PHOTOELECTRIC SIGNALS FROM DRIED ORIENTED PURPLE MEMBRANES OF *HALOBACTERIUM HALOBIUM*

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ABSTRACT In dried oriented samples of purple membrane isolated from *Halobacterium halobium*, the photoelectric activity decreases and the light adaptation vanishes when the water content of the sample is lowered. In the photocycle the first steps of the proton movement were accelerated with decreasing humidity, while the last steps of the photocycle could not be observed. From the analysis of the photoelectric signal we conclude that at low humidities the protons move forward in the L decay and return to their original place during M decay.

INTRODUCTION

Dried oriented samples of purple membranes (pm) isolated from *Halobacterium halobium* can be prepared (Váró 1982). The dried bacteriorhodopsin molecules (BR) preserve some features of their activity for light excitation. The samples can be stored for a long time (>2 yr) without a significant loss of activity and show a large electric response signal (>10 V) when continuously illuminated. They may have a practical application as photoelements.

Previously, the light-dark adaptation of the BR (Korenstein and Hess, 1977*a*), the photocycle (Korenstein and Hess, 1977*b*); and the photoelectric activity in continous light (Nagy, 1978) were studied in dried or in partially oriented dried samples, respectively. Here we report a detailed study of the photovoltage in continuous light, the kinetic parameters of the electric response signals, and the photocycle as a function of the humidity of the sample.

MATERIALS AND METHODS

The pm used in the preparation of dried samples were obtained by the standard procedure from *Halobacterium halobium* strain NRL R_1M_1 (Oesterhelt and Stoeckenius, 1974). The suspension was further purified by washing in triple distilled water until the conductivity of the suspension stabilized at a small value (<50 μS). Then the pH of the suspension was set with NaOH or HCl. Generally we used BR suspension of 300 μM with pH set between 6 and 7.

The preparation of dried oriented samples has already been described in detail elsewhere (Váró, 1982). Here we review only the major points. A SnO_2 covered glass slide served as an anode, a small quantity of pm suspension was placed on it and covered by a Pt cathode. The distance between the electrodes in contact with the suspension was 0.5–2 mm. An electric field of 20–40 V/cm oriented the pm by their permanent dipole moment and deposited them on the anode by electrophoresis due to their negative charge. The samples were dried by first carefully removing the conducting glass with the deposited pm, then the excess water was pipetted off the top and the samples air dried. The absorbance of the sample at λ = 570 nm was 1. Such samples are stable for years.

The photoelectric measurements were made with a vibrating plate electrometer (homemade, with a sensitivity of 0.5 mV and response time 0.1 s) under continuous light illumination by a mercury lamp (HBO 200; Carl Zeiss, Inc., Jena, German Democratic Republic) through heat and light filters. For flash-induced fast electric signal measurement, a second electrode (Sn or Al) was evaporated onto part of the sample. The measuring circuit is shown in Fig. 1. The sample, shunted with a resistor, was connected through a 5-MHz homemade amplifier to a transient recorder (NEO 200-B; Central Research Institute for Physics, Budapest, Hungary; shortest conversion time 0.1 μs) and the data were stored in a multichannel analyzer (ICA 70; Central Research Institute for Physics, Budapest, Hungary). We could also measure the optical changes on the same sample across that part that was not covered by the second electrode. To initiate the photocycle an Opton dye laser (λ = 580 nm, pulse length = 1 μs, energy ~ 10 mJ; Lambdaphysics, Göttingen, Federal Republic of Germany) was used.

The lifetimes obtained from the optical measurements depended on the intensity of the measuring light. The population of an excited state (n, normalized to one) changes in time

$$\frac{dn}{dt} = \phi_\lambda \sigma_\lambda (1 - n) - kn,$$

where ϕ_λ is the flux of photons in the measuring light, σ_λ, the cross section for excitation, and k is the rate constant of the decay from this state. We are interested in the decay when a laser flash at $t = 0$ excited n_g molecules additionally. The solution of the equation is

$$n = \frac{\sigma_\lambda \phi_\lambda}{k + \sigma_\lambda \phi_\lambda} + n_g e^{-(k+\sigma_\lambda\phi_\lambda)t}.$$

In the solution there is an apparent decay rate $k' = k + \sigma_\lambda \phi_\lambda$. To have a real comparison of the lifetimes from optical and electrical measurements, the same monitoring white light with constant intensity was applied. The wavelength for optical measurements was filtered behind the sample by a monochromator (Carl Zeiss, Inc.) (Fig. 1). In the results we give the apparent lifetimes.

