LIGHT-INDUCED CHANGES OF THE pH GRADIENT AND THE MEMBRANE POTENTIAL IN *H. HALOBIUM*

H. MICHEL and D. OESTERHELT
Institut für Biochemie der Universität Würzburg, Röntgenring 11, D-87 Würzburg, West Germany

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1. Introduction

The purple membrane of Halobacteria containing the retinal protein complex bacteriorhodopsin [1] was proposed to function as a light-driven proton pump [2,3]. Photophosphorylation mediated by bacteriorhodopsin has been observed under anaerobic conditions [4–6] and is best explained on the basis of the chemiosmotic hypothesis of Peter Mitchell [7]. Bacteriorhodopsin as an electrogenic proton pump creates transmembrane potentials and pH gradients in artificial systems such as liposomes and planar phospho lipid membranes [8–10]. For the measurement of membrane potentials and pH gradients across bacterial cell membranes (e.g. *S. faecalis*) the distribution of lipophilic ions and weak acids (e.g. DMO) has been introduced [11–14]. In this paper we report experiments demonstrating light-induced changes of the electrochemical proton gradient in intact cells of *Halobacterium halobium*.

2. Materials and methods

Halobacterium halobium R_1M_1 [15] was grown in Erlenmeyer flasks (2 litres) containing 700 ml growth medium [16] on a rotatory shaker (New Brunswick, N. J.; 105 rev/min, 40°C, 5 days, inoculum 5%). Cells were pelleted and resuspended in basal salt (BS = the growth medium without peptone and citrate). Radiochemicals were from NEN, CCCP and DMO from Sigma, $TPMP^+$ from K & K-laboratories (Plainview, N.Y.). The other chemicals were analytical grade and from Merck (Darmstadt) or Riedel de Haen (Hannover).

$[^{14}C]TPMP^+$ was synthesized from $[^{14}C]CH_3Br$ and triphenylphosphin [17]. The product gave one single radioactive spot on TLC plates (silica gel, propanol/acetone 1:1).

Accumulation of radioactively-labelled substances was measured by silicone layer filtering centrifugation [18]. 100 µl silicone oil (CR 500, Wacker, Munich) containing 15–20% bromobenzene (v/v) were layered on 50 µl CsCl solution (26% w/v) in polypropylen tubes (Greiner, Nürtingen). 200 µl cell suspension (in BS containing 75 mM Tris/Maleat, O.D. 5.5 at 578 nm, Eppendorf M 1) were added. This cell density corresponds to 4.39 mg intracellular water/ml cell suspension or 2.75 mg protein/ml (biuret method [19]) under the growth conditions used.

After one hour at room temperature (23 ± 2°C) under N_2 in the dark the tubes were centrifuged in a Beckman Microfuge 152 for 5 min. More than 99.5% of the cells had passed the silicone layer after that time (50% after 15 sec). The polypropylen tubes are cut through the silicone layer and the aqueous phases mixed with 1.5 ml water and subsequently with 10 ml of Bray's solution [20]. Quench was corrected by addition of an internal standard.

The cell suspensions were illuminated in the centrifuge by light from a projector (150 W, Rollei, Braunschweig) filtered through an OG 515 filter (Schott, Mainz). Light above 700 nm was removed by

Abbreviations: DCCD = *N,N'*-dicyclohexylcarbodiimide; DMO = 5,5-dimethyloxazolidine-2,4-dione; $TPMP^+$ = triphenylmethylphosphonium-ion; CCCP = carbonylcyanide *m*-chlorophenylhydrazone; ΔpH = pH-gradient across the cell membrane; S_{13} = 3-*t*-butyl, 5-chloro, 2'-chloro, 4'-nitrosalicylanilide; TTFB = tetrachloro-2-trifluoromethylbenzimidazole. Δψ = transmembrane potential.

a mirror (Schott, typ 213). The samples were centrifuged under illumination (7.3 mW/cm^2).

