Primary charge separation in halorhodopsin

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1. INTRODUCTION

Halorhodopsin (hR) is a retinal-binding protein in the cell membrane of *Halobacterium halobium* [1–4]. On the basis of light-induced volume change measurements on cell envelope vesicles, hR has been suggested to act as a light-driven Cl^- pump [5]. Direct demonstration of Cl^- pump activity has been obtained by measuring steady-state Cl^--dependent photocurrents with cell membrane fragments, and with proteoliposomes containing the purified chromoprotein, attached to black lipid membranes [6,7].

The structure and transport mechanism of hR, however, are less characterized than those of bacteriorhodopsin [8]. After flash-excitation hR undergoes a photocycle which can be followed by flash spectroscopy [9]. On the other hand, no experiments investigating the kinetics of electric events during the photocycle of hR have been carried out so far.

Here, we report the kinetic measurement of a photoelectric signal of cell membrane fragments containing hR. The fast part of the signal is assigned to a primary charge separation. Another component corresponds probably to the second step of the hR photocycle.

2. MATERIALS AND METHODS

Tween-washed membrane fragments containing hR we prepared from the bacteriorhodopsin-deficient mutant strain OD2 by the procedure in [6]. They were oriented by an electric field and immobilized in acrylamide gel as described elsewhere for purple membranes [11]. From dynamic light scattering measurements an average diameter of the membranes of about 100 nm was determined. Therefore, a 25-times higher electric field was necessary to obtain the same degree of orientation of the Tween-washed membranes as of purple membranes (assuming a comparable permanent dipole moment per unit area). On the other hand, the electric current in the sample had to be limited in order to avoid bubble formation, as a consequence of electrolysis, and warming up of the sample. Taking this into account, the orientation was achieved by applying to the sample (resistance > 1 kΩ) a series of rectangular pulses, of 20 ms duration and 7 Hz repetition rate with an amplitude of 80 V. The degree of the orientation was monitored by measuring the light scattered by the membrane suspension, as described [12]. Under these conditions the degree of orientation of Tween-washed membranes achieved was 15–20-times lower than that of purple membranes.

During the application of the electric field the membranes in their oriented state were immobilized by polymerization of the acrylamide gel. The time of polymerization was limited to 20 s to avoid electrophoretic migration of the membranes to one of the electrodes. The gel containing oriented Tween-washed membranes was then washed and bathed in 100 mM NaCl (pH 6.0) for 1 day.

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Pieces of 6 × 3 × 2 mm were cut from the gel, put in the middle of a 20 mm wide cuvette, and filled up to 3 mm with 100 mM NaCl. Platinum electrodes were immersed at a distance of 20 mm. The whole cuvette was in a shielded cage with a hole of 2 × 2 mm for the light. The sample was excited by a 575 nm laser pulse of 10 ns length (Lambda Physics EMG 100, FL 2001). The signals were amplified by a fast current amplifier, recorded with a Gould Transient Recorder 4500 and processed by computer. The rise time of the whole system depended slightly on sample resistance and was approx. 0.5 μs.

3. RESULTS AND DISCUSSION

The measured time dependence of the photoelectric signal of hR can be observed in fig.1. To exclude laser and electrode artifacts, the sample was turned to the opposite direction leaving the electrodes unchanged. The signal changed sign, indicating that the photoresponse is due to a charge movement in the oriented membrane.

The signal is characterized by a fast rise and a biexponential decay with two time constants $\tau_1 \simeq 0.4\,\mu$s and $\tau_2 \simeq 2\,\mu$s. Its amplitude is about 10^3-times smaller in the hR-containing sample than that in bR measured under the same conditions. However, this can be explained by the much smaller degree of orientation and the higher leakage current around the smaller Tween membrane patches.

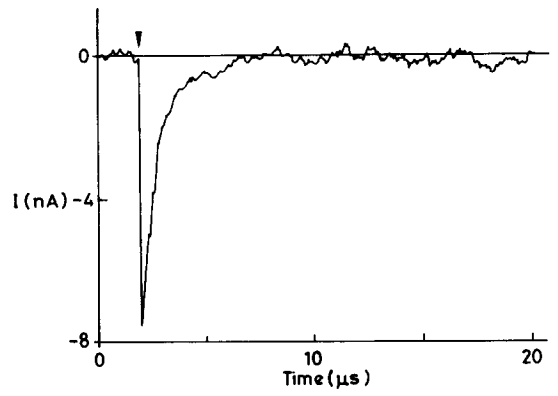

Fig.1. Transient photoelectric signal of hR oriented and immobilized in gel. 100 mM NaCl, pH 6.0, room temperature. The arrow shows timing of the laser flash.

The rise and the fast decay (τ_1) of the signal have to be considered as the response of the amplifier to a charge displacement within the sample faster than the rise time of the amplifier. To investigate this, a fast charge pulse (pulse length 100 ns) was applied to the amplifier-sample system, yielding a signal decaying with a single exponential of time constant equal to τ_1. These results lead to the conclusion that the charge displacement reflected in the fast part of the signal is faster than τ_1 and must be related to the submicrosecond steps in the hR photocycles.