Different humidities were obtained by incubation of the sample in the atmosphere over various saturated salt solutions (Weast, 1979). During measurements the temperature and humidity of the sample was kept at the necessary value by a continuous flow of nitrogen gas at the given humidity and temperature. The quantity of water uptake for a given relative humidity was determined by the method described by Jendrasiak and Hasty (1974) using a microbalance (Beckman Instruments, Inc., Cedar Grove, NJ) that continuously registered the weight of the sample in the incubation chamber. Equilibrium was reached within 1 h.

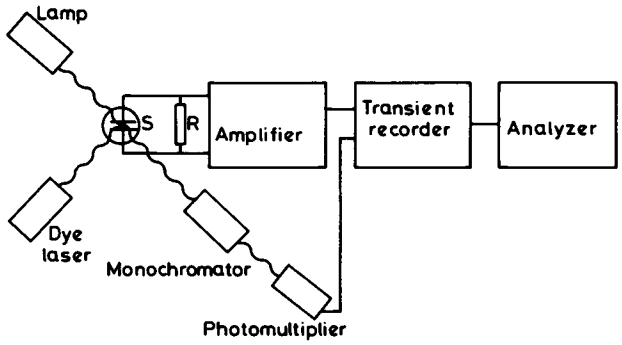

FIGURE 1 Scheme of the measuring system.

RESULTS AND DISCUSSION

First the water uptake of the dried oriented sample, which was incubated in a humidity controlled atmosphere, was determined (Fig. 2). The curve N shows the number of water molecules per BR molecule while the curve S denotes the average area per H_2O on the surface of the pm. The number of BR molecules was determined by an absorption measurement using $\epsilon_{BR} = 63{,}000$ at $\lambda = 570$ nm while the surface area for one BR molecule on one side of the pm was calculated from the known crystalline array (Stoeckenius et al., 1979). The shape of the N curve is very similar to the adsorption isotherms of water uptake by pure bulk phospholipids (Jendrasiak and Hasty, 1974). The curves between relative humidity $P/P_o = 0.6$–0.7 tend to a saturation of $N \simeq 100$ H_2O/BR or $S = 0.10$ nm^2, which is aproximately the area of one water molecule. Therefore we conclude that at $P/P_o = 0.6$–0.7, a single continuous water layer is formed on the pm. Above this relative humidity value a rapid water uptake occurs.

In continuous green light illumination large photoelectric signals (up to 30 V) were measured with the vibrating plate electrometer. The electrode at the glass surface (i.e., the anode during orientation) is the negative pole in the case of this photoelement-like behavior of the dried oriented sample. Light-driven protons move in the direction of the positive charge of the pm dipole. As we increased P/P_o above 0.6, the photovoltage rapidly decreased due to the shunting effect of the increased water content of the sample. This observation corroborates the above explanation of the adsorption isotherm. When water is adsorbed on the pm in one layer the resistance of the sample is very large ($>10^{11}$ Ω) and only the additional water taken up lowers the resistance.

In Fig. 3 the effect of light and dark adaptation on the photovoltage is shown. First the relative humidity of the sample was set. Then the photovoltage on the dark-adapted sample was measured with a light pulse of 1-s duration. The sample was light adapted by illumination for 30 min with 75 W/m^2 green light and the photovoltage measured with a 1-s pulse. Both curves tend to the same value at low humidity. The photoelectric response of the dark-adapted sample remains constant below $P/P_o = 0.6$, while the response of the light-adapted sample increases. Above $P/P_o = 0.6$ both curves fall for the reason explained above, i.e., lowering of the sample resistance. When we extrapolate the photoelectric response from the data below $P/P_o = 0.6$ to $P/P_o = 1$, the light-adapted sample has approximately twice the response as that of the dark-adapted sample.

Accepting that the BR molecules with all-*trans* retinal are the only ones photoelectrically active (Stoeckenius et

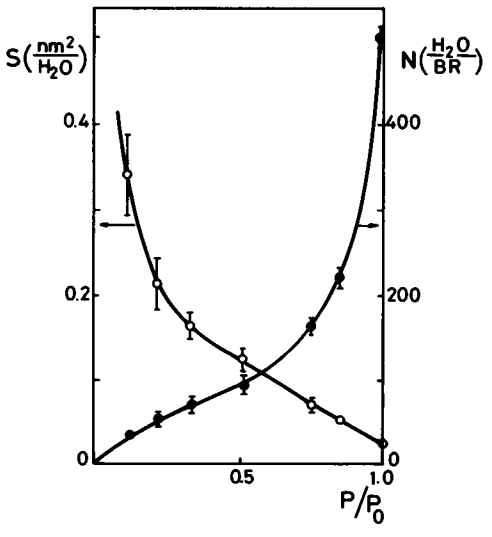

FIGURE 2 The number of water molecules per bacteriorhodopsin molecule (●) and the average area per H_2O on the surface of the pm (O) at different relative humidities.

