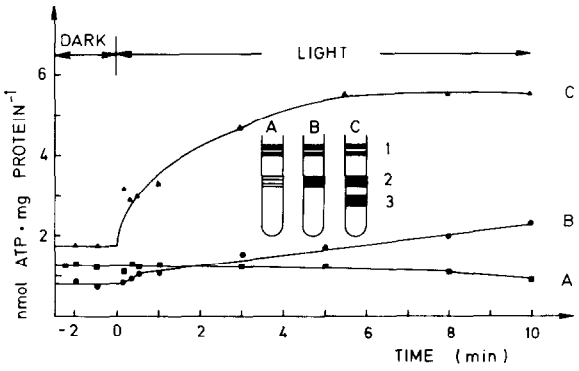

Fig.4. Photophosphorylation mediated by brown and purple membrane. 10 ml cell suspension in basal salt with 0.2% alanine and 1 mM nicotine were used for each experiment (2 mg protein/ml). Cells were pretreated as described in Materials and methods. Cell suspensions were first aereated for 10 min, kept in the dark for 20 min under nitrogen and then illuminated at a light intensity of 12.5 mW/cm^2. Samples for ATP determination were taken at times indicated. (A) Control cells without bacteriorhodopsin. (B) Bacteriorhodopsin reconstituted and localized in brown membrane. (C) Bacteriorhodopsin reconstituted and crystallized in purple membrane. Sample C contained the same total amount of bacteriorhodopsin as sample B. All three cell suspensions had the same capacity for oxidative phosphorylation. The phosphorylation experiments were carried out at 25°C and fractionation of cell membrane was as described [9]. (1) Red membrane-340. (2) Brown membrane. (3) Purple membrane.

bacteriorhodopsin to the purple membrane state is slower under these conditions. Bacteriorhodopsin in the brown membrane state is apparently able to act as a proton pump. An interesting question is whether or not bacteriorhodopsin in the brown membrane is equally well-suited to drive photophosphorylation as in the purple membrane state. Figure 4 clearly demonstrates that purple membrane is much more effective than brown membrane in supporting photophosphorylation. However, further experiments are required in order to exclude rigorously the possibility that traces of purple membrane formed during illumination mediate the observed photophosphorylation in the brown membrane sample. Such minute amounts might not be visible on our gradient. Nevertheless, crystallisation (and modification) appears to be a necessary step in transformation of bacteriorhodopsin into an efficient light energy converter.

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References

[1] Oesterhelt, D. and Stoeckenius, W. (1973) Proc. Natl. Acad. Sci. USA 70, 2853–2857.
[2] Blaurock, A. E. and Stoeckenius, W. (1971) Nature, New Biol. 233, 152–154.
[3] Danon, A. and Stoeckenius, W. (1974) Proc. Natl. Acad. Sci. USA 71, 1234–1238.
[4] Oesterhelt, D. (1975) in: Energy Transformation in Biological Systems Ciba Foundation, Symposium 31, pp. 147–167, Elsevier, Amsterdam.
[5] Danon, A. and Caplan, S. R. (1976) Biochim. Biophys. Acta 423, 133–140.
[6] Hartmann, R. and Oesterhelt, D. (1977) Eur. J. Biochem. 77, 325–335.
[7] Wagner, G. and Oesterhelt, D. (1976) Ber. Dtsch. Botan. Gesellsch. 89, 289–292.
[8] McDonald, R. E. and Lanyi, J. K. (1977) Fed. Proc. Fed. Am. Soc. Exp. Biol. 36, 1828–1832.
[9] Sumper, M., Reitmeier, H. and Oesterhelt, D. (1976) Angew. Chemie Int. Ed. Engl. 15, 187–194.
[10] Oesterhelt, D. and Christoffel, V. (1976) Biochem. Soc. Trans. 4, 556–559.
[11] Sumper, M. and Herrmann, G. (1976) FEBS Lett. 69, 149–152.
[12] Milanytch, M. (1973) Diplomarbeit. Universität München.
[13] Michel, H. and Oesterhelt, D. (1976) FEBS Lett. 65, 175–178.
[14] Oesterhelt, D., Gottschlich, R., Hartmann, R., Michel, H. and Wagner, G. (1977) in: Microbial energetics (Haddock, B. A. and Hamilton, W. A. eds) pp. 333–349.
[15] Weichselbaum, T. E. (1946) Am. J. Clin. Pathol. Suppl. 10, 40–45.
[16] Reitmeier, H. (1976) Doctoral Thesis, University of Würzburg.
[17] Oesterhelt, D. and Krippahl, G. (1973) FEBS Lett. 36, 72–76.
[18] Bogomolni, R. A. (1977) Fed. Proc. Fed. Am. Soc. Exp. Biol. 1833–1839.
[19] Oesterhelt, D. and Hess, B. (1973) Eur. J. Biochem. 37, 316–326.
[20] Michel, H. (1977) Doctoral Thesis, University of Würzburg.
[21] Belyakova, T. N., Kadzyauskas, Y. P., Skulachev, V. I., Smirnova, I. A., Chekulayeva, L. N. and Yasaytis, A. A. (1975) Dokl. Akad. Nauk SSSR 223, 483.
[22] Cherry, R., Heyn, M. and Oesterhelt, D. (1977) FEBS Lett. 78, 25–30.
[23] Henderson, R. and Unwin, P. N. T. (1975) Nature 257, 28–32.
[24] Razi Nagri, K., Gonzalez-Rodriguez, J., Cherry, R. J. and Chapman, D. (1973) Nature New Biol. 245, 249–251.