The amount of medium dragged into the tip of the tube was estimated with [^{14}C]dextran. Intracellular water content was calculated from the difference of the amount of ^3H_2O and [^{14}C]dextran found in the tips after centrifugation. The obtained values correlated very well with the cell volume determined by microhematocrit centrifugation. pH was measured with a glass electrode (405-M3, Ingold, Frankfurt/M) calibrated by a hydrogen electrode (Ingold). Since the pH of concentrated salt solutions depends on the ionic species present [21–23] a difference in pK of DMO in BS and a salt solution, which corresponds to the ionic conditions inside the halobacterial cell (80 mM $MgSO_4$, 3 M KCl, 1.3 M NaCl–K-BS) has to be expected. Titration of DMO in BS and K-BS gave pK-values of 6.04 (pK_e) and 6.22 (pK_i) respectively (water: 6.32 [24] at 25°C. The usual equation for the calculation of pH [14, 24, 25] has to be modified according to this difference in pK of DMO inside and outside the cells. This leads to:

$$\Delta pH = pH_i - pH_e = pK_i + \log \frac{A_i^{to}\, 10^{-pK_e} + (A_i^{to} - A_e^{to})\, 10^{-pH_e}}{A_e^{to}} \quad (1)$$

pH_i and pH_e are internal and external pH; pK_i and pK_e internal and external pK of DMO (see above); A_i^{to} and A_e^{to} are the sums of the concentrations of the undissociated and the dissociated DMO and were measured as dpm/kg water.

Equilibration of [^{14}C]DMO (22 μM, 8.8 Ci/mol) occurred in less than 30 sec. In the concentration range used the degree of accumulation was independent of the DMO-concentration.

The precision of this method is limited by [^{14}C] DMO dragged with extracellular water into the lower aqueous phase of the tubes. At small pH differences (below ΔpH of 0.5) the amount of this extracellular DMO becomes larger than intracellular DMO and increases experimental error.

Equilibration of [^{14}C]TPMP$^+$ (3.5 μM, 5 Ci/mol) is only complete after 10 min and therefore does not allow kinetic measurements. No correction for different activity of TPMP$^+$ in the cytoplasma and the medium was made.

3. Results and discussion

Lipophilic ions like the tetraphenylboron anion or the triphenylmethylphosphonium cation as probes for membrane potentials have been introduced some years ago [26]. For a given membrane potential, e.g. positive outside of the cell, TPMP$^+$ will accumulate inside the cell until its diffusion potential will have the same size as the membrane potential. This accumulation would destroy the membrane potential if it is not kept constant by an internal energy source, e.g. a proton pump. In fact a 1:1 exchange of H^+ or Na^+ and TPMP$^+$ during the accumulation of the latter ion has been observed [11,13]. At a given membrane potential, the necessary net uptake of TPMP$^+$ will depend on its concentration in the medium. The energy requirement for this accumulation will increase with the external concentration and eventually exhaust the internal energy pool of the cell. This is expected for the cells used in the experiment of fig.1 which are kept anaerobically in the dark. Increasing TPMP$^+$ concentrations decrease the degree of accumulation. In all the further experiments we used therefore 3.5 μM TPMP$^+$.

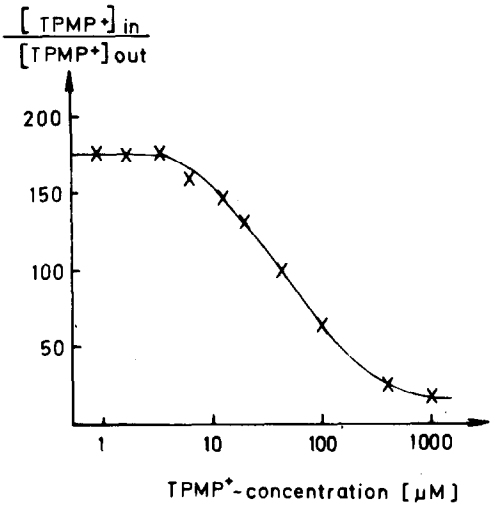

Fig.1. Dependence of TPMP$^+$ accumulation on its concentration in the medium. The experiment was carried out as described under Material and methods. External pH was 8.

Table 1
Effect of an uncoupler (CCCP) on ΔpH and accumulation of [^{14}C]TPMP$^+$

CCCP		0	10^{-6} M	10^{-5} M
ΔpH	at pH 6.05	1.02	0.45	0.22
$\frac{[TPMP^+]_{in}}{[TPMP^+]_{out}}$	at pH 6.05	35	15.5	a
$\frac{[TPMP^+]_{in}}{[TPMP^+]_{out}}$	at pH 7.11	77.9	22.5	a
$\frac{[TPMP^+]_{in}}{[TPMP^+]_{out}}$	at pH 8.05	184.0	32.1	a

[a]An increase of the accumulation of TPMP$^+$ is observed at concentrations of 10^{-5} M and higher. No interpretation of this effect can be given at present.