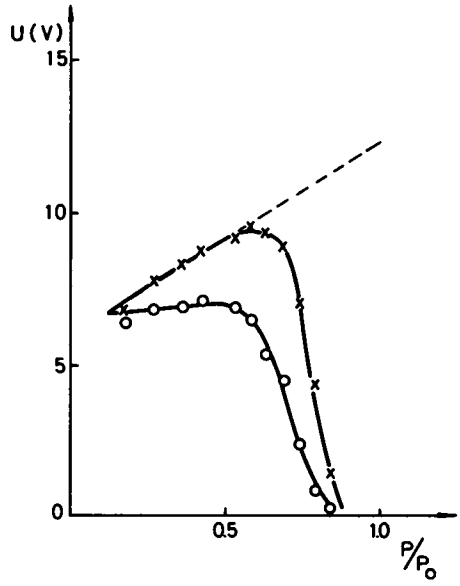

FIGURE 3 The effect of the humidity on the photoresponse of the dark-adapted (O) and light-adapted (x) bacteriorhodopsin.

al., 1979), we may conclude that the light adaptation decreases at low humidities. This is consistent with the results of Korenstein and Hess (1977*a*) obtained from optical measurements.

The decay time of a light-adapted sample to a dark-adapted one was also measured, and a marked increase with decreasing relative humidity was observed. This result shows a dependence of the energy barrier on humidity for the spontaneous *trans-cis* isomerization involved in dark adaptation. We note, however, that the light-driven adaptation (*cis-trans* isomerization) and the thermally activated dark adaptation (*trans-cis*) are different processes (Casadio and Stoeckenius, 1980).

The photovoltage depends strongly on the pH of the starting pm suspension. The largest responses were obtained at pH 6–7. Below pH 6 the signal diminished and disappeared around pH 5. At lower pH's the signal reappeared but with an opposite sign. This observation is easily understood by the fact that the direction of the pm dipole moment changes its sign at this pH (Barabás et al., 1983; Fisher et al., 1978).

The protein electric response signal (PERS) evoked by flash excitation of BR in dried oriented sample has three main components (Fig. 4). The first and second components have features similar to PERS in pm suspension (Keszthelyi and Ormos, 1980) but with different lifetimes. The third component differs both in sign and lifetime. The first component of PERS is negative related to the direction of the proton pump. The fast signal cannot be further resolved into components at all humidities because of the 1-μs laser pulse and the time resolution of the electronics (~0.5 μs). This signal is the electric associate of all transitions before L formation. The second component is positive and may be resolved into two exponentials with lifetimes $\tau_1 \simeq 6$ μs and $\tau_2 \simeq 40$ μs at a relative humidity $P/P_0 = 0.5$. The same lifetimes were found in light absorption measurements at $\lambda = 400$ nm and 525 nm corresponding to the L-M transition. Therefore this PERS component is assigned to the M formation. The third component is negative and can also be decomposed into two exponentials: $\tau_3 \simeq 40$ ms and $\tau_4 = 800$ ms (at $P/P_0 = 0.5$).

The PERS and absorption signals were measured on samples of different water content and the determined lifetimes are compared in Figs. 5–8. Figs. 5 and 6 show data for the L-M transition. All decay curves could be fitted by two components of different time constants (τ_1 and τ_2). The curves show excellent agreement between the PERS and absorption time constants. With increasing water content, τ_1 and τ_2 get longer, τ_2 approaches the lifetime of the L-M transition measured in pm suspension (Keszthelyi and Ormos, 1980), and the amplitude of the τ_1 component is reduced.

