NONLINEAR VOLTAGE DEPENDENCE OF THE LIGHT-DRIVEN PROTON PUMP CURRENT OF BACTERIORHODOPSIN

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ABSTRACT The light-driven proton pump current generated by bacteriorhodopsin reconstituted in asymmetric planar bilayer membranes was investigated. The current-voltage dependence was found to be nonlinear and can be approximated by an exponential at least below +50 mV. The current changed e-fold when the membrane potential was changed by 80 mV. The voltage dependence was analyzed in terms of a barrier model. This analysis revealed an effective displacement of 0.63 elementary charges across the membrane during the rate-limiting step. Comparison of this value with the results from flash-induced photovoltage signals suggests that one proton is pumped per cycle.

INTRODUCTION

Bacteriorhodopsin (bR) is an integral membrane protein of *Halobacterium halobium* which acts as a light-driven proton pump. Light energy is converted into an electrochemical proton gradient across the plasma membrane of these bacteria, which in turn can be utilized to produce ATP and to drive other processes. This protein is a convenient model system to study the molecular properties of ion pumps. We have reconstituted bR into planar lipid bilayers to investigate the voltage dependence of the photocurrent which directly reflects the pump activity. In contrast to previous results (Bamberg et al., 1981) we found that the current-voltage relationship is nonlinear. There was no evidence for a reversal potential down to −160 mV. The current–voltage relationship reported here is in good agreement with the predictions from a barrier model of the proton pump (Läuger, 1979) and with kinetic data from flash-induced photoelectric signals (Rayfield, 1985; Holz et al., 1987). The present results suggest that bR pumps one proton during the photocycle under our conditions.

MATERIALS AND METHODS

bR containing planar membranes were formed over a hole in a septum separating two compartments of a teflon cuvette. Buffer containing 20 mM NaCl, 20 mM KCl, 5 mM HEPES/NaOH pH 7.4, was first filled into both compartments. 250 μl of a bR-lipid vesicle suspension (lipid concentration 3 mg/ml) were then added to one compartment (*cis*). At this stage the solution levels were just below the hole (diameter 150–200 μm) in the teflon film (thickness 6 μm) separating the two compartments. The vesicles had been prepared from 95% DMPC and 5% PS as described (Heyn and Dencher, 1982) at a molar lipid/bR ratio of 90/1. The vesicles were suspended in a buffer containing 100 mM Na-acetate at pH 5. 10 μl of lipid dissolved in hexane (6 mg/ml DMPC, 6 mg/ml PS) were added to the other compartment (*trans*). A small amount (~3 μl) of hexane was then added to the 1 cm^2 surface of the *cis* compartment. After 30–45 s the solution levels were elevated above the hole by injecting ~0.5 ml of buffer into both compartments resulting in formation of a bilayer membrane as monitored by capacitance measurements (typically 150 pF, 5–100 pS). Final concentrations in the *cis* compartment were ~18.5 mM NaCl, 18.5 mM KCl, 7 mM Na-acetate, and 4.6 mM Hepes/NaOH pH 7. Both membrane formation and measurements of photocurrents were carried out at the same temperature. White light from a tungsten-halogen lamp was applied through a heat filter onto the membrane at 40–120 mW/cm^2. Currents were measured with a Keithley model 427 (Keithley Instruments, Cleveland, OH) or a EPC 5 (List Electronics, Darmstadt, FRG) current amplifier through light-shielded Ag/AgCl electrodes.

RESULTS AND DISCUSSION

Planar membranes were formed according to the methods developed by Montal and Mueller (1972) and Schindler (1980) after spreading bR containing vesicles on one side (*cis*) and pure lipid vesicles on the other side (*trans*) of a septum. Significant incorporation of bR into the membrane generating a steady-state photocurrent was observed only if a small amount of hexane was added before the solution levels were raised above the hole in the teflon septum. With this method photocurrents of up to 40 pA were measured at 0 mV membrane potential. In most experiments lipid (dimyristoylphosphatidylcholine (DMPC) with up to 50% of phosphatidylserine (PS) dissolved in hexane was added to the *trans* compartment instead of vesicles. The membranes formed by this method thus contained much more negatively-charged lipids on the *trans* side than on the *cis* side. The current response evoked by the onset of steady-state illumination was the superposition of a transient and a steady-state component (Fig. 1 *a*) if the membrane was formed at ~30°C, well above the transition temperature of the lipids used. Under these conditions the voltage dependence of the steady-state