Uncouplers decrease the degree of accumulation of TPMP$^+$ but do not induce the expected equal distribution of the ion (table 1). This is independent of the chemical nature of the uncoupler. S_{13} and TTFB show the same effect as CCCP. The presence of an uncoupler makes a residual membrane potential carried by protons unlikely. Because of the very high ionic strength a Donnan potential or differences in surface charge of the two sites of the cell membrane also hardly account for this accumulation. We therefore assume that a small but constant portion of TPMP$^+$ is absorbed by cell components. This must lead to a large error in the calculation of the membrane potential at low values of the ratio $TPMP^+_{in}/TPMP^+_{out}$. For instance, an observed ratio of 10 would give a membrane potential of 50 mV if it is due to an accumulation, but zero if it is due to absorption. Under the same condition a ratio of 300 gives a $\Delta\psi$ of 146 mV but 144 mV after correction for the absorption artifact. This means that most of the membrane potentials in table 2 have low errors. In addition, the listed changes in membrane potential and pmf. are the minimal values because the data of table 2 were not corrected for the absorption artifact.

pH can be calculated from the accumulation of [^{14}C]DMO as given be equation [1]. In tables 1 and 2 the pH differences found under different conditions are summarized. The internal pH is always more alkaline than the external pH (see also [6]). At pH 6

Table 2
Light-induced enhancement of the electrochemical proton gradient in *H. halobium* cells and the influence of DCCD treatment

pH_e	Light 7.3 mW/cm^2	DCCD treated	$\Delta\psi$ (mV)	ΔpH	pmf. (mV)	Change of pmf. by light
6.03	–	no	87.0	1.08	150.7	35.5
6.02	+	no	113.0	1.24	186.2	
5.99	–	yes	88.4	0.46	115.5	171.4
5.95	+	yes	155.9	2.17	283.9	
6.76	–	no	102.4	0.60	137.8	30.7
6.74	+	no	126.0	0.72	168.5	
6.83	–	yes	90.5	0.41	114.7	153.7
6.76	+	yes	150.4	2.00	268.4	
8.02	–	no	138.7	0	138.7	22.3
8.01	+	no	151.5	0.14	159.8	
8.08	–	yes	96.2	0.30	113.9	11.7
8.05	+	yes	141.2	1.43	225.6	

Cell suspensions ($O.D_{578}$ = 5.5) were incubated overnight at 30°C with and without 10^{-4} M DCCD, centrifuged and resuspended in BS containing 75 mM Tris maleat buffer. The uptake of [^{14}C]TPMP$^+$ and [^{14}C]DMO were measured as described under Materials and methods. Time of illumination 15 min. $\Delta\psi$ (mV) is calculated from $\Delta\psi = RT/nF \times \ln [TPMP^+]_{in}/[TPMP^+]_{out}$, ΔpH from equation (1). The electrochemical proton gradient pmf. (mV) equals $\Delta\psi + 59\ mV \times \Delta pH$.

ΔpH is 1 and drops to zero at pH 8. The ΔpH of cells treated with DCCD (DCCD-cells) is smaller but independent of external pH. This result is in accordance with data obtained with *Cl. pasteurianum* and *S. faecalis* [12,14] but no explanation of this effect can be given.

The aim of the experiments in table 2 is to demonstrate the large changes in the electrochemical proton gradient induced by the action of the light-driven proton pump bacteriorhodopsin. The results from DCCD-cells and untreated cells are compared. At pH 6 (table 2, first block) a change of pmf. upon illumination of 35 mV occurs in untreated cells, whereas a change of 171 mV is observed after DCCD treatment. The enhancement of pmf. by illumination is mainly due to a change in membrane potential (increase by 26 mV but only 0.16 pH units) for untreated cells whereas DCCD-cells show an increase of 67.5 mV in $\Delta\psi$ and a large change in ΔpH (1.7 pH units) upon illumination. An enlargement of the acidification in unbuffered medium after DCCD treatment has already been described [6] and interpreted as the uncoupling of a proton pump from the ATP synthase system which is blocked by DCCD. For accurate measurement of $\Delta\psi$ however it is important to use buffered media in order to exclude changes in $\Delta\psi$ introduced by changes in external pH. Changing pH_e from 6 to 8 results in a decrease of ΔpH and an increase of $\Delta\psi$ so that the resulting pmf. remains constant. This effect and its relation to the ATP level in the cell will be discussed in a following paper.

Independent of the external pH (table 2, last column) the change of pmf. induced by light in DCCD is 5 times higher than in untreated cells. The largest electrochemical proton gradient we measure is 284 mV (table 2, 6th column). The electromotive force of bacteriorhodopsin must therefore have at least that size. This corresponds to a free energy change which is large enough to explain all changes in ATP-level we observe in the halobacterial cell.

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