While the photocycle in the dried samples is similar to the photocycle in suspension until the M state is formed, during the subsequent transitions both the PERS and optical signals show different behavior. The most striking difference is the slow negative PERS in contrast to the positive components in pm suspension (Keszthelyi and Ormos, 1980) and the lack of the characteristic O absorption peak at 640 nm (Stoeckenius et al., 1979). The changes of the τ_3 and τ_4 lifetimes with the water content of

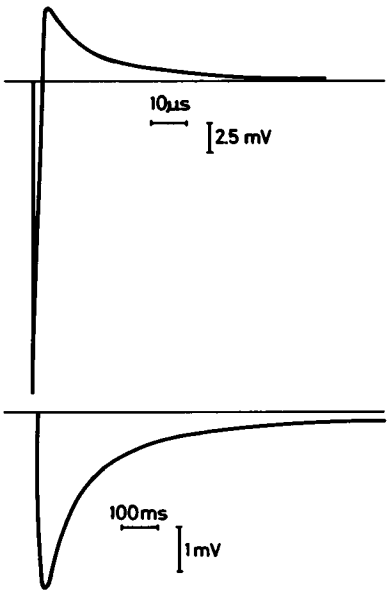

FIGURE 4 The protein electric response signals (PERS) measured in dried samples. The values of the shunting resistance were 1 kΩ for the fast signal and 5 MΩ for the slow one.

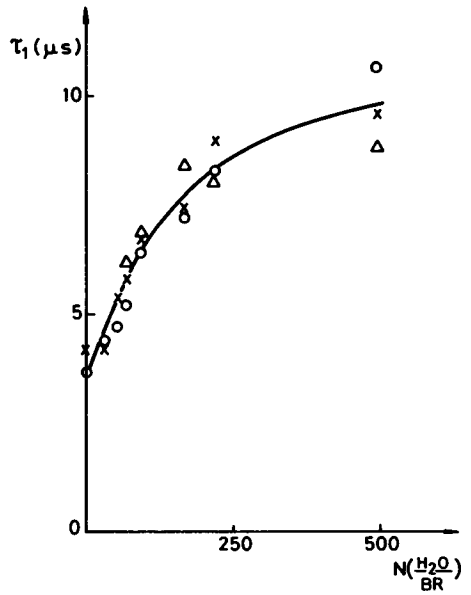

FIGURE 5 The shorter lifetime of the L-M transition measured in PERS (O), and of the optical changes at 400 nm (x) and 525 nm (△) at different water content of the sample. The errors are $< \pm 10\%$.

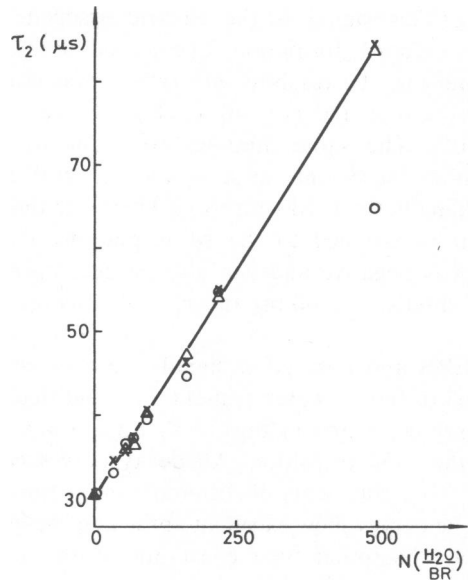

FIGURE 6 The longer lifetime of the L–M transition measured in PERS (O), and of the optical changes at 400 nm (x) and 625 nm (△) at different water content of the sample. The errors are < ± 10%.

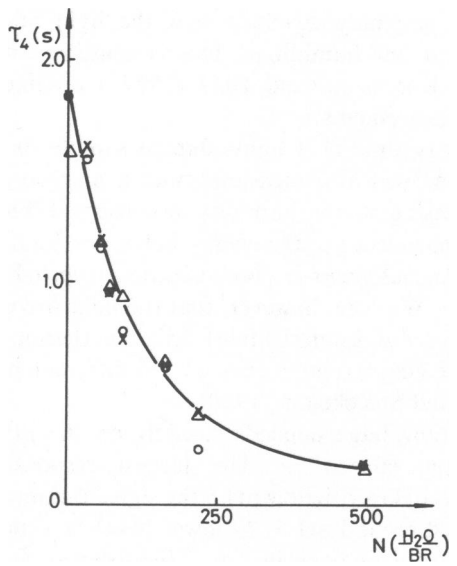

FIGURE 8 The longer lifetime of the M decay measured in PERS (O), and of the optical changes at 400 nm (x) and 590 nm (△) at different water content of the sample. The errors are < ± 10%.

the sample are shown in Figs. 7 and 8. The correlation of the values from electric and optical measurements is fairly good for the whole humidity range.