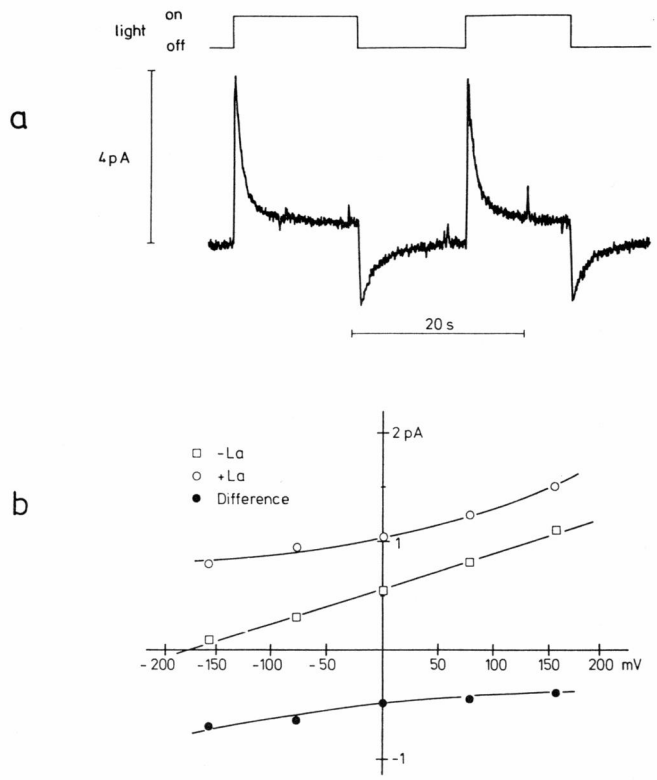

FIGURE 1 Typical photocurrents recorded from a membrane formed at 30.5°C. (*a*) Photocurrents at 0 mV membrane potential induced by saturating light pulses. The current is characterised by a transient on-component which decays to a much lower steady-state level and a smaller and slower off-component. (*b*) Voltage dependence of the photocurrent. The photocurrent was measured as the difference between the dark current (*light off*) and the steady-state photocurrent (*light on*). The voltage was always set before applying a light pulse. Each data point is an average of at least 5 determinations. Squares: photocurrents measured after formation of the membrane. Open circles: photo-currents measured at least 20 min. after addition of La^{3+} to the *trans* compartment at a final concentration of 7.5 mM (I_{La}). Filled circles: Difference between the data points measured before and after La^{3+} addition (I_D). The solid line describing I_D has been calculated from that describing I_{La} using the scaling function $I_D(V) = -0.48 \cdot I_{La}(-V)$ suggesting that this membrane contained two populations of bR molecules oriented in opposite directions.

photocurrent was found to be linear (Fig. 1 *b*) as described before for purple membrane sheets incorporated into planar membranes (Bamberg et al., 1981). To determine the degree of orientation of the protein in the membrane we used La^{3+} which has been demonstrated to block the proton pump if added to the cytoplasmic side of the protein (Drachev et al., 1984*a*; Seigneuret and Rigaud, 1985). In our experiments, the blocking effect of La^{3+} only occurred if the membrane contained several percent of PS as a negatively-charged lipid. Fig. 1 *b* shows that the steady-state photocurrent increased if 7.5 mM La^{3+} was added to the *trans* compartment. The shape of the current-voltage relationship also changed and became nonlinear. The difference between the photocurrents measured in the absence and presence of La^{3+} is also given in Fig. 1 *b*. The results indicate that bR was not perfectly oriented in this experiment. Two populations oriented in opposite directions, both having the voltage dependence determined in the presence of La^{3+}, would exactly reproduce the experiment of Fig. 1 *b*. The degree of orientation in this case was calculated to be ~2:1. Addition of La^{3+} to both compartments totally abolished the photoresponse.

When the membrane was formed at 24–25°C photocurrents without a transient component, but with a much higher steady-state value were observed in some but not all membranes (Fig. 2 *a*). At this temperature (as at 30°C) bR is most likely in the monomeric state (Heyn et al., 1981). The voltage dependence of the photocurrent was found to be nonlinear and much steeper as shown in Fig. 2 *b*. The photocurrent changed by a factor of ~3 when the voltage was varied between −50 and +50 mV. There was no indication of a reversal of the proton current down to −160 mV (data not shown). In support of our results is the report of a steep nonlinear voltage dependence of the pump current for purple membranes attached to a permeabilized lipid bilayer support, but no quantitative data were published (Szabo and Bamberg, 1985).