A second compound could be distinguished in the hR photoresponse with a lifetime of $\tau_2 \simeq 2\,\mu$s. This seems to correspond to the second step in the hR photocycle ($hR_{632} \longrightarrow hR_{520}$) [13] which, at room temperature, takes place within a few microseconds.

In the ground state of hR 2 spectroscopically distinct forms of the retinal protein exist, hR_{578} and hR_{565}. Their relative populations are strongly dependent on Cl^- concentration. Measurements performed on samples with lower NaCl concentrations (10^{-3} M), which decrease the amount of the Cl^--transporting form hR_{578}, did not reveal changes in the amplitudes or time constants of the signal. Therefore we conclude that the fast photoreactions reflected by our experiment are nearly the same for hR_{565} and hR_{578}.

Recently it was shown [19] that the photocycle of hR branches at hR_{520} into the active transport cycle and the metastable side product hR_{410}. Azide catalyses the formation of hR_{410}, whereas blue light drives it back to the ground state hR_{578} [19].

In accordance with those findings it has been demonstrated that yellow light in the presence of azide abolishes the pump current [20]. On the other hand, blue light reactivated the pump. The same azide effect could be observed for the fast electrical response of hR as shown in fig.2. This provides strong evidence that the electrical signal is related to the hR photocycle and that the photochromic behaviour of hR is also reflected in the fast photoelectric response of the protein.

The models attempting to explain the function of hR are based on the main role of retinal [14]. Accepting this picture, one can expect a close relationship between the photocycle and photoelectrical signal in hR similar to that in bR. From this it follows that the fast charge displacement (τ_1)

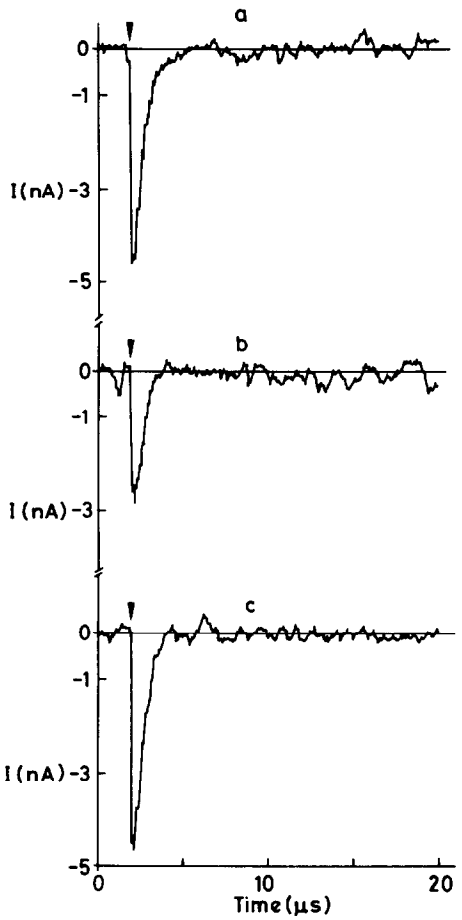

Fig.2. Transient photoelectric signal of hR oriented and immobilized in gel. Conditions as in fig.1 except that 5 mM azide was added to the solution. (a) Without background light. (b) With yellow background light, which accumulated hR_{410} thereby diminishing the population of the photoactive ground state of hR. (c) With yellow and blue background light. Note that the additional blue light restores the original signal.

described above corresponds to the only sub-microsecond step in both hR photocycles, the light-induced $hR_{578} \xrightarrow{h\nu} hR_{632}$ and $hR_{565} \xrightarrow{h\nu} hR_{630}$ transitions, which take place within ~5 ps [15]. The first light-induced step in the bR photocycle (τ = 12 ps) reflects the primary charge separation caused by the *trans-cis* isomerization of the retinal molecule [16]. It is probable that the signal we report is the first direct electrical indication of an analogue process in hR. It is in accordance with the results of Lanyi [17] pointing out the retinal isomerization in hR by optical methods.

The second component of the photoresponse $\tau_2 \simeq 2\,\mu$s was tentatively assigned to the first thermally induced steps of the hR photocycles. Because the signal is Cl^- independent, these steps apparently do not involve Cl^- translocation.

All the following steps of the hR photocycle are in the millisecond range. According to the idea of coupling between the electric and optical signals, we would also expect electrical components corresponding to these transitions. Assuming similarity between the primary processes of hR and bR, and provided that the main charge displacement in hR takes place in the millisecond range as in bR, we can estimate the amplitude of these components [18]. The calculated amplitudes are in the picoampere range, far below the noise level of the present experiments. Improvements in the sample preparation procedure are expected to provide data on the long-lived components of the photoelectric signal.

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