The negative sign means a backflow of protons in our interpretation of PERS (Keszthelyi and Ormos, 1980; Ormos et al., 1980). If the protons move forward in the L-M transition and flow back from this position, then the positive and negative areas of the signals representing forward (S_2) and backward (S_3) motions should reflect this. The areas were determined by a planimeter and are given in Table I for different humidities. The mean value of the area ratio below 0.5 humidity is ~ 0.73. The areas are proportional to the distances that the proton moves. It has been determined by Keszthelyi and Ormos (1980) that the very first charge displacement is negative $d_1 = -0.15$ nm and the distance in the L-M transition is $d_2 = 0.5$ nm. The protons from M intermediate can reach the orginal place by a displacement of $d_3 = -0.35$ nm. (The nanometer values of the displacements were determined with the assumption that only one proton was displaced per photocycle.) These values $|d_3|/|d_2| = S_3/S_2 = 0.7$ are in good agreement with the mean value found in our experiment.

The S_3/S_2 ratio rapidly decreases when the humidity is above 0.75. The appearance of the absorption signal of the O form at $\lambda = 640$ nm in this humidity range was also observed. These data can be interpreted as the consequence of the partial recovery of the transmembrane proton pump

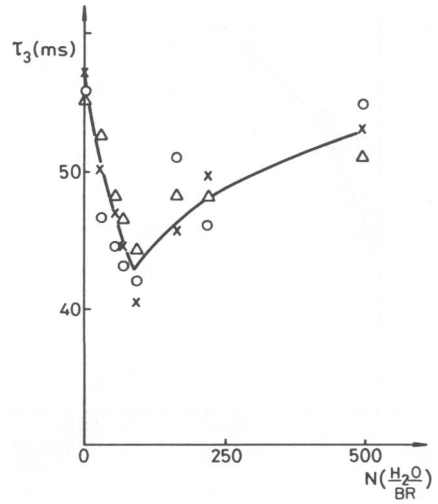

FIGURE 7 The shorter lifetime of the M decay measured in PERS (O), and of the optical changes at 400 nm (x) and 590 nm (△) at different water content of the sample. The errors are < ± 10%.

TABLE I
AREA OF THE PERS SIGNAL FOR M FORMATION (POSITIVE SIGNAL) AND M DECAY (NEGATIVE SIGNAL) AND THEIR RATIO AT DIFFERENT RELATIVE HUMIDITIES*

P/P_0	Formation of M S_2‡	Decay of M S_3‡	S_3/S_2
0	0.225	0.154	0.68
0.12	1.27	0.86	0.68
0.22	1.47	1.24	0.84
0.33	1.01	0.867	0.86
0.51	1.7	1.04	0.61
0.75	2.94	1.38	0.47
0.85	5.89	1.18	0.1
1	8.94	0.0325	0.0036

*Estimated accuracy, S_3/S_2 ± 15%.
‡Arbitrary units.

of BR. In the pm suspension d_3 was positive and $|d_3|/|d_2| \simeq 6$ showing the large displacement needed for proton release at the external surface of pm (Keszthelyi and Ormos, 1980).

Note the effect of water content on the lifetimes of the transitions. The L-M transition was accelerated with a factor of two by drying the sample while the M-O transition was hindered and consequently the proton was not released by the BR. It is very probable that the L-M transition occurs deep in the protein while in the M-O transition, the proton reaches the membrane surface (Stoeckenius et al., 1979; Kalisky et al., 1981; Keszthelyi et al., 1982). That is why in the photocycle the water molecules can only slightly modify the processes leading to M formation. The next steps of the photocycle need water molecules in abundance. The water may take part directly in this step of the proton-pumping mechanism.

The nonexponential decay of the L and M intermediates was previously approximated by two exponentials. To explain the data in terms of branching in the photocycle, we assume that the branching starts with the decay of the K intermediate and results in two different L and M intermediates. The fit of the M-decay curve with two exponential components is not very good; a third component improves it significantly, which would force us to add additional branches.

We are reluctant to accept such a complicated photocycle and prefer a linear sequence of transitions: BR–K–L–M–BR with an alternative explanation for the nonexponential behavior of the decay curves. We assume that the thermal barriers determining the rate constants have a distribution around a mean value. During drying of the sample the slightly different conformations of the BR proteins become stabilized similarly to myoglobin molecules at low temperature (Austin et al., 1975). Austin et al. have shown that the stabilized protein conformations manifest themselves in a continuous distribution of the barriers and consequently in nonexponential decay curves. Further studies to illuminate this very important point are in progress.

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