The weak voltage dependence of Fig. 1 *b* appears to be due to a reduction of the voltage which was actually present across the current generator in this experiment. The current–voltage relationship in the presence of La^{3+} is reproduced from the voltage dependence of Fig. 2 *b*, if we

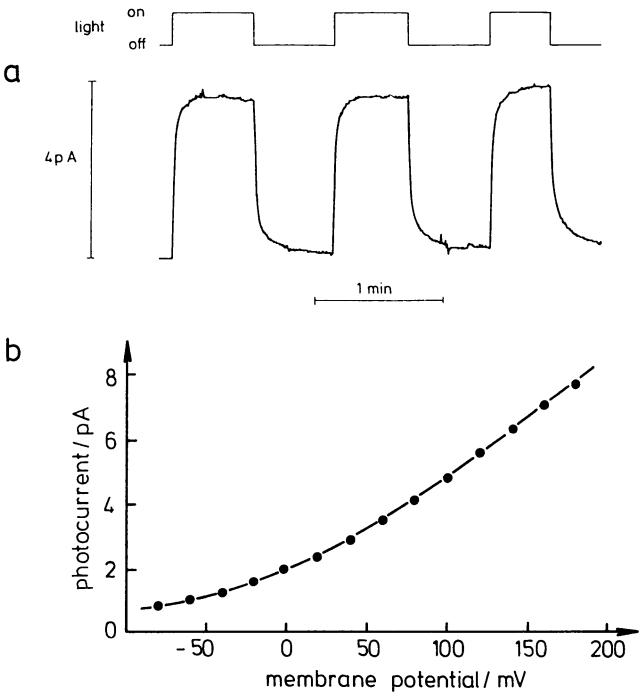

FIGURE 2 Photocurrents recorded from a membrane formed at 24.4°C. (*a*) Photocurrents in response to saturating light pulses measured at +60 mV showing no transient components. (*b*) Voltage dependence of the photocurrent recorded from the same membrane. Measurements were done as described in Fig. 1. The same current–voltage relationship was observed in four separate experiments.

assume that the effective voltage across the proton pump was only ~20% of the voltage applied to the whole system, suggesting imperfect incorporation of bR in the experiment of Fig. 1.

It has recently been demonstrated, that the formation of planar membranes from vesicles does not always lead to real bilayers but may result in more complicated structures. It has been suggested that these structures might consist of a planar membrane having incorporated numerous partly fused vesicles (Kolomytkin, 1987). The simplest equivalent circuit describing such a partly fused system is given in Fig. 3. This equivalent circuit is sufficient to describe the observed response. Our treatment is thus similar to the analysis of photocurrents recorded from permeabilized membranes with attached purple membrane fragments (Bamberg et al., 1979). The peak current of the response depends on the capacitance values C_m and C_s whereas the steady-state current measured externally is affected by the conductances g_m and g_s, which also determine the effective voltage drop across the proton pump. For perfect incorporation C_s should be very small, which would result in a response without a transient and g_s should be large compared with g_m to obtain short circuit conditions with a well defined potential across C_m. The absence of a transient in Fig. 2 *a* shows that C_s was negligible in this experiment. The series conductance g_s was determined to be >100 nS by the capacitive current measured in response to an externally applied voltage pulse, whereas g_m was only 0.1 nS. Thus, it can be concluded that the current–voltage relationship shown in Fig. 2 *b* represents the true voltage dependence of the proton pump incorporated into a lipid membrane. The previously reported linear voltage dependence (Bamberg et al., 1981) is very similar to the result shown in Fig. 1 *b*, which was probably a consequence of imperfect incorporation and poor orientation in the planar membrane.

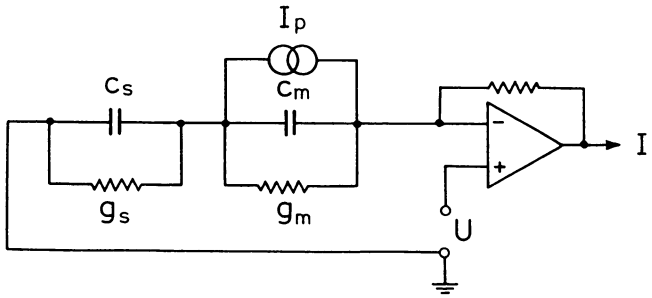

FIGURE 3 Equivalent circuit which accounts for the observed photoresponses. C_m and g_m are the capacitance and conductance of the bR-containing membrane. I_p denotes the light-driven current generator. To account for the observed transient components a bR-free membrane has to be added in series (C_s and g_s). If the voltage drop across C_m generated by the pump current I_p is small, then the voltage dependence of I_p can be approximated by a linear relationship and the expected photoresponse can be calculated from the circuit parameters. The response shown in Fig. 1 *a* is in qualitative agreement with these predictions.

We cannot give a conclusive answer why the perfect incorporation was obtained only in ~10% of the experiments at room temperature and was never observed at 30°C. If the formation of a planar membrane from vesicles starts with a partly fused system, our results would suggest that the transition to a completely fused membrane may be more likely at lower temperatures. Alternatively, the completely fused membrane may not form from the partly fused structure. We speculate that at the lower temperatures the hexane might not have evaporated completely and some residual hexane might be necessary for the formation of a real bilayer. Further work will be necessary to elucidate the requirements for perfect incorporation.

The proton current at saturating light intensity should be inversely proportional to the bR cycle time. If back reactions are neglected the current should essentially be a function of the time constants of the photocycle steps:

$$I = \text{const.}/\sum_i \tau_i.$$

If the light intensity is not saturating we have to add another term (Läuger, 1979):

$$I = \text{const.}/\left(\sum_i \tau_i + 1/\gamma J\right),$$

where J is the light intensity and γ is the reaction cross section. $1/\gamma J$ is thus the average time between completion of a photocycle and the beginning of the next one. The voltage dependence of any step within the photocycle depends on the charge movement associated with this step (Läuger, 1979) according to

$$\tau_i = \tau_i^0 \cdot \exp\left(-\frac{Q_i \cdot V_m}{2kT}\right),$$

where τ_i^0 is the time constant of the *i-th* step at 0 mV, V_m the membrane potential, Q_i is the charge effectively translocated across the membrane during this step, k and T are the Boltzmann constant and absolute temperature respectively. If the slowest (rate limiting) step involves a large charge movement, the photocurrent should be strongly voltage dependent.

Q_i is the total charge transferred during the photocycle multiplied by the fractional displacement during the *i-th* step or, equivalently, the charge effectively moved across the membrane within this step (Lindau and Rüppel, 1985). The steady-state photocurrent at saturating light intensity is roughly proportional to the rate constant of the slowest (rate limiting) step of the photocycle. The voltage dependence of this step thus determines the voltage dependence of the pump current. A logarithmic plot of the photocurrent versus membrane potential should give a straight line with a slope of $Q_i/2$ kT. This behavior is seen in Fig. 4 below +50 mV. The voltage dependence becomes much weaker at more positive potentials indicating that another process associated with a smaller charge displacement

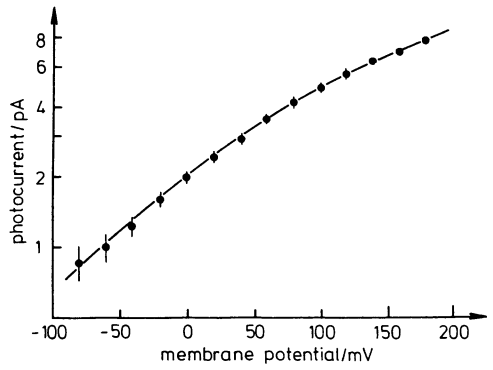

FIGURE 4 Semilogarithmic plot of the current–voltage relationship shown in Fig. 2 *b*. The solid line is the result of a nonlinear least squares fit assuming one voltage dependent and one voltage independent step (see text).

becomes rate limiting. To reproduce the whole current-voltage relationship between −80 and +180 mV, we have fitted the reciprocal photocurrent as a function of voltage with a sum of exponentials according to the equation

$$\frac{1}{I} = \frac{\sum_i \tau_i^0 \exp(-Q_i \cdot V_m/2\,kT) + 1/\gamma J}{I_0 \cdot \left(\sum_i \tau_i^0 + 1/\gamma J\right)},$$

where I_0 is the pump current at 0 mV.

The best fit (solid line in Fig. 4) revealed one exponential and a nonzero baseline. According to the above equation the result corresponds to the existence of one voltage-dependent process with an effective charge displacement of 0.63 +/− 0.02 elementary charges and another voltage independent step which is ~5 times faster at 0 mV. This step would eventually lead to saturation at high voltages reaching a current which is 6 times larger than the current measured at 0 mV. The light intensity could not be measured directly at the membrane during the experiments and could only be estimated to be in the range 40–120 mW/cm^2 from separate measurements. When the pump cycle is accelerated by voltage, the current will eventually be limited by the light intensity determining the $1/\gamma J$ term. At 0 mV membrane potential half-saturation was observed at ~8 mW/cm^2. From these values the rate of light activation is expected to be 5–15 times larger than the reciprocal bR cycle time at 0 mV. The saturation of the photocurrent at high positive membrane potentials thus may be simply due to the limited light intensity. Further experiments will be necessary to identify the effect of other, faster steps within the photocycle on the voltage dependence of the pump current.

In principal, the observed photocurrents could be the net current resulting from two bacteriorhodopsin populations of opposite orientation (fractions α and 1-α). If we assume the voltage dependence to be exponential, the total current will be:

$$I = I^+ + I^-$$
$$= I_0^* [\alpha \cdot \exp(V_m/V_0) - (1-\alpha) \cdot \exp(-V_m/V_0)].$$

The reversal potential V_R of the current is thus given by

$$1/\alpha = 1 + \exp(2V_R/V_0)$$

From the fit shown in Fig. 4 and the analysis of the previous paragraphs, we obtain for V_0 = 2 kT/Q = 2 kT/0.63 e the value of 80 mV. This might be an underestimate if the population was not perfectly oriented. The reversal potential was found to be < -160 mV. With these values we obtain $\alpha > 0.98$. The fraction of oppositely oriented bR molecules was thus <2% under these conditions. The observed current-voltage dependence thus reflects the activity of a highly oriented population.

The number of protons pumped per cycle has been a matter of controversy and has been claimed to be either two (Ort and Parson, 1979) or one Drachev et al., 1984*b*; Grzesiek and Dencher, 1986). The effective movement of 0.63 elementary charges would thus correspond to a movement of one proton across 63% of the membrane or to the movement of two protons by only half of that distance during the slowest (i.e., rate limiting) step of the cycle. The relative charge movements associated with certain time constants have been studied in purple membranes attached to a supporting membrane. Such flash relaxation experiments have shown that ~60% of the total charge movement is associated with the slowest component having a time constant of 15–25 ms at room temperature (Rayfield, 1985; Holz et al., 1987). The absolute size of the total charge movement, however, could not be determined from such measurements. The above value of 0.63 elementary charges corresponding to ~60% of the total charge movement provides additional independent evidence that one proton is pumped per cycle under the conditions of our experiments.

The voltage dependence of the ATP-driven Na/K pump current has been measured in heart cells using the whole-cell configuration of the patch-clamp technique (Gadsby et al., 1985). Recently kinetic information has also been obtained from the same system for Na translocation in response to voltage steps (Nakao and Gadsby, 1986). We anticipate that the analysis of the steady-state current-voltage relationship in terms of the voltage-dependence of the rate constants of the individual steps involved will provide a more detailed understanding of the properties and molecular mechanisms of the Na/K pump as well as other energy-driven ion pumps.

The results presented here demonstrate that bR can be inserted into asymmetric planar lipid bilayers and that the pump current has a steep exponential voltage dependence at least below +50 mV. The membrane potential will thus strongly affect the efficiency of the pump giving the cell an

effective way of regulating proton extrusion. The observed current-voltage relationship can be attributed to the voltage dependence of the slowest steps during proton translocation. Our results provide strong evidence that bR pumps one rather than two protons during the photocycle under the conditions employed here. The stoichiometry may, however, vary when these conditions are changed with respect to pH, ionic strength, or surface charge of the membrane. It will be particularly interesting to see if the stoichiometry increases at different pH. More information about the validity of the barrier model may be obtained from the influence of light intensity on the current-voltage relationship. This system is also well suited to investigate the effect of a pH gradient across the membrane; such experiments are in progress.

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